A PURSUIT OF GENES ACTIVE IN DEFENSE OF UPLAND COTTON AGAINST BACTERIAL BLIGHT

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ABBREVIATIONS

α	alpha
Avr	avirulence protein
B	bacterial blight resistant gene
bp	base pairs
β	beta
C	degrees Celsius
cDNA	complementary DNA
Ca^{2+}	calcium ions
CDPK	calcium-dependent protein kinases
CO_2	carbon dioxide
cfu	colony-forming units
Cy	cyanine
Da	Daltons
δ	delta
ds	double stranded
GFP	green fluorescent protein
GUS	β-glucuronidase
HMGR	3-hydroxy-3-methylglutaryl CoA reductase
hpi	hours post inoculation

HR	hypersensitive response
H_2O_2	hydrogen peroxide
Im216	Immune 216, a resistant cotton line
K^+	potassium ions
kDa	kilodalton
LRR	leucine rich repeat
mL	milliliters
mRNA	messenger RNA
NBS	nucleotide binding site
ng	nanograms
NTPs	nucleotide triphosphates
OH^-	hydroxyl ions
P	*Pseudomonas*
PCR	polymerase chain reaction
PLC	phospholipase C
PR	pathogenesis-related
POX	peroxidases
pv	pathovars
PUFA	polyunsaturated fatty acids
R	plant resistance gene
RT	reverse transcription
ROS	reactive oxygen species
SA	salicylic acid

SAR	systemic acquired response
SSC	sodium saline citrate
SSH	suppression subtractive hybridization
tRNA	transfer RNA
μL	microliters
μg	micrograms
μM	micromolar
UV	ultra violet
Vd	*Verticillium dahliae*
WbM*gl*	Westburn M gl_1 gl_1 gl_2 gl_2, a glandless cotton line
Xcm	*Xanthomonas campestris* pv. *malvacearum*

CHAPTER I

LITERATURE REVIEW

HOST-PATHOGEN INTERACTIONS

Plants constitute the largest and the most important group of autotrophic life forms on earth. They are the nutritional source for all heterotrophic organisms. Over the years, pathogens have developed various ways to penetrate plant cells. Many fungal pathogens gain entrance into their hosts by direct penetration of the cuticle. Some fungi as well as bacteria synthesize cutinase, that enables them to penetrate into the host. Most bacterial pathogens enter their hosts through stomata or wounds. Gram-negative phytopathogenic bacteria use an evolutionarily conserved type-III secretion system to deliver effector proteins, including AVR proteins, into the cytosol of host plant cells. Bacterial pilus structures unique to phytopathogenic bacteria ease the passage of effector proteins across the plant cell wall (Galan and Collmer, 1999). The pathogen adapts, or tends to adapt, itself to the host. It is this adaptation that is the basis of disease.

A complex array of interactions between plants and their pathogens has evolved that reflects both the nutrient acquisition strategies of pathogens and defense strategies of plants. It is this study of pathogen-induced genes and the plant-pathogen interactome that will enable us to understand signaling mechanisms taking place within the host across various plant species. It will also lead to practical solutions for the control of plant disease in crops of agricultural and horticultural importance.

a. Dynamics of Plant Defense Systems

Animals have the advantage of mobility to evade their attackers. Higher plants on the other hand do not, and therefore have developed other diverse strategies to ward off pathogen attack. Some pathogens are able to penetrate the plant, but many are overcome by the defense mechanisms activated by pathogenic infection. Only those pathogens with the ability to circumvent the defense mechanisms are able to successfully infect and colonize the plant. Thus, plant defenses in general are sound, and plant disease is the exception and not the rule (Campbell *et al.*, 1980). When a plant is successful in protecting itself, the

interactions are commonly known as host-pathogen incompatibility or non-host resistance. Compatible interactions result in disease symptoms in the host plant.

Elicitors are signal molecules that evoke host defense responses. They may be abiotic or biotic. Abiotic elicitors include heavy metal ions, air pollutants, and UV light (Sandermann *et al.*, 1998). Biotic elicitors may be exogenous, i.e. derived externally, either from a microbe, plant or animal, or endogenous, i.e. structural components or metabolites of a plant that are capable of eliciting defense responses in that particular plant. Inducers are signal molecules that initiate compatible host-parasite interactions (Huang, 2001).

Plant disease resistance mechanisms can be divided into two categories: preformed resistance and induced resistance. Preformed defenses include characteristics of normal, uninfected plants such as thickness of cuticle, opening of stomata, number of trichomes, and presence of constitutive antimicrobial compounds. Induced resistance is expressed after pathogen attack, in the form of fortification of cell walls, biosynthesis of phytoalexins, or accumulation of pathogenesis-related (PR) proteins, for example β-1, 3 glucanases, thaumatin and chitinases. Resistance responses vary with plant species and also with the pathogens to which they respond.

Recognition is an early event in plant-microbe interactions that activates an array of resistance mechanisms. Some resistance responses are induced by invasion of the pathogen and accompanied by localized host cell death, known as the hypersensitive response (HR) (Goodman and Novacky, 1994a). HR is characterized by a rapid loss of membrane integrity in the infected host cells and the accumulation of brown phenolic oxidation compounds. There are two applications of HR: one is general resistance against non-pathogens of a particular plant species; another is specific resistance against certain races of a pathogen by certain cultivars of a host plant species carrying particular disease resistance genes (Keen, 1999). Systemic acquired resistance (SAR) is another type of resistance that is induced following pathogen challenge of a plant. SAR does not inhibit the primary infection, but within a few days, the infected plant will exhibit varying degrees of resistance

to subsequent inoculation of other plant parts with the original pathogen or a completely different pathogen. SAR will be discussed in detail along with relevant examples later in this chapter.

Other responses that are generated in the plant, and that have been postulated to be involved in defense signal transduction include calcium and other ion fluxes, specific changes in protein phosphorylation, the generation of activated oxygen species such as superoxide and the production or release of salicylic acid (Dixon *et al.*, 1994). These will be discussed in more detail in various other sections in this chapter.

b. Genetics and Plant Pathogenesis

H. H. Flor studied the inheritance not only of plant resistance, but also of pathogen virulence in a flax-rust fungus pathogen system (Flor, 1971). His work proposed the classic 'gene-for-gene' model that describes the conditions for resistance to occur. Complementary pairs of dominant genes, one in the host and the other in the pathogen, are required. A loss or alteration to either member of the pair - the plant resistance gene (*R*) or the pathogen avirulence gene (*avr*) - leads to disease. A single plant can have many *R* genes, and a pathogen can have many *avr* genes. A resistance response is induced when an *avr* gene and an *R* gene of matched specificity are expressed.

The first *avr* gene was identified from the pathogen *Pseudomonas syringae* pv *glycinae* (Staskawicz *et al.*, 1984). Since then, several disease-resistance genes have been cloned and sequenced. The first gene cloned, *Pto* from tomato, encodes a rather conventional serine/threonine protein kinase. Experimental evidence has shown that the protein product of *Pto* directly interacts with the cognate avirulence gene protein AvrPto (Martin *et al.*, 1993).

The majority of *R* genes cloned so far encode proteins with a nucleotide-binding site (NBS) and leucine-rich repeat (LRR) region. LRRs have been implicated in protein-protein interactions and ligand binding in a diverse array of proteins (Kobe and Deisenhofer, 1994).

So far, *R* genes have been isolated from three monocots (maize, barley and rice) and eight dicots (*Arabidopsis*, tobacco, tomato, potato, pepper, flax, lettuce and beet) (Hulbert *et al.*, 2001; Bent *et al.*, 1994; Grant *et al.*, 1995).

With the advent of the isolation of plant *R* genes, immense opportunities now unfold for protein biochemists, biologists, physiologists, and geneticists alike to elucidate how these gene products function and the gene families evolve.

c. Molecular Mechanisms of Pathogen Recognition and Signal Transduction by Plants

Highly sophisticated and complex biological programs underlie the interactions between pathogens and the host plants. Defense responses in plants can be separated into three steps: (a) recognition of the pathogen, (b) signal transduction, and (c) execution of the defense programs such as HR cell death, oxidative burst, transcriptional activation of defense genes, and subsequent induction of systemic acquired resistance (SAR).

Bacterial speck in tomato is caused by the pathogen *P. syringae* pv. *tomato.* The *Pto* gene which confers resistance to bacterial speck and is responsible for the resistance trait of *L. pimpenellifolium* to *P. syringae* was isolated by map based cloning (Martin *et al.*, 1993). The physical interaction between the Pto kinase and AvrPto provides a molecular explanation for gene-for-gene specificity in plant disease resistance. The Pto-AvrPto recognition event is postulated to activate the Pto kinase and induce phosphorylation of downstream components in signaling pathways leading to defense responses (Sessa and Martin, 2000). In their model, the investigators postulated that during pathogenesis, the avirulence factor AvrPto is delivered by *P. syringae* directly into the plant cell by a type III secretion system. Within the plant cell, AvrPto is specifically recognized by the Pto kinase through determinants located in the Pto activation domain. The binding of AvrPto to Pto might cause a conformational change in the structure of the Pto molecule, which results in induction of its activity. Pto phosphorylation of the Pti1 kinase is a first step toward the

induction of the HR, while phosphorylation of the transcription factors Pti4, Pti5 and Pti6 activates signaling which determines the transcription of PR genes.

If a pathogen challenges a resistant plant, quite often the first infected plant cell(s) dies rapidly via HR. The HR is accompanied by transcriptional activation of defense genes that encode or enable synthesis of anti-microbial metabolites and proteins (Graham and Graham, 1991; Lamb *et al.*, 1992). Early and local molecular responses that are indicative of a hypersensitive response include the production of reactive oxygen species, transient opening of ion channels, cell wall fortifications, production of antimicrobial phytoalexins, and synthesis of pathogenesis-related (PR) proteins (Hammond-Kosack *et al.*, 1996; Somssich and Hahlbrook, 1998).

Potassium is the most rapidly lost ion during HR as seen during HR of tobacco and soybean to *P. syringae* pathovars. Although chloride ions are present at high concentrations in the cytosol, they are not lost from plant cells (Atkinson, 1993). The specificity of potassium ion efflux suggests that it is channel - or transporter-mediated. Potassium efflux is accompanied by extracellular alkalinization and intracellular acidification which may be due to direct H^+ uptake or to the outward transport of a base, such as OH^-, HCO^{3^-}, or electrons (Atkinson, 1993). The combined response is known as the K^+/H^+ response or the exchange response (XR). The external medium around the affected plant cell also alkalinizes correspondingly (Goodman and Novacky, 1994b).

Calcium acts as an intracellular second messenger, coupling extracellular stimuli to intracellular and whole plant responses (Hepler and Wayne, 1985; Sanders *et al.*, 1999). Normally, calcium is actively pumped out to the apoplast and deposited in the cell walls (Goodman and Novacky, 1994b). HR-dependent calcium influx may, in part, be activated by phospholipase C (PLC)-generated inositol phosphates. A role for inositol phosphates in HR-dependent calcium influx is strongly supported by (a) the accumulation of IP_3 during the exchange response and, (b) the inhibition of calcium influx across the plasma membrane by phospholipase inhibitors (Atkinson *et al.*, 1993).

In tomato (*Lycopersicon esculentum* L.; Gelli *et al.*, 1997) and parsley (*Petroselinum crispum*; Zimmermann *et al.*, 1997) distinct types of plant defense, elicitor-activated Ca^{2+} influx currents, have been described. Whole-cell and single-channel experiments on tomato protoplasts revealed a race-specific fungal elicitor-induced activation of a plasma membrane Ca^{2+}-permeable channel (Gelli *et al.*, 1997). The presence of the fungal elicitor resulted in a greater probability of the channel opening. Guanosine 5′-[β-thio]diphosphate, a GDP analog that locks heterotrimeric G-proteins into their inactivated state, abolished the channel activation induced by the fungal elicitor, whereas guanosine 5′-[γ-thio]triphosphate (GTP[γ]S), a nonhydrolyzable GTP analog that locks heterotrimeric G-proteins into their activated state, produced an effect similar to that observed with the fungal elicitor. Mastoparan, which stimulates GTPase activity, mimicked the effect of GTP[γ]S. The addition of HA1004 (a protein kinase inhibitor) in the presence of the elicitor totally abolished channel activity, whereas okadaic acid (a protein phosphatase inhibitor) moderately enhanced channel activity, suggesting that the activation of the channel by fungal elicitors is modulated by a heterotrimeric G-protein-dependent phosphorylation of the channel protein. Under asymmetric ionic conditions designed to resolve Ca^{2+}-inward currents, Zimmermann and colleagues were able to detect a channel (LEAC) that exhibited openings that lasted for some hundred milliseconds or even seconds (Zimmermann *et al.*, 1997). They also observed that a reduction of the external Ca^{2+} concentration toward physiological concentrations resulted in an increased Ca^{2+} permeability of LEAC. In addition, in the physiological range of plant membrane potentials (more negative than the reversal potential of LEAC), currents mediated by LEAC largely corresponded to Ca^{2+} influx.

Calcium-dependent protein kinases (CDPK) are calcium-binding serine/threonine protein kinases. CDPKs have a catalytic kinase domain in the N-terminal half of the protein directly tethered via an autoinhibitory junction domain to a regulatory calmodulin-like domain (Romeis *et al.*, 2001). CDPKs may function as a potential sensor that decodes and

translates the elevation of calcium concentration into enhanced protein kinase activity and subsequent downstream signaling events (Romeis *et al.*, 2001).

Living systems have developed defense mechanisms that utilize active oxygen species. Of these, the formation of hydrogen peroxide, superoxide, and possibly nitric oxide are ubiquitous in plant systems. They are often formed as by-products of normal metabolism as a result of leaky electron transport systems, but it has also become apparent that there is rapid production of these in defense against pathogens. Vanacker and associates (Vanacker *et al.*, 2000) detected reactive oxygen species (ROS) less than five minutes after pathogen attack. Plasma membrane localized NADPH and NADH oxidases, apoplastic peroxidases, amine oxidases and oxalate oxidases, are some of the existing enzymes currently known to generate ROS, along with protoplastic sources from mitochondria, chloroplasts, and peroxisomes (Bolwell and Wjtaszek, 1997).

In some plants, peroxidases have been shown to generate hydrogen peroxide through a superoxide-binding intermediate that requires neutral to alkaline pH and the presence of a suitable reductant (Wojtaszek, 1997; Bolwell and Wjtaszek, 1997; Murphy *et al.*, 1998). At least two ROS generating systems have been identified in cotton (Martinez *et al.*, 1998). These are discussed in more detail in the cotton-*Xcm* interaction section later in this chapter.

There is some evidence that hydrogen peroxide may be directly toxic to pathogens in the presence of iron by the production of reactive hydroxyl radicals (Chamnongpol *et al.*, 1998). Hydrogen peroxide may also play a role in the structural fortification of cell walls to resist parasitic intrusion and enzymatic degradation. This is brought about by cross-linking various compounds to the polysaccharide matrix or by increasing the rate of lignification due to high peroxidase activity (Karkonen *et al.*, 2002).

Peroxidases (POX) exist as isozymes with diverse expression profiles. They participate in various physiological processes such as lignification, auxin catabolism, wound healing, and defense mechanisms against infection (Hiraga *et al.*, 2001). Hydrogen

peroxide is an electron-accepting substrate for a wide variety of POX-dependent reactions, thus POXs are generally considered to be merely ROS-detoxifying enzymes. The breakdown of hydrogen peroxide by the POX reaction is highly active, especially in the presence of ROS-scavenging POX substrates such as flavonoids (Yamasaki *et al.*, 1997).

Nitric oxide (NO) is a signal molecule that regulates various biological processes and is known to induce host plant cell death by ROS (Hancock *et al.*, 2001). Nitric oxide binds to heme, thereby inhibiting catalase and ascorbate peroxidase, both of which detoxify H_2O_2. Studies have also shown that the addition of a NO-generating compound to cell suspension cultures lead to the accumulation of defense - related transcripts, indicating that NO may play a role in plant defense systems (Bolwell, 1999).

Wu and coworkers provide evidence that the H_2O_2-mediated disease resistance in potato is effective against a broad range of plant pathogens (Wu *et al.*, 1997). They investigated mechanisms underlying the H_2O_2-mediated disease resistance in transgenic potato plants. They found that elevated levels of H_2O_2 induced the accumulation of total salicylic acid several fold in the leaf tissue of transgenic plants, although they did not detect a significant change in the level of free salicylic acid. The mRNAs of two defense-related genes encoding an anionic peroxidase and acidic chitinase were also found to be induced. In addition, an increased accumulation of several isoforms of extracellular peroxidase was observed. This was accompanied by a significant increase in the lignin content of stem and root tissues of the transgenic plants. Their results suggest that constitutively elevated sub-lethal levels of H_2O_2 are sufficient to activate an array of host defense mechanisms, and these defense mechanisms may be a major contributing factor to the H_2O_2-mediated disease resistance in transgenic plants.

It has been reported that over-expression of *Pto* in tomato activates defense responses in the absence of the Pto–AvrPto interaction (Tang *et al.*, 1999). Leaves of three transgenic tomato lines carrying the cauliflower mosaic virus 35S::*Pto* transgene exhibited microscopic cell death, salicylic acid accumulation, and increased expression of

pathogenesis-related genes. Cell death in these plants was limited to palisade mesophyll cells and required light for induction. Mesophyll cells of 35S::*Pto* plants showed the accumulation of auto-fluorescent compounds, callose deposition, and lignification. When inoculated with *P. s. tomato* without avrPto, all three 35S::*Pto* lines displayed significant resistance and supported less bacterial growth than did non-transgenic lines. Similarly, the 35S::*Pto* lines also were more resistant to *Xanthomonas campestris pv. vesicatoria* and *Cladosporium fulvum.* These results demonstrated that defense responses and general resistance can be activated by the over-expression of an *R* gene.

VU-3 transgenic tobacco was used by Harding and Roberts to investigate the relationship between calmodulin signaling, the production of active oxygen species and cell death in response to infection with an incompatible pathogen (Harding and Roberts, 1998). VU-3-transgenic tobacco are *Nicotiana tabacum* L. cv. Wisconsin 38 lines expressing a mutant calmodulin (VU-3). Following *P. syringae* pv. *syringae 61* infection, suspension cells derived from VU-3 transgenic plants exhibited a stronger oxidative burst (3- to 4-fold higher primary and secondary burst reactions), greater media alkalinization (3-fold) and more rapid cell death (4-fold greater mortality at 20 hours post infection) than did infected control tobacco cells. Infection of leaf tissues with *P. syringae* pv. *syringae 61* also resulted in an enhanced cell death response compared to control tobacco tissues. This cell death response of VU-3 leaf tissues, but not control leaf tissues, was observed to be further enhanced by the presence of 50 M salicylic acid, suggesting that this transgenic line is more sensitive to the effects of this agent. Overall, their data supported the model that calmodulin signaling pathways are involved in the plant oxidative burst and contribute to the regulation of cell death in infected plant tissues undergoing the hypersensitive response.

Transgenic tobacco deficient in the H_2O_2-removing enzyme catalase (Cat1AS) was used as an inducible and noninvasive system to study the role of H_2O_2 as an activator of pathogenesis-related (PR) proteins in plants (Chamnongpol *et al.*, 1998). Sustained exposure of Cat1AS plants to excess H_2O_2 provoked tissue damage, stimulated salicylic

acid and ethylene production, and induced the expression of acidic and basic PR proteins with a timing and magnitude similar to the hypersensitive response against pathogens. Salicylic acid production was biphasic, and the first peak of salicylic acid as well as the peak of ethylene was observed to occur within the first hours of high light, which is long before the development of tissue necrosis. Under these conditions, accumulation of acidic PR proteins was also seen in upper leaves that were not exposed to high light, indicating systemic induction of expression. Short exposure of Cat1AS plants to excess H_2O_2 did not cause damage, induced local expression of acidic and basic PR proteins, and enhanced pathogen tolerance. However, they observed that the timing and magnitude of PR protein induction was in this case more similar to that in upper uninfected leaves than to that in hypersensitive-response leaves of pathogen-infected plants. Together, their data demonstrates that sub-lethal levels of H_2O_2 activate expression of acidic and basic PR proteins and lead to enhanced pathogen tolerance. However, rapid and strong activation of PR protein expression, as seen during the hypersensitive response, occurs only when excess H_2O_2 is accompanied by leaf necrosis.

d. Defense Programs

The term pathogenesis-related proteins was coined in 1980 to define a group of plant polypeptides that accumulate in pathological situations (Cutt and Klessig, 1992). PR-proteins are very stable at low pH and remain soluble, while most other plant proteins are denatured, and they resist proteolytic cleavage (Stintzi *et al.*, 1993). In 1994, a nomenclature for PRs was proposed based on their grouping into families sharing amino acid sequences, serological relationship, and enzymatic or biological activity (Van Loon and Van Strien, 1999). PR protein groups were divided into two subclasses (Kitajima and Sato, 1999). The two subclasses were: an acidic subclass of proteins that are secreted to the extracellular space and a basic subclass of proteins that are transported to the vacuole. Genes for basic PR proteins have been found to be expressed constitutively in organs such as roots, limited

parts of seedlings, and in cultured cells (Kitajima and Sato, 1999). Of the PR-protein families, PR-1 proteins are the most abundantly accumulated after pathogen infection and belong to the acidic subclass (Kitajima and Sato, 1999; Shewry and Lucas, 1997). The PR-1 proteins are represented by tobacco PR-1a. They are extracellular, acidic proteins with a molecular mass (M_r) of about 16 kD (Antoniw *et al.*, 1980). Their biological function is unknown. The PR-2 family consists of endo-β-1, 3-glucanases, with molecular weights of 33 kD, and four acidic enzymes and a major basic enzyme have been isolated (Stintzi *et al.*, 1993). The latter is a class I enzyme, PR-2, -N and –O are of class II, and another extracellular enzyme , PR-Q′ has been considered in a separate class II according to its sequence data (Stintzi *et al.*, 1993). Acidic glucanases (type II and type III) are isolated from intercellular fluids, whereas basic isoforms (type I) are isolated from vacuoles (Huang, 2001).

Family 3 PR proteins are chitinases. They consist of basic chitinases (M_r = 32,000 and 34,000; Huang, 2001) and acidic chitinases II, for which clones encoding two isoforms (chitinase, EC 3.2.1.14) have been isolated from tobacco following infection of the leaves with tobacco mosaic virus (PR-P and PR-Q with M_r=28,000; Payne *et al.*, 1990). Cotton (*Gossypium hirsutum* L.) chitinase and 1, 3-β-glucanase genes have been isolated, though no increase in 1, 3-β-glucanase mRNA expression was observed after treatment of cotton leaves with SA (Hudspeth *et al.*, 1996). Levels of mRNA transcripts of genes for several PR-3 proteins, such as acidic chitinase, basic chitinase and 1, 3-β-glucanase were observed to be increased in cotton stems after infection of the plants with *Verticillium dahliae* (McFadden *et al.*, 2001).

PR-4 proteins are small with M_r of 15, 000, and their biological function is not known. (Payne *et al.*, 1990). PR-5 proteins, sometimes called thaumatin-like proteins because their amino acid sequences, are highly similar to those of thaumatin, a sweet-tasting protein isolated from the fruit of *Thaumatococcus danielli*. The PR proteins belong to the acidic subclass as characterized by the presence of an acidic cleft in their 3D-structures

(Kitajima and Sato, 1999). Antifungal activity of PR-5 proteins has also been reported (Vigers *et al.*, 1992). In 1989, it was demonstrated that pathogenesis-related gene transcripts were induced in potato during a hypersensitive-like response (Matton and Brisson, 1989).

PR-6 proteins are known to be protease inhibitors that play a role in defense against insects, nematodes and microorganisms (Koiwa *et al.*, 1997; Ryan, 1990). The only PR-7 protein to be isolated to date has been from tomato and is known to act as an endoproteinase (Van Loon and Van Strien, 1999). Family 8 PR proteins are types III and IV chitinases. They possess lysozyme activity and have sequences very different from those in family 3. The type members are cucumber chitinase (Lawton *et al.*, 1994) and bean P4 (Margis-Pinheiro *et al.*, 1991). A cDNA encoding the bean P4 chitinase has been cloned. The open reading frame is 810 bp, encoding a polypeptide of 270 amino acids. The mature P4 chitinase is an acidic protein consisting of 255 amino acids. This chitinase has been located in the extracellular space. Family 9 PR proteins are a class of peroxidases that mediate lignin biosynthesis (Van Loon and Van Strien, 1999), while PR-10s are structurally related to ribonucleases (Moiseyev *et al.*, 1997). Levels of mRNA for at least one member of the family of cotton *Ypr10* gene homologues coding for potential PR 10 proteins were found to increase in cotton stems after inoculation of the plants with *V. dahliae* (McFadden *et al.*, 2001). A cDNA, *GaPR-10*, encoding a PR-class 10 protein was isolated from *G. arboreum* (Zhou *et al.*, 2002). RNA blot analysis detected some *GaPR-10* transcripts in roots of untreated seedlings, and observed that the transcript level increased after the seedlings were treated with *V. dahliae.* In *G. arboreum* suspension cells, induction of *GaPR-10* transcription by the fungal elicitor was gradual and prolonged, and the transcription was also inducible by jasmonate, but not by SA and 1-aminocyclopropane-1-carboxylate.

Van Loon and Van Strien have now proposed the inclusion of three additional families to PR-proteins: pathogen-induced plant defensins (PR-12), thionins (PR-13) and

lipid transfer proteins (LTPs) (PR-14) (Van Loon and Van Strien, 1999). Thionins were first purified from wheat flour in the early 1940s (Balls *et al.*, 1942a; Balls *et al.*, 1942b) and called purothionins. The toxicity of thionins to microorganisms has been well documented (Garcia-Olmedo *et al.*, 1989; Florack and Stiekema, 1994).

Phytoalexins are low molecular weight, antimicrobial compounds synthesized by the plant after exposure to a pathogen (Paxton, 1981). These compounds are undetectable or present in very small amounts prior to infection, but accumulate at the site of infection and may inhibit further development of most attacking pathogens. Phytoalexins have been reported in at least 17 plant families (Kuc and Rush, 1985). They have been frequently detected in angiosperms and dicotyledons but rarely in gymnosperms and monocotyledons, and have not been detected at all in vascular plants (Kuc and Rush, 1985). An extremely broad spectrum of substances and environmental conditions elicit the accumulation of phytoalexins. Elicitors of phytoalexins accumulation include ethylene, mercuric chloride, sodium fluoride, UV radiation, some fungicides, environmental stress, cell constituents of bacteria and fungi, damage by insects and nematodes, and infection by viruses, fungi and bacteria (Kuc and Rush, 1985).

The Carbon Nutrient Balance (CNB) hypothesis (Bryant *et al.*, 1983) is based on the concept that increasing photosynthesis or decreasing available nitrogen (N) should result in an increase in carbon-based defenses, such as phytoalexin production, whereas the opposite should increase reliance on N-based defenses. When insects feed on plants with a high C:N ratio, the hypothesis predicts that they will develop more slowly on such plants due to the increase in carbon defenses and a reduction in the amount of N per unit of food. Coviella and co-workers (Coviella *et al.*, 2002) aimed at discovering if allocation patterns of transgenic cotton plants containing genes for defensive chemicals that had not evolved in the species would respond as predicted by the CNB hypothesis. They grew a transgenic *G. hirsutum* line, producing *Bacillus thuringiensis* (Bt) toxin and a near isogenic line without the Bt gene. They observed a strong CO_2 effect on the N content in the plants. Plants

grown in elevated CO_2 showed a 16% decrease in N content compared to plants grown in ambient CO_2 atmosphere. They also found a significant CO_2 x N interaction effect on plant allocation to phenolic compounds. When grown in elevated CO_2, plants in the low nitrogen treatments allocated significantly more resources to phenolics than plants grown in ambient CO_2. There was a significant increase in condensed tannins from ambient to elevated CO_2 when plants were grown in low nitrogen. A strong CO_2 interaction effect was also found on Bt toxin production. In the high nitrogen treatments, exposure to elevated CO_2 produced lower levels of Bt toxin than in ambient CO_2. Thus, it is anticipated that as CO_2 concentrations increase, plants growing in nutrient-poor environments will show a similar shift in allocation from nitrogen-based compounds to carbon-based defenses. The observation that isoprenoid-derived compounds were not affected by changes in CO_2 levels demonstrated the complex biochemical apparatus that regulates synthesis of secondary compounds.

e. Systemic Acquired Resistance

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. After the formation of a necrotic lesion, either as a part of the HR or as a symptom of disease, the SAR pathway is activated throughout the plant. SAR can be distinguished from other disease resistance responses by both the spectrum of pathogen protection and the associated changes in gene expression. In tobacco, SAR activation results in a reduction of disease symptoms caused by the fungi *Phytophthora parasitica*, *Cercospora nicotianae*, and *Peronospora tabacina*, the viruses tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV), and the bacteria *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* (Vernooji *et al.*, 1995). Associated with SAR is the expression of a set of genes called SAR genes (Ward *et al.*, 1991). A protein is classified as a SAR protein when its presence or activity correlates with maintenance of the resistant state. Analyses of SAR proteins showed

that many belonged to the class of pathogenesis-related (PR) proteins (Ryals *et al.*, 1996). Hypersensitive resistance (HR) and systemic acquired resistance (SAR) are generally accompanied by elevated levels of endogenous salicylic acid (SA) (Malamy and Klessig, 1992; Metraux *et al.*, 1990). Strong evidence has been shown that SA plays a central role in HR and SAR (Malamy *et al.*, 1990; Dorey *et al.*, 1997).

The phenolic compound salicylic acid (SA) seems to be central to SAR signaling. Plants can synthesize SA and activate SA-dependent physiological programs (Dangl *et al.*, 1996; Greenberg, 1997). SA is an inducer of disease resistance and has been shown to accumulate in tobacco (Malamy *et al.*, 1990; Enyedi *et al.*, 1992) and *Arabidopsis* (Summermatter *et al.*, 1995), inducing systemic acquired resistance. To study the effect of SA peaks in HR, the SH-L isoforms of salicylate hydroxylase from *P. putida* were placed under the control of two different promoters, AoPR1 (inducible by H_2O_2) or PR1a (inducible by SA), and introduced into tobacco plants harboring the N-resistance gene (Mur *et al.*, 1997). After infection with TMV, both lines showed a 2-fold reduction in the SA levels in a second phase, although it still accumulated. Only the AoPR1::SH-L plants were found to strongly suppress SA accumulation in the pre-necrotic phase. Lesion formation was observed to be abnormal only in the AoPR1::SH-L plants where it initiates later and extends faster and longer than in wild-type plants. While the inefficient second phase of SA accumulation may contribute to such an effect, the lack of the first phase appeared to be crucial. Thus, in wild-type plants early enhancement of SA in HR may speed up both initiation and limitation of cell death.

Given the importance of SA in disease resistance, the pathway of SA biosynthesis may represent a major control point in plant defense responses. The biosynthetic pathway to SA appears to begin with the conversion of phenylalanine to *trans*-cinnamic acid (*t*-CA) catalyzed by phenylalanine ammonia lyase (PAL). The conversion of *t*-CA into SA has been proposed to proceed via chain shortening to produce benzoic acid (BA), followed by hydroxylation at the C-2 position to derive SA (Yalpani *et al.*, 1993). The latter step is

likely to be catalyzed by a cytochrome P450 monoxygenase, called benzoic acid 2-hydroxylase (BA2H), the activity of which is induced by either pathogen infection or exogenous BA application (Leon *et al.*, 1993). BA and SA can be conjugated to glucose, and regulation of SA levels through SA or BA conjugation may be important (Ryals *et al.*, 1996). In healthy tobacco plants, a large pool of conjugated BA that decreased transiently in size after pathogen infection was found (Yalpani *et al.*, 1993). This decrease in conjugated BA levels correlated with an increase in free BA and SA. Leon *et al.*, (1993) found that once SA accumulates, it is quickly converted to β-*O*-D-glucosylsalycylic acid (SAG), a compound that does not play a role in disease resistance. Conversion of SAG to free SA represents a potential mechanism for increasing levels of free SA.

BACTERIAL PLANT PATHOLOGY

a. Bacterial Pathogenesis

Higher plants are a nutrient source for the myriad bacterial species present in their environment. Bacteria are small enough to pass through stomates and other natural openings to take advantage of this nutrient source. Symptoms of plant pathogenic bacterial infection include chlorotic and necrotic spots on stems and leaves, death of flowers, wilts, destruction of root systems, soft rots, gall formation, stunting of organs, and other physiological changes.

Most gram-negative plant pathogens in the *Pseudomonadaceae* and *Enterobacteriaceae* are commonly known to colonize in the apoplast (Alfano and Collmer, 1996). It is these kinds of pathogens that cause rots, spots, wilts, cankers and blights afflicting virtually all the crop plants. Examples of some aggressively phytopathogenic gram-negative bacteria are: *Erwinia carotovora, E. chrysanthemi, E. amylvora, E. stewartii, Ralstonia solanacearum, Xanthomonas campestris* pathovars, and *Pseudomonas syringae* pathovars. While all are aggressive, the degree of aggressiveness of the pathogens varies. *Erwinia* species, known for being necrotrophic pathogens, attack the parenchymatous tissue of the host. On the other hand, biotrophic pathogens such as *Pseudomonas* and *Xanthomonas*, multiply in the host tissue for some period before causing necrosis (Collmer and Bauer, 1994). Gram-negative bacteria are potential pathogens because their mureins are well protected by lipopolysaccharides and lipoproteins, thus preventing toxic chemicals from the plant from penetrating this sheath to reach the murein layer (Verma and Formanek, 1981).

Virulence of a plant pathogen relates to those bacterial characteristics that determine the speed of pathogen growth and spread in the host, and the extent of destruction of host tissue. Major virulence factors include extracellular polysaccharides, cell wall degrading enzymes, and toxins.

Gram-negative bacteria use a type III secretion pathway to secrete proteins across

their inner and outer membranes (Salmond and Reeves, 1993). Gram-negative plant pathogenic bacteria use this pathway to transfer elicitors and other effector proteins into host plant cells (He *et al.*, 1993). *E. amylovora* (the causal agent of fire blight of pear, apple, and many other rosaceous plants) produces harpin, an acidic heat-stable protein (He *et al.*, 1993). The gene encoding harpin (*hrpN*) was located in the 40-kilobase *hrp* gene cluster of *E. amylovora*, sequenced, and mutated with Tn5tac1. The hrpN mutants were not pathogenic to pear, did not elicit a hypersensitive response, and did not produce harpin. Harpin causes tobacco cells to produce active oxygen and to increase a K^+/H^+ exchange response, two early events associated with HR (Huang, 2001). The transcriptional activation of a number of bacterial avirulence (*avr*) genes is controlled by hypersensitive reaction and pathogenicity (Hrp) regulatory proteins. *Hrp* genes were first described for *P. s.* pv. *phaseolicola* (Lindgren *et al.*, 1986), and have since been identified in many gram-negative phytopathogenic bacteria, including pathovars of *P. syringae* (Cuppels, 1986; Huang *et al.*, 1988) and *Xanthomonas campestris* (Arlat *et al.*, 1991; Bonas *et al.*, 1991).

Toxins are also produced by some pathogens, mainly *P. syringae* pathovars. These are secondary metabolites and often do not contribute to bacterial multiplication in plants, though they are highly diffusible and often produce characteristic symptoms spreading beyond developed lesions (Gross, 1991). The actual role of these toxins is still unclear. Some *Pseudomonas syringae* pathovars synthesize the pathogenicity factor coronatine (Bender *et al.*, 1987), an analog of the 18-carbon jasmonate family signal 12-oxo-phytodienoic acid (Weiler *et al.*, 1994). Coronatine to some extent mimics jasmonic acid (JA), inducing a number of JA-inducible proteins (Feys *et al.*, 1994). An advantage for the bacteria might be that a consequence of the induction of a jasmonate pathway is the inhibition of the SA-dependent pathway, thus permitting the bacteria to resist the plant's defensive gene products (Reymond and Farmer, 1998).

E. carotovora and *E. chrysanthemi* secrete pectic enzymes that cleave α-1,4-galacturonsyl linkages in plant cell wall polymers by hydrolysis (polygalacturonases) or β-

elimination (pectate or pectin lyases) (Perombelon and Kelman, 1980; Barras *et al.*, 1994).

b. Cotton – *Xcm* Interaction

Xanthomonas campestris pv. *malvacerum* (Smith) Dye (*Xcm*) is the causal agent of bacterial blight of cotton, an economically important crop. The genus *Xanthomonas* consists of plant pathogens with varying degrees of aggressiveness. The genus as a whole is known to have a wide host range, and the various pathovars attack a vast number of plant species in diverse plant families, although individual pathovars may attack only one or a few species.

Symptoms of bacterial blight of cotton include lesions that have a water soaked appearance, dark green or translucent depending on whether viewed with reflected or transmitted light, that later turn brown or black. Some bacteria are transported through the xylem and cause lesions in the leaf petiole, stem or leaf veins; in young expanding leaves, the attack may be limited to the tissue on either side of the main veins (Munro, 1987). Leaf spots and other lesions produce a bacterial exudate, which is readily dispersed in rainwater, and serves as an inoculum to spread the disease. Therefore, bacterial blight is usually associated with cold wet weather, as low temperatures slow down the growth rate of the crop and moist conditions favor infection and development of the disease (Munro, 1987).

Until the late 1980's, a gene-for-gene pattern of interaction had been suggested in bacterial pathogen-plant interactions (Brinkerhoff, 1970), but this has not been demonstrated even though at least 16 resistance genes against *Xcm* had been identified in cotton (Brinkerhoff, 1970). Six avirulence genes have been isolated from pXcmH (a plasmid found in XcmH), separately cloned, and localized to regions of 5-10 kb in the cloned DNA fragments (De Feyter and Gabriel, 1991). Four of the genes were designated $avr\mathrm{B}_4$, $avr\mathrm{b}_6$, $avr\mathrm{b}_7$ and $avr\mathrm{B}_{In}$ based on the HR elicited by *Xcm* transconjugants carrying the *avr* genes on cotton lines $\mathrm{Ac}B_4$, $\mathrm{Ac}b_6$, $\mathrm{Ac}b_7$ and $\mathrm{Ac}B_{In}$, respectively. These interactions were line-specific.

Cotton breeders have been successful in developing "immune" cotton lines that have been effective for over 30 years against all races of *Xcm* found in North America (Brinkerhoff *et al.*, 1984). We choose Im216 for this study because it is known for its superior resistance to bacterial blight (Brinkerhoff *et al.*, 1984).

Martinez and coworkers (Martinez *et al.*, 2000) detected SA in cotton cultivar (cv) Reba B50 cotyledon petioles six hours after infection with *Xcm* race 18 and after 24 hours in cotyledons and untreated leaves. In an earlier study, during an incompatible interaction between cv Reba B50, and the avirulent race 18 of *Xcm*, Martinez and colleagues observed a sharp production of superoxide at HR sites three hours after infection. This superoxide production was followed by an accumulation of H_2O_2 between four and six hours post inoculation (Martinez *et al.*, 1998). Martinez and coworkers also showed that plants of the cv Reba B50 when challenged by the virulent *Xcm* race 20, did not display any HR symptoms nor did they accumulate SA in cotyledons or in leaves even though they showed symptoms of bacterial blight (Martinez *et al.*, 2000). Surprisingly, they also detected a systemic accumulation of peroxidase activity 48 hours after infection, and this was associated with a relative inhibition of the bacterial population in cotyledons.

