Progress Towards the Development of Transgenic Disease Resistance in Citrus

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Abstract
Florida is the world’s third largest producer of citrus, behind Brazil and China. This 9 billion dollar industry is now under siege by two important diseases - citrus greening, also known as huanglongbing (HLB), and citrus canker. Both these diseases are caused by gram negative bacteria. Canker is primarily affecting the grapefruit industry and poses problems with the establishment of new sweet orange groves. It can be managed by following a canker suppression program, whereas HLB affects all cultivated citrus varieties and cannot currently be controlled. Resistance to either HLB or canker is also not present in commercial orange and grapefruit cultivars. A major strategy is to develop resistant cultivars via genetic engineering by incorporating resistance genes not found in citrus. We have successfully cloned several natural and synthetic antibacterial genes and genes that have the potential to turn on SAR (Systemic Acquired Resistance) and made significant progress in introducing them into commercial sweet orange and grapefruit cultivars. We have regenerated hundreds of transgenic citrus plants, using both the standard Agrobacterium-mediated transformation system and the protoplast/GFP transformation system developed previously in our program. Genetic constructs containing promoters that target expression of the above gene(s) exclusively to the phloem tissue, where HLB resides, are also being utilized in efforts to minimize foreign gene expression in fruit or juice subsequently going to market. Progress towards the development and testing of such transgenic disease resistance in commercial citrus cultivars will be discussed.

INTRODUCTION
Florida has been dealing with several common citrus diseases for many years, including CTV-induced quick-decline, citrus blight (unknown pathogen) and Phytophthora root rot. The impact of these diseases has been minimized by rootstock selection and more rigorous nursery regulations. More recently, two much more serious diseases have found their way into Florida. The first to arrive was citrus canker, a disease that is severely damaging the Florida fresh grapefruit industry. It does not kill trees, but can reduce yield and marketability of fruit. An effort by the state government to eradicate this disease (Schubert et al., 2001) failed, and the disease has since been widespread by hurricanes. The second disease to enter Florida, along with its insect vector the citrus psyllid, is citrus greening, also known as huanglongbing (HLB), caused by the phloem-limited bacterium Candidatus Liberibacter asiaticus. This disease is truly industry threatening because it spreads quickly and renders trees worthless. It has already spread to all citrus producing areas of the state and devastated entire counties. Both diseases are caused by gram negative bacteria, and the development of resistant sweet orange and grapefruit cultivars by conventional methods is not an option. Thus, several biotechnology-based approaches are being utilized in efforts to develop medium- and long-term solutions to both of these diseases. We are using somatic cybridization in
efforts to transfer organelles potentially containing gene(s) for canker resistance from kumquat to commercial grapefruit cultivars (Guo et al., 2004; Viloria et al., 2004; Grosser and Gmitter, 2005). Genetic engineering approaches offer potential long-term solutions to both diseases, thus the focus of this report.

Genetic engineering of citrus, resulting in the introduction of a single trait through genetic transformation, offers an opportunity for improvement of an already successful cultivar with no change in cultivar integrity, i.e., maintaining genotypic and phenotypic makeup. Genetic engineering also presents the possibility to produce transgenic citrus plants of commercial cultivars with resistance to bacterial disease(s) by incorporation of gene(s) from sexually compatible or incompatible plant species or other organisms.

ANTIMICROBIAL PEPTIDE CONSTRUCTS

A group of potent “bacteria killers” are the antimicrobial peptides, small protein molecules (12 to 50 amino acids) which form the first line of defense against pathogenic infection among all classes of life, including mammals (Tollin et al., 2003). These peptides selectively interact with bacterial membranes, resulting in pore formation and subsequent lysis (Izadpanah and Gallo, 2005). Antimicrobial peptides function by utilizing their cationic charge, allowing the peptides to be attracted to anionic components on the surface of the lipid membranes of the invading pathogen. Subsequently, the antimicrobial peptides, by various mechanisms, result in disruption of the membrane, leading to cell lysis and/or death of the pathogenic bacteria.

