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Production of Disease Resistance in Citrus by Understanding Natural Defense Pathways and Pathogen Interactions

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ABSTRACT

Molecular tools have facilitated the discovery and study of genes associated with natural defense pathways in a number of model systems. In our laboratories, citrus homologues of key genes have been identified (for instance, *NPR1* and *PR1*) using comparative analysis and their expression characterized. In addition, differential gene expression during infection with citrus canker has been examined. Both approaches have facilitated the study of defense responses in citrus. The improved understanding of these natural defense pathways in model species has allowed plant-derived genes to be used to induce disease resistance. These recent discoveries as well as strategies for their practical application in citrus breeding are discussed in this review.

Keywords: breeding, citrus canker, systemic acquired resistance, transformation
Abbreviations: ACRE, Avr9/Cf-9 rapidly elicited protein; **ATGB2**, *Arabidopsis thaliana* GTP-binding 2; **AtRAP**, *Arabidopsis thaliana* RNA-biding domain protein abundant in Apicomplexans; **Avr**, avirulence; **BTB/POZ**, broad-complex, Tramtrack and Bric-a-brac/ poxvirus and zinc finger protein domain; **bZIP**, basic leucine zipper protein domain associated with DNA binding; **CC**, coil coil; **Cf-9**, *Cladosporium fulvum* resistance gene 9; **DCL**, DICER-like ATP-dependent helicase/ ribonuclease III; **dsRNA**, double-stranded RNA; **EDR1**, enhanced disease resistance 1; **EDS1**, enhanced disease susceptibility 1; **EDS5**, enhanced disease susceptibility 5; **EST**, expressed sequence tag; **HSP90**, heat shock protein 90; **HR**, hypersensitive response; **ISC**, isochorismate synthase; **LRR**, leucine-rich repeats; **MAPK**, mitogen-activated protein kinase; **MATE**, Multi Antimicrobial Extrusion family of proteins that function as drug/sodium antiporters; **MeSA**, Methyl salicylate; **nat-siRNA**, natural antisense siRNAs (derived from overlapping regions from two genes with antisense transcripts, they downregulate one of the transcripts); **NBS**, nucleotide binding site; **NDR1**, non race-specific disease resistance 1; **NIMIN**, NIM1 (same as NPR1)-interacting; **NPR1**, non-expresser of PR genes 1; **PAD4**, phytoalexin deficient 4; **PAMPs**, pathogen-associated molecular patterns; **PPRL**, pentatricopeptide repeats-like; **RdRp**, RNA-dependent RNA polymerase; **PR**, pathogenesis-related; **RACE**, rapid amplification of cDNA ends; **ROS**, reactive oxygen species; **RPP5**, recognition of *Peronospora parasitica* 5; **RPS2**, resistant to *Pseudomonas syringae* 2; **RT-PCR**, reverse transcription followed by polymerase chain reaction; **SA**, salicylic acid; **SAG101**, senescence-associated gene 101; **SAR**, systemic acquired resistance; **SGT1**, suppressor of G2 (Two) 1; **SID2**, salicylic acid induction deficient 2; **siRNA**, small interfering RNA; **ssRNA**, single-stranded RNA; **TIR**, Toll/interleukin-1-receptor; **WRKY**, a family of regulatory proteins containing the WRKY domain, a conserved WRKYGQK sequence followed by a zinc finger motif; **Xac**, *Xanthomonas axonopodis* pv *citri*; **XB3**, XA21 binding protein 3

CONTENTS

INTRODUCTION

Developing disease resistance is an important component of any plant breeding program. Citrus is no exception. It is affected by a variety of pathogens: viroids, viruses, bacteria, fungi and nematodes. Some of these diseases are very destructive and have had great economic impact in diverse regions of the world; examples include tristeza decline (caused by *Citrus tristeza virus*, CTV), canker (caused by *Xanthomonas axonopodis* pv. c*itri*), and Huanglongbing or greening (caused by *Candidatus* Liberibacter spp.) (Timmer *et al.* 2000). Genetic resistance is an ideal approach to controlling diseases because it is cost effective and can be long-lasting. However, with citrus this is not always possible using traditional breeding techniques due to long reproductive periods, incompatibility, apomixis and/or the absence of resistance genes to many of these important diseases. A viable alternative to conventional breeding in citrus is the use of genetic engineering. However, consumers and producers have shown some reluctance to embrace this technology in food products due to real or perceived fears. One strategy that can help ease those fears is to enhance the natural mechanisms of defense already present in the plant by using genes or other components derived from the same or

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Invited Review

other plant species. But, whether we use traditional breeding or biotechnology we need to have an understanding of the natural defense mechanisms in citrus. Ideally we could modify these defenses to achieve wide-spectrum disease resistance. In other words, with the manipulation of one or a few genes we could generate plants that exhibit resistance to several of the important diseases that citrus production faces.

Over the past few years the understanding of plant defense mechanisms has advanced, mostly in model systems such as *Arabidopsis thaliana*, rice and tobacco. Sequencing of the complete genomes of some of these and other plant species has also facilitated this work. In addition, there have been several efforts in our labs and others around the world to generate *Citrus* ESTs (expressed sequence tags) that are publicly available in GenBank and other databases (Talon and Gmitter 2008). Using these tools we have identified specific genes associated with the response of citrus to canker and also other genes associated with the general defense response. In this review we will describe these advances and how they can be used to better control some of the most important diseases of citrus.

PLANT DEFENSE PATHWAYS

Plants possess several levels of defense against potential pathogens. One level consists of preformed barriers such as wax, cell walls, secondary metabolites and antimicrobial enzymes. Another level corresponds to an active response following the recognition of the pathogen (Thordal-Christensen 2003; Jones and Takemoto 2004). Two kinds of active responses have been identified in plants. The first one is often referred to as basal defense and it is activated by broadly conserved and slowly evolving structural molecules from the pathogen, such as cell wall components, chitin fragments, flagellins, and lipopolysacharides that are referred to as "general elicitors" or "pathogen-associated molecular patterns" (PAMPs) (Thordal-Christensen 2003; Nurnberger and Lipka 2005; Jones and Dangl 2006; Ryan *et al*. 2007). Some pathogens have evolved mechanisms to derail basal defense and deploy "effectors" that are used to promote virulence. To counteract this attack, resistant plants have evolved more specific detection systems to recognize pathogen effectors using a complex array of constitutively expressed *R* (for resistance) genes (Martin *et al*. 2003; Rathjen and Moffett 2003; Jones and Dangl 2006). The effectors or virulence genes expressed by the pathogen are referred to as avirulence (*Avr*) genes when specifically recognized by a corresponding *R* gene and this mechanism of defense is also known as gene-for-gene resistance.

