Progress towards Incorporation of Antimicrobial Peptides for Disease Resistance in Citrus

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Abstract. Antimicrobial peptides play important roles in the innate immune response and can be found to occur in all classes of life, including humans. These peptides are usually small proteins and have an ability to associate with bacterial cell membranes. Antimicrobial peptides are also characterized by their broad spectrum antibiotic property. Citrus canker and Huanglongbing (HLB) are the two major diseases threatening the global citrus industry. All commercial grapefruit, sweet orange and tangerine cultivars are susceptible to these diseases, and it is difficult to create tolerant cultivars via conventional breeding. Incorporation of one or more genes encoding for antimicrobial peptides into the citrus genome via genetic engineering could potentially result in development of cultivars resistant to these diseases, without modifying the varietal fidelity.

Using Agrobacterium mediated as well as protoplast transformation of citrus, we have successfully incorporated several genes encoding for antimicrobial peptides into the citrus genome of several citrus cultivars. Two different promoters were used for direct expression of the antimicrobial peptide gene (s); a constitutive Cauliflower Mosaic Virus 35S promoter and a phloem specific Arabidopsis sucrose synthase promoter. It is hoped that by targeting gene expression using phloem specific promoters, we will be able to minimize the expression of the transprotein in fruit and juice. Several transgenic plants have been challenged with the disease organism (s) and results look promising.

Florida is the largest producer of grapefruits and the second largest producer of sweet oranges. This large citrus industry has been threatened in recent years by citrus canker (caused by Xanthomonas citri ssp. citri) and citrus greening or Huanglongbing (HLB associated with Candidatus Liberibacter asiaticus), two bacterial diseases caused by gram negative, non-indigenous but now endemic pathogens. Both of these diseases result in substantial economic losses to the industry. While canker can be managed by following a canker suppression program, HLB, affecting all cultivated citrus varieties, cannot be currently controlled. Resistance to either HLB or canker is also not present in commercial orange and grapefruit cultivars cultivated in Florida.

Our strategy in producing disease-resistant citrus is to incorporate resistance genes not found in citrus by employing genetic engineering. This process of insertion of a single gene without otherwise altering the genetic makeup of the plant also maintains the varietal fidelity and does not alter its genotype and phenotypic makeup. Genetic engineering therefore presents the possibility to produce citrus plants with resistance to bacterial disease (s) by incorporation of resistant gene(s) from sexually compatible or incompatible plant species or other organisms.

A group of small peptides, present in all classes of living organisms, provide a first line of defense against invading pathogens are the antimicrobial peptides (Tollin, et al., 2003). These peptides play important roles in the innate immune response, and are usually secreted in response to infection. These peptides have been found to be involved in the antimicrobial defense systems of a wide range of animal species, including insects (Hultmark, et al., 1980), crustaceans (Nakamura, et al., 1988); amphibians (Zusloff, 1987) and even mammals (Ganz, et al., 1985). Such peptides are usually small negatively charged proteins which have an ability to associate with the positively charged bacterial cell membranes. They function by selectively interacting with the bacterial membranes resulting in pore formation and subsequent lysis (Izadpanah and Gallo, 2005). These peptides are also characterized by their broad spectrum antibiotic property and are bacteriocidal (bacteria killer) in nature instead of being bacteriostatic (bacteria growth inhibitor).

Several antimicrobial peptides have been
demonstrated to neutralize bacteria and fungi. An important group of antimicrobial peptides are cecropins. These lytic peptides were originally isolated from the haemolymph of the giant silk moth, *Hyalophora cecropia* and have been found to be present in other insects also (Moore, et al., 1996). In general, gram-negative bacteria are more sensitive to cecropins than gram-positive ones. Several cecropins and their derivatives have been used in disease resistance studies. Modified cecropin peptides conferred disease resistance to *Pseudomonas syringae* pv. tabaci in tobacco (Huang et al., 1997). Other 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, attacin E, an inducible antibacterial protein present in *Hyalophora cecropia* pupae, resulted in increased resistance against Erwinia amylovora, the causal organism for fire blight in apple (Norelli, et al., 1994). Also, transgenic potatoes containing N-terminally modified temporin A were resistant to late blight caused by Phytophthora infestans and pink rot caused by Phytophthora erythrosepta (Osusky, et al., 2004). Antimicrobial peptides have also been used in research involving human diseases in order to combat drug resistant bacteria (Juyne, et al., 1989). In view of the increasing importance of antimicrobial peptides and their efficacy against gram negative bacteria, we decided to clone and introduce several of these genes—both natural and synthetic into citrus in order to combat canker and HLB.