Antimicrobial peptides have a track record of neutralizing pathogenic bacteria and fungi. Modified cecropin peptides conferred disease resistance to \textit{Pseudomonas syringae pv. tabaci} (Huang et al., 1997). Cecropin derivatives such as CEME and CEMA were shown to permeabilize bacterial outer membranes of gram negative bacteria. Also, CEMA had a strong binding affinity for bacterial endotoxin (Piers et al., 1994). Another antimicrobial peptide, attacin E, conferred resistance against \textit{Erwinia amylovora}, the causal organism for fire blight in apple (Norelli et al., 1994). Transgenic potatoes containing N-terminally modified temporin A were resistant to late blight caused by \textit{Phytophthora infestans} and pink rot caused by \textit{Phytophthora erythroseptica} (Osusky et al., 2004).

The identification, characterization and incorporation of antimicrobial peptide gene(s) which have been shown to provide resistance to diseases in other plant species could lead to the development of citrus cultivars resistant to both HLB and canker. In this report, we detail our progress for the development of transgenic citrus cultivars containing antimicrobial peptide genes and which have the potential to provide resistance to HLB and canker.

SYSTEMIC ACQUIRED RESISTANCE INDUCTION

Systemic acquired resistance (SAR) is defined as a defense response resulting in the systemic expression of a subset of defense genes, and causing the plant to become systemically “immunized” so that further infection will either exhibit increased resistance or reduced disease symptoms (Kuc, 1982). SAR has been shown to provide protection against a broad spectrum of microorganisms due to the accumulation of pathogenesis-related (PR) proteins (Ward et al., 1991). This defense response has been shown to be induced by salicylic acid (SA) (Malamy et al., 1990). Plants that fail to produce salicylic acid also fail to develop SAR and do not express pathogenesis-related (PR) genes in the uninoculated leaves (Durrant and Dong, 2004). Such plants also are very susceptible to pathogen infection (Forouhar et al., 2005). For example, transgenic \textit{Arabidopsis} plants overexpressing the nahG gene encoding an SA hydroxylase gene degrade SA to catechol. This results in SA being unavailable to the plant. Such plants are very susceptible to infection by \textit{Pseudomonas syringae} and \textit{Peronospora parasitica}. Anecdotal evidence suggests that the application of products containing salicylic acid in HLB remediation programs such as the ‘Maury Boyd’ program enhances the longevity and productivity of HLB-infected trees, although scientific validation of this response is still pending.
Several genes from plants and bacteria that play a role in SAR have been identified. Amongst them, two plant genes showing potential are the tobacco Salicylic Acid Binding Protein 2 (SABP2) and the Arabidopsis non-expressor of pathogenesis related genes 1 (NPR1). The SABP2 gene is a high-affinity SA-binding protein from tobacco. It was shown previously that silencing the expression of this gene via RNA interference suppressed the ability of the tobacco plant to provide local resistance to Tobacco Mosaic Virus and SA-induced PR-1 gene expression. This also blocked the development of SAR (Forouhar et al., 2005) and demonstrated the critical role of this gene in the development of SAR following infection. Further, it was also demonstrated that this gene converted methyl salicylate (MeSA) into salicylic acid (SA), which is subsequently required for SAR signal perception in systemic tissue (Park et al., 2007). The NPR1 gene is a key regulator in the signal transduction pathway that leads to SAR. In Arabidopsis, an npr1 mutant fails to respond to various SAR-inducing agents, exhibits very low expression of several PR genes and is susceptible to a range of bacterial and fungal pathogens (Nawrath and Métraux, 1999). It has been hypothesized that this gene may act as a regulator of the transcription factor or factors that control PR gene expression (Kinkema et al., 2000). The NPR1 gene has also been shown to mediate the salicylic acid induced expression of pathogenesis-related (PR) genes and SAR (Clarke et al., 1998). Plants over expressing NPR1 have shown enhanced resistance to several pathogens (Cao et al., 1998). Utilization of SAR via overexpressing SA, or using genes that induce defense gene expression, is a novel method that employs the plant’s own inherent immune system to combat disease development and spread. Thus, the application of these technologies in citrus in the fight against citrus canker and HLB is warranted. Our approach is to produce transgenic citrus plants over expressing the SAR genes either in the phloem tissues (where HLB resides) via utilization of a phloem specific Arabidopsis SUC2 promoter (for HLB only) or systemically using a constitutive CaMV 35s promoter, and their subsequent evaluation against canker and HLB.