The R-mediated defense is similar to basal defense and depends on some of the same genes, but it occurs more quickly and with larger effects (Jones and Dangl 2006; Tsuda *et al*. 2008). It is often associated with a hypersensitive response (HR), an area of cell necrosis at the site of invasion that normally stops pathogen invasion and is linked to systemic acquired resistance (SAR) (**Fig. 1**). Cell death, however is not necessary to activate SAR (Glocova *et al*. 2005; Mishina and Zeier 2007). Seven distinct classes of *R* proteins that mediate resistance against different pathogen taxa have been identified (Lahaye 2002; Tameling and Takken 2008). The majority of the known *R* genes encode putatively cytoplasmic proteins with a nucleotide binding site (NBS) and a carboxy-terminal leucine-rich repeat (LRR) domain (Lahaye 2002). NBS-LRR proteins are further divided into two subclasses depending on whether the N-terminal domain is a Toll/interleukin-1-receptor (TIR) or a coiled coil (CC) motif. Based on the genomic sequences available,

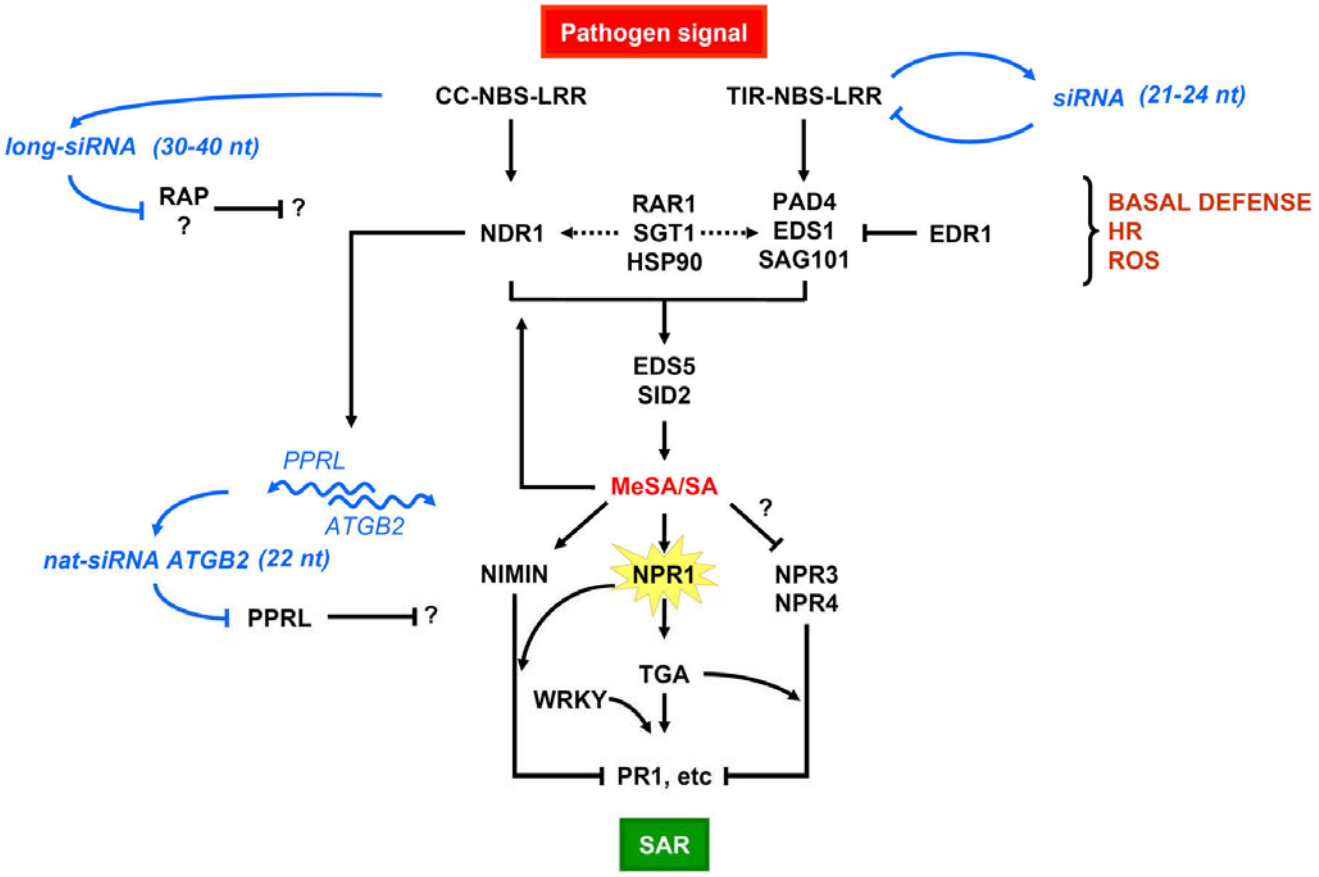

Fig. 1 Overview of the systemic signaling in *R*-mediated disease resistance that leads to systemic acquired resistance (SAR). Some of the genes that are known to be required for pathogen-induced SA accumulation and full resistance are shown. Solid arrows indicate positive effect, dashed arrows indicate partial requirement and lines with a bar indicate inhibitory effect. Some genes (within the bracket) are also implicated in basal defense, the hypersensitive response (HR) and production of reactive oxygen species (ROS). Small RNA-mediated regulatory pathways are shown in blue. A question mark indicates that the exact target of the regulatory pathway is not known (RAP and PPRL proteins), that other genes may also be implicated in the same regulatory pathway (RAP) or that the effect may sometimes be positive instead of negative (NPR3, NPR4). See text for details.

the number of NBS-LRR encoded in the genome of plants is relatively large and varies widely between species: 50 in papaya, 150 in *Arabidopsis*, 350 in grape, 400 in poplar and 600 in rice (Meyers *et al*. 2003; Zhou *et al*. 2004; Tuskan *et al*. 2006; Velasco *et al*. 2007; Ming *et al*. 2008).

