Proc. Fla. State Hort. Soc. 121:100–103. 2008.

Progress in Manipulating Citrus Defense Pathways in Favor of Citrus Resistance against Greening and Canker

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Additional index words. *Xanthomonas axonopodis* pv. *citri*, breeding, citrus canker, systemic acquired resistance, transformation

Understanding the mechanisms of plant–pathogen interaction is believed to be key to resolve the existing crop disease crisis. Molecular advances 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 vital defense genes have been identified using comparative analysis and their expression has been 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 better understanding of these natural defense pathways 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.

Florida produces 76% of the total citrus in the United States with an impact of about $9 billion to the state's economy. The industry also employs more than 100,000 people statewide. However, due to the devastation of major citrus-producing areas in Florida during the 2004 and 2005 hurricane seasons, production was significantly reduced (an estimated 10.6 million tons). Grapefruit exports in particular, which are very important to the Florida citrus industry, are down over 40% from the 2003–04 level. In addition, the increasing cost of control and lost revenue from reduced production caused by the newly emerging citrus greening disease and the previously existing citrus canker is shifting Florida's citrus research resources toward new approaches to combat both diseases. Understanding the molecular basis of tolerance of certain citrus types to these diseases is an important step in developing new control measures. For instance, we have determined that upon infection, a large number of genes are expressed early in canker-resistant kumquat, but not in susceptible grapefruit. These differentially expressed genes include pathogenesis related (PR) genes, transcription factors as well as other defense genes that are probably responsible for the observed resistance in kumquat (Khalaf et al., 2007a, 2007b). Components of this pathway are being tested for their ability to enhance tolerance to canker and greening disease in economically important rootstocks and scions.

Plant diseases have had disastrous effects on crop production as well as an enormous economic impact in different regions of the world. Commercial citrus varieties are always challenged with very destructive diseases due to their vulnerable genetic structure. During the 1980s tristeza (caused by *Citrus tristeza virus*, CTV) induced the decline and death of trees on sour orange rootstocks. This was followed by asiatic citrus canker (caused by *Xanthomonas axonopodis* pv. *citri;* Xac), considered a very severe disease of several citrus species and cultivars until the spread of huanglongbing (HLB, greening) caused by *Candidatus* Liberibacter spp. and now considered to be the most serious exotic citrus disease introduced to the US.

Enormous progress has been made during the last decade, not only in the fields of molecular biology and bioinformatics, but also in the field of plant pathology and its linking to the genetic organization of both the plant and the pathogen. The use of resistant cultivars will remain an excellent long-term strategy to control disease. Growers and breeders have been utilizing this approach by crossing resistant genotypes with different resistance for thousands of years. Unfortunately, citrus breeding is a long process obstructed by a long juvenile period, heterozygosity, and nucellar embryony. Furthermore, orange and grapefruit breeding by conventional strategies is virtually impossible because of their hybrid nature (Gmitter et al., 1992). In the past, breeding was the only means to introduce a desirable trait but there are genetic engineering approaches that can achieve the exact same goal, possibly more efficiently and in less time. Concerns about genetically modified crops among consumers as well as producers set off significant controversy in the past. This controversy may become less consequential with the newly produced genetically engineered crops that are more productive while allowing decreased use of pesticides. In the meantime, more acceptable feasible alternatives have been found, for instance, by introducing natural resistance genes from wild relatives to achieve disease resistance to a broad range of pathogens. In addition, application of the plant's own defense mechanisms can lead to more effective protection against plant pathogens. In fact, a better understanding of the nature of the host–pathogen interactions has emerged recently through the design of molecular strategies to improve disease resistance.

The genomes of more than 20 plant species have or are currently being sequenced, including *Citrus*, in what is considered to be an

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effort to add to the already existing genomic resources available. There have been several efforts in our labs and others around the world to generate citrus expressed sequence tags (ESTs) that are publically available in GenBank and other databases (Talon and Gmitter, 2008). This will facilitate comparative plant genome sequencing as well as discoveries of new genes that might be a part of yet unknown plant pathways. In this work, we will describe these advances and how they can be used to better control some of the most important diseases of citrus.

