Combating Huanglongbing and Canker via Genetic Engineering of Citrus

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ADDITIONAL INDEX WORDS. Agrobacterium, antimicrobial, canker, citrus transformation, greening, Green Fluorescent Protein (GFP), protoplast, Candidatus Liberibacter asiaticus, Xanthomonas citri ssp. citri

Florida is the world’s third largest producer of citrus, behind Brazil and China. In recent years, this 9 billion dollar industry has been affected by two important diseases—citrus greening, also known as huanglongbing (HLB) and citrus canker. Both these diseases are caused by gram negative bacteria. Although canker can be managed by following a canker suppression program, HLB affects all cultivated citrus varieties and cannot currently be controlled, except in the absence of the psyllid insect vector. Resistance to either HLB or canker is also not present in commercial orange and grapefruit cultivars. A strategy to produce resistant citrus is through genetic engineering by incorporating resistance genes not found in citrus. We have successfully cloned several natural and synthetic antibacterial genes and made significant progress in introducing them into commercial sweet orange and grapefruit cultivars using both the standard Agrobacterium-mediated transformation system, and the protoplast/Green Fluorescent Protein (GFP) transformation system developed previously in our program. Genetic constructs containing promoters that target genes exclusively in 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. Techniques for construction and incorporation of the genes into citrus are discussed.

In Florida, citrus greening [huanglongbing (HLB, caused by Candidatus Liberibacter asiaticus)] and citrus canker (caused by Xanthomonas citri ssp. citri) are the two most important diseases that affect citrus. Both diseases are caused by gram negative bacteria and result in substantial economic losses for the citrus industry. Also, none of the commercial citrus cultivars are resistant to HLB, whereas mandarins are tolerant and kumquat is resistant to canker (Brunings and Gabriel, 2003; Schubert et al., 2001). However, the two most important citrus groups in Florida, grapefruits and sweet oranges, are highly susceptible. Genetic improvement of citrus via conventional plant breeding, to introgress resistant gene(s) into susceptible cultivars, is currently not possible for HLB. Research to characterize, isolate and incorporate the canker resistance gene(s) from kumquat is underway, but it will be several years before any acceptable canker resistant cultivar can be developed.

Genetic engineering of citrus, resulting in the introduction of a single trait through genetic transformation, offers an opportunity for improvement of a desirable cultivar without otherwise changing its genotypic and phenotypic makeup. Genetic engineering also presents the possibility to produce citrus plants with resistance to bacterial disease(s) by incorporation of resistance gene(s) from sexually compatible or incompatible plant species or other organisms.

A group of potent “bacteria killers” are the antimicrobial peptides. These peptides are usually 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). They form a key component of the innate immune system. 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 which allows the peptide 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 and leading to cell lysis and/or death.

Several antimicrobial peptides have been demonstrated to neutralize bacteria and fungi. Modified cecropin peptides conferred disease resistance to Pseudomonas syringae pv. tabaci (Huang et al., 1997). Cecropin derivatives like 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, atacin E, conferred resistance against Erwinia amylovora, the causal organism for fire blight (Norelli et al., 1994). Transgenic potatoes containing N-terminally modified temporin A were resistant to late blight caused by Phytophthora infestans and pink rot caused by Phytophthora erythroseptica (Osusky et al., 2004).

The identification, characterization, and incorporation of gene(s) which have been shown to provide resistance to diseases in other plant species can 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 peptides genes and which have the potential to provide resistance to HLB and canker.

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Materials and Methods

Construction of plasmid vectors. We obtained the vector containing the LIMA antimicrobial peptide gene from Dr. Dennis Gray, MREC, UF/IFAS. To generate the other vectors used in this study, both synthetic as well as natural antimicrobial peptide genes were used (Table 1). Synthetic genes were codon optimized for Citrus sinensis L. Osbeck using the data available at the Codon Usage Database (www.kazusa.or.jp/codon/). This database is maintained using data compiled and available at the GenBank DNA sequence database. The synthetic genes were designed using the Vector NTI Advance™ Software (Invitrogen Corporation, Carlsbad, CA) and oligonucleotides corresponding to the designed gene were ordered from Integrated DNA Technologies, Coralville, IA. The assembly of the synthetic gene(s) was carried out by stepwise ligation of the gene segments as described by Matsubara et al. (2003).