After pathogen recognition by the R proteins, several metabolic changes occur that ultimately lead to the activation of the defense response and resistance: ion fluxes across the plasma membrane, a burst of oxygen metabolism that produces reactive oxygen intermediates (ROIs), protein kinase activation, transcriptional reprogramming with the activation of defense gene expression and in some cases HR (McDowell and Dangl 2000; Bent and Mackey 2007). How exactly R proteins relay the pathogen signal and activate defense is not known. However, a picture is emerging in which R proteins, once activated, interact in the nucleus with transcriptional regulators, such as WRKY proteins to start reprogramming (Bent and Mackey 2007; Tameling and Takken 2008). Subsequently, plants show elevated accumulation of salicylic acid (SA) and induced expression of pathogenesis-related (PR) genes (**Fig. 1**). One or more molecular signals are then transported through the plant from the point of attack and confer SAR, which renders distant parts of the plant more resistant to the invader and to infection by a broad range of other pathogens. It has been proposed recently that methyl salicylate (MeSA) acts as the mobile systemic signal but needs to be converted into SA in the target systemic tissue to become biologically active and trigger SAR (Park *et al*. 2007). In addition, plants possess SA-independent defense pathways. Jasmonic acid and ethylene function as signaling molecules in what is believed to be a signal transduction network that acts independently but coordinately with the SA defense pathway (Pieterse and van Loon 1999).

Several genes required for proper *R* gene function and induction of the SA pathway have been identified (**Fig. 1**). *EDS1*, *PAD4* and *SAG101* encode lipase-like proteins that interact with each other and mediate the downstream signaling of TIR- but not CC-type R protein receptors (Wiermer *et al*. 2005). In contrast, NDR1, a membrane-associated protein, is required to trigger resistance by many CC R proteins, but not by TIR proteins (Aarts *et al*. 1998). The chaperon protein HSP90 and its interacting cochaperons, RAR1 and SGT1, are also required for the accumulation, stabilization and function in modulating disease resistance of many R proteins (Takahashi *et al*. 2003; Liu *et al*. 2004; Boter *et al*. 2007). On the other hand, EDR1, a MAPK kinase kinase, functions as a negative regulator of the SA defense pathway (Frye *et al*. 2001). These proteins are highly conserved in sequence and function between different species (Kim *et al*. 2003; Zhang *et al*. 2004; Pajerowska *et al*. 2005; Tuskan *et al*. 2006; Bhaskar *et al*. 2008; Wang *et al*. 2008), indicating that this defense pathway is conserved among plants. In addition, these proteins are also part of the basal defense pathway (Wiermer *et al*. 2005; Ham *et al*. 2007; Zhou *et al*. 2008).

The proteins described above lead to the induction of SA production. There are two pathways associated with the biosynthesis of SA in plants. One is the phenylpropanoid pathway that involves phenylalanine ammonia lyase (PAL) as the first enzyme (Lee *et al*. 1995). The second uses chorismate to synthesize SA. Evidence suggests that the second pathway is the most important source of SA during SAR (**Fig. 1**). In *Arabidopsis*, expression of *SID2*, which encodes an isochorismate synthase (ICS, the first enzyme in the chorismate pathway) is localized to the chloroplast and is activated by pathogen infection (Wildermuth *et al*. 2001). Plants with a mutant *sid2* fail to accumulate SA after pathogen inoculation and are more susceptible to both virulent and avirulent pathogens (Nawrath and Metraux 1999). A second gene associated with the SA pathway is *EDS5,* which encodes a protein with sequence similarity to the multidrug and toxin extrusion (MATE) family of transporter proteins (Nawrath *et al*. 2002). This protein may be involved in moving phenolic compounds that are precursors of SA biosynthesis into and out of the chloroplast. *EDS5* expression increases early after pathogen infection or SA application, suggesting its involvement in a positive feedback loop. Furthermore, *eds5* mutant *Arabidopsis* plants fail to accumulate SA, establish SAR and are more susceptible to pathogens (Nawrath *et al*. 2002).

Downstream of SA the *NPR1* gene is central in transmitting the SA signal and activation of SAR (Cao *et al*. 1997) (**Fig. 1**). In uninduced, healthy cells, NPR1 exists as an oligomer formed by intermolecular disulfide bonds. After the initial oxidative burst following R-mediated pathogen recognition and the subsequent accumulation of antioxidants, the cell becomes a more reductive environment and NPR1 is converted to a monomeric state (Mou *et al*. 2003). In this reduced state NPR1 is translocated to the nucleus where it interacts with members of the basic leucine zipper (bZIP) family of transcription factors (TGA factors) to induce the activity of PR genes (Kinkema *et al*. 2000; Chern *et al*. 2001; Fan and Dong 2002; Johnson *et al*. 2003). Certain WRKY transcription factors are also induced by NPR1/TGA and further activate transcription of defense genes and the resistance response (Wang *et al*. 2006a). SA increases the expression of the *NPR1* gene via other SA-induced WRKY factors (Yu *et al*. 2001). In addition, some SA- and NPR1-induced WRKY factors are negative regulators of basal plant defense (Kim *et al* 2008). NPR1 homologs have been identified in a variety of economically important plants, including rice, soybean, maize, apple and citrus.

In addition to TGA factors, NPR1 interacts with NIMIM proteins in the nucleus to negatively regulate PR gene expression in distal parts of the plant before the full onset of SAR (Weigel *et al*. 2005; Zwicker *et al*. 2007). This mechanism of regulation contributes to systemic priming, preparing the plant for further pathogen attack, while reducing the energetic cost of defense.