**Materials and Methods**

Construction of plasmid vectors: Antimicrobial genes corresponding to antimicrobial peptides were synthetically created and codon optimized for sweet orange (*Citrus sinensis* L. Osbeck) using the data available at the Codon Usage Database (www.kazusa.or.jp/codon/) and the Vector NTI Advance™ Software (Invitrogen Corporation, Carlsbad, CA). Oligonucleotides corresponding to the designed gene were obtained from Integrated DNA Technologies, Coralville, IA. The oligonucleotides contained a NcoI restriction site at the 5′ of the gene while the 3′ end contained a NotI site. 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 Arabidopsis thaliana SUC2 (AtSUC2) gene promoter (Truemit and Sauer, 1995) was cloned from Arabidopsis genomic DNA.

Different antimicrobial genes driven by a double enhanced CaMV 35S promoter (d35S) containing a CaMV 35S terminator (3′ CaMV) were cloned into a pBIN19-derived binary vector. Variations of this cloning vector containing the phloem specific AtSUC2 promoter were also produced. This vector, containing a bifunctional nptII/egfp fusion gene 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 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).

Genetic transformation and selection of regenerants: Nucellar seedlings of various citrus cultivars as outlined in Table 1 were used for genetic transformation studies. The seeds from each cultivar were processed for generation of etiolated epicotyls as described by Orbovic and Grosser (2006). Generation of embryogenic callus and establishment of suspension cultures for protoplast transformation were as described by Grosser and Gmitter (1990).

Genetic transformation was carried out using a standard Agrobacterium-mediated method (Orbovic and Grosser, 2006) as well as a protoplast mediated method (Omar, et al., 2007). Following Agrobacterium mediated transformation, putative transgenic shoots regenerating on a selective medium containing kanamycin were evaluated for GFP-specific fluorescence by viewing with a Zeiss SV11 epifluorescence stereomicroscope (light source consisting of a 100 W mercury bulb and a FTTC/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). Transgenic shoots expressing GFP were transferred onto MSG medium (MS salts and vitamins supplemented with 1mg/L GA3) for shoot elongation. Similarly, transgenic embryos produced using the protoplast transformation method were selected from the non-transgenic embryos and transferred onto B + germination medium. Germinated seedlings were transferred into RMAN rooting medium for subsequent growth and root development (Grosser and Gmitter, 1990). To expedite the growth and development of the transgenic plants obtained from both methods, the elongated shoots were micro grafted in vitro onto Carrizo citrange [ *Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] nucellar rootstock seedlings. 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.
Table 1 Results of transformation experiments to transfer antimicrobial genes into several citrus cultivars

<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>Carriazo</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>Carriazo</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>CEMA</td>
<td>18</td>
</tr>
<tr>
<td>Hamlin</td>
<td>CEMA</td>
<td>6</td>
</tr>
<tr>
<td>Valencia</td>
<td>CEAD</td>
<td>14</td>
</tr>
<tr>
<td>Carriazo</td>
<td>CEAD</td>
<td>12</td>
</tr>
<tr>
<td>Carriazo</td>
<td>LIMA under AtSuc2 promoter</td>
<td>25</td>
</tr>
<tr>
<td>Valencia, Hamlin, OLL-8</td>
<td>LIMA under AtSuc2 promoter</td>
<td>23</td>
</tr>
<tr>
<td>Key Lime</td>
<td>LIMA under AtSuc2 promoter</td>
<td>17</td>
</tr>
<tr>
<td>Misc Grapefruit</td>
<td>LIMA under AtSuc2 promoter</td>
<td>12</td>
</tr>
</tbody>
</table>

* Misc Grapefruit includes Duncan, Marsh and Flame cultivars.

PCR and RT-PCR analysis: Citrus genomic DNA, used as template for PCR, was isolated from 100mg 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 to amplify the gene of interest. 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 100mg leaf tissues using a RNeasy Mini Kit (Qiagen Inc., Valencia, CA). cDNA was synthesized from 500 ng total RNA using Oligo (dT) primer and a RETROscript® RT-PCR kit as described by the manufacturer (Applied Biosystems, Austin, TX). The cDNA product was used as a template for PCR as described above. An additional primer set based on plant cytochrome oxidase (COX) gene was used as a positive internal control. 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. The images were recorded and digitized.

