

BREEDING CITRUS FOR HLB RESISTANCE AT THE USDA/ARS U.S. HORTICULTURAL RESEARCH LABORATORY, FT. PIERCE, FLORIDA

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Abstract

Citrus breeding has been conducted by the USDA since 1893. The initial objectives included improved disease-resistance, cold hardiness, and easy peeling fruit, which are still important breeding objectives today. The emergence of huanglongbing (HLB) in the US has propelled the development of HLB resistance to the forefront of our breeding objectives. Transgenic strategies offer the potential to develop truly resistant citrus even when such a phenotype has yet to be clearly identified in citrus and associated to a specific citrus gene. Strategies being employed in the lab include genetic transformation to create trees producing antimicrobial peptides and other transgenes targeting *Candidatus Liberibacter asiaticus* (CLAs) gene products, virulence mechanisms, or induce host plant resistance mechanisms. Both constitutive and phloem-specific promoters are being utilized. Tests of HLB-resistance in transgenic lines are currently underway. While all tested commercial citrus scion cultivars are reported to be susceptible to HLB, evidence suggests some cultivars are slower to develop severe symptoms. In recent grove surveys where most sweet orange and 'Minneola' trees have HLB symptoms and high CLAs titers, fewer trees of 'Temple' display HLB symptoms or CLAs associated with HLB disease. These observations have precipitated numerous experiments at the USHRL, including replicated trials of cultivars with high exposure to CLAs, assessment of CLAs/HLB in breeding populations exposed to high disease pressure, and controlled psyllid challenges. Since varieties showing lower CLAs or their parents are used frequently within our breeding program, we hope to identify many citrus scion types with greater HLB tolerance for near-term deployment. Continued observation that *Poncirus trifoliata* and its hybrids are among the most resistant material to HLB and Asian citrus psyllid (ACP) offers the more distant promise of identifying advanced intergeneric hybrids with even greater resistance, and suitable fruit quality. Theoretically, slower development of HLB/CLAs could be due to alteration in a number of components: attractiveness of trees to ACP, CLAs establishment at ACP feeding, CLAs proliferation following ACP inoculation, systemic movement of CLAs with subsequent further proliferation, and development of plant responses observed as HLB symptoms. Reduction or slowing of any of these steps may slow disease development and spread, but have different implications in overall management and commercial significance. Careful consideration needs to be given to the value and implications of such tolerance.

Introduction

The USDA citrus breeding program was initiated in 1893 by Walter T. Swingle and Herbert J. Webber at the U.S. Subtropical Laboratory in Eustis, Fla. Their initial objective was to develop varieties with resistance to the numerous diseases that plagued citrus in Florida. The severe freeze in the winter of 1894-1895 killed most of the initial USDA hybrids and prompted Swingle and Webber to focus on breeding for cold hardy citrus using trifoliolate orange (*Poncirus trifoliata*) as a parent. To date, trifoliolate-derived hybrids have not been released as scion varieties, though they now represent a substantial proportion of world citrus rootstocks. The USDA program has many low-poncirin trifoliolate hybrid selections that may serve as parents or grandparents for

future cold-hardy citrus cultivars (e.g. Barrett, 1990) or may offer opportunities as parents of HLB-resistant new varieties, as *P. trifoliata* is among the most resistant species in several reports (e.g. Folimonova et al., 2009).

The presence of HLB in Florida, first documented in 2005 (Halbert 2005), and the threat of spread to other citrus growing states in the US, has now redirected the USDA breeding program perhaps even more emphatically than freeze-tolerance motivated Swingle and Webber. Control of HLB, through production of disease-free nursery stock, roguing infected trees and suppressing the psyllid vector (Bove, 2006), along with increased tree replacement markedly increase production costs where HLB is endemic. There is further concern that sustainability of entire industries may be threatened by HLB-induced production losses, and therefore the need for resistance is of paramount importance. Since sweet orange and grapefruit represent the vast majority of Florida citrus which is currently at greatest risk, HLB resistance in these cultivars are of highest priority.

Even in the absence of overt threats, regular introduction of new citrus scion cultivars is highly desirable to maintain a healthy citrus industry. Distinctive and novel traits are also important to attract consumers who are confronted with an increasingly broad array of produce choices throughout the year. Enhanced fruit quality through better flavor and easier consumption (easy peeling, seedlessness, and low juice release when segments are separated) provide major market incentives for return purchases, while extension of harvest season, and resistance to pests are also valuable considerations in considering new varieties (Hearn, 1973). All of these factors are considered in developing and releasing new varieties and have been discussed in the context of the USHRL breeding program in earlier papers (Cooper et al., 1962; Hearn, 1973; McCollum, 2007). These efforts remain underway with the added need for HLB tolerance or resistance.