Damage to membranes during HR has been correlated with polyunsaturated fatty acid (PUFA) hydroperoxide production (van Ginkel and Sevanian, 1997; Mittler *et al.*, 1996) and associated with active oxygen species (AOS) generation (Adam *et al.*, 1989). AOS are suspected to trigger the oxidative degradation (PUFA peroxidation) in membranes via a propagation process linked to the production of fatty acid free radicals by autoxidation (Porter *et al.*, 1995). Peroxidation of the membrane lipids may also result from lipoxygenase activity (LOX, EC 1.13.11.12) (Brash, 1999). Jalloul and coworkers (Jalloul *et al.*, 2002) demonstrated that during an incompatible interaction between cotyledon cells of the Reba B50 cotton cultivar containing the B_2B_3 resistance genes and the avirulent race 18 of *Xcm*, lipid peroxidation was caused by 9S-LOX activity and was associated with both water loss from inoculated tissues and HR cell death. During disease induced by the

virulent *Xcm* race 20, lipid peroxidation resulted from a late and weak 9S-LOX activity; this was associated with tissue chlorosis and preceded water loss and complete necrosis of inoculated tissue. These investigators' results also indicated an association between LOX activity and accumulation of LOX transcripts.

For cotton plants, accumulation of sesquiterpenoids in subepidermal, lysigenous glands of the aerial organs provides an effective protection against insects and pests. In roots, the active transcription of *cad1*-A and *cad1*-C genes, and accumulation of sesquiterpene aldehydes form a chemical barrier against invading organisms (Tan *et al.*, 2000). Gossypol and related compounds are made and stored by cotton plants in subepidermal pigment glands as a deterrent to insects and other herbivores. Bell and associates (Bell *et al.*, 1993) found gossypol and its precursors, including hemigossypol (HG), desoxyhemigossypol (dHG), and their 3-hydroxyl methyl ethers (MHG and dHMG) are also phytoalexins, as they appear in vascular extracts of young cotton seedlings when challenged by *Verticillium dahliae* conidia. Low concentrations of several of these gossypol precursors inhibit the growth of *Verticillium* and other fungi, and were therefore assumed to play a role in defense of the host plant. Gossypol and its precursors are derived from mevalonic acid (MVA), the product of a reaction catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR, EC 1.1.1.34). By aligning previously cloned plant HMGR sequences in GenBank, Joost *et al.*, (1995) at Texas A& M University identified regions of high homology that were used to synthesize degenerate primers for PCR amplification of the equivalent gene from cotton. The cloned PCR product, when used as probe in Northern blots, showed a rapid induction of HMGR mRNA within 10 hours of introducing *V. dahliae* spores into the vascular system of a resistant *G. barbadense* cotton; in a susceptible *G. hirsutum*, a more gradual increase of HMGR mRNA was observed. The amount of HMGR transcripts returned to near control levels in four days in the resistant variety, but continued to accumulate in the susceptible one. They also showed that the specific enzyme activity of HMGR increased more rapidly in *G. barbadense* cotton than it did in *G. hirsutum*.

More recently, Liu *et al.*, (2002) reported the cloning of a gene from *G. barbadense* that encodes the enzyme that methylates the phenolic group of dHG exclusively at the 6-position, dHG-6-*O*-methyltransferase (dHG-6-OMT). Partial peptide sequences from digests of purified dHG-6-OMT were used to design primers for RT-PCR amplification of cDNA fragments from poly(A) mRNA. The full length clone was obtained using 5' and 3' RACE. The resulting cDNA codes for a 365-residue polypeptide with a calculated molecular weight of 40.6 kD. The investigators also expressed the cDNA in *E.coli*, and bacterial lysates showed a high specificity for the methylation of dHG, differentiating the cloned gene from other pathogen-induced methyltransferases.

Numerous other *O*-methyltransferases occur in plants, and many of them are involved in secondary metabolism (Wang and Pichersky, 1999). In addition, both caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl CoA 3-*O*-methyltransferase (CCOMT) are typically induced in stressed plants (Grimmig *et al.*, 1999; Ni *et al.*, 1996). An *O*-methyltransferase presumed to be involved in lignin synthesis has also been shown to be induced in cotton following inoculation with *V. dahliae* (Cui *et al.*, 2000).

The role of phytoalexins in resistance of cotton to bacterial blight has been well documented (Pierce *et al.*, 1996; Mace *et al.*, 1985). Sesquiterpene phenols 2,7-dihydroxycadalene (DHC) and lacinilene C (LC) as well as their 7-methyl ethers have been identified in inoculated resistant cotton leaves and cotyledons (Essenberg *et al.*, 1982; Abraham *et al.*, 1999). It was observed earlier that in leaves of resistant cotton lines, multiplication of each *Xcm* colony is inhibited by a local resistance response during which the mesophyll cells closest to the bacterial colony collapse and turn brown (Essenberg *et al.*, 1979). It was also observed that phytoalexins are localized in the HR cells of resistant cotton leaves, which exhibit green fluorescence of lacinilene C (LC) and lacinilene C 7-methyl ether (LCME) (Essenberg *et al.*, 1992). This observation indicated that, if they play a role in resistance by inhibiting bacterial growth and division, the phytoalexins accumulate close to the site of their action. A study of *Xcm*-inoculated OK 1.2 cotyledons showed that 45-90

hours-post inoculation was the period of most rapid increase in the fluorescent cell numbers, and phytoalexin accumulation peaked during this period when the rate of bacterial multiplication was declining (Gorski *et al.*, 1995).

A key enzyme in the terpenoid biosynthesis pathway in cotton is the sesquiterpene cyclase, δ-cadinene synthase. From an elicitor-induced cDNA library of *G. arboreum*, a diploid A-genome species, four different clones have been isolated. On the basis of sequence similarities, these cDNAs have been grouped into two subfamilies: *cad1*-C and *cad1*-A. The *cad1*-C is plural; of the four cDNAs characterized, three, isolated from *Verticillium* elicitor treated *Gossypium arboreum* suspension culture belong to this subfamily: (*cad1*-C1, *cad1*-C14 and *cad1*-C2; Chen *et al.*, 1995; Meng *et al.*, 1999), whereas only one member of *cad1*-A has been isolated by screening of a *G. arboreum* L. cv. Nanking cDNA library (Chen *et al.*, 1996). This type of gene encodes a protein that is 80% identical to the *cad1*-C. In 2000, it was found that there were high levels of *cad1*-A mRNAs in cotton sepals and petals before anthesis, and that the transcription ceased after anthesis (Tan *et al.*, 2000).

By chiral GC-mass spectroscopy, Davis and Essenberg (1995) showed that the δ–cadinene synthase was a (+)-δ–cadinene synthase and catalyzed syntheis of an early enzymatic intermediate in the biosynthesis of sesquiterpenoid phytoalexins by upland cotton. Davis and co-workers (Davis *et al.*, 1996) also isolated (+)-δ–cadinene synthase from *Xcm*-inoculated WbM*gl* cotyledons and observed maximum sesquiterpene cyclase activity at 60 hours following inoculation with *Xcm*. Qualitative RT-PCR data has shown that the transcripts were induced in cotyledons 24 hours post-inoculation with *Xcm,* while mock-inoculated controls showed no accumulation of the transcripts (Davis, 1998). Northern blots performed by Ed Davis and Theresa Haan also showed *cdn1*-A and *cdn1*-C to be induced after infection of bacterial blight resistant WbM*gl* cotyledons with *Xcm* (Davis and Haan; unpublished results).

Cytochrome P450 enzymes represent a super family of heme-containing proteins,

most of which catalyze NADPH- and O_2-dependent hydroxylation reactions. The proteins of this gene family catalyze a variety of reactions in plant secondary metabolism, including phytoalexin biosynthesis. Many P450 genes have been isolated and characterized (Chapple, 1998; Koopman and Hahlbrook, 1997; Frey *et al.*, 1997; Nelson *et al.*, 1996; Schuler, 1996; Halkier *et al.*, 1995; Kraus and Kutchman, 1995; Bolwell *et al.*, 1994; Funk and Croteau, 1993). Involvement of cytochrome P450s in host-plant allelochemical resistance is also well documented (Danielson *et al.*, 1997). A great number of P450 genes have been isolated from *Arabidopsis* (Winkler *et al.*, 1998).

A cytochrome P450 was found to be expressed in aerial tissues of glanded cotton cultivars (Luo *et al.*, 2001). After expression in yeast it was found to catalyze the hydroxylation of (+)-δ-cadinene, forming 8-hydroxy-(+)-δ-cadinene. This P450 has been classified as CYP706B1. Cloning of this enzyme is a step in elucidation of the gossypol biosynthetic pathway. It catalyzes an early step in gossypol biosynthesis, directing (+)-δ-cadinene into toxic sesquiterpene aldehydes. It is encoded by a single-copy gene in *G. arboreum* and this makes it a good target for suppression of gossypol formation in cotton seeds through genetic engineering.

Farnesyl diphosphate synthase (FPS) is involved in the biosynthesis of sesquiterpenes, and has been isolated from *G. arboreum* (Liu *et al.*, 1999). FPS was found to be induced by a fungal elicitor preparation, resulting in elevated levels of immunologically detectable FPS proteins and of catalytic activity (maximum activity detected at ~24 hours in elicitor treated cells), and in the subsequent accumulation of sesquiterpene aldehydes.

The role of sesquiterpene phytoalexins produced in response to bacterial infection in upland cotton has been the focus of research in the laboratory of Margaret Essenberg and Margaret Pierce for a number of years. However, the response of cotton to bacterial infection probably includes other induced defenses as well. Work was begun in 1999 to survey the genes induced in a resistant upland cotton line in response to *Xcm*. The research in this dissertation will give an overview of genes induced during HR in upland cotton

leaves (Im216) in response to *Xcm*. It is by no means an exhaustive collection of all the genes involved, but rather an attempt to provide an overview of the processes that may take place in cotton during HR. It will make an effort to provide answers to questions such as: What genes are induced in upland cotton when challenged by *Xanthomonas*? At what time points are these genes induced, and what is their expression profile like? Are the genes identified in cotton similar to defense related genes isolated from other plant species? What kind of role do these genes play in the whole scheme of defense responses? Are the defense genes induced by *Xcm* similar to those induced by *V. dahliae* in cotton?

GENERATION OF DIFFERENTIALLY EXPRESSED GENES BY SUBTRACTIVE HYBRIDIZATION

a. History of Subtractive Hybridization

Defense against pathogens is controlled by an array of biological processes that are in part mediated by programs of differential gene expression. There are however exceptions: some resistance genes may be constitutively expressed, and the very quick oxidative wall cross-linking discovered by Lamb and Dixon is independent of transcription (Lamb and Dixon, 1997). In order to understand the molecular regulation of these processes, the relevant subsets of differentially expressed genes of interest must be identified, cloned, and studied in detail. Subtractive hybridization is a powerful tool for enriching differentially expressed transcripts and was first used in 1966 by Bautz and Reilly to purify T4 mRNA (Bautz and Reilly, 1966). Since then subtractive cDNA hybridization has been widely used to identify and study cDNAs of differentially expressed genes (Ye and Connor, 2000; Agron *et al.*, 2002; Yang *et al.*, 1999).

Numerous cDNA subtraction methods have been reported for isolating differentially expressed mRNA sequences. Subtraction involves hybridization of cDNA from one population (tester) to an excess of cDNA from the other population (driver) and then separation of the unhybridized fraction (target) from hybridized sequences (Diatchenko *et al.*, 1996). The large amount of mRNA required and minute quantities of remaining cDNA are a few limitations of the pure subtractive methodologies, such as representational difference analysis (RDA). Lisitsyn and coworkers developed RDA in 1993 for cloning DNA fragments that differ in size between two genomes (Lisitsyn *et al.*, 1993). They developed a system in which subtractive and kinetic enrichment was used to purify restriction endonuclease fragments present in one population of DNA fragments but not in another. Application of this method to DNA populations of reduced complexity ("representations") resulted in the isolation of probes to viral genomes present as single

copies in human DNA, and probes that detect polymorphisms between two individuals (Lisitsyn *et al.*, 1993). RDA is useful for studying a large number of biological problems in a wide variety of organisms. It is exceptionally effective for isolating DNA fragments present in insertions or removed by deletion. However, wide differences in the abundance of individual mRNA species is a problem. As a consequence, multiple rounds of subtraction are needed to overcome these differences in mRNA abundance (Hubank and Schatz, 1994).

Other methods for identification of differentially expressed genes include mRNA differential display (Liang and Pardee, 1992), RNA fingerprinting by arbitrary primed PCR (RAP-PCR) (Welsh *et al.*, 1992), and serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995). The essence of the mRNA differential display is to use reverse transcription for an anchored oligo-dT primer which anneals to the beginning of a subpopulation of the poly (A) tails of mRNAs (Liang and Pardee, 1992). The anchored oligo-dT primers consist of 11 or 12 T residues plus two additional 3′ bases which provide specificity. These are used in conjunction with a decamer oligodeoxynucleotide of arbitrarily defined sequence for subsequent PCR amplification. Amplification of 3′ termini of mRNAs are separated by size on a denaturing polyacrylamide gel. The RAP-PCR method begins with conversion of RNA into cDNA, followed by arbitrarily primed PCR. The technique uses arbitrarily primed PCR to amplify cDNA stretches lying between sequences that, by chance, match arbitrarily chosen oligonucleotide primers well enough to initiate primer extension. The complex mixture of products is resolved by polyacrylamide gel electrophoresis, yielding highly reproducible fingerprints characteristic of the RNA source. Differences between fingerprints resulting from differentially expressed genes can be verified by Northern blot analysis or reverse transcription (RT)-PCR (Welsh *et al.*, 1992).

In SAGE, short sequence tags (~10 bp) are isolated from mRNA at a defined position, ligated to long multimers, cloned and sequenced. The frequency of each tag in the

cloned multimers directly reflects transcript abundance. In addition, the short tags are long enough to uniquely identify the corresponding transcript in database searches. Thus, SAGE results in an accurate picture of gene expression at both the qualitative and quantitative level (Velculescu *et al.*, 1995). A major drawback of SAGE is the requirement of large amounts of input RNA [2.5 – 5 μg poly(A)$^+$ RNA]. Although SAGE potentially has applications in many fields of research, its use is thus restricted to situations in which the amount of starting material is not limiting. In addition, the analysis of expression profiles in complex tissues composed of highly heterogeneous cell populations is difficult, since transcriptional changes in a specific subtype of cells will be diluted by expression profiles of other cell types present in the tissue, thus masking relevant changes in expression. In such cases, it is preferable to specifically isolate the cell population of interest for expression profiling, rather than using the complex tissue as a whole. SAGE is also characterized by a large number of sequential reactions and purifications, which can give rise to a significant loss of material. To overcome some of these problems, the method has been modified and called microSAGE, which allows use of very limited amounts of starting material [total RNA from a single punch of 300 μm tissue slice (10^5 cells; 1-5 ng poly A$^+$ RNA)] (Datson *et al.*, 1999). A 'single-tube' procedure has been incorporated for the all steps from RNA isolation to tag release. Furthermore, a limited number of additional PCR cycles (8-15 cycles in addition to 28 cycles) are performed. Other modifications to the original SAGE protocol include the addition of a heating step that helps to break up contaminating aggregates (Kenzelmann and Mühlemann, 1999) and utilization of biotinylated PCR primers that help to remove unwanted linkers that bind to streptavidin-coated beads at a later stage (Powell, 1999).

One strategy that emerged in the late 1990s, suppression subtractive hybridization, is a highly popular technique among researchers today. This technique, developed by Diatchenko and coworkers, requires small amounts of starting RNA and selectively amplifies differentially expressed transcripts, while simultaneously normalizing the mRNA

population (Diatchenko *et al.*, 1996). This technique is discussed in detail in the next section.

b. Suppression Subtractive Hybridization (SSH)

SSH is a powerful tool that enables scientists to compare two populations of mRNA and isolate transcripts that are expressed in one population but not in the other. This method is based on a technique called suppression PCR (Siebert *et al.*, 1995). SSH has now been commercialized by Clontech and made available to investigators worldwide as a PCR-Select™ DNA Subtraction kit (CLONTECH Laboratories, Palo Alto, CA, USA).

Figure 1-1 presents the details of molecular events occurring during this the SSH procedure. cDNA is synthesized from 0.5 to 2 μg of poly A^+ RNA from the two types of populations being compared. The experimental cDNA is known as "tester" and the control cDNA is known as "driver". Prior to hybridization of the tester and driver, the cDNAs of both populations are digested with *Rsa*I, a four-base-recognition restriction enzyme that yields blunt ends. *Rsa*I was chosen because it generated the largest average size of fragments (~600 bp). The tester cDNA is divided into two parts, each part ligated with different cDNA adaptors (Adaptor1 or 2R). The ends of the adaptors are designed without phosphate groups, so that only the longer strand of each adaptor can be covalently attached to the 5' ends of the ds cDNA. Excess driver cDNA is added to each batch of tester. The mixture is heat denatured and allowed to anneal. This process results in the preferential production of *a* type molecules (Figure1-1) from differentially expressed sequences, because cDNAs that are not differentially expressed form *c* type molecules with the driver (Figure1-1). Abundant tester cDNAs will form *b* type molecules (homo-hybrids), as they hybridize with each other. Abundant driver cDNAs also form hybrids, *d* type molecules.

A second round of hybridization follows this, where the first two hybridization samples are mixed together. This process enables the *a* type single-stranded cDNA

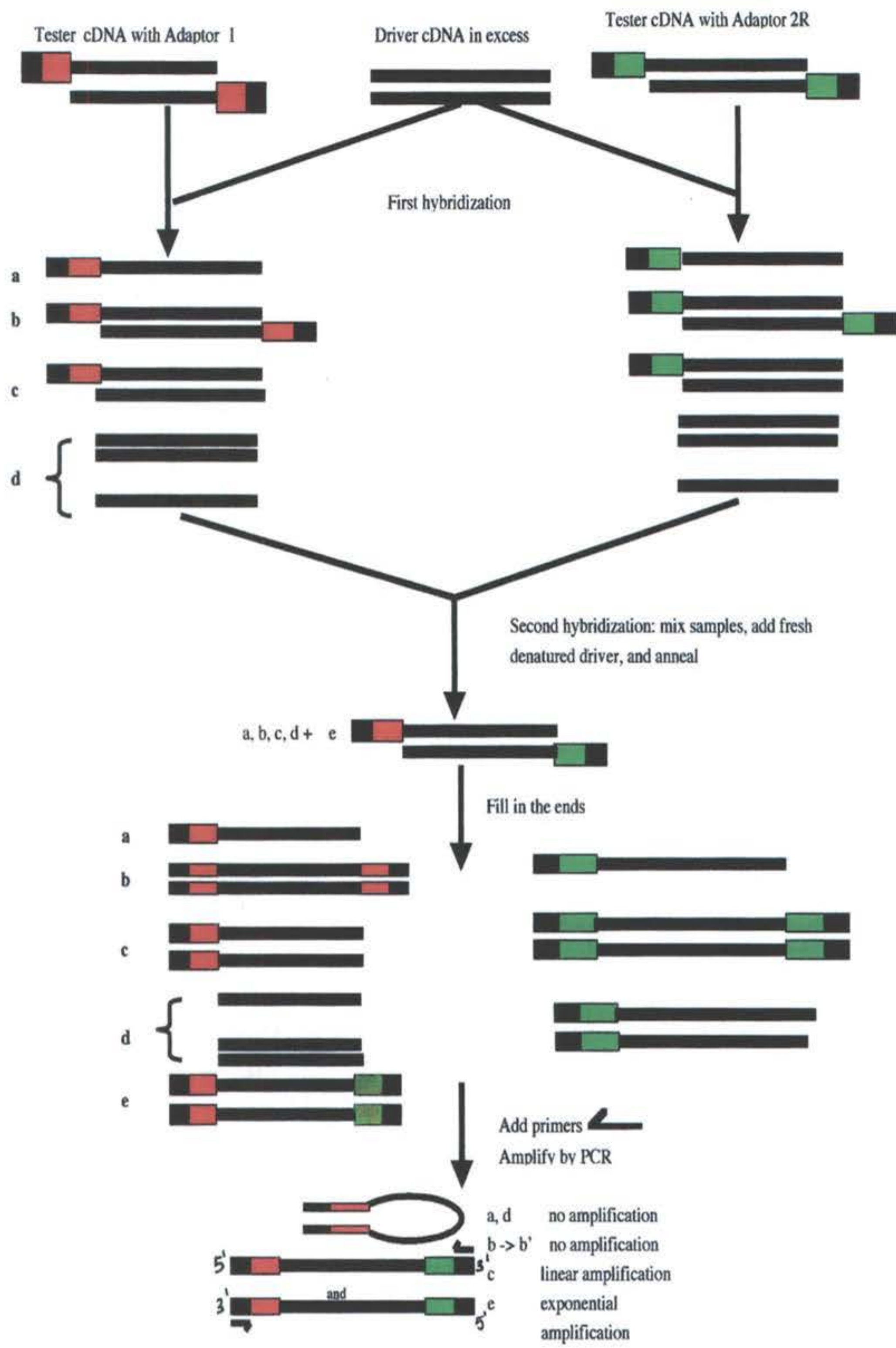

Figure 1-1: Scheme for PCR-Select cDNA subtraction (Diatchenko, 1996).

molecules to associate and form *e* type hybrids. These new hybrids are double stranded cDNA molecules with adaptors 1 and 2R on their two ends. Addition of freshly denatured driver cDNA helps to further enrich the *e* molecules for differentially expressed sequences, as only the remaining normalized and subtracted tester cDNAs are present for reassociation. DNA polymerase is used to fill in the ends, and the cDNAs are subjected to PCR using both adaptors 1 and 2R to amplify the differentially expressed sequences. The *b* type molecules have the same adaptor on both ends and hence tend to form a panhandle-like structure. This is known as the suppression effect, and these types of molecules will not be amplified during PCR. Type *c* molecules have only one primer-annealing site and therefore are amplified only linearly. The type *e* molecules possess the two different adaptors and hence can be amplified exponentially.

A secondary PCR using nested primers, complementary to the distinct 1 and 2R adaptors, further reduces the possibility of non-specific priming taking place and thereby enriches 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 1 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. The differentially expressed RNAs can then be identified by sequencing and hybridization analysis.

The stringency of subtraction can be altered by changing the ratio of driver to tester. Increasing driver:tester ratio will allow for preferential enrichment of those genes that are most differential in the tester compared to the driver. However, this may result in loss during subtraction of cDNAs with smaller differences in expression. SSH combines subtraction and normalization in a single procedure, in which the normalization equalizes sequence abundance during the course of subtraction by standard hybridization kinetics. Normalization occurs because the reannealing process generating homo-hybrid cDNAs (*b* type molecules; Figure 1-1) is faster for the more abundant molecules, due to second order kinetics (Diatchenko *et al.*, 1996). It eliminates any intermediate step(s) requiring physical

separation of ss and ds DNA, and requires only one round of subtraction to achieve greater than 1,000-fold enrichment for differentially expressed cDNAs (Diatchenko *et al.*, 1996). The subtracted cDNA mixture can be used directly for screening libraries or can be cloned to generate a cDNA differential library.

There are however some drawbacks of the procedure. First a few micrograms of poly A^+ RNA are required from each population. In special cases, these amounts may be difficult to obtain. Second, *Rsa* I was the chosen restriction enzyme because it generates fragments of an average size of 600 bp. This is a distinct disadvantage when full-length cDNAs are desired. A high level of enrichment and normalized abundance of cDNAs do however make SSH an ideal method for cloning of cDNAs of differentially expressed genes (Diatchenko *et al.*, 1996).

SSH may result in the formation of chimeras as demonstrated by Zhang and colleagues (Zhang *et al.*, 2000). They found that the *Rsa* I sites had been regenerated, thereby creating chimeras. They observed that in one group from the adaptor ligation, two *Rsa*I-digested tester cDNAs that represented two different genes (at least one of which was differentially expressed in tester mRNA population, allowing for enrichment after SSH) were ligated together, effectively regenerating the *Rsa*I site (Figure 1-2). The chimeric sequence was also ligated to adaptor 1 (or adaptor 2). They also proposed a simple method for the identification of such chimeras. Chimeras can also be generated if the restriction enzyme used during cloning has a recognition site in the adaptors used for SSH (Angela Phillips, personal communication).

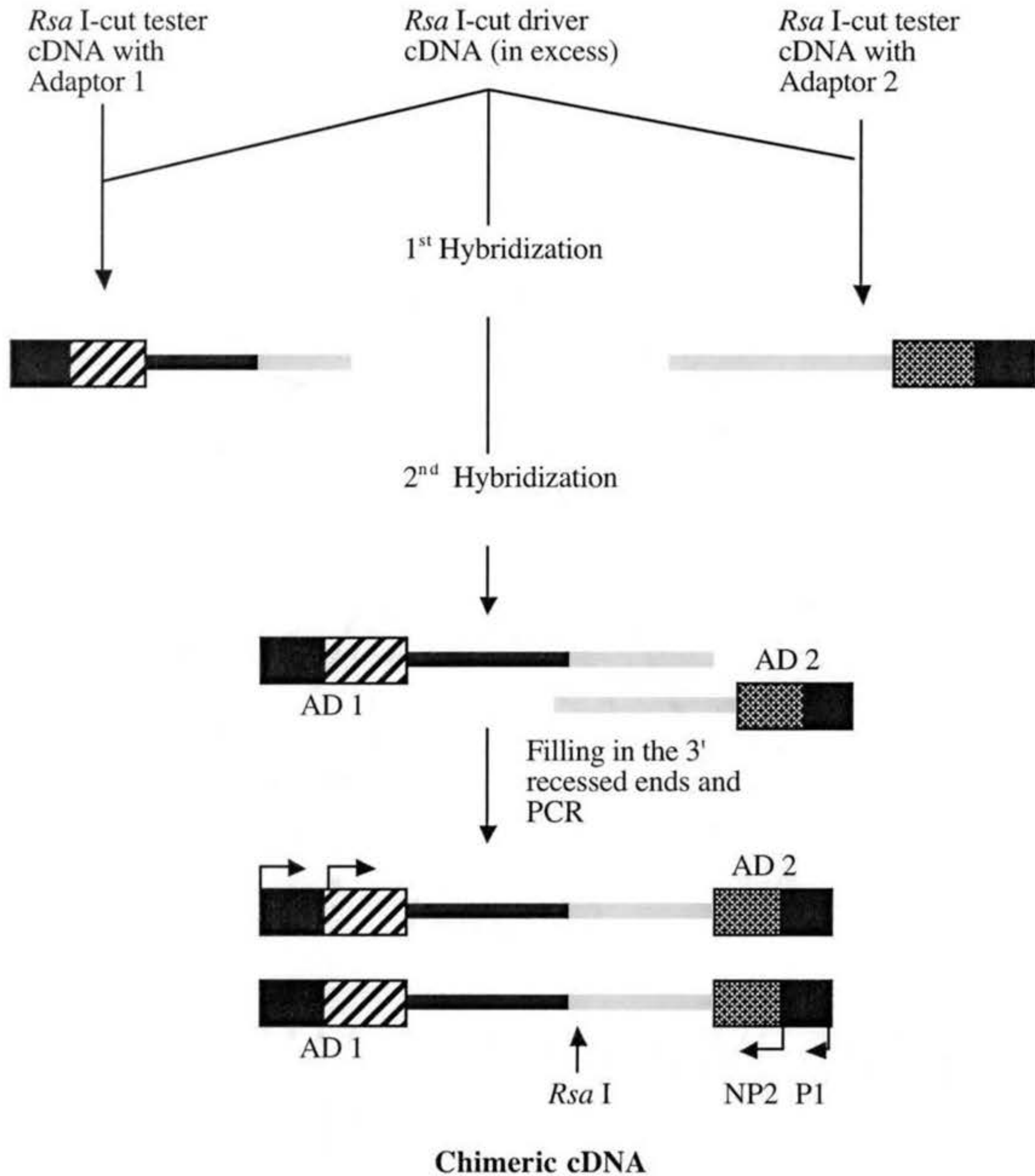

Figure 1- 2: A simplified diagram illustrating the formation of chimeric cDNA clones during SSH and the subsequent PCR. This sequence outlines the events when chimeric cDNA molecules can be formed. Refer to figure 1-1 for the complete scheme of SSH (Zhang *et al.*, 2000).

c. Identification of Differentially Expressed Genes by SSH

The main purpose of the SSH technique is to enrich for differentially expressed genes. SSH has been widely used by scientists in the medical field (Gardmo *et al.*, 2002; (Porkka and Visakorpi, 2001; Petkov *et al.*, 2000). More recently, plant biologists and pathologists alike have been utilizing this technology to study genes induced under various stress conditions as well as at various developmental stages.

Scientists in Germany have utilized SSH to identify a number of arbuscular mycorrhiza (AM)-regulated genes in *Medicago truncatula* (Wulf *et al.*, 2003). None of the genes they isolated was expressed in nonmycorrhizal roots or leaves of non-infected plants. Electronic data obtained by comparison of the cDNA sequences to expressed sequence tag (EST) sequences from a wide range of cDNA libraries in the *M. truncatula* EST database (Gene Index, MtGI) supported the mycorrhizal specificity of the corresponding genes because sequences in the MtGI that were found to match the identified SSH-cDNA sequences originated exclusively from cDNA libraries of AM-infected plants.

Watt (2003) employed the SSH technology to gain preliminary insights into gene expression induced by phytotoxic aluminum species – (Al^{3+}) in sugarcane roots. In this study, only forward subtractions were performed in which cDNA derived from control and challenged root tips served as driver and tester populations, respectively. The efficiency of subtraction was assessed by means of reverse northern hybridizations of the 288 fragments isolated, of which 182 were seen to be upregulated by Al^{3+}. From these 182 fragments, the investigators selected 50 cDNAs with the most obvious Al^{3+}-inducible expression patterns for characterization. Database comparisons revealed that of a these 50 cDNAs ostensibly up-regulated by the metal in the root tips, 14 possessed putative identities indicative of involvement in signaling events and the regulation of gene expression, while the majority (28) were of unknown function.

In order to identify nitrate-induced genes in rice roots, scientists constructed a

nitrate-induced root subtracted library using the SSH method and a split-root experiment (Wang *et al.*, 2002). They prepared the driver for subtraction by pooling total RNA from nitrate-deficient side roots at 1, 4 12 and 24 hour after split-root treatment. Tester RNA was prepared by pooling total RNA from nitrate-supplied side roots at the same time points after treatment as the driver RNA. Subtracted cDNA screening was performed, where clones that showed positive signals in the hybridization with forward subtracted probes (nitrate-supplied side roots cDNA as tester) and negative signals in the hybridization with reverse subtracted probes (nitrate-deficient side roots cDNA as tester) were selected. From a total of 10,000 clones, 864 positive clones were selected. The investigators further confirmed the positive clones from the forward and reverse screening by reverse Northern, using [α-^{32}P] dCTP labeled first strand cDNA probes that were reverse transcribed from the mRNA of the different treated roots. After sequencing, 37 known genes and 55 novel genes were identified to be up-regulated in roots on the nitrate-supplied side. The known genes were involved in nitrogen uptake and assimilation, sugar transport and organic acid metabolism, signal transduction, protein synthesis and degradation, plant resistance, hormone metabolism, and cell division.

Resistance of wheat to powdery mildew was studied by constructing a conventional library from a wheat line (Bai Nong 3217/Mardler BC5F4) with resistance to powdery mildew, and a suppression subtractive hybridization cDNA library from wheat leaves inoculated by *Erysiphe graminis DC* (Luo *et al.*, 2002). Three hundred and eighty-seven non-redundant ESTs from the conventional cDNA library and 760 ESTs from the SSH cDNA library were obtained. The ESTs were then compared to genes submitted to GenBank. Results showed that the redundancy of some kinds of genes such as photosynthesis-related genes and ribosome-related genes was higher in the conventional cDNA library and the varieties and quantities of disease resistance genes were less than in the SSH cDNA library. The SSH cDNA library was found to have obvious advantages in gene expression profiling of disease resistance such as simple library construction

procedure, enrichment in specific DRR (disease-resistance-related) genes, and decreased sequencing costs. In order to acquire genes that were involved in the powdery mildew resistance of wheat, hybridization with high-density dot membranes was used to screen the two libraries. About 50% function-known ESTs in the SSH cDNA library were identified to be DRR genes by screening. There were 247 out of 760 clones (32.5%) of the SSH cDNA library that had a positive signal in the repeated hybridizations with the uninfected probe.

Cloning parasitism genes encoding secretory proteins expressed in the esophageal gland cells is one key to understanding the molecular basis of nematode parasitism of plants. SSH has been used as a tool for reaching this goal. Gao and colleagues (Gao *et al.*, 2001) prepared an SSH library by subtracting intestinal region cDNAs as the driver and gland-cell cDNAs as the tester. Initially, 20 were picked randomly by the investigators and assessed by *in situ* hybridization in *H. glycines* sections. These were used as probes to hybridize to macroarrays containing the SSH gland-cell cDNA library. Twenty additional cDNAs from the membranes that did not hybridize were selected for *in situ* hybridization. This direct screening of the SSH cDNA library by in situ hybridization identified 13 unique clones. A total of 23 unique cDNA sequences from the SSH cDNA library were hybridized to the genomic DNA of *H. glycines* in Southern blots. *In situ* hybridization showed that four of the predicted extracellular clones were expressed specifically in the dorsal gland cell, one in the subventral gland cells and three in the intestines of *H. glycines.*

Capsaicinoids responsible for the pungency of chili pepper are synthesized exclusively in the placental tissue of the fruit. An attempt to understand the molecular basis of capsaicinoid biosynthesis was embarked upon by employing the SSH technique (Kim *et al.*, 2001). An SSH cDNA library (*Capsicum chinense* cv. Habanero and *Capsicum* cv. Haehwa III used as testers and drivers, repectively) was constructed from the placenta of *Capsicum chinense cv. Habanero,* a highly pungent pepper. They prepared

ca. 400 subtracted cDNAs, blotted them onto nylon membranes and screened by differential hybridization with three types of [α-^{32}P]dCTP-labeled total cDNA probes synthesized from placenta tissues of Habanero at 30, 10 days after flowering (DAF) and Haehwa II at 30 DAF. Thirty-nine cDNA clones were highly expressed in the placenta of Habanero at 30 DAF, and not in the placenta of Haehwa III or Habanero at 10 DAF. Based on the hypothesis that transcription levels of capsaicinoid biosynthetic genes are proportional to the degree of pungency, the subtracted cDNA clones showing highly differential expression levels were regarded as pungency-related genes. The nucleotide sequences of these selected cDNA clones were determined by single-run partial sequencing. Sequence information of the chosen clones was evaluated by comparing it with DNA and protein databases. Comparison of sequences in GenBank resulted in categorizing the clones into four groups according to their putative identities: cDNAs with similarities to 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 to genes of known function (Group IV). Northern blot analyses (of 39 clones) confirmed that all the clones were differentially expressed in pungent pepper. In addition, the cDNA clones of Groups I and IV were differentially or preferentially expressed in the placenta of pungent pepper.

GENOMICS ERA OF PLANT PATHOLOGY

a. Overview of Microarrays

Analysis of gene expression plays an essential role in many biochemical studies. These analyses were conventionally performed by Northern blot analyses, RNase protection assays, and reverse transcription-polymerase chain reaction (RT-PCR). These techniques require the visualization of the data as specific bands of expected sizes following gel electrophoresis. These techniques, however, allow only a small number of genes to be analyzed. To analyze the expression of thousands of genes simultaneously, new techniques like DNA arrays have been developed.

Global profiling of gene expression is one attractive approach to assessing function. A variety of techniques including SAGE (Velculescu *et al.*, 1995), differential display (Liang and Pardee, 1992), oligonucleotide arrays (Lockhart *et al.*, 1996), and cDNA macro- and microarrays (Desprez *et al.*, 1998; Schena *et al.*, 1995) have been developed that allow mRNA expression to be assessed on a global scale and the parallel assessment for hundreds and thousands of genes in a single experiment.

Pioneering work on microarrays was first published in 1995 (Schena *et al.*, 1995). Since then, it has made an impact on many fields including oncology (Wang *et al.*, 1999; (Khan *et al.*, 1998), pharmacology (Scherf *et al.*, 2000; Marton *et al.*, 1998), cellular physiology (Richmond *et al.*, 1999), and more recently on host-pathogen interactions (Wan *et al.*, 2002). The latter will be discussed in detail in the following sections.

The microarray process can be broadly divided into three stages: (a) probe preparation and array fabrication, (b) target preparation and hybridization, and (c) data collection, normalization and analysis.

Microarrays are constructed by arraying PCR-amplified cDNA clones or genes (probe preparation) at high density on derivatized glass microscope slides (array fabrication). Generally, cDNA clones are selected to represent as many unique transcripts as possible. The cDNA clone inserts can be amplified by PCR from plasmid DNA or

directly from clones in culture. The arrays are then prepared by printing the amplified products, which have been suspended in either a high salt or other denaturing buffer, to poly-L-lysine or aminosilane-coated glass slides. This technology was first developed in the laboratories of Patrick Brown and collaborators (Schena *et al.*, 1995) at Stanford University. The process is described in detail and available to the public (http:cmgm.Stanford.edu/pbrown/mguide/index.html). Researchers also use commercially prepared oligo arrays such as those offered by Affymetrix.

Both the slide surface and spotting buffer are critical components for reproducible, high fidelity microarray analysis (Hegde *et al.*, 2000). One of the most widely used spotting buffers is 3X sodium saline citrate (SSC). Some investigators also use 50% dimethyl sulfoxide (DMSO) to print their PCR products. Advantages of DMSO over 3X SSC include: DMSO denatures the DNA, allowing better binding to the slide and providing more single-stranded targets for hybridization. It is also hygroscopic and has low vapor pressure which allows DNA prepared for arrays to be stored for long periods of time without significant evaporation (Hegde *et al.*, 2000). Advantages of 3X SSC are that: it is a commonly used aqueous solution and produces spots of small diameter, thus allowing high printing density. Glass slides are the solid support for immobilizing probes for reasons of availability, low fluorescence, transparency, resistance to high temperature, physical rigidity, and the variety of surface chemical modifications possible (Holloway *et al.*, 2002). There are numerous vendors that supply aminosilane-coated slides, including Corning and TeleChem.

Differential gene expression is assayed by competitive hybridization of two targets that are prepared from two different mRNA sources, each being labeled with a different fluorophore. The purity and quality of starting RNA have a significant effect on subsequent results of the assay. It is also vital that products of the labeling reaction be purified to remove unincorporated, labeled nucleotides which can be a source of background on slides following hybridization. Finally, hybridization temperature, buffer, and washing conditions

must be optimized to provide high specificity and to minimize cross-hybridization. The ratio of the fluorescence readings of the two fluorophores bound to each DNA spot following hybridization correlates with the relative abundance of its mRNA in the two samples.

Initial protocols used for target labeling were based on direct labeling, whereby reverse transcription of mRNA is primed using a poly(dT) primer in the presence of fluorescently labeled nucleotides. Cy3- or Cy 5-conjugated dCTP or dUTP are bulky, making their incorporation using standard enzymes inefficient (Holloway *et al.*, 2002). An alternative method to direct labeling is indirect or amino allyl labeling that circumvents the need to incorporate bulky dyes. In this method, an amino allyl modified dUTP is used instead of a prelabeled nucleotide. After reverse transription, the free amine group on the amino allyl dUTP can be coupled to a reactive *N*-hydroxysuccinimydl ester fluorescent dye. This method takes longer than the direct labeling, but has advantages such as better sensitivity, reduced cost, and absence of dye bias.

