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

Vicente J. Febres1* · Abeer Khalaf1,2 · Frederick G. Gmitter Jr.2 · Gloria A. Moore1

1 Plant Molecular and Cellular Biology Program (PMCB), Horticultural Sciences Department, University of Florida, Gainesville, FL 32611, USA
2 Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, USA

Corresponding author: * vjf@ufl.edu

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/Cl-9 rapidly elicited protein; ATGB2, Arabidopsis thaliana GTP-binding 2; AtRAP, Arabidopsis thaliana RNA-binding domain protein abundant in Apicomplexans; Avr, avirulence; BTB/POZ, broad-complex, Tramtrack and Bric-a-brac/POZ zipper 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; MsSA, 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-expressor of PR genes 1; PAD4, phytoalexin deficient 4; PAMPS, pathogen-associated molecular patterns; PPRPL, 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 pv syringae; RT-PCR, reverse transcription followed by polymerase chain reaction; SA, salicylic acid; SAG101, senescence-associated gene 101; SAR, systemic acquired resistance; SST1, 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

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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. citri), 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
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,
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 plant membranes, a burst of 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 Tacken 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 repeatedly that production of reactive oxygen intermediates (ROIs), protein peroxidase and other pathogen-related proteins (PRPs) is required for defense activation and/or fine-tuning of defense.

At the onset of SAR (Weigel and Pena 1999; Loon 1999), a process of 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), which in turn leads to the induction of 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. 2006). SA increases the expression of the NPR1 gene via other SA-induced WRKY factors (Yu et al. 2001). In addition, some NIMIM gene products and NPR1 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 5) 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 PRPs, such as WRKY and TGA. Several classes of small RNAs have been found in plants 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 (siRNAs), are produced by the Dicer cleaving machinery and function posttranscriptionally to suppress target genes. siRNAs are produced from endogenous or 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 (RdRs) are involved in the production of siRNAs by converting single stranded RNA (ssRNA) into dsRNA, the
substrate for Dicer-like (DCL) ribonucleases (Dalmay et al. 2004). DCLs are RNAse III enzymes that cleave the precursor dsRNA into siRNAs (Xie et al. 2000; Xie 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 transcriptional activators are constitutively upregulated upon pathogen infection by transgenic Arabidopsis carrying 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. Consequently, 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 Cys22 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://bioinfo.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, SGTL1* 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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number used for the tblastn search</th>
<th>E value</th>
<th>Citrus UniGene accession number</th>
<th>ESTs representing UniGene</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDR1</td>
<td>AT1G08720</td>
<td>8e-148</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EDS1</td>
<td>AT3G48090</td>
<td>3e-107</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EDS5</td>
<td>AT4G39070</td>
<td>4e-57</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NDR1</td>
<td>AT3G20600</td>
<td>1e-122</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NPR1</td>
<td>AT1G64200</td>
<td>1e-110</td>
<td>ccl.1315</td>
<td>DY273835.1, DY293454.1</td>
</tr>
<tr>
<td>NPR3</td>
<td>AT5G45110</td>
<td>2e-93</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NPR4</td>
<td>AT4G19660</td>
<td>7e-97</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PAD4</td>
<td>AT3G52430</td>
<td>2e-59</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>RAR1</td>
<td>AT5G51700</td>
<td>1e-70</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SGTL1b</td>
<td>AT4G11260</td>
<td>2e-99</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SID2</td>
<td>AT1G74110</td>
<td>9e-92</td>
<td>Csl.6935</td>
<td>CK935726.1</td>
</tr>
</tbody>
</table>

GenBank accession number used for the tblastn search, E value for amino acid similarity. The closer the E-value is to zero, the more significant the match is. GenBank UniGene accession number. Each UniGene entry is a set of transcribed sequences that appear to come from the same locus. N/A, no citrus UniGenes available in GenBank.
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...
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 \textit{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 \textit{Avr9/Cf-9} rapidly elicited (ACRE) \textit{284} gene homolog was induced within 30 min after kumquat was challenged with Xac. \textit{ACRE} genes code for regulatory proteins with diverse functions important in \textit{Cf-9} (an \textit{R} gene)-mediated resistance, HR and basal defense (Durant 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 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 \textit{Citrus viroid III} (CVdIII) in citron (\textit{C. medica} L.) and CTV in Mexican lime \textit{[C.aurantifolia} (Christm.) Swingle\textit{] (Gandia et al. 2007; Tessitori et al. 2007). CVDIII induces dwarfism when infec-

![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, \textit{Arabidopsis thaliana}; Bn, \textit{Brassica napus}; Ca, \textit{Capsicum annuum}; Cp, \textit{Citrus paradisi}; Hv, \textit{Hordeum vulgare}; Le, \textit{Lycopersicon esculentum}; Nt, \textit{Nicotiana tabacum}; Os, \textit{Oryza sativa}. Red, 100 \% amino acids conserved; Blue, 80\% amino acids conserved; Green, 60\% amino acids conserved.](image-url)

![Fig. 4 Northern blot analysis of selected differentially-regulated cDNAs from \textit{F. margarita} tissue inoculated with \textit{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 \textit{F. margarita} subtractive library from the following clones: ET2 F11_H2, a sequence of unknown function; LT2 F113_A1, a sequence homologous to a phospholipid hydroperoxide glutathione peroxidase gene; ET2 F1_E12, a sequence homologous to a phospholipid hydroperoxide glutathione peroxidase gene; ET2 F11_E12, a 1-aminoacyclopropane carboxylic acid oxidase homologue; LT2 F112_A5, a sequence of unknown function; mock, leaves inoculated using sterile water (negative control); 18S rRNA, ribosomal RNA used as a loading control.](image-url)
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 PR, spontaneous lesion formation and stunting (Copininger 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 (Copininger 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 overriding 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.

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 this fruit crop remains to be determined. In addi-
tion, 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 condi-
tions.

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 path-
ogenic mechanisms may need to be induced in more susceptible
types to obtain a similar level of resistance. How we ac-
cumulate knowledge about the pathogenesis of citrus is further
analysis which reveals 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. Addition-
ally, a few proteins may ultimately be responsible for the
demise of the pathogen in the plant (for instance, PR pro-
teins). Modifying the expression (earlier or to higher levels)
of these genes in susceptible plants may provide the resis-
tance necessary to control the disease.

In conclusion, the better understanding of plant defense
facilitates the development of more effective ways to con-
trol 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 genes) will be more acceptable to consumers.

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