PAPAYA SYSTEMIC ACQUIRED RESISTANCE

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ABSTRACT

Challenge by a pathogen induces systemic acquired resistance (SAR) in plants, a state marked by the elevated expression of pathogenesis related (*PR*) genes and enhanced resistance to a broad spectrum of pathogens. SAR requires the endogenous accumulation of salicylic acid (SA), and can be induced by exogenous application of SA or related molecules such as benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH). All tested plants have a SAR response, but some important aspects of SAR differ between species. In this work, four (partial) *PR-1* cDNAs were cloned from papaya. One of these, *PR-1d*, was shown to be induced by BTH. This data, together with previous data showing the induction of SAR related enzymes and enhanced tolerance to a pathogen in response to BTH, demonstrates that papaya has a SAR response and it is induced by BTH. With this knowledge, global profiling of papaya genes induced by BTH was carried out by suppression subtractive hybridization. 25 unique expressed sequence tags (ESTs) induced by BTH were identified, including homologs of numerous genes known to be defense related, and some genes previously unknown to have defense functions. A papaya homolog of *NPR1*, shown to be required for SAR signal transduction in *Arabidopsis*, was isolated and found to contain all three structural domains required for activity in *Arabidopsis*. This data, together with the profile of BTH induced genes, and induction kinetics for some of these genes, shows that papaya SAR is similar in many important aspects to SAR in the model system *Arabidopsis*. Additionally, tobacco plants over-expressing *Arabidopsis NPR1* were produced and found to produce elevated (compared to wild-type) levels of *PR-1a* mRNA in response to SA treatment. This

demonstrates that even in a heterologous system, over-expression of *NPR1* may confer an enhanced SAR response.

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3' RACE	3' Rapid Amplification of cDNA Ends
3'UTR	3' untranslated region
5' RACE	5' Rapid Amplification of cDNA Ends
5'UTR	5' untranslated region
AIP	aminoindan-2-phosphonic acid
AFPs	antifreeze proteins
ATP	adenosine triphopsphate
A. tumefaciens	*Agrobacterium tumefaciens*
Avr	avirulence
BA	benzoic acid
BA2H	benzoic acid 2-hydroxylase
BAC	bacterial artificial chromosome
BD	benzaldehyde
bp	base pair
BSA	bovine serum albumin
POZ	poxvirusand zinc finger
BTH	benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
CA	trans-cinnamic acid
cDNA	complementary DNA
CPBI	*Carica papaya* BTH-induced gene
cpr	constitutive expresser of PR genes

CMV	cauliflower mosaic virus
DEPC	diethyl pyrocarbonate
DEX	dexamethasone
dicot	dicotyledon
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ds	double-stranded
ESTs	expressed sequence tags
EtOH	ethanol
EtBr	ethidium bromide
GluNAc	β-1,4-linked-N-acetylglucosamine
GUS	β-glucuronidase
HBD	glucocorticoid receptor hormone biding domain
HR	hypersensitive response
hsp	heat shock protein
ICS	isochorismate synthase
IPL	isochorismate pyruvate lyase
INA	2,6-dicholoroisonicotinic acid
KAc	potassium acetate
LiCl	lithium chloride
min	minute
ml	milliliter

mM	millimolar
MOPS	3-[N-Morpholino]propanesulfonic acid
monocot	monocotyledon
mRNA	message RNA
NaAc	sodium acetate
nahG	bacterial salicylate hydroxylase
nim1	noninducible immunity
NPR1	Nonexpressor of *PR* genes
NLSs	nuclear localization signals
NOS	3' terminus of *Agrobacterium tumefaciens* synthase
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
P. palmivora	*Phytophthora palmivora*
Phe	phenylalanine
POZ	poxvirus and zinc finger
PR	pathogenesis-related
PRSV	papaya ring spot virus
R	resistance
Rb	retinoblastoma gene product
ROIs	reactive oxygen intermediates
RT-PCR	reverse transcriptase polymerase chain reaction
SAR	systemic acquired resistance

ss	single-stranded
ssi	suppressor of SA insensitivity
TCV	turnip crinkle virus
TL	thaumatin-like
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus

CHAPTER 1

INTRODUCTION

Papaya (*Carica papaya L.*) is an important fruit industry in Hawaii and in other tropical and subtropical regions of the world. Hawaii State Statistics showed the total acreage for papaya was 4,500 acres, with a value of 16 million dollars in 2000 (2001). Because Hawaii agriculture has been in transition for the past several decades from large-scale plantation operations growing sugarcane to smaller, entrepreneurial farms growing diversified products, papaya may gradually become a more important crop for Hawaii.

To the papaya industry, the disease problem has been the biggest constraint on production. Papaya engineered for resistance to papaya ringspot virus (PRSV) was proven to be a very effective remedy for that disease (Lius *et al.* 1997; Gonsalves 1998), and has spurred interest in biotech approaches to solving other disease problems. In Hawaii the papaya industry moved from the island of Oahu to the island of Hawaii in the 1940's to avoid devastation by PRSV. PRSV became a devastating disease again when it destroyed 500 farms on more than 3,000 acres in early to mid 1990's on the island of Hawaii. In 1997, production had fallen by nearly 40 percent. There is no naturally resistant germplasm, however the transgenic papaya cultivars Rainbow and Sunup carrying the PRSV coat protein gene are resistant to PRSV (Lius *et al.* 1997). They have helped save the Hawaii papaya industry from PRSV. In 2001, 39% of the papaya planted was Rainbow, the major transgenic cultivar (2001). The transgenic papaya is still the only source of resistance to PRSV.

Fungal diseases such as Phytophthora blight and root rot, anthracnose, and leaf spot remain major problems for papaya growers. They also can cause significant losses. For example, *Phytophthora palmivora*, the causal agent of Phytophthora blight and root rot, can cause the death of young seedlings especially in a cool and wet environment. *Colletotrichum gloeosporioides*, the causal agent of anthracnose, can damage the fruit and cause market value loss (Dickman and Alvarez 1983). These fungal diseases usually are controlled by frequent and timely applications of fungicides including EBDC (ethylene bis-dithiocarbamate; e.g. Dithane, Mancozeb, Maneb), acylalanine (Ridomil or Ridomil + Maneb), or Aliette (Fosetyl-Al)(Zhu *et al.* 2002). The application of these pesticides increases production costs for farmers. The potential environmental damage caused by pesticides plus the possibility of losing registration for some of them make it imperative to find alternative methods to address these fungal diseases.

Intelligent use of systemic acquired resistance may provide an alternative control measure. There are many examples in which plants initially infected with one pathogen become more resistant to subsequent infection by the same or another pathogen. This state is called systemic acquired resistance (SAR). It is a sustained and broad-based resistance to secondary infection by a wide variety of pathogens. Synthetic activators of SAR have been used commercially (Schweizer *et al.* 1999) to control diseases in many plants including dicots and monocots.

The SAR signal transduction pathway has been intensively studied in model plants such as tobacco and *Arabidopsis* for the past 15 years. The engineering of individual

components and regulators in this transduction pathway in diverse transgenic plants led to enhanced resistance against a variety of fungal and bacterial diseases. SAR is quite conserved among diverse plant species. Furthermore, BTH, a chemical activator of SAR in model species, was shown to activate enzymes which are markers of SAR in *Arabidopsis* and tobacco and to reduce disease symptoms of papaya seedlings inoculated with *Phytophthora palmivora* (Zhu *et al.* 2002). This result is similar to that obtained from *Arabidopsis* and tobacco. Therefore, approaches used to enhance SAR in model systems may also prove useful in papaya. In the present study we dissect papaya SAR by isolating a number of genes involved in the response. The genes obtained in this work may then be used to further analyze SAR in papaya, to analyze treatments or varieties designed to enhance disease resistance, and perhaps for use in papaya engineered for broad-spectrum disease resistance.

1.1 Systemic Acquired Resistance (SAR) and *PR* Genes in Plants

1.1.1 Activation and Persistence of SAR

SAR has been recognized as a plant response to pathogen infection for almost 100 years (Chester 1933). After the local formation of a necrotic lesion, either as a part of hypersensitive response (HR) or as a symptom, the SAR pathway is activated and it provides systemic, broad-spectrum resistance in a resistance (R) gene independent manner. Although most studies of SAR involve induction by avirulent pathogens and accompany a HR, there is evidence to show that HR is not required for the induction of SAR (Klarzynski *et al.* 2003). SAR can be induced by chemical activators in susceptible

varieties without the presence of the R gene (Friedrich *et al.* 1996). SAR has been observed in many plant species including dicots and monocots such as *Arabidopsis*, tobacco, cucumber, rice, and wheat. The SAR state can persist for extended time periods. A single inducing infection protects cucumber against all pathogens tested for 4 to 6 weeks. When a second, booster inoculation is given 2 to 3 weeks after the primary infection, the plant acquires season-long protection to all tested pathogens (Agrios 1997).

1.1.2 **Spectrum of SAR**

SAR can be characterized by both the spectrum of pathogen protection and the expression of the marker genes. SAR can be induced by various pathogens in a few days and results in the development of broad-spectrum systemic resistance against a variety of, but not all, pathogens. Localized infection of young cucumber with either a fungus (*Colletotrichum lagenarium*), a bacterium (*Pseudomonas lachrymans*), or a virus (tobacco necrosis virus), leads within a few days to systemic acquired resistance to at least 13 diseases caused by fungi, bacteria, and viruses. In tobacco, SAR can provide resistance against seven out of nine tested pathogens (Ryals *et al.* 1996). These pathogens include three fungi, *Phytophthora parasitica, Cercospora nicotianae,* and *Peronospora tabacina*, two viruses, tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV), and two bacteria, *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora.* However, SAR is not effective at providing resistance against either *Alternaria alternata* or *Botrytis cinerea* in tobacco (Vernooij *et al.* 1995). In *Arabidopsis*, SAR has been shown to be effective against *Peronospora parasitica*, turnip crinkle virus (TCV), and *Pseudomonas syringae* pv. *tomato* (*Pst*) (Lawton *et al.* 1996).

1.1.3 **Pathogenesis-related (PR) Proteins in SAR**

The activation of SAR correlates with the expression of the pathogenesis-related (*PR*) genes. In plants PR proteins comprise four families of chitinases (PR-3, -4, -8 and –11), one of β -1,3-glucanase (PR-2), one of a proteinase inhibitor (PR-6), one endoproteinase (PR-7), one specific peroxidase (PR-9), the PR-1 family, the thaumatin-like PR-5 family, and the birth allergen betv1-related PR-10 family (Van Loon 1997). Not all families are present in every plant species, but each family may contain several members (Van Loon 1997). There is a tight correlation between the onset of SAR and the enhanced accumulation of the mRNA encoding these different PR protein isoforms. The PR proteins are either extremely basic or extremely acidic. Therefore, they are quite soluble and reactive (Agrios 1997). Although the function of many PR genes is unknown, some of them have *in vitro* antimicrobial activity (Ryals *et al.* 1996). For example, the chitinases catalyze the hydrolysis of chitin, a biopolymer of N-acetyl-D-glucosamine. Chitinase, together with β-1,3-glucanase, could be directed against fungal cell wall components. PR-6 are proteinase inhibitors implicated in defense against microorganisms and nematodes (Van Loon and Van Strien 1999). PR-7 has so far only been reported in tomato as a major PR protein and it is believed to act as an endoproteinase (Van Loon and Van Strien 1999). It is speculated to degrade fungal cell wall proteins and may function as an accessory to antifungal action. The PR-8 chitinases also possess lysozyme activity, and they may inhibit bacteria. The PR-9 peroxidase is of the lignin-forming type and could be involved in the strengthening of the plant cell wall (Van Loon and Van Strien 1999). The PR-1 and –5 proteins possess activity against oomycetes, which do not have chitin in their cell walls. The antimicrobial activity of PR

proteins is believed to partially contribute to plant SAR, and the variety of different *PR* genes may explain the broad-spectrum resistance. None of the known PR proteins has been shown to possess antiviral activity (Cutt *et al.* 1989; Linthorst *et al.* 1989).

1.1.4 Engineering of Plants Using Pathogenesis-related (PR) Genes

Constitutive expression of individual *PR* genes in transgenic plants has in some cases led to reduced disease symptoms. Constitutive high-level expression of *PR-1a* in transgenic tobacco results in tolerance to infection by two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander *et al.* 1993), but not to infections caused by the non-oomycete *Cercospora nicotianae,* or the bacterial pathogen *Pseudomonas syringae* pv. *tabaci.* Apparently, the overexpression of *PR-1a* alone did not achieve the same broad-spectrum resistance displayed during tobacco SAR. A synergistic effect between different PRs has also been proven repeatedly. Antifungal activity of chitinase can be synergistically enhanced by β-1,3-glucanase, both *in vitro* and *in vivo* (Sela-Buurlage *et al.* 1993) (Zhu *et al.* 1994). Co-expression of chitinase and glucanase genes in tobacco increased resistance against *C. nicotianae* (Zhu *et al.* 1994; Jach *et al.* 1995).

1.1.5 Other Genes Upregulated During SAR

Besides designated *PR* genes, there are other genes upregulated transcriptionally during SAR. After surveying 25% to 30% of all *Arabidopsis* genes using microarrays, 31 genes were found to have expression induction similar to *PR-1* under various SAR-inducing conditions including pathogen inoculation and chemical SAR activators. Besides *PR*

genes, these genes included glutamine synthetase and glutamine dehydrogenase (Maleck *et al.* 2000). Some of them may be involved in the synthesis of signal molecules. Although quite a few genes are transcriptionally upregulated during SAR, it is still possible that some resistance-determining genes are downregulated during SAR or they are regulated at translation or post-translationally (Dong 2001).

1.1.6 **Other Functions of Pathogenesis-related Proteins**

In addition to the role in SAR, *PR* genes also may function in a variety of plant physiological processes such as seed germination, pollen germination and fruit ripening. In potato the protein level of a β-1,3-glucanase gene increases during flower development, reaching a maximum in stigmatic cells during flowering while usually it is undetected in mature leaves of uninfected plants. This β-1,3-glucanase expression increases significantly after infection with potato virus Y (PVY), treatment with *Phytophytora infestans* culture filtrate, and salicylic acid (Hennig *et al.* 2002). This gene could play a role in both plant development and in SAR against pathogen infection. In another study, two closely related genes encoding a basic and an acidic PR1 protein (*PR1b1* and *PR1a2*) from tomato (*Lycopersicon esculentum*) were isolated. The comparative study of the mode of transcriptional regulation of these two genes in transgenic tobacco plants using a series of promoter-GUS fusions was carried out. The chimeric *PR1a2/GUS* gene was not induced by pathogen but instead displayed constitutive expression under developmental regulation. It was expressed in shoot meristems, trichomes, and cortical cells as well as in vascular and nearby tissues of the mature stem. This constitutive expression pattern may indicate preemptive defenses

against potential pathogens (Tornero *et al.* 1997). Conversely, the chimeric *PR1b1/GUS* gene did not show any constitutive expression in the plant, but it was transcriptionally activated in response to pathogen attack (Tornero *et al.* 1997). Upon infection by tobacco mosaic virus, the *PR1b1* gene was strongly activated locally in tissues surrounding the local lesion but not systemically in uninoculated systemic tissues. Furthermore, its expression was induced by both salicylic acid and ethylene precursors, two signal molecules that coexist and mediate the activation of local defenses during the HR (Tornero *et al.* 1997). Interestingly there are other *PR1* genes induced in systemic, uninoculated tissues during SAR response in tomato (Van Kan *et al.* 1992; Tornedo *et al.* 1994). Based on these data, *PR1* genes in tomato have at least three different expression patterns.

Under some circumstances, there is a synchronized accumulation of various PR proteins not induced by pathogen infection. In response to cold temperatures, winter rye secretes six antifreeze proteins (AFPs) into the apoplast (Hon *et al.* 1995). These AFPs have the ability to retard ice crystal growth, and are similar to PR proteins normally secreted upon infection by pathogens in SAR. Two of the rye AFPs are glucanases, two are chitinases, and two are thaumatin-like proteins (Hon *et al.* 1995). In another study, accumulations of glucanases, chitinases, and osmotin-like proteins are shown to be partly correlated with the somatic embryogenesis process (Helleboid *et al.* 2000).

PR proteins with demonstrated hydrolytic activity on specific pathogen cell wall components can not automatically be assumed to be inhibitory to this pathogen. Tobacco

chitinase V had proven chitinase activity on a soluble dye-labeled chitin substrate (Melchers *et al.* 1994) . It significantly inhibited growth of *Alternaria radicina* at 5-10 μg per well while 100 μg per well had no effect on *Fusarium solani.* There is a distinction between enzymatic activity and specific fungal inhibition (Melchers *et al.* 1994). The inhibitory activity provided by one PR protein may be pathogen specific. This may explain why the onset of SAR is characterized by the accumulation of so many different PR proteins, as many may be required to provide broad-spectrum resistance. Tobacco class II chitinase and β-1,3-glucanase did not exhibit any antifungal activity. But the class II chitinase could synergistically inhibit fungal growth in combination with class I β-1,3-glucanase while the class II β-1,3-glucanase showed no inhibitory effect in any tested combination (Sela-Buurlage *et al.* 1993). Therefore, the inhibitory activity of any specific PR protein against a particular pathogen may not be apparent without the presence of other PR proteins.

1.1.7 **PR-1 Proteins**

Some *PR-1* genes are systemically induced by pathogen attack or salicylic acid, and they are often used as markers of SAR. Some PR-1 proteins have a limited antifungal activity. Various tomato and tobacco PR-1 proteins exhibited inhibitory activity on the growth of *Phytophthora infestans* in tomato leaf disc assays, with tomato PR-1c and tobacco PR-1g being the most effective members (Niderman *et al.* 1995). But the biochemical mode of action or function of PR-1 proteins is unclear. In tobacco, PR-1 proteins belong to two small multigene families, acidic forms of PR-1 (PR-1a, PR1b, and PR-1c) and the basic isoforms. The primary translation product of *PR-1* genes contains an N-terminal

hydrophobic signal sequence, which is cleaved off after entering the endoplasmic reticulum. The mature proteins of *PR-1* genes are mostly about 135 amino acids long and contain six conserved cysteine residues, which form three disulfide bridges. The structure of tomato PR-1b was resolved by nuclear magnetic resonance, and this protein contains four α-helices and four β-strands arranged antiparallel between helices I, III and IV and II, respectively (Fernandez *et al.* 1997). It is believed that all plant PR-1 proteins share a similar structure (Van Loon and Van Strien 1999).

1.1.8 Chitinases

Chitinases catalyse the hydrolysis of chitin, a linear homopolymer of ß-1,4-linked N-acetylglucosamine (GlcNAc) residues. Accumulation of chitinases is believed to be a defense response against fungi, which have chitin as a common component of their cell wall. Alternative substrates for plant chitinases are bacterial peptidoglycan, the soluble chitin derivative glycol chitin, and in some cases, chitosan (deacetylated chitin) (Collinge *et al.* 1993). Since many chitin-containing fungi are not inhibited by chitinase, probably the chitin components are shielded by other protective components. According to sequence similarities and subcellular location, plant chitinases have been grouped into 5 classes (Busam *et al.* 1997). The mature class I chitinase proteins have a cysteine-rich N-terminal region, which is responsible for chitin-binding (Shinshi *et al.* 1990). Next there is a variable hinge region, which is followed by a highly conserved main structure. Finally a C-terminal extension, found to be necessary and sufficient for the vacuolar subcellular location of type I chitinase (Neuhaus *et al.* 1991). Class I chitinases are basic proteins. Class II chitinases share high homology with the major structure of class I

chitinases except that they lack the N-terminal cysteine-rich motif. They are extracellular and acidic proteins (Collinge *et al.* 1993). Class III chitinases are characterized by lysozyme activity, which means that they can hydrolyse ß-1,4-linkages between N-acetylmuramic acid and GlcNAc residues in peptidoglycan, a component of bacterial cell walls. Class III exhibits no sequence similarity with either class I chitinase or II chitinase. Class III chitinase appear to be located extracellularly. Class I, II and III chitinases are believed to exist ubiquitously in plants. Class IV chitinase contains a cysteine-rich domain and a major structure resembling those of type I chitinase but are significantly smaller due to four deletions (Collinge *et al.* 1993). Therefore, the class IV chitinases contain only 241-255 amino acid residues in the mature protein compared with about 300 amino acid residues for class I chitinase (Collinge *et al.* 1993). Class V chitinase does not fit in any of the above classes and shares significant sequence similarity with bacterial exo-chitinases (Melchers *et al.* 1994).

In tobacco there are at least four classes of chitinases. The acidic class III chitinase, the basic class III chitinase and acidic class II chitinase have been shown to play a role in the SAR response (Ward *et al.* 1991). The steady-state level of their mRNA increased in both TMV-infected leaves and uninfected systemic leaves of TMV-preinoculated plants. The tobacco basic class I mRNA did not accumulate in uninfected, systematic leaves even though it did in the infected leaves. Therefore, the acidic class III chitinase, the basic class III chitinase, and acidic class II chitinase can function as SAR markers and tobacco basic class I chitinase can not. Interestingly, there is no evidence that any one of these three tobacco SAR marker chitinases does show antifungal activity alone *in vitro*

(Sela-Buurlage *et al.* 1993; Melchers *et al.* 1994). But the possibility that they may function synergistically against microorganisms with other PR proteins in the SAR response can not be ruled out. In contrast, the basic class I tobacco chitinase was the most active against *Fusarium solani* germlings, resulting in lysis of the hyphal tips and in growth inhibition (Sela-Buurlage *et al.* 1993). Apparently, there is a difference between the enzymatic and antifungal activities of the different chitinases. Tobacco class V chitinase has endo-chitinase activity determined by *in vitro* assays and shows antifungal activity toward *Trichoderma viride* and *Alternaria radicina* (Melchers *et al.* 1994).

1.1.9 Peroxidases

Most higher plants possess a number of different peroxidase isozymes that play roles in secondary cell wall synthesis, auxin catabolism, wound healing, and defense against pathogen attack. In the tobacco plant there are at least 12 distinguished isozymes, which fall into three subgroups: the anionic, the moderately anionic, and cationic. One of them, tobacco PR-9 is induced systemically after SAR is induced following the hypersensitive response in response to tobacco mosaic virus preinoculation. It is an anionic type. PR-9 protein is able to polymerize cinnamyl alcohol into lignin and could be involved in the strengthening of cell walls during the SAR response (Van Loon *et al.* 1994). The strengthening of cell wall mediated by peroxidases may account for part of resistance displayed during SAR. Among all peroxidases, eight cysteine and three histidine residues are located in similar positions in the primary sequences (Welinder 1992). These cysteines were shown to form intramolecular disulfide linkages. Three domains are also highly conserved: a domain involved in acid/base catalysis; a domain containing

the consensus sequence VSCADIL; and a domain involved in heme stabilization (Welinder 1992).

1.1.10 Thaumatin-like (TL) proteins

PR-5, one marker of systemic acquired resistance in dicots (Uknes *et al.* 1992) as well as in monocots (Morris *et al.* 1998), is a thaumatin-like (TL) protein. Among TL proteins, it is possible to distinguish several subgroups such as osmotins, permatin, PR-5 proteins and other TL proteins. TL proteins are low molecular mass (15-30 kDa) acidic or basic proteins. They accumulate extracellularly or in cell vacuoles of various organs. TL proteins can be constitutive or inducible. They are resistant to heat or acidic treatments and also relatively resistant to proteolytic degradation. In addition to playing a role in the SAR response, some TL isoforms called thaumatin are intensely sweet proteins isolated from thaumatin (*Thaumatotococcus daniaelli)* ripe fruits. A TL protein (Cherry CHTL) was isolated from ripe cherry fruit as the most abundant soluble protein. It does not have a sweet taste and was found not to be antifungal against *Botrytis cinerea* and *Monilia laxa* (Fils-Lycaon *et al.* 1996). Some TL proteins including Cherry CHTL were demonstrated to have endo-β-1,3-glucanase activity, which appears different from other β-1,3-glucanase (Grenier *et al.* 1999). Some TL isoforms are believed to function as cold-tolerance proteins in response to cold weather (Kuwabara *et al.* 1999).