Procedures commonly used require 0.5 to a few micrograms of mRNA or 50-200 μg total RNA from each source to attain sufficient sensitivity to detect a few copies of transcript per cell. In some cases RNA may be limiting, and there are several methodologies now available to circumvent this restriction. Examples include antisense RNA (aRNA) amplification with *in vitro* transcription alone (Eberwine *et al.*, 1992) or in conjunction with a template-switching effect (Wang *et al.*, 2000). The first step in aRNA amplification is the synthesis of an oligo (dT) primer that is extended at the 5′ with a T7 RNA polymerase promoter (Eberwine *et al.*, 1992). This oligonucleotide can be used to prime poly $(A)^+$ mRNA populations for cDNA synthesis. After the first-strand of cDNA is synthesized, the second-strand is made using "RNA-nicking and priming" for RNA in solution or "hairpinning" for tissue sections. This is followed by a brief S1 nuclease treatment and "blunt-ending" with T4 DNA polymerase. The cDNA can now be amplified using the T7 RNA polymerase promoter to direct synthesis of RNA. Since it is difficult to isolate RNA

from a single cell, Eberwine and coworkers amplified aRNA from defined single cells by microinjecting (with a patch pipette) primer, nucleotides, and enzyme into acutely dissociated cells from a defined region of rat brain (Eberwine *et al.*, 1992). Another widely used emerging technique is the dendrimer technology; it enhances detection sensitivity, can be used with limiting starting material, and does not require an RNA amplification step (Stears *et al.*, 2000).

After hybridization, arrays are scanned with a confocal laser scanner that is capable of differentiating between the two fluorescent (usually Cy-5 and Cy-3) labeled targets and producing separate TIFF images for each. There are numerous commercial scanners (Examples: Axon instruments, GSI Lumonics, Genetic Microsystems and Molecular Dynamics) available for detecting Cy3 and Cy5. With a large number of experiments it is prudent to scan all arrays using the same unit. Once the images have been generated, they are analyzed to calculate the relative expression levels of each gene and to identify differentially expressed genes. The analysis process can be divided in to two steps, image processing and data analysis.

Image processing involves identifying the spots of arrayed genes and distinguishing them from spurious signals generated by artifacts. Once this has been accomplished, the background-subtracted hybridization intensities for each spot are calculated in both channels. Measured intensities can be entered into the Molecular Analysis of Gene Expression (MAGE) database, a database specifically designed to capture gene expression data.

The process by which data from different channels or different chips are equalized before analysis is known as normalization, and the value that is used to normalize different datasets is known as the normalization factor. If performed properly, the normalization process does not alter the content of the data, but rather corrects for minor imbalances that arise during the imaging process owing to differences in labeling, hybridization efficiency, washes and differential quantum yield of dyes, variations in laser power, and detector

sensitivities (Schena, 2003). Global intensity normalization uses the sum of signals in multiple images to provide equalized signals. Global intensity matching works well for samples that share common signal intensities. Normalizing data from different biological tissues or from many different microarray experiments presents a greater normalization challenge, owing mainly to the fact that global intensities and median intensities may differ substantially for a large amount of data points on each array (Schena, 2003). Normalizing signals to a set of housekeeping genes which are expressed approximately at the same level in many tissues is one approach to normalizing data derived from different tissues or chips. Another approach is to spike small quantities of control samples from a foreign source into each labeling reaction, and normalize the images using the signal intensities from the control spots.

The examination of gene expression using microarrays holds great promise for the identification of candidate genes involved in a variety of processes. Hierarchical clustering and self-organizing maps have now been applied to the analysis of microarray expression data across multiple experiments. However, microarray experiments can result in false-positive results, i.e. some genes may appear to be differential, but are not. One way to determine whether the differential expression observed in an experiment is real and not due to artifact signals is to generate a ratio-intensity (R-I) plot for a sample labeled with one dye against itself labeled the another dye (Quackenbush, 2002). The R-I plot can reveal intensity-specific artifacts in the log_2 ratio measurements. Therefore, it is necessary to analyze multiple independent experiments in order to eliminate spurious results. It is also important to validate the differentially expressed genes by independent methods such as Northern blots, quantitative real time-PCR, or protein expression. Future challenges for microarray researchers will include developing databases and algorithms to manage and analyze vast genomic-scale datasets.

b. Using Microarrays to Study Gene Expression in Plants

Microarrays are a revolutionary tool that has been most popularly used to monitor differences in transcript abundance and expression patterns of thousands and tens of thousands of genes simultaneously. It allows a comprehensive and high throughput survey of DNA or RNA molecules on a genome-wide scale (Wu *et al.*, 2001).

Now that the *Arabidopsis* genome has been completely sequenced, plant biologists have a wealth of information for further understanding the biological processes of this model organism. Earlier Schena and collaborators (Schena *et al.*, 1995) used *A. thaliana* as a model organism to study differential expression patterns between root and leaf tissues grown in the light and dark, using a small 45-element array. The year 2000 saw DNA microarrays consisting of ~11,500 elements, mostly from the EST collection at Michigan State University with a few hundred clones donated by individual researchers (Wu *et al.*, 2001). The number of genes arrayed is rapidly growing day by day. Affymetrix now has a GeneChip with *ca* 25,000 genes arrayed.

Arabidopsis-based arrays have also been utilized to screen plant genes responsive to mechanical wounding and insect feeding (Reymond *et al.*, 2000), to plant defense signaling molecules (Schenk *et al.*, 2000), to cold and drought stress (Seki *et al.*, 2001), and for genes related to seed development (Girke *et al.*, 2000). More recently, *Arabidopsis* arrays have been used to identify conserved and differentially expressed genes involved in shoot growth and development from distantly related plant species such as wild oat (*Avena fatua*), poplar (*Populus deltoidsies*) and leafy spurge (*Euphorbia esula*) (Horvath *et al.*, 2003).

Gene expression studies using microarrays have not been limited to *Arabidopsis* chips. They have been used to identify a novel gene involved in flavor biogenesis in strawberry (Aharoni *et al.*, 2000), as well as to study strawberry achene and receptacle maturation (Aharoni and O'Connell, 2002). Scientists have also used this cutting edge technology to evaluate responses to ethanol or herbicide treatment by maize glutathione S-transferase gene family members (Mcgonigle *et al.*, 2000), to characterize salt stress

responses in the halophytic ice plant *Mesembryanthemum crystallinum* (Bohnert *et al.*, 2001) and in rice (Kawasaki *et al.*, 2001), as well as to identify reproductive organ-specific gene expression in *Lotus japonicus* (Endo *et al.*, 2002). Microarrays have also been applied to gene discovery in commodity crops such as cacao (Jones *et al.*, 2002). In a novel application, DNA microarrays were used to classify various highly repetitive sequences by their presence or absence in genomic DNA from twenty legume species (Nouzova *et al.*, 2001).

Transcript regulation in response to high salinity has been investigated for salt-tolerant rice (var Pokkali) using microarrays including 1728 cDNAs from libraries of salt-stressed roots (Kawasaki *et al.*, 2001). Hybridizations of fluorescence-labeled targets to microarray slides probed for changes in transcripts from 15 minutes to 1 week after salt shock. Beginning 15 minutes after the shock, Pokkali showed upregulation of transcripts. Approximately 10% of the transcripts in Pokkali were significantly upregulated or down regulated within one hour of salt stress.

Microarrays provide a measure of steady state transcript levels or relative steady-state transcript levels only (Gygi *et al.*, 1999). Biological processes regulated by RNA degradation or protein modification will be inert to the microarray approach.

Microarrays are a powerful tool for studying host-microbe interactions and downstream signaling pathways. However, as mentioned by Wan and colleagues in their review article (Wan *et al.*, 2002), there are a few points to be kept in mind while analyzing microarray data:

i. DNA microarrays measure the abundance of mRNA and not the rate of transcription.

ii. Some studies have suggested a correlation between protein and transcript abundance, but this was unexpectedly low, as shown recently (Gygi *et al.*, 1999).

iii. Microarrays have yet to accurately monitor genes that are expressed

transiently at low levels or in minute number of cells.

iv. Due to high sequence similarities within gene families, microarrays may not be able to differentiate these genes.

v. It is important to have data replicated in experiments, as well as with independent biological samples.

vi. Currently there are no set tools for data analyses, and use of different data analysis software can often lead to different results.

Availability of full-genome microarrays will greatly expand our knowledge of the inter-connections and similarities among defense response pathways, and will aid in the identification of genes previously unknown in defense responses.

c. Profiling Defense Response Genes

Active disease resistance in plants depends on the ability of the host to recognize pathogens and initiate defense mechanisms that limit infection. In the past, signaling processes and their interactions in plants have been studied only one gene or a few genes at a time (Alonso *et al.*, 1999). These studies have not been able to assess the extent of overlap of gene activation by different signals and pathogens in the defense response for tens and thousands of genes simultaneously. Recently developed methods, such as cDNA microarray analysis, are quantitative methods for global and simultaneous study of expression profiles and will enable the world to better understand the molecular basis of plant defense responses.

Since the advent of this genomic tool, several plant biologists have undertaken research projects to decipher the complex network of signaling pathways involved in plant defense responses as well as to understand virulence factors in the pathogen. Genes regulated in *Erwinia chrysanthemi* 3937 during infection of African violet have been examined (Okinaka *et al.*, 2002). This study revealed that several genes were down regulated in the presence of the plant, most of which were homologous to well-known

housekeeping genes. On the other hand, almost all of the genes up-regulated during infection were likely to be involved in specialized functions such as virulence, anaerobiosis, iron uptake, and stress responses to reactive oxygen species and heat.

Maleck and coworkers (Maleck *et al.*, 2000) provided a comprehensive description of SAR in *Arabidopsis thaliana.* They used a chip containing 10, 000 ESTs, representing approximately 7000 genes, to profile gene expression in plants under fourteen different SAR-related conditions, including plants that had been infected with an avirulent bacterium. The authors compared several experiments in which SAR was induced, and scored as SAR-related those 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 7000) were involved in the SAR response.

Researchers at the Boyce Thompson Institute at Cornell, used GeneCalling, an mRNA-profiling technology, to identify genes that are either induced or suppressed in leaves four hours after bacterial infection in the Pto- and Prf-mediated tomato-*Pseudomonas (AvrPto)* system (Mysore *et al.*, 2002). They examined over 135, 000 individual cDNA fragments representing an estimated 90% of the transcripts (~150,000) expressed in tomato leaves. Of these 432 differentially expressed genes were identified. GeneCalling (Bruce *et al.*, 2000), is an open-architecture, gel based assay that reproducibly measures changes in RNA amounts of known and novel genes.

The availability of *Arabidopsis* mutants is a major asset to the study of plant science. Reymond (Reymond, 2001) found that jasmonate does not induce a set of genes that are normally induced in wild-type plants in *coil-1* mutant plants that are insensitive to jasmonate. Earlier Reymond used a small array containing only 150 genes, but this was already large enough to permit the detection of a gene (*HEL*) induced by feeding *P. rapae* larvae but not by mechanical wounding in *Arabidopsis* leaves (Reymond *et al.*, 2000).

Systemic infections of plants by viruses require that viruses modify host cells in order to faciltate infections. Examples of some modifications are induction of host factors

that are required for replication, propagation and movement, and suppression of host defense responses that are likely to be associated with changes in host gene expression. *Arabidopsis* leaves, either mock-inoculated or inoculated with cucumber mosaic cucumovirus, oil seed rape tobamovirus, turnip vein clearing tobamovirus, potato virus X potexvirus, or turnip mosaic potyvirus, enabled investigators to gain an insight into the responses elicited by viruses in susceptible hosts (Whitham *et al.*, 2003). Total RNA isolated from inoculated leaves collected at 1, 2, 4 and 5 days after inoculation were hybridized to *Arabidopsis* GeneChip microarrays (Affymetrix). The hybridizations revealed co-ordinated changes in gene expression in response to infection by diverse viruses. The changes included both virus-general and virus-specific alterations in the expression of genes associated with distinct defense or stress response.

Torres and coworkers (Torres *et al.*, 2003) investigated the changes in transcription in leaves of *A. thaliana* challenged with strains of *P. syringae* pv. *tomato* DC3000 to allow for differentiation of basal resistance (*hrpA* mutants), gene-specific resistance (*RPM1*-specified interactions) , and susceptibility (wild-type pathogen). They observed that within the first 2 hours, host transcriptional changes were common to all the challenges, indicating that the Type III effector function did not contribute to early events in host transcriptome re-programming. *R*-gene specific transcripts were not observed until three hours after inoculation. Significantly, this induction occurred prior to the appearance of any macroscopic symptoms. Initial signs of tissue collapse were not observed until 5 hours post inoculation. They also observed three distinct transcriptional phases in the transition to induced defense responses. The authors found a threefold higher proportion of genes encoding proteins involved in translation machinery in Phase II than in Phase I. In the continued presence of the pathogen, genes with functions in transport and cellular organization increased proportionately 2.5 fold over Phase I. In Phase II, genes of unknown function decreased four fold relative to their representation in Phase I.

Using the Affymetrix GeneChip, researchers were able to study *Arabidopsis*

reponses to the bacterial pathogen *P. synringae* (Tao *et al.*, 2003). The interactions were compatible (virulent bacteria) or incompatible (avirulent bacteria), including nonhost interactions mediated by two different avirulence gene-resistance (*R*) combinations. The *Arabidopsis* GeneChip used in this study represented ~8000 genes, approximately one-third of the *Arabidopsis* genome. They had multiple probes per probe set to allow statistical validation of the hybridization data for a particular probe set using a single array. The authors found that the results for the incompatible interaction showed relatively small variation between two experiments, especially at 6 and 9 hours. By contrast, the results for the compatible interaction showed a fair amount of variation at the same time. They speculated that the major reason for this effect is that the behavior of the compatible interaction as a biological system is not as robust as that of the incompatible interaction. This would mean that the level of biological variation would depend heavily on an intrinsic characteristic of the biological system: the degree of robustness of the system.

As more data is accumulated on the genes involved in plant defense, the same "boutique arrays" may be used for studying responses, such as wound, induced systemic resistance, insect-specific, or pathogen specific due to the overlap in signaling pathways, and will be useful for fundamental and applied research. However, different arrays may be required for the study of genes involved in flower development or fruit development.

d. To the Future: Proteomics and Gene Silencing for Studying Defense Responses

Proteomics is a tool for the systematic identification of differentially expressed proteins or protein populations within a tissue, cell or sub-cellular compartment (Ramonell and Somerville, 2002). When combined with microarrays, proteomics provides a powerful device to indicate whether gene regulation is controlled transcriptionally, translationally or post-translationally. Proteomics involves the separation of proteins using two-dimensional gel electrophoresis, which are then excised from the gel. Mass spectroscopy is then used to identify the proteins by comparison of peptide masses to predicted peptides publicly

available in the database.

Over the past few years more and more investigators are applying proteomics to plant pathology, to gain insight into the early signaling events involved in plant perception of microbial and fungal elicitors. Two-dimensional electrophoresis and mass spectroscopy were utilized to identify proteins in *Arabidopsis* that were rapidly phosphorylated upon treatment with flagellin and chitin (Peck *et al.*, 2001). Peck and coworkers (Peck *et al.*, 2001) also showed that the chitin- and flagellin-induced phosphorylation was independent of SA and the presence of enhanced disease susceptibility 1 protein (EDS1), a putative lipase involved in defense signaling.

More recently, a study conducted by Cooper *et al.*, (2003) utilized two-dimensional electrophoresis along with high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to identify an unknown plant virus from infected plants. Protein extracts were first prepared from leaf tissue of uninfected tobacco plants, and the proteins were visualized with two-dimensional electrophoresis. Matching gels were then run using protein extracts of a tobacco plant infected with tobacco mosaic virus (TMV). After visual comparison, the protein spots that were differentially expressed in infected plant tissues were cut from the gels and analyzed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Tandem mass spectrometry data of individual peptides were searched with SEQUEST. Using this method, they successfully demonstrated that TMV proteins present in a total cell protein extract could be identified correctly. They then applied this strategy to tobacco plants infected with a laboratory viral isolate of unknown identity. Several of the differentially expressed proteins were identified as proteins of potato virus X (PVX), thus successfully identifying the causative agent of the uncharacterized viral infection.

Proteomics has also been applied to study proteins expressed during gibberellin controlled leaf-sheath elongation response (Shen *et al.*, 2003). Out of the 352 protein spots detected on 2-D PAGE, 32 proteins showed modulation in their expression levels in GA_3-

treated leaf-sheath for 48 hours as compared to the control; among them was calreticulin. Over-expression of calreticulin in rice inhibited the callus regeneration and seedling growth. From these results, they concluded that calreticulin was an important component in the GA signaling pathway that regulates rice seedling leaf-sheath elongation.

Scientists at the Noble Foundation in Ardmore, Oklahoma, are interested in application of proteomics. Most recently, they have surveyed six organ-/tissue-specific proteomes of *Medicago truncatula* (Watson *et al.*, 2003). They excised 551 proteins and identified 304 using peptide mass-fingerprinting and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The tissue-specific expression of proteins and the levels of identified proteins were compared with their related transcript abundance. It was estimated that approximately 50% of the protein levels appeared to correlate with their corresponding mRNA levels. These data will provide the basis for future proteome comparisons of genetic mutants and biotically-and abiotically-challenged plants.

Other examples include verification of identities of plant proteins in maize that cross-reacted with mammalian nitric oxide synthase antibodies (Butt *et al.*, 2003) and functional identification of a monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in *Arabidopsis thaliana* (Faldt *et al.*, 2003). (*E*)-β-Ocimene is one of the most commonly found monoterpenes of the volatile blends that are emitted from leaves in response to damage by herbivores. The gene encoding (*E*)-β-ocimene synthase was cloned and characterized using GC-MS analysis.

Plant proteomics has included the analyses of proteins isolated from the leaf blades of rice plants that were infected with the blast fungus and fertilized with various levels of nitrogen (Konishi *et al.*, 2001). It is known that rice plants grown with high levels of nitrogen nutrient are more susceptible to infection by blast fungus (Long *et al.*, 2000). Konishi and co-workers identified twelve proteins whose accumulation decreased with different levels of nitrogen nutrient. They also suggested that the twelve proteins might be involved in the incompatible interaction in rice plants infected with the blast fungus.

Mehta and Bomura-Rosato (2001) studied the protein profiles of leaf extracts from resistant and susceptible host plants that had been exposed to *Xanthomonas axonopodis* pv. *citri*. Five differentially expressed proteins were sequenced and putative identities assigned by homology searching.

Availability of full-genome arrays in conjunction with proteomics will aid in the study of pathogen responses in plants. This will lead to the development of plant pathology-specific databases, which will be used to integrate the array information and provide it in a public repository. In the future, genes discovered in these experiments may provide new insights into disease resistance in multiple crops.

Another emerging technique to enable investigators to identify genes of interest during host-pathogen interaction is virus-induced gene silencing (VIGS). When a plant virus infects a host cell it activates an RNA-based defense that is targeted against the viral genome. By analogy with RNA interference in animals it is thought that this mechanism involves processing of double stranded (ds) RNA into short interfering (si) RNAs. The dsRNA in virus-infected cells is thought to be the replication intermediate that causes the siRNA/RNase complex to target the viral single-stranded (ss) RNA (Lu *et al.*, 2003). During later stages of infection as the rate of viral replication increases, the viral dsRNA and siRNA become more abundant. Eventually, the viral ssRNA would be targeted intensively and virus accumulation slows down (Lu *et al.*, 2003). Many plant viruses encode proteins that are suppressors of this RNA silencing process (Brigneti *et al.*, 1998; Voinnet *et al.*, 1999). VIGS is a virus vector technology that exploits this RNA defense. Genes encoding metabolic enzymes have been targeted by VIGS. In one such example, the insert in a tobacco rattle virus (TRV) vector was from a gene (EDS1) that is required for N-mediated resistance to TMV. The virus vector-infected N-genotype plant exhibited compromised TMV resistance (Peart *et al.*, 2002). Most of the applications of VIGS have been in tobacco, but this method is now also being applied in other species. For example, it has been used in barley, *Arabidopsis*, tomato and *N. benthamiana* (Lu *et al.*, 2003).

Insertion of a transposon or T-DNA into a structural gene (whether into an exon or an intron) will disrupt gene expression completely and give a null mutation. This is commonly referred to as a 'knock-out'. Insertional mutagenesis helps target individual genes within a family of closely-related genes, so that the functions individual members can be investigated. To date, several hundred thousand T-DNA and transposon insertion lines have been generated for *Arabidopsis* (Bouche and Bouchez, 2001). In the next few years, it can be expected that these populations will cover the disruption of all *Arabidopsis* genes, and that the search for specific knockout mutants will be greatly facilitated by the use of knockout databases.

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CHAPTER II

A PURSUIT OF GENES ACTIVE IN DEFENSE OF UPLAND COTTON AGAINST BACTERIAL BLIGHT

ABSTRACT

Cotton is a cash crop grown worldwide and has many uses. Bacterial blight, caused by *Xanthomonas campestris* pv. *malvacearum (Xcm),* is a major disease of cotton. Overall damage to the cotton crop in the U.S. is only 1 to 2% due to cultivation of resistant varieties. Im216 is a pyramided cotton line, possessing several resistance genes. Expressed sequence tags (ESTs) were used to identify genes expressed during the cotton-*Xcm* interaction. We generated approximately 2000 ESTs from a suppression subtractive hybridization (SSH) cDNA library prepared from upland cotton Im216 foliage leaves infected with *Xcm.* These ESTs were composed of a total of 161 unique non-redundant contiguous sequences, consisting of 110 (68%) with similarity to sequences submitted to Genbank and 51 (32%) with no homology to sequences in GenBank. They were classified into seven categories according to their putative functions. Amplified cDNA products of these non-redundant sequences were arrayed onto glass slides. An additional 30 clones whose single-pass sequences were not good were also arrayed, as they might be new transcripts worth adding to the Im216 database. These microarrays were used to analyse the time course of expression of the corresponding genes in Im216 leaves following *Xcm* inoculation. Expression profiles revealed 83% were induced 2-fold at 8 hpi. At 14 hpi, 88% displayed a 2-fold induction, while at 20 and 30 hpi, 86% and 92% of the genes were induced 2-fold respectively. Interestingly, at later time points, 45 hpi and 60 hpi, ~88% and ~96% of the genes on the array were induced 2-fold. Seventeen percent, 12%, 14%, 8%, 11% and 4% of the arrayed genes were less than 2-fold induced at 8, 14, 20, 30, 45 and 60 hpi respectively. Using K-means clustering, the genes were grouped according to their expression profiles.

Additional keywords: bacterial blight, upland cotton, Xcm, SSH, expression profiling

INTRODUCTION

Bacterial blight of cotton is an economically important, world-wide disease caused by the bacterium *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (*Xcm*). Symptoms of bacterial blight of cotton include water-soaked lesions. The disease is common in the semi-humid and humid areas of the world.

Plants contain numerous genes encoding defense-related proteins. These include resistance genes involved in gene-for-gene interactions as demonstrated by Flor (1971). This interaction leads to hypersensitive cell death and upregulation of various defense genes such as those encoding enzymes involved in the generation of phytoalexins, pathogenesis-related proteins, the enzymes of oxidative stress, signal transduction proteins, and enzymes involved in tissue repair and lignification. Many of these genes are activated both when the host plant is challenged by a pathogen and when it is attacked by herbivores.

De Feyter and Gabriel demonstrated in cotton that four avirulence (*avr*) genes of *Xcm* ($avrB_4$, $avrb_6$, $avrb_7$ and $avrB_{In}$) interacted in a gene-for-gene manner with the corresponding host plant resistance genes, as well as two other *avr* genes (*avrB101* and *avrB102*) that interacted with more than one resistance gene (De Feyter and Gabriel, 1991; De Feyter *et al.*, 1993). The latter indicated a possible exception to the gene-for-gene hypothesis (De Feyter *et al.*, 1993).

An economical way of controlling bacterial blight in cotton was to develop resistant plants through breeding. Bird and Brinkerhoff developed resistant varieties of *Gossypium hirsutum* by crossing lines carrying several resistance genes (Bird, 1982; Brinkerhoff *et al.*, 1984). Brinkerhoff developed the resistant cotton line Im216 in Oklahoma (Brinkerhoff *et al.*, 1984). The pedigree of Im216 includes several resistance genes including B_2, B_3, b_7, and the polygenic complex B_{Sm}. Immunity in Im216 is inherited as a completely dominant trait (Brinkerhoff and Verhalen, 1976).

The role of phytoalexins in resistance of cotton to pathogens has been well documented (Pierce *et al.*, 1996; Mace *et al.*, 1985). A key enzyme in the terpenoid

biosynthesis pathway in cotton is the sesquiterpene cyclase, (+)-δ-cadinene synthase (Benedict *et al.*, 1995; Davis *et al.*, 1996). Chen and colleagues (Chen *et al.*, 1995; Chen *et al.*, 1996) cloned, expressed and characterized (+)-δ-cadinene synthase from *Gossypium arboreum* suspension culture cells. Qualitative RT-PCR using *G. hirsutum* inoculated with *Xcm* in the cotyledons has shown an induction of both *cdn1*-A and *cdn1*-C transcripts to occur by 24 hours post-inoculation (hpi), while mock-inoculated controls showed no accumulation of these transcripts (Davis, 1998). These data were consistent with the observed CDN1 activity time course, which revealed the greatest rate of increase of cyclase activity in *Xcm*-inoculated cotyledons to occur between 24 and 36 hpi (Davis, 1998). (+)-δ-Cadinene synthase activity has also been detected in Im216 at 12 to 84 hpi (Davis, Romero, McCollough, unpublished results), and in WbM*gl* it was observed to increase from 18 to 60 hpi (Davis *et al.*, 1996).

Several time course studies have shown correlations between phytoalexin production, appearance of the fluorescent cells in which they accumulate, and inhibition of bacterial multiplication. Sesquiterpene phenols 2,7-dihydroxycadalene (DHC) and lacinilene C (LC) and their 7-methyl ethers have been detected in inoculated resistant cotton leaves and cotyledons (Essenberg *et al.*, 1982, Abraham *et al.*, 1999). It has also been observed that in leaves of resistant cotton lines, multiplication of each *Xcm* colony is inhibited by a local resistance response, during which the mesophyll cells closest to the bacterial colony collapse and turn brown (Essenberg *et al.*, 1979). It was also observed that phytoalexins are localized in the HR cells of resistant cotton leaves, which exhibit green fluorescence of lacinilene C (LC) and lacinilene C 7-methyl ether (LCME) (Essenberg *et al.*, 1992). This observation indicated that if they play a role in resistance by inhibiting growth and cell division, the phytoalexins accumulate close to the site of their action.

A study of *Xcm*-inoculated OK1.2 cotyledons showed that 45-90 hpi was the period of most rapid increase in fluorescent cell numbers, and phytoalexin accumulation peaked during this period while the rate of bacterial multiplication was declining (Gorski *et al.*,

1995). A sharp rise in phytoalexin levels 24 to 48 hpi in Im216 cotyledons was also observed by Grover and Essenberg (unpublished results). Fifty percent of infection sites in Im216 have been shown to respond hypersensitively by day three, while all sites showed a reaction by day four (Pierce *et al.*, 1996). Therefore, sample times for this study were taken at times preceding and in the early phases of the various observations cited earlier to ensure that all the times of induction of the various defense responses were covered.

In addition to the above mentioned cyclase, other defense or stress related genes have been identified in cotton. These include a lipoxygenase (Jalloul *et al.*, 2002), bacteria-induced peroxidase (Delannoy, E., unpublished results), alcohol dehydrogenases (Millar *et al.*, 1994; Millar and Dennis, 1996), PR-10 proteins (Zhou *et al.*, 2002; McFadden *et al.*, 2001), chitinases and β,1, 3- glucanases (McFadden *et al.*, 2001). Most of these have been isolated from *V. dahliae*-challenged cotton species (except for the peroxidase). Therefore, this is one of the first studies undertaken to investigate genes induced by *Xcm* in cotton.

Suppression subtractive hybridization (SSH) is a procedure that enables the comparison of two mRNA populations and isolation of differentially expressed transcripts. SSH has been widely used in plants to identify various genes of interest. SSH has been utilized to identify genes in *Medicago truncatula* that are regulated by an arbuscular mycorrhiza (Wulf *et al.*, 2003). Luo and his coworkers have used it to study resistance of wheat to powdery mildew (Luo *et al.*, 2002). Microarrays have become a popular method for identifying gene sets expressed by organisms under different environments. They have the advantage of producing expression data for hundreds and thousands of genes in the target organism, and no prior knowledge of the genes or their regulation is required. Microarrays, coupled with the SSH protocol, represent an appealing approach to identifying plant gene sets that are induced when challenged by a pathogen.

In this study, we have used SSH to identify differentially expressed transcripts in Im216 that are induced by the bacterium *Xanthomonas campestris* pv. *malvacearum* (*Xcm*). We have used cDNA microarrays to examine the abundance and expression

changes of 192 Im216 transcripts over a time-course. Our results demonstrate the presence of potential networks of regulatory interactions between genes in response to the pathogen. This study provides some insights into the genes induced in upland cotton when challenged by *Xcm* and whether these genes are similar to defense related genes isolated from other plant species.

RESULTS

Im216 SSH cDNA library construction

Im216 leaves spot-inoculated with a widely avirulent, race 1 strain 3631 of *Xcm* inoculum (5 X 10^6 cfu/mL) displayed a hypersensitive resistant response after five days (Figure 2-1). No tissue collapse was observed and red anthocyanin was observed in the adaxial epidermis. To capture a wide spectrum of differentially expressed genes, leaf tissue from ~ five-week-old plants, was collected at different time intervals after spray-infiltration with *Xcm*, and simultaneously, leaf tissue was harvested from control (non-inoculated) plants. Leaves were harvested at 8, 14, 20, 30, 45 and 60 hours after inoculation.

We prepared an SSH cDNA library using pooled mRNA from *Xcm*-infected Im216 leaves as the tester and pooled mRNA from non-infected Im216 leaves as the driver, and isolated 2337 clones. Initially 138 clones were subjected to single-pass sequencing (from the 5′ end of the vector's cloning site). Trace files of the sequences were processed using PipeOnline (POL, Ayoubi *et al.*, 2002) and a database containing contiguous sequences (contigs) was assembled. Contigs sequenced seven times or more were selected for making probes for a redundant screen (Figure 2-2). Clones with a signal intensity equal to or above that of the lowest positive control printed on the membrane (75 ng cDNA identical to the probe) were eliminated. Adaptors (same as those used during the SSH procedure) and their complements were added to the prehybridization solution to prevent cross-hybridization of the adaptor sequences. Preliminary experiments were also performed by hybridizing probes to the vector and adaptors printed on the membranes (results not shown). These

experiments demonstrated that the signals observed by chemiluminescent detection were from the probe hybridizing to its complement on the membrane and not due to cross-hybridization from the adaptors or vector. However, we did observe some cross-hybridization between clones. Fourteen and a half percent of the clones in the library were eliminated using P4-A10 (putative senescence associated protein) exclusively as a probe (Figure 2-3). Clones P4-F9 (putative PR protein R major precursor) and P6-H10 (putative PR protein) eliminated *ca.* 10% and 3% of the clones respectively in the library (Figure 2-3). It was also observed, that in spite of the redundant screening, 7% of the library still constituted clone P4-A10 (putative senescence associated protein). Hence we observe that a few genes account for a large percent of the SSH library.

Two thousand one hundred and ninety-nine clones were screened using the nine highly redundant clones. One thousand one hundred and fifty-seven (53%) of these clones were sent for single-pass sequencing. Trace files submitted to POL yielded a total of 161 unique non-redundant contigs, consisting of 110 (68%) contigs with similarity to sequences in Genbank and 51 (32%) contigs with no homology to sequences in GenBank. The sequences of these contigs are possibly novel, i.e. genes not found in other species. We did not set a cut off value for claiming similarity. The higher the high score pair value (HSP), the more significant the similarity. Most of the sequences in our library (except those with no protein alignment) had HSP values ≥ 100 (Table 1).

In order to check whether genes isolated in the SSH library were truly differential, we performed 13 northern blot analyses using pooled total RNA (Figure 2-4). Clones were picked for making RNA probes based on their putative functions, as these were of interest to the investigators (Table 2). Of the 13 clones picked, nine (69%) were differential, while four transcripts were found to be expressed equally in *Xcm*-infected tissue and in control tissue.

Functional annotations

Sequences of transcripts in the database were grouped into eight categories (Figure 2-5) according to Schenk and coworkers (Schenk *et al.*, 2000). The two largest categories were genes involved in antimicrobial activity (30%) and sequences with no homology to any sequence in GenBank (30%). Thirteen percent were involved in oxidative burst/stress or apoptosis, and ten percent in cell maintenance and plant development. Only small percentages of the genes in the SSH library were found to be involved in signal transduction (4%) and in hormone production (3%). Six percent of the clones were genes in GenBank whose function is still unclear.

Manufacture of Im216 SSH microarrays

From each contig, a single clone that fitted the following criteria was selected to be amplified and printed on the array: longest insert sequence, lowest percentage of N's (no definite base assigned, resulting from poor quality of sequencing), and highest %Phred 20 score (the percentage of bases in a sequence with PHRED values greater than or equal to 20). The clone was aligned with sequences from the database using the MyBlast feature in POL. If the best-quality sequence was ≥90% as long as the longest clone in that contig, it was considered a good candidate for PCR. All 161 unique non-redundant clones were PCR-amplified as described in the materials and method section. PCR products were checked for quality and quantitated by electrophoresis (Figure 2-6). In addition, 30 clones whose single-pass sequences were not good were also PCR amplified and electrophoresed, as they may be new transcripts worth adding to the Im216 database. If the band of interest was not amplified successfully, PCR was repeated; if the PCR still did not yield good product, another candidate clone from that contig was picked.

To obtain good probes on the glass slides for hybridization, we tested various printing buffers such as 50% DMSO, 3X SSC and TeleChem proprietary printing buffer. 3X SSC was the printing buffer of choice, since it gave us good compact spots (features).

Probes printed in DMSO and the TeleChem proprietary buffer gave larger features than those printed using 3X SSC. Probes were printed twelve times within a slide (technical replicates; Appendix B; Section L). From preliminary experiments we decided to hybridize the arrays at 42°C instead of 37°C or 47°C, since (1) at this temperature we obtained the best signal intensities from the probes on the array, (2) cross-hybridization between the cotton probes and spiking controls was seen at 37°C, and (3) loss of lowly expressed genes was observed at 47°C. Other genes identified in cotton by Kent Chapman, Thea Wilkins and Michel Nicole were given to the investigators and printed on the array. These are additional genes whose expression profiles are of interest to us and were not isolated during the SSH and screening procedure. Vector sequences of the clones on the array, GUS and GFP clones (given by Jean-Marie Verchot), and 3X SSC were printed as negative controls and did not cross-hybridize with Im216 targets in a preliminary experiment, thereby confirming that signal intensities being measured were from the cotton probes.

Intensities of hybridization with targets from the same *Xcm*-infected total RNA (self versus self) labeled with Cy 5 and Cy 3 were compared using a ratio intensity (R-I) plot (Figure 2-7). Six features with $\log_2$ (ratio) greater than 1 or less than –1 were identified, all of which were below the range of the bulk of the signal intensities. There are 2,568 features (214 features replicated 12 times on the slide) on the plot. No spikes were used for this experiment and global normalization was applied.

Experiments were also conducted using just the spiking controls (Sp1, Sp3, Sp4 and Sp5 at 0.45 ng, 0.05 ng, 0.15 ng and 0.015 ng, respectively) from the Arabidopsis Functional Genomics Consortium (AFGC) with filler RNA (yeast t-RNA) to check for cross-hybridization between cotton probes and spiking controls. We decided to use Sp4 and Sp5 for data normalization, as these two clones hybridized the least with the cotton probes. The same cotton probes hybridized to the individual spikes at different intensities. Some of the spikes on the array also hybridized to the spike in the RNA mixture; Sp 6 was found to cross-hybridize the most. Most of the signal from cross-hybridization for Sp 4

and Sp 5 came from the Cy 3 channel, so we conducted an experiment using just the Cy 3 dendrimer and found that it did somehow bind to the cotton probes on the array. Fortunately, this signal was not so high that it biased the Cy 3 data.

Differential gene expression in response to *Xcm*

Im216 is a highly resistant cotton line. To better understand its resistance response, gene expression profiles were studied with three biological replicates. To investigate differences in gene expression between an Im216 non-infected plant and an Im216 *Xcm*-infected plant, targets were prepared from the two treatments at six different time points post-inoculation. The background corrected and normalized signal intensities for the three biological replicates and twelve technical replicates per slide for each time point were averaged. Changes in gene expression were observed (Figure 2-8). By inspecting the $\log_2$(ratios) that were more than +1.0 and less than –1.0 , we observed that 83% were induced 2-fold at 8 hpi. At 14 hpi, 88% displayed a 2-fold induction, while at 20 and 30 hpi, 86% and 92% of the genes were induced 2-fold respectively. Interestingly, at the later time points, 45 hpi and 60 hpi, ~88% and ~96% of the genes on the array were induced two fold. Seventeen percent, 12%, 14%, 8%, 11% and 4% of the arrayed genes were less than 2-fold induced at 8, 14, 20, 30, 45 and 60 hpi respectively.

GenePix Pro AutoProcessor (GPAP) software (http://darwin.biochem.okstate.edu /gp/) (Weng and Ayoubi, 2003) was used to analyse the microarray data. Its algorithm calculates the $\log_2$(ratio) value for each spot on the slide, sums up for the number of replicate spots on a slide for a particular gene (12 on our slides), and divides by that number. GPAP allows one to upload up to 4 data files that are generated by the GenePix Pro results ("gpr"). The data from this work were analysed two ways; one by uploading data for each biological replicate and time point separately, and then by merging three "gpr" files from the three biological replicates for a given time point. The data obtained from the three individual biological replicate time points were used to compare the

reproducibility of the data among biological replicates.

There are many algorithms to cluster sample data based on nearness or a similarity measure, such as K-means, fuzzy C-means, self-organizing maps, hierarchical Euclidean-distance-based and correlation-based clustering (Dougherty *et al*. 2002). In the K-means algorithm, each datum is placed into a specific cluster during iteration, and the means are updated based on the classified samples (Dougherty *et al*. 2002). Given a set S of n data, those data are to be placed into k clusters with k means $m1$, $m2$, $m3$, ... mk. The genes expressed at various time points were clustered into five groups according to their expression profiles using K-means clustering (Figure 2-8).

Many of the genes in Cluster 1 (Figures 2-8A and 9A), are genes that we expect to be up-regulated in Im216 after being challenged by *Xcm.* Putative identities of some genes in this cluster are: a bacteria induced peroxidase (P4-C8; Table 1), a sensescence associated protein (1-C8; Table 1), putative 1,3-β-glucanase precursor (P21-F8; Table 1) and PR 1 a and b precursor proteins (P17-B3 and P19-F6; Table 1). Interestingly, a putative cytochrome P450 (P1-C4; Table 1) as well as P450 #64 characterized from cotton previously (X-Y Chen's group, China) are clustered together.

Some genes in cluster 2 (Figure 2-8B) are more highly induced at 8 hpi than the genes in cluster 1 while others have the same fold induction at 8 hpi as genes in cluster 1. Putative identities of examples of genes in cluster 2 are: a gene encoding for signal transduction and a protein kinase. Comparing the expression profiles of *cdn1*-C (Figure 2-9B), previously isolated by Davis (Davis *et al.*, 1996) and a (+)-δ-cadinene-8-hydroxylase (P450 #132; Figure 2-9C), previously characterized in cotton (Luo *et al.*, 2001), we observe two different patterns. *Cdn1*-C is observed to be induced almost 6-fold at 8 hpi compared to the (+)-δ-cadinene-8-hydroxylase, which is induced *ca.* 3-fold at the same time point. The (+)-δ-cadinene-8-hydroxylase also appears to peak twice during the time course, once at 14 hpi and again at 30 hpi. In contrast, *cdn1*-C shows a decrease in its induction at 14 hpi and then steadily rises after 20 hpi. Its induction levels appear not to increase very much after

30 hpi. Expression profiles for all three biological replicates for these two genes agree well with each other, therefore the patterns we observe are real for these genes.