TARGETING TRANSGENE EXPRESSION IN THE PHLOEM

The acceptance of transgenics by consumers is still a significant issue, especially in the European Union and Japan. Chances for future acceptance of transgenic citrus may be enhanced if the expression of the foreign transgene is minimized or prevented in the subsequent fruit or juice product. In efforts to achieve this, we have collected phloem-limited promoters identified in other plants and tested them in citrus. For example, the DNA sequence of the AtSUC2 gene phloem-specific promoter (Trueheart and Sauer, 1995) was retrieved from GenBank (accession number X79702). This promoter has been demonstrated to direct expression of trans-protein activity with high specificity to the phloem of all green tissues including rosette leaves, stems and sepals. Primers were designed for the AtSUC2 gene promoter based on this sequence and included the restriction enzyme sites for HindIII and EcoRI. Following PCR amplification of the AtSUC2 promoter from Arabidopsis genomic DNA, the amplified AtSUC2 promoter was cloned into the binary vector pCAMBIA1391Z. We have previously demonstrated that the At-SUC2 promoter-GUS fusion gene construct is active in citrus and expresses high levels of trans-protein in the phloem tissue (Omar et al., 2008). Other phloem-specific promoters we have shown to work in citrus include the rice sucrose synthase promoter and the Agrobacterium rolC promoter. Constructs attaching the described disease resistance genes to the At-SUC2 promoter have been built and transgenic plants have been produced (Tables 1 and 2).

CITRUS TRANSFORMATION SYSTEMS

Agrobacterium-Mediated Transformation

Transformation of etiolated sweet orange epicotyl segments from nucellar seedlings of the cultivars ‘Hamlin’, ‘Valencia’ and OLL#8 sweet oranges, ‘Duncan’ and ‘Flame’ grapefruits, Carrizo citrange rootstock and ‘Key’ (Mexican) lime were carried
out with the commonly used protocol as described by Orbović and Grosser (2006) with slight modifications (Dutt and Grosser, 2009).

Protoplast/GFP Transformation

The protoplast transformation protocol was slightly modified from a PEG-mediated protoplast fusion protocol (Grosser and Gmitter, 1990) used for somatic hybridization in citrus. The protoplast/GFP transformation protocol, described in detail by Omar et al. (2007), utilizes newly initiated embryogenic suspension cultures with high totipotency. Regenerating transformed colonies are identified by gfp expression. This method of selection does not require the use of any antibiotic resistance gene for selection at the cellular level, thereby providing an advantage over standard citrus transformation methodology using *Agrobacterium*, in which antibiotic resistance genes are used for selection and to kill *Agrobacterium* following transformation. More recently, we have developed an efficient transformation protocol that allows for the transformation of embryogenic culture cells without going to the protoplast level. This latter procedure is attractive for polyembryonic mandarin fresh fruit cultivars such as ‘Ponkan’ or ‘W. Murcott’ that are recalcitrant in the *Agrobacterium*-mediated epicotyl segment transformation system.

TRANSGENIC PLANT RECOVERY AND TESTING

The presence of the transgene in the citrus genome of transgenic plants was confirmed using Polymerase Chain Reaction (PCR), followed by Southern analysis on selected plants. Production of mRNA in the transgenic plants was confirmed by RT-PCR analysis. Micro-grafting to Carrizo citrange rootstock or other promising rootstocks from our breeding program was utilized to expedite transgenic plant recovery and propagation for greenhouse and field evaluation. Recovered transgenic plants from independent transformation events are listed in Table 1 for those with anti-microbial genes and Table 2 for those with SAR-induction genes. To date, we have a few thousand transgenic plants that are in all stages of development, i.e., from plants that have been recently grafted in vitro to plants that are currently being challenged with canker and HLB bacteria. Our main objective, currently, is to produce a large population of transgenic lines to evaluate for disease resistance. This is in part because gene integration into the plant genome by *Agrobacterium* or protoplast transformation is a random event. Also, rearrangements or gene silencing in transgenic plants can result in low or no protein production. Therefore, a large population of plants must be screened in order to find resistant lines. Even with the use of potent antibacterial genes, which have protected other species from diseases, this strategy of screening a large population of transgenic lines remains vital.