The *Arabidopsis* (At) and rice (Os) genomes contain 6 and 5 *NPR1*-like genes (named NPR1 through 6), respectively, based on protein sequence and structure (Liu *et al*. 2005; Yuan *et al*. 2007). All of these proteins contain ankyrin repeat domains, protein-protein interaction domains, BTB/POZ domains, and nuclear localization signals. AtNPR3 and 4 and OsNPR1, 2 and 3 are also associated with plant defense (Liu *et al*. 2005; Zhang *et al*. 2006; Yuan *et al*. 2007). AtNPR3 and 4 interact with TGA factors in the nucleus, but negatively regulate *PR* gene expression (Zhang *et al*. 2006) as another regulatory layer to prevent untimely activation and/or fine-tuning of defense.

The role of small RNAs in defense gene regulation

In recent years, evidence has accumulated indicating that gene expression in diverse cell pathways is regulated by gene silencing via interaction with small RNA molecules. Correspondingly, the plant defense response is regulated by RNA silencing in addition to transcriptional regulation by factors, such as WRKY and TGA. Several classes of small RNAs have been found in plants and other eukaryotes and they are classified based on their origin, biosynthetic pathway, length and function. Here, we will discuss only those classes that have been implicated in pathogen defense (**Fig. 1**).

One class of small RNAs present in plants, small interfering RNAs (siRNA), are produced by the Dicer cleaving machinery and function postranscriptionally to suppress target genes. SiRNAs are produced from endogenous and exogenous (for instance, transgenes, transposons and viral RNA) long double stranded RNA (dsRNA) (Hamilton and Baulcombe 1999; Meins *et al*. 2005). Such dsRNA precursors typically yield many 21-22 nt siRNAs derived from both strands (Hamilton *et al*. 2002). SiRNAs target for degradation the same sequences that generated them. Inducible endogenous plant RNA-dependent RNA polymerases (RdRps) are involved in the production of siRNAs by converting single stranded RNA (ssRNA) into dsRNA, the

Table 1 Citrus ESTs with the highest similarity to *Arabidopsis* (At) defense genes using tblastn (search of translated nucleotide database using a protein query).

At gene	At locus	At protein accession #[a]	Citrus EST[b]	E value[c]	Citrus UniGene[d]	ESTs representing Unigene[e]
EDR1	AT1G08720	NP_563824	EY716340.1	8e-148	N/A[f]	N/A
			CX545910.1	3e-107	N/A	N/A
EDS1	AT3G48090	NP_190392	DY258747.1	4e-57	N/A	N/A
EDS5	AT4G39030	NP_195614	DY283467.1	1e-122	N/A	N/A
NDR1	AT3G20600	NP_188696	EY699625.1	1e-52	N/A	N/A
NPR1	AT1G64280	NP_176610	DY293454.1	2e-110	Ccl.13135	DY273835.1, DY293454.1 DY297212.1, DY300900.1
NPR3	AT5G45110	NP_199324	EY706882.1	2e-93	N/A	N/A
NPR4	AT4G19660	NP_193701	EY706882.1	7e-97	N/A	N/A
PAD4	AT3G52430	NP_190811	EY738335.1	2e-59	N/A	N/A
RAR1 (PBS2)	AT5G51700	NP_568762	CF831306.1	1e-70	N/A	N/A
SGT1b	AT4G11260	NP_192865	DY294557.1	2e-99	N/A	N/A
			CK935726.1	9e-92	Csi.6935	CK739844.1, CK935726.1 CK937258.1, CF504851.1 CF504865.1, CF509856.1 CF509932.1, CF510149.1 CF510154.1, CF835052.1 CX676280.1, CX676281.1 DY305896.1
SID2 (ISC1)	AT1G74710	NP_974143	EY798239.1	7e-69	N/A	N/A

[1] GenBank protein accession number used for the tblastn search
[b] GenBank accession number for the most homologous citrus ESTs
[c] E value for amino acid similarity. The closer the E-value is to zero, the more significant the match is.
[d] GenBank UniGene accession number. Each UniGene entry is a set of transcribed sequences that appear to come from the same locus.
[e] GenBank accession number of all ESTs in the database forming one citrus UniGene.
[f] N/A, no citrus UniGenes available in GenBank

substrate for Dicer-like (DCL) ribonucleases (Dalmay *et al*. 2000; Xie *et al*. 2001). DCLs are RNase III enzymes that cleave the precursor dsRNA into siRNAs (Xie *et al*. 2004). SiRNAs are part of an important plant defense mechanism against viruses (Vaucheret *et al*. 2001). Further, siRNAs can be translocated systemically through the phloem facilitated by a small RNA-binding protein (PSRP1) (Yoo *et al*. 2004), suggesting that they are also part of the systemic regulatory pathway.

A cluster of *R* genes, RPP5, that recognize the fungal pathogen *Peronospora parasitica*) in *Arabidopsis*, are coordinately up-regulated upon pathogen infection by transcriptional activators through SA accumulation. These same *R* genes are negatively regulated in a feedback mechanism by siRNAs and silencing once the levels of mRNA reach a certain threshold (Yi and Richards 2007). Also, the steady state levels of these *R* genes are maintained by silencing. Thus, RNA silencing possibly functions to optimize the expression levels of these *R* genes and their SA-mediated defense response and to also sense any disruptions induced by the pathogen to the silencing pathway of the host (Yi and Richards 2007).

Natural *cis*-antisense transcript-associated siRNAs (nat-siRNAs) are derived from partially overlapping genes that lie on opposite strands (and in opposite orientation) of the genomic DNA. In the cases studied, one of the genes is constitutively transcribed while the other is induced by pathogens or abiotic stress. Once the induced gene is expressed, the antisense transcripts form a dsRNA precursor in the overlapping regions and RNA silencing of the constitutive gene is triggered with the production of 22-24 nt nat-siRNAs (Xie and Qi 2008). A protein that functions in *Arabidopsis* as a negative regulator of R-mediated defense, PPRL, is repressed via this pathway by a pathogen-induced nat-siRNA derived from the overlapping genes *PPRL* and *ATGB2*. The induction of the nat-siRNA ATGB2 is dependent on NDR1 and the R protein (RPS2) that detects the bacterial pathogen, *Pseudomonas syringae*, carrying the effector *avrRpt2* (Katiyar-Agarwal *et al*. 2006).