Cluster 3 (Figures 2- 8C & 9D) consists of 30 genes, putative identities of which include dicyanin, putative cold-inducible protein, *cdn1*-A (Davis *et al.*, 1996), senescence associated protein, as well P450 #125 (Chen, unpublished). A lipid transfer protein and a PR protein, osmotin, also fall into this cluster. A plot of just one gene in all three biological replicates (Figure 2-9D) shows a peak at 14 hpi. It is possible that, since the hierarchical clustering was performed using data obtained by averaging $\log_2$(ratio) signal intensities from all three replicates, the pattern observed for *cdn1*-A is not seen in figure 2-8C.

Cluster 4 (Figures 2-8D) is the largest, consisting of 62 genes. Genes in this cluster have similarities to known defense genes such as another putative P450, an oxidase, class II chitinase, as well as genes that encode for proteins whose function in defense is unclear, such as RNA-binding protein, 60 S ribosomal protein L21 and a transcript antisense to rRNA. The hierarchical clustering shows the genes to have the same fold induction as genes in clusters 1 and 2, with some genes being induced at a greater degree at 30 hpi. Another bacteria-induced peroxidase (P21-F11; Table 1) from the Im216 library is in this cluster. A putative PR class 10 protein (P16-F5) (Figure 2-9E) has a lower degree of induction at 8 hpi, peaks at 14 hpi and then increases again at 30 hpi, where it plateaus out by 60 hpi, a pattern also observed in *cdn1*-A (Figure 2-9D). This indicates that genes in clusters 2, 3 and 4 all have similar patterns of induction. Reducing the number of clusters may group all these genes into one cluster.

The last cluster of genes, cluster 5 (Figures 2-8E & 9F), is those genes whose transcripts are hardly induced above control values until ~ 20 hpi and then show a slight increase after that. It contains genes such as a putative cytokinin binding protein that is down-regulated until 20 hpi and is slightly induced at 60 hpi, and carbonic anhydrase (given by K. Chapman) that is down-regulated after infection. A putative jasmonate induced protein (Figure 2-9F) is induced slightly at 8 hpi and after 14 hpi, its induction level drops

and the gene appears to be induced again after 20 hpi, and continues to rise at 60 hpi.

In order to make a correlation between the mRNA transcript level induction as determined by northern blot analysis and microarray results, two clones, P1-C4 (putative cytochrome P450) and P5-D7 (putative 1, 3-β glucanase precursor) were picked for northern blot analysis. RNA preparations from the first biological replicate were used for the northern blots. P1-C4 transcripts were observed to be induced at 30 hpi, with increasing levels at 45 and 60 hpi in *Xcm*-inoculated leaves (Figure 2-10). This agrees somewhat with the expression profile obtained from microarray data (Figure 2-11A), except that we observe a slight drop in induction levels at 60 hpi. In the case of P5-D7 microarray data, we observe the dip at 20 hrs, both in the array experiment, as well as in the northern blot, and that the dip is more dramatic in the case of the latter (Figure 2-11B). Induction levels of the gene in both experiments agree somewhat at 45 and 60 hpi. The northern analysis was just a preliminary experiment done once, and the quality of RNA is suspect. These results will need to be confirmed by additional time course northern blots. We can also pick a few other genes and compare their microarray gene profiles with the profiles obtained from northern blots.

Total RNA from the second biological replicate was electrophoresed and blotted onto a nylon membrane and then stained with methylene blue (Figure 2-12A) to see the integrity of the sample, while total RNA from the third biological replicate was electrophoresed and stained with ethidium bromide and observed under ultra-violet light (Figure 2-12B). By comparing the banding pattern in the northern blot of sample P5-D7 (Figure 2-10) and that of the methylene blue stained membrane (Figure 2-12A), we conclude that both of those preparations were partially degraded, whereas the total RNA from the third biological replicate was of the best quality as determined by the compactness of the rRNA bands.

DISCUSSION

The redundant screening was a sensible procedure to follow as it helped to eliminate *ca.* 50% of the SSH library prior to sequencing, hence being cost effective. It also demonstrated that the normalization step during the SSH procedure was not optimal. It can also be observed that a large number of clones from the library hybridized to the nine clones used during the redundant screening (Figure 2-3).

Nine transcripts that were observed to be differentially expressed in the northern blots (Figure 2-4A) were also found to be induced in the microarray experiments. However, four of the transcripts found to be constitutive in the northern blots (Figure 2-4B) were found to be induced in the microarray experiment. Once induced at 8 hpi, the levels of induction for 1-E2, P3-F6 and P3-H10 do not change very much (Figures 2-13 A, B and C). However, P6-D10 (represented by clone P5-D9 in Table 1) is not induced at 8 hpi, but steadily rose at later time points (Figure 2-13D).

For the self versus self experiment (Figure 2-7), we did not use any spiking controls, but instead applied global normalization to the data. If some of the probes on the array were biased toward one of the dyes or the other, we would see more data above the 1.0 and/or below the –1.0 mark (Figure 2-7). Since most of our data lie within this range and the signal intensities were high, we know that the probes are not biased. The data observed to lie outside the range are individual features and were not regarded during data analysis as they are of a lower intensity; the higher data outside the specified range were regarded as artifactual signal and also eliminated (flagged as bad) during data analyses.

In our SSH library, the largest category of identified sequences consisted of genes encoding proteins with antimicrobial activity such as pathogenesis-related (PR) proteins. The PR proteins were mainly from the family 1 PR proteins, family 2 PR proteins, family 5 PR proteins, and family 10 PR proteins. The putative PR 10 class gene isolated from the Im216 SSH library (P18-A12; Table 1) had high homology (HSP value =824) with a PR 10 protein previously isolated from cotton stems in response to infection by *Verticillium*

dahliae (McFadden *et al.*, 2001). They found induction from 9 hpi to 11 dpi in *Verticillium*-inoculated CS50 and Sicala V2 cotton stems. No evidence for induction of the mRNA was found in samples from mock-inoculated or chemically treated plants. We see P18-A12 (Table 1) to be induced *ca.* 8-fold at 8 hpi (Figure 2-14A). This is not surprising as it is known that PR proteins are generally induced in response to pathogen attack and are not pathogen specific. In the same study, investigators studied the transcript expression of 1,3-β-glucanase, and basic and acidic chitinases. They found induction of an mRNA (RT-PCR analysis) for 1,3-β-glucanase from 24 hpi to eight days post inoculation, but not at the earlier times of 9 and 12 hpi.

Three genes (P5-D7, P21-F8, P24-C10; Table 1) isolated from the library encoded 1,3-β-glucanase precursor proteins that have some identity (HSP value = 483, 339 and 556 respectively) with 1,3-β-glucanase previously isolated from *G. hirsutum* (Hudspeth *et al.*, 1996). They used PCR amplification to obtain a probe for the glucanase. Primers were designed based on conserved regions derived from a 1,3-β-glucanase obtained from soybean. Since *Xcm* has no 1,3-β-glucan, it is possible that the putative1,3-β-glucanase precursors isolated from our SSH library are a part of a generalized defense response. Interestingly, these two putative 1 ,3-β-glucanase precursors have different expression profiles (Figures 2-14 B and C). In both cases, biological replicates 1 and 2 agreed, whereas biological replicate 3 had a different profile. Analyses of proteins associated with SAR (SAR proteins) suggest that many of them belong to the class of PR proteins (Ryals *et al.*, 1996). Interestingly though, in the study done by Hudspeth, not much change was seen in the transcript levels of the 1, 3-β-glucanase in one-month-old plants sprayed with SA.

Thirteen percent of the genes in our library encode putative proteins with functions in oxidative burst, stress responses, and apoptosis. Two putative bacteria-induced peroxidases were found in the library, one (gene) of which has been isolated by the Nicole group in France (unpublished results). This is expected, since peroxidases have been identified in other plant-pathogen interactions such as wheat infected with *Erysiphe*

graminis (Rebmann *et al.*, 1991) and rice seedlings infiltrated with a non host pathogen *P. syringae* pv. *syringae* (Smith and Metraux, 1991). Peroxidases are known to catalyze a number of reactions that fortify plant cell walls. These reactions include the incorporation of phenolics into cell walls and lignification of the plant cell walls. This is an important process of the plant in defending itself against pathogen attack (Kolattukudy *et al.*, 1992). Interestingly, the expression profiles of the two peroxidases are not present in the same K-means cluster. One peroxidase (clone P4-C8) belongs to cluster 1, where transcript expression rises sharply from 14 to 20 hpi, then rises more gradually to 60 hpi (Figure 2-9A). The second bacteria-induced peroxidase (P21-F11, Figure 2-14D) is clustered in group 4. No consensus pattern is observed for this gene, except that data from biological replicates 1 and 2 agree after 20 hpi. It is possible that these two peroxidases (P4-C8 and P21-F11) may be isozymes behaving differently and are probably involved in the lignification of the cell wall.

From the above data (Figure 2-14), it seems that biological replicates 1 and 2 demonstrated expression patterns similar to each other but different from biological replicate 3. Biological replicates 1 and 2 are from two different growth chambers (though of similar size; 15 square feet), but both sets of plants used in this study were grown and harvested in Summer 2002. Biological replicate 3 samples are from plants grown and harvested in Summer 2003, using a larger growth chamber (30 square feet) than the ones used a year earlier. Larger growth chambers have a larger air space and may result in less humidity. Lower humidity in biological replicate 3 may have contributed to the difference in induction patterns for these particular genes. Light intensities in growth chambers used for plants grown for biological replicate 1 and 2 were *ca.* 475 nmol $m^{-2}s^{-1}$, whereas the light intensity of the chamber used for biological replicate 3 was *ca.* 284 nmol $m^{-2}s^{-1}$. It is possible that the difference in light intensities could cause a difference in the expression profile. Plants used for all three replicate experiments were approximately the same age. Kawasaki and coworkers observed higher variability in repeat experiments using RNA from

root tissues exposed to the same experimental conditions and harvested over a period of a year (Kawasaki *et al.*, 2001). However, some genes behaved in a similar pattern in all of our three biological replicates (Figure 2-9Ato F). Another reason for the variability we observed could be due to the differences in RNA quality.

Though some genes (4%) involved in defensive secondary metabolism were isolated from the library, previously identified *cdn1*-A, *cdn1*-C and *FPS* were not among the genes isolated. *Cdn1*-A and *cdn1*-C were both printed on the array and hybrization signals were obtained from both genes. *Cdn1*-A and *cdn1*-C were grouped separately in clusters 3 and 2, respectively. Both genes gave robust signals (Figures 2-9B & D), suggesting that they are present in Im216 and induced when the plant is challenged by *Xcm*, but were just not isolated by the SSH technique. Induction of these mRNA transcripts was seen in WbM*gl* cotyledons 24 hpi with *Xcm* (Davis, 1998), but in our microarray experiments we see them induced as early as 8 hours. Im216 is the most resistant line we know of and it contains a number of *B* genes, therefore it is possible that the induction is quicker in Im216 than in WbM*gl* when challenged by the pathogen. P450s #64, #125 and #132 identified earlier in cotton (Luo *et al.*, 2001) were all induced as early as 8 hpi (data not shown for #s 64 and 125). The (+)-δ-cadinene 8-hydoxylase (P450 # 132) has a similar expression profile to that of *cdn1*-C after 20 hpi (Figure 2-15). It was also observed that *cdn1*-A and *cdn1*-C have different expression profiles. These expression profiles were reproducible in all three biological replicates (Figures 2-9B and D).

Alcohol dehydrogenases (ADH) are induced by anaerobic stress in various plants. Alcohol dehydrogenases have been previously isolated from anaerobically stressed root tips of cotton plants (Millar and Dennis, 1996). Nine of the twelve cDNAs they isolated fell into one class while each of the other three cDNAs fell into separate classes. A putative alcohol dehydrogenase gene (P18-F2; Table 1) is present in the SSH library. Examination of its expression profile (Figures 2-8D and 2-14E) reveals that it belongs to cluster 4 and is induced more at 14 and 45 hpi, in biological replicates 1 and 2. The data at each time point

are fairly similar for biological replicates 1 and 2. This agrees somewhat with studies performed in roots and shoots of five-day old etiolated seedlings of *G. hirsutum* cv, Siokra (Millar *et al.*, 1994). To determine whether ADH enzyme activity was anaerobically inducible, the authors subjected 5-day old etiolated seedlings of Siokra to anaerobic conditions, and the level of ADH activity was determined in shoots and roots. In both, a linear relationship was observed between the increase in specific activity of ADH and the duration of the stress for the first 48 hours of anaerobiosis, at which time the ADH activity level peaked. After 72 hours of anaerobic conditions, the tissue of the seedlings began to dry, suggesting that they were beginning to die, thus explaining why ADH levels began to decline. The fact that this group studied the enzyme activity whereas we are studying transcript levels, which are not necessarily translated into protein, could lead to the decrepancies in correlation of the two data. Also, the fact that all three biological replicates do not agree means that we will have to confirm our microarray data further by northern blots analyses and/or further microarray analysis.

Several of the genes in the Im216 library have been identified during stress in other plant species such as *A. thaliana* (Seki, 2001; Reymond *et al.*, 2000; Santos *et al.*, 2003), rice (Kawasaki *et al.*, 2001), and tomato (Torres *et al.*, 2003; Mysore *et al.*, 2002), suggesting that some of the same genes are induced during plant defense mechanisms irrespective of the host or pathogen, with differences being the times at which the genes are induced and the fold-inductions. PR proteins have been isolated after challenge from other plants such as tobacco, potato and sorghum (Payne *et al.*, 1990; Matton *et al.*, 1989; Lo *et al.*, 1999). In this study, we found genes for putative PR-proteins are turned on as early as 8 hpi, though they have various expression profiles. This suggests that they may all be components of similar defense-related mechanisms.

The microarray experiment was conducted using targets from three different biological replicates on the same arrayed set of probes at the same hours post inoculation. It was observed that across the three replicates at 8 hpi (Figure 2-16A), the induction levels

are spread from lowly induced genes to some highly induced genes, and therefore the signal is spread out along a straight line. In contrast, at 60 hpi (Figure 2-16F), more genes were highly induced and are clustered. Also, the percentage of genes up-regulated increased as the time points increased, suggesting more genes take part in defense processes occurring as late as 60 hours. The dotted line on the figure explains the ideal identical data from two replicates. This implies that maybe the response is more predictable at 8 hours than at 60 hours.

Fold induction of the genes in this work ranged from *ca.* two-fold to *ca.* 16-fold across the various time points selected. In some cases (for example, genes in cluster 1; Figure 2-8A), the induction appears to get higher and higher and we would have been able to learn for how long this trend continued if we continued the study to later time points.

Standard error is used to compare a sample mean to the distribution of possible means. Table 3 is a list of all the valid cDNAs on the arrays along with their standard error values for each biological replicate at each time point. The standard error was calculated by dividing the standard deviation of $\log_2$(ratio) by the square root of the number of replicates for each array (i.e. $\sqrt{12}$). This lets us know how reliable our data are for a given cDNA. All the standard error values are below 1.0, and most are below 0.1 (about 22% of the valid cDNAs have standard error values above 0.1) indicating that the data are reliable.

The coefficient of variation (CV) of replicate spots expresses the standard deviation of a set of data as a proportion of its mean. It can be expressed as a percentage. When the CV is small, the data scatter compared to the mean is small. When the CV is large compared to the mean, the amount of variation is large. Table 4 is a summary of the number of cDNAs in this study that fall within CV%$\log_2$(ratio) ranges; <10%, 10-25%, 25-50%, 50-100% and >100%. Approximately 80% of the valid cDNAs on the array have a CV%$\log_2$(ratio) value between 0- 25%. From this one can infer that the data scatter is small in this study.

This study aimed at identifying genes that are involved during the cotton-*Xcm*

interaction. Many genes have already been identified in other plant species, but there is also a large portion of the SSH library (30%) containing genes with no similarity to previously reported genes. These are interesting, as they may be novel. Cyclases previously known to be induced by *Xcm* in cotton line WbM*gl* (Davis, 1998) were not identified in the library. It would be possible to make specific primers for genes of interest, such as the ADHs and PR proteins. It is possible to isolate the full length clone by 5' and 3' RACE and express the gene of interest and characterize the resulting protein. There may be many more genes involved in defense pathways in cotton, and in order to get these, one can go back and repeat the SSH procedure and mine for more interesting genes. Hence, this study opens up the avenue for more interesting research.

Four near-isogenic lines have been reported in the literature that are homozygous for the B_2, B_4, B_{In} and b_7 genes and were derived using at least six backcrosses to the parent 'Acala 44', followed by single plant-progeny row selection for uniformity (Essenberg *et al.*, 2002). Two other near-isogenic lines, AcB_5 and AcB_6 also exist (Essenberg *et al.*, unpublished). B_2 is not race-specific and is weakly resistant, B_4 is very specific, and strongly resistant, B_{5A} is not race specific and highly resistant, B_6 is specific and very weakly resistant, while B_{In} and b_7 have intermediate levels of resistance and high specificity. By screening the Im216 SSH array with RNA-derived targets made from these near-isogenic lines, it will be interesting to study the different responses conditioned by the resistant genes. It will also be interesting to see if the genes are induced at earlier time points (quicker response to challenge) in Im216 than the near-isogenic single-*B*-gene lines. We would also be able to observe if the differences in responses triggered by *B* genes individually will shed light on resistance mechanisms that are *B* gene specific.

MATERIALS AND METHODS

Plant and bacterial material for SSH library

Delinted Im216 seeds coated with a mixture containing three-parts Arasan 70-S and

ten parts Demosan 65 W to prevent the seeds from rotting, were grown in Jiffy-Mix Plus (Park Seed Company) in a Conviron E15 growth chamber as described by Pierce and associates (Pierce *et al.*, 1993). Bacterial cultures of *Xcm* strain 3631 were subcultured from frozen glycerol stocks in nutrient broth medium and incubated at 30°C with agitation (300 rpm) overnight. The next day, dilutions were made to ensure that the bacteria were growing in the logarithmic phase. *Xcm* 3631 was the pathogen of choice as it is known to trigger a vigorous hypersensitive response in resistant cultivars of cotton (Pierce *et al.*, 1993).

Im216 inoculations and RNA isolations for SSH library

Four- to five-week-old plants were spray infiltrated (Chapin Yard Sprayer Plus) with an *Xcm* suspension at a concentration of $5x10^6$ cfu/mL. This inoculum concentration is high enough to elicit an HR from the plant, yet at the same time, not so high as to cause collapse of the leaf tissue, causing it to dry and/or abscise. The inoculum was made in sterile saturated calcium carbonate ($CaCO_3$) solution to maintain viable bacteria. After inoculation, the plants were kept outside the chamber until the water of inoculation had been absorbed or transpired, then they were returned to the chamber. Leaves were harvested from *Xcm*-inoculated plants and non-inoculated plants at 8, 14, 20, 30, 45 and 60 hpi. RNA was isolated according to Thompson and associates (Thompson *et al.*, 1983). RNA preparations from the six different timepoints were combined in equivalent quantities to create two RNA pools: "*Xcm*-inoculated, RNA" and "non-inoculated, RNA", 480 μg each. Poly $(A)^+$ RNA was purified from these mixtures using oligo-dT cellulose columns (Poly (A) Pure™ mRNA isolation kit; Ambion, Austin, TX, USA).

SSH cDNA library preparation

cDNA was prepared from each Poly $(A)^+$ pool and SSH was carried out using the PCR-Select™ cDNA subtraction kit according to the manufacturer's instructions

(Clontech, Palo Alto, CA, USA). During subtraction, ds cDNA populations derived from control and *Xcm*-challenged leaves served as driver and tester cDNA, respectively. The SSH cDNA products were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and transformed into *E. coli* (TOP10 chemically competent cells). Transformants were picked by hand and stored as15% glycerol stocks in Luria Bertani (LB)-ampicillin (150 μg/mL) medium in 96-wells at -80°C.

Redundant screening and sequencing

Plasmid DNA was isolated from the library using a 96-well alkaline lysis miniprep format (Edge Biosystems, Gaithersburg, MD, USA). Quality and quantity of the DNAs were evaluated on 1% agarose gels. DNA concentrations ranged from 75 to 400 ng/μL. Plasmids were denatured in a 0.3M NaOH, 6X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) mixture and printed on 8 cm x 12 cm nylon membranes (GeneScreen, NEN LifeSciences) using a Seiko D-Tran Cartesian robot, that was assembled at Oklahoma State University and programmed by Jerry M. Merz. Each plasmid was printed in triplicate on a membrane. Also printed on the membranes in triplicate were the probes used for hybridization in three different amounts; 300 ng, 150 ng and 75 ng. Membranes were UV-crosslinked twice (Stratalinker, Stratagene, Ja Lolla, USA) and stored at room temperature. Probes used for redundant screening were fluorescein-labeled using *Gene Images* random prime labeling module (Amersham Pharmacia Biotech). The Southern-Light™ Chemiluminescent Detection System (Tropix, Applied Biosystems) was used for detection. Hybridization was carried out at 65°C overnight. Clones that did not hybridize to any of the probes were sequenced by dye terminator cycle chemistry (BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA) and automated capillary electrophoresis (ABI Prism 3700 Genetic Analyser, Applied Biosystems, Foster City, CA, USA). The universal reverse primer (at the 5' end of the vector's cloning site) was used to generate single-pass sequences. Clones that hybridized with equal or greater intensity than

the lowest positive control (75 ng) on the membrane were eliminated.

Sequence data analysis

Trace files of clones sequenced were submitted to PipeOnline (POL) (Ayoubi *et al.*, 2002), a web-based resource designed by the Oklahoma State University Bioinformatics Group. POL is a processing module consisting of a series of script-linked programs that process multiple (up to several thousand) raw DNA sequence files and produce a new set of contiguous-assembled files. The first program, PHRED (Ewing *et al.*, 1998), accepts trace files, evaluates the quality of the DNA sequence, and generates an output file containing this information. XMATCH (Green, 1999) accepts PHRED output or text files, runs a comparison against a local vector database file, and crosses out matches. PHRAP (Green, 2000) accepts text, PHRED, and/ or XMATCH output files and assembles the entered DNA sequence files into contigs. PHRAP or text input files are compared against a local NCBI non-redundant nucleotide database using Blastx (Altschul *et al.*, 1997). The contigs from POL were further condensed manually by the investigator, since PHRAP parameters were too stringent, and some nearly identical sequences were not assembled correctly. All the Im216 ESTs generated from this study will be deposited in dbEST at NCBI and made available to the public at http://www.ncbi.nlm.nih.gov.

Preparation of Im216 SSH arrays

Plasmid DNA isolated from all 192 SSH clones was amplified in 50 μL reactions in a 96 well format using the following PCR program: 95°C for 3 minutes; followed by 40 cycles of 95°C for 1 minute, 55°C for 30 seconds, 72°C for 2 minutes; then 72°C for 10 minutes. The Multi Screen PCR 96 Well filtration system (Millipore, Bedford, MA, USA) was used to remove unwanted primers, dNTPs and salt contaminants. The cleaned PCR products were eluted in 50 μL sterile water. Five microliters of the eluate was run on a 1.5% agarose gel to check quality and quantity of the PCR products. The PCR reaction for

a particular clone was repeated if no PCR product or multiple bands were observed on the gel. PCR products were dried completely in a speed vacuum and resuspended in 5 μL of sterile water and equal volume of 6 X sodium saline citrate (SSC) solution in 96-well plates. Spiking controls Sp 1, 2, 3, 4, 5, 6, 7, 9 and 10 were also PCR-amplified using specific pairs of primers (sequences made available by the Arabidopsis Functional Genomics Consortium). The PCR products were processed in manner identical to the PCR products from the SSH library. The PCR products were resuspended at 4°C overnight with gentle horizontal agitation. The PCR products were transferred to a 384-well titer plate before printing. The PCR products (~ 360 ng/μL) were printed on Corning GAPS II slides (Corning Life Sciences, NY, USA) using a GeneMachines OmniGrid 100 arrayer. Slides were left to dry overnight on the arrayer, rehydrated using a humid chamber (Sigma Chemicals) for 30 seconds, and snap dried by placing the slide (DNA side up) on a hot plate heated to 65°-70°C for a few seconds (till condensation on the slide dries). PCR products were immobilized onto the slide by baking at 85°C overnight. Before use, PCR products were denatured in boiling water for two minutes and snap cooled by immersing the slides in –20°C ethanol. Slides were stored in a vacuum desiccator at room temperature.

Plant and bacterial material for microarray experiments

For the first and second biological replicates, delinted Im216 seeds (treated as mentioned above), were grown in Metro Mix 702 (American Plant Products) in two separate Conviron E15 growth chambers (15 square feet) as described by Pierce and associates (Pierce *et al.*, 1993). Light intensities in both chambers were *ca.* 475 nmol $m^{-2}s^{-1}$. For the third biological replicate,treated, delinted Im216 seeds were grown in a mixture of Metro Mix 702 and Metro Mix 366 (v/v) (American Plant Products) in a larger Conviron (30 square feet). Light intensity in the chamber was *ca.* 284 nmol $m^{-2}s^{-1}$. Bacterial cultures of Xcm strain 3631 were cultured as described for the SSH library.

Im216 inoculations and RNA isolations for microarray experiments

Fifth or sixth leaves of *ca.* four-week-old plants (biological replicates one and two) were spray infiltrated (Chapin Yard Sprayer Plus) with an *Xcm* suspension at concentrations of 5.02 x 10^6 cfu/mL and 6.68 x 10^6 cfu/mL respectively. Fifth or sixth leaves of *ca.* five-week-old plants (third biological replicate) were spray infiltrated (Chapin Yard Sprayer Plus) with an *Xcm* suspension at a concentration of 4 x 10^6 cfu/mL. Total RNA from Im216 foliage leaves harvested at 8 hpi, 14 hpi, 20 hpi, 30 hpi and 60 hpi from the three biological replicates, was isolated as previously described by Chang and coworkers (Chang *et al.*, 1993). In addition, after isolation of total RNA, it was cleaned by precipitation overnight at -20°C with one-tenth volume 3M sodium acetate and three volumes of 75% ACS grade ethanol. RNA pellets were washed thrice with 75% ACS grade ethanol, air dried and rehydrated in 20 μL DEPC-treated water. RNA was quantitated using a GeneQuant RNA/DNA Calculator (Amersham Pharmacia). All A_{260}/A_{280} ratios of RNA samples were > 2.0. RNA was stored at -80°C before use.

cDNA target synthesis for microarrays

For target labeling, 50μg of total RNA was used for Cy3 (non-inoculated) as well as Cy 5 (*Xcm*-treated) reactions, using the 3 DNA Array 350™ kit (Genisphere, Hatfield, PA, USA). External spikes, Sp 1, Sp 3, Sp4 and Sp5 (non-plant spikes) from the Arabidopsis Functional Genomics Consortium (AFGC) were generated by *in vitro* transcription of the PCR-amplified (see next section) products using a Riboprobe In vitro Transcription Systems Kit (Promega, Madison, WI). Sp 4 and Sp5 RNA were used at 0.5 ng and 0.05 ng respectively in each labeling reaction. Slides were hybridized with the target for 18 hours during the first hybridization and for 3 hours with the capture sequence for the second hybridization. Washes for both days were followed as per the manufacturer's protocol.

Data collection and processing

Slides were scanned using the ScanExpress Scanner (Perkin Elmer Biosystems). Channels were balanced so that the ratio of intensities (Cy5/Cy3) for spike Sp4 was close to 1. Signal extraction and spot quantification were conducted using GenePix Pro 4.0 (Axon Instruments, Union City, CA, USA). Global background was subtracted, and Sp4 features with ratios between 0.7 and 1.3 were used for normalization of the data; if the ratio for the spike was not within this range, it was not included in normalization. Normalization of the array is performed to correct for minor imbalances that may arise due to technical variations owing to differences in labeling, hybridization efficiency, washes, variation in laser power and detector sensitivities. The normalized data was stored as "gpr" files and uploaded to the GenePix Pro AutoProcessor (GPAP) (http://darwin.biochem.okstate.edu/gp/) (Wang and Ayoubi, 2003). This software calculates valid and invalid features (a feature being an individual spot on the array; there are 12 features for each gene on a slide), and for each gpr file uploaded, four result reports are generated: the first is a detailed report of the valid features, i.e., the averaged replicate background-corrected normalized signal intensity in each channel for each valid gene on the array. It calculates the coefficient of variation (CV) of replicate spots, expressed as a percentage. The second report gives the average ratio value and its standard deviation associated with each gene. Thirdly, a similar summary report for valid gene gives the x-fold increase or decrease in gene expression and number of outliers for each gene. The final report is of the individual invalid features on the array.

RNA gel blot hybridization

RNA gel blotting was done as previously described (Elliot *et al.*, 1989). RNA samples were separated by gel electrophoresis and blotted to GeneScreen membranes (NEN Life Sciences). Membranes were prehybridized in hybridization buffer (Frances *et al.*, 1992) for at least two hours at 68°C. Gene-specific probes (as shown in the results) were synthesized with the Promega (Madison, WI, USA) *in vitro* RNA transcription system, the

appropriate polymerase, and ^{32}P-UTP. The radiolabeled probe was purified on a Sephadex G-50 column and added to prehybridized membrane for hybridization overnight at 68°C. Blots were washed twice for 15 minutes each at 68°C in 2X SSC and 0.1% SDS and washed twice for 15 minutes each at 68°C in 0.5X SSC and 0.1% SDS. Blots were wrapped in Saran wrap and exposed to X-ray film for 12- 96 hours at –70°C.

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Table 1: The unique non-redundant contigs arrayed.

Clone Name	Length (bp)	Top Blast Hit	HSP Score
1-C2	561	Expressed protein	231
1-C8	293	Senescence associated protein	877
1-E2	550	No homology	-
P10-B1	542	No homology	-
P10-B12	790	No homology	-
P10-B3	716	No homology	-
P10-B5	396	Putative transposon protein	98
P10-C1	1307	No homology	-
P10-C10	999	No homology	-
P10-C3	743	Triose phosphate translocator precursor	85
P10-C5	820	Hypothetical protein XP (homosapiens)	85
P10-C6	719	Gag-pol	284
P10-D2	812	No homology	-
P10-D9	1333	No homology	-
P10-E1	407	No homology	-
P10-E10	625	Hypothetical protein	118
P10-E12	259	60S Ribosomal protein L21	185
P10-F8	745	Similar to *A. thaliana* DNA-directed RNA poly	92
P10-H6	386	No homology	-
P11-A2	638	Glycosyl hydrolase family 19	581
P11-C11	528	In2-1 protein	308
P11-E4	337	Transcript antisense to rRNA	162
P12-A11	395	Cytochrome p450	494
P12-A4	736	NADH dehydrogenase subunit 5	85
P12-B1	437	Protease homolog	146
P12-E6	675	Protein subunit 2	442
P12-G5	22	No homology	-
P12-H1	1109	No homology	-
P13-A8	616	No homology	-
P13-C3	461	No homology	-
P13-D10	454	Putative kafirin cluster	405
P13-E5	706	Succinate dehydrogenase subunit3	712
P13-F9	735	Alpha-amylase/trypsin inhibitor	100
P13-H11	24	No homology	-
P14-H11	446	No homology	-
P15-B4	624	Dicyanin	284
P15-C12	162	No homology	-
P15-C6	1418	No homology	-
P15-C7	432	No homology	-
P15-F5	275	No homology	-
P15-F7	690	Polyubiquitin	848

P15-H1	500	Cyclosporin A binding protein	479
P15-H11	504	Zinc finger protein 2	207
P16-E4	538	Similar to non-phototropic hypocotyl-like protein	640
P16-F5	680	PR protein class 10	829
P16-F6	169	20S proteasome beta subunit A	114
P16-G11	708	Cyteine proteinase precursor	111
P17-B3	480	PR1a precursor	559
P17-D9	788	No homology	-
P17-E3	598	No homology	-
P17-F12	397	No homology	-
P17-F4	534	Putative aquaporin	478
P17-F9	532	Hypothetical protein (soybean)	155
P17-G7	569	Glutathione S-transferase	597
P17-G9	209	No homology	-
P18-A12	703	PR protein 10	821
P18-A2	375	Lipid transfer protein precursor	194
P18-B1	707	Hevamine A	695
P18-D4	264	Probable, small nuclear ribonucleoprotein	333
P18-D7	511	B2 protein	538
P18-F11	692	RNA-binding protein	403
P18-F2	708	Secoisolariciresinol dehydrogenase (alcohol dehydrogenase)	587
P18-F3	309	No homology	-
P18-H6	166	No homology	-
P18-H9	378	Jasmonate induced protein	98
P19-A8	203	Expressed protein	140
P19-B1	136	60s ribosomal protein	199
P19-C1	507	Osmotin like protein	456
P19-E12	431	Photoassimilate responsive protein	225
P19-F1	56	No homology	-
P19-F6	187	PAR-1b precursor	272
P19-G8	556	Ketol-acid reductase isomerase	519
P19-H2	256	No homology	-
P19-H3	262	Cold induced protein	381
P1-A4	675	Caffeoyl-CoA *O*-methyltransferase	1026
P1-A8	306	No homology	-
P1-B4	665	60S ribosomal protein L10	491
P1-C2	493	Protein kinase (LRR transmembrane protein)	443
P1-C4	664	Cytochrome P450	201
P1-C7	671	Oryza sativa protein	106
P1-D11	198	No homology	-
P1-D6	689	No homology	-
P1-D8	759	PglA	84
P1-G1c	252	Similar to drosophila lethal malignant tumor	78
P20-A4	515	No homology	-
P20-B12	309	Putative metal ion transporter	789

P20-C12	613	PR protein 5-1	279
P20-C2	488	Class III chitinase	230
P20-C6	693	No homology	-
P20-E6	706	Chitinase	89
P20-G7	793	No homology	-
P20-H7	79	No homology	-
P20-H9	337	Hypothetical protein (Soybean)	149
P21-A10	707	Putative glutathione peroxidase	768
P21-B7	459	No homology	-
P21-C12	545	AP2-related transcription factor	395
P21-D11	676	IS1 protein InsB	146
P21-F11	575	Bacteria-induced peroxidase	963
P21-F8	201	1,3-beta glucanase precursor	339
P22-A2	825	No homology	-
P22-B3	280	ORF122 (involved in photosynthesis)	204
P22-C7	692	PRprotein 4A	498
P22-E8	282	Auxin-induced protein	420
P22-E9	1369	No homology	-
P22-H2	365	T26F17.11(*A. thaliana*; Genomic sequence, chromosome 1)	156
P23-B10	249	Digalactosyldiacylglycerol synthase	186
P23-C8	388	Hypothetical protein	89
P23-G2	606	PR protein	559
P24-A8	848	No homology	-
P24-B9	755	No homology	-
P24-C10	540	1,3-beta glucanase precursor	544
P24-E1	527	Glutathione transferase	385
P24-F3	635	Ammonia-lyase	598
P25-A10	675	Unknown protein (A. thaliana)	302
P2-B12	943	No homology	-
P2-C12	698	Hevamine A (plant endochitinase)	998
P2-D11	613	Retrotransposan dell-46	158
P2-E1	682	Retrotransposan dell-46	254
P2-F3	767	No homology	-
P3-A3	616	Mucin (dog)	98
P3-A6	185	No homology	-
P3-B6	410	Protein	93
P3-C11	553	Finger protein	117
P3-D1	315	Ubiquinol cytochrome c reductase	424
P3-D3	93	No homology	-
P3-D4	738	Photosystem I reaction center subunit XI	140
P3-E3	88	No homology	-
P3-E7	272	No homology	-
P3-E9	681	Protein product	85
P3-F2	616	PR protein class 10	536
P3-F6	377	No homology	-
P3-G3	512	Oxidase	630

P3-G4	193	No homology	-
P3-G7c	695	Major occlusion body protein	972
P3-G9	393	Expressed protein	146
P3-H10	497	No homology	-
P3-H3	392	DNA packaging protein	574
P3-H5	693	No homology	-
P3-H6	894	Protein (N. crassa)	90
P3-H7	655	AP2 domain protein	198
P4-A3	467	RNA binding protein	83
P4-A6	820	No homology	-
P4-B5	720	No homology	-
P4-C8	320	Bacteria-induced peroxidase	552
P4-D7	675	2-oxoglutarate dependent dioxygenase	425
P4-F7	540	No homology	-
P4-F9	835	No homology	-
P4-G10	319	Protein	340
P4-G2	279	Acidic endochitinase	322
P4-H1	454	Aldehyde dehydrogenase	731
P5-A7	563	Senescence associated protein	877
P5-B9	700	No homology	-
P5-C12	839	No homology	-
P5-D5	247	No homology	-
P5-D7	283	1,3- beta glucanase	483
P5-D9	352	Hypothetical protein	182
P5-H12	384	Dehydrogenase like protein	339
P5-H7	687	Thaumatin-likeprotein 1 precurosr	155
P5-H8	304	Dioxygenase	100
P6-A4	1168	No homology	-
P6-A7	750	No homology	-
P6-B6	724	Thaumatin-like protein 1 precursor	124
P6-B6	738	No homology	-
P6-C2	685	Latent protein	102
P6-C5	685	No homology	-
P6-D5	685	No homology	-
P6-D6	97	No homology	-
P6-D8	680	Expressed protein	83
P6-E4	20	No homology	-
P6-E7	686	Senescence associated protein	877
P6-F1	219	Putative protein kinase	154
P6-F5	70	No homology	-
P6-F6	506	Mitochondrial dependent malate dehydrogenase	277
P6-F9	556	Cytokinin binding protein	151
P7-E10	648	Hypothetical protein (Rice)	83
P7-G2	213	MATE efflux family	79
P8-A9	715	Cold inducible protein	241
P8-D6	389	Glycosyl hydrolase family 19	178

P8-E3	444	No homology	-
P8-E5	460	PR protein 5	379
P8-H12	474	PR protein 5	380
P9-A12	549	No homology	-
P9-B12	715	Cysteine proteinase	785
P9-E2	753	Retinitis pigmentosa,GTPase regulator	85
P9-F3	914	No homology	-
P9-F6	603	No homology	-
P9-G3	246	No homology	-

Table 2: List of clones used as probes for the Northern blots, their putative identities, HSP values and contiguous sequence group they belong to.

Clone Name	Putative Identity	HSP Value	Contig #
1-B1	Senescence associated protein	877	#119
1-B6	PR protein Class 10	824	#101
1-C1	PR 1a precursor	95	#116
1-D2	Glycosyl hydrolase family 19 (Class IV chitinase)	583	#111
1-D4	Preprotein	559	#118
1-E6	PR protein 5-1	460	#117
P3-A9	Expressed protein	393	# 23
P3-G3	Oxidase	630	# 202
P3-H3	DNA packaging protein	367	# 224
P3-F6	No protein alignment	-	# 200
P3-H10	No protein alignment	-	# 43
P6-D10	Hypothetical protein	182	# 68
1-E2	No protein alignment	-	# 249

Table 3: List of standard error values for each valid cDNA on the array for the three biological replicates (BR 1, 2, and 3) at each time point. (#N/A = no valid data obtained for that sample).