Plants have multiple levels and mechanisms to protect themselves against different assaults. Protection from the initial invasion of a pathogen is achieved primarily via constitutive defenses, such as physical and chemical barriers. These are mainly properties of the plant surface, for example, cell walls, cuticle and wax layer (Vorwerk et al., 2004). If the pathogen survives all the passive barriers employed by the plant, the plant can deploy a second line of defense. Active inducible defenses include both the basal defense that is a general immune response to all types of stresses and the R-mediated (for resistance) defense that relies on the compatibility between the pathogen and the host (Cohn et al., 2001, 2005; Zeidler et al., 2004). Active plant defense mechanisms employ pathogen associated molecular patterns (PAMPs) perception, and R gene mediated recognition leading to plant resistance against specific pathogens (Mishina and Zeier, 2007; Nurnberger and Lipka, 2005; Ryan et al., 2007; Thordal-Christensen, 2003). A large array of constitutively expressed R genes has evolved to counteract pathogen attack (Jones and Dangl, 2006; Martin et al., 2003; Rathjen and Moffett 2003).

Plant Defense Pathways

R proteins mediate rapid recognition of the pathogen, and this frequently leads to the hypersensitive response (HR), usually characterized by an oxidative burst followed by plant cell death. Initiated with ion fluxes across the plasma membrane caused by an increase in cytosolic Ca^{++}, the production of nitrous oxide (NO) and a burst of oxygen metabolism is triggered (Grant et al., 2000). The oxygen burst in turn, produces reactive oxygen intermediates (ROIs), protein kinase(s) activation, transcriptional reprogramming with the activation defense gene(s) expression and in some cases HR (Bent and Mackey, 2007; McDowell and Dangl, 2000).

A subsequent cascade of responses takes place as a result of this recognition: the activation of defense-related genes all the way through the plant resulting in a broad-spectrum resistance to pathogens known as the systemic acquired resistance (SAR) (Ryals et al., 1996; Vlot et al., 2008). Cell death, however, is not required to activate SAR (Glocova et al., 2005; Mishina and Zeier, 2007).

A network of multiple interconnected signaling pathways mediates the transduction of these signals. The plant-signaling molecules, salicylic acid (SA), jasmonic acid (JA), and ethylene play an important role in this systemic signaling network. SAR is accompanied by an increase of salicylic acid (SA) levels throughout the plant and the concomitant upregulation of a large set of genes, including genes that encode PR proteins (Ryals et al., 1996; Spoel and Dong, 2008; Ward et al., 1991). Several studies have indicated that a large number of plant genes are transcriptionally-regulated upon challenge by a pathogen (Khalaf et al., 2007; Maleck et al., 2000; Schenk et al., 2000; Zeidler et al., 2004).

The activity of certain R proteins (referred to as TIR) requires the genes EDS1, PAD4, and SAG101 which encode lipase-like proteins that interact with each other and mediate the downstream signaling (Wiermer et al., 2005). Other R proteins (referred to as CC) require NDR1, a membrane-associated protein (Aarts et al., 1998). Chaperon (HSP90) and co-chaperon (RAR1 and SGT1) proteins are also required for the function of many R proteins (Boter et al., 2007; Liu et al., 2005; Takahashi et al., 2003). Other proteins, such as EDR1, a MAPKK kinase, function as a negative regulator of the SA defense pathway (Frye et al. 2001). The sequence and function of the proteins mentioned above are conserved between different species (Bhaskar et al., 2008; Pajerowska et al., 2005; Tuskan et al., 2006; Wang et al., 2008; Zhang et al., 2004) suggesting this defense pathway is conserved among plants. NPR1 is a gene that controls the onset of the SAR, it operates downstream of the SA and is involved in crosstalk inhibition of jasmonic acid (JA)-mediated defense responses.

