

# `Awa, a Potential Nutraceutical for Hawaii

Mel C. Jackson

Recently, interest in herbal supplements throughout the United States and Europe has grown at a significant pace. The U.S. imports many botanical products from around the world to meet herbal supplement demand

**A** good example of this is Kava (*Piper methysticum*) or `awa as it is known in Hawaii. The main center for `awa production is the Pacific Islands, in particular Vanuatu, Fiji, Samoa and Tonga. However, `awa has also been grown in Hawaii since the arrival of the Polynesians and is now an agricultural enterprise throughout Hawaii.

Hawaiian `awa, although originating in the South Pacific, now comprises approximately 13 cultivars, which are morphologically and chemically distinct from their South Pacific counterparts. This is due to the selection pressures applied by generations of astute growers who, owing to `awa's propensity to spontaneously mutate, carefully selected mutations with the desired pharmaceutical effects. The result is that Hawaiian `awa today is of high quality.

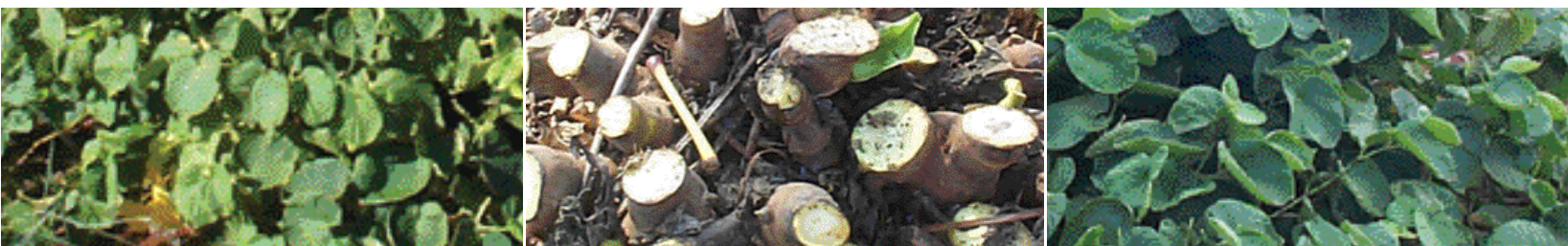
With the gradual diversification of agriculture in Hawaii, the HARC Analytical Chemistry Laboratory has moved to develop research projects that service the needs of the `awa farmers. We now analyze the bulk of the commercially grown `awa. Clients need accurate,



*The `awa cultivar "green mai" was grown in full sunlight with drip irrigation at Kunia substation —  
High Kavalactone yield potential was demonstrated*

dependable information on overall kavalactone content and the relative concentrations of each of the six major kavalactones. Kavalactones are the primary active components in `awa.

Traditional cultural practices would indicate that `awa should be grown in partial shade. No clear practices for nutrition and other cultural practices have been established. Therefore, together with collaborators at the University of Hawaii, College of Tropical Agriculture and Human Resources (Dr. H. C. Bittenbender, Dr. C. S. Tang), we were awarded a grant from the USDA T-STAR



(Tropical and Subtropical Agriculture Research) program, to evaluate the effects of parameters such as light, pruning and fertilization rate on kavalactone content in the root. The work began in September 2000 and will continue through 2002. The project is based at the University of Hawaii Magoon horticultural facility and utilizes a novel basket cultivation system based upon experimentation by Hawaii `awa growers. This system allows for the close monitoring of irrigation, fertilizer rates and the amount of sunlight. Its greatest advantage is that it facilitates the sampling of roots. The basket is simply opened, pieces of root excised and then the basket re-sealed. At the end of the project, the importance of light, fertilization and pruning will be established and cultural practices optimized so that kavalactone content can be maximized. HARC's analytical laboratory is analyzing all of the samples, which will total more than a thousand.

Independently, we have studied the cultivation of `awa under difficult environmental conditions. `Awa was planted at our dry, leeward Kunia substation, in full sun and exposed to northeast tradewinds. Two-year-old `awa plants were harvested resulting in root plus crown weights exceeding 30 lb/plant. Kavalactone content was at the higher end of the normal range.

The major costs associated with the production of `awa in Hawaii have been propagation and preparation of plants for field planting. Even today, to ensure rooting of cuttings, nursery misting systems are used. Once rooted, plants are generally kept in a greenhouse until

they are strong enough to be transplanted to the field. To try to reduce propagation costs for farmers, we have begun some preliminary work to determine if rooting from cuttings similar to a sugarcane planting can be done efficiently in the field. At HARC's Kunia substation, we have planted stem pieces from `awa plants. Planting was done in shallow furrows and covered with soil, and drip irrigation was installed. The best propagation material was obtained from the youngest nodes.