Name	BR1-8 hpi	BR1-14 hpi	BR1-20 hpi	BR1-30 hpi	BR1-45 hpi	BR1-60 hpi	BR2-8 hpi	BR2-14 hpi	BR2-20 hpi	BR2-30 hpi	BR2-45 hpi	BR2-60 hpi	BR3-8 hpi	BR3-14 hpi	BR3-20 hpi	BR3-30 hpi	BR3-45 hpi	BR3-60 hpi
1-C2	0.058	0.121	0.055	0.100	0.093	0.107	0.061	0.047	0.063	0.156	0.024	0.086	0.037	0.052	0.017	0.000	0.047	0.069
1-C8	0.130	0.323	0.067	0.069	0.073	0.058	0.129	0.133	0.201	0.053	0.130	0.135	0.167	0.102	0.080	0.047	0.051	0.028
1-E2	0.051	0.101	0.065	0.076	0.088	0.090	0.077	0.047	0.133	0.173	0.066	0.062	0.019	0.049	0.058	0.078	0.045	0.083
cdn1-A	0.045	0.099	0.055	0.091	0.092	0.060	0.078	0.038	0.076	0.064	0.047	0.059	0.029	0.000	0.072	0.098	0.035	0.062
cdn1-C	0.032	0.134	0.017	0.103	0.102	0.058	0.058	0.045	0.089	0.117	0.044	0.044	0.041	0.029	0.044	0.067	0.041	0.063
F8K1RC	0.069	0.123	0.057	0.044	0.069	0.058	0.078	0.094	0.157	#N/A	0.070	#N/A	0.033	0.000	0.059	0.062	#N/A	#N/A
GhCA2	0.025	0.037	0.013	0.109	0.057	0.096	0.055	0.000	0.000	0.158	0.084	0.101	0.000	0.013	0.011	0.056	0.025	0.057
hmg 2	0.050	0.135	0.047	0.077	0.105	0.091	0.089	0.065	0.133	0.122	0.043	0.096	0.035	0.034	0.022	0.097	0.045	0.085
hmg1	0.041	0.113	0.067	0.085	0.060	0.042	0.074	0.112	0.242	0.087	0.039	#N/A	0.029	0.000	0.063	0.039	0.032	0.022
P10-A6	0.031	0.094	0.074	0.040	0.079	0.046	0.061	0.073	0.117	0.092	0.054	0.097	0.058	0.054	0.030	0.059	0.056	0.057
P10-B1	0.045	0.087	0.035	0.104	0.064	0.059	0.082	0.082	0.243	0.126	0.071	0.081	0.037	0.039	0.051	0.110	0.021	0.091
P10-B12	0.137	0.237	0.195	0.119	0.130	0.097	0.192	0.079	0.188	0.164	0.225	0.072	0.035	0.052	0.100	0.090	0.031	0.071
P10-B3	0.057	0.118	0.060	0.054	0.111	0.080	0.079	0.048	0.503	0.026	0.000	#N/A	0.046	0.057	0.057	0.094	0.055	0.079
P10-B5	0.060	0.211	0.402	0.287	0.066	0.120	0.122	0.103	0.217	0.171	0.119	0.094	0.240	0.286	0.176	0.142	0.059	0.046
P10-C1	0.542	0.381	0.415	0.297	0.230	0.218	0.523	0.361	0.469	0.189	0.159	0.163	0.401	0.470	0.313	0.273	0.274	0.228
P10-C10	0.038	0.105	0.060	0.083	0.102	0.048	0.058	0.059	0.077	0.117	0.035	0.082	0.044	0.050	0.390	0.087	0.192	0.071
P10-C3	0.055	0.129	0.046	0.064	0.095	0.076	0.205	0.055	0.164	0.065	0.036	0.093	0.048	0.047	0.017	0.068	0.024	0.037
P10-C5(1)	0.046	0.091	0.081	0.060	0.046	0.130	0.061	0.063	0.169	0.078	0.061	0.083	0.037	0.072	0.038	0.075	0.030	0.046
P10-C5(3)	0.043	0.216	0.028	0.077	0.103	0.081	0.030	0.057	0.067	0.090	0.091	0.099	0.052	0.055	0.025	0.072	0.053	0.069
P10-C5(5)	0.069	0.099	0.034	0.070	0.070	0.078	0.051	0.049	0.089	0.117	0.067	0.082	0.060	0.077	0.022	0.013	0.038	0.084
P10-C6	0.059	0.127	0.010	0.125	0.054	0.067	0.098	0.115	0.220	0.054	0.061	0.077	0.037	0.086	0.000	0.042	0.058	0.069
P10-D2	0.035	0.081	0.049	0.055	0.041	0.031	0.078	0.134	0.129	0.144	0.055	0.083	0.048	0.056	0.047	0.048	0.041	0.045
P10-D9	0.169	0.086	0.090	0.153	0.133	0.081	0.140	0.160	0.104	0.103	0.119	0.193	0.068	0.091	0.069	0.146	0.047	0.065
P10-E1	0.050	0.087	0.027	0.062	0.167	0.093	0.062	0.031	0.079	0.063	0.061	0.102	0.034	0.029	0.028	0.142	0.053	0.051
P10-E10	0.042	0.090	0.041	0.103	0.075	0.073	0.055	0.035	0.084	0.098	0.053	0.051	0.031	0.037	0.026	0.123	0.049	0.084
P10-E12	0.027	0.092	0.050	0.071	0.166	0.083	0.049	0.034	0.082	0.077	0.053	0.087	0.031	0.029	0.033	0.115	0.057	0.062
P10-F8	0.041	0.116	0.058	0.059	0.111	0.082	0.039	0.046	0.082	0.118	0.046	0.054	0.037	0.033	0.036	0.116	0.055	0.057
P10-H6	0.051	0.068	0.031	0.059	0.078	0.047	0.063	0.056	0.108	0.074	0.047	0.104	0.045	0.048	0.032	0.047	0.085	0.057
P11-C11	0.045	0.074	0.027	0.048	0.109	0.086	0.029	0.059	0.000	0.141	0.057	0.041	0.019	0.019	0.016	#N/A	0.036	0.078
P11-E4	0.061	0.090	0.046	0.077	0.069	0.081	0.048	0.040	0.178	0.003	0.071	0.116	0.044	0.047	0.067	0.128	0.041	0.057
P12-A11	0.086	0.100	0.065	0.283	0.153	0.067	0.065	0.102	0.155	0.114	0.057	0.198	0.053	0.053	0.028	0.129	0.042	0.071
P12-A4	0.032	0.061	0.007	0.089	0.068	0.066	0.064	0.085	0.000	0.070	0.051	0.054	0.032	0.000	0.036	0.080	0.031	0.073
P12-B1	0.038	0.123	0.080	0.099	0.072	0.115	0.051	0.091	0.237	0.231	0.095	0.127	0.038	0.021	0.031	0.045	0.079	0.120
P12-E6	0.051	0.124	0.063	0.099	0.054	0.092	0.048	0.029	0.197	0.213	0.000	0.127	0.047	0.057	0.020	0.055	0.035	0.077
P12-G5	0.030	0.065	0.022	0.076	0.088	0.082	0.058	0.159	0.352	0.087	0.054	0.094	0.055	0.000	0.041	0.071	0.058	0.038
P12-H1	0.030	0.265	0.154	0.084	0.183	0.133	0.193	0.143	0.228	0.160	0.137	0.129	0.093	0.039	0.058	0.042	0.017	0.042
P13-A8	0.053	0.090	0.065	0.121	0.084	0.079	0.058	0.092	0.335	0.092	0.067	0.124	0.050	0.035	0.039	#N/A	0.064	0.066
P13-C3	0.137	#N/A	#N/A	0.091	0.023	0.000	#N/A	#N/A	#N/A	#N/A	#N/A	0.000	0.000	#N/A	#N/A	#N/A	0.000	0.000
P13-D10	0.046	0.087	0.059	0.082	0.082	0.079	0.047	0.041	0.155	0.071	0.010	0.075	0.046	0.032	0.052	0.111	0.035	0.049
P13-E5	0.034	0.095	0.043	0.071	0.090	0.069	0.069	0.046	0.080	0.045	0.051	0.083	0.037	0.063	0.021	0.092	0.059	0.077
P13-F9	0.072	0.117	0.077	0.089	0.056	0.076	0.066	0.358	0.173	0.070	0.036	0.067	0.028	0.021	0.047	0.072	0.055	0.085
P14-H11	0.055	0.000	#N/A	0.000	#N/A	#N/A	0.000	0.000	0.267	0.000	0.000	0.000	0.073	0.052	0.261	#N/A	0.147	0.000
P15-B4	0.054	0.120	0.053	0.084	0.088	0.087	0.058	0.034	0.170	0.104	0.066	0.056	0.035	0.057	0.043	0.085	0.051	0.087
P15-C12	0.038	0.073	0.037	0.089	0.082	0.070	0.065	0.045	0.081	0.099	0.069	0.097	0.046	0.050	0.019	0.068	0.063	0.099
P15-C6	0.029	0.093	0.037	0.045	0.077	0.079	0.029	0.050	0.084	0.177	0.055	0.114	0.031	0.025	0.019	0.092	0.067	0.127
P15-C7	0.020	0.069	0.047	0.078	0.049	0.057	0.055	0.072	0.224	0.158	0.032	0.073	0.035	0.023	0.021	0.067	0.038	0.061
P15-F5	0.032	0.073	0.027	0.031	0.081	0.093	0.035	0.063	0.090	0.154	0.051	0.084	0.023	0.032	0.023	0.071	0.056	0.080
P15-F7	0.023	0.043	0.032	0.055	0.055	0.085	0.029	0.022	0.051	0.081	0.042	0.092	0.020	0.067	0.077	0.043	0.074	0.312
P15-H1	0.032	0.063	0.046	#N/A	0.046	#N/A	0.054	0.043	0.163	0.098	0.065	0.074	0.033	0.027	0.023	#N/A	0.051	0.067
P15-H11	0.037	0.077	0.043	0.068	0.070	0.073	0.032	0.049	0.154	0.126	0.000	0.092	0.036	0.032	0.029	0.060	0.073	0.070
P16-E4	0.029	0.054	0.060	0.047	0.079	0.075	0.064	0.067	0.161	0.156	0.072	0.087	0.029	0.023	0.029	0.057	0.026	0.052
P16-F5	0.025	0.103	0.034	0.075	0.089	0.066	0.053	0.065	0.165	0.127	0.034	0.121	0.056	0.027	0.028	0.123	0.033	0.064

Name	BR1-8 hpi	BR1-14 hpi	BR1-20 hpi	BR1-30 hpi	BR1-45 hpi	BR1-60 hpi	BR2-8 hpi	BR2-14 hpi	BR2-20 hpi	BR2-30 hpi	BR2-45 hpi	BR2-60 hpi	BR3-8 hpi	BR3-14 hpi	BR3-20 hpi	BR3-30 hpi	BR3-45 hpi	BR3-60 hpi
P16-F6	0.199	0.198	0.114	0.302	0.285	0.307	0.241	0.121	0.388	0.187	0.221	0.204	0.203	0.167	0.107	0.145	0.334	0.287
P16-G11	0.032	0.133	0.066	0.061	0.061	0.207	0.133	0.000	0.247	0.109	0.186	0.137	0.029	0.039	0.056	0.119	0.031	0.055
P17-B3	0.079	0.121	0.053	0.172	0.062	0.053	0.073	0.078	0.249	0.237	0.197	0.045	0.035	0.042	0.053	0.093	0.024	0.057
P17-D9	0.047	0.091	0.045	0.070	0.075	0.090	0.066	0.044	0.075	0.124	0.105	0.072	0.032	0.049	0.035	0.090	0.044	0.075
P17-E3	0.104	0.120	0.059	0.097	0.180	0.111	0.036	0.077	0.064	0.053	0.058	0.053	0.059	0.048	0.053	0.183	0.060	0.096
P17-F12	0.040	0.078	0.047	0.047	0.192	0.075	0.026	0.041	0.060	0.083	0.045	0.070	0.023	0.042	0.021	0.146	0.067	0.064
P17-F4	0.146	0.147	0.200	0.217	0.258	0.268	0.171	0.191	0.172	0.128	0.221	0.221	0.154	0.131	0.159	0.201	0.206	0.167
P17-G7	0.179	0.285	0.061	0.113	0.063	0.000	0.249	0.146	0.127	0.071	0.047	0.057	0.064	0.099	0.076	0.152	0.103	0.142
P17-G9	0.080	0.118	0.035	0.097	0.140	0.089	0.043	0.037	0.071	0.129	0.060	0.055	0.041	0.049	0.042	0.122	0.069	0.065
P18-A12	0.039	0.079	0.057	0.062	0.063	0.067	0.083	0.076	0.205	0.072	0.053	0.071	0.037	0.038	0.038	0.042	0.049	0.051
P18-A2	0.035	0.081	0.025	0.085	0.143	0.096	0.061	0.000	0.073	0.099	0.047	0.062	0.039	0.029	0.036	0.155	0.062	0.094
P18-B1	0.042	0.093	0.015	0.078	0.103	0.070	0.046	0.037	0.056	0.078	0.044	0.044	0.016	0.040	0.030	0.131	0.055	0.093
P18-D4	0.021	0.087	0.016	0.056	0.108	0.102	0.077	0.027	0.046	0.094	0.065	0.045	0.049	0.023	0.011	0.301	0.049	0.077
P18-D7	0.024	0.084	0.024	0.073	0.065	0.080	0.052	0.029	0.000	0.089	0.033	0.074	0.022	0.020	0.021	0.091	0.045	0.058
P18-F11	0.020	0.085	0.041	0.107	0.134	0.103	0.046	0.033	0.095	0.187	0.080	0.127	0.033	0.035	0.050	0.100	0.032	0.092
P18-F2	0.099	0.161	0.176	0.228	0.107	0.085	0.107	0.129	0.153	0.131	0.133	0.078	0.113	0.105	0.138	0.187	0.109	0.094
P18-F3	0.047	0.110	0.041	0.067	0.104	0.086	0.050	0.058	0.000	0.123	0.050	0.089	0.019	0.037	0.030	0.113	0.044	0.061
P18-H6	0.062	0.191	0.049	0.082	0.099	0.114	0.030	0.035	0.331	0.166	0.055	0.058	0.039	0.029	0.061	0.076	0.040	0.062
P18-H9	0.018	0.080	#N/A	0.033	0.072	0.073	0.077	0.086	0.320	0.114	0.057	0.129	0.030	0.022	0.029	#N/A	0.053	0.047
P19-A8	0.093	0.059	0.105	0.044	0.039	0.057	0.058	0.040	0.202	0.143	0.060	0.083	0.037	0.045	0.046	0.032	0.033	0.050
P19-B1	0.049	0.111	0.027	0.059	0.058	0.086	0.055	0.000	0.200	0.148	0.068	0.097	0.019	0.016	0.035	0.030	0.031	0.036
P19-C1	0.055	0.098	0.045	0.058	0.091	0.097	0.060	0.023	0.148	0.134	0.018	0.059	0.025	0.037	0.031	0.065	0.069	0.096
P19-E12	0.029	0.099	0.145	0.054	0.061	0.071	0.057	0.000	0.237	0.131	0.024	0.100	0.053	0.047	0.039	0.075	0.033	0.047
P19-F1	0.181	0.153	0.066	0.125	0.073	0.095	0.062	0.053	0.000	0.065	0.041	0.071	0.143	0.049	0.071	0.058	0.027	0.000
P19-F6	0.034	0.075	0.045	0.041	0.027	0.057	0.044	0.074	0.261	0.097	0.068	0.000	0.022	0.039	0.038	0.061	0.026	0.043
P19-G8	0.031	0.063	0.025	0.000	#N/A	#N/A	0.051	0.088	0.204	0.054	0.048	0.075	0.048	0.089	0.036	0.026	0.034	0.030
P19-H3	0.040	0.112	0.037	0.080	0.079	0.073	0.078	0.054	0.080	0.080	0.044	0.083	0.045	0.038	0.038	0.084	0.063	0.078
P1-A4	0.048	0.069	0.032	0.045	0.096	0.053	0.102	0.078	0.115	0.099	0.031	0.186	0.115	0.083	0.061	0.099	0.068	0.050
P1-A8	0.059	0.087	0.017	0.133	0.091	0.000	0.000	0.078	0.160	0.077	0.154	0.108	0.212	0.035	0.070	#N/A	0.101	0.080
P1-B4	0.130	0.133	0.058	0.186	0.075	0.045	0.137	0.102	0.269	0.097	0.047	0.086	0.178	0.028	0.115	0.122	0.061	0.033
P1-C2	0.117	0.073	0.035	0.079	0.056	0.041	0.153	0.155	0.079	0.034	0.027	0.043	0.079	0.073	0.167	0.097	0.030	0.037
P1-C4	0.064	0.096	0.055	0.124	0.063	0.044	0.079	0.128	0.121	0.041	0.033	0.079	0.062	0.077	0.156	0.093	0.044	0.055
P1-C7	0.037	0.164	0.263	0.173	0.000	#N/A	0.080	0.173	0.121	#N/A	#N/A	0.000	0.034	0.042	0.043	0.091	0.049	0.077
P1-D11	0.110	0.146	0.097	0.133	0.106	0.087	0.053	0.098	0.204	0.133	0.081	0.099	0.060	0.084	0.147	0.155	0.033	0.109
P1-D6	0.039	0.000	0.000	0.164	0.069	0.067	0.160	0.000	0.000	0.105	0.164	0.157	0.051	0.041	0.000	#N/A	0.166	0.250
P1-D8	0.034	0.077	0.065	0.092	0.107	0.049	0.049	0.080	0.119	0.102	0.033	0.066	0.030	0.036	0.021	0.115	0.047	0.052
P1-G1c	0.113	0.150	0.044	0.043	0.063	0.060	0.077	0.000	0.137	0.110	0.117	0.077	0.207	0.087	0.081	#N/A	0.053	0.043
P20-A4	0.043	0.159	0.094	0.067	0.054	0.075	0.151	0.048	0.209	0.111	0.042	0.030	0.000	0.083	0.000	0.000	#N/A	#N/A
P20-B12	0.031	0.082	0.027	0.073	0.073	0.083	0.062	0.096	0.149	0.079	0.062	0.048	0.025	0.000	0.023	0.096	0.059	0.078
P20-C12	0.045	0.122	0.062	0.086	0.086	0.077	0.070	0.047	0.200	0.133	0.091	0.065	0.032	0.049	0.065	0.054	0.035	0.100
P20-C2	0.035	0.109	0.039	0.058	0.075	0.095	0.068	0.067	0.199	0.128	0.067	0.170	0.025	0.030	0.032	0.079	0.047	0.078
P20-C6	0.032	0.126	0.062	0.052	0.087	0.104	0.228	0.126	0.135	0.138	0.061	0.077	0.036	0.050	0.052	0.083	0.041	0.087
P20-E6	0.056	0.097	0.030	0.084	0.140	0.129	0.041	0.036	0.142	0.195	0.048	0.088	0.025	0.034	0.049	0.097	0.045	0.114
P20-G7	0.031	0.088	0.047	0.051	0.051	0.108	0.065	0.040	0.114	0.141	0.045	0.093	0.029	0.022	0.028	0.049	0.059	0.044
P20-H7	0.046	0.057	0.066	0.079	0.089	0.142	0.037	0.039	0.169	0.156	0.049	0.107	0.059	0.047	0.055	0.075	0.067	0.032
P20-H9	0.065	0.116	0.076	0.060	0.060	0.085	0.075	0.065	0.145	0.126	0.034	0.079	0.053	0.033	0.052	0.039	0.033	0.038
P21-A10	0.050	0.104	0.065	0.045	0.044	0.041	0.038	0.038	0.000	0.070	0.030	0.129	0.039	0.049	0.046	0.069	0.022	0.062
P21-B7	0.027	0.087	0.038	0.076	0.064	0.089	0.043	0.061	0.171	0.113	0.061	0.083	0.030	0.039	0.038	0.094	0.062	0.090
P21-C12	0.000	#N/A	#N/A	0.183	0.073	0.097	#N/A	0.000	0.077	0.006	0.000	#N/A	0.045	#N/A	0.014	0.013	0.039	0.098
P21-D11	0.042	0.062	0.045	0.106	0.052	0.051	0.228	0.047	0.207	0.073	0.042	0.073	0.047	0.054	0.113	0.048	0.026	0.066
P21-F11	0.051	0.093	0.029	0.075	0.073	0.109	0.050	0.000	0.071	0.099	0.048	0.061	0.075	0.027	0.060	0.110	0.047	0.055
P21-F8	0.049	0.259	0.070	0.079	0.077	0.262	0.049	0.077	0.105	0.121	0.073	0.159	0.060	0.104	0.051	0.048	0.043	0.067

Name	BR1-8 hpi	BR1-14 hpi	BR1-20 hpi	BR1-30 hpi	BR1-45 hpi	BR1-60 hpi	BR2-8 hpi	BR2-14 hpi	BR2-20 hpi	BR2-30 hpi	BR2-45 hpi	BR2-60 hpi	BR3-8 hpi	BR3-14 hpi	BR3-20 hpi	BR3-30 hpi	BR3-45 hpi	BR3-60 hpi
P22-A2	0.032	0.041	0.035	0.156	0.054	0.098	0.028	0.060	0.130	0.090	0.055	0.164	0.058	0.062	0.016	0.095	0.070	0.067
P22-B3	0.053	0.103	0.073	0.051	0.063	0.071	0.071	0.070	0.000	0.081	0.039	0.045	0.042	0.061	0.112	0.106	0.044	0.042
P22-C7	0.028	0.075	0.018	0.077	0.109	0.031	0.063	0.057	0.031	0.066	0.043	0.074	0.032	0.030	0.015	0.115	0.047	0.055
P22-E8	0.175	0.475	0.100	0.058	0.067	0.284	0.057	0.082	0.053	0.092	0.064	0.086	0.082	0.051	0.051	0.213	0.058	0.142
P22-E9	0.026	0.079	0.015	0.060	0.076	0.041	0.037	0.029	0.052	0.052	0.035	0.040	0.033	0.036	0.019	0.103	0.042	0.063
P22-H2	0.195	0.200	0.172	0.103	0.000	0.015	0.021	0.270	0.088	0.067	0.072	0.066	0.075	0.087	0.000	0.000	#N/A	#N/A
P23-B10	0.030	0.127	0.086	0.196	0.055	0.056	0.073	0.047	0.066	0.057	0.041	0.074	#N/A	#N/A	#N/A	0.031	#N/A	#N/A
P23-C8	0.006	0.243	0.107	0.071	0.078	0.084	0.107	0.050	0.069	0.107	0.069	0.103	0.051	0.055	0.051	0.098	0.060	0.048
P23-G2	0.129	0.255	0.131	0.116	0.067	0.113	0.099	0.118	0.000	0.117	0.057	0.056	0.077	0.076	0.095	0.097	0.060	0.071
P24-A8	0.028	0.336	0.057	0.139	0.124	0.191	0.042	0.062	0.099	0.124	0.026	0.074	0.042	0.032	0.024	0.083	0.045	0.081
P24-B9	0.035	0.131	0.033	0.181	0.122	0.115	0.035	0.043	0.160	0.131	0.041	0.096	0.035	0.040	0.017	0.301	0.059	0.082
P24-C10	0.031	0.070	0.037	0.060	0.089	0.074	0.049	0.032	0.000	0.149	0.057	0.062	0.028	0.029	0.016	0.088	0.053	0.064
P24-E1	0.029	0.039	0.047	0.082	0.093	0.047	0.020	0.030	0.056	0.103	0.059	0.085	0.021	0.036	0.015	0.097	0.032	0.086
P24-F3	0.039	0.091	0.039	0.077	0.063	0.066	0.079	0.045	0.000	0.137	0.044	0.067	0.035	0.057	0.033	0.070	0.041	0.046
P2-B12	0.042	0.107	0.035	0.117	0.346	0.108	0.167	0.130	0.270	0.000	#N/A	0.000	0.032	0.027	0.055	0.056	0.401	0.360
P2-C12	0.023	0.082	0.101	0.065	0.060	0.055	0.094	0.118	0.067	0.135	0.054	0.054	0.049	0.037	0.107	0.073	0.018	0.032
P2-D11	0.025	0.061	0.076	0.111	0.048	0.053	0.064	0.148	0.211	0.081	0.059	0.061	0.035	0.038	0.025	0.079	0.021	0.054
P2-E1	0.067	0.076	0.053	0.263	0.067	0.389	0.131	0.125	0.189	0.081	0.054	0.020	0.097	0.000	0.051	0.076	0.092	0.122
P3-A3	0.121	0.105	0.051	0.060	0.099	0.067	0.076	0.000	0.000	0.244	0.159	0.182	0.160	0.086	0.144	0.000	0.145	0.084
P3-A6	0.043	0.240	#N/A	0.292	0.215	0.097	0.067	0.118	0.184	0.372	0.265	0.056	0.057	0.053	0.076	0.091	0.057	0.239
P3-B6	0.015	0.314	#N/A	0.196	0.175	0.123	0.076	0.000	#N/A	#N/A	0.040	#N/A	0.073	0.031	0.187	0.055	0.129	0.146
P3-D1	0.128	0.175	0.082	0.091	0.167	0.338	0.096	0.086	0.339	0.111	0.084	0.073	0.107	0.077	0.163	0.048	0.059	0.027
P3-D3	0.058	0.250	0.010	0.108	0.103	0.077	0.054	0.134	0.118	0.095	0.047	0.074	0.076	0.085	0.056	0.052	0.052	0.045
P3-D4	0.089	0.062	0.020	0.051	0.127	0.064	0.081	0.035	0.121	0.038	0.038	0.084	0.057	0.048	0.026	0.060	0.053	0.045
P3-E3	0.031	0.058	0.025	0.046	0.054	0.049	0.051	0.049	0.175	0.082	0.036	0.051	0.060	0.000	0.052	0.068	0.028	0.082
P3-E7	0.037	0.096	0.085	0.047	0.052	0.054	0.058	0.110	0.244	0.126	0.077	0.039	0.108	0.031	0.029	0.036	0.051	0.056
P3-E9	0.038	0.128	0.080	0.070	0.053	0.108	0.088	0.170	0.338	0.081	0.052	0.093	0.077	0.044	0.140	0.034	0.058	0.067
P3-F2	0.042	0.103	0.047	0.054	0.027	0.082	0.079	0.168	0.183	0.156	0.054	0.062	0.041	0.058	0.070	0.037	0.053	0.067
P3-F6	0.075	0.061	0.025	0.047	#N/A	#N/A	0.101	0.080	0.185	0.086	0.087	0.067	0.059	0.032	0.060	0.056	0.033	0.050
P3-G3	0.027	0.070	0.036	0.095	0.055	0.061	0.042	0.028	0.136	0.048	0.057	0.045	0.000	0.038	0.030	0.054	0.056	0.048
P3-G4	0.051	0.119	0.036	0.061	0.056	0.059	0.083	0.083	0.084	0.009	0.052	0.049	0.041	0.047	0.031	0.041	0.046	0.063
P3-G7c	0.051	0.220	0.136	0.194	0.071	0.119	0.056	0.093	0.214	0.159	0.089	0.184	0.160	0.083	0.097	0.053	0.032	0.051
P3-G9	0.055	0.071	0.071	0.051	0.010	0.031	0.057	0.051	0.211	0.107	0.045	0.032	0.028	0.025	0.055	0.062	0.030	0.047
P3-H10	0.040	0.065	0.044	0.041	0.072	0.047	0.101	0.071	0.121	0.102	0.064	0.057	0.059	0.046	0.031	0.055	0.042	0.067
P3-H3	0.204	0.152	0.109	0.066	0.077	0.221	0.079	0.206	0.116	0.064	0.132	0.091	0.113	0.226	0.185	0.060	0.043	0.033
P3-H5	0.024	0.255	0.069	0.049	0.093	0.142	0.054	0.084	0.049	0.124	0.045	0.074	0.034	0.036	0.000	0.074	0.071	0.035
P3-H6	0.043	0.048	0.016	0.069	0.061	0.032	0.052	0.034	0.097	0.037	0.059	0.091	0.025	0.039	0.019	0.030	0.068	0.046
P3-H7	0.064	0.092	0.013	0.087	0.043	0.023	0.100	0.104	0.324	0.185	0.082	0.085	0.097	0.065	0.150	0.053	0.047	0.047
P450 #125	0.173	0.093	0.021	0.087	0.137	0.105	0.041	0.044	0.000	0.102	0.041	0.070	0.035	0.035	0.018	0.141	0.066	0.077
P450 #132	0.046	0.063	0.057	0.065	0.068	0.059	0.064	0.125	0.208	0.106	#N/A	0.073	0.012	0.032	0.031	0.051	0.078	0.055
P450 #64	0.021	0.058	0.037	0.034	0.029	0.034	0.115	0.113	0.217	0.099	0.085	0.047	0.018	0.022	0.000	0.052	0.025	0.051
P4-A3	0.038	0.089	0.063	0.051	0.045	0.041	0.095	0.090	0.254	0.072	0.000	0.057	0.019	0.037	0.044	0.081	0.036	0.035
P4-A6	0.035	0.050	0.029	0.051	0.043	0.055	0.093	0.151	0.124	0.057	0.054	0.049	0.040	0.042	0.042	0.052	0.077	0.043
P4-B5	0.036	0.060	0.037	0.056	0.068	0.047	0.102	0.111	0.208	0.104	0.071	0.065	0.075	0.034	0.030	0.037	0.063	0.069
P4-C8	0.057	0.117	0.037	0.038	0.058	0.058	0.111	0.169	0.317	0.119	0.075	0.060	0.073	0.063	0.095	0.076	0.040	0.029
P4-D7	0.056	0.047	0.035	0.045	0.051	0.070	0.039	0.099	0.281	0.089	0.047	0.037	0.070	0.000	0.070	0.067	0.049	0.060
P4-F7	0.000	#N/A	#N/A	#N/A	#N/A	0.000	0.000	0.000	0.000	#N/A	0.000	#N/A	0.000	0.106	0.216	0.086	#N/A	#N/A
P4-F9	0.036	0.136	0.039	0.082	0.017	0.103	0.040	0.047	0.219	0.117	0.035	0.073	0.015	0.057	0.072	0.088	0.039	0.058
P4-G10	0.063	#N/A	0.155	0.196	0.110	#N/A	0.000	0.205	0.234	0.206	0.134	0.232	0.083	0.102	0.109	0.079	#N/A	#N/A
P4-G2	0.046	0.289	0.078	0.100	0.138	0.089	0.089	0.162	0.180	0.076	0.054	0.104	0.063	0.044	0.062	0.073	0.061	0.048
P4-H1	0.039	0.146	0.074	0.094	0.109	0.088	0.076	0.073	0.000	0.074	0.049	0.047	0.034	0.067	0.029	0.123	0.037	0.100
P5-A7	0.107	0.154	0.096	0.086	0.114	0.160	0.178	0.039	0.061	#N/A	#N/A	#N/A	0.048	0.103	0.061	0.062	#N/A	#N/A

Name	BR1-8 hpi	BR1-14 hpi	BR1-20 hpi	BR1-30 hpi	BR1-45 hpi	BR1-60 hpi	BR2-8 hpi	BR2-14 hpi	BR2-20 hpi	BR2-30 hpi	BR2-45 hpi	BR2-60 hpi	BR3-8 hpi	BR3-14 hpi	BR3-20 hpi	BR3-30 hpi	BR3-45 hpi	BR3-60 hpi
P5-B9	0.033	0.057	0.027	0.119	0.089	0.024	0.057	0.048	0.078	0.087	0.067	0.089	0.034	0.027	0.017	0.117	0.049	0.059
P5-C12	0.026	0.091	0.037	0.103	0.100	0.250	0.118	0.056	0.102	0.056	0.054	0.061	0.038	0.000	0.030	0.087	0.032	0.066
P5-D5	0.018	0.052	0.027	0.071	0.090	0.057	0.091	0.113	0.109	0.180	0.159	0.243	0.098	0.069	0.150	0.094	0.067	0.313
P5-D7	0.054	0.328	0.153	0.535	0.403	0.032	0.096	0.085	0.115	0.151	0.163	0.248	0.042	0.031	0.067	0.053	0.081	0.130
P5-D9	0.036	0.192	#N/A	0.113	0.139	0.111	0.097	0.087	0.240	0.135	0.076	0.079	0.167	0.000	0.101	0.121	0.061	0.083
P5-H12	0.055	0.047	0.011	0.077	0.044	0.069	0.063	0.154	0.112	0.079	0.093	0.053	0.021	0.045	0.036	0.058	0.044	0.066
P5-H8	0.044	0.088	0.038	0.081	0.052	0.155	0.094	0.055	0.073	0.037	0.095	0.048	0.027	0.037	0.083	0.044	0.018	0.053
P6-A4	0.070	0.242	0.086	0.066	0.390	0.000	0.064	0.071	0.205	0.117	0.155	0.068	0.040	0.000	0.029	0.047	0.032	0.052
P6-A7	0.028	0.089	0.018	0.093	0.105	0.048	0.044	0.078	0.123	0.086	0.021	0.081	0.042	0.031	0.000	0.095	0.053	0.069
P6-B6	0.026	0.095	0.031	0.111	0.097	0.087	0.081	0.049	0.086	0.090	0.044	0.063	0.049	0.037	0.024	0.086	0.041	0.070
P6-C2	0.017	0.072	0.041	0.100	0.070	0.058	0.047	0.037	0.102	0.155	0.051	0.059	0.030	0.024	0.045	0.064	0.030	0.093
P6-C5	0.185	0.105	0.072	0.255	0.589	#N/A	0.034	0.057	0.105	0.063	0.040	0.050	0.042	0.055	0.071	0.117	0.253	0.578
P6-D5	0.042	0.087	0.036	0.114	0.148	0.083	0.056	0.000	0.000	0.066	0.035	0.090	0.019	0.022	0.032	0.121	0.033	0.089
P6-D6	0.030	0.063	0.029	0.063	0.161	0.056	0.047	0.066	0.112	0.068	0.051	0.079	0.027	0.022	0.014	0.127	0.073	0.060
P6-D8	0.039	0.191	0.043	0.113	0.123	0.085	0.071	0.094	0.152	0.113	0.085	0.088	0.029	0.039	0.063	0.092	0.029	0.071
P6-E4	0.036	0.101	0.037	0.131	0.141	0.056	0.000	0.062	0.123	0.088	0.042	0.086	0.021	0.000	0.035	0.129	0.082	0.041
P6-E7	0.027	0.067	0.032	0.056	0.131	0.062	0.057	0.054	0.068	0.069	0.061	0.059	0.031	0.030	0.039	0.121	0.046	0.053
P6-F1	0.046	0.093	#N/A	0.267	0.000	0.000	0.064	#N/A	#N/A	#N/A	0.000	0.000	0.037	0.039	#N/A	0.067	#N/A	#N/A
P6-F5	0.125	0.131	0.102	0.129	0.185	0.133	0.000	0.091	0.210	0.067	0.105	0.110	0.146	0.120	0.181	0.101	0.108	0.229
P6-F6	0.119	0.228	0.123	0.073	0.061	0.089	0.142	0.049	0.353	0.101	0.066	0.072	0.064	0.016	0.152	0.058	0.031	0.071
P6-F9	0.027	0.089	0.066	0.127	0.062	0.103	0.084	0.000	0.199	0.133	#N/A	0.094	0.000	0.021	0.017	0.108	0.045	0.172
P6-G10	0.072	0.119	0.058	0.074	0.043	0.069	0.159	0.145	0.223	0.041	0.047	0.060	0.069	0.051	0.036	0.050	0.040	0.029
P7-E10	0.133	0.154	0.143	0.111	0.058	0.050	0.080	0.087	0.353	0.207	0.108	0.053	0.099	0.052	0.049	0.075	0.078	0.060
P7-G2	0.037	0.061	0.042	0.086	0.052	0.060	0.132	0.073	0.119	0.126	0.035	0.077	0.044	0.027	0.045	0.055	0.057	0.065
P8-A9	0.173	0.195	0.208	0.097	0.073	0.075	0.128	0.120	0.146	0.218	0.085	0.036	0.118	0.101	0.108	0.059	0.084	0.069
P8-E3	0.148	0.118	0.133	0.061	0.048	0.053	0.141	0.102	0.115	0.083	0.114	0.137	0.101	0.125	0.183	0.043	0.052	0.037
P8-H12	0.097	0.333	0.103	0.438	0.373	0.387	0.017	0.070	0.276	0.037	0.076	0.151	0.041	0.038	0.043	0.080	0.046	0.000
P9-A12	0.084	0.052	0.106	0.074	0.073	0.048	0.109	0.084	0.243	0.015	0.037	0.072	0.031	0.038	0.037	0.037	0.056	0.047
P9-B12	0.040	0.068	0.037	0.051	0.109	0.071	#N/A	0.066	0.120	0.087	0.049	0.099	0.033	0.048	0.031	0.014	0.058	0.039
P9-E2	0.041	0.093	0.055	0.093	0.098	0.086	0.043	0.026	0.000	0.109	0.055	0.085	0.033	0.033	0.020	0.093	0.038	0.040
P9-F3	0.031	0.049	0.015	0.046	0.069	0.047	0.051	0.109	0.195	0.088	0.057	0.080	0.043	0.033	0.064	0.085	0.057	0.047
P9-F6	0.032	0.115	0.023	0.068	0.025	0.064	0.044	0.026	0.163	0.139	0.045	0.058	0.017	0.025	0.049	0.055	0.047	0.099
P9-F6(6)	0.029	0.045	0.036	0.058	0.050	0.070	0.102	0.065	0.141	0.047	0.041	0.079	0.016	0.030	0.044	0.077	0.053	0.073
P9-G3	0.026	0.093	0.027	0.063	0.079	0.073	0.066	0.057	0.069	0.088	0.036	0.104	0.022	0.106	0.046	0.067	0.041	0.063
PATE	0.046	0.074	0.076	0.080	0.051	0.050	0.060	0.106	0.075	0.053	0.085	0.096	0.046	0.033	0.056	0.079	0.062	0.037
PLD b-#2	0.034	0.097	0.046	0.069	0.050	0.129	0.080	0.091	0.179	0.170	#N/A	0.096	#N/A	#N/A	0.017	0.067	#N/A	#N/A
PLD delta	0.029	0.054	0.038	0.072	0.062	0.082	0.041	0.067	0.198	#N/A	#N/A	#N/A	0.027	0.020	0.026	0.069	0.053	0.049
PLD delta #	0.056	0.081	0.076	0.083	0.134	0.111	0.041	0.067	0.198	0.123	0.056	0.105	0.033	0.042	0.000	0.168	0.024	0.089

Table 4: A distribution of CV% $\log_2$(ratios) among technical replicates on the arrays.

Range of CV% $\log_2$(ratio)s	Number of features present
< 10%	1485
10 – 25%	1255
25 – 50%	417
50 – 100%	183
> 100%	95

Each CV was computed from the valid replicate features (spots) of a single cDNA on a given slide. If all features were valid, the number of spots was 12. Ninety-three (3435/3690) percent of the cDNAs on the arrays were valid. (Note: This does not include the spiking controls on the array; 205 cDNAs x 6 time points x 3 biological replications = 3690).

Figure 2-1: The resistant response of Im216 in a growth chamber to a dilute ($5 X 10^6$ cfu/mL) inoculum five days after spot inoculation. With dilute inocula, microscopic hypersensitive responses do not result in tissue collapse. Red anthocyanin appears in the epidermis.