Various PR-5 proteins showed antifungal properties *in vitro* and *in vivo* (Liu *et al.* 1994; Abad *et al.* 1996). PR-5 has a broad-spectrum of antifungal activity and also displays specificity for certain fungal pathogens. For example, tobacco osmotin inhibited the

hyphal growth of *Bipolaris*, *Fusarium*, *Phytophthora* and especially of *Trichoderma longibrachiatum* (Abad *et al.* 1996). It also caused spore lysis, inhibited spore germination, or reduced germling viability of the seven fungal species whose hyphal growth was inhibited by osmotin. Tobacco osmotin was shown to cause leakage of intracellular components of target fungal cells (Abad *et al.* 1996). The binding site of PR-5 proteins is suggested be the polysaccharide constituent of fungal cell wall mannoprotein and cell wall mannosylphosphate is required for osmotin toxicity to *Saccharomyces cerevisiae* (Ibeas *et al.* 2000).

1.2 Salicylic acid (SA) and Other Chemical Activators in SAR

1.2.1 SA is Required For SAR Response in *Arabidopsis* and Tobacco

Activation of PR gene expression and the establishment of SAR require salicylic acid (SA). In both infected and uninfected plant tissues after pathogen attack, the endogenous concentration of SA has been shown to increase (Malamy *et al.* 1990; Metraux *et al.* 1990; Rasmussen *et al.* 1991). The exogenous application of SA leads to expression of *PR* genes and activation of SAR (White 1979; Ward *et al.* 1991; Gorlach *et al.* 1996; Lawton *et al.* 1996). The bacterial nahG gene encodes salicylate hydroxylase, an enzyme that catalyzes the conversion of SA to inactive catechol. Transgenic tobacco and *Arabidopsis* expressing the bacterial enzyme nahG cannot accumulate SA. This defect makes the plants unable to induce SAR and reduces SAR gene expression in response to pathogens (Gaffney *et al.* 1993; Delaney *et al.* 1994). This evidence demonstrates that SA plays an essential role in SAR.

Although salicylic acid is required in signal transduction, SA is not the translocated signal responsible for inducing SAR (Vernooij *et al.* 1994). A transgenic tobacco rootstock expressing nahG does not accumulate SA, but is fully capable of delivering a signal that renders nontransgenic grafted scions resistant to further pathogen infection. A wild-type scion grafted onto a nahG rootstock shows SAR in response to TMV inoculation of the rootstock. TMV pretreatment on the nahG rootstock decreases the average lesion size in challenge leaves of the wild-type normal scion to the same extent as observed in the control, a wild-type scion grafted onto a wild-type rootstock. Reciprocal grafts demonstrate that SA presence in tissue distant from the infection site is required to induce SAR. It is proposed that reactive oxygen intermediates (ROIs) mediate a systemic signal network in SAR (Alvarez *et al.* 1998).

1.2.2 Variation of the SA Role in Other Plants' SAR

There are some plants in which SA may play a different mechanism in the SAR response. In rice the basal levels of SA are at least one hundred-fold higher than those found in tobacco and *Arabidopsis* without any deleterious effect and are not further inducible upon pathogen infection (Chen *et al.* 1997). However, a general correlation between SA levels and resistance to rice blast disease was established (Silverman *et al.* 1995). The potato also contains high basal levels of SA (40- to 100 fold higher than those in tobacco and *Arabidopsis*). The exogenous application of SA does not increase resistance against *Phytophthora infestans,* the causal pathogen of potato late blight. This disease usually serves as the indicator of SAR in potato. Potato SAR against *P. infestans* can be activated by the local preinoculation of this fungus (Doke *et al.* 1987) or the pathogen

elicitor arachidonic acid (Coquoz *et al.* 1995). During potato SAR response the endogenous level of SA does not increase in the upper, untreated leaves in contrast to that observed in tobacco and *Arabidopsis*. However, in the transgenic potato plants that express the bacterial hydroxylase gene (*nahG*) and consequently have a drastic reduction of total SA levels, resistance to *P. infestans* induced by arachidonic acid is abolished, in contrast to nontransformed control plants (Yu *et al.* 1997). Therefore, SA still plays an essential role in arachidonic acid-induced potato SAR. It is proposed that the induction of SAR in potato may involve the enhanced sensitivity of the plant to SA since it already has a relatively high SA pool. In contrast, *Arabidopsis* and tobacco SAR involves induced biosynthesis and enhanced concentration of SA while sensitivity may already be relatively high (Yu *et al.* 1997).

1.2.3 Biosynthesis of SA

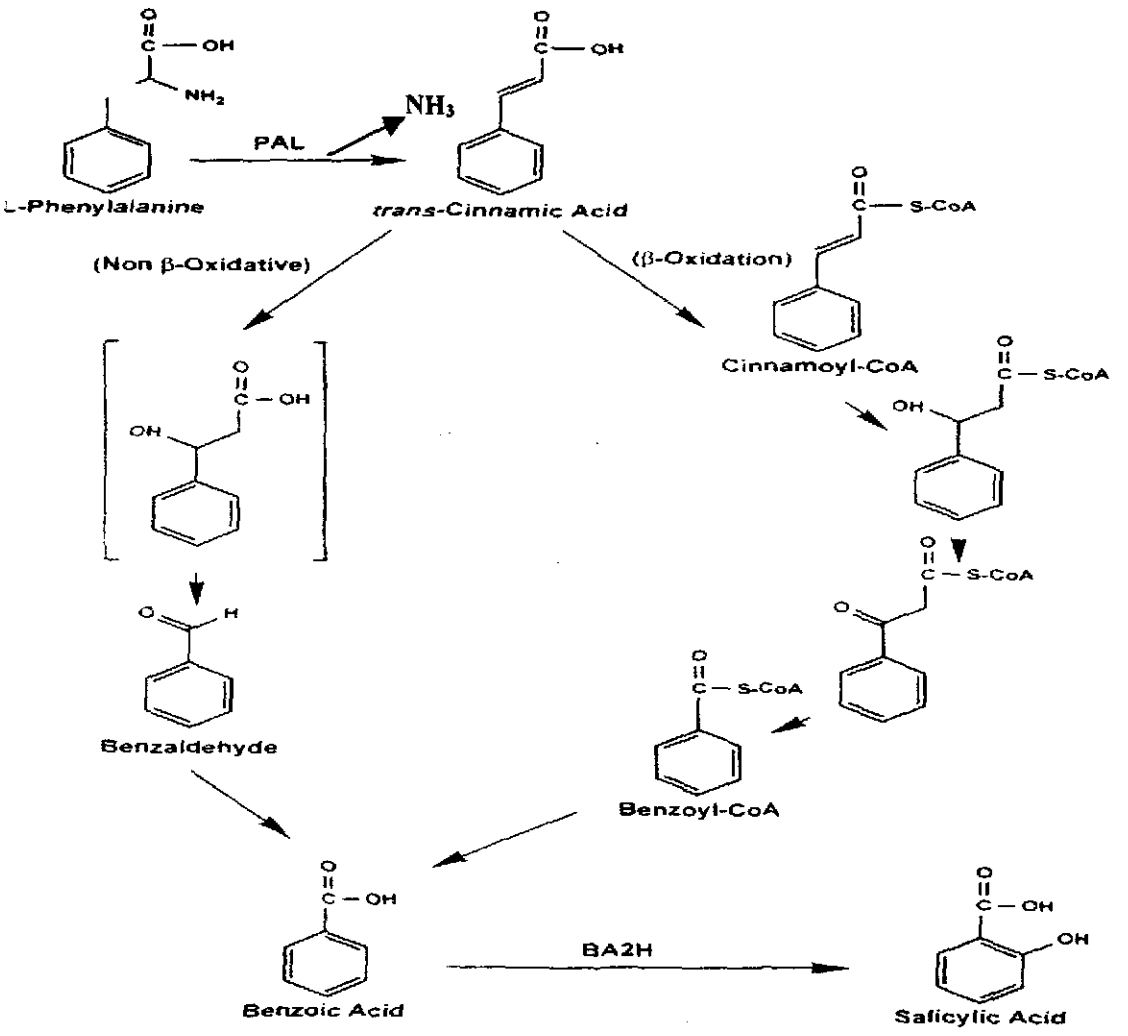
SA is synthesized from phenylalanines (Phe) as part of the phenylpropanoid pathway and requires phenylalanine ammonia lyase (PAL). Inhibition of PAL by 2-aminoindan-2-phosphonic acid (AIP) leads to a reduction in the amount of SA in SAR-inducing conditions in both *Arabidopsis* (Mauch-Mani and Slusarenko 1996) and potato (Coquoz *et al.* 1998). The inhibition of PAL by AIP makes *Arabidopsis* completely susceptible, and this effect is suppressed by exogenous SA application (Mauch-Mani and Slusarenko 1996). These inhibitor experiments indicate that a major function of PAL in plant disease resistance is to provide precursors for the production of SA.

In tobacco SA can be synthesized from Phe via *trans*-cinnamic acid (CA) and benzoic acid (BA). First Phe is converted to CA mediated by PAL. Then there are two possible synthesis pathways from CA to BA. It could occur by way of β-oxidation in which benzoyl-CoA is the intermediate or by a non-β-oxidation route in which benzaldehyde (BD) serves as the intermediate (Figure 1, A). At least in tobacco, β-oxidation is the most likely mechanism for the biosynthesis of BA from CA (Ribnicky *et al.* 1998). By either route, the rate-limiting step in SA biosynthesis is this conversion process. In addition, there are data to show that some SA may be synthesized from *trans*-cinnamic acid through a route that does not involve BA (Ribnicky *et al.* 1998).

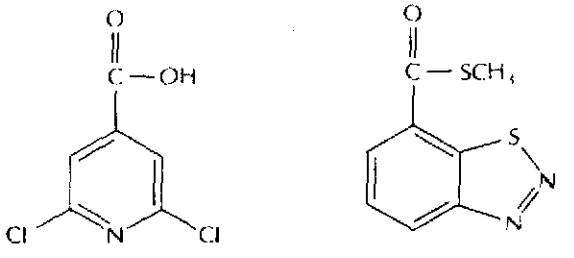
1.2.4 Other Chemical Activators of SAR

Analogs of SA, such as 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Figure 1, B), can activate SAR in many plants. BTH -treated *Arabidopsis* plants were resistant to infection by turnip crinkle virus, *Pseudomonas syringae* pv. 'tomato' DC3000 and *Peronospora parasitica*, the same spectrum of pathogens inhibited during SAR induced by pathogen preinoculation or exogenous SA treatment. BTH or INA treatment also induced upregulation of the same set of *PR* genes as pathogen preinoculation or exogenous SA treatment in both monocots and dicots (Friedrich *et al.* 1996; Gorlach *et al.* 1996; Lawton *et al.* 1996; Morris *et al.* 1998). Further, neither BTH, INA, or their major metabolites have direct antifungal activity. All the above three criteria can be used to search for novel SAR activators. In tobacco, BTH is less toxic than SA. BTH treatment protected a susceptible tobacco line against TMV infection (Friedrich *et al.* 1996). In rice, BTH treatment of seedlings gives

A



B



INA **BTH**

Figure 1. Salicylic acid biosynthesis in tobacco and synthetic SAR-inducing compounds. A. Possible pathways of salicylic acid biosynthesis in tobacco. PAL, Phenylalanine ammonia lyase; BA2H, benzoic acid 2-hydroxylase. after Ribnicky *et al.*, 1998. B. Chemical structures of two synthetic SAR-inducing compounds. INA, 2,6-dichloroisonicotinic acid; BTH, benzo-(1,2,3)-thiodiazole-7-carbothionic acid S-methylester (BTH).

season-long protection against *Magnaporthe grisea*, the causal agent of rice blast (Gorlach *et al.* 1996). It has been commercially released to the market (Schweizer *et al.* 1999). In both dicots and some moncots, BTH treatment enhances plant resistance and may activate a similar range of *PR* genes (Morris *et al.* 1998).

Neither INA nor BTH treatment causes SA concentration to increase (Hammond-kosack and Jones 2000). In *Arabidopsis* and tobacco transgenic NahG plants which are SA-free, both BTH and INA still can activate both SAR marker gene induction and resistance against pathogens (Vernooij *et al.* 1995; Friedrich *et al.* 1996; Lawton *et al.* 1996). Therefore, the activation of SAR by BTH or INA is not dependent on SA. Therefore, BTH and INA may function to induce SAR at the site of or downstream of SA.

The sulfated fucans are a common structural scomponents of marine brown algae. Sulfated fucans are hydrolyzed by a fucan-degrading hydrolase isolated from a marine bacterium to produce sulfated fucan oligosaccharides. Sulfated fucan oligosaccharides do not share structural similarity with SA. They can activate the expression of two SAR marker genes in tobacco and stimulate SAR to tobacco mosaic virus (TMV). However, unlike INA and BTH, this oligofucans-induced resistance is SA-dependent. In NahG transgenic tobacco plants not accumulating SA, the oligofucan-induced resistance is abolished. Because fucan oligosaccharides do not induce HR in tobacco and do not share molecular structure with SA, these results indicate that HR is not a prerequisite for the induction of SAR (Klarzynski *et al.* 2003).

Harpin is the product of the *hrpN* gene of *Erwinia amylovora* (Wei *et al.* 1992). It can elicit HR response and induces SAR in many plants (Wei *et al.* 1998). Harpin induces SAR in *Arabidopsis* indicated by pathogen resistance and the activation of SAR genes. The resistance induced by Harpin is mediated by SA and the *NPR1/ NIM1* gene (Dong *et al.* 1999).

1.3. Relationship between SAR Response and Plant Genotype

Usually plant resistance is mediated by dominant resistance (*R*) genes possessed by the plant and the complementary dominant avirulence genes (*Avr*) expressed by the pathogen. The activated defenses can lead to the hypersensitive response (HR), a rapid, localized cell death that prevents pathogen spread. SAR activation often follows HR, but is not necessarily linked to HR. Necrotic lesions can activate SAR, whether they form as part of HR in an incompatible interaction or as a symptom of disease in a compatible interaction (Ryals *et al.* 1996). As in dicots, infection of maize with an avirulent pathogen resulted in HR and a rapid and enhanced expression of *PR-1* and *PR-5*, both of which are SAR molecular markers. When maize plants are infected with a compatible pathogen, *PR-1* and *PR-5* induction is significantly slower and weaker than when induced by an incompatible interaction (Morris *et al.* 1998). The delayed and low PR protein accumulation in compatible interactions compared to incompatible ones was previously described in tomato (P.J.G.M. *et al.* 1986)

Most of the knowledge about SAR has been obtained from dicots such as *Arabidopsis* and tobacco. There has been some evidence to show the similarity between dicots and monocots such as maize in terms of the SAR response. For example, BTH induced resistance to downy mildew in maize. Furthermore, the expression of maize *PR-1* and *PR-5* genes was coordinately activated by BTH, INA, SA or pathogen infection, as in dicots. Furthermore, lesion mimic mutants in *Arabidopsis* showed increased *PR* gene expression and elevated SA levels when lesions were present (Weymann *et al.* 1995). The same phenomenon was also reported in maize mutants (Morris *et al.* 1998). All

these similarities reinforce the notion that between dicots and at least some monocots, signal transduction of SAR is highly conserved.

On the other hand, rice and wheat show differences from dicots. In rice, BTH did not induce any designated *PR* genes. There was no overlap between genes induced by BTH and genes induced by *P. syringae*, which induced the HR (Schweizer *et al.* 1999). During SAR following HR upon infection by *Pseudomonas syringae* pv. *syringae*, the activities of peroxidase, β-1,3-glucanase or chitinase increased only locally but not systemically (Smith and Metraux 1991). In wheat, a prior infection by *Erysiphe graminis* led to enhanced protection against a second infection by *E. graminis*, the causative agent of powdery mildew (Hwang and Heitefuss 1992). BTH protected wheat systemically against *E. graminis*, but none of the BTH-induced genes isolated from wheat were designated *PR* genes (Gorlach *et al.* 1996). Furthermore, *PR1.1* and *PR1.2* expression was induced upon infection with *E. graminis*, but neither of them was activated by SA, INA (Molina *et al.* 1999), or BTH. A set of *PR* genes (PR-1, PR-2, PR-3, PR-4, PR-5 and peroxidase) are induced by the nonhost pathogen *Erysiphe graminis* f. sp. *hordei* or the compatible pathogen *F. graminearum* but none of these *PR* genes are induced by BTH (Schaffrath *et al.* 1997) (Yu and Muehlbauer 2001). However, another seperate study indicated in wheat *PR-4* can be induced by SA, BTH, wounding and specifically induced in seedlings infected with *Fusarium culmorum* (Bertini *et al.* 2003). The contradicting conclusions about whether BTH induces *PR-4* in wheat may result from different experimental designs. In one study (Yu and Muehlbauer 2001), barley PR-4 was employed as the probe to study wheat *PR-4* gene expression. For another, wheat

PR4b cDNA was used as a probe that could not distinguish among *PR-4* homologues. Therfore, the results may reflect different expression pattern changes of one or more members of the multigenic PR-4 family (Bertini *et al.* 2003). Overrall, the molecular response in wheat to BTH appears to differ from *Arabidopsis*, tobacco and maize, although BTH does induce systemic resistance against some pathogens in wheat. Because the most thorough profiling of genes upregulated during SAR only covered 25-30% of all *Arabidopsis* genes, all current efforts to document genes induced by pathogen infection or upon treatement by BTH is incomplete. More comprehensive studies of both the model systems and the exceptions like wheat will be required to assess how fundamental the interspecific differences in SAR responses really are.

1.4 NPR1 , Other Regulatory Proteins and Transcription Factors Involved in SAR

1.4.1 Isolation of *Arabidopsis NPR1* (*Nonexpressor of PR genes*) gene

A transgenic *Arabidopsis* line was generated that contains a β-glucuronidase reporter gene (*GUS*) under the control of the SA- and INA-responsive *BGL2* gene promoter. Several mutants were isolated based on the absence of *GUS* expression in the presence of the SA and INA. The *npr1-1* mutant displays almost complete abolishment of expression of the *BGL2-GUS* reporter gene and the endogenous *PR1*, *BGL2*, and *PR5* genes in response to SA, INA, and avirulent pathogen treatments (Cao *et al.* 1994). The *NPR1* gene then was isolated using a map-based approach (Cao *et al.* 1997). Other mutant alleles of *npr1* (also known as *nim1* and *sai1*) have been isolated by different genetic screening strategies (Delaney *et al.* 1995; Glazebrook *et al.* 1996; Shah *et al.* 1997). In

nim1 (for noninducible immunity) mutants, the resistance against *P. parasitica* induced by INA, a SAR chemical activator, was abolished. The fact that multiple mutants isolated by several groups using different genetic screening approaches involved the same gene indicates that NPR1 is a critical component in SAR signal transduction. Comparison of the cDNA sequence and the genomic sequence revealed that there are four exons and three introns in the *NPR1*gene (Cao *et al.* 1997). There is only one copy of *NPR1* in the *Arabidopsis* genome, located on chromosome 1 (Cao *et al.* 1997; Ryals *et al.* 1997). Expression studies show that *NPR1* is constitutively expressed in plants although its transcript level can be further elevated by about 2-fold after SA or INA treatment (Cao *et al.* 1997) or by pathogen infection (Ryals *et al.* 1997).

1.4.2 Molecular Structure and Functions of NPR1

NPR1 is a novel protein containing ankyrin repeats. The ankyrin repeat motif has been found in a diverse group of proteins involved in cell structure, transcription regulation, cell differentiation, and enzymatic and toxic activities. These repeats, which occur as a minimum of four copies, have been known to fold cooperatively and to play a role in protein-protein interactions. The *Arabidopsis* NPR1 protein contains four contiguous ankyrin repeat regions (Cao *et al.* 1997). In addition, the NPR1 protein has a POZ (poxvirus and zinc finger) domain (Aravind and Koonin 1999), which also is involved in protein-protein interactions. In several proteins, POZ domains were showed to mediate homodimerization, multimerization, or heterophilic interactions (Aravind and Koonin 1999). In various proteins, POZ is combined with a variety of additional protein-protein interaction domains, such as C-terminal zinc fingers (Hu *et al.* 1995), kelch repeats (Bork

and Doolittle 1994), MATH domains (Uren and Vaux 1996), ankyrin repeats, etc. The importance of the ankyrin repeat domain and POZ domain in NPR1 was verified by the isolation of loss-of-function point mutations in these domains.

NPR1 is most similar to the mammalian transcription regulator IkBα. This may indicate that NPR1 is involved in the transcriptional regulation of *PR* genes. In mammals, IkBα protein represses the pathways leading to inflammatory and immune responses by binding the transcription factor NF-kB and thereby preventing it from entering the nucleus. In response to certain stimuli, IkBα protein is phosphorylated and degraded. This enables NF-kB protein to be released into the nucleus, where it activates defense gene expression and these defense pathways are turned on. But unlike IkBα, NPR1 appears to function as a limiting positive regulator of defense responses. In seperate studies, overexpression of *NPR1* in *Arabidopsis* conferred enhanced disease resistance to bacterial and oomycete pathogens (Cao *et al.* 1998; Friedrich *et al.* 2001). These transgenic plants overexpressing *NPR1* had increased or earlier accumulation of the *PR* gene transcripts following pathogen infection. Overexpression of *NPR1* did not cause constitutive activation of defense responses (Cao *et al.* 1998; Friedrich *et al.* 2001). High expression levels of NPR1 enhanced the sensitivity of plants to BTH (Friedrich *et al.* 2001). Overexpression of *Arabidopsis NPR1* in rice increased resistance against *Xanthomonas oryzae* pv. *oryzae (Xoo)*, the rice bacterial blight pathogen and there was a correlation between *NPR1* mRNA expression and the enhanced resistance phenotype (Chern *et al.* 2001).

The fact that NPR1 lacks any known DNA binding domains suggests that it may either play an indirect role in *PR* gene regulation or may function as a regulator of the transcription factor or factors that control *PR* gene expression. Using NPR1 as bait in a yeast two-hybrid screen, the TGA subclass of transcription factors in the basic leucine zipper protein family was identified to interact specifically in yeast and *in vitro* with NPR1 (Zhang *et al.* 1999; Despres *et al.* 2000; Zhou *et al.* 2000). Point mutations that abolished NPR1 function in *Arabidopsis* also impaired the interactions between NPR1 and TGA2 and TGA3 in the yeast two-hybrid assay. TGA2 and TGA3 were found to bind the SA-responsive element of the *Arabidopsis* PR-1 promoter (Zhou *et al.* 2000). At least part of the TGA binding site of NPR1 is located in the ankyrin repeat region. In an *in vitro* gel mobility shift assay, the DNA binding activity of TGA2 was shown to be enhanced by NPR1 (Despres *et al.* 2000). The TGA transcription factor has three distinct domains: N-terminal domain for stability and possibly transactivation activity of the protein; the bZIP domain functioning in DNA binding and dimerization ; and C-terminal domain sufficient for interaction with NPR1 in yeast. Transgenic *Arabidopsis* lines expressing high levels of the truncated form of TGA2, in which the N-terminal and bZIP domains were deleted, had a defect in SAR gene expression similar to those of *npr1* mutants. The truncated form of TGA2 competed with the wild type TGA2 for interaction with NPR1 but failed to activate *PR* genes during SAR since it had no DNA-binding domain. In response to SA the chimeric TGA2GAL4 transcription factor, in which the bZIP domain of TGA2 was replaced by the DNA binding domain of the yeast GAL4 transcription factor, activated a UAS ^{GAL}:: GUS, in which the GUS was under the control of minimal CaMV 35S promoter sequence and six GAL4 binding sites. This activation

was absent in the *npr1* mutant. These data prove that TGA2 is an NPR1-dependent transcription activator which responds to SA (Fan and Dong 2002).