A third class of siRNAs are the long-siRNAS (30-40 nt), also derived from protein-coding genes by a DCL (Katiyar-Agarwal *et al*. 2007). Long-siRNAs are induced by bacterial infection. At least one, AtlsiRNA-1, targets and represses the expression of another negative regulator of defense, AtRAP (Katiyar-Agarwal *et al*. 2007), thus facilitating the activation of defense genes. Given the number of still unstudied small RNAs of all classes present in plants, it is likely that knowledge of their role as regulators of plant defense will only increase.

CITRUS GENES HOMOLOGOUS TO THE *ARABIDOPSIS* DEFENSE GENES

We have used the BLAST utility at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as a first step to identify citrus ESTs with homology to some of the potential genes in the defense pathway (**Table 1**). Subsequently, we have cloned and further analyzed these sequences for the presence of conserved motifs, regions and amino acids known to be important for the activity of these proteins in plant defense. For instance, the pummelo [*C. grandis* (L.) Osbeck] *NPR1* homologous gene that we cloned has the BTB/POZ and ankyrin repeat domains, the nuclear localization signal and the conserved Cys82 and Cys216 amino acids critical for the activity of NPR1 (**Fig. 2**).

In the case of proteins with low or no homology to available citrus ESTs, we have designed degenerate primers based on conserved motifs or regions from protein sequence alignments to amplify the homologous sequences from citrus. Two free programs available online are useful for the design of degenerate primers: 1) CodeHop (http://blocks.fhcrc.org/blocks/codehop.html) and 2) GeneFisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/welcome.html). We cloned the grapefruit (*C. paradisi* Macf.) *PR1* gene, considered a marker for SAR, in this manner (**Fig. 3**).

Using these strategies we have obtained full-length sequences for *EDR1*, *EDS1*, *EDS5*, *NDR1*, *NPR1*, *NPR3*, *PR1*, *RAR1*, *SGT1* and *SID2*. We cloned these genes from cDNA extracted from SA-treated plants (1 mM solution applied to the soil) to guarantee their expression. Because ESTs and degenerate primers from conserved regions provide only partial sequences of the gene, we used RACE (Rapid amplification of cDNA ends) RT-PCR to clone the full length sequences. Once the genes were cloned we analyzed their expression to confirm their role in defense. For example, *CgNPR1* was constitutively expressed and only slightly induced by SA application. On the other hand, *CpPR1* and *CpEDS5* were only expressed after SA application. These results are similar to what is observed in *Arabidopsis* and other model systems.

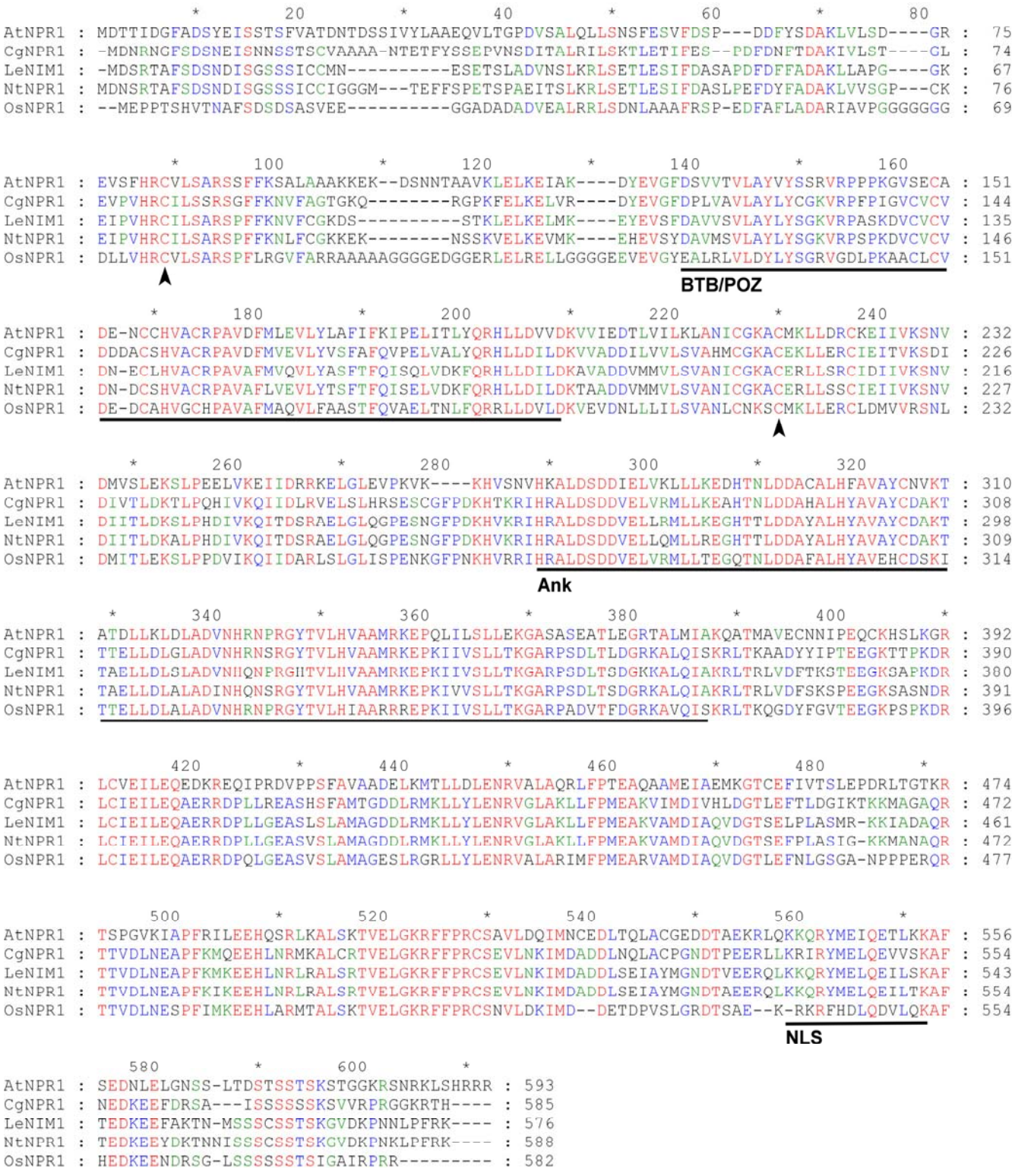

Fig. 2 Alignment of *bona fide* NPR1 homologous proteins from various species. Bars below the alignment indicate conserved domains. BTB/POZ, broad-complex, Tramtrack and Bric-a-brac/poxvirus and zinc finger protein domain; Ank, Ankyrin repeat domain; NLS, nuclear localization signal. Arrowheads indicate AtNPR1 cysteines 82 and 216, critical in oligomer formation and protein function. Two letter prefixes: At, *Arabidopsis thaliana*; Cg, *Citrus grandis*; Le, *Lycopersicon esculentum*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*. Red, 100 % amino acids conserved; Blue, 80% amino acids conserved; Green, 60% amino acids conserved.