The DNA sequence of the Arabidopsis thaliana SUC2 (AtSUC2) gene promoter (Truernit and Sauer, 1995) was retrieved from GenBank (accession number X79702). 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. Variations of this cloning vector containing the phloem specific AtSUC2 promoter were also produced. A HindIII DNA fragment containing the expression cassette d35S (or AtSUC2) - antimicrobial peptide gene-3′CaMV was isolated and cloned into the unique HindIII site of a pBIN19-derived binary vector. This vector, containing a bifunctional nptII/egfp fusion gene (Fig. 1) has been described earlier (Li et al., 2001). Standard techniques for plasmid manipulation and cloning were as described by Sambrook and Russell (2001). E. coli strain DH5α was used for the cloning of all plasmids and all constructions were verified first by restriction analysis and then by DNA sequencing. Each binary plasmid was introduced into A. tumefaciens strain EHA105 (Hood et al., 1993) by the freeze-thaw method (Burrow et al., 1990).

Plant materials. Nucellar seedlings of various citrus cultivars were used (Table 1). The seeds were extracted from fruit, the outer seed coat removed, surface-sterilized for 10 min in a 0.6% (v/v) sodium hypochlorite solution and rinsed with sterile deionized water. Seeds were germinated in 15-cm-long glass culture tubes containing 15 mL of solid MS medium [consisting of MS salts and vitamins (Murashige and Skoog, 1962) supplemented with sugar 30 g·L⁻¹ and agar 7 g·L⁻¹, pH 5.8]. Generation of embryogenic callus and establishment of suspension cultures for protoplast transformation were as described by Grosser and Gmitter (1990).

Transformation and selection of regenerants. Genetic

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Plasmid</th>
<th>Gene</th>
<th>No. of plants in soil</th>
</tr>
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<tbody>
<tr>
<td>Duncan</td>
<td>d35s-attacin E</td>
<td>attacin E</td>
<td>27</td>
</tr>
<tr>
<td>Misc. grapefruit</td>
<td>d35s-LIMA</td>
<td>LIMA</td>
<td>43</td>
</tr>
<tr>
<td>Misc. grapefruit</td>
<td>d35s-PTA</td>
<td>N-Terminal modified Temporin A gene</td>
<td>18</td>
</tr>
<tr>
<td>Valencia, Hamlin, OLL8</td>
<td>d35s-CEMA</td>
<td>Cecropin-Melittin fusion gene</td>
<td>14</td>
</tr>
<tr>
<td>Key lime</td>
<td>d35s-CEMA</td>
<td>Cecropin-Melittin fusion gene</td>
<td>6</td>
</tr>
<tr>
<td>Misc. grapefruit</td>
<td>d35s-CEME</td>
<td>Cecropin-Melittin fusion gene; differs from CEMA by containing different amino acids in the C terminus</td>
<td>14</td>
</tr>
<tr>
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<td>d35s-CEAD</td>
<td>Cecropin A-Cecropin D fusion gene</td>
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<tr>
<td>Valencia, Hamlin</td>
<td>Suc2-LIMA</td>
<td>LIMA</td>
<td>13</td>
</tr>
<tr>
<td>Carrizo</td>
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<tr>
<td>Key lime</td>
<td>Suc2-LIMA</td>
<td>LIMA</td>
<td>11</td>
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</table>

Miscellaneous grapefruit include cultivars Duncan, Marsh, and Flame.
OLL8 is a ‘Valencia’-like somaclone.