If NPR1 interacts with transcription factors to regulate *PR* gene expression, there should be a mechanism to direct it to the nucleus after its synthesis in the cytoplasm. An NPR1-green fluorescent protein fusion, which was functionally equivalent to endogenous NPR1 protein, was shown to accumulate in the nucleus in response to SA or INA. To demonstrate the cause-and- effect relationship between the NPR1 subcellular localization and its activity in controlling *PR* gene expression, a fusion protein of NPR1 with the glucocorticoid receptor hormone binding domain (HBD) was made. In untreated cells, NPR1-HBD fusion protein expressed in *npr1* mutant plants were contained in the cytoplasm in association with Hsp90 and the activation of *PR-1* by INA was abolished. Treatment with the steroid hormone dexamethasone (DEX) caused NPR1-HBD to be relased from Hsp90, and to be translocated into the nucleus in *npr1* mutant plants and the activation of *PR-1* by INA was restored. Use of this steroid-inducible system showed that nuclear localization of NPR1 is essential for its activity in inducing *PR* genes. Homology analysis of the NPR1 protein sequence pointed out three potential NLSs (nuclear localization signals). Mutation study indicated further that the second NLS (residues 541 to 554) may be the functional one (Kinkema *et al.* 2000).

1.4.3 NPR1-independent Resistance Downstream of SA

Interestingly, NPR1 is a limiting factor in resistance signal transduction, and overexpression of NPR1 leads to enhanced resistance to bacterial and oomycete

pathogens (Cao *et al.* 1998; Chern *et al.* 2001). But there are no reports of enhanced virus resistance due to enhanced level of NPR1. Also, none of the PR proteins under the regulation of NPR1 exhibits antiviral activity. NPR1 is not required for resistance to some virus pathogens. *Arabidopsis* SAR is effective against turnip crinkle virus (TCV) (Lawton *et al.* 1996) and this resistance to TCV in *Arabidopsis* is salicylic acid dependent but NPR1 independent (Kachroo *et al.* 2000). In terms of SA-induced resistance to Turnip vein clearing virus (TVCV), the *npr1* mutant did not show any difference from the wild type (Wong *et al.* 2002). But recently, the tobacco NPR1 was found to be required for N-mediated resistance to tobacco mosaic virus (Liu *et al.* 2002). Therefore, both NPR1-independent and NPR1 -dependent virus resistance appear to be branches of defense signal transduction downstream of SA.

Furthermore, there is evidence that SA-dependent resistance to certain bacterial and fungal-like pathogens is also NPR1-independent. One *Arabidopsis* mutant *ssi1* (suppressor of SA insensitivity), obtained by screening of mutations that restore SA-mediated signaling in the *npr1* mutant background, constitutively expressed SA-dependent defense responses independently of NPR1. *ssi1 npr1* plants constitutively express the *PR* genes and exhibit enhanced resistance to *Pseudomonas syringae (P.syringae)* and the oomycete *Peronospora parasitica (P.parasitica).* All *ssi1*-phenotypes are dependent on the ability to accumulate SA to elevated levels, suggesting that ssi1 confers resistance via SA (Shah and Nandi 2002). The mutant *cpr6* (constitutive expresser of PR genes 6) also has elevated levels of SA and can restore resistance in the *npr1* background. In *cpr6* mutant nahG transgenic plants, which do not accumulate SA,

cpr6-mediated resistance against both *P. syringae* and *P. parasitica* is blocked while in the double mutant *cpr6 npr1* only the resistance to *P. syringae* is abolished. Therefore, it is proposed that in the *cpr6* mutant, a SA-dependent but NPR1-independent resistance may be activated (Clarke *et al.* 2000). However, SAR against *P. syringae* and *P. parasitica* induced by SA or avirulent pathogen infection is blocked in *npr1* (Dong 2001). The reconciling explanation of these observations has yet to be found. Based on all the studies on mutants, there appears to be four branches of resistance downstream of SA in SAR signal transduction: NPR1-independent viral resistance, NPR1-dependent viral resistance, NPR1-independent fungal or bacterial resistance, and NPR1-dependent fungal or bacterial resistance. It is likely the details and relative importance of these four branches vary in different plant species and pathogens.

1.4.4 Other Factors Involved in *PR* Gene Regulation

Other transcription factors are also involved in SAR-related gene regulation in addition to TGA transcription factors. The WRKY transcription factors are believed to be involved in SAR signal transduction (Maleck *et al.* 2000). They have the characteristic WEKYGQK amino acid sequence at the N-terminal and a distinct zinc finger DNA-binding domain at the C-terminal. The WRKY proteins have been proved to function as transcription factors in both yeast and plants (de Pater *et al.* 1996; Eulgem *et al.* 1999) and are encoded by a large gene family, with more than 70 members in *Arabidopsis* (Chen and Chen 2002). They specifically bind to the W-box (TTGAC) in the promoter region or transcribed but untranslated region of genes to regulate transcription.

The WRKY transcription factors are believed to be involved in transcriptional regulation of *NPR1*, *PR* genes, their own genes, and to play a role in plant response to pathogen infection (Yang *et al.* 1999; Yu *et al.* 2001). There are a number of separate studies to support this notion. First, several WRKY transcription factors are rapidly induced by pathogens, pathogen elicitors, or treatment by SA. SA-induced expression of at least some of them is independent of NPR1 (Yu *et al.* 2001). Secondly, the W-boxes have been found in genes upregulated during SAR. Transcript profiling of 25% to 30% of all *Arabidopsis* genes during SAR found 31 genes with expression patterns similar to that of *PR-1*, a reliable marker for SAR. They may encode proteins functioning during SAR and they form a regulon with *PR-1*. An average of 4.3 copies of the W-box per promoter are present in genes of this regulon while a random set of genes contains fewer than two W-boxes per promoter (Maleck *et al.* 2000). Thirdly, AtWRKY18, a pathogen- and salicylic acid-induced *Arabidopsis* WRKY transcription factor, is able to potentiate developmentally regulated *PR* gene expression and resistance to a bacterial pathogen. AtWRKY18 expressed at high levels caused deleterious effects on transgenic plants. When expressed at moderate level, AtWRKY18 enhanced developmentally regulated defense responses in transgenic plants without causing substantial negative effects on plant growth (Chen and Chen 2002). The W-boxes found in AtWRKY18 functioned as a negative cis-acting element for the induction of AtWRKY18. These WRKY binding sites may prevent overexpression of AtWRKY18 during the activation of plant defense that could be detrimental to plant growth (Chen and Chen 2002). Fourthly, the W-box sequences in the NPR1 promoter were identified to be required for NPR1 function. Mutation in these W-boxes abolished the binding by WRKY transcription factor,

inactivated the expression of the downsream reporter gene, and compromised the ability of NPR1 to complement *npr1* mutants for SA-induced *PR1* expression and disease resistance against *P. syringae*. These results indicate that certain WRKY transcription factors positively regulate *NPR1* gene expression upstream during the activation of SAR (Yu *et al.* 2001). Therefore, some WRKY factors may act as positive regulators of SAR and some as repressors.

Mutations that can restore resistance in the *npr1* background are likely those that activate SA-dependent but NPR1-independent pathway(s) in SAR. Mutants such as *cpr6* and *ssi1* all have increased levels of SA and they are not considered as true suppressors of *npr1*. Until now only one mutant, *sni1* (suppressor of npr1, inducible 1) that may be a true *npr1* suppressor has been isolated. The *sni1* mutant had an endogenous SA level comparable to those of the wild type in both noninducing and SAR-inducing conditions. This recessive mutant restored the wide-type level of SA- and INA –inducible *PR* gene expression and disease resistance in the *npr1* background, suggesting that the wild-type SNI1 protein is a negative regulator of SAR. The wild-type SNI1 encodes a novel, leucine-rich nuclear protein and has no obvious homology with any known transcription factors (Li *et al.* 1999). SNI1 does share limited homology with the mammalian tumor suppressor RB (the retinoblastoma gene product). RB negatively regulates gene expression by sequestering transcriptional activators such as E2F transcription factor and by recruiting histone deacetylase, which functions in chromatin remodeling. The mechanism by which SNI1 negatively regulates SAR genes and the relationship between NPR1 and SNI1 are not clear.

1.4.5 Phylogenetic Conservation of NPR1

NPR1 homologs have been identified in many plants including canola, cabbage, broccoli, tobacco, tomato, potato, corn, wheat and rice (Cao *et al.* 1998). The NPR1 homologs from tobacco and tomato share ~70% deduced amino acid sequence similarity to *Arabidopsis* (Cao *et al.* 1997). Overexpression of *Arabidopsis* NPR1 in rice enhanced resistance against *Xanthomonas oryzae* pv. *oryzae (Xoo)*, the rice bacterial blight pathogen. There was a correlation between *NPR1* mRNA expression and the enhanced resistance phenotype in transgenic rice plants. Rice proteins interacting with *Arabidopsis* NPR1 also share high degrees of sequence similarity with the *Arabidopsis* TGA2 protein, which was shown to interact with *Arabidopsis* NPR1 (Chern *et al.* 2001). Overexpression of the endogenous rice NPR1 also can enhance resistance against *Xoo* (Campbell *et al.* 2002). These experiments suggest that monocot and dicot plants share a conserved signal transduction pathway and the study of *NPR1* in *Arabidopsis* generally can be applicable to other species.

Tobacco *NPR1* is shown to be essential for N-mediated resistance against TMV but its mode of function is not clear (Liu *et al.* 2002). The *NPR1*-mediated regulation of *PR* genes has only been demonstrated in *Arabidosis* to date. The exact function of *NPR1* homologues in other plants is yet to be addressed. In addition, unlike in *Arabidopsis*, rice overexpressing *NPR1* grown under suboptimal condition display an abnormal growth phenotype (Campbell *et al.* 2002).

1.5 Profiling of Genes Differentially Expressed During Plant SAR

A number of technologies have been applied to profiling gene expression during plant SAR response. cDNA microarrays allow the analysis of transcripts of more than 10,000 genes simultaneously and it can detect one RNA molecule per cell. However, the requirement for comprehensive sequence data makes the cDNA microarray mostly suited to model organisms, for which large-scale expressed sequence tags (ESTs) are available. Until now two microarray experiments have been done to document the genes differentially expressed during plant SAR (Maleck *et al.* 2000; Schenk *et al.* 2000). After surveying 10,000 expressed sequence tags (ESTs) representing 25% to 30% of all *Arabidopsis* genes using microarrays, 31 genes were found to have expression induction similar to *PR-1* under various SAR-inducing conditions. The expression of these co-regulated genes repeatedly were correlated with the establishment of SAR, as opposed to genes activated during single SAR or defense-response conditions. That led to the discovery of a common promoter element in these genes to which WRKY transcription factor binds (Maleck *et al.* 2000).

Using differential screening and differential display, five BTH-induced wheat cDNAs designated as *WCI* genes (for wheat chemical induction) were isolated. The induction of resistance by BTH in wheat correlates with the expression of the *WCI* genes with respect to both timing and dose-response of the chemical. These five *WCI* genes are also coordinately expressed in response to SA and INA. But none of them shares homology with any known dicot SAR genes (i.e. *PR* genes upregulated in *Arabidopsis* and tobacco

SAR) (Gorlach *et al.* 1996). Therefore, the conclusion that BTH induces a similar set of genes in wheat as those found in *Arabidopsis* and tobacco could not be reached.

Suppression subtractive hybridization enables researchers to compare two populations of mRNA and clone genes that are expressed at higher level in one population (Diatchenko *et al.* 1996). The purpose of the present study is to obtain global profiling of papaya genes induced by BTH, which has been shown in this system to activate some aspects of SAR (Zhu *et al.* 2002).

First, double-stranded (ds) cDNA is synthesized from two populations of mRNA being compared. The tester cDNA contains the differentially expressed transcripts (eg. cDNA from BTH-treated sample), and the driver cDNA is the reference (eg. cDNA from water-treated sample). The tester and driver ds cDNAs are digested with *Rsa 1*, a four-base-recognition restriction enzyme, to generate blunt-ends. Then, two portions of the digested tester ds cDNA are ligated with two different adaptors 1 and 2R in separate reactions. The first 20 nucleotides at the 5' end of the two adaptors are identical. Because the ends of the adaptor do not have phosphate groups, only one strand of each adaptor attaches to the 5' ends at both strands of the tester cDNA. Two first hybridations are set up. In one, after both are denatured, the excessive driver cDNA is hybridized with the tester cDNA ligated with adaptor 1. In the other, denatured driver cDNA in excess is hybridized with the tester cDNA ligated with adaptor 2R. In each first hybridization, there are four types of molecules ***a***, ***b***, ***c*** and ***d*** (Figure 2). The single-stranded (ss) type ***a***

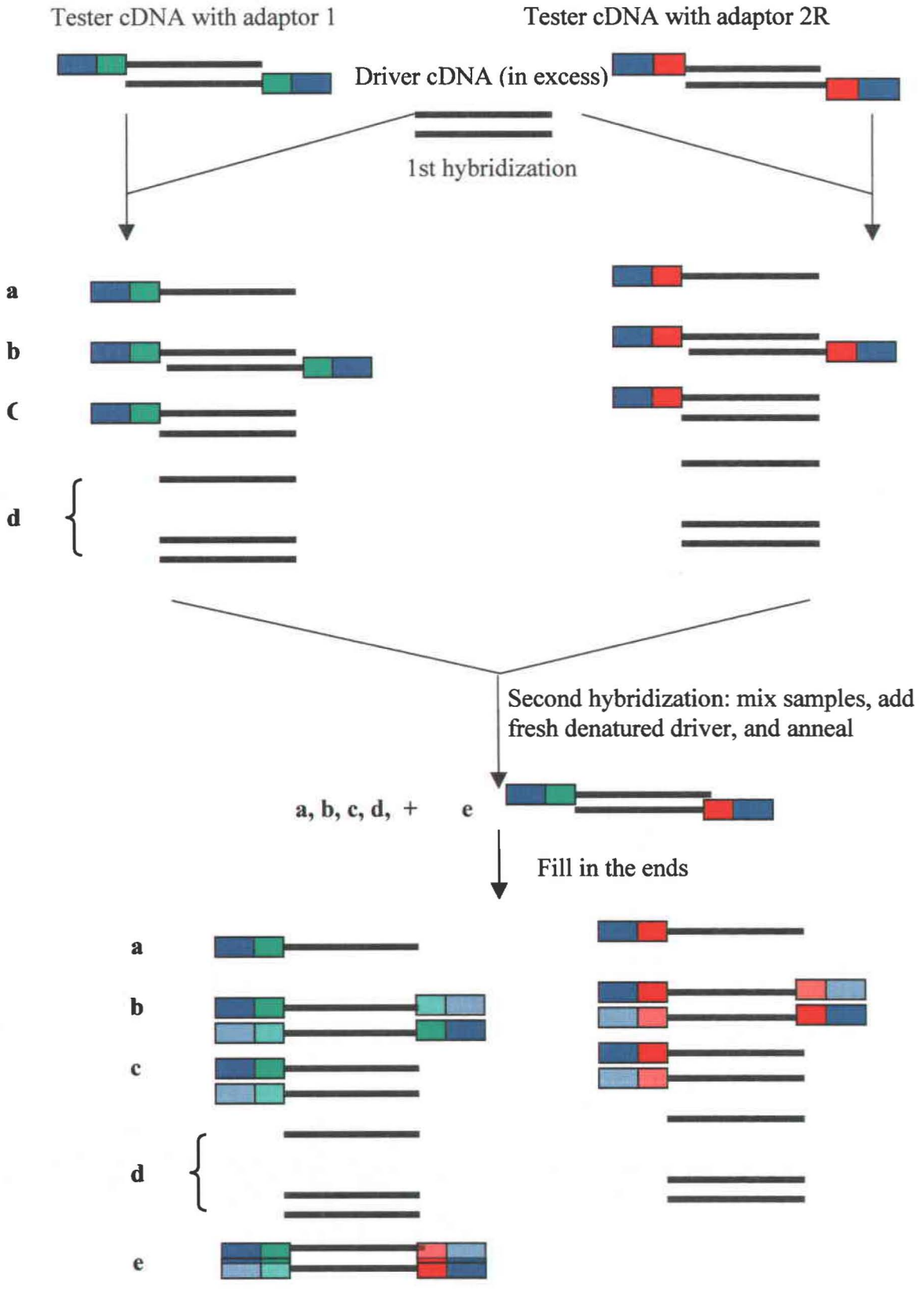


Figure 2. Schematic diagram of suppression subtractive hybridization, after Diatchenko *et al.*, 1996.

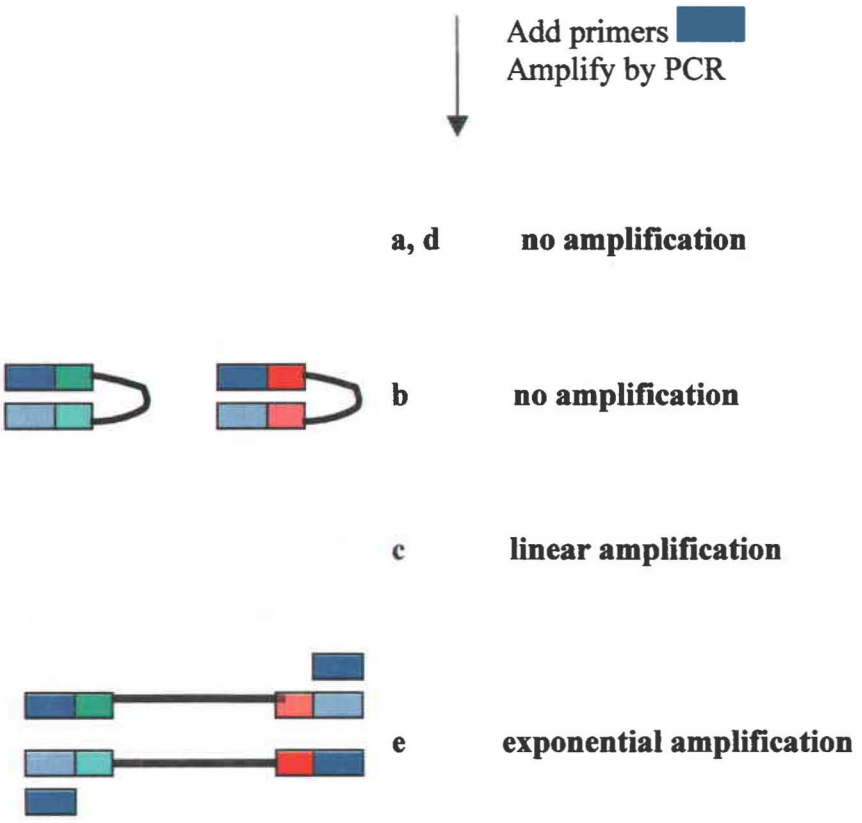


Figure 2. (continued) Schematic diagram of suppression subtractive hybridization, after Diatchenko *et al.*,1996.

molecules are greatly enriched for differentially expressed sequences since sequences not differentially expressed form type ***c*** molecules with the driver cDNA. The high- and low-abundance ss type ***a*** is equalized because reannealing of homo-hybrid cDNAs is faster for the high-abundance sequences.

During the second hybridization, the two first hybridization reactions are mixed together ,without being denatured, in the presence of excessive freshly denatured driver cDNA. After this, in addtion to type ***a***, ***b***, ***c***, and ***d*** molecules, there is a new type of molecule: ***e*** hybrids. The ***e*** hybrids form from two ss type ***a*** molecules and therefore, they are enriched and equalized for differentially expressed sequences. The type ***e*** hybrids are ds tester molecules with different ends corresponding to the sequences of adaptors 1 and 2R.

A small portion of the second hybridization is subjected to a suppression PCR to specifically amplify the type ***e*** molecules using a primer, which can anneal to part of both adaptors. The type ***a*** and ***d*** molecules are not amplified because of the absence of annealing sites. The type ***c*** only can be amplified linearly. For type ***b*** molecules, they have long inverted repeats on the ends designed to form a pan-like structure that prevents their exponential amplification (Diatchenko *et al.* 1996). Only type ***e*** molecules, which have two different adaptors, can be amplified exponentially. They represent the abundance-equalized, differentially expressed sequences. A secondary nested PCR amplification is conducted to obtain more differentially expressed

sequences, which are then cloned into a T/A cloing vector to obtain the subtracted cDNA library.

1.6 Strategies to Manipulate SAR to Improve Disease Resistance

As mentioned in previous sections, individual *PR* genes and the *NPR1* gene have been overexpressed in transgenic plants to achieve increased disease resistance. The moderate level expression of AtWRKY18 transcription factor also leads to enhancement of developmentally regulated defense responses without any observable detrimental effect on plant growth. Since the NPR1 and WRKY transcription factors are usually involved in regulation of multiple *PR* genes and there is a synergistic effect among different PR proteins, the engineering of plants using these regulatory genes may give plants more broad-spectrum resistance than overexpression of a single *PR* gene.

Considering that application of salicylic acid can activate SAR in *Arabidopsis* and tobacco, overproduction of salicylic acid in plants might lead to constitutive SAR. Microorganisms can produce SA as building blocks for iron-chelating siderophores. In these microorganisms chorismate is converted to isochorismate, catalyzed by isochorismate synthase (ICS) and subsequently isochorismate is converted to SA catalyzed by isochorismate pyruvate lyase (IPL). Tobacco accumulates chorismate in chloroplasts. When both bacterial ICS and IPL were expressed and targeted to chloroplasts, the transgenic plants showed 500- to 1,000-fold increased accumulation of SA and SA glucoside compared to the nontransgenic tobacco. This enhancement led to constitutive expression of *PR* genes and a resistance to viral and fungal infection resembling SAR in nontransgenic plants triggered by TMV preinoculation. The constitutive expression of *PR* genes did not change the plant phenotype. As expected, the

resistance of the ICS-IPL transgenic plants was not further increased by TMV preinoculation (Verberne *et al.* 2000).

Usually the SAR response follows HR. If we induce HR with an avirulence gene (Avr) product, we can expect SAR activation in plants. But the simultaneous expression of both an *R* gene and the cognate *Avr* gene product in an entire plant would induce systemic HR and plant death. There are some novel approaches to address this problem. The first one is to place the *Avr* under the control of a pathogen-inducible promoter in the plant genome while the *R* gene is constitutively expressed. The expression of the *Avr* gene is activated by nonspecific elicitors from the attacking pathogen. The *Avr* gene product then can interact with the *R* gene product to activate HR and SAR (Hammond-kosack and Jones 2000). The second approach is to insert a transposable element in the coding region of the *R* gene. At low frequency transposition restores functional *R* expression. This technology creates a plant that is a genetic mosaic for cells with and without restored *R* gene function. The R-Avr recognition event occurring in a small number of cells triggers a systemic SAR response (Hammond-kosack and Jones 2000). The third approach is to use feedback regulation to control the expression of an *Avr* gene in plants (Xu *et al.* 2002). The LacI repressor was placed under the control of a *PR-1b* promoter in *Arabidopsis*. Additionally, these lines contained two *Avr* genes, *HrmA* (from bacterium) and *Pap1* (from yeast), both driven by a 35S promoter engineered to contain two LacO sites. Initially the *Avr* genes are expressed constitutively, which in turn induces *PR* gene expression. This includes expression of LacI repressor. The LacI repressor then binds to the two Lac O sites in the 35S promoter and turns off expression

of *Avr* genes. This feedback can keep Avr proteins high enough to turn on SAR but not so high as to trigger systemic HR and the resulting transgenic *Arabidopsis* plants show enhanced resistance against *Pseudomonas syringae* pv. maculicola strain ES4326 and turnip crinkle virus (TCV). All the above approaches utilize R-Avr recognition to trigger SAR and work upstream of SA. They may provide more broad and sustainable resistance against pathogens than overexpression of any single *PR* gene.