DEFENSE RESPONSE TO *CITRUS* CANKER IN KUMQUAT

Citrus canker is a destructive disease caused by the bacterium *Xanthomonas axonopodis* pv *citri* (Xac). It infects all *Citrus* species and many citrus relatives. The disease is highly contagious and can spread widely if the environment is favorable for bacterial proliferation (high temperatures, humidity and rain) as the pathogen enters the plant through wounds and natural openings, promoted by water splashing. Infection causes lesions on the green parts of the plant including leaves and stems as well as fruits. Citrus canker disease has caused serious losses in citrus trees as well as in citrus fresh fruit production all over the world.

Early experiments using natural inoculation indicated that kumquat (*Fortunella* sp., a *Citrus* relative) and some of its hybrids were resistant to canker (Reddy 1997). Further testing using injection inoculation confirmed these observations, suggesting that a genetic component must be part of the resistance observed in kumquat and that resistance

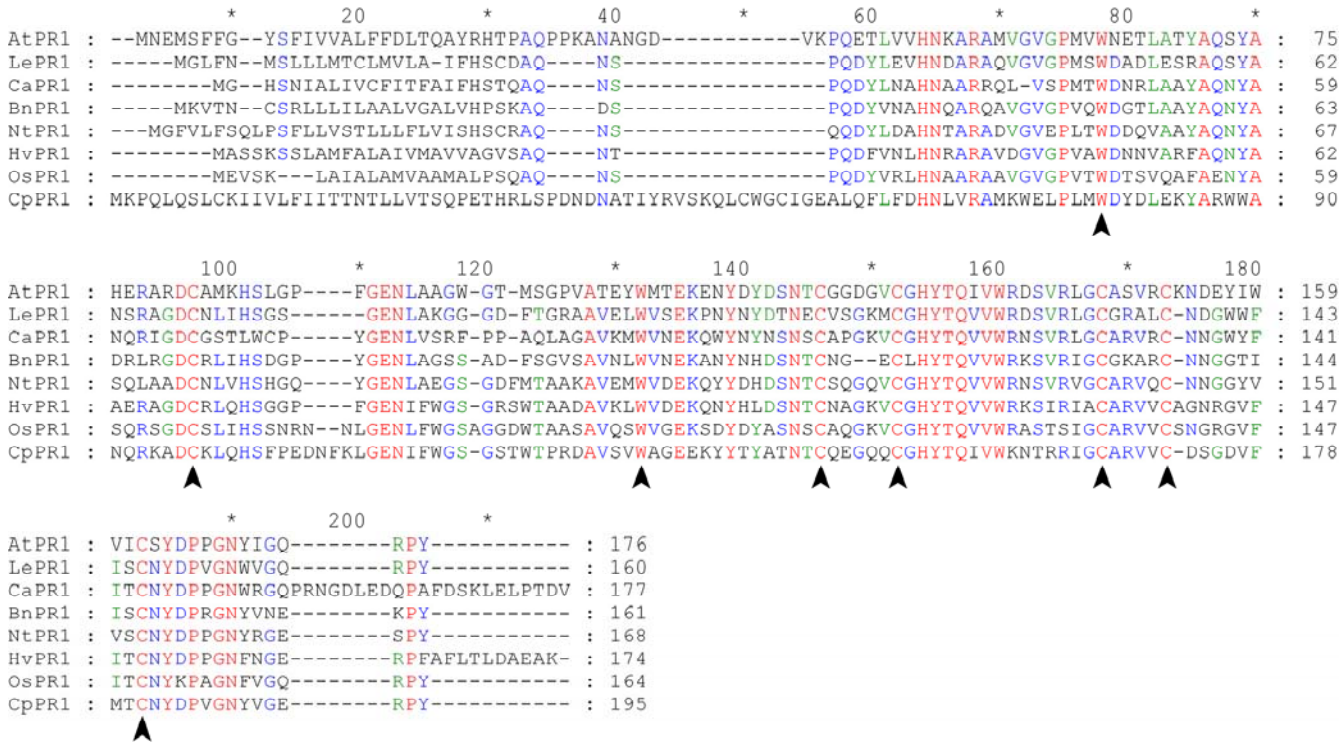

Fig. 3 Alignment of PR1 proteins from several species, including grapefruit. The arrow heads indicate conserved amino acids among all PR1 proteins including six cysteines that form three disulphide bonds (van Loon and van Strien 1999). Two letter prefixes: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Ca, *Capsicum annuum*; Cp, *Citrus paradisi*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*. Red, 100 % amino acids conserved; Blue, 80% amino acids conserved; Green, 60% amino acids conserved.

could potentially be incorporated into certain citrus types by conventional breeding (Viloria *et al*. 2004; McCollum *et al*. 2006). More recently, kumquat was shown to have an active response upon inoculation with canker (Khalaf *et al*. 2007a). We began investigating this phenomenon by comparing the bacterial population inside resistant kumquat [*Fortunella margarita* (Lour.) Swing.] leaves versus those of susceptible grapefruit after injection inoculation. 'Duncan' grapefruit supported a 2.5-fold higher bacterial population than kumquat, indicating the ability of kumquat to restrict the growth of Xac. In addition, kumquat leaves developed sudden necrosis, followed by leaf abscission about 5 days after inoculation, a response similar to HR. In contrast, grapefruit leaves developed typical canker lesions.