Discussion

Transgenics: Genetic transformation or genetic engineering (GE) offers the opportunity to incorporate genes and traits which cannot be found in cross-compatible species. Even when possible to use hybridization for gene transfer, GE permits inclusion of one or a few identified genes into an otherwise desirable cultivar without the need for repeated backcrossing and selection. No strong HLB-resistance has been identified within cultivated citrus, making transgenic solutions the priority to develop both scions and rootstocks which will permit economic citrus production where HLB is endemic. Deregulation of HLB-resistant GE citrus is likely to pave the way for many additional GE solutions for citrus improvement. *Agrobacterium*-mediated transformation of seedling epicotyl tissue is the standard technique for citrus transformation and typically provides 0.1-5% of explants producing a transformed shoot. The procedures followed at the USHRL are slight modifications of those published by Orbovic and Grosser (2006). Fortunately, many important citrus genotypes are highly apomictic and the seedlings reflect maternal genotypes and thus maintain cultivar identity.

Little is known about the HLB pathosystem and thus antimicrobial peptides (AMPs) have been the primary focus for current GE work. AMPs have been identified from numerous organisms and are among the first defenses against microbial infections. AMPs disrupt bacterial membrane integrity, but do not damage plant cells, and confer some resistance to a wide array of disease-producing pathogens (e.g. Montesinos, 2007). Related efforts are underway at Texas A&M University, the University of Florida, and the Sylvio Moreira Citrus Center in Brazil, and ongoing discussions ensure that these efforts are complementary rather than duplicative. In the USHRL program, priority AMPs are those that are from plants or synthetic rather than animal origin, are already reported to be effective against gram-negative bacteria, and have negligible potential for human health problems. There are numerous bottlenecks in efficiently mobilizing AMPs to confer HLB-resistance. Due to the urgency for identifying solutions, we are moving full speed with best available information, while also attempting to improve transformation efficiency and screen AMPs for important traits. D4E1, a 17 amino acid synthetic AMP which forms a beta sheet (Lucca et al., 1998), is active against *Agrobacterium tumefaciens* in poplar (Mentag et al.,

2003) and has been utilized extensively in our initial efforts. More than a 4000 putative transformants have been developed with AMPs driven by several promoters (both constitutive and phloem-limited), representing a range of rootstock and scion genotypes. In vitro assessment of minimum inhibitory concentration (MIC) has been conducted using *Sinorhizobium* and *Agrobacterium* as surrogates for *Liberibacter* as they are closely related alpha proteobacters (Bastianel et al., 2005). The causal agent of citrus bacterial canker (CBC), *Xanthomonas citri* pv. *Citri*, is also included in these analyses, in the hope that HLB and CBC resistance can be achieved with the same AMP transgene driven by a constitutive promoter. Thus far, D4E1 is among the most active AMPs tested, with an MIC less than 1 μ M. Initial scion challenges have been conducted with CBC, and some Hamlin, Valencia transformants show negligible infection (similar to 'Nagami' kumquat) and have been replicated in the greenhouse for further CBC and HLB testing.

Additional transgenes will be used as opportunities are identified to target CLas gene products, virulence mechanisms, and host plant resistance pathways. Peptides have been identified, using a phage display library, which bind a CLas epitope projected to extend into the host cell on the bacterial membrane surface. Following verification that the CLas binding peptides disrupt essential bacterial processes, transgenes will be developed and introduced to test this strategy in citrus phloem. Studies of host plant gene expression in response to CLas infection have provided several leads for target genes that may interrupt disease development (Albrecht and Bowman, 2008).

In collaboration with WRRRC (Albany, CA) existing citrus genomic data has been used to identify citrus sequences closely matching the L and R border regions of *A. tumefaciens*. Constructs using these have been tested with tobacco and used to produce transgenics. Ubiquitin promoter and terminator sequences have been identified in citrus and used to create constructs. New citrus sequence is being generated from Carrizo citrange and will be mined for defensins to provide an all citrus construct for transformation which will be tested in the next year and may provide HLB and CBC resistance, perhaps with an anthocyanin marker gene.

Key steps in the production of transgenics are being studied to enhance throughput. Experiments are underway and include optimization of *Agrobacterium* growth phase and inoculation density, cocultivation parameters including those that reduce tissue necrosis, use of thin cell layer explants, evaluating parameters to enhance shoot regeneration and growth such as light, type and concentration of cytokinins, antibiotic selection, carbon source, gelling agents, mineral nutrition, and liquid culture systems to simultaneously improve selection and shoot regeneration. In addition, efforts are underway to transform mature citrus tissue using proliferating mature tissue in vitro cultures. In the first year of mature tissue transformation the first transformed shoot buds have been observed; these results are extremely promising.

Resistance in conventional cultivars: There are anecdotal reports from growers that some minor citrus cultivars are much slower to develop HLB. To investigate this, eight Indian River region (East Florida) groves with four or more diverse scions planted in close proximity were surveyed for CLas and HLB. No cultivar displayed a low % of CLas infection (as measured by % trees with Ct <36 in qPCR using CLas 16S rDNA primers) in every grove sampled. 'Minneola', followed by sweet orange, tended to be most uniformly high in incidence of CLas infection. 'Temple' was among the lowest in CLas incidence in 6 of 7 groves where it was tested. Grapefruit were highly variable in CLas incidence. Sweet Orange had an average of 25 X the level of CLas observed in 'Temple'. Since observed differences in HLB infection may also be influenced by attractiveness of plants to Asian Citrus Psyllid (ACP), numerous choice and no-choice greenhouse tests as well as field trials are being conducted or initiated to explore this component of HLB resistance.