Preliminary studies have begun to determine if `awa is a viable secondary crop planted under koa (*Acacia koa*) trees grown for timber. Typically a grower plants trees and then waits 20 or more years to see a return on investment. `Awa may be able to provide a source of income while the koa trees mature.

`Awa represents only one of a number of "new" crops that make up Hawaii's transition to a more diversified agriculture.

# Sugarcane Research

## Breeding and Selection

Sugarcane crossing began on November 22, 1999, and was completed in six weeks. We used 2004 flowering stalks from 369 parents to make 222 polycrosses. In 2000, we evaluated 294 clones in FT7 yield tests with 12.5% (37 clones) yielding more sugar per acre compared to the standard cultivars.

H78-7750 was identified as the standard potential commercial cultivar for a shorter cropping cycle. Six FT7 tests of short-cropped H78-7750 were hand harvested. The estimated yield per acre was 79.4 tons of cane, 9.5 tons of sugar and 31.9 tons of dry matter. When harvested by a billet harvester from a nearby unburned commercial field, H78-7750 produced 68.1 tons of cane, 6.4 tons of sugar and 27.2 tons of dry matter per acre.

During the past two years (1999 and 2000), we have tested 201 Hawaii clones and 108 foreign clones for potential short cropping cycle cane for Hawaii. Using the sugar yield percent cane (YD/C) of H78-7750 as the standard selection criteria, we have identified five Hawaii clones (83-6179, 86-6180, 87-4319, 87-4442 and 95-4318) and 13 foreign clones (CP70-1133, CP86-1791, LF172, LF80-1205, LF80-2690, LF80-3443, LF80-5304, Q123, Q127, Q131, Q135, ROC1 and SP79-1169) as superior. They were propagated for further evaluation.

This year, we transplanted 30,848 bunch seedlings (about five seedlings per bunch) to the field for our regular FT1 selection. By the end of 2000, we selected 1062 clones from FT4 that were planted in FT5A tests. We selected 814 clones having better than average yield for inclusion in advanced yield testing.

In 2000, we harvested 22 FT7 yield trials and evaluated 294 new clones of which about 12.5% (37 clones) yielded more sugar than the commercial standard. During the year, we installed 8 FT7 tests on Kauai and 12 on Maui.

The top four cultivars based on acres planted at the end of 2000 were H78-7750, H78-4153, H77-4643 and H78-3567. H78-7750 was planted on 13,758 acres or 32% of the total cane area. Acreage of H78-4153 continued to decline occupying about 30% of the total cane area.

New clones with commercial potential are: H91-4392, H92-5867 and H93-6002 for the Makaweli soil on Kauai; H79-6503 and H90-7435 for the rocky, dry areas on Maui; H82-3569 and H83-4501 for the sandy areas on Maui; H82-3569, H85-4501, H85-1605, H86-3792, H87-4394 and H88-6401 for windward



*Maunawili breeding facility. Sugarcane seedlings are grown in the greenhouses and shipped to Maui where the selection process begins.*

Maui. H87-5794 had the best FT7 records in the leeward region and H87-4319 performed well in mill water fields.

*K. K. Wu, E. Gamatero*

Sugarcane was received in three shipments in 1999 at the Kunia quarantine facility. They were primarily from Louisiana but two were received from Israel through the USDA as tissue-cultured plantlets. During 2000, all the quarantined sugarcane was graded and evaluated for YLS, ratoon stunting disease and other disease symptoms. Most were selected and shipped to Maui for field trials and also planted at Maunawili. Of the two Israeli cultivars, one flowered heavily and set seed. Seed was collected and some was planted. The seedlings are self progeny since no other flowering cane was present in the vicinity. They are currently in flats at Maunawili and will be planted in the field. *S. Schenck*

## Alternate Hosts of the Sugarcane Yellowleaf Virus.

Sugarcane Yellowleaf Virus (SCYLV) is not mechanically transmitted. It is a member of the Luteovirus group of viruses that are confined to the phloem tissue in their plant hosts and are transmitted from plant to plant by phloem-feeding aphid vectors. In Hawaii, the main vector for SCYLV is the sugarcane aphid, *Melanaphis sacchari*. In order to determine whether there are alternative host plants for SCYLV, transmission studies were carried out using the sugarcane aphid. Viruliferous aphids were reared on sugarcane plants infected with SCYLV. The aphids were transferred one by one from the colony plant to the test plants using a small watercolor brush.

Depending on the test plant, between 4 and 100 aphids were transferred per plant. Plants were then placed in insect-proof cages for a period of time to allow the virus to multiply.