In order to study the molecular components of kumquat resistance to Xac, genes differentially expressed in response to canker infection were isolated using the suppression subtractive hybridization method (Diatchenko *et al*. 1996). This method enriched those transcripts associated with the response by reducing or eliminating transcripts also present in uninoculated plants. We first confirmed that the cDNAs isolated showed significant differential expression levels by northern (**Fig. 4**) or dot blot hybridization. Subsequently, approximately 3500 cDNAs from the library were selected for sequencing. The ESTs generated could be assembled into 738 distinct contigs (consensus sequences derived from overlapping ESTs). Further comparisons using blastx (search of the protein database with translated sequences) identified some contigs as homologous to genes associated with pathogen defense pathways in other systems. For example, contig125, an *Avr9/Cf-9 rapidly elicited* (*ACRE*) *protein 284* gene homolog was induced within 30 min after kumquat was challenged with Xac. *ACRE* genes code for regulatory proteins with diverse functions important in *Cf-9* (an *R* gene)-mediated resistance, HR and basal defense (Durrant *et al*. 2000; Navarro *et al*. 2004). We are looking into the potential role of this and other genes in the kumquat HR response. In addition, we have analyzed the expression profiles of more than 2300 kumquat ESTs using microarrays (Khalaf *et al*. 2007b). Approximately 54% of the ESTs were differentially regulated in infected vs. uninfected kumquat

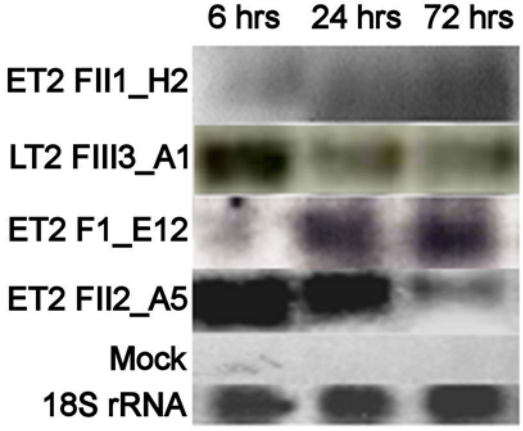

Fig. 4 Northern blot analysis of selected differentially-regulated cDNAs from *F. margarita* tissue inoculated with *Xanthomonas axonopodis* pv. *citri*. Ten micrograms of total RNA were isolated at 6, 24, and 72 hrs after inoculation and separated on a 1.5% denaturing agarose gel, and transferred to nylon membranes. Subsequently, the membranes were hybridized with digoxigenin-labeled DNA probes prepared by PCR labeling amplification from the *F. margarita* subtractive library from the following clones: **ET2 FII1_H2**, a sequence of unknown function**; LT2 FIII3_A1**, a sequence homologous to a phospholipid hydroperoxide glutathione peroxidase gene; **ET2 F1_E12**, a 1-aminocyclopropane carboxylic acid oxidase homologue, **LT2FII2_A5**, a sequence of unknown function; **mock**, leaves inoculated using sterile water (negative control); **18S rRNA**, ribosomal RNA used as a loading control.

starting 6 hrs after inoculation. Not surprisingly, given that cell death is observed during the response of kumquat to Xac, many of the genes induced early on were associated with ROS production, the HR and general defense pathways.

Other studies of this kind have been carried out in citrus but with compatible interactions such as *Citrus viroid III* (CVdIII) in citron (*C. medica* L.) and CTV in Mexican lime [*C. aurantifolia* (Christm.) Swingle] (Gandia *et al*. 2007; Tessitori *et al*. 2007). CVdIII induces dwarfism when infec-

ting certain rootstocks but does not cause detrimental effects so it is not considered a disease (Timmer *et al*. 2000). On the other hand, a comparison between a CTV-susceptible type (such as Mexican lime) and CTV-resistant *Poncirus trifoliata* (L.) Raf. could have revealed specific pathways that lead to resistance.

HOW TO INCORPORATE THIS INFORMATION IN THE IMPROVEMENT OF *CITRUS*

The defense pathways described (induced by PAMPs or pathogen effectors) are deployed against a wide variety of pathogens: viruses, bacteria, fungi and nematodes. They also seem to be conserved in most plant species since homologous genes in sequence and function have been identified in a variety of species. This means that what has been found for model systems, such as *Arabidopsis*, can potentially be applied to less studied crops like citrus. Also, manipulating the expression levels of one or a few genes could lead to the simultaneous improved resistance to various diseases. One has to be careful, however, because there is a reason why these defense pathways are not constitutively turned on and are regulated and fine-tuned at so many levels: there is an energy cost for the activation of defense.

Constitutive expression in *Arabidopsis* of the *EDS5* gene led to a more rapid accumulation of SA and activation of PR genes as well as improved resistance against three different virulent viruses, however the plants were severely dwarfed (Ishihara *et al*. 2008). Similarly, overexpression of *NDR1* in *Arabidopsis* led to enhanced resistance against bacterial pathogens but it also led to constitutive expression of *PR1*, spontaneous lesion formation and stunting (Coppinger *et al*. 2004). On the other hand, constitutive overexpression of *NPR1* in *Arabidopsis*, tomato, rice, wheat and apple did not result in constitutive *PR* gene expression in the absence of pathogens; however, it did lead to enhanced disease resistance to bacterial and fungal pathogens with no obvious detrimental effect on the transgenic plants (Cao *et al*. 1998; Chern *et al*. 2001; Friedrich *et al*. 2001; Makandar *et al*. 2006; Malnoy *et al*. 2007). Additionally, at least in *Arabidopsis*, the NPR1 plants also showed enhanced effectiveness to three fungicides suggesting that combining chemical treatments with transgenics could result in more effective control strategies (Friedrich *et al*. 2001).

Experiments in rice transformed with *AtNPR1* showed contradictory results. Although researchers found increased resistance against a variety of foliar, root and seed pathogens (both bacterial and fungal) (Chern *et al.* 2001; Quilis *et al.* 2008), some observed normal growth and development (Chern *et al.* 2001), while others observed spontaneous lesion formation, reduced growth and higher susceptibility to viral pathogens and abiotic stress (Fitzgerald *et al.* 2004; Quilis *et al.* 2008).