Fig. 1. Physical map of the plasmids used for transformation. (A) T-DNA region of the binary vectors used in Agrobacterium-mediated transformation. (B) Gene cassette region of the vectors used for protoplast transformation. d35S/AtSUC2, double enhanced (2 × –419 to –90) CaMV 35S promoter with the Ω leader sequence of TMV or the Arabidopsis sucrose synthase phloem specific (AtSUC2) promoter; dCsVMV, double enhanced (2 × –443 to –123) cassava vein mosaic virus promoter; egfp/nptII, bifunctional enhanced green fluorescent protein and neomycin phosphotransferase III fusion gene; egfp, enhanced green fluorescent protein gene; 35S-3′, termination site and polyadenylation signal of the CaMV 35S transcript; RB, right border; LB, left border.
transformation was carried out using the standard Agrobacterium-mediated method (Orbovic and Grosser, 2006) as well as the protoplast mediated method (Omar et al., 2007). Briefly, the culture tubes were kept in the dark for 3 weeks at 25 °C, and subsequently moved to the light for an additional week, allowing the etiolated explants to turn green. Seedling epicotyls were cut into 0.5- to 1-inch-long segments, incubated in a suspension of the appropriate Agrobacterium strain (OD 0.3 to 0.5, depending on the cultivar—lower OD for sweet orange and higher for grapefruit) and placed on co-cultivation medium (CM) for 2 d at 25 °C in the dark, after which they were transferred to selective regeneration medium (RM; Orbovic and Grosser, 2006) until shoots developed and selected for presence of GFP.

The protoplast transformation protocol was slightly modified from a polyethlene glycol (PEG) mediated protoplast fusion protocol (Grosser and Gmitter, 1990) for somatic hybridization in citrus. A protoplast/Green Fluorescent Protein (GFP) transformation protocol, described in detail by Omar et al. (2007) was followed. Briefly, suspension cells used for protoplast isolation were taken 4–10 d into a 2-week sub-culture cycle. Suspension cell tissues were digested overnight in the enzyme mixture and the protoplasts were purified by centrifugation on a sucrose-mannitol gradient. The protoplast pellet was resuspended in 0.6 M BHJ3 protoplast liquid culture medium. The plasmid DNA containing the antimicrobial gene(s) were added at a concentration of 25 µg DNA per 100 µL protoplast suspension and mixed well. Protoplast, plasmid DNA and PEG were incubated for 25–30 min at room temperature. Following incubation, PEG was eluted and protoplasts were washed three times. Protoplasts were cultured in liquid medium, transferred to solid medium and transformed colonies were microscopically selected based on the expression of GFP. Transformed colonies were physically separated from non-transformed tissue, and cultured on somatic embryogenesis induction medium as described previously by Grosser and Gmitter, 1990.

GFP-specific fluorescence was evaluated using a Zeiss SV11 epi-fluorescence stereomicroscope equipped with a light source consisting of a 100-W mercury bulb and a FITC/GFP filter set with a 480-nm excitation filter and a 515-nm longpass emission filter producing a blue light (Chroma Technology Corp., Brattleboro, VT). GFP expressing transgenic shoots were transferred onto MG medium (MS salts and vitamins supplemented with 1 mg L−1 GA3, 1 µM NAA) for shoot elongation. Elongated shoots were micrografted in vitro onto Carrizo citrange [Citrus sinensis (L.) Osbeck × Poncirus trifoliata (L.) Raf.] or sour orange [Citrus aurantium L.] nucellar rootstock seedlings as described by Navarro (1992) and Orbovic and Grosser (2006) to expedite the recovery of transgenic plants. After a month of growth in vitro, the grafted shoots were potted into a peat based commercial potting medium (Metromix 500, Sun Gro Horticulture, Bellevue, WA) and acclimated to greenhouse conditions.

**Molecular analysis of transformants.** To confirm the presence of the transgene in the citrus genome, we used the Polymerase Chain Reaction (PCR). Citrus genomic DNA, used as template for PCR, was isolated from 100 mg of young leaves of transgenic citrus plants using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Corp., St. Louis, MO). PCR was carried out in a thermal cycler (MJ Research, Watertown, MA) using GoTaq® Green Master PCR Mix (Promega Corp, Madison WI) and appropriate primers. Amplified DNA fragments were electrophoresed on a 1% agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA) and visualized under UV light. All images were recorded and digitized.

Reverse transcription polymerase chain reaction (RT-PCR) was used to detect the presence of the mRNA in all transgenic plants before plants were transferred into the greenhouse. Briefly, total RNA was isolated from 100-mg leaf tissues using a RNeasy Mini Kit (Qiagen Inc., Valencia, CA). cDNA was synthesized from total RNA using Oligo (d)T primer and a RETROscript® RT-PCR kit (Applied Biosystems, Austin, TX). The cDNA product was used for PCR as described above, and images were recorded and digitized.