1.7 Research Strategies and Hypotheses

Disease is the most serious problem facing the Hawaii papaya industry. SAR has been observed in many plant species to provide a broad-spectrum resistance against pathogens. BTH has been commercially used in crop fields as an activator of SAR. A previous study demonstrates that BTH root treatment protects papaya against *P. palmivora* and increases the activities of β-1,3-glucanases and chitinases systemically. (Zhu *et al.* 2002). These results are consistent with SAR induction, however, because plant species differ significantly in the details of SAR induction, it is important to obtain additional data so the applicability of SAR models derived from *Arabidopsis* and tobacco can be assessed for papaya.

PR-1 genes were proven to be upregulated by BTH in dicots, *Arabidopsis* (Lawton *et al.* 1996) and tobacco (Friedrich *et al.* 1996), and a monocot, wheat (Gorlach *et al.* 1996). *PR-1* expression is the most used molecular marker for plant SAR. Four PR1 cDNAs were isolated from papaya, based on a region conserved in *PR-1* genes of other plants. One of these is systemically induced by BTH, which provides new evidence to indicate

that BTH induces SAR in papaya. Then, a cDNA library based on suppression subtractive hybridization was constructed to isolate genes upregulated in leaves by BTH root treatment. Because the response occurred in untreated tissue, the observed gene induction can be defined as systemic. Isolated papaya genes shown to be BTH induced can serve as tools for further study of disease resistance in papaya, and in the evaluation of treatments or varieties intended to enhance disease resistance. Some novel genes, not known in other systems to be involved in disease resistance, were also cloned.

NPR1 protein was repeatedly shown to be a positive regulatory protein in *Arabidopsis* SAR. It occurs as a single copy gene in the *Arabidopsis* genome, and homologs have been identified in numerous plant species. It is a limiting factor in *Arabidopsis* SAR, and its overexpression can lead to increased broad-spectrum resistance (Cao *et al.* 1998; Chern *et al.* 2001; Friedrich *et al.* 2001). In the present study, an NPR1 gene was isolated from papaya and the structure of the deduced protein was compared to those domains required for activity in Arabidopsis. Additionally, overexpression of the *Arabidopsis NPR1* gene was tested in tobacco, to see if an altered SAR response was observed in a heterologous system.

The following hypotheses are to be tested in the present study:

- papaya has a SAR response and it is fundamentally similar to that described in *Arabidopsis*
- papaya has a *PR-1* gene which can be used as a marker of SAR, as has been established in *Arabidopsis* and tobacco

- suppression subtractive hybridization can be used for global analysis of gene expression in papaya SAR, with the potential for isolating genes not currently known to be defense related
- papaya has a *NPR1* homologue in its genome and it conserves all the domains critical for *Arabidopsis NPR1* gene function
- engineering tobacco using *Arabidopsis NPR1* cDNA under the control of the 35S promoter enhances the tobacco SAR response

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All enzymes for cloning were purchased from Promega. The radioactive P^{32} dCTP-labeled probes used for Southern blot analysis and Northern blot analysis were synthesized using Amersham Biosciences Rediprime™ II Random Prime Labeling System kit. Benzo-(1,2,3)-thiodiazole-7-carbothionic acid S-methyl ester (BTH) was obtained from Novartis Crop Protection, Inc. as the formulated wettable power (50% active ingredient). All other reagents & chemicals used for this study were purchased from Sigma unless otherwise specified.

2.1.2 Plant Material

Papaya (*Carica papaya L.*) seeds were germinated in the lab at 25°C, and approximately two weeks after emergence seedlings were transferred to 4X 4 $inch^2$ pots containing Sunshine® Mix No. 4 potting soil (pH 6.0-6.5). Plants were grown in the greenhouse at Hawaii Agriculture Research Center (HARC) Aiea, Hawaii with a temperature range of 20-26^0C.

2.2 Methods

2.2.1 Preparation and Sequencing of Plasmid DNA and Bacterial Artificial Chromosome (BAC) DNA

E. coli strain DH5α or DH10B carrying plasmids mentioned below were grown overnight in LB medium with 50 μg/ml kanamycin or 60-100 μg/ml carbenicillin as selection. BAC clones were cultured overnight in LB medium (see below) with 12.5 μg/ml chloramphenicol. Plasmid DNA and BAC DNA were purified using DNA-binding matrix kits (Qiagen). DNA quantity and quality was determined spectrophotometrically and its final concentration was adjusted to 1 μg/μl. DNA sequencing was performed by the University of Hawaii Biotechnology and Molecular Biology Instrumentation and Training Facility. All sequencing analysis was done with programs available at the NCBI web site (http://www.ncbi.nlm.nih.gov/blast) unless otherwise specified.

LB medium, pH 7.0 (1L):

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

Plasmid Sequencing Preparation:

0.5-1.0 μg template
3.0-4.0 pmol primer
Adjust with diH_2O to a final volume of 12 μl

BAC DNA sequencing Preparation:

1.0 μg template
30 pmol primer
Adjust with diH_2O to a final volume of 12 μl

2.2.2 Small Scale RNA Isolation from Plant Leaves

Plant leaves were ground in liquid N_2 to achieve full homogeneity. Approximately 100 mg ground sample was combined in an 1.5 ml eppendorf tube with 500 µl RNA Extraction Buffer (see below), which had been preheated to 80^0C and well mixed. After vortexing for 30 sec, 250 µl of chloroform: isoamyl alcohol (24:1, V:V) was added to each tube. After an additional vortex of 30 seconds, the mixture was centrifuged at 14,000 rpm at room temperature for 5 min. The supernatant was removed to a fresh RNase-free eppendorf tube and mixed well with an equal volume of 4 M lithium chloride (LiCl). The tubes were left overnight at 4^0C. The mixture was centrifuge at 4^0C at 14,000 rpm for 30 min. The supernatant was discarded and the pellet was washed with absolute EtOH. The pellet was air dried and resuspended in 250 µl DEPC-treated water. 25 µl of 3M pH5.5 sodium acetata (NaAc) and 2 volumes of the absolute EtOH were added, mixed well, and the mixture was kept in -20^0C for 2 hr to precipitate the RNA. Then the tubes were centrifuged at 4^0C at 14,000 rpm for 30 min and the resulting pellet was washed again with 100 % EtOH. After air drying, the pellet was dissolved in 50 µl DEPC-treated water. The dissolved RNA was stored at -80^0C. RNA concentration was determined using RiboGreen RNA Quantitation kit (Molecular Probes) and FLUOROLITE 1000 microplate fluorometer (DYNEX TECHNOLOGIES).

RNA Extraction Buffer

0.1 M	LiCl
100 mM	Tris.HCl, pH8.0
100 mM	EDTA
1%	SDS

The above four-item buffer was mixed with an equal volume of phenol (pH4.3) to make the RNA extraction buffer before use.

2.2.3 Large Scale RNA Isolation by Acid Guanidinium thiocyanate-phenol-chloroform Extraction

Large scale RNA isolation was carried out according to the single-step method by acid guanidinium thiocyanate-phenol-chloroform extraction with minor modification (Chomczynski and Sacchi 1987). 30 gram of papaya leaf was ground in liquid N_2 and homogenized at room temperature with 90 ml solution D. The following reagents were added to the homogenate and mixed by inversion after the addition of each reagent.

1.5 ml 2M NaAc pH4
15ml phenol pH6.7
3ml Chloroform-isoamyl alcohol (49:1)

After the addition of the final reagent, the mixture was shaken vigorously for 10 s and cooled on ice for 15 min. The mixture was centrifuge at 10,000 g for 20 min at 4^0C. The supernatant was transferred to a fresh tube and an equal volume of isopropanol was added to precipitate the RNA. The extract was kept at -20^0C for 1 hr and then centrifuged at 10,000 g for 10 min at 4^0C. The supernantant was discarded, and the pellet was dissolved in 15 ml solution D. After extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, V:V:V) twice, the supernant containing RNA was mixed with an equal volume of isopropanol. After incubating at -20^0C for 1 hr, the precipitate was centrifuged at 10,000 g for 10 min at 4^0C. The supernant was discarded. The resulting pellet was washed in 75% EtOH and air dried. It was then dissolved in 4 ml DEPC-treated water. The dissolved RNA was stored at -80^0C. RNA concentration was determined using RiboGreen RNA Quantitation kit (Molecular Probes).

Stock Solution of Solution D

250 g Guanidinium thiocyanate dissolved in 293 ml DEPC-treated Water
17.6 ml 0.75 M sodium citrate, pH7
26.4 ml 10% sodium lauroyl sarcosine
dissolved at 65^0C

Stock solution of solution D was stored at room temperature.

Solution D

0.36 ml 2-mercaptoethanol was added per 50 ml stock solution D to make the solution D prior to using.

2.2.4 Northern Blot Analysis and Stripping the Northern Blot

For each sample 10 μg of total RNA was precipitated and resuspensed in 10 μl formaldehyde gel loading buffer. The samples were heated at 70^0C for 10 min and quenched on ice for 5 min. The denatured RNA was separated on a formaldehyde denaturing gel (Sun 1994) using 1 X MOPS buffer with 1.85% formaldehyde (5ml 37% formaldehyde per 100ml solution). After electrophoresis, the gel was rinsed in DEPC-treated water twice and soaked in 10 X SSC (pH7.0) for 30 min on a gyrotory shaker (Model-2, New Brunswick Scientific Co.) at 50 rpm RT. The 10 X SSC (pH7.0) buffer was poured off and new 10 X SSC (pH7.0) was added to soak the gel in the same shaking condition for 30 more min. The RNA then was blotted to Hybond-N^+ membrane (Amersham) in the presence of 10 X SSC (pH7.0) by capillary blotting. After overnight blotting, the membrane was treated by UV crosslinking of 1200uJoules in a Stratalinker™ 1800 (STRATAGENE). Northern hybridization was conducted according to the same procedure used for Southern hybridization (see below).

Formaldehyde gel loading Buffer
0.72 ml formamide
0.16 ml 10 X MOPS Buffer
0.26 ml formaldehyde (37%)
0.18 ml H_2O
0.1 ml 80% glycerol
0.08 ml bromophenol blue (saturated solution)
0.015 ml 10 mg/ml EtBr
Total: 1.5 ml, stored at -20^0C

10 X MOPS

41.8 g MOPS
4.1g sodium acetate
Dissolved in 800 ml DEPC-treated water and the pH adjusted to 7.0
20 ml 0.5 RNase-free 0.5 M EDTA (pH8.0)
Adjust the volume to 1 liter using DEPC-treated water and autoclave

Before re-hybridization with the loading control probe, the first probe was stripped according to the manufacture's manual (Amersham Pharmacia Biotech). Boiling 0.1% (w/v) SDS solution was poured onto the moist membrane in a tray and allowed to cool to RT on a gyrotory shaker (Model-2, New Brunswick Scientific Co.) at 50 rpm. The solution was discarded and the membrane was rinsed briefly in 2 X SSC (pH7.0) twice. The stripped membrane was kept at 4^0C until ready for re-hybridization.

20 X SSC (pH7.0)

3.0 M NaCl
0.3 M sodium citrate
Adjust to pH7.0 with 1 N HCl

2.2.5 Isolation of Four *PR-1* Genes from Papaya

Three-month-old papaya seedlings were sprayed with 0.5 mM BTH water solution. Total RNA was isolated 24 hours later using the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). First strand cDNA was synthesized using the 3' RACE kit for rapid amplification of cDNA ends (cat. No. 18373-019, Life technolgies). According to the conserved region among all PR-1 type proteins: GHYTQVW (Van Loon and Van Strien 1999), two degenerate primers SPPR1 (5'-GWR TGY SGW CAC TAY ACT CA-3') and SPPR2 (5'-TGY SGW CAC TAY ACT CAG RT-3') were designed to do a 3' RACE and a nested 3' RACE respectively in combination with AUAP primer included in the kit.

1X 3' RACE reaction

di H_20:	29.25 µl
25 mM $MgCl_2$	8.0 µl
100 µM Primer SPPR1	1.25 µl
100 µM Primer AUAP	1.25 µl
10 X Buffer	5.0 µl
2mM dNTP (each)	5.0 µl
Taq DNA Polymerase	0.5 µl

3' RACE Thermal Cycle

Step 1	94^0C	30 S
Step 2	54^0C	30 S
Step 3	72^0C	30 S
34 more cycles of the above three steps		
Then 72^0C		5 min
4^0C		store

1 X 3' RACE nested PCR reaction

di H_20:	29.25 µl
25 mM $MgCl_2$	8.0 µl
100 µM Primer SPPR2	1.25 µl
100 µM Primer AUAP	1.25 µl

10 X Buffer	5.0 µl
2mM dNTP (each)	5.0 µl
Taq DNA Polymerase	0.5 µl

3' RACE nested PCR Thermal Cycle

Step 1	94^0C	30 S
Step 2	56^0C	30 S
Step 3	72^0C	30 S
34 more cycles of the above three steps		
Then 72^0C		5 min
4^0C		store

The resulting PCR product was cloned using the pPCR-Script plasmid vector using the PCR-Script Cloning Kit (#211190, STRATAGENE) and eight clones were sequenced.

2.2.6 Northern Blot Analysis of ESTs (Expressed Sequence Tags) in Papaya

Total RNA was isolated from papaya leaves after 50 ml 100 µM BTH and 50 ml water root drench treatments per three-month-old papaya plant, respectively, following the method described in section 2.2.2. For each sample 10 µg of total RNA was separated on a formaldehyde denaturing gel (Sun 1994). Northern blot analysis was performed according to the method described in section 2.2.4. After stripping, the transcription level was normalized against a partial papaya actin gene. This partial papaya actin gene was released from the plasmid pFA1-2 kindly provided by Dr. Kabi R. Neupane (University of Hawaii at Manoa) via double digestion of *EcoR1* and *Xho1*.

2.2.7 Induction of Pathogenesis-related (*PR*) Gene Expression by BTH Treatment

Total RNA was extracted from papaya leaves 0, 1, 4, 7, 10, 14 days after 50 ml 100 µM BTH root drench per three-month-old plant following the method described in section

2.2.2. For each sample 10 μg of total RNA was separated on a formaldehyde denaturing gel (Sun 1994). Northern blot analysis was performed according to the method described in section 2.2.4. The template to make the probe for *PR* genes was obtained by PCR amplification following the method described in section 2.2.9. After stripping, the transcription level was normalized against the mitochondrial small subunit rRNA gene using 532 bp PCR fragment of the tobacco mitochondrial small subunit rRNA gene kindly provided by Dr. Bob Bugos (University of Hawaii).

2.2.8 Construction of the Suppression-Subtraction cDNA Library

From three-month-old papaya seedlings sprayed with 0.5 mM BTH or water on leaves respectively, two populations of the total RNA were isolated from leaves three days after treatment using the guanidinium thiocyanate-phenol-chloroform extraction method described in section 2.2.3 (Chomczynski and Sacchi 1987). The messenger RNA then was isolated from the two populations of total RNA using the Promega PolyATract mRNA Isolation System IV kit (cat. No. Z5310). 2 μg of mRNA from the BTH treated population served as the starting material to synthesize tester cDNA and 2 μg of mRNA from the water treatment served as the starting material to synthesize driver cDNA. To test this system's ability to identify differences between two RNA populations, 2 μg human skeletal muscle mRNA provided in the kit was used to synthesize control driver cDNA. A portion of this cDNA was mixed with *HaeIII*-digested ΦX 174 DNA (0.2%) to serve as control tester cDNA. The PCR-Select Subtraction cDNA library kit (K1804-1, CLONTECH) was used to construct the subtracted cDNA library following the manufacturer's instruction except that to eliminate salts, enzymes, oligomers, linkers of

the previous reaction, GENECLEAN SPIN kit (BIO101) was employed instead of phenol:chloroform extraction and ethanol precipitation. All PCR amplification was conducted using the Advantage cDNA Polymerase Mix (#8417-1, CLONTECH) and Perkin-Elmer GeneAmp PCR System 9600 thermal cycler. The final PCR products were cloned into the plasmid vector pCR 2.1-TOPO hosted by TOP10F'*E. coli* (Catalog No. 45-0641, Invitrogen). LB plates containing 100 ug /ml carbenicillin and covered by 200 ul water solution of 1 % X-gal and 5 mM IPTG were used to select for the white (insert containing) colonies.

2.2.9 Reverse Northern Screening

Nested PCR primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and nested PCR primer 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') were used to amplify the inserts from white colonies in the subtracted cDNA library by PCR (CLONTECH PCR-Select cDNA Subtraction kit).

1X PCR reaction

di H_20:	16.0 µl
25 mM $MgCl_2$	1.5 µl
10 µM nested PCR primer 1	1.0 µl
10 µM nested PCR primer 2R	1.0 µl
10 X Buffer	2.5 µl
2mM dNTP (each)	2.5 µl
Taq DNA Polymerase	0.5 µl

Thermal Cycle

Step 1	94^0C	30 S
Step 2	58^0C	30 S
Step 3	72^0C	1 min 30 S

29 more cycles of the above three steps

Then 72^{0}C	5 min
4^{0}C	store

3 ul of the PCR reaction was dot blotted on each of two Nylon membranes after being denatured at 100^{0}C for 5 minutes and immediately cooled on ice for 5 minutes. After air drying, these two sets of identical dot blot membranes were treated by UV crosslinking of 1200 μJoules in a Stratalinker^{TM} 1800 (STRATAGENE) and kept at 4^{0}C for future hybridization.

0.5 μg mRNA isolated from papaya leaves 3 days after 50 ml water and 50 ml 100 μM BTH root drench treatments respectively was precipitated and resuspended in 6 μl DEPC-treated water in an RNase-free microcentrifuge tube. 1μl cDNA synthesis primer (10 μM) was added to the tube. The tubes were incubated at 70^{0}C in a thermal cycler for 2 min and cooled on ice for 2 min. After a brief centrifugation, the following items were added to each reaction tube:

5 X M-MLV Reverse Transcriptase Reaction Buffer:	4 μl
Rnasin	1μl
dNTP Mix (10mM for each of dATP, dTTP, and dGTP)	1μl
dCTP (60μM)	1μl
dCTP 32^P-ladeled (6,000 μCi/mmol)	5μl
M-MLV Reverse Transcriptase	1μl

The tubes were vortexed gently and centrifuged briefly. The reverse transcription reactions were conducted at 42^{0}C for 1.5 hr in an air incubator. Equal activity of ^{32}P-labeled first-strand cDNA was determined by TCA precipitation and used for each hybridization.

The ^{32}P-labeled first-strand cDNA synthesized from 0.5 μg mRNA isolated from papaya leaves from two treatment groups were denatured in the boiling water for 5 min and

cooled on ice for 5 min. Each pair of identical dot blot membranes was hybridized with each denatured probe. Hybridization and washing at 65^0C were carried out by the method described in section 2.2.14.

2.2.10 Inoculation with *Phytophthora palmivora*

Phytophthora palmivora was isolated from the roots of diseased papaya seedlings and cultured on V8 juice agar for one week under continuous fluorescent light. A culture was generously provided by Dr. Susan Scheck (Hawaii Agricultural Research Center). Characteristic sporangia and zoospores of *P. palmivora* were observed under the microscope. In order to release zoospores, the plates were flooded with sterile water, kept at 4^0C for 20 min and then at room temperature for 30 min. Then a sample of the overlaid water was vortexed violently to remove flagella to stop the movement of zoospores, and the number of zoospores per ml was determined microscopically. Four-month-old papaya seedlings roots were inoculated with 10,000 zoospores in 10 ml H_20. Pathogenicity was confirmed by the appearance of symptoms. As the negative control, 10 ml H_20 was used to treat roots of another group. After six days, all the leaves from treated plants were collected in liquid N_2 for RNA extraction according to the method described in section 2.2.2.

V8 Juice Agar 1 liter

V-8	200 ml
$CaCO_3$	3 g
agar	20 g
Water	800 ml
Streptomycin	0.5 g

2.2.11 Agrobacterium-mediated Genetic Transformation of Tobacco

The pKExNPR1 containing the entire coding region of the *Arabidopsis NPR1* cDNA was a gift from Dr. Xinnian Dong. The full-length *Arabidopsis NPR1* cDNA was released from pKEXNPR1 by *SalI* digestion, made blunt with Klenow fill-in and followed by *SacI* digestion. The GUS gene of pBI 121 was removed as a *Sma I-Sac I* fragment and replaced by the *Arabidopsis* cDNA sequence. The resulting plasmid pXQ1 (Figure 3) used Cauliflower mosaic virus (CaMV) 35S promoter to drive the *NPR1* cDNA, followed by the 3' terminus of *Agrobacterium tumefaciens* synthase (NOS). This construct was used to transform competent Agrobacterium strains EHA105 and LBA4404. The resulting Agrobacterium transformants were used to transform tobacco WI38 according to (Mathis and Hinchee 1994). Kanamycin resistant tobacco plants were screened by PCR using primer NPRf (5'-TACTCACCGGACCTGATGTA-3') and NPRr (5'-TCTCAGCAGTGTCGTCTTCT-3'). PCR positive plants were further confirmed using Southern blot analysis.