There are a number of wild sugarcane relatives growing at the Maunawili breeding station. These field plots were tested using the tissue blot immunoassay for SCYLV infection. In addition, some of the uninfected plants were put in pots and inoculated by means of the aphids. It was found that all of the sugarcane relatives were susceptible to SCYLV in varying degrees. The percentage of infected clones in each tested species was as follows: *Saccharum officinarum* 52%, *S. robustum* 11%, *S. spontaneum* 21% and *S. sinensis* 71%. None of the *Erianthus* species were infected in the field, but subsequently a few were infected by aphid inoculation.

Several grain crops were grown from seeds and the seedlings in pots were then inoculated with viruliferous *M. sacchari* aphids. Nearly 100% of the wheat, oats and barley seedlings subsequently tested positive for SCYLV. Rice and corn were less susceptible and became infected at 8.5% and 10.5%, respectively. These crops could become infected with SCYLV in the field if they were planted near infected sugarcane and the aphid vectors were present. Since luteoviruses are not seed-borne, there is no danger of the virus being transmitted to the next generation of grain crops. Unlike the grains, sugarcane is propagated vegetatively by stalk pieces that were proven to carry the virus and to spread it to all fields planted with infected seed pieces.

*S. Schenck and A.T. Lehrer (Bayreuth University, Germany)*

## Augmentative Biocontrol of Lesser Cornstalk Borer

The lesser cornstalk borer (LCB), *Elasmopalpus lignosellus* has been the most destructive pest of young sugarcane plants in Hawaii since the mid 1980s. To control this pest and protect sugarcane production, especially the shorter cycle cane, an augmentative biological control project including mass rearing and release of the LCB parasitoid, *Horismenus elineatus* was carried out.

LCB larvae were collected from plantations on Kauai and Maui. *H. elineatus* parasitoids were collected from LCB pupae. LCB and parasite colonies were reared in the entomology laboratory and parasitism of LCB by *H. elineatus* was assessed. The assessment provided the information necessary for the decisions about the parasite releases and collection sites. Three mass releases of parasitoids were made on Maui and Kauai.



*Sugarcane showing symptoms of yellow leaf syndrome (YLS)*

## Assessment of parasitism

Three collections on Maui were made in HC&S fields 605, 510 and 818 on August 8 and 16, and September 13, respectively. A collection on Kauai was made in G&R field number 12 on September 6. The numbers of adult LCB that emerged, numbers of LCB larvae that were parasitized, the rates of parasitism and the mean numbers of parasites per LCB larva were recorded. The results showed that the rates of parasitism were higher in Maui fields than in Kauai indicating higher parasite populations in Maui fields. The means of parasites per LCB larva were similar in all collections.

## Field releases

There were two field releases of 1000 to 2000 parasitoids each at HC&S and one release of 2000 individuals at G&R. Changes in the rate of parasitism were not measurable owing to the low populations of LCB in the fields. Both LCB and parasitoid colonies continue to thrive in the laboratory. Parasitoid colonies will be maintained in the laboratory and releases made when the periodic field inspections indicate a need. Releases will continue in 2001.

*H. Chen and A. Ota*



## Improving Sugarcane Transformation

Transformation methods for sugarcane have been improved through modifications of several steps in the transformation process. Transgenic plants were generated by particle gun bombardment, where gold particles coated with DNA were used to transfer genes into plant cells. This enabled recovery of as many as six to seven potentially transgenic plants per culture plate bombarded with DNA, a significant increase in success rate.

Modifications to the transformation system include changes in tissue culture medium and more frequent transfers, leading to generation of larger numbers of vigorous plants. Sugarcane cells are now placed on a nutrient medium that helps protect against stresses of handling and DNA bombardment. Addition of the growth regulator thidiazuron during the plant regeneration phase has resulted in greater numbers of plants which are often more vigorous. Higher concentrations of selection agents are now used to screen potentially transgenic cultures, minimizing the number of plants that escape the selection process. Focus on establishment of a healthier and more developed root system prior to transition from sterile culture to soil has lead to improved survival.

Several sugarcane cultivars have been transformed, including two commercial sugarcane cultivars, H78-3567 and H77-4643. The short planting cycle cultivar H78-7750 has also been included in the most recent transformation experiments. In earlier experiments, the GUS reporter gene was used to allow detection of successful transformation. Plants expressing the GUS gene turn blue in the assay system, giving direct visual evidence that plants are positive for the introduced gene. In more recent experiments, a gene for a high value pharmaceutical protein, granulocyte macrophage colony stimulating factor (GMCSF), was introduced. Experimentation is also underway with a new selection system based on mannose tolerance to screen for transgenic plants.