**Acclimatization and transgenic plant challenge.** Transgenic plants that had been confirmed to contain the gene by PCR and producing mRNA using RT-PCR were subsequently transferred into the greenhouse for growth. Plants were grown in a greenhouse under 40% shade cloth and approximately 31 °C daytime and 21 °C nighttime temperatures.

Vigorously growing transgenic plants were pruned to initiate new flushes. Young, fully expanded but immature leaves were harvested early in the morning for challenge with canker using the detached leaf assay method (Francis and Graham, 2008). An overnight culture of a pathogenic canker strain growing in nutrient broth medium was centrifuged to harvest cells. The cells were diluted with sterile phosphate buffer to an OD600 of 0.1 corresponding to 10⁶ colony forming units (CFU)/mL. The 10⁶ CFU/mL sample was again twice serially diluted 1:10 to obtain a 10⁴ CFU/mL suspension for inoculating the transgenic leaves. Control leaves were from 1-year-old greenhouse grown non-transgenic Duncan trees. Leaves were surface sterilized using 70% alcohol for 1 min and rinsed three times in sterile water before being infiltrated with the bacterial cells. Infiltration was carried out using a 1-cm³ syringe. Bacterial cells were forced out of the syringe into the adaxial (top) surface of the leaf. The leaves were subsequently placed aseptically on thin 1% water-agar petri plates with the adaxial surface of the leaf exposed. Leaves were incubated in a growth chamber maintained at 30 °C and observed for the development of tissue hyperplasia after 7 d.

Once the transgenic plants reached a minimum trunk caliper of 6 mm, they were challenged by side grafting with a 4- to 8-cm-long stem segment taken from a PCR-positive HLB-infected field tree. The infected graft union and twig were completely wrapped with budding tape and maintained in a greenhouse under 26 °C daytime and 21 °C nighttime temperatures. After 21–28 d, the twig was unwrapped, leaving the graft union wrapped. The graft union was unwrapped after 4 months post-inoculation. Plants were visually monitored for any HLB symptom development and frequently assayed by PCR for the presence of the HLB organism in the transgenic plant and in the inoculum twig.

**Results and Discussion**

We have successfully developed vectors for several antimicrobial peptide genes (Table 1). Each antimicrobial gene (with the exception of LIMA and the natural attack E gene) has been codon optimized for C. sinensis. The pattern of codon usage is similar among closely related species, but differs among distantly related organisms (Murray et al., 1998). Codon optimization has also been demonstrated to significantly increase the protein production (Sinclair and Choy, 2002), since the codon frequency of the gene under study is matched to that of the host expression system. We optimized the codon for the antimicrobial genes to a citrus consensus codon usage in order to maximize the production of the trans-protein in citrus. This can potentially have benefits...
in enhancement of disease resistance as have been seen in other systems (Zhou et al., 2003).

Constructs driven by a constitutive 35S promoter as well as an AtSUC2 promoter have been produced. The AtSUC2 promoter is a phloem specific promoter. In Arabidopsis, this promoter directs expression of trans-protein activity with high specificity to the phloem of all green tissues such as rosette leaves, stems, and sepals (Truernit and Sauer, 1995). The promoter-GUS fusion gene construct is active in citrus and expresses high levels of trans-protein in the phloem tissue (Omar et al., 2008). AtSUC2 and other phloem specific promoters currently under evaluation (e.g., rice sucrose synthase promoter and the Agrobacterium rolC promoter), can potentially resolve issues of the potential presence of trans-protein in fruit and juice considering expression of trans-protein takes place only in the phloem tissue.

We utilized an Agrobacterium-mediated and a protoplast mediated transformation approach to incorporate the transgene into the citrus genome. Both transformation protocols have their own unique advantages. The basic idea behind Agrobacterium-mediated transformation is use of disarmed strains of A. tumefaciens (biovar 1) and exploitation of its natural transformation process to incorporate foreign DNA into plant tissue (Tinland, 1996; Birch 1997). Agrobacterium-mediated transformation (Orbovic and Grosser, 2006) also has the unique advantage in that it is possible to get stable transgene integration without rearrangement of either host or transgene DNA and the integration of one or few gene copies into the plant’s genome minimizes transgene silencing.