1 X PCR Screening reaction

di H_20:	34.5 µl
25 mM $MgCl_2$	3.0 µl
100 µM Primer NPRf	1.0 µl
100 µM Primer NPRr	1.0 µl
10 X Buffer	5.0 µl
2mM dNTP (each)	5.0 µl
Taq DNA Polymerase	0.5 µl

Thermal Cycle

Step 1	94^0C	30 S
Step 2	58^0C	30 S
Step 3	72^0C	1 min 30 S

34 more cycles of the above three steps

Then 72^0C	5 min
4^0C	store

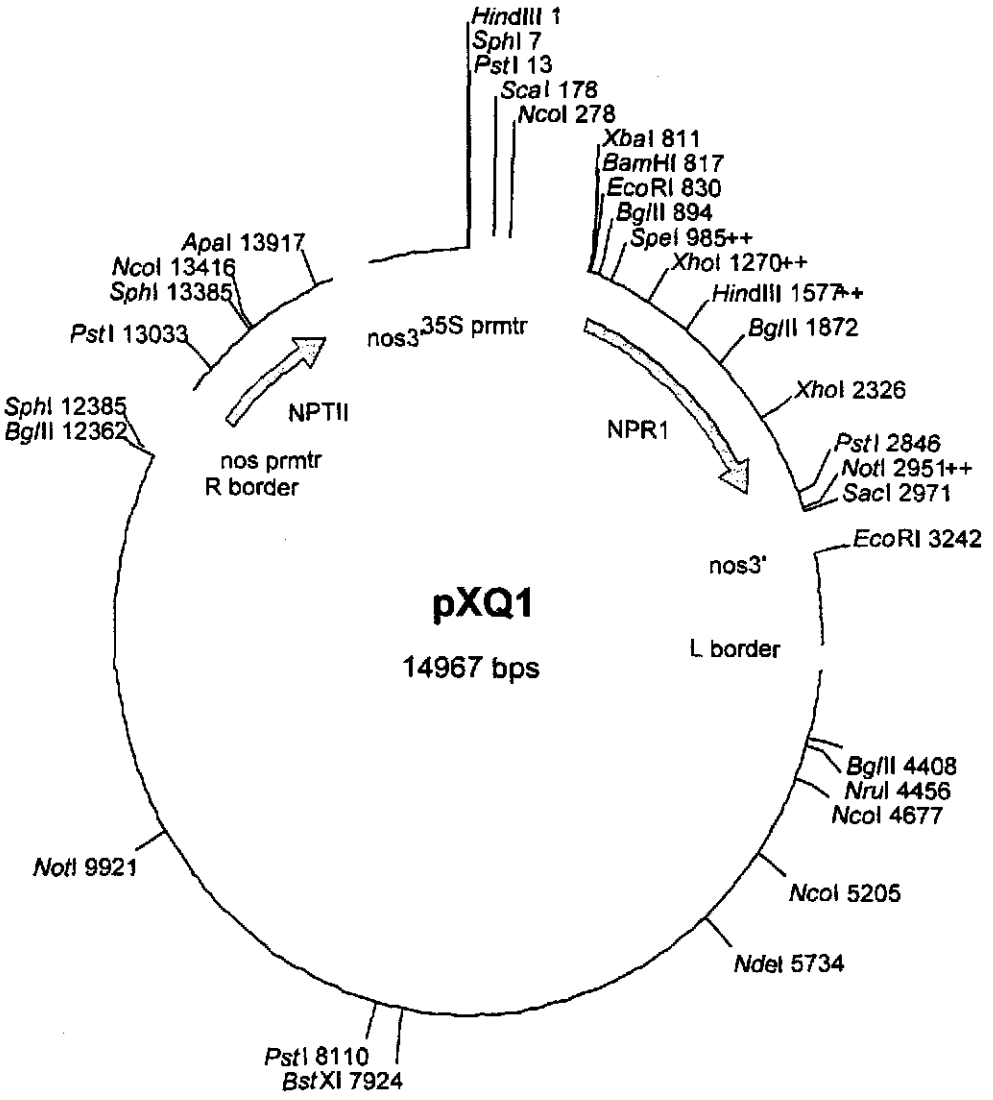


Figure 3. The restriction map of pXQ1, the binary vector used to transform tobacco with *Arabidopsis NPR1* cDNA driven by the cauliflower mosaic virus 35 S promoter.

2.2.12 Genomic DNA Isolation from Tobacco Leaves

Immediately before extraction, 1.14 g $NaHSO_3$ was added to 300 ml Extraction Buffer. 6-7 g of fresh leaf tissue from tobacco plants was ground into power in liquid N_2 and transferred to a 50 ml Falcon tube containing 20 ml extraction buffer. The slurry was thoroughly mixed by inversion and the mixture was incubated in 65^{0}C waterbath for 3 hrs. Six ml of 5 M potassium acetate (KAC) was added per tube, and the tubes were inverted gently until thoroughly mixed. After storing on ice for 20 min, the mixture was centrifuged at 3,500 rpm, 4^{0}C for 20 min. The supernatant was filtered through Miracloth into a set of new Falcon tubes containing 20 ml of –20^{0}C isopropanol without mixing. The tubes were kept untouched at –20^{0}C for 1 hr. DNA was spooled out on glass rods and washed in microfuge tubes containing 1 ml purifying buffer. The purifying buffer was poured off and DNA was washed in –20^{0}C 70% EtOH for 1 min. 70% EtOH was poured off and DNA was air dried. 300 µl TE (pH 8.0) were added to dissolve genomic DNA overnight at 4^{0}C.

<u>300 ml Extraction Buffer</u>

30 ml 1M Tris, pH8.0
30 ml 0.5 EDTA, pH8.0
30 ml 5M NaCl
18.75 ml 20% SDS
Use diH_2O to adjust the final volume to 300 ml

<u>Purifying Buffer</u>

70% EtOH
0.3 M NaAc

2.2.13 Southern Blot

Genomic DNA (10 μg) or BAC DNA (1μg) was digested with restriction enzymes overnight. Approximately 5 units of each enzyme per μg DNA were used in the reaction. Digested DNA was separated on a 0.8 % (w/v) agarose gel buffered with 1 X TAE and 0.5 μg/ml ethidium bromide. After electrophoresis, the gel was visualized on a UV transilluminator (FOTODYNE) and photographed by using Gel Print system (Biophotonics Corp). After soaking in 2 % HCl (v/v) for 10 min on a gyrotary shaker (Model-2, New Brunswick Scientific Co.) at 50 rpm at RT, the gel was transferred to a solution of 0.4 M NaOH and 1.5 M NaCl for 30 min with the same shaking condition to denature DNA. The denatured DNA was transferred to Hybond-N^+ membrane (Amersham) by capillary blot in a tray containing 0.25 M NaOH and 1.5 M NaCl overnight. After blotting, the membrane was air dried.

2.2.14 Southern Hybridization

The membrane was prehybridized in pre-hybrization buffer for one hour at 65^0C. The labeling reaction was carried out generally following the Rediprime™ II Random Prime Labeling System kit (Amersham) except that the probe was denatured in boiling water for 5 min and immediately quenched on ice for 5 min. The pre-hybridization buffer was discarded and hybridization was conducted overnight in 10ml fresh pre-hybridization buffer with the addition of denatured labeled probe. Then the membrane was washed with 50 ml first washing buffer 2 X 10 min and 50 ml second washing buffer 2 X 20 min. All the hybridization and washing were carried at 65^0C. The washed membrane was

exposed to Kodak X-Omat RP XRP-5 film for a few min to several days at -80^0C, as required for adequate exposure.

Prehybridization Buffer

7 % SDS
1% BSA (A-3912, SIGMA)
1mM $Na_2EDTA.2H_2O$
0.25 M $Na_2HPO4.7H_2O$ (pH7.4)

First Washing Buffer

0.5% BSA
1mM $Na_2EDTA.2H_2O$
40mM $Na_2HPO4.7H_2O$ (pH7.4)
5% SDS

Second Washing Buffer

1mM $Na_2EDTA.2H_2O$
40mM $Na_2HPO4.7H_2O$ (pH7.4)
1% SDS

2.2.15 Northern Analysis of *PR-1a* Gene in Tobacco

Total RNA were isolated from wild type nontransgenic plants and 15 independent transgenic lines before and 24 hours after treatment with 50 mM sodium salicylate. RNA concentration was determined using RiboGreen RNA Quantitation kit (Molecular Probes). For each sample 10 μg of total RNA was separated on a formaldehyde denaturing gel (Sun 1994) and blotted to Hybond-N^+ membrane (Amersham) in the presence of 1.5 M NaCl and 0.15 M sodium citrate (pH7.0) following the protocol. Northern hybridization was carried out according to the same procedure done for Southern hybridization. A partial tobacco *PR-1a* genomic clone was used as a probe to

perform the Northern hybridization. The transcription level was normalized against the level of tobacco 18S RNA.

2.2.16 Isolation of NPR1 Homologue Gene from Papaya

Total RNA was isolated from papaya seedlings 24 hours after treatment with 0.5 mM BTH water solution, according to the method described in section 2.2.3 (Chomczynski and Sacchi 1987). Using the 3' RACE system for rapid amplification of cDNA ends (cat. No. 18373-019, Life technolgies) first strand cDNA was synthesized. The comparison of *Arabidopsis*, tobacco and tomato NPR1 amino acid sequences found two shared regions (X. Dong, unpublished data). One is in the ankyrin repeat (from *Arabidopsis* amino acid 332 to 342) and another is C-terminal to the ankyrin repeats (from *Arabidopsis* amino acid 500 to 512). Based on these conserved regions, two degenerate primers F2 (5'-CTK CAT GTT GCW GCS ATG –3') and R2 (5' AAG AAB CGT TTY CCR AGT TC-3') provided generously by Drs. Wayne B. Borth and John Hu (University of Hawaii) were employed to carry out RT-PCR using the first strand cDNA as the template.

1X RT-PCR reaction

di H_20:	26.75 µl
25 mM $MgCl_2$	8.0 µl
50 µM Primer F2	2.5 µl
50 µM Primer R2	2.5 µl
10 X Buffer	5.0 µl
2mM dNTP (each)	5.0 µl
Taq DNA Polymerase	0.25 µl

RT-PCR Cycle

Step 1	94^0C	30 S
Step 2	54^0C	30 S
Step 3	72^0C	40 S

31 more cycles of the above three steps
Then 72^0C 5 min
4^0C store

10 pg pKExNPR1 containing the *Arabidopsis* NPR1 full length cDNA was used as a positive control. The amplification product size from the pKExNPR1 was 527 bp, as expected. A same size amplification product was obtained using the papaya first strand cDNA as the template. This PCR product derived from papaya was then recovered from the gel and cloned into the pPCR-Script plasmid vector using the PCR-Script Cloning Kit (#211190, STRATAGENE). Sequence analysis indicated that it was a partial papaya *NPR1* homologue cDNA. To clone its C-terminal two gene specific primers 5'-AGA ATC CAA GAG GAG CAC CT-3' and 5'-ACT CTA ACC GCG AGC AGA AC-3' were used for 3' RACE (Life Technologies). To clone the N-terminal of the Papaya *NPR1* homologue cDNA, the cloned partial cDNA was used to screen a papaya BAC (bacterial artificial chromosome) genomic library (Ming *et al.* 2001) (see the below for details). Three subclones were isolated to get the whole coding region sequence.

2.2.17 Screening the Papaya BAC (bacterial artificial chromosome) Genomic Library

Two BAC filters covering approximately 13.7 X papaya-genome equivalents (Ming *et al.* 2001) were prehybridized in 100 ml prehybridization buffer described in section 2.2.14 at 65^0C for 4 hrs. Then prehybrization buffer was poured out. 100 ml fresh buffer was added with denatured P^{32} dCTP-labeled probe to hybridize for 48 hrs at 65^0C. The hybridized BAC filter was then washed following the protocol described in 2.2.14 and exposed to Blue Sensitive Autoradiographic film (Marsh Bio Products, Inc.) at -80^0C. The positive BAC clones were identified by BAC-DMS 2.1 software (Universite

Catholique de Louvain, University of Georgia and Texas A & M University XD, August, 1999).

CHAPTER 3

RESULTS

3.1 Loading Controls for RNA Gel Blot Hybridization

Before separating RNA samples by electrophoresis, RNA concentrations were determined by the RiboGreen fluorescence assay (see methods above). In most cases, RNA samples visualized by EtBr fluorescence after gel electrophoresis appeared highly similar. However, occasionally a sample appeared abnormally high or low. To normalize for possible sample loading differences, papaya actin (Dr. Kabi R. Neupane, unpublished data) and tobacco mitochondrial small subunit rRNA (GenBank accession number: AF161095) genes were employed as probes to hybridize with blots to serve as loading controls, by comparing EtBr fluorescence to hybridization signals. Actin was found to be unaffected by BTH treatment, so actin hybridization was used to normalize expression of genes being tested for BTH induction (Figure 4, I). In these experiments, BTH treated and control plants were sampled at the same age and time after treatments; however, in time course experiments some change in actin levels was observed, perhaps reflecting developmental differences (Figure 4, II). Mitochondrial small subunit rRNA did not vary significantly over the time course of an experiment (Figure 4, II), so this gene was used for normalization of expression in experiments testing the kinetics of induction.

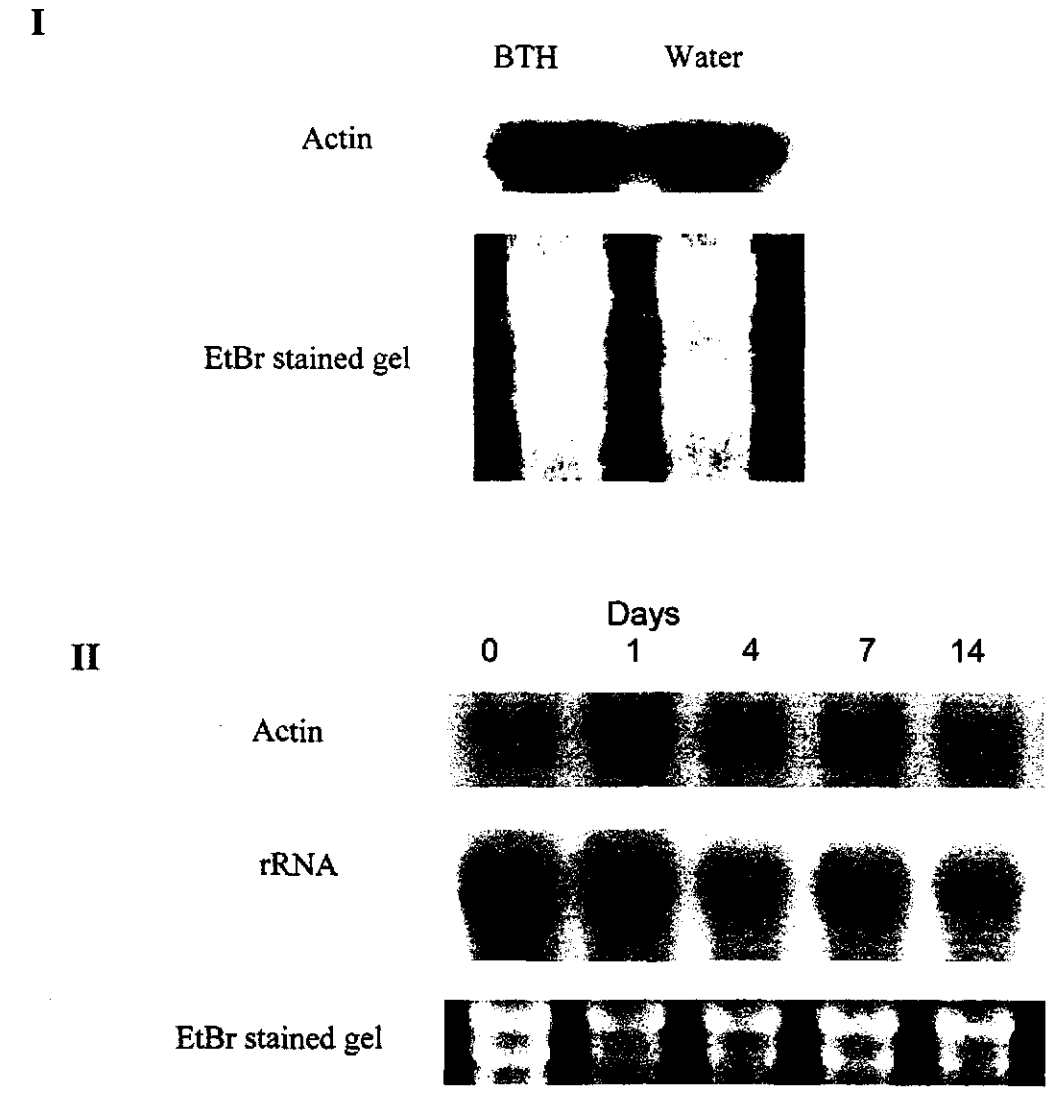


Figure 4. Actin and mitochondrial small subunit ribosomal RNA used as loading controls. (I) Hybridization to a papaya actin probe on a blot made from the gel shown with EtBr stain. (II) Hybridization to a papaya actin and tobacco mitochondrial small subunit ribosomal RNA (rRNA) probes on a blot made from the gel shown with EtBr stain.

3.2 Four *PR-1* Genes in Papaya

PR-1 is a group of PRs transcriptionally induced by pathogens, SA or BTH, and is a commonly used marker for SAR in a variety of plants including dicots, such as *Arabidopsis* and tobacco, and monocots, such as maize (Friedrich *et al.* 1996; Gorlach *et al.* 1996; Lawton *et al.* 1996). *PR-1* mRNA level has been used as a quantitative indicator of SAR strength in *Arabidopsis* (Cao *et al.* 1998). To clone a reliable molecular marker for papaya SAR, degenerate primers were designed according to the region GHYTQVW (Van Loon and Van Strien 1999) highly conserved among all PR-1 proteins, and used to carry out 3' RACE RT-PCR using first strand cDNA synthesized from the papaya plants sprayed by BTH as the template. Sequence analysis of the cloned RT-PCR products showed that four different *PR-1* partial cDNAs had been cloned. The cloned partial cDNA sequence includes 135 nucleotides in the coding region and 122 to 145 nucleotides in the 3' untranslated region (3' UTR).

The four *PR1* homologues were designated *PR-1a, b, c,* and *d.* Using northern blot analysis, *PR-1d* mRNA level in leaves was found to increase more than 17 fold three days after 100 μM BTH root drench (data not shown). Steady state mRNA pools for the other three related genes did not consistently increase in response to this treatment. Already at six hours after BTH root drench, *PR-1d* mRNA showed a significant increase in leaves (Figure 5). Similar rapid induction of *PR-1* transcription by BTH was reported in *Arabidopsis* (Lawton *et al.* 1996). Papaya *PR-1d* mRNA levels continued increasing for 14 days (Figure 6). This result is roughly consistent with that obtained

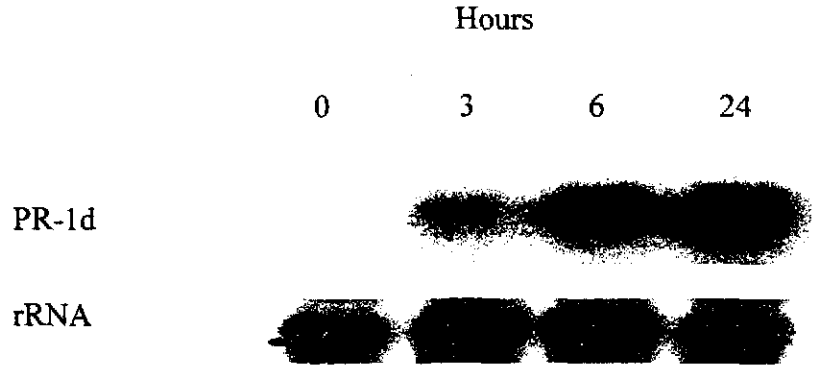


Figure 5. Accumulation of *PR-1d* mRNA in leaves 0-24 hours following 100 μM BTH root drench treatment in papaya. The tobacco mitochondrial small subunit rRNA gene was used as a probe for the loading control. Six hours of autoradiography.

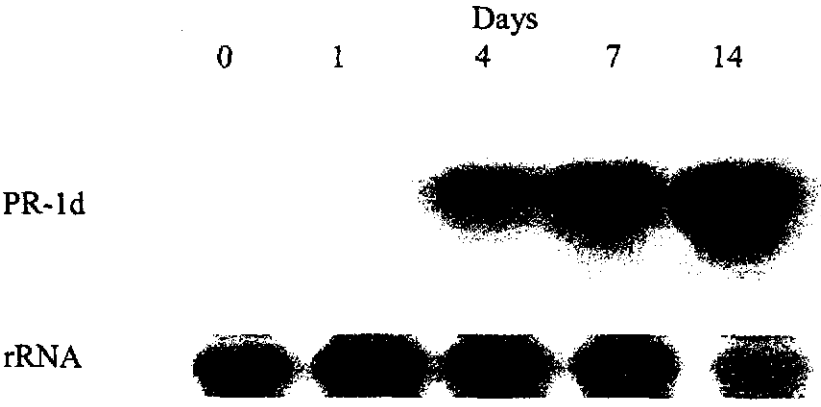


Figure 6. Accumulation of *PR-1d* mRNAs in leaves 0-14 days following 100 μM BTH root drench treatment in papaya. The tobacco mitochondrial small subunit rRNA gene was used as a probe for the loading control. One hour of autoradiography.

from *Arabidopsis* and tobacco (Friedrich *et al.* 1996; Lawton *et al.* 1996). Because BTH root drench can activate SAR-related enzyme activities, and increases tolerance to *P. palmivera* in papaya (Zhu *et al.* 2002) and this same treatment induces the accumulation of *PR-1d* mRNA, *PR-1d* can be used as a functional marker of SAR in papaya. Its cloned partial cDNA and deduced amino acid sequences are listed in Figure 7. This clone contains sequence encoding the fourth α-helix (α IV) and the third & fourth β-strands of the PR-1 protein (βC and βD), as showed in Figure 8 (Van Loon and Van Strien 1999).

Most *PR* genes belong to multi-gene families, and some members of the same family may not be inducible by pathogen challenge. In this study, only one out of four papaya *PR-1* genes is believed to be involved in the SAR response, since its expression is induced by BTH. The other three *PR-1* genes may have a constitutive defense function, or may play a role in other biological processes. Tobacco *PR-1a*, which is BTH inducible and widely used as a marker for SAR, shares similar levels of identity with papaya *PR-1b* (76%) and *PR-1d* (75%) (Table 1). Therefore, the degree of similarity with known *PR-1* genes involved in SAR can not be used to distinguish newly cloned *PR-1* genes induced during SAR from others having different expression patterns.

Table 1. Homology between four papaya *PR-1* genes with *Nicotiana tabacum PR-1a*. *PR-1a* is a molecular marker for tobacco SAR. Alignment of the 135 bp cloned cDNA with the corresponding coding region of tobacco *PR-1a* was performed using Align plus 3 for Windows (Scientific & Educational Software)

papaya *PR1* genes	Nucleotide identity with *Nicotiana tabacum PR-1a* (GenBank accession number: X12737)
PR-1a	47 %
PR-1b	76 %
PR-1c	67 %
PR-1d	75 %

```
5'-TGCGGTCACTATACTCAGGTTGTGTGGAAAAACTCGGTTCGTTTAGGGTGT
   C  G  H  Y  T  Q  V  V  W  K  N  S  V  R  L  G  C

   GCAAAAGTGACGTGCAACAATGGTGGAACTTTCATCACTTGCAACTATGAT
   A  K  V  T  C  N  N  G  G  T  F  I  T  C  N  Y  D

   CCTCCAGGCAACTTTATTGGCCAGAAACCTTACTAACCTACCATTCCTATCT
   P  P  G  N  F  I  G  Q  K  P  Y  *

   ATATATTATCAATAAAAGTTTCCCAATAATCCAGAGATAAAAAAAAAAAAAA

   AAAAAAAAA-3'
```

Figure 7. Partial nucleotide and deduced amino acid sequence of papaya *PR-1d* cDNA.

```
Identity = 33/45 (73%), Similarity = 40/45 (88%)

Tobacco  CGHYTQVVWRNSVRVGCARVQCNNGGYVVSCNYDPPGNYRGESPY
         CGHYTQVVW+NSVR+GCA+V CNNGG  ++CNYDPPGN+ G+ PY
Papaya   CGHYTQVVWKNSVRLGCAKVTCNNGGTFITCNYDPPGNFIGQKPY
             αIV       βC             βD
```

Figure 8. Amino acid sequence alignments of papaya *PR-1 d* deduced amino acid sequence with PR-1a protein from tobacco (GenBank accession number: X12737). The positions of the αIV, βC, and βD are underlined (Van Loon and Van Strien 1999).