Acclimatization, and transgenic plant challenge: Transgenic plants that had been confirmed to contain the gene of interest by PCR and producing mRNA using RT-PCR were propagated into multiple clones. For small transgenic plants, multiplication was done by micro budding while buds from larger trees were used for an inverted T budding. Several rootstocks including 'Carriazo' citrange, 'Swingle' citrumelo, or the tetrazyg 'orange 16' (hybrid between two promising tetraploid rootstock parents obtained from the rootstock improvement program directed by Dr. J. W. Grosser) were used for the production of clones. These clones were grown in a greenhouse under 40% shade cloth and approximately 31°C daytime and 21°C nighttime temperatures.

Transgenic plants were screened for their resistance to citrus bacterial spot (CBS) caused by Xanthomonas citri pv. citrulina; citrus canker (CC) caused by Xanthomonas citri ssp. citri and HLB associated with Candidatus Liberibacter asiaticus. Vigorously growing transgenic plants were pruned to initiate new flushes. Screening for CBS was done using young fully expanded but immature citrus leaves using an attached leaf assay test. Briefly, a syringe was used without the needle to infiltrate citrus leaves with the bacterial suspension. 106 cfu/ml cell suspension in phosphate buffer was infiltrated into the abaxial leaf surface by gently pressing the blunt end of the syringe against the abaxial leaf surface. The leaves were then observed for development of tissue hyperplasia after 7 – 14 days of inoculation. Similarly, for the citrus canker assay, young leaves at a similar stage of growth as described above were harvested early in the morning for challenge using the detached leaf assay method as described by Francis and Graham (2008) as outlined in Fig. 1. For evaluation of resistance to HLB, transgenic trees that had reached a minimum trunk...
caliper of 6mm were tested. These trees were challenged by side grafting with a 4 – 8cm long stem segment taken from a PCR positive HLB infected field tree. In additions, an infected bud was T-budded a few inches above or below the side graft union. 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 days, the twig was unwrapped, leaving the graft union wrapped. The graft union was unwrapped after 4 months post-inoculation. Trees were visually monitored for any HLB symptom development and assayed by Real Time PCR every 6 months post inoculation for the presence of the HLB organism in the transgenic plants and in the inoculum budsticks.

![Diagram of the process](Image)

**Fig. 1** Schematic representation of detached leaf assay for canker resistance studies

### Results and Discussion

In the present study, we make our effort to transform various citrus cultivars with several antimicrobial genes and challenge them with bacterial pathogens in an attempt to evaluate their resistance. Several antimicrobial genes were cloned into binary vectors and incorporated into Agrobacterium tumefaciens strain EHA105. These binary vectors vary in the antimicrobial gene incorporated into them and the promoter driving the gene. We have used a constitutive double enhanced 35s promoter as well as a phloem specific Arabidopsis thaliana sucrose synthase promoter to drive the cloned genes. Each of the constructed binary vectors contained a bifunctional gfp/nptII fusion gene which acted as a dual purpose selectable marker and reporter gene. The presence of green fluorescent protein or GFP enabled us to perform a non-destructive assay by shining blue light to identify putative transformed shoots. Under blue light, transformed shoots appeared bright green while non transformed shoots appeared red due to chlorophyll autofluorescence.

Each antimicrobial gene (with the exception of LIMA and the natural attacin E gene) was codon optimized for C. sinensis. Codon optimization has 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 codons of the antimicrobial genes (with the exception of LIMA and the natural attacin E gene) 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).

In addition to genetic transformation using Agrobacterium, we also used a protoplast mediated transformation approach to incorporate the antimicrobial gene into the citrus genome. This system also utilized the green fluorescent protein as a reporter gene and allowed non destructive visualization of the transformed shoots (using Agrobacterium) or embryos (using protoplasts). GFP positive shoots were obtained from several cultivars as outlined in Table 1. Several transgenic plants from different citrus cultivars and containing different antimicrobial genes have been obtained using the Agrobacterium mediated method. We also have transgenic callus containing the CEMA gene.
and stably expressing the GFP protein from mandarin cultivars using the protoplast transformation technique.

We decided to use both transformation systems as each has 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 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. A standard transformation protocol, described in detail by Orbović and Grosser (2006) was followed to regenerate a large number of transgenic plants. In contrast, the protoplast mediated transformation system does not require the use of any selectable marker gene like NPTII as used in the Agrobacterium mediated approach. Another advantage of this transformation system is the nature of starting material used (protoplasts derived from callus), which allowed transformation of recalcitrant cultivars to Agrobacterium infection or seedless polyembryonic citrus cultivars that produce inadequate quantities of nucellar seed needed for Agrobacterium-mediated transformation.