Greenhouse testing of selected lines against canker and HLB has been limited due to a lack of available certified greenhouse space, but results seem promising. Canker screening in collaboration with J.H. Graham, using a detached leaf assay method, has suggested that some transgenic grapefruit lines are significantly more resistant to canker infection when compared to non-transgenic control grapefruit. Such plants showed fewer and smaller canker lesions, and a more robust evaluation is in progress. Challenging transgenic plants with HLB by graft inoculation with diseased budwood in an approved quarantine greenhouse facility is also underway in collaboration with R.H. Brlansky. Preliminary results are encouraging as some plants have remained HLB symptom free for more than 16 months; however, optimal greenhouse conditions and time required for HLB disease development in this assay are still under investigation.

Finally, we have initiated the most important aspect of this research, which is field testing of our transgenic plants under commercial conditions affected by canker and HLB. We have acquired a permit from APHIS to plant genetically engineered organisms in the field (also approved by the University of Florida Biological Safety Office). In the past few months, approximately 900 transgenic citrus trees with 15 different constructs have been planted at two field sites with severe canker and HLB pressure. These trials are expected to identify any successful constructs (if any) in the battle against these two
devastating diseases. Once the best construct is identified, it can then be quickly transferred into all pertinent commercial cultivars.

Several steps remain in the successful production of transgenic citrus and future commercialization of disease resistant cultivars. This paper provides a preliminary progress report on our genetic transformation strategies using antibacterial peptides and genes with potential to induce SAR.

ACKNOWLEDGEMENTS

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Literature Cited


### Tables

Table 1. Transgenic citrus plant regeneration with lytic peptide constructs.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Gene</th>
<th>No. of plants in soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duncan</td>
<td>AttacinE</td>
<td>27</td>
</tr>
<tr>
<td>Hamlin</td>
<td>AttacinE</td>
<td>15</td>
</tr>
<tr>
<td>Misc Grapefruit</td>
<td>LIMA</td>
<td>45</td>
</tr>
<tr>
<td>Valencia, Hamlin, OLL-8*</td>
<td>LIMA</td>
<td>56</td>
</tr>
<tr>
<td>Carrizo</td>
<td>LIMA</td>
<td>8</td>
</tr>
<tr>
<td>Flame</td>
<td>LIMA-SN</td>
<td>10</td>
</tr>
<tr>
<td>Misc Grapefruit</td>
<td>PTA</td>
<td>12</td>
</tr>
<tr>
<td>Valencia, Hamlin, OLL8</td>
<td>CEMA</td>
<td>21</td>
</tr>
<tr>
<td>Carrizo</td>
<td>CEMA</td>
<td>20</td>
</tr>
<tr>
<td>Key Lime</td>
<td>CEMA</td>
<td>6</td>
</tr>
<tr>
<td>Misc Grapefruit</td>
<td>CEME</td>
<td>18</td>
</tr>
<tr>
<td>Hamlin</td>
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<tr>
<td>Valencia</td>
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<tr>
<td>Carrizo</td>
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<tr>
<td>Carrizo</td>
<td>LIMA under AtSuc2 promoter</td>
<td>25</td>
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<tr>
<td>Valencia, Hamlin, OLL-8</td>
<td>LIMA under AtSuc2 promoter</td>
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</tr>
<tr>
<td>Key Lime</td>
<td>LIMA under AtSuc2 promoter</td>
<td>17</td>
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<tr>
<td>Misc Grapefruit</td>
<td>LIMA under AtSuc2 promoter</td>
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</tbody>
</table>

*OLL-8 is a high quality Valencia-like sweet orange selection.

Table 2. Transgenic citrus plant regeneration with SAR-induction constructs.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Gene construct</th>
<th>No. of transgenic plants in soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valencia, Hamlin, Flame</td>
<td>35s - SABP2</td>
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</tr>
<tr>
<td>Valencia, Hamlin</td>
<td>35s - NPR1</td>
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</tr>
<tr>
<td>Hamlin</td>
<td>AtSUC2 - SABP2</td>
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<tr>
<td>Hamlin</td>
<td>AtSUC2 - NPR1</td>
<td>10</td>
</tr>
<tr>
<td>Hamlin</td>
<td>35s - NPR1 + 35s - LIMA</td>
<td>2</td>
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