3.3 Isolation of BTH-upregulated ESTs Using the Subtracted cDNA Library

3.3.1 Characterization of the Subtracted cDNA Library

Genes induced by BTH have been profiled in *Arabidopsis* using microarrays (Maleck *et al.* 2000; Schenk *et al.* 2000). In papaya, a BTH root drench increases the activities of chitinases and β-1,3-glucanases systemically and enhances resistance to *P. palmivora* (Zhu *et al.* 2002). One papaya *PR-1* gene (i.e. *PR-1d*) is induced by BTH like *Arabidopsis PR-1,* the SAR marker gene. All these indicate the activation of SAR by BTH in papaya. Cloning of more genes induced by BTH should further elucidate the similarity between papaya and *Arabidopsis* and may also shed light on unique aspects of papaya response to BTH.

A method termed suppression subtraction hybridization was used to construct a subtracted cDNA library to clone genes induced by BTH. First, mRNA was isolated from the leaves of BTH and water control root-drenched papaya seedlings. They were converted into cDNA. The cDNA derived from BTH treatment was referred to as "experimental tester cDNA" and cDNA from water treatment as "experimental driver cDNA". After restriction digestion of tester and driver cDNAs and the ligation of two aliquots of the tester cDNAs with adapters 1 and 2R, modified tester and driver cDNAs were hybridized. Only sequences unique/more abundant in the tester population, i.e. genes induced by BTH in this study, would lead to the production of a ds cDNA hybrid containing different (and hence non-suppressing) adapators at each end. Only these could be exponentially amplified in the suppression PCR; all others would be suppressed or amplified only in a linear mode. During suppression PCR, the very short cDNAs

(under 200 nucleotides) were not amplified because of the formation of pan structures. Then a second nested PCR was conducted to get more amplification products representing genes induced by BTH. The amplified products were cloned into a plasmid vector to form a subtracted cDNA library enriched for sequences induced by BTH.

To estimate the subtraction effect, a side-by-side control was included in this study. The control driver cDNA was synthesized from human skeletal muscle mRNA and the control tester was simulated by spiking a small amount of the *Hae* III-digested ΦX 174 DNA (0.2%) to an aliquot of the control driver cDNA. A complete control subtraction with the skeletal muscle driver and tester cDNAs in parallel with the experimental subtraction was performed. The spiked genes differentially expressed between skeletal muscle driver and tester cDNAs should be individual *Hae III* digested ΦX 174 DNA ligated with adapter 1 and 2R at each end. After the subtraction and two PCRs, the secondary PCR products were checked on an agarose/EtBr gel. The *Hae III* digested ΦX 174 DNA bands above 200 nucleotides, increased by the adaptor length, were indeed observed. This confirmed the method was able to detect unique sequences in the control tester at an abundance of 0.2%.

The secondary PCR products of the subtracted experimental cDNA were cloned into the plasmid vector pCR 2.1-TOPO. On IPTG /X-gal plates, 28 white colonies were chosen randomly. PCR was performed to amplify the insert between adapter 1 and adaptor 2R. Suppression subtractive hybridization is intended to isolate genes that are differentially expressed between two populations, and to equalize the abundance of different transcript

(cDNAs) within the tester population (Diatchenko *et al.* 1996). Sequence analysis found that among these 28 clones, 24 unique sequences were obtained. As the 28 randomly selected clones were not dominated by many copies of a few highly expressed genes, it appears the method was at least partially successful in abundance equalization. Northern blot analysis confirmed 12 of the 24 unique clones were upregulated by BTH. This showed that the subtracted cDNA library was enriched for differentially expressed genes, in this case genes induced by BTH.

Six of the 24 clones did not exhibit any hybridization signal in Northern blot analysis in either control or BTH treated plants. With the high sensitivity of RT-PCR and the abundance equalization, it is possible to clone genes by subtraction-suppression which are expressed at levels too low to be detected by Northern blot. With the current analysis, these low abundance clones can not be confirmed or rejected as differentially expressed (Diatchenko *et al.* 1996). Out of the other six, five are equally expressed between BTH and water treatments and one actually is downregulated by BTH slightly. They are nontargeted genes in the present study and are the background.

To screen for more genes induced by BTH, reverse Northern of the dot blot of 332 inserts amplified by PCR was carried out, and 89 of them were indicated to be upregulated by BTH. One example of the reverse Northern screening is given in Figure 9. In reverse Northern, a chitinase gene, previously shown to be induced by BTH, was employed as the positive control for genes induced by BTH, and the actin gene, which had been found to be equally expressed in both water and BTH treatments, was used as the loading or

negative control. Conventional Northern blot analysis confirmed the reverse Northern in every case. A total of 25 unique partial cDNAs or expressed sequence tags (ESTs) were confirmed by Northern blot analysis to be induced by BTH at least 1.5 fold in papaya leaves after BTH root drench treatment. These 25 unique clones were designated *CPBI*s (*Carica papaya* BTH-induced genes) (Table 2).

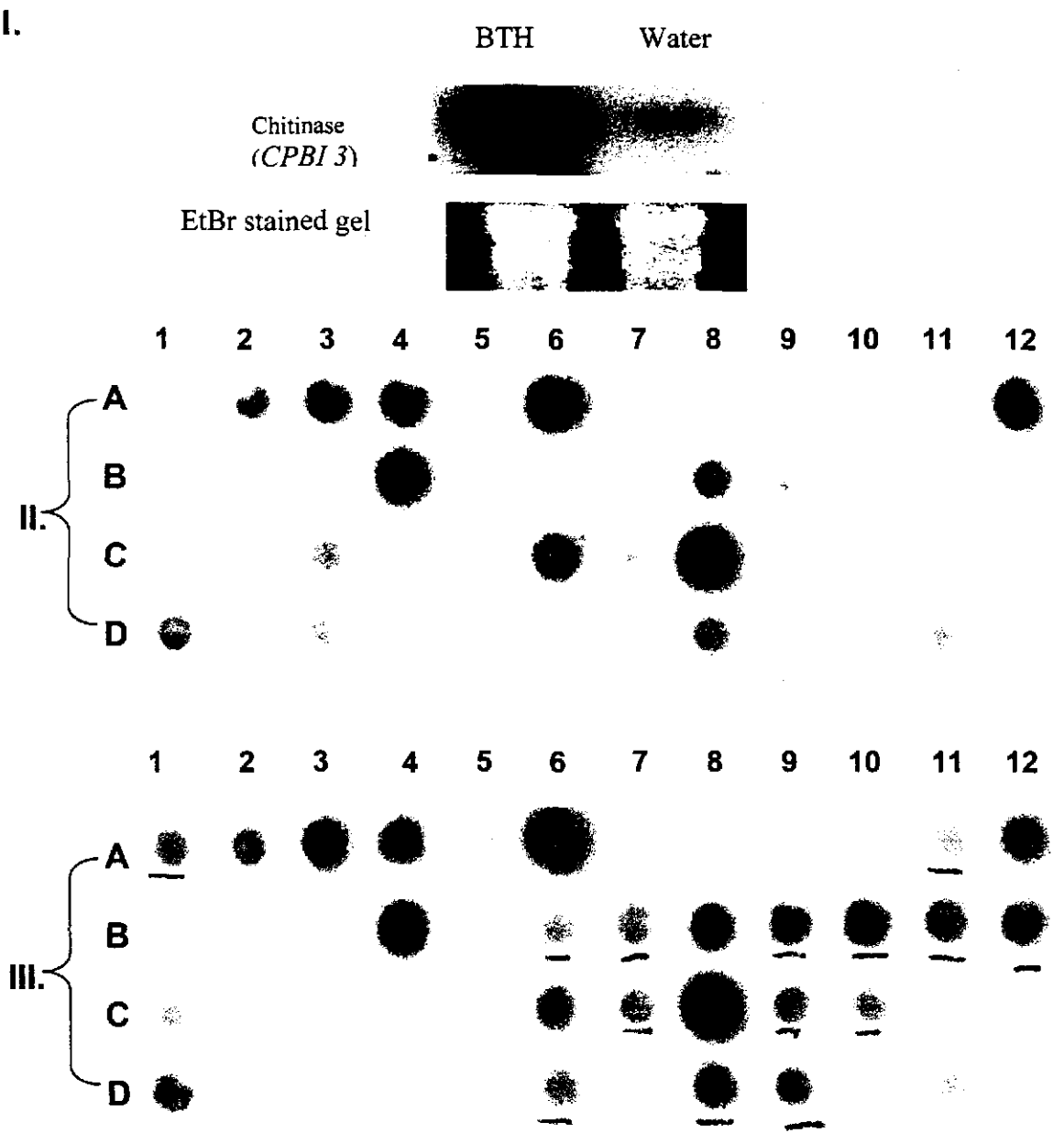


Figure 9. An example of the reverse Northern screening. Two series of identical dot blots were hybridized with P^{32} -labeled first strand cDNA respectively synthesized from mRNA of (II) water and (III) BTH root drench treatments. (I). Chitinase *CPBI 3* mRNA was induced by BTH. EtBr stained gel indicated equal loading of RNA from each treatment. In (II) and (III), A1 was *CPBI 3* clone, a positive control induced by BTH and A2 was an actin gene, a control not expressed differentially between two treatments.

Table 2. Genes induced in papaya leaves after BTH root drench treatment. Induction (BTH/ Water): genes transcript increase in BTH treatment compared with water treatment determined by Northern bot analysis after the normalization using the actin gene as a probe.

	Induction (BTH/Water)	Homologue	Others
CPBI 1	4.53	*Arabidopsis thaliana* chromosome 5 (NM_125936)	
CPBI 2	12.2	Citrus blight-associated protein p12 precursor (AAD03398)	
CPBI 3	16.7	Rape chitinase precursor (S25311)	CPBI 3 and CPBI 8 may be different regions of the same gene
CPBI 4	14.6	Tomato defense-related peroxidase precursor (T07008)	CPBI 4 and CPBI 25 may be different regions of the same gene
CPBI 5	Differential splicing or cross hybridization	*Arabidopsis* cytochrome P450, putative(NM_113538)	
CPBI 6	Water treatment not detectable	*Arabidopsis* putative Fe(II)/ascorbate oxidase (NM_117118)	CPBI 6 and CPBI 23 may be different regions of the same gene
CPBI 7	1.54	soybean ribosome-associated protein p40 (T05733)	
CPBI 8	2.77	Rape chitinase precursor (S25311)	CPBI 8 and CPBI 3 may be different regions of the same gene
CPBI 9	1.8	*Arabidopsis* RPT2 (AF181683)	
CPBI 10	1.54	*Arabidopsis* putative protein (NM_123878)	
CPBI 11	1.94	Nepenthes alata aspartic proteinase 3 (AB045893)	
CPBI 12	1.65	Tomato heat shock cognate protein 80 (M96549)	
CPBI 13	8.23	tobacco acidic chitinase class III (CAA77656)	
CPBI 14	8.9	*Ricinus communis* NADP-dependent malic protein (AF262997)	
CPBI 15	3.38	*Lavatera thuringiaca* stress-induced cysteine proteinase (AAB62937)	
CPBI 16	4.34	*Arabidopsis* 4-hydroxyphenylpyruvate dioxygenase (AAL61936)	
CPBI 17	6.66	common tobacco osmotin (X65700)	
CPBI 18	1.54	Tomato polyubiquitin (S34285)	
CPBI 19	1.52	*Arabidopsis* pectinesterase - like protein (T45827)	
CPBI 20	6.1	*Arabidopsis* callose synthase 1 catalytic subunit (AF237733)	
CPBI 21	3.73	upland cotton bacterial-induced gaiacol peroxidase (AF485268)	
CPBI 22	1.99	*Arabidopsis* putative membrane protein (NP_567130)	
CPBI 23	Water treatment not detectable	*Arabidopsis* putative Fe(II)/ascorbate oxidase (NM_117118)	CPBI 6 and CPBI 23 may be different regions of the same gene
CPBI 24	1.58	*Arabidopsis* cytosolic ribosomal protein L11 (T04865)	
CPBI 25	1.51	Tomato defense-related peroxidase precursor (T07008)	CPBI 25 & CPBI 4 may be different regions of one gene

Based on sequencing analysis some ESTs may just represent different parts of the same genes as detailed in Table 2, however, as induction levels varied these ESTs, they may represent different (and differently regulated) members of a gene family.

3.3.2 Four Papaya *PR* Genes Isolated from the Subtracted cDNA Library

At least 4 ESTs for chitinase (*CPBI 3*), peroxidase (*CPBI 4*), a second chitinase (*CPBI 13*) and osmotin-like proteins (*CPBI 17*) have been identified in these 25 papaya ESTs. As shown in Figure 10, their mRNA increased in leaf in response to BTH root drench. Presumably the induction was systemic. BTH root treatment resulted in the coordinate induction of multiple *PR* genes with different kinetics in papaya, as previously demonstrated in maize, *Arabidopsis*, and tobacco (Friedrich *et al.* 1996; Gorlach *et al.* 1996; Lawton *et al.* 1996; Morris *et al.* 1998). These 4 PR genes and PR1d can function as molecular markers for papaya SAR. Considering that each type of PR protein usually has multiple functions, multiple *PR* genes instead of a single *PR-1d* could serve as a more accurate indicator of SAR and a better tool to understand the various interactions between papaya and pathogens of different nature.

In plants, class I and class IV chitinases share a conserved main structure, although class IV chitinases lack four domains present in class I. Class II chitinases lack the N-terminal cysteine-rich domain but have a high amino acid sequence identity to the main structure of class I chitinases (Collinge *et al.* 1993). The deduced amino acid sequence of clone *CPBI 3* shows highest similarity with class I and IV plant chitinases (Figure 11); however

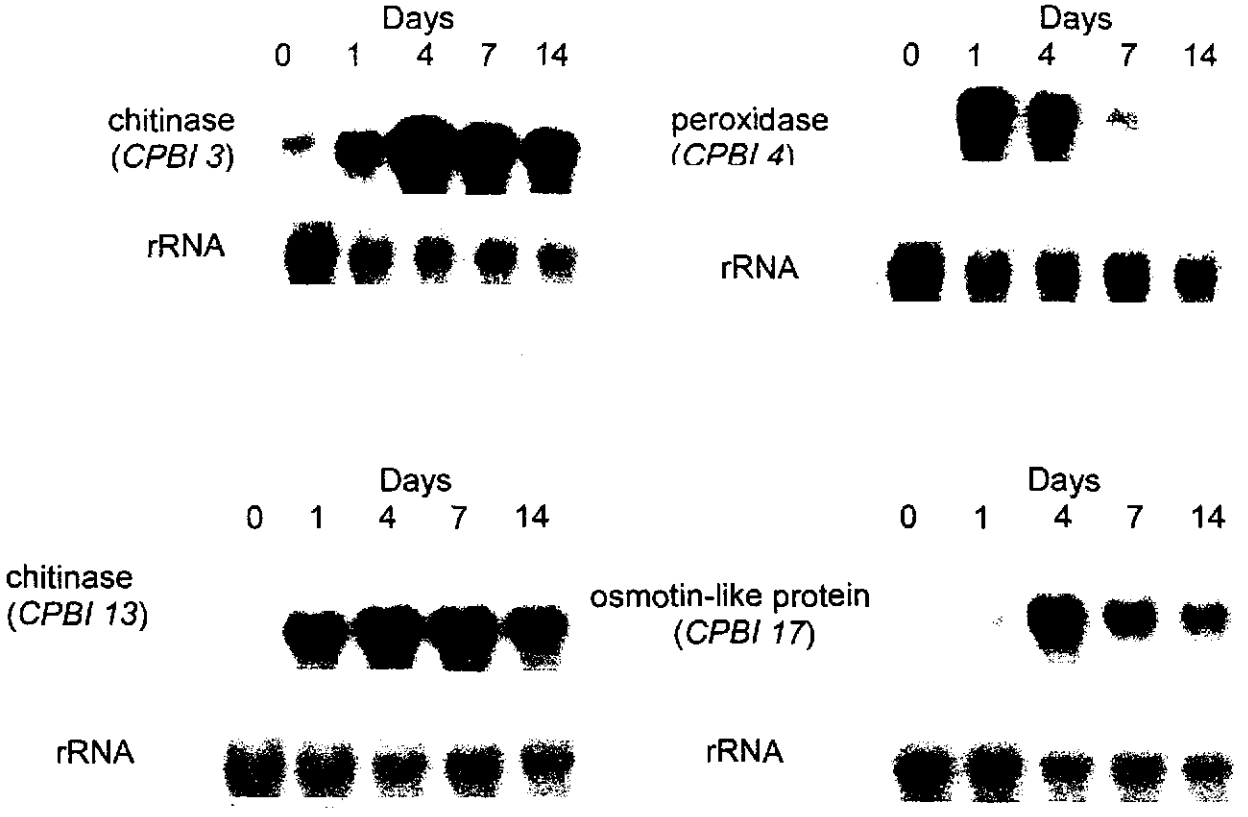


Figure 10. Accumulation of 4 *PR* gene mRNAs in papaya leaves following 100 μM BTH root drench treatment. The tobacco mitochondrial small subunit ribosomal RNA (rRNA) gene was used as a probe to normalize loading.

it does not contain the N-terminal cysteine-rich domain possessed by them. Therefore, clone *CPBI 3* most likely encodes a class II chitinase.

From the time course of induction by BTH and sequence analysis, it is clear that *CPBI 3* and *CPBI 13* encode different chitinases. The deduced amino acid sequence encoded by *CPBI 13* showed highest similarity with plant class III chitinases (Figure 12). Because the class III chitinases do not exhibit sequence similarity to chitinases in class I, II or IV (Collinge *et al.* 1993), clone *CPBI 13* most likely encodes a class III chitinase.

A search with the deduced amino acid sequences of *CPBI 4* revealed extensive homology to plant peroxidases. The alignment of deduced amino acid sequences of *CPBI 4* with the tomato defense-related peroxidase is shown in Figure 13. The partial cDNA *CPBI 4* encodes seven cysteine residues and three histidine residues in positions that have been conserved in the active-site structure of all peroxidases (Welinder 1992). Three domains found in other peroxidases are also present in the *CPBI 4* amino acid sequence: the histidine containing domain involved in acid/base catalysis (at tomato defense-related peroxidase position 71); the domain containing the sequence VSCSDIL; and the motif that includes the histidine residue involved in heme stabilization (at tomato defense-related peroxidase position 196) (Welinder 1992; Gadea *et al.* 1996).

Identity = 58/88 (65%), similarity = 68/88 (76%)

```
Papaya      SVDGTVTPAFFDGIIGQASASCAGKRFYSRNAFLSAARQYPQFGD--TKREIAAFFAHVT
            SV   VT AFF+GII QA   CAGK FY+R++F++AA  +P F +  T+REIA  FAH T
Rape    71  SVGSIVTQAFFNGIINQAGGGCAGKNFYTRDSFINAANTFPNFANSVTRREIATMFAHFT

Papaya      HETGHFCYIEEINGASLNYCDPNNRQYP
            HETGHFCYIEEINGAS +YCD NNRQYP
Rape    131 HETGHFCYIEEINGASRDYCDENNRQYP 158
```

Figure 11. Comparison of deduced amino acid sequence of *CPBI 3* with rape chitinase precursor (GenBank accession number: S25311)

Identity = 151/250 (60%), similarity = 187/250 (74%)

```
Papaya    SSGNYGIKTXLFSX-FRKRSDPQCSISXATVTHQLPNS--CIRFADQIKTCQRLGIKVLL
          ++ NY I    F   F    +P  +++     H  PN+  C   ++ I+ CQ  GIKV+L
Tobacco   ATNNYAIVNIAFLVVFGNGQNPVLNLAG----HCDPNAGACTGLSNDIRACQNQGIKVML

Papaya    SLGGGVGTYTXSSADDARQVANFLWNTFLGGQSSSRPFGNAVLDGIDFDIELGTNQHWDV
          SLGGG G+Y  SSADDAR VAN+LWN +LGGQS++RP G+AVLDGIDFDIE GT QHWD
Tobacco   SLGGGAGSYFLSSADDARNVANYLWNNYLGGQSNTRPLGDAVLDGIDFDIEGGTTQHWDE

Papaya    LARALRGFSGGRKVLLSAAPQCPFPDAKLDTAIRTGLFDYVWVQFYNNPPCQFSGNGA-S
          LA+ L  FS  RKV L+AAPQCPFPD  L+ A+ TGLFDYVWVQFYNNPPCQ+SG  A +
Tobacco:  LAKTLSQFSQQRKVYLTAAPQCPFPDTWLNGALSTGLFDYVWVQFYNNPPCQYSGGSADN

Papaya    LKSAWNTWTSMPAGKIFLGLPASPDAAGSGFVPANVVISQILPDIKRSPKYGGVMLWSRF
          LK+ WN W ++ AGKIFLGLPA+  AAGSGF+P++V++SQ+LP I  SPKYGGVMLWS+F
Tobacco   LKNYWNQWNAIQAGKIFLGLPAAQGAAGSGFIPSDVLVSQVLPLINGSPKYGGVMLWSKF
278

Papaya    HDRGFSSAIK
          +D G+SSAIK
Tobacco   YDNGYSSAIK
```

Figure 12. Comparison of deduced amino acid sequence of *CPBI 13* with common tobacco class III acidic chitinase (GenBank accession number: CAA77656)

Identity = 140/210 (66%), similarity = 173/210 (81%)

```
                          *                                          ▼ ▼*    *
Papaya      RGTRVGFYSTTCPQVESIVRSAVQARFNTDPTIAPGLLRMHFHDCFVRGCDGSILIEGSN
            +GTRVGFYS+TCP+ ESIV+S V++ F +DPT+APGLLRMHFHDCFV+GCDGSILI G+
Tomato 32   QGTRVGFYSSTCPRAESIVQSTVRSHFQSDPTVAPGLLRMHFHDCFVQGCDGSILISGTG

                                        *     *
Papaya      TEQTAIPNSNLRGFDVIQNAKTQLDSICPGVVSCSDILALAARDSVVLTGARSWPVPTGR
            TE+TA PNSNLRGF+VI +AK Q++++CPGVVSC+DILALAARDSV++T   +W VPTGR
Tomato 92   TERTAPPNSNLRGFEVIDDAKQQIEAVCPGVVSCADILALAARDSVLVTKGLTWSVPTGR

                                                          ▼     *
Papaya      RDGRVSLASEVGPNIPAFTDSVNEQKRKFSIKNLNTQDLVVLAGGHTIGTAACTVITGRL
             DGRVS AS+   N+P FT+SV  QK+KF+ K LNTQDLV L GGHTIGT+AC   + RL
Tomato 152  TDGRVSSASDTS-NLPGFTESVAAQKQKFAAKGLNTQDLVTLVGGHTIGTSACQFFSYRL

                                       *
Papaya:      FNFNGTAGASDPSISPSFLPVLKSLCPQDG
             +NFN T G  DPSI  +FL  L++LCPQ+G
Tomato: 211 YNFNST-GGPDPSIDATFLSQLQALCPQNG 239
```

Figure 13. Comparison of deduced amino acid sequence of *CPBI 4* with tomato defense-related peroxidase precursor (GenBank accession number: T07008). Conserved domains are underlined and conserved cystein and histidine residues are indicated with asterisks and triangles, respectively.

Identity = 34/65 (52%), similarity = 45/65 (68%)

```
Papaya:     MSPLKTTLPILGLLFVTLSFRLANAATFQVRNNCPFTVWAAGVP-GGGRQLNRGETWIIN
            M  L+++     L  VT ++    AAT +VRNNCP+TVWAA  P GGGR+L+RG+TW+IN
Tobacco: 1  MGNLRSSFVFFLLALVTYTY----AATIEVRNNCPYTVWAASTPIGGGRRLDRGQTWVIN
56

Papaya:      ANPGT
             A  GT
Tobacco: 57 APRGT 61
```

Figure 14. Comparison of deduced amino acid sequence of *CPBI 17* with osmotin from *N. tabacum* (GenBank accession number: X65700)

The alignment of the partial deduced amino acid sequence of *CPBI 17* with tobacco osmotin (amino acid 1-69) is shown in Figure 14. Amino acids 1-80 of tobacco osmotin are the amino-terminal signal peptide, which is cleaved off during post-translational processing (Melchers *et al.* 1993). This amino-terminal signal sequence is highly conserved in osmotins that accumulate during plant SAR. Because *CPBI 17* mRNA accumulated in response to BTH, it most likely encodes an osmotin-like protein.