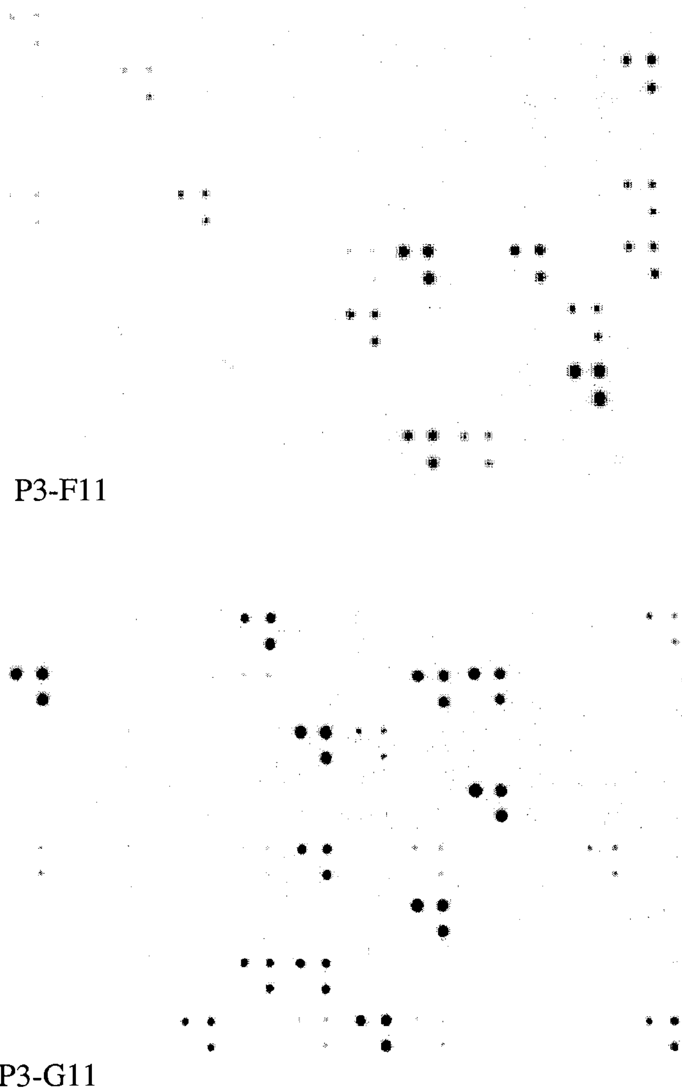


Figure 2-2: Screening of the forward SSH cDNA library for redundant clones. Fluorescein-labeled probes of two previously sequenced, abundant clones were hybridized to a set of clones from the SSH library arrayed in triplicate. Clones that did not hybridize to the previously sequenced probes were picked for sequencing, and their sequences were added to the database.

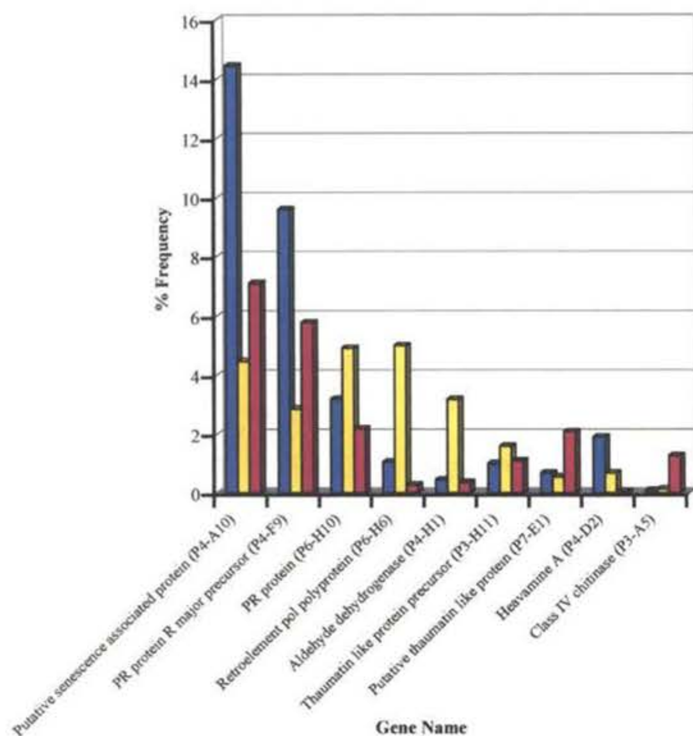


Figure 2-3: Histogram showing the frequency of clones in the Im216 SSH library used in the redundant screening procedure. The blue bar indicates the percent of clones eliminated by hybrdization to a single probe, the yellow bar indicates the percent of clones eliminated in concert with one or another of the eight probes, while the pink bar indicates the percent of clones that were sequenced.

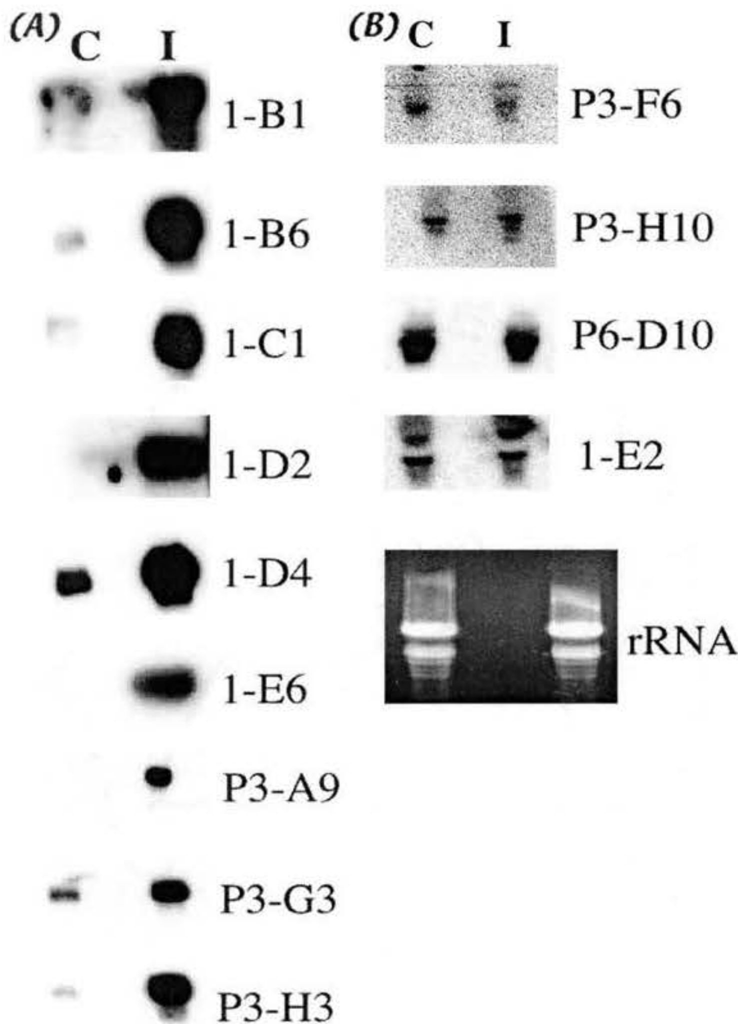


Figure 2-4: RNA blot analyses with clones from the SSH cDNA library. RNA blots were prepared from total RNA (10 μg electrophoresed in each lane) isolated 8, 14, 20, 30, 45 and 60 hours after inoculation and pooled from non-inoculated (C) and *Xcm*-inoculated (I) Im216 leaves. The blots were hybridized individually with ^{32}P-UTP labeled antisense RNA probes made from the indicated clones. Nine transcripts were *Xcm*-induced (A); four were constitutive (B).

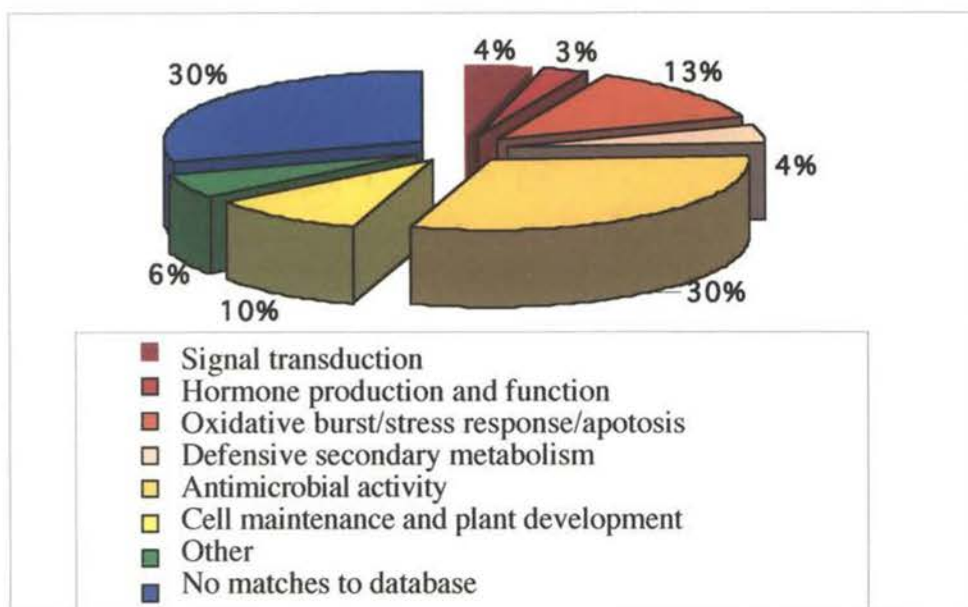


Figure 2-5: Percentages of 161 non-redundant sequences from this study, grouped according to the functions of plant genes with which they have sequence similarity. Signal transduction: proteins such as protein kinases, with a defined role in signaling pathways; Hormone production or function: e.g., cytokinin binding protein; Oxidative burst/stress response/apoptosis: e.g., oxidoreductases, peroxidases and glutathione peroxidases; Defensive secondary metabolism: e.g., cytochrome P450s; Antimicrobial function: different classes of PR proteins; Maintenance of the plant and cell: e.g., ribosomal proteins and RNA binding proteins; Other: proteins whose function is still unclear and which do not belong to any of the previously mentioned categories.

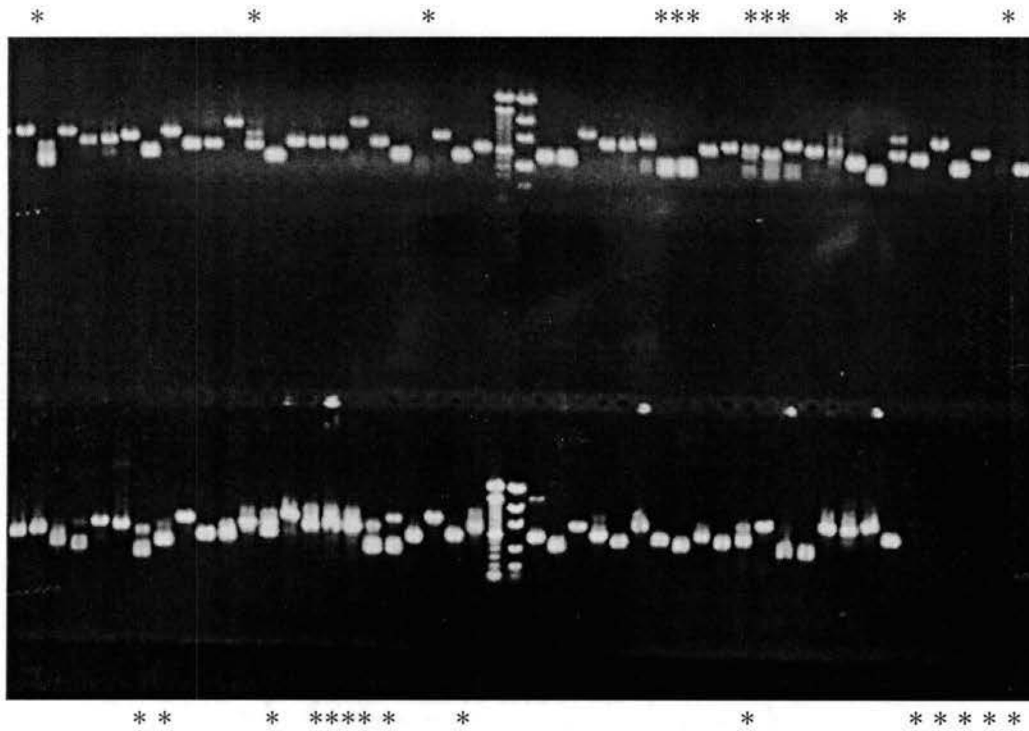


Figure 2-6: Ninety-six PCR products prepared for printing on the arrays were electrophoresed on a 1% agarose gel and stained with ethidium bromide. * Samples with multiple or missing bands whose PCR reactions were repeated.

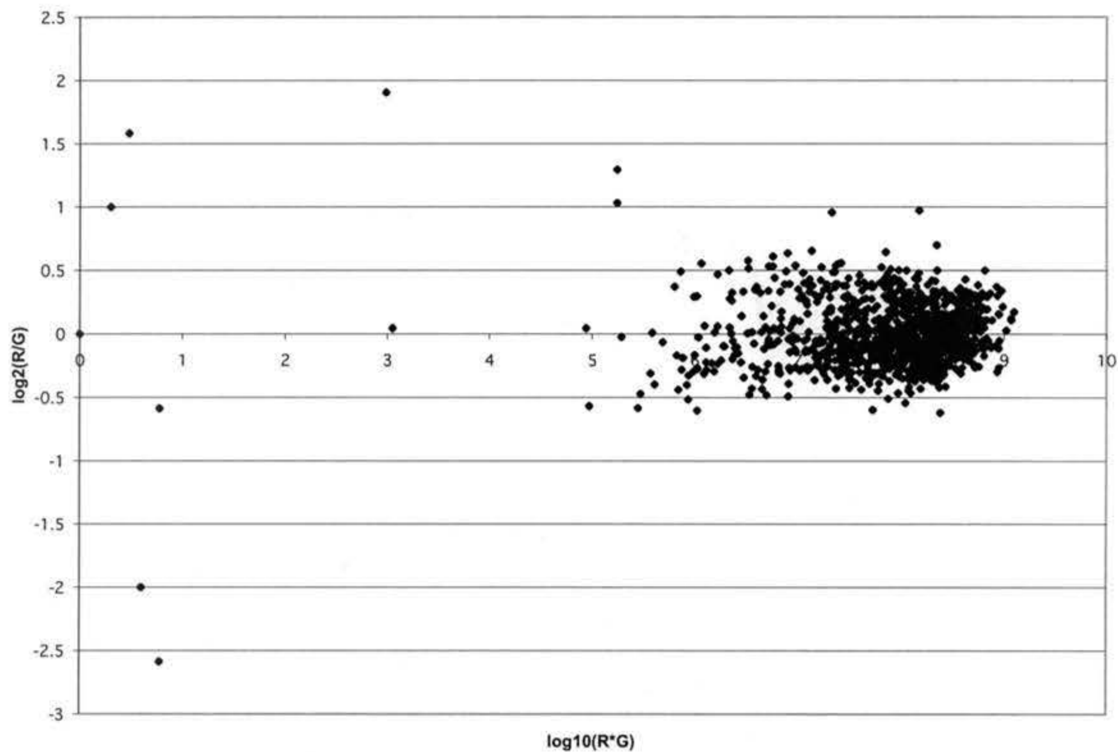
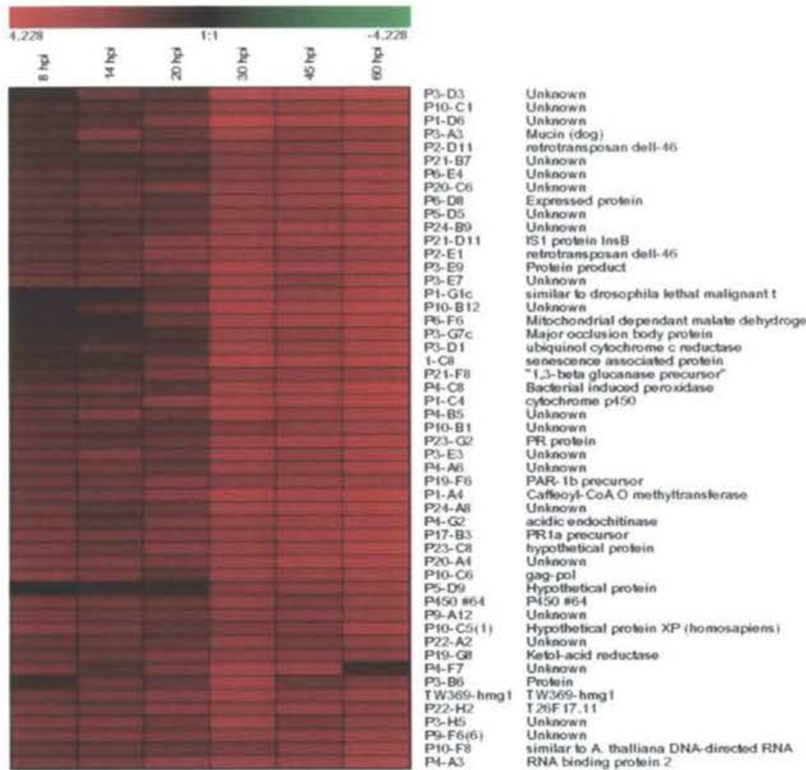
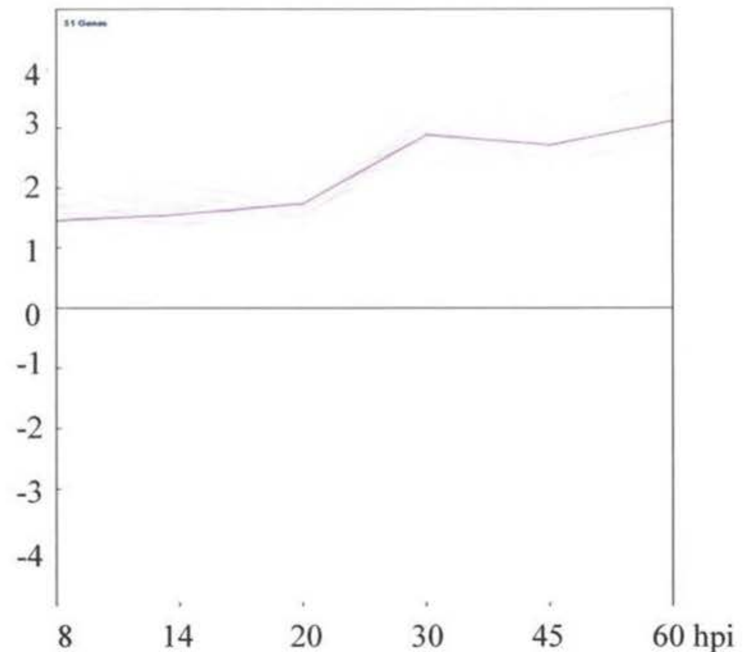


Figure 2-7 : A ratio-intensity plot displaying the $\log_2(R/G)$ ratio for each element in the array as a function of the $\log_{10}(R*G)$ product of intensities, revealing any systemic, intensity-dependent effects in the measured $\log_2$(ratio) values. Data shown here are for elements on the Im216 SSH array in a self versus self hybridization experiment.

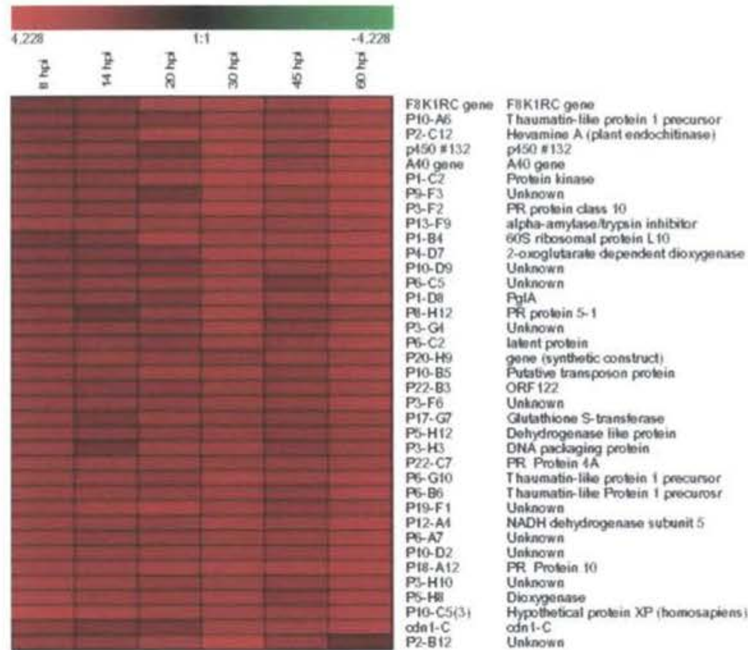
A. Cluster 1- 51 genes

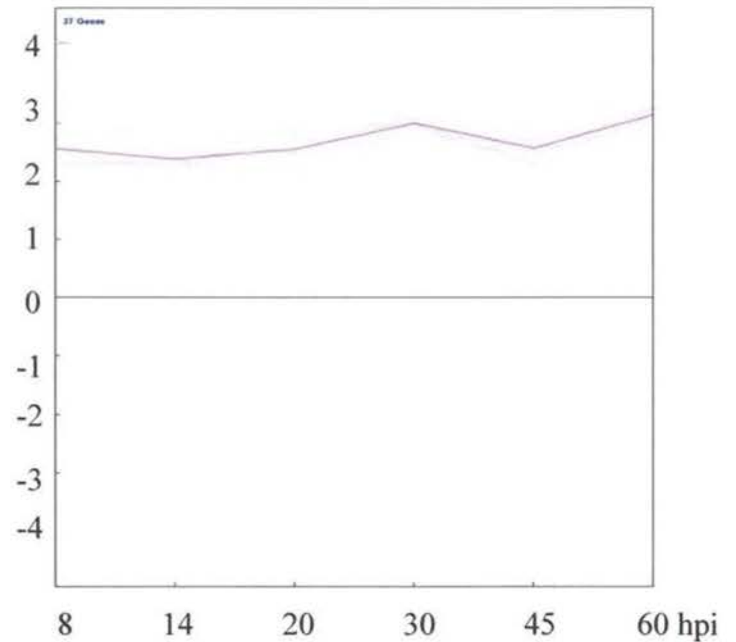




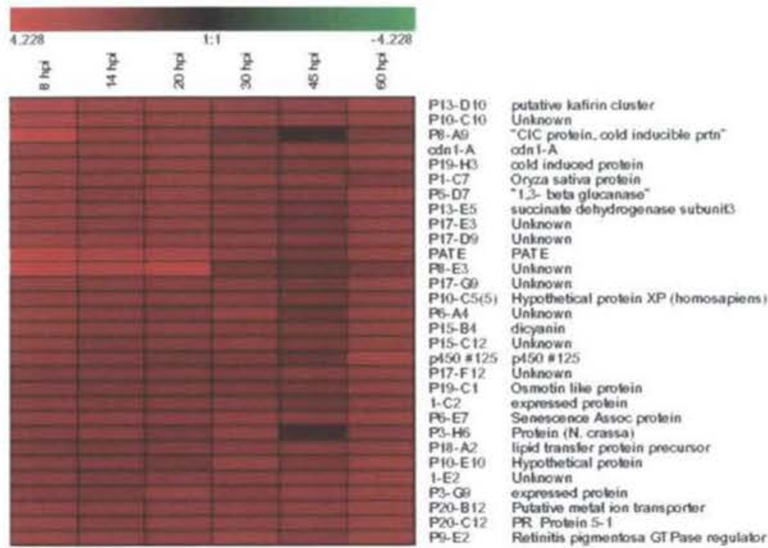
(Figure legend on page 144)

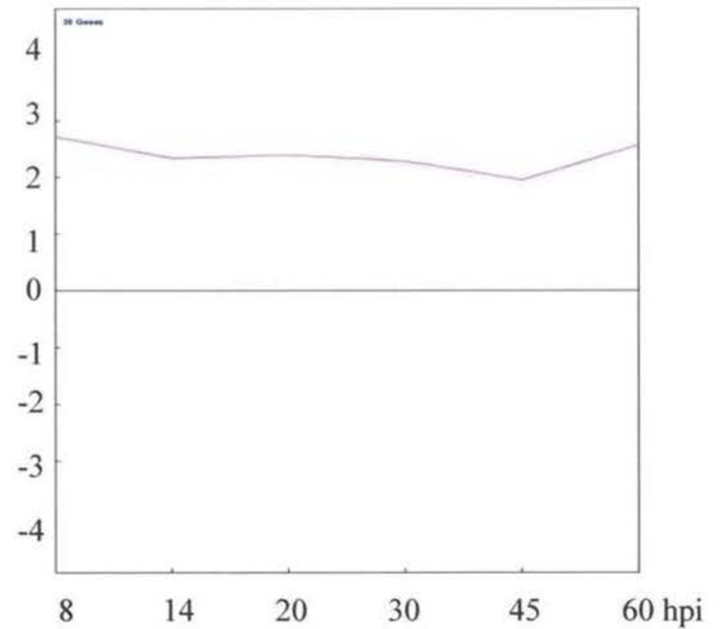
B. Cluster 2 – 37 genes



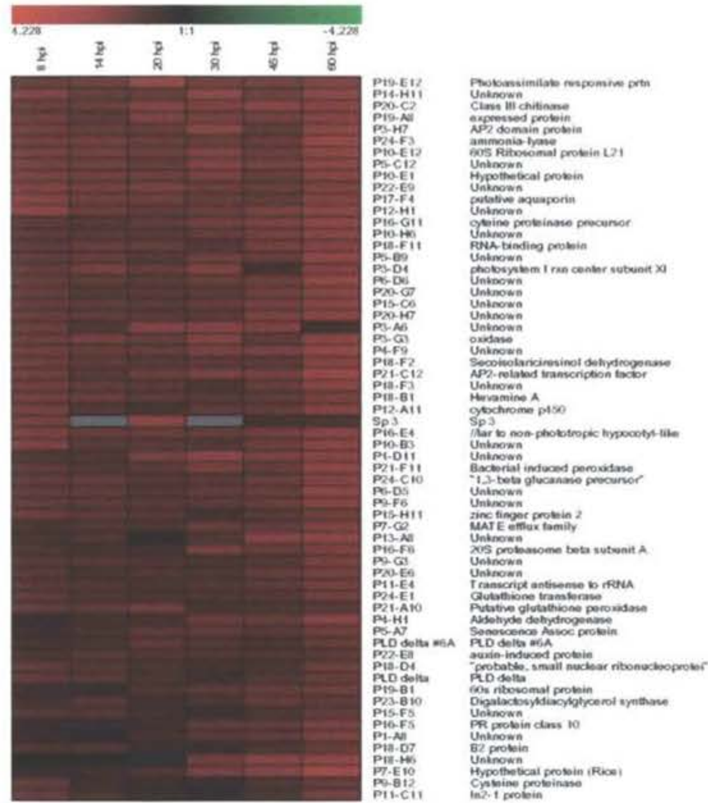


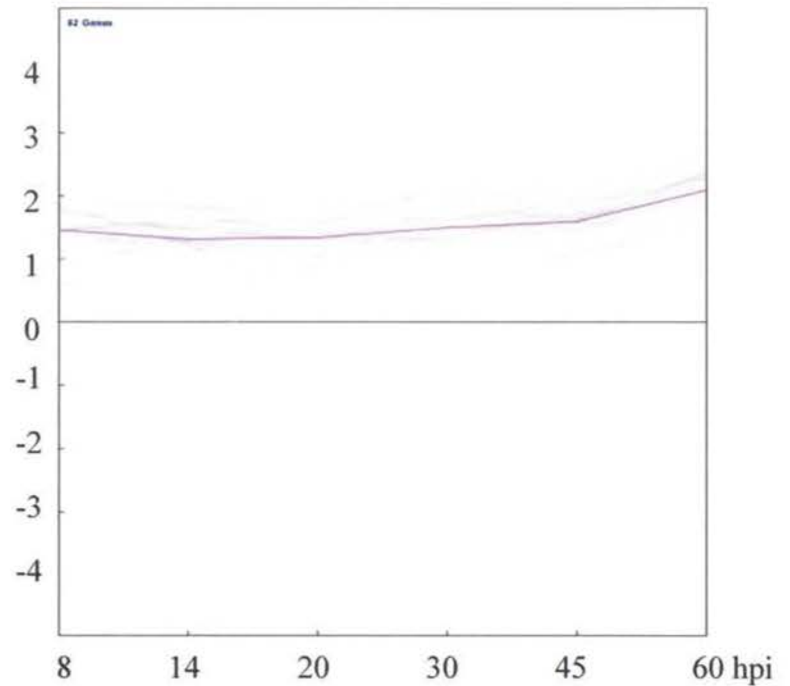
C. Cluster 3 – 30 genes





D. Cluster 4 – 62 genes





E. Cluster 5 – 16 genes

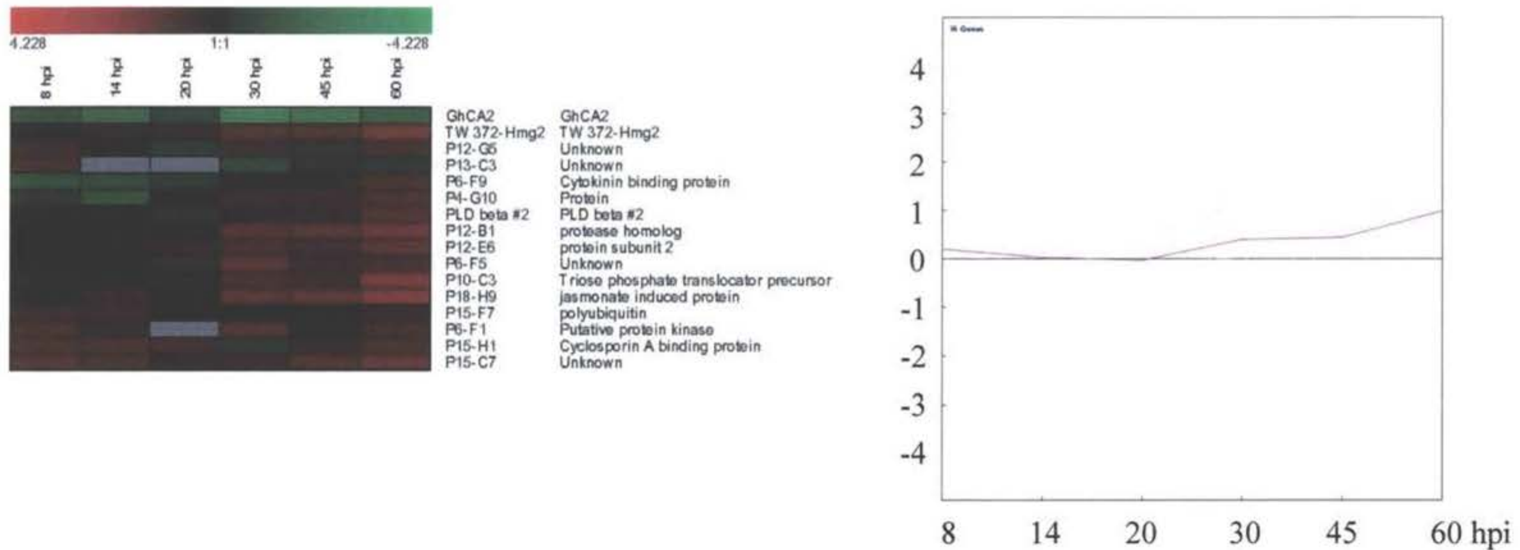
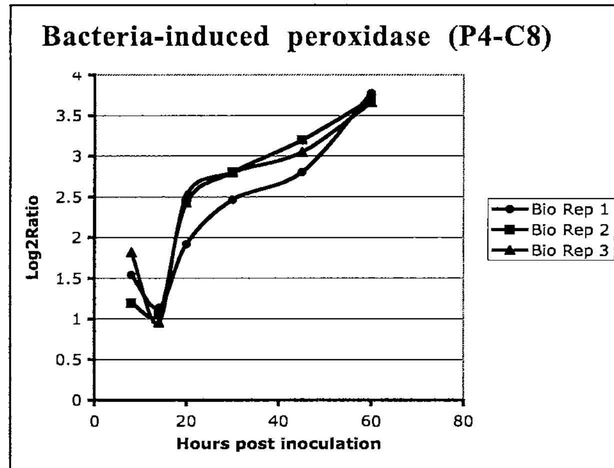
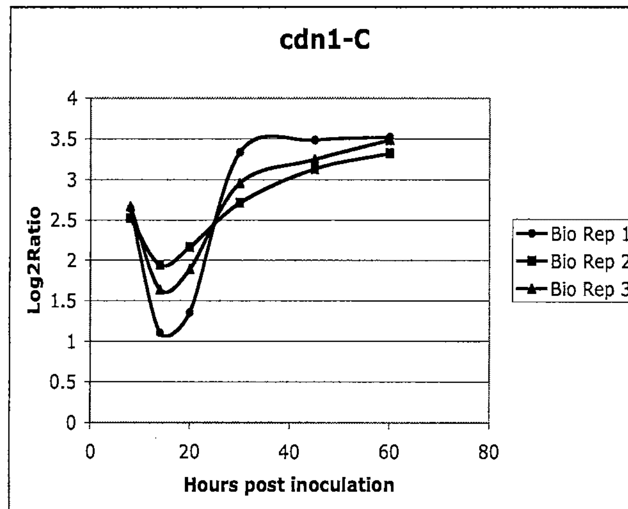


Figure 2-8: Induction of resistance-related genes in response to *Xcm* infection of cotton line Im216 (average of 3 biological replicates). K-means clustering was used to group transcripts by their expression profiles (Also A to D *on previous pages*). Each gene is represented by a single row of colored bars, and the time point is represented by a single column (left). Colored bars, (red, increased transcript abundance; green, decreased transcript abundance; grey, missing or incomplete data; black, no change in transcript levels) represent the ratio of hybridization measurements between corresponding time points in the *Xcm*-infected and uninfected Im216 samples. In the cluster expression plots (right), the abscissa shows the hours after inoculation, and the ordinate shows the $\log_2$(ratio) values.

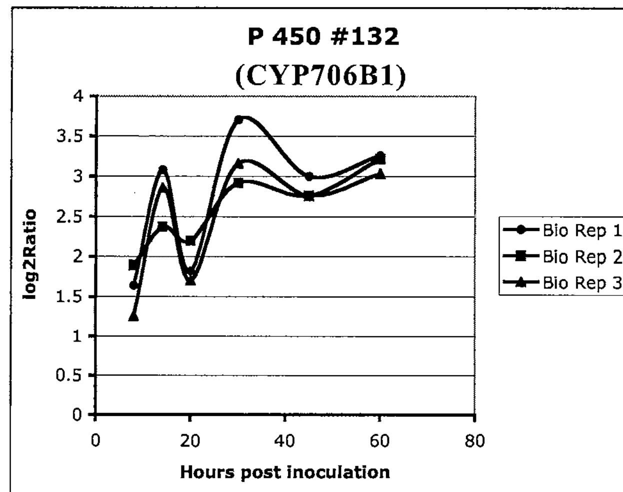
A. Cluster 1



B. Cluster 2

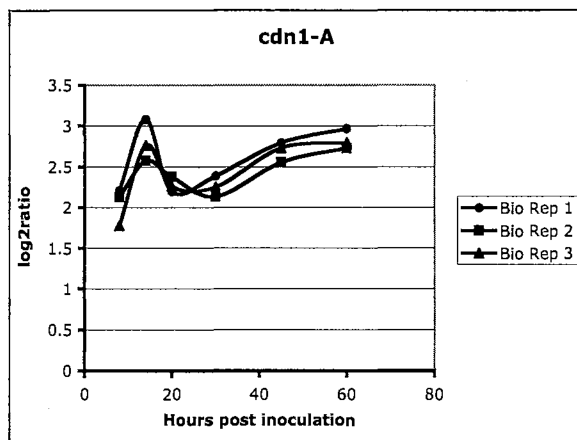


C. Cluster 2

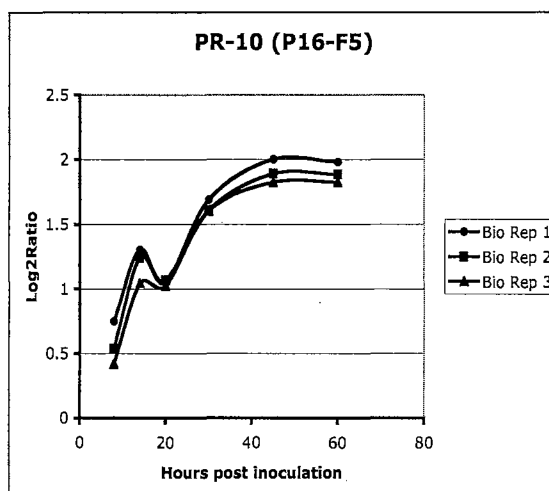


(Figure legend on next page)

D. Cluster 3



E. Cluster 4



F. Cluster 5

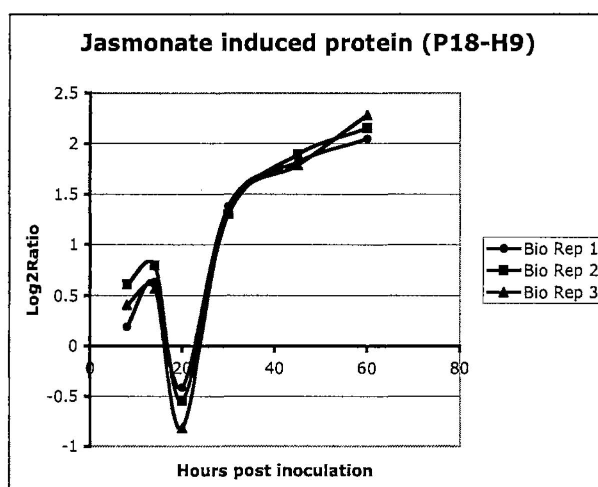


Figure 2-9: Expression profiles of genes from eachof the clusters (B& C are both genes from cluster 2). Each line is the profile of the gene for one biological replicate.

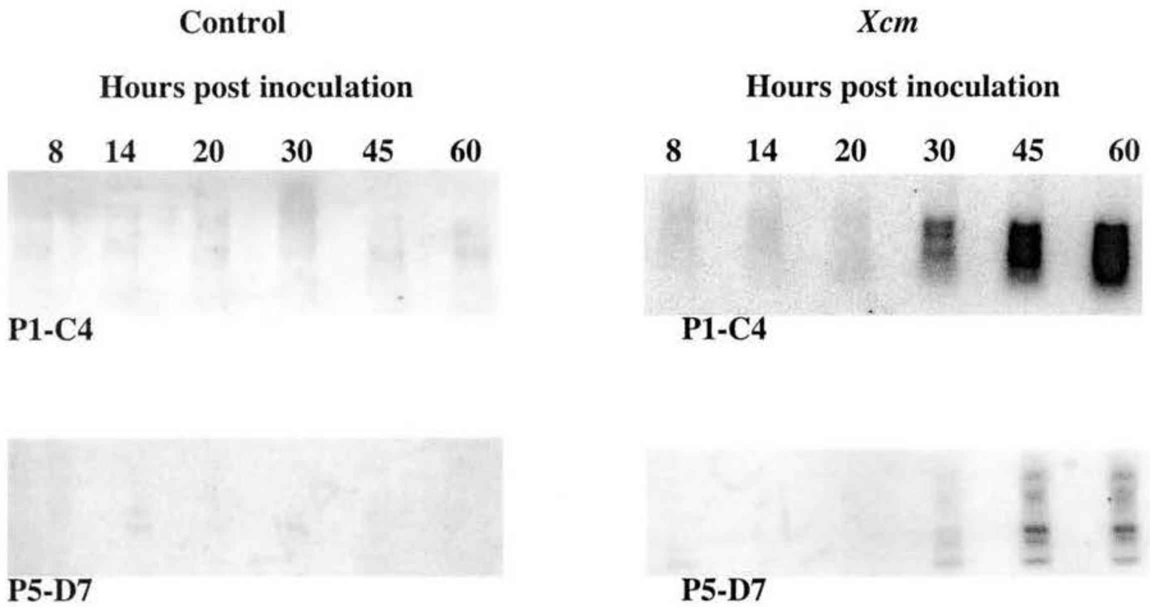
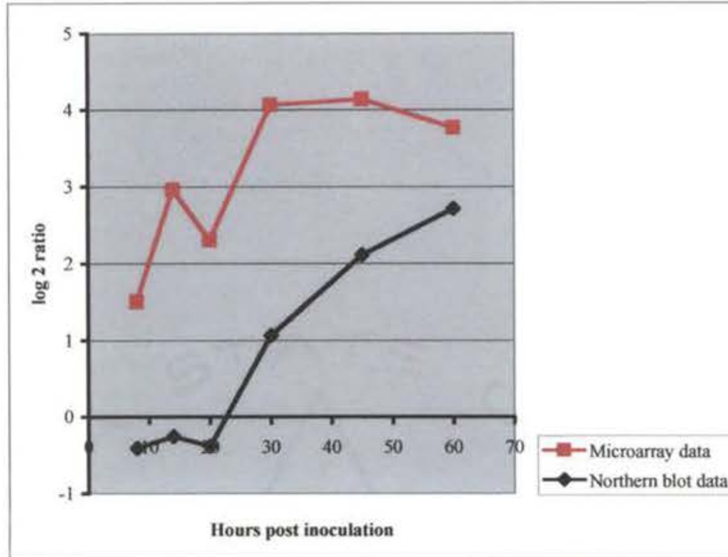


Figure 2-10: RNA blot analyses with two clones from the Im216 SSH library. RNA blots were prepared from total RNA from the first biological replicate (10 μg electrophoresed in each lane) isolated from non-inoculated (control) and *Xcm*-inoculated leaves. The blots were hybridized with ^{32}P-UTP labeled antisense RNA probes made from the indicated clones; for each probe, the blots for control and *Xcm*-inoculated samples were hybridized together in the same bottle and exposed for detection for the same duration.