We have generated a series of transgenic 'Duncan' grapefruit and 'Carrizo' citrange [*C. sinensis* (L.) Osbeck x *P. trifoliata* (L.) Raf.] plants that express the *Arabidopsis NPR1* gene. Carrizo is commonly used as a rootstock and it is easy to transform and regenerate and it also has a relatively fast growth rate. 'Duncan' grapefruit is also relatively easy to transform and regenerate. Both of these citrus types are economically important and are susceptible to a variety of diseases. We are currently evaluating several of the transgenic lines for their resistance to CTV, canker and greening. These experiments are underway, however, the transgenic lines are phenotypically normal and we have evidence that some of them show an enhanced response, in terms of levels of PR1 (a marker of SAR) induction compared to wild type plants (**Fig. 5**). This is a promising result since it suggests that the heterologous AtNPR1 protein is working properly in citrus and is capable of overinducing SAR.

In a separate effort to produce canker resistant plants an *R* gene from rice, *Xa21*, was transformed into 'Hamlin' sweet orange (Omar *et al*. 2007). This gene confers resistance to *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight of rice. The authors do not report the evaluation of the transgenic plants for resistance to canker. However, in rice, XA21 requires the interaction with another protein, XB3, for proper function and mediation of resistance (Wang *et al*. 2006b). Whether this protein or a protein equivalent in function is present in sweet orange it is not known, although a BLAST search revealed a number of citrus ESTs highly homologous to rice XB3 (V. Febres unpublished). In addition, the effectors in *X. axonopodis* pv. *citri* may not be recognized by XA21.

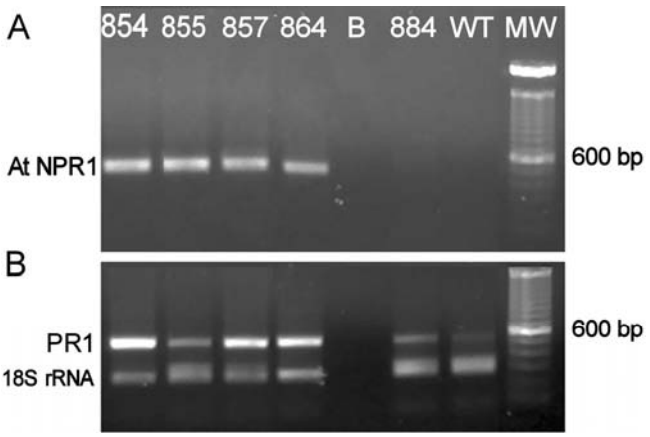

Fig. 5 Expression of transgenic *NPR1* (A) and endogenous *PR1* (B) in Carrizo plants transformed with the *Arabidopsis thaliana NPR1* gene (*AtNPR1*). Semi-quantitative reverse transcription (RT) reactions were used to detect and compare the expression levels of the *AtNPR1* and to correlate them with the endogenous *PR1* expression levels. The amplification of the 18S rRNA in the same RT reaction was used as a control in the amplification, for both integrity of the RNA and efficiency of the RT-PCR. The intensity of the PR1 band is directly proportional to the expression levels. Transgenic line numbers are indicated above; B, blank (no RNA used as a negative control); MW, molecular weight marker. Lines 854, 857 and 864 exhibited *AtNPR1* expression and enhanced PR1 expression compared to the WT and a transgenic plant that did not express the *AtNPR1* gene (884).

Another group of authors used pathogenesis related protein 5 (PR5) from tomato to transform 'Pineapple' sweet orange. PR5 is induced by pathogen infection in tomato and has antifungal activity. At least one transgenic line showed enhanced resistance (90% survival rate) against *Phytophthora citrophthora* when compared to wild type plants (50% survival rate) (Fagoaga *et al*. 2001). Other transgenic lines showed increased, although not statistically significant, survival rates of 70-80%.

CONCLUDING REMARKS

The recent and continuing discoveries in model systems, such as *Arabidopsis* and rice, have greatly improved our understanding of the molecular basis of plant defense and how defense pathways operate and interrelate. In addition, the advent of genomics has facilitated comparisons between these model systems and lesser studied crops. Research has shown mechanistic conservation in defense pathways between species and how some of their components are compatible. Databases, such as GenBank, provide a multitude of information as well as a nearly infinite source of genes potentially useful for crop improvement. Researchers have already started to use these resources to improve disease resistance in citrus.

One promising gene is *NPR1*. The NPR1 protein is involved in resistance against a wide variety of pathogens in several defense pathways. This protein is also only activated in the presence of an invading pathogen, minimizing the unnecessary activation of defense and the energy costs associated with it. Indeed NPR1 has been transformed in several species and has been shown to provide wide-spectrum disease resistance. Whether this holds true for citrus in controlling some of the economically important pathogens

that affect this fruit crop remains to be determined. In addition, field experiments with transgenic NPR1 plants, to our knowledge, have not been carried out. Thus we do not know of the efficacy of this strategy under real growth conditions.

Important molecular studies of citrus and its response to pathogens have also been completed providing a better understanding of specific plant-pathogen interactions. These studies also provide new insights on potential mechanisms for the control of important diseases. For instance, the study of the kumquat-canker bacteria interaction revealed that the resistance was the product of an active rather than a passive response and that ROS, HR and general defense-associated genes were induced during this response. These same pathways may need to be induced in more susceptible citrus types to obtain a similar level of resistance. How we accomplish this is the next challenge. Further analyses may reveal which genes are responsible for the activation of the response and/or transduction of the pathogen signal and they could be added (if not present) or more efficiently activated (if present) in susceptible citrus types. Additionally, a few proteins may ultimately be responsible for the demise of the pathogen in the plant (for instance, PR proteins). Modifying the expression (earlier or to higher levels) of these genes in susceptible plants may provide the resistance necessary to control the disease.

In conclusion, the better understanding of plant defense facilitates the development of more effective ways to control important citrus diseases. The use of plant-derived genes and regulatory sequences (promoters) together with improved transformation methods that do not rely on or subsequently eliminate exotic genes (antibiotic or herbicide resistance used for selection, see for instance Ballester *et al*. 2008) has the potential of producing new and more resistant citrus types that will be more acceptable to consumers.

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