3.3.3 Two Novel Genes Isolated from the Subtracted cDNA Library

The deduced amino acid sequence of *CPBI 2* shares highest homology with citrus blight-associated protein p12 precursor (Figure 15, I). Citrus p12 proteins accumulate in leaves, roots and stems of plants infected by citrus blight while it is not detectable in healthy plants or trees affected with other disorders (Derrick and Timmer 2000). Citrus p12 transcripts are present in root and stem cambium, but not in leaves of citrus blight-affected trees, suggesting transport of the protein to leaves (Ceccardi *et al.* 1998). The cause of citrus blight remains an unsolved mystery (Derrick and Timmer 2000).

Expansins are extracellular proteins that loosen plant cell walls and are encoded by two multigene families in *Arabidopsis* (Cosgrove 2000). Both p12 and the putative papaya protein that CPBI 2 encodes show similarity with plant expansins. But neither of them contains all conserved residues and HFD motif that are found in all expansins (Cosgrove 2000). Expansins are nearly three times larger than citrus p12 and bind to cell walls while p12 is a soluble protein (Derrick and Timmer 2000). Moreover, p12 does not

display any wall-loosening activity. The activity of p12 protein, and its relationship to expansin function is uncertain (Cosgrove 2000). The identity between papaya CPBI 2 and citrus p12, 51% based on the partial deduced amino acid, is considerably higher than between papaya CPBI 2 and expansins, which have no more than 31% identity. Citrus p12 was shown to be a citrus DNA-encoded protein and proposed be a plant-derived PR or stress protein which is translocated to perform or induce an unknown resistance function (Ceccardi *et al.* 1998). Since papaya *CPBI 2* transcripts are induced by BTH and accumulate specifically during SAR coordinately with PR genes, it supports a defense role for p12 proteins. Unlike citrus p12, *CPBI 2* transcripts were found to accumulate in leaves of papaya seedlings.

The *CPBI 6* transcript was not detectable in the water control treatment, but accumulated significantly three days after BTH root drench treatment (Figure 16, I). The partial deduced amino acid sequence of *CPBI 6* shares similarity with *Arabidopsis* putative Fe (II)/ ascorbate oxidase and *Arabidopsis* putative flavanone 3-beta-hydroxylase, neither of which is functionally defined (Figure 16, II and III). The function of either gene in plant defense is not obvious, however the very strong induction of *CPBI 6* by BTH argues for such a role.

I

Identity = 35/68 (51%), similarity = 48/68 (70%)

```
Papaya:     SVSRPRYRVRCIGGAN-AAPHPCKAGSVVVKVVDYCRSVCYGDINLSQYAFSQIADVNAG
            +V    +RV+C G  N   PHPC+ GSV+VK+VD C + C   I+LSQ AFSQIA+ +AG
p12   : 63  AVCNKSFRVKCTGATNQGTPHPCRGGSVLVKIVDLCPAGCQATIDLSQEAFSQIANPDAG

Papaya:     KVRIEYYQ
            K++IE+ Q
p12:    123 KIKIEFNQ 130
```

II

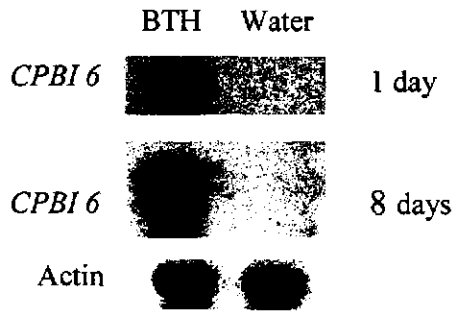
Identity = 21/67 (31%), similarity = 33/67 (48%)

```
Papaya:     YRVRCIGGANAAPHPCKAGSVVVKVVDYCRSVCYGD-----INLSQYAFSQIADVNAGKV
            Y++RC+       P  C  GS++V   ++C    + D       +LSQ  F +IA   AG V
Apple: 78   YQIRCVND----PQWCLPGSIIVTATNFCPPGGWCDPPQQHFDLSQPVFLRIAQYKAGVV

Papaya:      RIEYYQV
              + Y +V
Apple: 134   PVSYRRV 140
```

Figure 15. Alignment of deduced amino acid sequence of *CPBI 2* with citrus p12 and apple expansin 3. **(I)** *Comparison of deduced amino acid sequence of CPBI 2* with blight-associated protein p12 precursor from citrus (GenBank accession number: AAD03398). **(II)** Comparison of deduced amino acid sequence of *CPBI 2* with apple expansin 3 (GenBank accession number: AF527800). In **(II)**, the conserved amino acid and the HFD motif as the key signatures of the expansin family are in bold and underlined. Not all of them are present in the deduced amino acid sequence of *CPBI 2*.

I



II

Identity = 51/79 (64%), similarity = 62/79 (78%)

```
Papaya:    INGILRLAREFFRLPESQRLENYSDDPAKTTRLSTSFNVKTEKFSNWRDFLRLHCYPVQD
           I  ++  AREFFR  ES+R+++YS D  KTTRLSTSFNV  EK SNWRDFLRLHCYP++D
Arab.: 84  IKKMMNAAREFFRQSESERVKHYSADTKKTTRLSTSFNVSKEKVSNWRDFLRLHCYPIED
  143

Papaya:    YIHEWPTNPPFFREDVAEY
           +I+EWP+ P  FRE  AEY
Arab.: 144 FINEWPSTPISFREVTAEY 162
```

III

Identity = 46/79 (58%), similarity = 65/79 (82%)

```
Papaya:    INGILRLAREFFRLPESQRLENYSDDPAKTTRLSTSFNVKTEKFSNWRDFLRLHCYPVQD
           I+ ++ +AREFF +   ++++ YSDDP KTTRLSTSFNVK E+ +NWRD+LRLHCYP+
Arab.: 77  IDEMVSVAREFFSMSMEEKMKLYSDDPTKTTRLSTSFNVKKEEVNNWRDYLRLHCYPIHK
  136

Papaya:    YIHEWPTNPPFFREDVAEY
           Y++EWP+NPP F+E V++Y
Arab.: 137 YVNEWPSNPPSFKEIVSKY 155
```

Figure 16. Papaya *CPBI 6*. (I) *CPBI 6* mRNA accumulation in leaves three days after BTH and water root drench treatments. 1 day and 8 days: time of autoradiography. After 8 days autoradiography in -80^{0}C, the *CPBI 6* transcript in the water treatment was still not detectable. (II) Comparison of deduced amino acid sequence of *CPBI 6* with *Arabidopsis* putative flavanone 3-beta-hydroxylase (GenBank accession number: AAO50563). (III) Comparison of deduced amino acid sequence of *CPBI 6* with *Arabidopsis* putative Fe(II)/ascorbate oxidase (GenBank accession number: CAB78173).

3.4 Effect of Pathogen Infection on Papaya *PR* Genes

Phytophthora root rot caused by *Phytophthora palmivora* is one of the most difficult diseases to control in papaya. To determine whether infection with this virulent pathogen induced changes in gene expression, papaya seedlings were challenged with high titers of zoospores. *P. palmivora* was cultured on V8 juice agar, and characteristic sporangia and zoospores were observed. Nine days after root inoculation with *P. palmivora*, the lateral roots of papaya seedlings died and a soft, dark-brown rot extended to the taproot (Figure 17, I). The leaves turned yellow and wilted. Seedling roots treated with water were free of any visible symptoms (Figure 17, I).

Northern blot analysis of the expression of 4 *PR* genes (for PR-1d, two chitinases, one osmotin) did not show any difference in the mRNA level after *P. palmivora* root inoculation (Figure 17, II). Only *CPBI4* (peroxidase) increased 1.4 fold (Figure 17, III). This is consistent with compatible reactions in other systems, and with work showing that BTH treatment is an effective treatment against *P. palmivora* in papaya (Zhu *et al.* 2002). BTH induces SAR in papaya, but *P. palmivora* infection slightly induces only one of five SAR related genes induced by BTH.

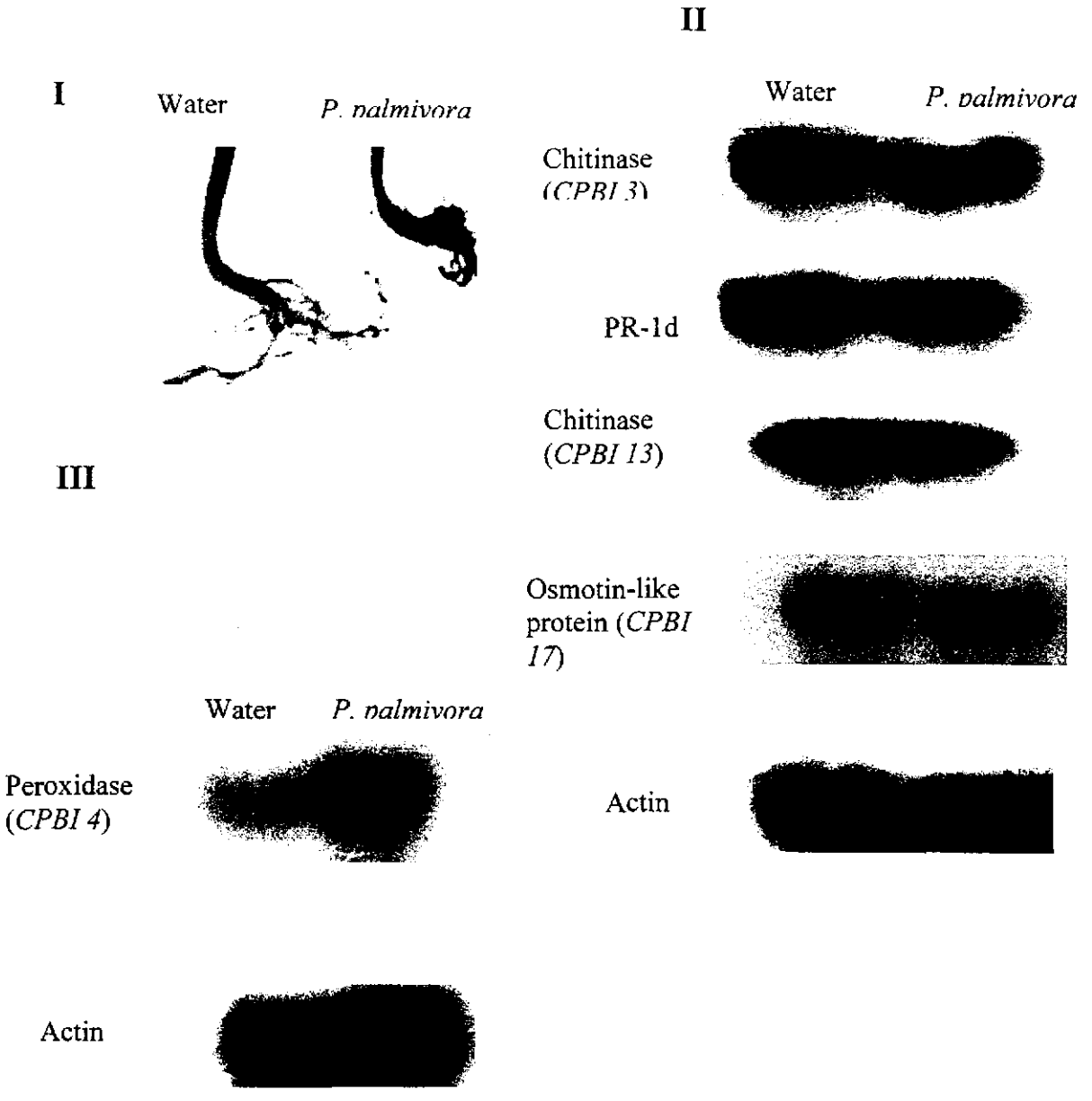


Figure 17. Accumulation of five PR gene transcripts in papaya leaves six days after root inoculation with *Phytophthora palmivora*

3.5 Heterologous Expression of the *Arabidopsis NPR1* cDNA in Tobacco

Tobacco (cv. WI38) was transformed with the binary vector pXQ1 (Figure 1), in which the *Arabidopsis NPR1* cDNA is under the control of the constitutive 35S promoter of the cauliflower mosaic virus. Putative transgenic plants were screened by PCR (Figure 18) and Southern blot. Fifteen independent transgenic lines were recovered. These plants were then analyzed by Northern blot for altered induction of *PR-1a* after SA treatment (Figure 19). Prior to SA treatment, *PR-1a* mRNA levels were unaltered relative to wild type plants. After SA treatment of 15 transgenic lines, 6 lines showed increased *PR-1a* expression in comparison to wild type (Table 3). Since *PR-1a* is an indicator of SAR in tobacco, it is expected that these six transgenic lines might have elevated SAR. *Arabidopsis* lines overexpressing *NPR1* with induced expression of *PR1* mRNA 1.5 to 3 times that of wild type level displayed significantly increased resistance against a bacterial pathogen and an oomycete fungal pathogen (Cao *et al.* 1998). Correspondingly, *NPR1* transgenic tobacco with elevated induction of *PR-1a* may have elevated resistance against pathogens. Altered *PR-1a* expression in these lines indicates the heterologous NPR1 protein functions in tobacco. Recent work has shown the *Arabidopsis* NPR1 protein also functions in rice (Chern *et al.* 2001).

Table 3. Six out of 15 transgenic plants show increased *PR-1a* mRNA accumulation after salicylic acid treatment. Mitochondrial small ribosomal RNA was used to normalize loading.

	Salicylic acid-induced PR-1a (mRNA)
Wild type	1.00
T_0-42	1.80
T_0-54	1.68
T_0-57	2.12
T_0-59	3.51
T_0-67	3.45
T_0-78	3.34

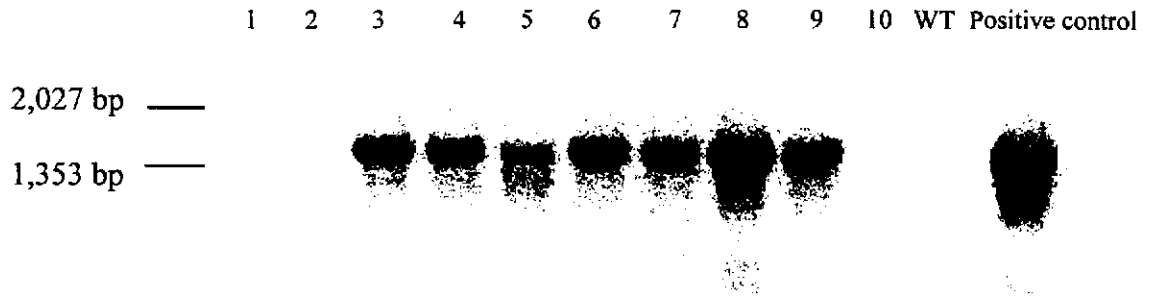


Figure 18. An example of the PCR screening of putative transgenic tobacco plants. 1-10: putative transgenic plants carrying *Arabridopsis NPR1*. WT: nontransformed tobacco plants. Positive control: 100 pg of pKEXNPR1.

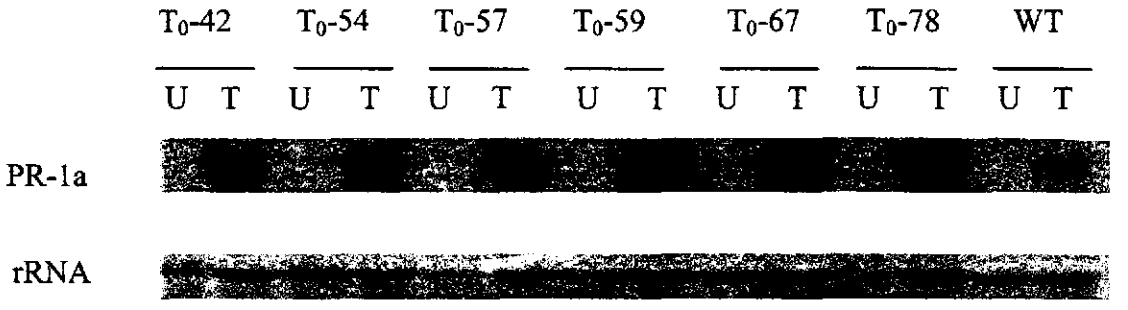


Figure 19. mRNA accumulation in 6 putative transgenic and wild type tobacco plant leaves 24 hr after water (U) and salicylic acid (T) treatments. The mitochondrial small ribosomal RNA (rRNA) was used to normalize loading.

3.6 Isolation and Characterization of the NPR1 Homologue from Papaya

The papaya NPR1 deduced peptide sequence has 71.04% and 66.84% similarity with rice and *Arabidopsis* respectively. A similar level of homology was reported between tobacco, tomato, and *Arabidopsis* (Cao *et al.* 1998). There are four exons and three introns in the papaya *NPR1* gene as marked in Figure 20. The position of the three introns is identical to that of the *Arabidopsis NPR1*, further supporting homology of these genes. Amino acid residues required for function of NPR1 in *Arabidopsis* are conserved in the papaya and rice NPR1 sequences (Figure 21).

As in *Arabidopsis*, exon 2 of papaya *NPR1* encodes four ankyrin repeats (amino acids 268 to 396). This ankyrin repeat region shows significantly higher identity and similarity with the *Arabidopsis* sequence than do other regions of the gene. For this region, the identity and similarity are 64.3% and 72.9% respectively while the identity & similarity of the whole sequence are 58.4% and 66.8%. This indicates the importance of the ankyrin repeat region to the normal function of NPR1. Exon 1 of papaya *NPR1* contains a POZ domain (Figure 22). For the consensus amino acids of the POZ, *Arabidopsis* and papaya share 85% similarity (28/33).

Analysis of the *Arabidopsis* NPR1 protein sequence identified three potential nuclear localization signals (NLSs) (NLS1, amino acids 252 to 265; NLS2 amino acids 541-554;

NLS3, amino acids 582 to 593). Mutations in the first possible NLS did not affect the nuclear localization of the NPR1-GFP fusion protein in *Arabidopsis*. Mutation study indicated that the domain from 541 to 554 (NLS2) may function as a NLS (Kinkema *et al.* 2000). Mutagenesis of five basic amino acids in this region resulted in localization of the fusion protein exclusively to the cytoplasm. Four out of these 5 amino acids required for nuclear localization of *Arabidopsis* NPR1 are also conserved in papaya, rice, and tobacco (Liu *et al.* 2002) (Figure 23, B). This conservation further supports that the second possible NLS is functional. Papaya and rice NPR1 homologues do not show high conservation with *Arabidopsis* NPR1 in the first possible NLS region (Figure 23, A). Therefore, this one is unlikely to be functional, as suggested by the mutagenesis study. Both papaya and rice NPR1 homologues lack the entire third possible NLS present in *Arabidopsis* (Figure 23, C), suggesting that this one is likely not functional, either.

```
5'- ATTTCCTCTGCTTCAAAAATTTCCCTAAATCCGTATTTTCTGCGTAGCTAT

    CTTCATTTCTCCTCTTAATCCTCTCATGGATTATAGAACAGGAACCTCAGA
                             M  D  Y  R  T  G  T  S  D

    TTCCAACGACATCAGCAACAACAGCAGTACTTGTTGCGTGGCAACTAACAC
     S  N  D  I  S  N  N  S  S  T  C  C  V  A  T  N  T

    AGACACGCTTTCGCATCCATTAGAGCCTCTAACGACTCCGGAGATCTCGGG
     D  T  L  S  H  P  L  E  P  L  T  T  P  E  I  S  G

    TCTGCAGCTGCTCTCTCGTAACCTGCTGACAATCTTTGACTCTTCTGACTT
     L  Q  L  L  S  R  N  L  L  T  I  F  D  S  S  D  F

    TGACTTCTTCAGTGACGCGAGGCTGATGCTCGGCTCCGGCCGTGAGATCCC
     D  F  F  S  D  A  R  L  M  L  G  S  G  R  E  I  P

    CGTGCACCGTTGCATTCTTTCCTCGAGGAGTCCCTTCTTCAAAGCCATCTT
     V  H  R  C  I  L  S  S  R  S  P  F  F  K  A  I  F

    CTCCGGCTCTGCGTTCAAGGAGAGAACCGCCAAGTTCCGCCTCAAGGAACT
     S  G  S  A  F  K  E  R  T  A  K  F  R  L  K  E  L

    TGCTGGAGACTATGATGTCGGTTTCGACGCGCTTGTTGCCGTTTTAGCTTA
     A  G  D  Y  D  V  G  F  D  A  L  V  A  V  L  A  Y

    TCTGTACACTGGCAAGGTTTGGCCGTTACCAAAGGGAGTTTGTGTTTGCGT
     L  Y  T  G  K  V  W  P  L  P  K  G  V  C  V  C  V

    GGACGAAGAGTGCTCGCACGTCGGCTGCAGGCCGGCGGTGGATTTCCTGGT
     D  E  E  C  S  H  V  G  C  R  P  A  V  D  F  L  V

    GGAGGTGCTCTACGTGGCTTTTACCTTCCAGATTTCGGAATTAGTGGCCCT
     E  V  L  Y  V  A  F  T  F  Q  I  S  E  L  V  A  L
           ▼
    TTATCAGCGGCACTTACTGGACATTATTGATAAAGTTGAGACGGACAATAT
     Y  Q  R  H  L  L  D  I  I  D  K  V  E  T  D  N  I

    TTTAGTGATTCTTTCTGTTGCAAATATATGTGGTAAAGTGTGTGACAGATT
     L  V  I  L  S  V  A  N  I  C  G  K  V  C  D  R  L
```

Figure 20. The nucleotide and deduced amino acid of the papaya *NPR1* cDNA. The start and stop codes are in bold and underlined. The nucleotide sequence of the three introns are not shown and their positons are marked by▼

```
GCTCGGCAGGTGTATGGATATTATTGTCAAATCTGATGTAGATGCAGTCAC
 L  G  R  C  M  D  I  I  V  K  S  D  V  D  A  V  T

CCTTGATAAATCGTTGCCCCTGAGCATTGTAAAACAAATCATGGATTTACG
 L  D  K  S  L  P  L  S  I  V  K  Q  I  M  D  L  R

AGCAGAATGCGACACACAAGGCCCTGAAGGTAGGAGTTTTCCAGATAAACA
 A  E  C  D  T  Q  G  P  E  G  R  S  F  P  D  K  H

TGTGAAGCGAATACACCGTGCTTTGGATTCAGATGATGTTGAATTAGTTAG
 V  K  R  I  H  R  A  L  D  S  D  D  V  E  L  V  R

GATGCTTCTGAAGGAGGCACGCACCAATCTGGATGATGCACATGCTCTCCA
 M  L  L  K  E  A  R  T  N  L  D  D  A  H  A  L  H

CTATGCTGTAGCATATTGTGATGCAAAGACAACAATAGAGCTCCTTGACCT
 Y  A  V  A  Y  C  D  A  K  T  T  I  E  L  L  D  L

TGGGCTTGCAGATGTTAACCATAGAAATTCAAGAGGCTATACTGTGCTACA
 G  L  A  D  V  N  H  R  N  S  R  G  Y  T  V  L  H

TATTGCTGCAATGCGGAAAGAGCCCAAACTCATAGTATCGCTTTTAACAAA
 I  A  A  M  R  K  E  P  K  L  I  V  S  L  L  T  K

AGGCGCTCGACCATCAGATCTTACCCCAGATGGGAGGAAAGCACTCCAAAT
 G  A  R  P  S  D  L  T  P  D  G  R  K  A  L  Q  I

ATCAAAACGGCTCACTAAAGCAGCTGATTATTATAACACTACAGAGGAAGG
 S  K  R  L  T  K  A  A  D  Y  Y  N  T  T  E  E  G

AAAGGCTGCACCCAAGGATCGGTTATGTGTAGAAATATTGGAGCAGGCAGA
 K  A  A  P  K  D  R  L  C  V  E  I  L  E  Q  A  E

AAGGCGAGATCCACTACTTGGAGAAGCTTCTCTCTCTCTTGCAAAAGCTGG
 R  R  D  P  L  L  G  E  A  S  L  S  L  A  K  A  G

                                                ▼
TGATGATTTCAGGATGAAACTGTTGTACCTTGAAAACAGAGTTGGGCTGGC
 D  D  F  R  M  K  L  L  Y  L  E  N  R  V  G  L  A
```