A.



B.

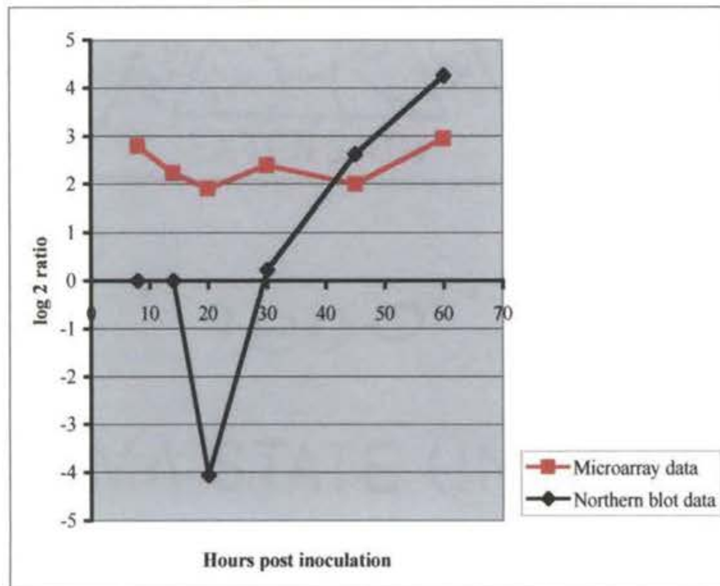
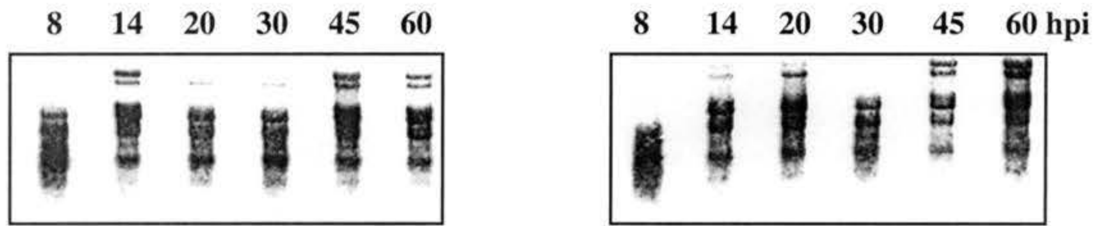


Figure 2-11: Plot comparing data obtained with DNA microarrays and northern blot for P1-C4 (A) and P5-D7 (B) from biological replicate 1. Log_2ratios (*Xcm*-infected/uninfected) of hybridization signals from samples taken at different hours post inoculation were compared between the two methods.

A.



B.

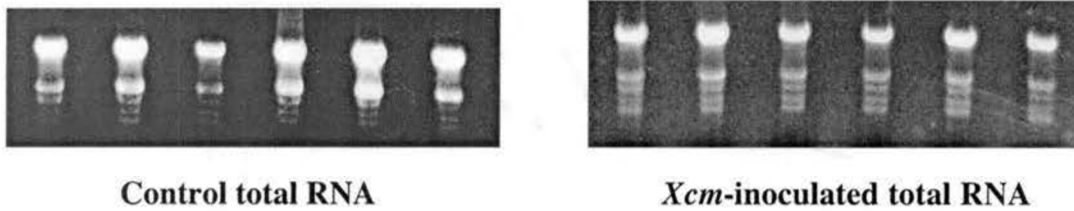
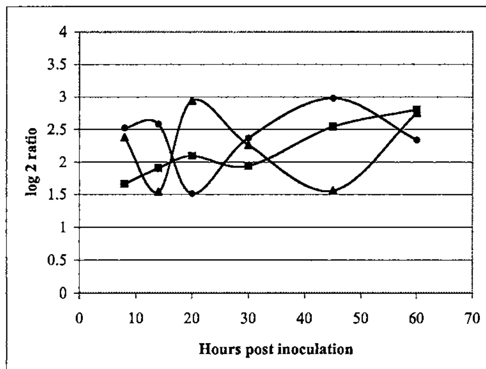
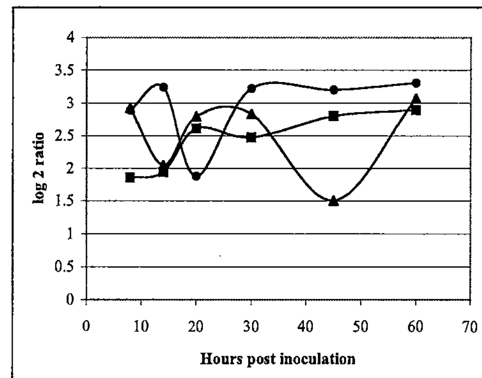


Figure 2-12: Quality check of total RNA samples used for the microarray experiments. Total RNA (10 μg) from a second biological replicate was electrophoresed and blotted onto a GeneScreen membrane. The membrane was stained with methylene blue (A). Total RNA (5 μg) from a third biological replicate was electrophoresed on a 1.2% agarose gel and stained with ethidium bromide solution (B).

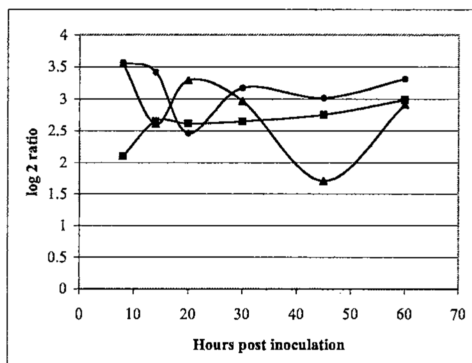
A. Putative unknown gene (1-E2)



B. Putative unknown gene (P3-F6)



C. Putative unknown gene (P3-H10)



D. Putative hypothetical protein (P5-D9)

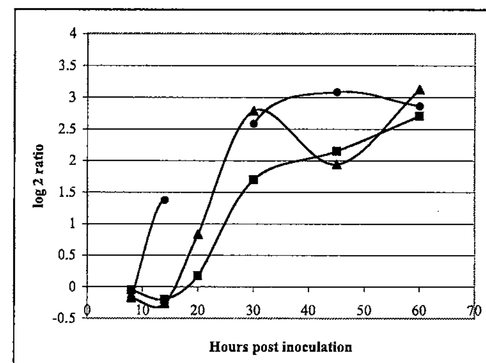
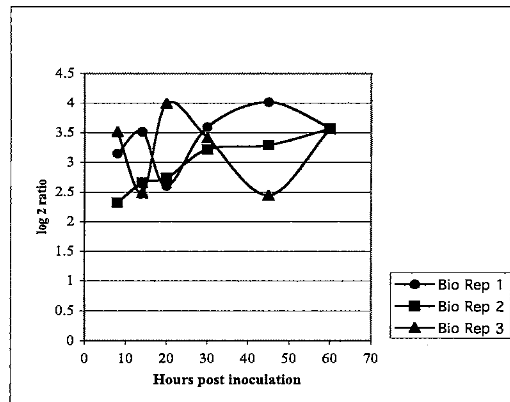


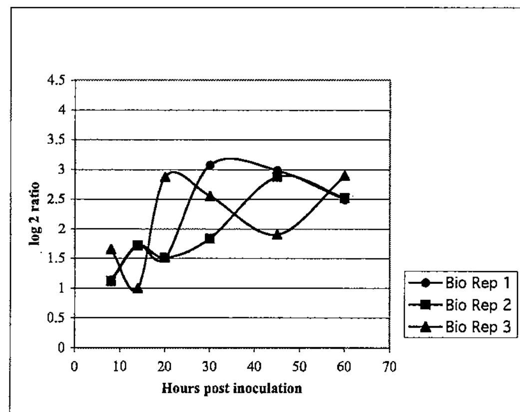
Figure 2-13 : Log_2(ratio) plots of the microarray data of those genes whose expression was shown to be constitutive in the northern blots. [(●) biological replicate 1, (■) biological replicate 2 and (▲) biological replicate 3].

Note: No valid datum was obtained for 20 hpi for biological replicate 3 for P5-D9.

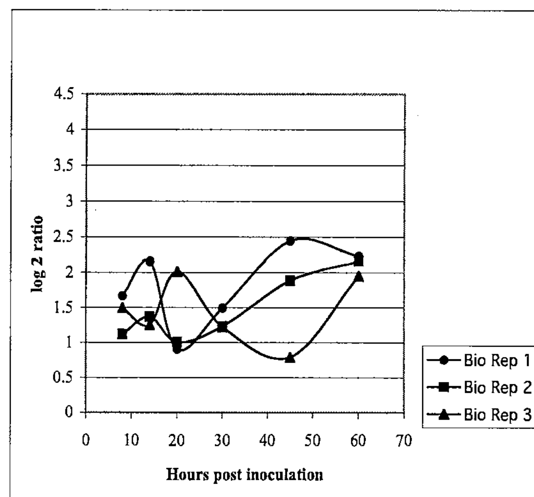
A. PR class 10 protein (P18-A12)



B. 1, 3-β glucanase precursor (P21-F8)



C. 1, 3-β glucanase precursor (P24-C10)



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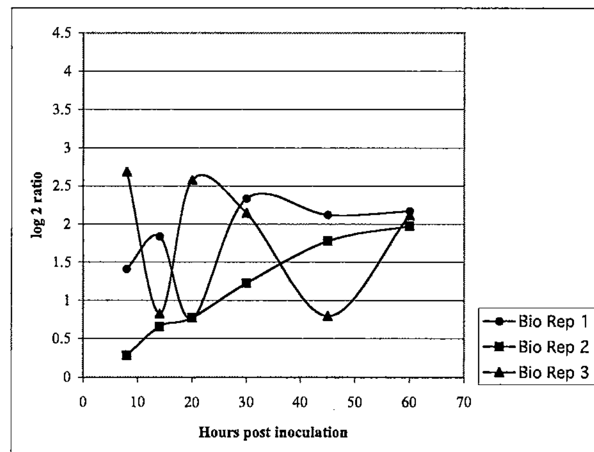
D. Bacteria induced peoxidase (P21-F11)



E. Alcohol dehydrogenase (P18-F2)

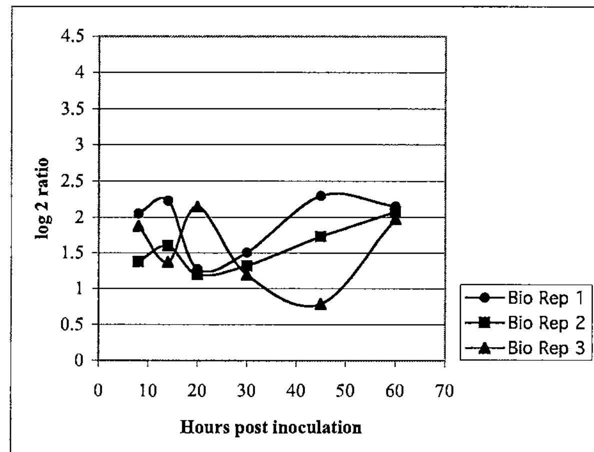


Figure 2-14: Expression profiles of some genes of interest. Each line is the profile of the gene for one biological replicate.

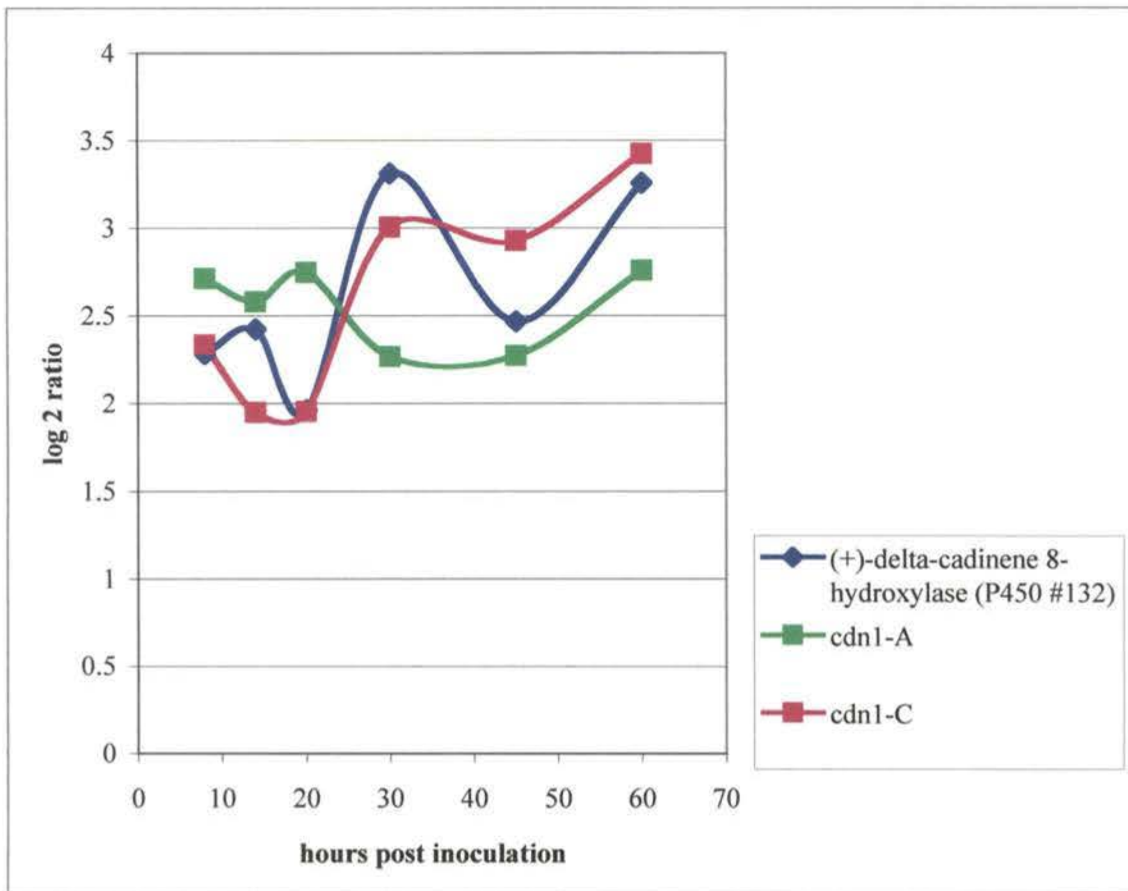
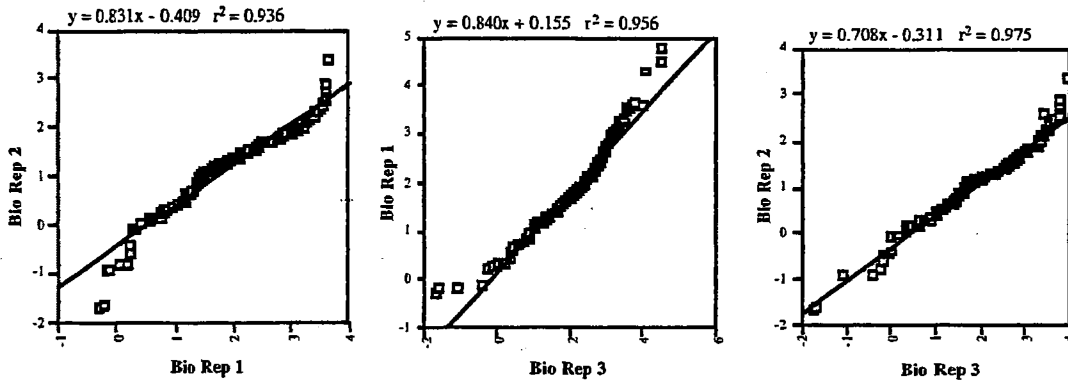
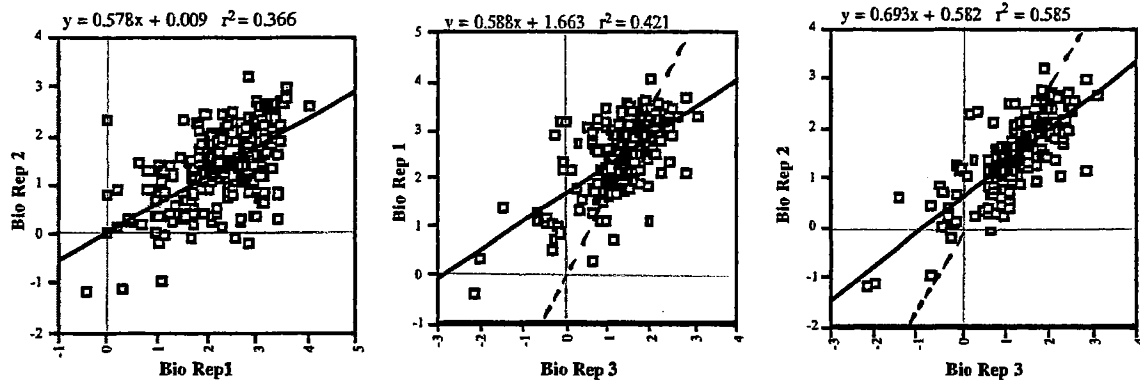


Figure 2-15: Change in expression profile of a P450 in Im216 that hybridizes to an element isolated from *G. arboreum*, and of *cdn1* transcripts in Im216. The abscissa shows the hours after inoculation with *Xanthomonas campestris* pv. *malvacearum*. The ordinate shows the $\log_2$(*Xcm*-infected/uninfected) values, i.e. differential gene expression. The plots are averages of biological replicates 1, 2 and 3.

A.



B.



C.

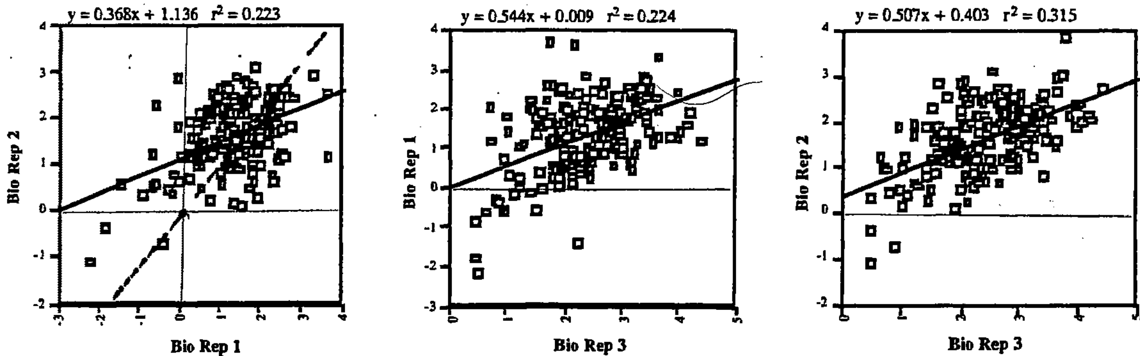
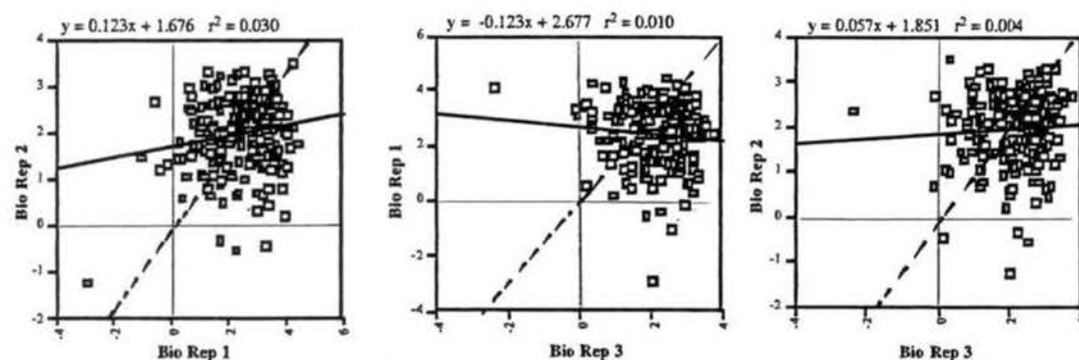
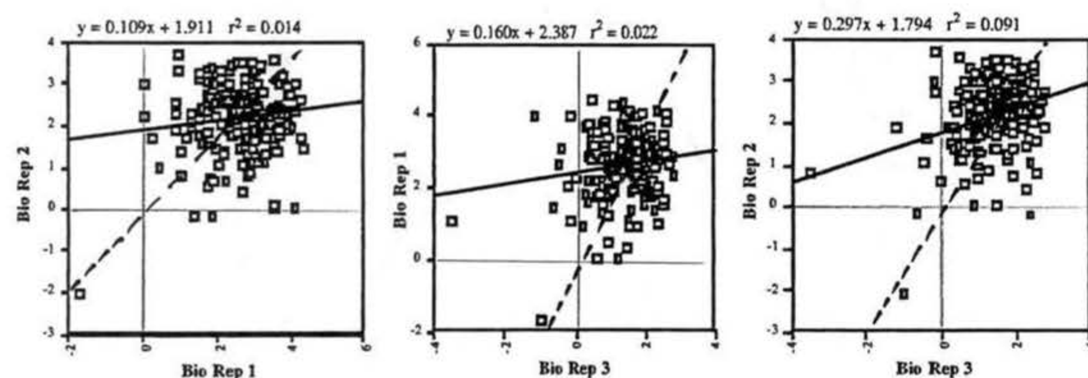


Figure 2-16 A to C: Scatter plot analyses of averaged $\log_2$ratio of array elements from one biological replicate versus averaged log 2 ratio of array elements from another biological replicate at the same time point. Panels A, B and C represent 8, 14 and 20 hours post inoculation.

D.



E.



F.

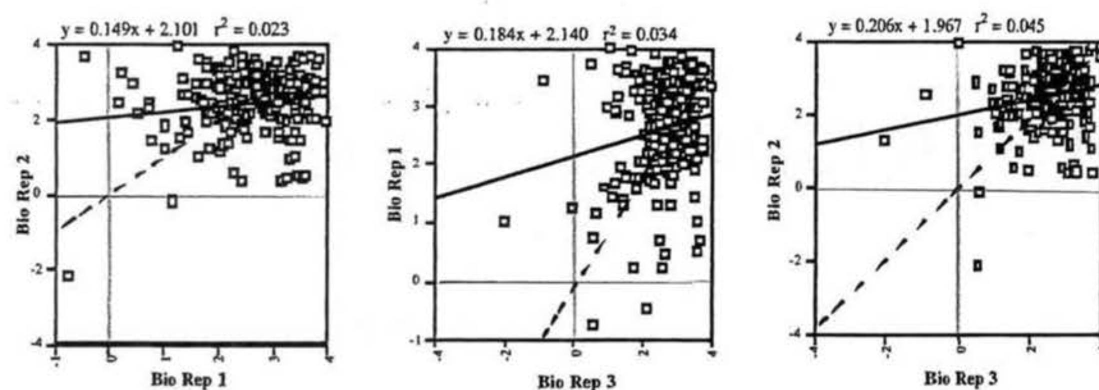


Fig 2-16 D to F: Scatter plot analyses of averaged $\log_2$ratio of array elements from one biological replicate versus averaged log 2 ratio of array elements from another biological replicate at the same time point. Panels D, E and F represent 30, 45 and 60 hours post inoculation respectively.

APPENDICES

APPENDIX A: PROTOCOLS

A. Introduction

Where possible the original source for a protocol is given. Basic solution recipes can be found in (Sambrook et al., 1989) and are not given here. If recipes are different or not found in this manual, they are specified.

B. Mini Total RNA Isolation (from Dr. Marie Petracek's protocol that originated in (Thompson et al., 1983)).

Day 1

1. Start with ~1 gm of tissue (~ half a fully expanded cotton leaf) at liquid nitrogen temperature in a 15 mL round bottom polypropylene tube (Fisher, Catalog # 149561J). Crush the leaves into small pieces using an Rnase-free spatula chilled in liquid nitrogen. Drain all the liquid nitrogen from tubes and, working quickly (it is important not to let the tissue thaw), add phenol/chloroform (USB, Catalog # US75831) and RNA extraction buffer; immediately homogenize with the Polytron. Use 1 mL of each solution per gram fresh weight of tissue. Grind at the maximum setting, until the sample is completely homogenized and a homogeneous emulsion is formed (approximately 45-50 seconds).[1]
2. Place sample on ice. Wash off Polytron probe in a beaker of deionized water at the maximum setting. Dry off probe using a Kimwipe.

[1] This step should be done in the hood, wearing protective clothing, eyewear and gloves.

3. Repeat steps 1-2 until there are enough samples to fit the centrifuge rotor. Centrifuge for 30 minutes at 9000 rpm at 4°C, using either SA600 or SS34 rotors in the Sorvall RC-5B (Refrigerated Superspeed centrifuge, in teaching lab or hallway on the first floor).
4. While samples are spinning, label a set of 1.5 mL microfuge tubes. Pipet 5 µL of 100 mM ATA (if you are making a cDNA library, use DEPC-water).[2]
5. Remove samples from centrifuge; be careful not to disturb the soft pellet. Place the samples on ice and in the hood; remove the aqueous (upper) phase to relabeled tubes. Avoid any loose tissue debris. Note the volume of supernatant removed, as you will need it to determine how much LiCl to add in the next step.
6. Add enough 12M LiCl to make the final concentration =1.5 M LiCl[3] (170 µL of 12M LiCl should be added to a 1 mL sample). Vortex to mix, then place on ice overnight (use an ice bucket in the cold room).

Day 2

7. Spin samples in the microfuge (30 min, 13K rpm) to pellet the RNA. **DO NOT LET THE SAMPLES SIT OR THE PELLET WILL LOOSEN!** Using a pasteur pipet, remove the supernatant.
8. Respin samples for 30 sec at 13K rpm (4°C) to bring down any remaining liquid in the tube. Using a 200 µL pipetman, pull off excess liquid

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[2] ATA is an effective Rnase inhibitor (see Hallick et al., Nucleic Acids Research 4, 3055-3064, 1977). It must be removed before attempting procedures such as S-1 or Rnase mapping, since it inhibits the enzymes involved. Removal involves extracting with phenol/$CHCl_3$ and then putting the RNA through a G-50 spin column.

[3] Good precipitation is obtained with final concentrations of LiCl between 1.5 and 2.0M.

(phenol/chloroform) trapped under the pellet. Push the tip under the pellet and slowly suck up the excess phenol.

9. Dissolve the pellet in 200 µL of 100 µM ATA (or DEPC-water if you are making a cDNA library) per sample. Depending on the size of the pellet you can use as little as 100 µL or as much as 600 µL. Pipet up and down until the pellet is completely in solution. If the sample remains cloudy, add more water or ATA until it becomes clear. (**NOTE:** if the pellet looks green or debris is present, dissolve the pellet in DEPC-water or 100 µM ATA and spin sample for 5 minutes at 13k rpm. Use the supernatant and discard the pellet.)
10. To each 200 µL of resuspended pellet, add 100 µL of 7.5M NH_4OAc and 600 µL of 95% cold EtOH. Place in -70°C for at least 1 hour (Alternatively you can leave the samples at -70°C for 5 hours to overnight).[4]
11. Centrifuge for 30 min at 13k rpm (at 4°C) and remove the supernatant with a pasteur pipet or 200 µL pipetman. Be careful, so as not to loosen the pellet. Spin tubes again for a few seconds to pull down excess EtOH from the sides. Pipet off all remaining EtOH.[5]
12. Dissolve the RNA pellet in 20 µL DEPC-water. You may need to use more if you have a lot more RNA. Mix by pipetting up and down to break up the pellet.[6] Check that the RNA has dissolved completely. If sample is thick, add more water until it is easy to pipet.

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[4] If you are in a hurry, leave the samples at -70°C for 5 hours, otherwise leave overnight.
[5] RNA will not dissolve in ethanolic solutions.
[6] Avoid having the pellet stick to the pipet tip, because if the pellet sticks, you may inadvertently discard it along with the tip! This can be done my mixing gently and a few microliters at a time.

13. Quantitate by measuring the OD 260, using the RNA/DNA quantitator (Pharmacia). We usually make a 1:40 or 1:80 dilutions of the samples. The GeneQuant is most accurate between 0.6 and 2.0 OD. Redilute if you are not in this range.

SOLUTIONS FOR RNA EXTRACTION

a. **RNA Extraction Buffer** (Store at 4°C)

1% SDS[7]

1mM Aurin tricarboxylic acid (ATA)[8], (Sigma, Catalog # A0885)

1% (w/v) tri-isopropylnapthalene-sulfonic acid (TPNS), (Acros Organics, Catalog # 421820250)

4% (w/v) p-aminosalicyclic acid (PAS), (Sigma, Catalog # A-3505)

1X TE (10mM Tris pH 7.5, 1mM EDTA)

2% (v/v) β-mercaptoethanol (BME)[9] (Sigma, Catalog # M-7154)

For 100mL:

10 mL 10% SDS

1 mL of 100mM ATA

1 gm TPNS

4 gm PAS

1 mL of 1.0 M Tris

0.2 mL of 0.5M EDTA

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[7] Wear a mask and gloves while weighing. Make the solution using the hood. An MSDS is on file, situated in the cabinet in the southwest corner of Room 150 NRC (near file extinguisher).
[8] Wear protective clothing and a gloves while weighing. An MSDS is on file, situated in the cabinet in the southwest corner of Room 150 NRC (near file extinguisher).
[9] Use only in the hood wearing protective clothing and gloves.

2 mL β-mercaptoethanol (BME), add to aliquot just before using for extraction.

85.8 mL sterile DEPC treated water

Stir at room temperature until dissolved; store at 4°C.

b. **100mM ATA**

Dissolve 4.22 gm in 100mL sterile DEPC treated water.

c. **12M LiCl**

50.9 gm dissolved in sterile DEPC treated water, up to a final volume of 200 mL.

Note: solution gets hot while dissolving! Filter sterilize once solution has cooled.

d. **7.5M Ammonium Acetate**

Dissolve 28.88 gm in 50mL of sterile DEPC treated water.

C. mRNA Isolation (From the Ambion Poly (A) Pure™ mRNA isolation kit).

Follow the procedure for isolation of mRNA as instructed in the manual.

D. Construction of the Im216 SSH Library (From Clontech's PCR-Select™ cDNA subtraction kit, and Invitrogen's TOPO TA cloning kit for sequencing).

The Clontech manual (Catalog # K1804-1, published 15 September 1999) was followed exactly, except for the primary and secondary PCR reactions (see below), to prepare SSH cDNA.

1° PCR Cycling Parameters		2° PCR Cycling Parameters	
35 cycles:		16 cycles:	
94°C	30 sec	94°C	30 sec
66°C	30sec	68°C	30sec
72°C	1.5 min	72°C	1.5 min

SSH cDNA products are stored at -20°C.

Similarly the Invitrogen manual (TOPO TA cloning kit for sequencing, Catalog # K4575-01, Version E) was followed to transform *E. coli* (TOP10[10] chemically competent cells) with the SSH PCR products.

E. Isolation of Plasmids in a 96-well format[11] (From Edge Biosystems, Catalog # 91528, V13).

Day 1

1. Fill 96-well blocks[12] (2mL growth block, comes with the kit) with 1.2 mL sterile Terrific-broth (TB) containing 150 μg/mL Ampicillin and 50 μg/mL Kanamycin (or any antibiotic marker(s) needed for plasmid selection[13]).
2. Inoculate wells with bacteria by poking a single colony on the agar plate with a sterile P200 pipet tip or take a stab of culture from the cryoplate with a sterile P200 tip whose end has been cut off. Leave the tip in the well until all

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[10] Genotype of TOP10: F^- *mcr*AΔ(*mrr-hsd*RMS-*mcr*BC)Φ80*lac*ZΔM15 Δ*lac*X74*rec*A1 *deo*R *ara*D139Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G.
[11] All solutions mentioned in this section come with the kit. The protocol mentioned here is more detailed than the one in the manual that comes with the kit.
[12] Mark the A1 corner of the block with a sharpie.
[13] Double selection using two antibiotics has shown to increase the plasmid yield.

96-wells have been finished and remove the tips together using an 8-channel or 12-channel pipetman after pipetting up & down several times in each well.

3. Place a polypropylene pad (Edge BioSystems, Catalog # 72344) over the growth block to prevent cross-contamination. If the pads are not available, use a plate sealer (Edge BioSystems, Catalog # 48461) and poke a hole for each well using a sterile needle.[14] Place a lid over the block and culture for 17hrs (grow cultures for no longer than 22 hrs) at 37°C at 350 rpm.

Day 2

1. Centrifuge the 96-well growth block in a microplate carrier (Core facility) at 1500 x g (~2800rpm). This is roughly between speed 5 & 4 on the centrifuge. Spin culture for 5 minutes at 4°C. If the pellets can be seen at the bottom of the block and the supernatant is clear, then the pellets are sufficiently compact.[15]
2. Remove the media immediately by inverting the block. Rap the block firmly over several paper towels to make sure that all excess media has been removed.
3. Add fresh 0.01 volumes Rnase solution to Resuspension buffer and mix.[16] Pour the solution in to a sterile pipet tip box lid.

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[14] Be careful so as not to touch the media, as this will also lead to cross-contamination of your samples.
[15] The pellets can be stored at this step at -80°C. When you are ready to proceed to the next step, thaw the block and spin once again to avoid dislodging of the pellet. Proceed to step 2.
[16] Pour 10mL resuspension buffer into a 15mL Falcon tube. Use a fresh Falcon tube for each solution.

4. Add 100 μL Rnase + Resuspension buffer into each well (use an 8-channel pipetman and gently pipet into each well. Be careful NOT to touch the wells, no need to change tips in between wells).
5. Resuspend the pellets by vortexing for no longer than 45 seconds between speeds 4 & 5, using a plate adaptor. Ensure that the wells are sealed with a plate sealer to avoid cross-contamination.
6. Add 100 μL Lysis buffer.[17]
7. Apply the adhesive plate sealer evenly to the top of the block.
8. Mix by shaking laterally (hold block with both hands and then rotate wrists gently in clockwise and anticlockwise direction) until the lysate is homogeneous and relatively clear. **DO NOT FULLY INVERT THE PLATE!!!!!** This will lead to cross-contamination of the samples.
9. Wait five minutes.
10. Add 100 μL Precipitation Buffer.[18]
11. Mix by vortexing at low speed for 50 seconds to 1 minute. Hold the block flat with both hands on the plate adaptor for even mixing.
12. Add 350 μL of absolute alcohol (at room temperature) to the wells of the receiver plate, using an 8-channel pipetman. Stack the filter plate on top of the receiver plate and secure with tape.

[17] Pipet buffer as described in step 4 of Day 2.
[18] Pipet buffer as described in step 4 of Day 2.

13. Transfer samples from the growth block to the filter plate using an 8-channel pipetman with the wide-bore tips provided with the kit.[19]

14. Centrifuge the contents of the filter plate into the receiver plate at 1500 x g (~2800rpm), this is roughly between speed 5 & 4 on the centrifuge. Centrifuge for 10 minutes.

15. Discard the filter plate. The DNA is pelleted in the ethanol in the receiver plate.

17. Quickly discard the ethanol (invert quickly over sink and gently tap on paper towels to get rid of remaining ethanol) and add 500 μL 70% ethanol to wash the DNA pellet, decant the supernatant immediately as before.

18. Dry the DNA pellets in the vacuum oven in the core facility (**DO NOT** turn on the heat, dry at room temperature, pellets will take approximately 1 to 1.5 hours to dry). Resuspend the pellets in 50 μL 10mM Tris Buffer (pH 8.0).

[19] Leave the white 'cheesy' looking clumps behind in the growth block. Try transferring all the supernatant only. The other "stuff" will only clog the filters.

F. Preparation of Plasmid Preps for Nylon Macroarrays

Denaturing Master Mix (for one 96-well plate, Fisher, Catalog # 262162)

	[Stock]	[Final]
125 µL	6M NaOH	0.3M
600 µL	25X SSC	6X

To each well add 7.25 µL of the master mix

Add 20.75 µL plasmid DNA (my concentrations ranged anywhere from 75 ng/µL to 400 ng/µL).

G. Protocol for Using Robot for MacroArrays

Materials Needed

96-well U-bottom plate (Fisher, Catalog # 262162)

Nylon membrane pre-cut to 8 cm x 12 cm (NEN LifeSciences, Catalog # NEF1018)

95% EtOH (Spray bottle)

Kimwipes

95% EtOH } To fill the wash stations

Sterile Water } To fill the wash stations

Forceps

Whatman Filter Paper

Preparing the Robot

1. Wipe down all membrane holders (including the gray plastic cut out 8 x 12s) with 95% EtOH.
2. With forceps, place the precut membranes in the membrane holders and place the gray weights. Make sure the membranes are completely flat.
3. Add ethanol (95%) and sterile water in their respective reservoirs; also add sterile water in the sonicator (**Note:** for stamping bacteria, replace the sterile water in the plastic container with a 10% bleach solution).
4. Place the stamping plate in the holder (orient the A1 position correctly).

NOTE: Before starting the robot, check and make sure that all the membranes, holders etc. are completely flat on the robot table. Remove all other materials from the area to be used by the robot arm or anything else that might hinder the movement of the robot head.

Starting the Robot

1. Before using the robot, sign the logbook by the computer and read the instruction manual provided.
2. Turn the **RED switch ON** located on the power strip.
3. Turn the **BLACK switch ON** located on the D-TRAN Controller Control Panel.
4. Once the cursor is blinking on the computer screen, push the **GREEN button ON** (D-TRAN Controller Control Panel).

5. Type, “HOME” and “ENTER” on the keyboard. This brings the robot head to the “home” position. (NOTE: This is at a slight angle, so do not worry if the head is not completely straight!).
6. Type “BANK2” and then “START” (Refer to the manual for the different stamping patterns stored in the various banks. Bank 2 is the program I used and generates the isosceles triangle pattern).
7. Type, “Y” if you want to proceed and press “ENTER”.
8. “# of membranes” is then seen on the screen . Type the number of membranes you want stamped of each plate. Press “ENTER”. (Max. # of membranes that can be stamped in one go is 5).
9. “ # of deposits” is then seen on the screen. (**Note:** this is the number of times the same well will be stamped in the same location. Usually one deposit is sufficient).
10. Once you press, “ENTER” the robot will start its stamping process by first rinsing the 96-pin head in the various reservoirs and then drying the pinhead for one minute.
11. According to the “BANK” chosen by you, it will begin printing.
12. Once all your membranes have been printed, place them with forceps gently on Whatman filter paper and proceed to UV-crosslinking the DNA[20] onto the nylon membrane.

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[20] See Protocol H.

Switching off the Robot

1. Press the RED **button OFF** (D-TRAN Controller Control Panel).

2. Turn the BLACK **switch OFF.**

3. Turn off the sonicator by switching off the power strip.

H. UV-Crosslinking DNA to Nylon Membrane

Transfer stamped membranes onto Whatman filter paper and UV crosslink DNA onto the membrane. Place membranes in UV Stratalinker 1800 (Stratagene), press 'Autocrosslink' (1200 μJoules x 1000) and start. Repeat again. Store membranes between filter paper in a box at room temperature.