In the case of protoplast transformation, following the introduction of foreign DNA, transgenic tissue was selected and physically separated from non-transformed tissue and transferred to solid medium approximately 2 months after the transformation procedure. This method of selection does not require the use of any antibiotic resistance gene for selection at the cellular level. This provided an advantage over standard citrus transformation methodology using Agrobacterium, in which antibiotic resistance genes are used for selection and to kill Agrobacterium following transformation. Another advantage of this protoplast/GFP system is the type of starting material used, which should facilitate transformation of seedless polyembryonic citrus cultivars that produce inadequate quantities of nucellar seed for Agrobacterium-mediated transformation.

Transformed embryogenic callus developed normal, bipolar, heart-shaped embryos that were proliferated and enlarged by subculturing on different media (Grosser and Gmitter, 1990, Omar et al., 2007). Once multiple shoots were induced, they were treated to induce roots and most of them produced roots successfully and were transferred to soil. Shoots failing to produce roots were either micrografted in vitro or shoot-tip grafted onto nucellar seedlings of Carrizo citrange or sour orange. Over 95% and 80% of the in vitro micrografts and shoot-tip grafts, respectively, successfully produced vigorous plants. We routinely micrograft the transgenic shoots onto Carrizo or sour orange rootstock, as this facilitates a rapid plant recovery and fast growth.

The use of GFP provided us with a strong non-destructive tool to visually select transgenic shoots from the non-transgenic shoots of no value. Transgenic plants observed under a blue light produced by an epi-fluorescent microscope glow green while non-transgenic plants are red. This allows us to efficiently discriminate between green transgenic and red non-transgenic plants. As a result, we are able to routinely evaluate a large number of putative transgenic shoots and quickly select for shoots expressing the GFP gene. Since our gene(s) of interest is linked to the GFP gene, there is a high probability that plants showing green fluorescence have the gene of interest incorporated into their genome. Data on the production of transgenic lines are presented in Table 1. We have been successful in generating over 100 independent transgenic plants in several citrus cultivars and in the popular Carrizo citrange rootstock. Integration of the gene was confirmed by PCR (Fig. 2). Only plants which were PCR positive (amplifying the gene of interest) were transferred to the greenhouse, the rest are discarded. However, PCR only tells us if the gene is present in the tissue of transgenic plants. To confirm gene transcription, we performed RT-PCR on plants which have been confirmed positive by PCR (data not shown). RT-PCR is usually performed on larger plants. We have had only one incident where the plant was positive for PCR, but did not amplify the cDNA following RT-PCR. This can happen as a result of transcriptional gene silencing.

To date we have transgenic plants that are in all stages of development, from plants that have been recently grafted in vitro, to plants that are currently being challenged with canker and HLB bacteria. Currently, our main objective 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 has to 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.

We have challenged some transgenic lines with canker, and results are promising. Using the detached leaf assay method outlined in the materials and methods, we have so far observed one of our transgenic lines to be significantly resistant to canker infection, when compared to non-transgenic control (Fig. 3). Numerous other transgenic lines remain to be challenged as soon as they are of a suitable size. Some of our transgenic lines, which show significantly smaller lesions, compared to controls may prove to be resistant under field conditions. Further analysis is in progress.

We have adopted the method for challenging transgenic plants with HLB by graft inoculation with diseased budwood in an ap-
proved quarantine greenhouse facility. Currently, we have several transgenic lines being challenged with HLB (Fig. 4). Preliminary results are encouraging; however, optimal greenhouse conditions and time required for HLB disease development in this assay are still under investigation. We have transgenic grapefruit trees containing the LIMA construct that were inoculated over 11 months ago with HLB infected sweet orange budsticks. Phloem tissue analyzed from the grapefruit trees are all still PCR negative for HLB with no visible symptoms, whereas the budsticks are alive and PCR positive for HLB.

In conclusion, several steps remain in the successful production of transgenic citrus and future commercialization of disease resistant cultivars. This paper provides a preliminary report on our genetic transformation strategies using antibacterial peptides.

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