Figure 20. (continued) The nucleotide and deduced amino acid of the papaya *NPR1* cDNA. The start and stop codes are in bold and underlined. . The nucleotide sequence of the three introns are not shown and their positons are marked by▼

AAAACTTCTTTTCCCCATGGAAGCAAAAGTTGCAATGGATATTGCCCAAGT
K L L F P M E A K V A M D I A Q V

GAATGGAACTTCTGAGTTCACATTTGATGGCATCAACTCTAACCGCGAGCA
N G T S E F T F D G I N S N R E Q

GAACACTATGGATTTGAATGAGGCGCCTTTCAGAATCCAAGAGGAGCACCT
N T M D L N E A P F R I Q E E H L

GAATAGACTCAGAGCACTCTCTAGAACTG▼TGGAACTAGGGAAACGGTTTTT
N R L R A L S R T V E L G K R F F

CCCTCGTTGTTCTGAAGTACTGAACAAAATCATGGATGCTGATGATTTGTC
P R C S E V L N K I M D A D D L S

ATTGCTTGCACGTCTGGAACATGATACCCCAGAGGAGCGACGCCTAAAAAA
L L A R L E H D T P E E R R L K K

ACGTAGGTACATGGAACTTCAGGACATTCTCAGCAAAGCGTTTAGTGAGGA
R R Y M E L Q D I L S K A F S E D

CAAAGAAGAGTTTGACAAATCAACATTTTCATCATCATCTTCTTCAAAATC
K E E F D K S T F S S S S S S K S

AGTAGGGCCGATAAAAATA**TAA**CGGTGAGCTCATCGATTANATGTAGGTAA
V G P I K I *

GCTTAGTTGAGAGTTTTTATATAGTTGATTTCTTCTTCCAATAATTATAAG

TTACTTTAGGAGGATTTATGAGTTAATAGTCAACATAAATGTAATTATTAC

ACTTAAAAAATTTTATGTTTATAATTTTTCACATAAATTTAGAATTACAGA

ATCACATTTAAAAAAAAAAAAA

Figure 20. (continued) The nucleotide and deduced amino acid of the papaya *NPR1* cDNA. The start and stop codes are in bold and underlined. The nucleotide sequence of the three introns are not shown and their positons are marked by ▼

```
Papaya  MDYRTGTSDS NDISNNSSTC CVATN..TDT LSHPLE...P LTTPEISGLQ
  Rice  ~~~~~~~~~~ ~~~~~MEPPT SHVTNAFSDS DSASVEEGGA DADADVEALR
 Arab.  ~~~MDTTIDG FADSYEISST SFVATDNTDS SIVYLAAEQV LTGPDVSALQ

Papaya  LLSRNLLTIF DS.SDFDFFS DARLML.... GSGREIPVHR CILSSRSPFF
  Rice  RLSDNLAAAF RSPEDFAFLA DARIAVPGGG GGGGDLLVHR CVLSARSPFL
 Arab.  LLSNSFESVF DSPDD..FYS DAKLVL.... SDGREVSFHR CVLSARSSFF

Papaya  KAIF...... .SGSAFKERT AKFRLKELAG ....DYDVGF DALVAVLAYL
  Rice  RGVFARRAAA AAGGGGEDGG ERLELRELLG GGGEEVEVGY EALRLVLDYL
 Arab.  KSALA..AAK KEKDSNNTAA VKLELKEIA. ...KDYEVGF DSVVTVLAYV

                        *
Papaya  YTGKVWPLPK GVCVCVDEEC SHVGCRPAVD FLVEVLYVAF TFQISELVAL
  Rice  YSGRVGDLPK AACLCVDEDC AHVGCHPAVA FMAQVLFAAS TFQVAELTNL
 Arab.  YSSRVRPPPK GVSECADENC CHVACRPAVD FMLEVLYLAF IFKIPELITL

Papaya  YQRHLLDIID KVETDNILVI LSVANICGKV CDRLLGRCMD IIVKSDVDAV
  Rice  FQRRLLDVLD KVEVDNLLLI LSVANLCNKS CMKLLERCLD MVVRSNLDMI
 Arab.  YQRHLLDVVD KVVIEDTLVI LKLANICGKA CMKLLDRCKE IIVKSNVDMV

Papaya  TLDKSLPLSI VKQIMDLRAE CDTQGPEGRS FPDKHVKRIH RALDSDDVEL
  Rice  TLEKSLPPDV IKQIIDARLS LGLISPENKG FPNKHVRRIH RALDSDDVEL
 Arab.  SLEKSLPEEL VKEIIDRRKE LGLEVPKVK. ...KHVSNVH KALDSDDIEL

Papaya  VRMLLKEART NLDDAHALHY AVAYCDAKTT IELLDLGLAD VNHRNSRGYT
  Rice  VRMLLTEGQT NLDDAFALHY AVEHCDSKIT TELLDLALAD VNHRNPRGYT
 Arab.  VKLLLKEDHT NLDDACALHF AVAYCNVKTA TDLLKLDLAD VNHRNPRGYT

          *         *
Papaya  VLHVAAMRKE PKLIVSLLTK GARPSDLTPD GRKALQISKR LTKAADYYNT
  Rice  VLHIAARRRE PKIIVSLLTK GARPADVTFD GRKAVQISKR LTKQGDYFGV
 Arab.  VLHVAAMRKE PQLILSLLEK GASASEATLE GRTALMIAKQ ATMAVECNNI

Papaya  TEEGKAAPKD RLCVEILEQA ERRDPLLGEA SLSLAKAGND FRMKLLYLEN
  Rice  TEEGKPSPKD RLCIEILEQA ERRDPQLGEA SVSLAMAGES LRGRLLYLEN
 Arab.  PEQCKHSLKG RLCVEILEQE DKREQIPRDV PPSFAVAADE LKMTLLDLEN

Papaya  RVGLAKLLFP MEAKVAMDIA QVNGTSEFTF .D.GIN..SN REQNTMDLNE
  Rice  RVALARIMFP MEARVAMDIA QVDGTLEFNL GS.GANPPPE RQRTTVDLNE
 Arab.  RVALAQRLFP TEAQAAMEIA EMKGTCEFIV TSLEPDRLTG TKRTSPGVKI
```

Figure 21. Alignment of papaya NPR1 with rice putative NPR1 (GenBank accession number: AP002537) and *Arabidopsis* NPR1 (GenBank accession number: U76707) (Cao *et al.* 1997). The alignment was conducted using GCG PileUp program (University Research Park, Madison). Ankyrin repeats are underlined. The amino acids in which mutation affects the function of NPR1 in *Arabidopsis* are marked with asterisks and shown in bold.

```
Papaya  APFRIQEEHL NRLRALSRTV ELGKRFFPRC SEVLNKIMDA DDLSLLARLE
  Rice  SPFIMKEEHL ARMTALSKTV ELGKRFFPRC SNVLDKIMD. DETDPVS.LG
 Arab.  APFRILEEHQ SRLKALSKTV ELGKRFFPRC SAVLDQIMNC EDLTQLACGE

Papaya  HDTPEERRLK KRRYMELQDI LSKAFSEDKE EFDKSTF..S SSSSSKSVGP
  Rice  RDTSAE...K RKRFHDLQDV LQKAFHEDKE ENDRSGL..S SSSSSTSIGA
 Arab.  DDTAEKRLQK KQRYMEIQET LKKAFSEDNL ELGNSSLTDS TSSTSKSTGG

Papaya  IKI~~ ~~~~~~~~~
  Rice  IRPRR ~~~~~~~~~
 Arab.  KRSNRKLSHRRR*
```

Figure 21. (continued) Alignment of papaya NPR1 with rice putative NPR1 (GenBank accession number: AP002537) and *Arabidopsis* NPR1 (GenBank accession number: U76707) (Cao *et al.* 1997). The alignment was conducted using GCG PileUp program (University Research Park, Madison). Ankyrin repeats are underlined. The amino acids in which mutation affects the function of NPR1 in *Arabidopsis* are marked with asterisks and shown in bold.

Identity = 57/116 (49%), similarity = 72/116 (62%)

```
Consensus       ch h  h           h spb  Ls   s hh  hh
Papaya    DFDFFSDARL ML....GSGR EIPVHRCILS SRSPFFKAIF    .....SGS
 Arab.    D..FYSDAKL VL....SDGR EVSFHRCVLS ARSSFFKSAL.A..AAKKEKD

Consensus       p                    p   h   hh hhb s  h       h
Papaya.   AFKERTAKFR LKELAG.... DYDVGFDALV AVLAYLYTGK VWPLPKGVCV
 Arab     SNNTAAVKLE LKEIA....K DYEVGFDSVV TVLAYVYSSR VRPPPKGVSE

Consensus hh   p h  h   h   h
Papaya    CVDEECSHVG CRPAVDFLVE VLYVAFT
 Arab.    CADENCCHVA CRPAVDFMLE VLYLAFI
```

Figure 22. Alignment of NPR1s with the POZ domain consensus sequence (Aravind and Koonin 1999). The consensus sequence uses the following convention: hydrophobic amino acid residues (AVLIMIFYW:h); polar amino acid residues (NQDEKRH:p); charged amino acid residues (KHRED:c); small side-chain amino acid residues (AGSTPVNDH:s); big side-chain amino acid residues (ILFYMRQEKW:b). Compared with the POZ consensus sequence, 28 conserved amino acids in NPR1s are in bold and underlined. Five non-conserved amino acids among NPR1 are only underlined.

A. papaya 246 LRAECDTQGPEGRSFPDK 263
Arabidopsis 252 RRKELGLEVPKVK....K 265
rice 252 ARLSLGLISPENKGFPNK 269

B. papaya 536 ERRL**KK**R**R**YMELQDILS**K** 553
Arabidopsis 537 KRLQ**KK**Q**R**YMEIQETL**KK** 554
tobacco 535 ERQL**KK**Q**R**YMELQEILT**K** 552
rice 537 E **KR**K**R**FHDLQDVLQ**K** 551

C. papaya 579 IKI 581
Arabidopsis 582 KRSNRKLSHRRR 593
tobacco 579 DKPN KLPFRK 588
rice 578 IRPRR 582

Figure 23. The possible NLS (nuclear localization sequence) in NPR1 proteins from papaya, *Arabidopsis*, and rice. **A.** For the region corresponding to the first possible NLS in *Aradidopsis,* the conservation is lower among three NPR1 proteins. **B.** The second possible NLS in NPR1 from papaya, *Arabidopsis*, tobacco, and rice. Five Amino acids required for nuclear localization of NPR1 in *Arabidopsis* are shown in bold. Four out them are conserved in papaya, tobacco and rice. **C.** The C-terminus of NPR1 from papaya, *Arabidopsis*, tobacco and rice. The absence of this potential NLS in papaya and rice indicates that this one may not be functional.

CHAPTER 4

DISCUSSION

4.1 Subtracted cDNA Library

The subtracted cDNA library based on suppression subtractive hybridization was designed to clone differentially expressed genes between two populations of mRNA with abundance equalization. This technology in combination with dot blot arrays of clones from the subtracted library (i.e. reverse Northern screening) allowed us to specifically clone genes induced by BTH, which induces SAR in dicots such as tobacco (Friedrich *et al.* 1996) and *Arabidopsis* (Lawton *et al.* 1996). An aliquot of the subtracted PCR products was cloned to produce 322 colonies. eighty-nine of these colonies were found to be upregulated by BTH in reverse Northern assays. From the subtracted cDNA library, 25 unique sequences were isolated which were shown by Northern blot analysis to be systemically upregulated by BTH more than 1.5 fold.

The subtracted cDNA library technology in combination with dot blot reverse Northern confirmation is a rapid, low-cost, and relatively accurate procedure to clone differentially expressed genes. Compared with microarray technology, it has several advantages. Microarray technologies usually need large-scale sequencing and the equipment is quite expensive. Considerable effort and expense is required to validate a microarray system. For minor crops, where funding is limited and large-scale sequencing of the genome has not been done, the subtracted cDNA library technology plus dot blot reverse Northern analysis conducted here should be an economical and powerful tool to pinpoint genes induced in response to pathogens, stresses, etc.

Two independent surveys of genes upregulated during *Arabidopsis* SAR response using microarrays have been done (Maleck *et al.* 2000; Schenk *et al.* 2000). In one study, approximately 25-30% of all *A. thaliana* genes was represented (Maleck *et al.* 2000). In the present papaya study, among twenty-five unique ESTs confirmed to be induced more than 1.5 fold by BTH, seven sequences encode at least four *PR* genes well known to be induced during SAR in other systems. Some of the BTH-induced ESTs isolated from papaya have not been reported to be involved in SAR in other plants. This procedure allowed simultaneous cloning of numerous genes involved in SAR, and rendered cloning of genes not previously known to be involved possible. These genes may elucidate novel aspects of SAR or may reveal differences between papaya and other plants.

Suppression subtractive hybridization enriches differentially expressed genes, but the resulting cDNA library still contains some cDNAs that correspond to mRNA equally present in both the tester and driver samples. This background mainly arises when the RNA population is not much different for the tester and driver samples. SAR marker mRNA accumulation increased in a dose-dependent manner in response to BTH in tobacco (Friedrich *et al.* 1996) and *Arabidopsis* (Lawton *et al.* 1996). In papaya, 100 μM BTH root drench is able to increase the activities of chitinases and β-1,3-glucanases systemically and enhance resistance against *P. palmivora.* Both of these suggest the establishment of SAR in papaya (Zhu *et al.* 2002). 500 μM BTH was used to spray entire papaya seedlings to maximize the difference between the tester sample (i.e. BTH treatment) and the driver sample (i.e. water treatment). When subtracted clones were

confirmed by Northern or reverse Northern blot analysis, the inducing treatment was 100 μM BTH root drench, which had been shown to have only minor phytotoxicity. RNA was extracted from leaves. This treatment ensured that the observed response was systemic and not only local, and was most likely not solely stress/phytotoxic response. With BTH and water controls, RNA was extracted 3 days after treatment. At this time, SAR is fully established in tobacco (Friedrich *et al.* 1996) and *Arabidopsis* (Lawton *et al.* 1996).

Suppression subtractive hybridization is essentially a sampling technique, and a single application of the method is not expected to recover all differentially expressed genes. Papaya *PR-1d* is induced by BTH, but did not show up in this subtracted cDNA library, confirming that this subtracted cDNA library did not represent all the genes induced by BTH in papaya.

4.2 BTH-induced Genes and SAR in Papaya

BTH has been reported to activate SAR in a variety of dicots, such as to tobacco and *Arabidopsis*, and in monocots, such as maize. It can induce plant resistance and mRNA accumulation for multiple *PR* genes in *Arabidopsis* and tobacco. BTH does not cause an increase of endogenous salicylic acid (SA) known to be required for SAR. In transgenic plants expressing the *nahG* gene, which cannot accumulate significant amounts of SA, BTH is still able to induce *PR* gene mRNA accumulation and disease resistance. BTH is unable to induce disease resistance in an *Arabidopsis npr1/nim1* mutant (Ryals *et al.*

1997). These experiments indicate BTH functions upstream of NPR1/NIM1 and at the site of or downstream of SA accumulation.

Rice and wheat responses to BTH are different from *Arabidopsis*, tobacco and maize. In rice, BTH is not known to induce any *PR* gene. The gene expression patterns induced by BTH and by *Pseudomonas syringae* pv. *syringae*, which triggers HR, are dissimilar (Schweizer *et al.* 1999). In wheat, two *PR-1* genes are induced upon infection by incompatible isolates of the pathogen *Erysiphe graminis*, which is suggested to activate SAR, but their expression is not induced by BTH, SA or INA (Molina *et al.* 1999). PR-1, PR-2, PR-3, PR-5 and peroxidase were induced by *F. graminearum* infection and none of them were induced by BTH (Yu and Muehlbauer 2001).

In papaya, BTH root drench enhanced chitinase & β-1,3-glucanase enzyme activities in leaves and roots and reduced significantly the symptoms caused by *P. palmivora*, the causal agent for papaya root rot disease (Zhu *et al.* 2002). Both are consistent with SAR response observed in tobacco (Friedrich *et al.* 1996) and *Arabidopsis* (Lawton *et al.* 1996). This study showed that the same treatment also induced the systemic expression of five *PR* genes in leaves. The papaya gene expression patterns in response to BTH resemble dicots such as *Arabidopsis* and tobacco. The expression patterns appear to be different from those in rice and wheat.

The induction kinetics of *PR* genes in papaya differs from many other plants, although they appear to be quite similar to tobacco. In many plants, BTH-induced *PR* gene

expression appears to be transient. The RNA level of all tested *PR* genes including *PR-1* begins to decrease to basal levels at about 10 days after treatment in maize and *Arabidopsis* (Lawton *et al.* 1996; Morris *et al.* 1998). The elevated transcript level of *PR-1* is maintained without any apparent decrease beyond 14 days in both papaya and tobacco (Friedrich *et al.* 1996). In contrast, tobacco *PR-5* remains elevated more than 20 days while papaya *PR-5* mRNA is reduced after 7 days. BTH-induced disease resistance in wheat lasts the entire growing season while induced defense-related gene expression goes down to basal levels only 10 days after treatment (Gorlach *et al.* 1996). The duration of BTH-induced resistance in papaya against *P. palmivera* has not been reported.

These *PR* genes are also believed to be at least partly responsible for BTH induced resistance in papaya, since the proteins they encode have anti-microbial activity or are involved in plant cell wall strengthening. They are believed to be defense-related genes. Their expression was monitored to study the interaction between the papaya cultivar Sunup and *P. palmivora.* Six days after root drench inoculation when disease symptoms were obvious, only one of five tested BTH induced genes was detectably induced by the pathogen. This absence of rapid induction for *PR* transcripts has been observed repeatedly in compatible interactions between pathogen and plant (Morris *et al.* 1998; Pritsch *et al.* 2000). A range of papaya genotypes with different levels of resistance against various pathogens should be included in future studies to assess the actual participation of these *PR* genes. Since the compatible and incompatible interactions

between papaya and its pathogens are not well characterized, this assessment could not be accomplished in this study.

The 5 *PR* ESTs isolated in this study could be used to monitor SAR in papaya. BTH has been commercially applied to many crops with protective doses, which do not have negative effects on crop growth (Lawton *et al.* 1996). Unfortunately, BTH caused a transitory loss of chlorophyll in papaya seedlings and at least temporarily affected normal growth (Zhu *et al.* 2002). It is not commercially used in papaya. Other SAR activators (eg. harpin protein and sulfated fucan oligosaccharides) have been reported (Dong *et al.* 1999; Klarzynski *et al.* 2003). With the established papaya *PR* genes as SAR markers, we can test these SAR activators in the laboratory, before conducting laborious and large-sample-size bioassays. In addition, papaya lines engineered to increase SAR can be tested with these markers.

Generally, each particular *PR* gene belongs to a gene family. One *PR* gene may be involved in SAR while other members in the same family may play roles in normal developmental processes or other protective mechanisms. The deduced proteins encoded by the four papaya *PR-1* genes have similar structures shared among plant PR-1 proteins. From the partial deduced amino acid sequence, it is not possible to know which one is induced during SAR. However, among 4 papaya *PR-1* genes, only the *PR-1d* transcript is induced by BTH and its enhanced accumulation is correlated with the onset of the SAR. None of the others shows BTH induction. This suggests that as in other plants, papaya has the *PR-1d* gene induced during SAR and others which are not.

The *Arabidopsis* NPR1/NIM1 protein has been shown to interact with members of the basic leucine zipper (bZIP) family of transcription factors like TGA2, TGA3 and TGA6. The interaction between TGA2 and NPR1 is required for activation of *PR* gene transcription by SA, and is necessary for *Arabidopsis* resistance to *Pseudomonas syringae* pv. *maculicola*. In a proposed model (Fan and Dong 2002), NPR1 forms a complex with TGA2 and other TGA factors under non-SAR conditions. Under SAR-inducing conditions, more NPR1 proteins are produced in response to endogenous increased SA to complex with TGA2. The association with NPR1 also increases the DNA binding activity of TGA2, resulting in *PR* gene induction. Some TGA factors may be able to bind to DNA in the absence of NPR1, but they do not play a role in inducing *PR* gene expression. The ankyrin region was shown to be required for functional NPR1-TGA interaction. The existence of the ankyrin region and nuclear localization signal in the deduced amino acid sequence of the papaya NPR1 supports a functional role for these domains. Function of the putative POZ domain of NPR1 has not been conclusively demonstrated, although the fact that a mutation in its consensus sequence abolishes NPR1 normal function indicates its importance (Cao *et al.* 1997).

Compared to intensively studied *Arabidopsis NPR1*, there has been very limited information about the function of *NPR1* homologues in other plants. Although the tobacco *NPR1* gene was isolated by 1998 (Cao *et al.* 1998), it has only recently been shown that the tobacco homologue shares close structural similarity with *Arabidopsis* NPR1 and is required for N-mediated resistance to tobacco mosaic virus (Liu *et al.* 2002).

The regulation of *PR* genes by tobacco *NPR1* has not been established. In another study, overexpression of the *Arabidopsis NPR1* in rice produced enhanced resistance to rice bacterial blight, and *Arabidopsis* NPR1 protein is able to interact specifically with rice TGA proteins belonging to the bZIP family. Overexpression of the endogenous rice gene can also provide resistance against rice bacterial blight (Campbell *et al.* 2002). These results suggest that monocot and dicot plants share a conserved signal transduction pathway controlling *NPR1*-mediated resistance.

In papaya, a BTH root drench increases the activities of β-1,3-glucanase and chitinase systemically and boosts the resistance to *P. palmivora* (Zhu *et al.* 2002). Simultaneously, it activates the expression of a set of *PR* genes. The *NPR1* homologue in papaya shares close similarity of *Arabidopsis NPR1*. Papaya NPR1 contains all essential domains and amino acids for the normal function of *Arabidopsis* NPR1. All the evidence indicates *Arabidopsis* and papaya SAR share high similarity. The function of *NPR1* in *PR* gene induction by BTH was not proven in papaya in this study. But, given that overexpression of *NPR1* in both *Arabidopsis* and rice led to the enhanced resistance to bacterial and oomycete diseases (Cao *et al.* 1998; Chern *et al.* 2001), efforts are under way to overexpress either *Arabidopsis* or papaya *NPR1* genes in trangenic papaya in order to increase resistance to pathogens. In addition, *PR* ESTs isolated in this study could also serve as a starting point to isolate the full-length cDNAs, which could be employed to engineer plants to improve disease resistance.

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