Characterization of *Citrus* homologues to the *Arabidopsis* defense genes. Using bioinformatics we have identified, cloned and further analyzed putative genes from citrus involved in SAR. In particular, EDR1, EDS1, EDS5, NDR1, NPR1, PR1, RAR1, SGT1 and SID2. The expression levels of these genes were also analyzed to determine their role in defense.

Defense Response in Kumquat against Citrus Canker

Until the appearance of citrus greening (HLB) in Florida, asiatic citrus canker caused by Xac was considered to be the most important disease of several citrus species and cultivars. Canker affects all *Citrus* species and some of the relatives. The disease 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 rain and wind. Infection causes lesions on the green parts of the plant including leaves and stem, as well as fruit. A significant quantity of citrus production in Florida is at high risk due to the favorable climatic conditions for spreading the bacteria and the susceptibility of the cultivated citrus. Attempts to eradicate citrus canker disease have caused serious losses in citrus trees as well as in citrus fresh fruit production all over the world.

Early field experiments and natural inoculations has shown that kumquat (*Fortunella margarita* Swingle) and some of its hybrids were resistant to canker (Reddy, 1997). Further testing using injection inoculation confirmed these observations (McCollum et al., 2006; Viloria et al., 2004). More recently, kumquat was shown to have an active response after canker inoculation, suggesting that a genetic element must be a part of the resistance observed and that resistance could potentially be incorporated into certain citrus types by conventional breeding (Khalaf et al., 2007a). We first started investigating this phenomenon by assessing the bacterial population inside both resistant Nagami kumquat [*Fortunella margarita* (Lour.) Swing.] and susceptible grapefruit leaves after injection inoculation. 'Duncan' grapefruit (*C. paradisi* Macf.) 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 d after inoculation, a response similar to HR. In contrast, grapefruit leaves developed the typical canker lesions.

In order to study the molecular components of kumquat resistance to Xac, suppression subtractive hybridization (SSH) libraries were constructed to generate cDNA libraries enriched

in sequences differentially expressed in kumquat leaves during Xac A infection (Diatchenko et al., 1996). This method enriched those transcripts associated with the response by reducing or eliminating transcripts present in un-inoculated plants. We first validated the significant differential expression levels of some of the cDNAs isolated by northern analysis. Subsequently, approximately 3500 cDNAs from the library were selected for sequencing. The ESTs generated assembled into 738 distinct contigs (consensus sequences derived from overlapping ESTs). Interestingly, SA- or JA-dependent signaling pathways were both activated in response to Xac infection according to microarray results (Khalaf et al., 2007b). Cross-talk between different plant defense signals has been described before in response to bacterial infection in different plant systems. For example, the biotrophic bacterial leaf-pathogen *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 can simultaneously trigger synthesis of both SA and JA (Spoel et al., 2003). In addition, the majority of R proteins contain a putative nucleotide binding site and a leucine rich repeats (LRR) domain, some of which have been characterized in the libraries. 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. un-infected kumquat beginning 6 h 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.

Concluding Remarks

Identifying genes involved in signaling and defense responses that result in the onset of resistance, has been the central goal of our research. The subsequent transformation of some of these genes into susceptible citrus varieties to render them resistant to both citrus and greening is our ultimate target. In the process of accomplishing both our short- and long-term goals, we have achieved some milestones that we are building on and adding to constantly. Two routes were chosen; the first is to isolate genes already identified in more than one plant species whereas the other is to identify novel citrus genes differentially expressed in response to either citrus canker or greening disease.

Certain defense pathways 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 the discoveries in model systems such as *Arabidopsis* can potentially be applied to the less studied citrus. Expression of defense genes represents a promising strategy for conferring genetic resistance against a broad range of plant pathogens. Several research groups have been successful in transforming exotic genes into citrus. 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 with the purpose of inducing resistance against bacterial, fungal and viral pathogens.

In the meantime, given the fact that the plant genome encodes hundreds of R proteins that play an indispensable role in plant defense, identifying R genes in some citrus relatives should be pursued as a major objective. The kumquat database of the canker responsive genes created through subtraction hybridization libraries will be employed for that purpose (Khalaf et al., 2007a).

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