Following production of transgenic plants, each of the putative transgenic lines was tested for the presence of antimicrobial gene using PCR and RT-PCR (Fig. 2A). All transgenic lines that amplified the gene of interest using PCR also. Each PCR positive transgenic line was subsequently acclimated and hardened in our greenhouses as explained in materials and methods. To confirm transcription of transgene, each line was subsequently tested for the presence of a functional mRNA using RT-PCR. Several transgenic lines have been tested using the two step RETROscript® RT-PCR (Fig. 2B). We incorporated amplification of a conserved plant cytochrome oxidase (COX) gene from citrus to verify production of cDNA in our samples. This serves as an internal check in cases where production of low or no cDNA results in amplification failure of the transgene of interest (data not shown).

![Amplification products obtained from transgenic citrus plants.](image)

**Fig. 2** Amplification products obtained from transgenic citrus plants with AttacinE gene specific primers which successfully amplified the expected 567bp AttacinE fragment.

M. λ-Std marker 1-10 are 10 individual transgenic lines. B. Reverse Transcriptase PCR of total RNA isolated from leaf tissues of putative transgenic plants. Equal loading of RNA was confirmed by measuring the 28S rRNA.

Many of the larger transgenic plants which are positive following RT-PCR have been propagated using micrografting or budding. We have adopted an attached leaf assay test to screen the large number of transgenic lines for disease resistance. Initial assays consist of inoculation with the citrus bacterial spot pathogen. The citrus bacterial spot pathogen is a weak pathogen affecting plants in the greenhouse in Florida. Also this pathogen is not under any state or federal regulations, which makes it easy to work with. It is, however, a gram negative bacterium and like citrus canker can cause disease symptoms if left untreated. Screening transgenic lines for resistance to citrus bacterial spot infection helps us narrow the number of potential transgenic lines that can be further evaluated for resistance to HLB or canker. The reasoning behind this approach is that gene integration into the plant genome by Agrobacterium or protoplast transformation is a random event and rearrangements or gene silencing in transgenic plants can result in low or no protein production. Therefore, a large population of plants have to be screened in order to find a few resistant transgenic 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. Several of our transgenic lines exhibited tolerance to the pathogen when compared to the control (Fig. 3).
Subsequently, a detached leaf assay for evaluation of disease resistance as outlined by Francis and Graham (2008) was performed. The process of the canker assay is outlined in Fig. 1 and consists of incubation of transgenic leaves with the bacterial suspension in a growth chamber and observed for water-soaking and tissue hypertrophy symptoms after 7 – 14 days. Based on the canker assay, we have evaluated a large number of transgenic lines for resistance to canker and several lines look promising (data not shown). Also, we are observing a wide range of symptoms ranging from a mild tissue hypertrophy in transgenic citrus leaves containing the LIMA gene to moderate tissue hypertrophy in transgenic citrus leaves containing the AttacinE gene (data not shown). Controls in all experiments exhibited severe water-soaking and tissue hypertrophy. Validation of these results by stomatal inoculation of whole trees with bacterial suspension in a secure greenhouse as outlined by Viloria et al. (2004) is ongoing. Field trials are also planned following evaluation of promising transgenic lines in the greenhouse.

Concurrently, we are challenging the same promising transgenic plants with HLB by graft inoculation with diseased budwood in an approved quarantine greenhouse facility (Fig. 4). Preliminary results are encouraging since we have transgenic lines that exhibit a wide range of disease symptoms—from transgenic lines that do not exhibit disease symptoms even after a year of inoculation with the HLB infected sweet orange budwood to lines which exhibit the characteristic HLB disease symptoms (yellowing on some of the new shoots in the canopy to irregular mottling elsewhere). The HLB negative transgenic plants produce healthy flushes as do the HLB positive budwood [verified by Real Time PCR using protocol and primers as outlined by Li et al., (2006)]. Such results are encouraging as we hope that the genes we used can potentially produce transgenic plants that are tolerant/resistant to HLB. Currently we are repeating our initial HLB studies by challenging multiple clones of selected transgenic lines to evaluate optimal greenhouse conditions and time required for HLB disease development.

**Literature Cited**

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