Resistance in related species and genera: Numerous reports have been made for HLB resistance of distant Citrus species and related genera. Field evaluations are underway for >50 diverse accessions of Citrus and related members of the Rutaceae obtained from the Citrus Clonal Germplasm Repository in Riverside, Calif. and seedlings are being challenged with HLB

in a replicated field plot. Few plants show HLB symptoms after one year of exposure, but a few accessions (including *P. trifoliata*) display significantly reduced ACP feeding.

Poncirus trifoliata cultivars and various *P. trifoliata* hybrids are maintained as scions in the USHRL Ft. Pierce farm variety block, all on Sun Chu Sha mandarin and planted in 2000. In November 2009 it was apparent that many unifoliate rootstock suckers had greater HLB symptom expression than the trifoliate scions. Leaves were randomly sampled from four quadrants of each of the trifoliate “scions”, selecting terminal leaves from the next to last flush, plus a diagnostic “worst sample”, and both random and “worst sample” for unifoliate rootstock suckers. These data provide evidence that *P. trifoliata* and some of its hybrids may “tolerate” and/or significantly suppress CLas even when grafted to high-titer source genotypes. It also appears that citranges may vary in their tolerance to CLas, suggesting that citranges may segregate for resistance/tolerance to HLB, presumably with resistance/tolerance derived from their *P. trifoliata* parent. In collaboration with the U of Florida, a replicated planting of a characterized citrange mapping population is being established at USHRL to identify genes associated with HLB/ACP resistance/tolerance.

Several approaches are being studied to reduce the time between generations in citrus breeding to as little as one to two years, permitting rapid introgression of traits from distant relatives into commercial citrus types. This may be critical for conventional breeding to produce high levels of HLB resistance in the next 20 years. An early flowering gene (FT) has been identified and sequenced in several plant genera, including *Citrus* (Endo et al., 2005). Based on studies with other woody perennials, over-expression of the FT gene should result in citrus plants that flower within a year of seed germination. An early flowering *P. trifoliata* has been reported in China, with enhanced expression of FT and alterations in several other flowering genes (Zhang et al., 2009), and efforts are being made to identify similar mutants at USHRL. Purely horticultural manipulations also show promise in producing citrus hybrids which flower just a few years after initial seed germination (Gmitter and Grosser, personal com.).

Conclusions

A wealth of citrus germplasm has been generated by the USDA using a wide assortment of breeding techniques, both conventional and transgenic. USDA citrus breeding material is being grown at the A.H. Whitmore Citrus Research foundation farm in Lake County, Florida and at the USDA, ARS, US Horticultural Research Laboratory farm in St. Lucie County, Florida. The Whitmore farm is at the northern edge of Florida commercial citrus production and no HLB has been identified at this site. It is our hope that vigilance will permit continued evaluation of hybrids at this farm without interference by HLB, as this will be essential for conventional breeding. HLB is rampant at the Ft. Pierce farm and the best use of this site is to test for HLB/ACP resistance and control and therefore HLB infected trees are not removed and many citrus blocks are not receiving psyllid control sprays. Therefore, this is an ideal site for testing transgenic and other citrus that offer potential for HLB or psyllid resistance. It is our hope that these diverse strategies will continue to provide the US and world citrus industry with useful new cultivars.

We are certainly not suggesting that the Florida industry markedly increase production of the cultivars with lower CLas titers (like ‘Temple’) in the study of field-infected commercial cultivars. If significant resistance/tolerance is found in conventional cultivars, we may find similar responses in related hybrids including some that are very sweet-orange or grapefruit like, and may be suited to broad scale production, processing, and marketing, and many such hybrids with diverse genetic makeup are under evaluation in citrus breeding programs.

We have initiated field and lab trials to compare HLB / ACP resistance and tolerance in diverse commercial varieties and rootstocks, and are evaluating HLB resistance in hybrid populations at the Picos Farm of USHRL USDA/ARS. If resistance or tolerance is confirmed, we need to evaluate how this may benefit the Florida citrus industry and how much of a delay in HLB symptom development or extension of useful commercial life is needed to be useful.

Theoretically, slower development of HLB/CLAs infection could be due to alteration in a number of components, such as: attractiveness of trees to ACP; CLAs establishment at ACP feeding; CLAs proliferation following ACP inoculation; systemic movement of CLAs with subsequent further proliferation; and development of plant responses observed as HLB symptoms. Reduction or slowing of any step may slow disease development and spread, but with different implications for overall management and commercial significance. Careful consideration is needed regarding the value and implications of such tolerance, which likely will still need to be coupled with use of clean nursery stock, aggressive psyllid control, and identification and roguing of HLB-infected trees. Cultivars less attractive to ACP may be particularly useful, since such trees might be valuable in ringing groves of high-demand highly-susceptible cultivars, such as sweet orange, and rapid development of symptoms would still permit ready identification and roguing of developing inoculum sources.

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