I. Making Probes for Redundant Screening

1. Choose the clones to make probes from. Follow the criteria: (a) have no (or least number of) *EcoR*I site(s) in the cotton insert and (b) have no (or least number of) *Rsa*I site(s) in the cotton insert.

2. Isolate plasmid from the clones using QIAprep® Spin Miniprep kit (Qiagen, Catalog # 27106). Follow the manual exactly.

3. Run plasmids isolated on a 1% agarose gel at 200V for 30 minutes (using the midsize gel apparatus). Quantitate the plasmid concentration by comparing the band intensities with the markers (pGEM, 200 ng and high mass ladder).

4. Digest plasmid using restriction enzyme *EcoR*I. This will cut out the cotton insert from the vector. Set up the following restriction digest:

Plasmid (10 μg)	10 μL
10X REACT3 buffer	2 μL
*EcoR*I enzyme (50 U/μL)	1 μL
Sterile water	7 μL

Incubate reaction at 37°C (water bath in teaching lab) for one hour.

5. Run products of the restriction digest on a 1.5% agarose gel at 200V for 30 minutes using a mid size gel apparatus). Run a low mass ladder and 100bp ladder as controls on the gel.
6. Extract the cotton insert band of interest from the gel using QIAquick® PCR Purification kit (Qiagen, Catalog # 28104). Follow the manual exactly for gel extraction.
7. Save behind 2 μL of the gel extracted product. The remaining, digest using *Rsa*I restriction enzyme (this will remove the adaptor sequences from the cotton insert).
8. Clean up the *Rsa*I digested product using using QIAquick® PCR Purification kit (Qiagen, Catalog # 28104). Follow the manual exactly for PCR purification. This will remove the small 20 and 22 bp adaptors.
9. Run an aliquote of the *Rsa*I digested and cleaned up product along with the aliquote saved in step 7 on a 1.5% agarose gel as before. Estimate the concentration of the *Rsa*I digested product by comparing the band intensities with that of the low mass ladder. You should notice a slight shift in the band size after *Rsa*I digestion.

10. Proceed with this sample for labeling a redundant probe as described in the *Gene Images* random prime labeling module manual (Amersham Pharmacia Biotech, Catalog # RPN3540). Follow the manual exactly (pages 12-15).
11. To quantitate the efficiency of incorporation of label, follow the protocol in the manual (pages 31-32) exactly.

J. Redundant Screening- Chemiluminescent Detection System for Fluorescein Labeled DNA with CSPD (modified Southern-Light™ protocol from Tropix (volumes mentioned in this protocol are for 12-14 membranes per hybridization bottle.)

Hybridization with Random-Primed Probes

Note: Drain well in between each solution change, holding the mouth of the hybridization bottle to prevent the membranes from sliding out.

1. Wet membranes[21] in 0.25M sodium phosphate, pH7.2 (50 mL per bottle). Rotate the bottle to make sure that the membranes are thoroughly wetted.
2. Prehybridize membranes with Hybridization Buffer (50 mL per bottle) for 1 hour at 65°C in hybridization oven.[22]
3. Dilute the stock probe to 0.33ng/mL[23] in Hybridization Buffer (50 mL per bottle). Denature this diluted probe by placing in a boiling water-bath for 10mins and

[21] Between each membrane and the next, place a nylon mesh cut slightly larger than the 8cm X 12cm membrane. This helps the probe to reach each membrane. Roll the membranes into the hybridization bottle. This must be done before the membranes are wetted as it is easier to do when dry. Once the membranes are wet, they should not be allowed to dry.
[22] This is the minimum time one should prehybridize membranes for. Longer than an hour does not cause a problem.
[23] This is the [probe] used for the redundant probes and was found to work the best for the redundant screening.

immediately place in ice for 2mins.[24] Add the denatured probe to the membranes and incubate overnight at 65°C (optimal hybridization temperature, achieve high stringency with this temperature) with the lights off in Room 149B (this is where the hybridization oven is).

4. Save the diluted probe solution in a 50mL Falcon tube (orange cap) covered in foil, labeled with name of probe, date dilution was made and # of times the diluted probe has been used along with initials of the person who made the diluted probe and store at -20°C (stand up freezer in Room 150).[25] Diluted probe can be used for a total of three times.
5. Next day: Wash membranes with 2X SSC/1%SDS (50 mL per bottle, R.T.) twice for 5 min each.[26]
6. Wash membranes with preheated to 65°C 0.1X SSC/1%SDS (50 mL per bottle). Wash at 65°C in hybridization oven for 22.5 min. REPEAT.
7. Wash membranes with 1X SSC (50 mL per bottle, R.T.) twice for 7.5 min each.[26]
8. Wash blots for 2 x 5 min in Blocking Buffer (50 mL per bottle). Incubate blots for 25 min in fresh Blocking Buffer (50 mL per bottle).[26]
9. Dilute Fluorx-AP ™ conjugate 1:10,000 in Blocking Buffer (50 mL per bottle).

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[24] Add some cold water to the ice so that heat transfer is quick.
[25] Do not tighten the cap of the tube, this prevents cracking of the cap when the tube freezes.
[26] This should be done in the hybridization oven at room temperature with the door of the oven open (otherwise the temperature will creep up) and bottles rotating. All further washings should be done like this unless mentioned otherwise.

10. Incubate blots in conjugate solution for 1 hr in hybridization oven at R.T. with bottles rotating and hybridization oven door open. Discard conjugate solution after incubation.
11. Wash for 5 min in Blocking Buffer (50 mL per bottle), then 3 X 5 min in Wash Buffer (50 mL per bottle).
12. Wash 2 X 2 min in 1X Assay buffer (dilute 10X Assay Buffer 1:10 in nanopure water, 50 mL per bottle).[26]
13. Drain membranes by touching a corner on a paper towel and place 5-7 membranes (with the nylon mesh in between them) in a hybridization bottle. It does not matter if membranes hybridized with different probes are in the same bottle. DO NOT LET THE BLOTS DRY!!!
14. Add 10 mL CSPD® Ready-to-Use substrate solution in each bottle and incubate for 15 minutes in the hybridization oven at room temperature with the oven covered with a black cloth. Save the used CSPD® solution in a dark bottle at 4°C.
15. Drain excess CSPD® and place membranes on saran-wrap. Five membranes can be arranged on a large piece of saran wrap (See Section K for layout). Smooth the plastic wrap and remove any bubbles. Seal the wrap just by folding to cover both sides.

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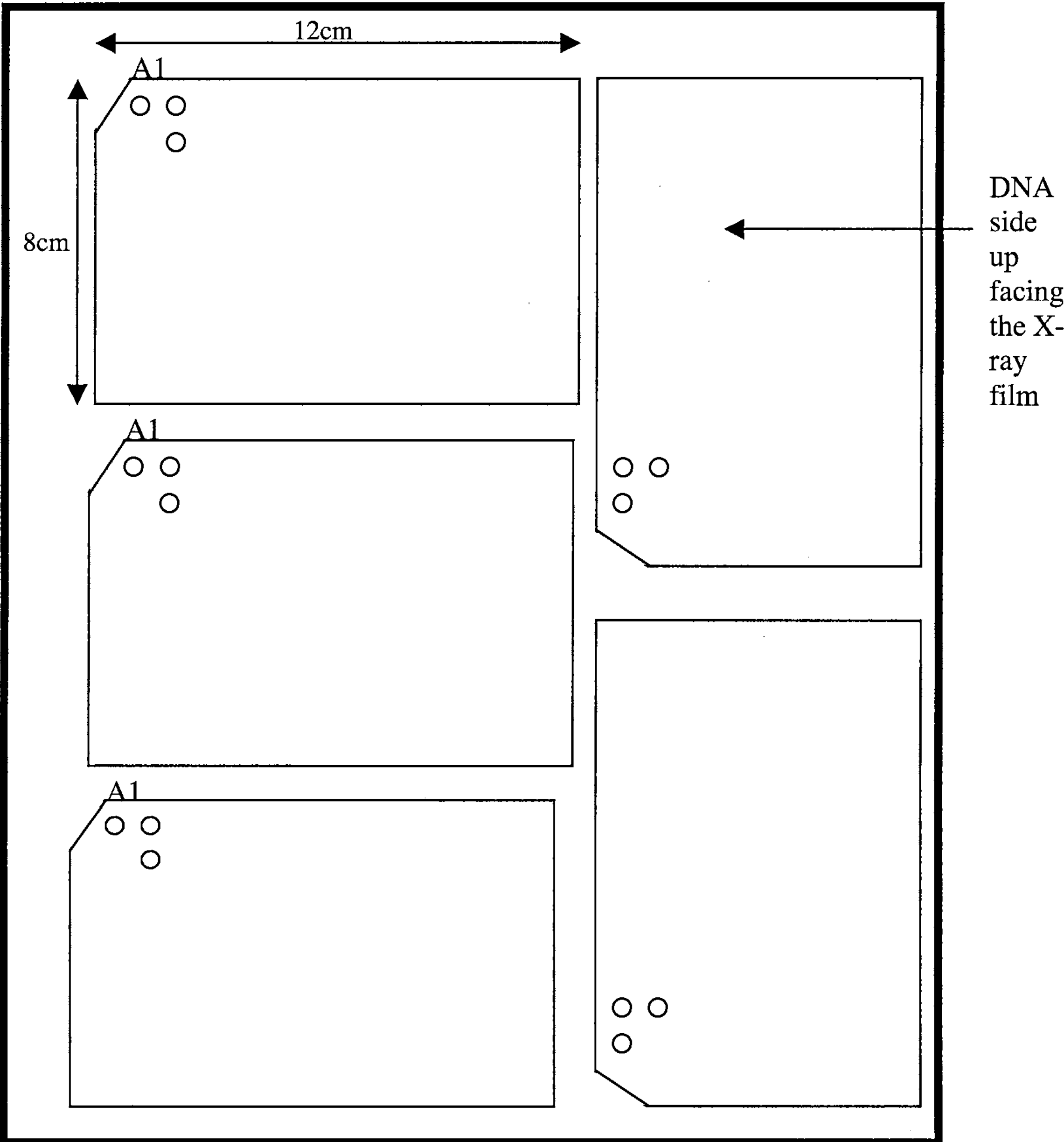
[26] This should be done in the hybridization oven at room temperature with the door of the oven open (otherwise the temperature will creep up) and bottles rotating. All further washings should be done like this unless mentioned otherwise.

16. Membranes are then imaged by placing them in contact with X-ray film (DNA side up exposed to the film) for desired exposure times.

NOTE: Make Blocking Buffer and Wash Buffer just before use. Do not store for long periods of time.

K. <u>Membrane Layout in X-ray Cassette</u> (Fisher Biotech, Catalog # FBXC 810)



L. Running a RNA Gel – Glyoxylation of Total RNA (Dr. Marie Petracek's Method)

1. Calculate the volume you will need for glyoxylation. Add 43 μL of glyoxal mix to every 30 μg of RNA you have. The total volume of RNA should be 11 μL before you add the glyoxal mix. So if you only need 5 μL of RNA for 30 μg, add 6 μL of DEPC-water to bring it to 11 μL, then add 43 μL glyoxal mix. This option is usually used when running a Northern blot. The 30 μg of RNA is then divided into six lanes with 5 μg RNA per lane on the gel.

OR

2. If your RNA is dilute and to glyoxylate you need more than 11 μL, use **option 2**: glyoxylate 5 μg and add enough glyoxal mix to keep the proportions relatively 2:7. So if the RNA is 1 μg/μL, use 5 μL of your RNA and 17.5 μL of the glyoxal mix. This is how high you want to go, since the maximum volume a well in a 100 mL gel will hold is 20 μL.

OR

3. If your RNA is very concentrated and you require less than 5 μL for glyoxylation, add DEPC-water to bring the final volume of the RNA equal to 5 μL and add 17.5 μL of the glyoxal mix.

4. Glyoxal Mix (to be made a Rnase-free 1.5 mL microfuge tube)

   242 μL glyoxal (Sigma, Catalog # G-3140)

   720 μL DMSO (Sigma, Catalog # D-2650)

   144 μL 0.1M PO_4 buffer (pH 7)

   179 μL of 0.2% bromophenol blue in DEPC-water

5. Glyoxylate samples for 1 hr at 50°C (water bath). **Note**: Try and stick pretty close to this time.

6. You can run the glyoxylated RNA samples on a 1.2% agarose gel immediately or store the glyoxylated samples at -80°C.

7. **1.2% RNA Agarose Gel (100 mL, Mid size gel)**

   1.2 g Rnase free agarose

   2 mL 0.5 M PO_4 buffer (pH 7)

   98 mL DEPC-water

   a. Heat or microwave till all the agarose has melted and pour into Rnase free gel apparatus (see end of this section).

   b. Run the gel in 10 mM PO4 buffer (pH 7) at 100 V at room temperature for 3-4 hours (for a midsize gel), recirculate the buffer periodically (place stir bars at the two ends of the gel apparatus and place two stir plates at those ends).

   c. Stain the gel in EtBr solution [5.0 μL stock (10mg/mL) per 100mL DEPC-water] for 30 minutes and then look at the bands using Gel Doc.

Recipe for 10 mM PO_4 buffer (make using autoclaved DEPC-water)

Na_2HPO_4 Mol. Wt. = 141.96 g/mole

NaH_2PO_4 Mol. Wt. = 137.99 g/mole

To make 10mM of Na_2HPO_4 (pH=~9)

$$\frac{141.96g}{mole} \times \frac{0.01mole}{L} \times 1 \text{ liter} = 1.42 \text{ g in one liter}$$

To make 10mM of NaH_2PO_4 (pH=~4.5)

$$\frac{137.99g}{mole} \times \frac{0.01mole}{L} \times 1 \text{ liter} = 1.38 \text{ g in one liter}$$

Titrate NaH_2PO_4 10 mM solution with Na_2HPO_4 solution to get pH=7

Recipe for 0.5 M PO_4 buffer (pH=7)

40.96 g Na_2HPO_4

29 g NaH_2PO_4

Make upto one liter using DI-water, check pH=7 and autoclave once.

Making Rnase-free gel apparatus

1. Wash the gel apparatus, combs and dams with detergent and rinse thoroughly with RO water.
2. Soak the entire apparatus for 20 minutes in 95% ethanol.
3. Discard ethanol and let the apparatus air-dry in the hood for 30 minutes.
4. Soak the entire apparatus for 20 minutes in 3% H_2O_2.
5. Repeat step 4.
6. Rinse with DEPC-treated water. If the apparatus is not being used immediately, leave the DEPC-treated water till use.

M. Northern Blot

1. Refer to section L above for running a RNA gel.
2. Using a large glass Pyrex tray, make the blot in the following order: place the gel support (glass plate) across the tray, two pieces of Whatman 3 MM paper as wicks

on top of the support (breadth of the wicks should be equal to the breadth of the gel, length should be long enough to be submerged in buffer solution for entire duration of the transfer), the gel (nick the left top corner for orientation purposes), a piece of GeneScreen membrane (NEN, Life Sciences, Catalog # NEF 984) cut to the size of the gel (nick the left top corner for orientation purposes), two pieces of Whatman 3 MM filter paper cut to the size of the gel, a stack of paper towel (balanced evenly across the gel) topped with the gel tray and weight.

3. Prewet all of the above with 25 mM PO_4 buffer,except for the paper towels. Roll out air bubbles between each layer using a glass rod. Allow 25 mM PO_4 buffer to soak up overnight.
4. Dismantle the blot the next day and lift membrane off the gel with filter paper and UV crosslink while membrane is still wet.
5. Proceed to prehybridization directly or store membrane between Whatman 3 MM filter paper at room temperature.

Hybridization

1. Make the following hybridization buffer:	[FINAL]
20 mL Formamide (Fisher Scientific)	50%
8 mL 25 X SSC	5 X
8 mL 5 X PE * (See below)	1 X
2.5 mL calf liver RNA (10 mg/mL)	625 µg/mL
1.5 mL DEPC-treated water	

*** 5 X PE**

[Stock]	[Final]	Amount/300 mL
1M Tris (pH 7.5)	250 mM	75 mL
0.5 M EDTA	25 mM	15 mL
0.5% w/v Na pyro PO4	1.5 gm	
5% SDS	15 gm	
1% PVP (40, 000)	3 gm	
1% Ficoll	3 gm	
Water	180 mL	
1 % BSA (see below)	30 mL	
	300 mL	

Autoclave solution. Once it has cooled add 30 mL 10 % BSA (made by adding 10 gm BSA to 100 mL water; filter sterilize the BSA solution through a 0.45 μM apparatus.

Aliquote hybridizatiob buffer into 50 mL Falcon tubes and store at 4°C.

2. In a hybridization bottle, add 10 mL hybridization buffer and blotted membrane. Prehybridize for at least 2 hours at 68°C. Add radiolabeled probe as prepared in Step 3 below and hybridize at 68°C overnight.

3. Preparation of Radiolabeled RNA Probe:

2.5 M NTP (minus UTP)	3 μL
0.1 M DTT	2 μL
DEPC-treated water	5 μL
Inhibitace	1 μL
5 X transcription buffer	5 μL

Plasmid (300-500 ng)	7μL
^{32}P-UTP	5 μL
T3, T7 or Sp6 RNA Polymerase (to make antisense transcript)	1 μL

4. Incuabate reaction at 37°C for 45 minutes and add 75 μL DEPC-treated water and apply entire solution to Sephadex G-50 column prepared in 0.5 μL eppendorf tube.

Washing

1. Prepare one liter of each solution. All washes to be done at 68°C.
2. Wash twice for 15 minutes each in 2X SSC and 0.1% SDS.
3. Wash twice for 15 minutes each in 0.5X SSC and 0.1% SDS.
4. Wrap blot in Saran wrap and expose blot to X-ray film at least overnight at -70°C.

APPENDIX B: MICROARRAY PROTOCOLS

A. Introduction

This section contains protocols used for the microarray experiments. Where possible the original source of the protocol is given. Solution recipes if not found in this section can be found in (Sambrook et al., 1989).

B. Total RNA Isolation (modifications made to the protocol found in (Chang et al., 1993)).

Day 1

1. Add 5 mL extraction buffer (5 mL/gm of tissue) to Rnase free 30 mL corex tubes and set them in a 65°C water bath.
2. Just before adding the tissue to the extraction buffer, add an equal volume (5 mL) of chloroform: isoamyl alcohol (24:1).
3. With the tissue in liquid nitrogen and using a liquid nitrogen cooled spatula rapidly break up the frozen tissue and transfer the pieces to the corex tube containing extraction buffer at 65°C before the tissue thaws. If you simply invert the pop-top tube and tap it against the top of the corex tube, the frozen tissue easily drops into the extraction buffer.
4. Immediately homogenize. Place the polytron probe down into the extraction buffer and turn the control knob to the highest setting.[1] Move the tube up and down, while simultaneously rotating the tube until a homogeneous emulsion is formed. Return the homogenized tissue to the 65°C water bath until all the samples are

[1] Be careful while homogenizing the tissue: The extraction buffer tends to foam a little in the beginning.

homogenized. Rinse the polytron probe with RO water. Several times between samples wipe the probe dry with a Kimwipe.

5. Spin samples (use thin walled adaptors for the Corex tubes) for 20 minutes at 10,000 rpm using a SS34 rotor in Sorvall RC-5B at room temperature.[2]
6. Carefully remove the supernatant to a fresh Rnase-free pop-top tube (Fisher, Catalog # 149561J).[3] Record the volume and color of supernatant removed.
7. Extract once with an equal volume (5 mL) of chloroform:isoamyl alcohol (24:1). Shake vigorously several times by sealing the mouth of the tube with your thumb.[4]
8. Spin for 10 minutes at 10,000 rpm at room temperature using a SS34 rotor in the Sorvall RC-5B (superspeed centrifuge in the teaching lab).[5]
9. Remove supernatant to a fresh Rnase free tube (Fisher, Catalog # 149561J). Record volume and color of the supernatant removed.
10. Add one-fourth volume of 10M LiCl to the supernatant and mix well. Record the volume LiCl added.
11. Vortex thoroughly and leave samples overnight at 4°C in an ice bucket.

Day 2

12. Centrifuge tubes at 10,000 rpm for 20 minutes at 4°C using a SS34 rotor in the Sorvall RC-5B (superspeed centrifuge in the teaching lab).[6]
13. Discard the supernatant. Touch spin and remove any remaining supernatant.
14. Record the pellets' appearance/size and dissolve the pellets in 500 μL of SSTE.

[2] It is vital to the quality and RNA yield that this centrifuging is done at room temperature.
[3] Avoid pipetting debris from the interphase.
[4] Avoid spilling the solutions while shaking and remember, chloroform eats away parafilm and latex!
[5] Need to use the thickwalled adaptors, remove tube caps during centrifugation.
[6] Need to use the thickwalled adaptors, remove tube caps during centrifugation.

When poly A (+) RNA is to be extracted, dissolve total RNA in 0.5% SDS instead of SSTE and proceed with selection directly.

15. Extract once with an equal volume (500μL) of chloroform: isoamyl alcohol (24:1). Shake vigorously and centrifuge samples for 10 minutes at 10,000 rpm at 4°C.
16. Transfer the supernatant to a 2 mL microfuge tube and add two volumes of 95% ethanol, precipitate at –70^{0}C for at least 2 hours. Record the volumes of supernatant obtained and 95% ethanol used.
17. Spin for 20 minutes at 13,000 rpm at 4^{0}Cusing a SS34 rotor in Sorvall RC-5B to pellet the RNA. Remove the supernatant and dry the pellet under the hood at room temperature.[7]
18. Resuspend pellets in 200 μL DEPC-treated water.
19. Cleanup
    a. Add one-tenth volume (20 μL) of 3M Rnase Free sodium acetate (Ambion) and three volumes (600 μL) of 75% Rnase free ethanol (ACS grade). Mix well.
    b. Precipitate overnight at –20°C.

Day 3

20. Wash 3X with 75% ethanol (ACS grade, dilution made in sterile DEPC-water).
21. Dry pellet for 10 to 20 minutes at room temperature under the hood until all the ethanol residue is gone and record the appearance/size of the pellet.
22. Add 20 μL of DEPC water, touch spin, and let samples sit on ice for an hour

[7] It takes approximately 10 to 15 minutes for the pellet to dry under the hood at room temperature.

to completely rehydrate.

23. Make dilutions in 1X TE buffer to read the absorbance.

24. Store at –80°C.

C. <u>PCR Reactions for SSH Clones</u> – 96 well format.

1. Set up the following PCR reactions in a 96 well plate (Fisher Scientific, Catalog # 05 500-68).

Reagents	**1X**	**100X**	**[FINAL]**
DNA Template	2 μL (5ng/μL)		10 ng
10X PCR buffer	5.0 μL	500 μL	1X
25mM dNTP each	0.4 μL	40 μL	0.2 mM
25mM MgCl2	3.0 μL	300 μL	1.5 mM
20μM Forward Primer	0.6 μL	60 μL	0.25 μM
20μM Reverse Primer	0.6 μL	60 μL	0.25 μM
Sterile Water	37.9 μL	3790 μL	
Taq B polymerase (5U/μL)	0.5 μL	50 μL	2.5 U
	50 μL	4800 μL	

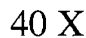
2. Add 48 μL of the master mix per well. Cover each well with a PCR strip (Fisher Scientific, Catalog # 05 407-4B). Vortex to mix well, quick spin to collect contents at the bottom of each well.

3. Cycling parameters:

Step 1: 3 min @ 95°C

Step 2: 1 min @ 95°C

Step 3: 30 sec @ 55°C



40 X

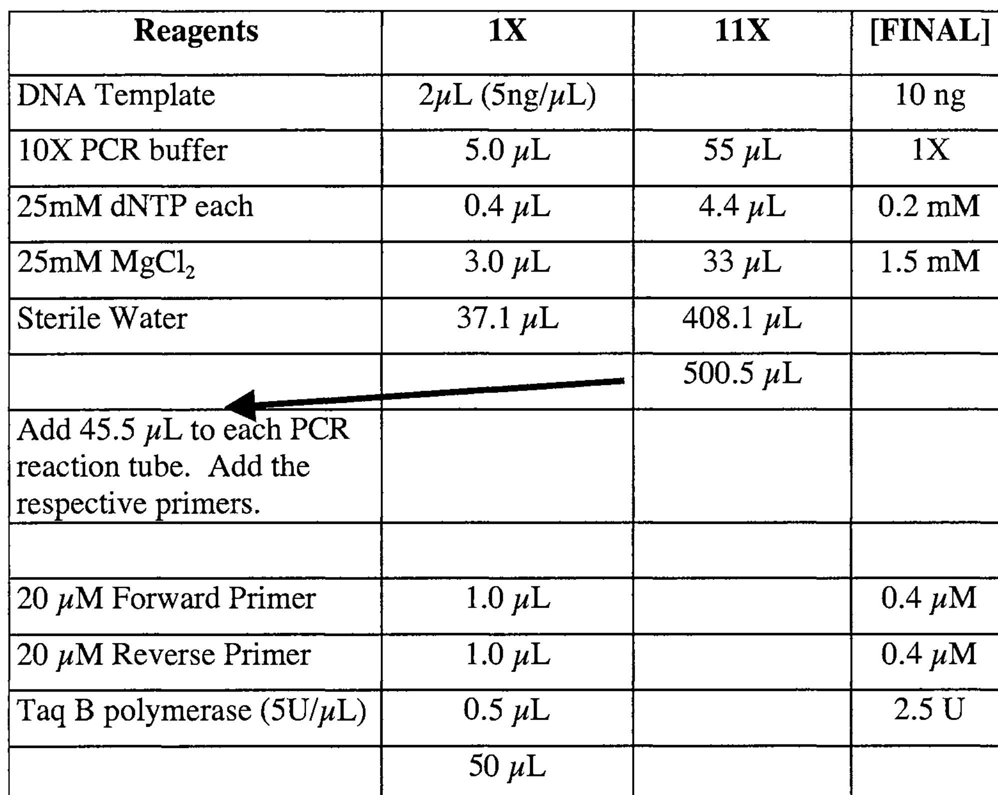
Step 4: 2 min @ 72°C

Step 5: 10 min @ 72°C

Step 6: ∞ @ 4°C

D. PCR Reaction for AFGC Non-Plant Controls.

1. Set up PCR reactions as shown below:



Reagents	1X	11X	[FINAL]
DNA Template	2μL (5ng/μL)		10 ng
10X PCR buffer	5.0 μL	55 μL	1X
25mM dNTP each	0.4 μL	4.4 μL	0.2 mM
25mM $MgCl_2$	3.0 μL	33 μL	1.5 mM
Sterile Water	37.1 μL	408.1 μL	
		500.5 μL	
Add 45.5 μL to each PCR reaction tube. Add the respective primers.			
20 μM Forward Primer	1.0 μL		0.4 μM
20 μM Reverse Primer	1.0 μL		0.4 μM
Taq B polymerase (5U/μL)	0.5 μL		2.5 U
	50 μL		

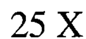
2. Vortex tubes to thoroughly mix. Briefly centrifuge to bring down the contents of each tube.

3. Cycling parameters:

Step 1: 1 min @ 94°C

Step 2: 30 sec @ 94°C

Step 3: 30 sec @ 65°C



Step 4: 1 min @ 72°C

Step 5: 2 min @ 72°C

Step 6: ∞ @ 4°C

E. PCR Reactions for p450 genes; #64, #125 and #132.

1. Set up the following PCR reactions:

Reagents	1X	5X	[FINAL]
DNA Template	2μL(5ng/μL)		10 ng
10X PCR buffer	5.0 μL	25 μL	1X
25mM dNTP each	0.8 μL	4 μL	0.4 mM
25mM MgCl2	4.0 μL	20 μL	2.0 mM
20μM pYeDO60 Primer 1	1.4 μL	7 μL	0.56 μM
20μM pYeDO60 Primer 2	1.4 μL	7 μL	0.56 μM
Sterile Water	34.4 μL	172 μL	
Taq B polymerase (5U/μL)	1.0 μL	5 μL	5 U
	50 μL	240 μL	

2. Add 48 μL to each PCR tube. Vortex thoroughly to mix and briefly centrifuge tube to collect contents.

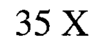
3. Cycling parameters:

Step 1: 2 min @ 94°C

Step 2: 45 sec @ 94°C

Step 3: 45 sec @ 50°C

Step 4: 50sec @ 72°C



Step 5: 10 min @ 72°C

Step 6: ∞ @ 4°C

F. Invitro transcription Reaction for RNA Spikes.

Riboprobe Invitro Transcription Systems Kit (Promega) was used to generate the RNA spikes. The reactions were scaled up as follows:

		[Final]
5 X Buffer	20μL	1X
100 mM DTT	10μL	10 mM
Recombinant Rnasin Riboprobe Inhibitor (400U/μL)	3μL	120 U
rATP + rGTP + rCTP + rUTP (2.5 mM each, 5 μL each)	20μL	
DNA template (40 ng/μL)	20μL	800 ng
T 3 polymerase (170 U)	3μL	51 U
Nuclease free water	21μL	
Final Volume =	100μL	

Set up reactions for each control at room temperature, mix well, briefly centrifuge and incubate for 1.5 hours at 37°C. After incubation, follow the protocol for DNA template removal (page 12 of manual) exactly.

G. Rehydration of Corning GAPS II slides

1. Fill a beaker of hot water (~ 39 °C) and pour into the humid chamber (Sigma, Catalog # H 6644) till a depth of ~ 5 mm. Place one slide (DNA side down) in the center and cover the chamber. Let the slide hydrate for 30 seconds (you will see features on your slides turn to small beads).
2. Immediately dry (snap dry) the slides over a heating plate, heated to 75 to 80°C (leave slides on hot plate for the count of three).

H. Immobilization of DNA onto Slides

1. After rehydrating and snap-drying the slides, bake the printed slides (DNA-side up) for 3 hours in an oven (Core facility) at 85°C.
2. To remove the excess unbound DNA (probe) from the slide, wash twice for 5 minutes each in 0.1% SDS at RT. Put the slides in the slots in a glass container (as shown in the picture below), and stir over a stir plate with a stir-bar. Make sure the stir-bar does not hit the slides, so as to damage them or scratch the slides. Alternatively you can wash each slide in 50 mL Falcon tubes individually.

Slide staining container (Fisher Scientific)

I. Denaturation of DNA on slides.

Boil a beaker of nanopure water; add the slides. You will notice that the bubbles stop; once the water begins to boil again, start the timer. Boil slides for two minutes. Slides are snap cooled by plunging them into a Falcon tube containing chilled 95% EtOH. Dry the slides by centrifugation. To centrifuge the slides, you can use a slide centrifuge, located in the microarray room (see below).

Slide Centrifuge (TeleChem)

Note: If you do not have a slide centrifuge, you can also put the slide in an empty 50 mL Falcon tube and centrifuge the tube.

J. Prehybridization of Slides.

1. Prepare the following prehybridization mix in a 50 mL Falcon tube:

		[Final]
BSA Fraction V	0.5 gm	1%
Formamide	12.5 mL	25%
25 X SSC	10 mL	5 X
20% SDS	250 μL	0.1%
Sterile water	Bring to Final Volume of 50 mL	

2. Prehybridize slide for a minimum of two hours at 42°C.
3. Rinse slides with sterile water and dry.

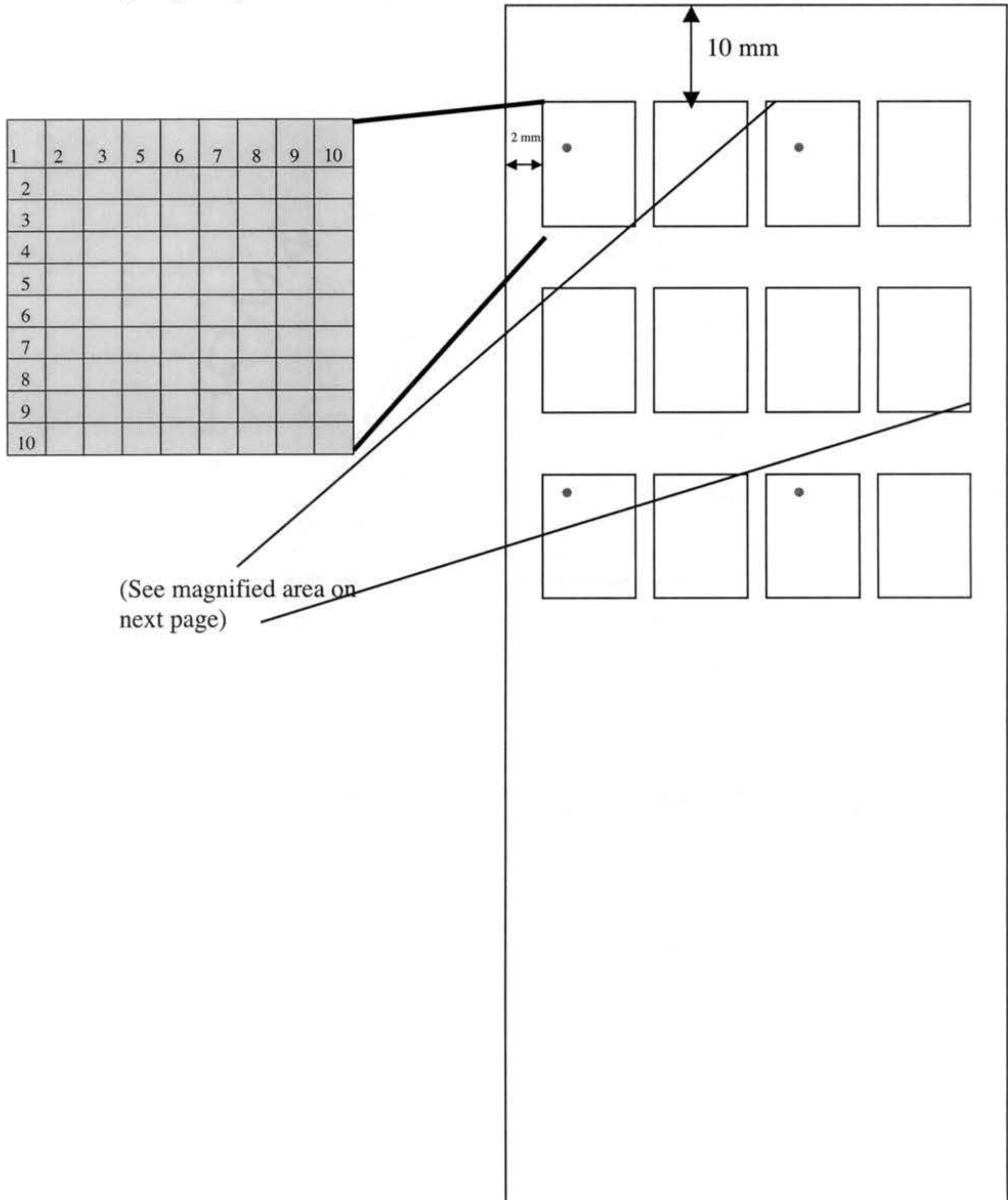
K. Hybridization of Total RNA to Array.

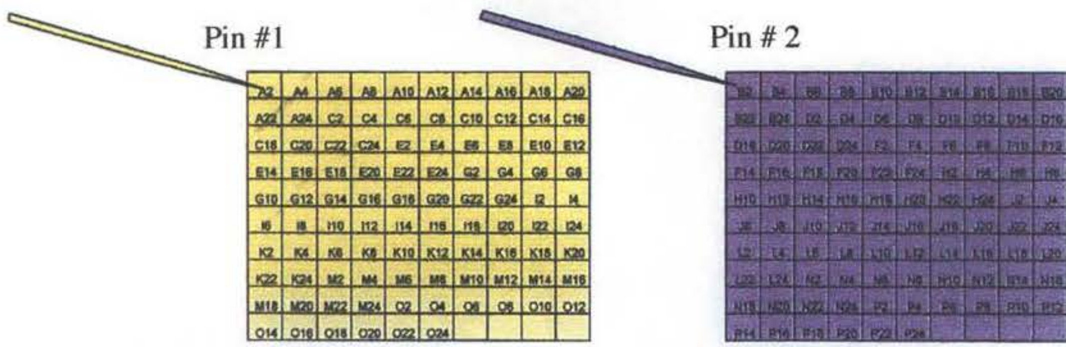
Genipshere's Array 350 kit was used for cDNA synthesis, labeling and detection. Starting material was 50 μg of total RNA for each condition. The manual was followed.

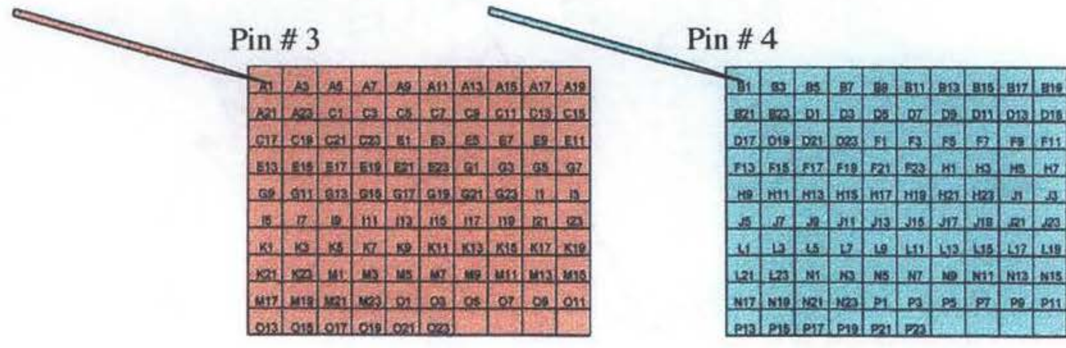
L. Array Layout.

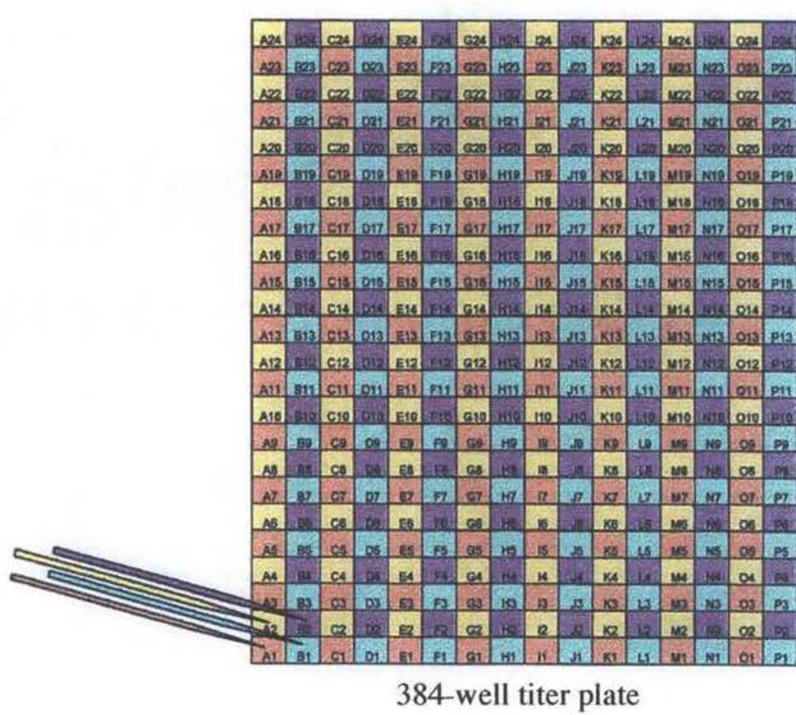
Sub array element; 10 x 10 array = 100 features

Spot spacing = 350 microns









384-well titer plate

VITA

Mohini Aparna Patil

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Doctor of Philosophy

Dissertation: A PURSUIT OF GENES ACTIVE IN DEFENSE OF UPLAND COTTON AGAINST BACTERIAL BLIGHT

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