*C. Goldstein*

## The Mechanisms of Gene Silencing in Sugarcane Transgenic Plants

Although the production of transgenic plants has become routine for a number of crop species, levels of transgene expression in these transgenic plants are often unpredictable and many transgenic plants become "silenced". Understanding the mechanisms that are responsible for transgene silencing has become increasingly important and is a prerequisite for making genetic engineering of crop plants efficient.

Previously, we produced numerous transgenic sugarcane lines containing a GUS reporter gene driven by either of the two



*Transgenic sugarcane plants regenerated from callus*

sugarcane polyubiquitin gene promoters (ubi4 and ubi9). Numerous lines were selected which produced high levels of GUS expression while still in the callus stage. However in all of these lines the GUS gene became silenced after plant regeneration. We have now performed several experiments to determine the nature of the transgene silencing process which has occurred in these plants. DNA gel blot (Southern) hybridizations indicated that many copies of the GUS reporter gene had inserted into the plant chromosomes and many of these copies had undergone rearrangements. This phenomenon is commonly observed in particle gun transformation, and is associated with transgene silencing. Additional Southern blots were used to detect DNA methylation in the GUS reporter genes, a phenomenon also frequently associated with transgene silencing. Our assays did not detect widespread methylation in the GUS genes. As silencing of the GUS genes occurred after plant regeneration, callus was generated from silenced plant lines to test for silencing reversibility. Some lines did undergo an increase in GUS expression in the reinitiated callus, however the best lines accumulated only 10-15% of the levels present in the original callus lines. To test whether the silencing occurred at the transcriptional or post-transcriptional level, two types of experiments were performed. In the first experiments, 23 nucleotide RNA molecules homologous to GUS were detected in the silenced plants. These small RNAs have been previously demonstrated to accompany

post-transcriptional gene silencing (PTGS). To confirm this, nuclei were isolated from silenced plant lines and used in nuclear run-off experiments. These experiments showed that GUS genes were being actively transcribed in silenced plant lines, confirming that the silencing was PTGS.

It is now recognized that PTGS represents an endogenous plant defense mechanism which has evolved to defend against viruses and or transposable elements. Current efforts to overcome PTGS in transgenic plants are focused in two areas: one is to partially inactivate the plants' PTGS system so that transgenes are unaffected. The other general approach is to construct transgenes designed for plants, so they are not recognized by the plant as being virus- or transposon-like.

Using particle gun bombardment, we have successfully transferred the GUS reporter gene into sugarcane calli. However, the high level of GUS expression measured at the callus stage was no longer detectable in the regenerated sugarcane transgenic plants, indicating that gene silencing was triggered. The objective is to unravel the underlying mechanisms that were responsible for this transgene inactivation. First, we examined the number of copies of our inserted transgene in different transgenic sugarcane lines by Southern hybridization. Using this method for detecting the presence of introduced DNA, it was revealed that most transgenic plants contained multiple copies at multiple loci and a few of them were observed to have multiple copies in one locus. Second, we checked methylation status of the DNA coding for the GUS gene because in some cases gene silencing has been associated with methylation of DNA. We found that most copies of the transgene were not methylated, ruling this out as the cause of the gene silencing we have observed in our sugarcane plants. To further elucidate the mechanism of gene silencing we regenerated calli from silenced transgenic plants and found that GUS gene expression was restored, even though the expression level was only 10-15% of the expression in original calli, indicating the silencing status was reversed. Based on these results, we proposed that the silencing in sugarcane is posttranscriptional gene silencing. In this case the introduced gene is still active, but the RNAs transcribed from the transgene are targeted for degradation by a currently unclear process. Therefore no protein product is produced from the transgene. To verify such a hypothesis, we isolated nuclei from transgenic plants and fed them with radioactive labeled materials that allow detection of transcripts from the gene. We were able to detect the presence of transcripts of our gene of interest, suggesting the transgene was active. In our final experiments to explain gene silencing in sugarcane, we isolated and detected 21 to 23 nucleotide pieces, small RNA molecules called

"small interfering RNA" (siRNA), which were the final products from degraded GUS RNA molecules. These small RNA molecules are now believed to incorporate into a ribonuclease complex, which then targets the introduced RNA sequence for degradation.

*H. Wei (University of Hawaii), H. Albert (USDA/ARS)*

## Alternative Promoters for Sugarcane Transformation

Two maize promoters for the reporter gene GUS were tested for transient expression in sugarcane calli. The promoter H2B with constitutive activity gave a strong response whereas the stalk specific promoter F3.7 showed no GUS expression in calli and young stalk tissues. Since the level of GUS expression could depend on the age of stalk tissue, stable transformants were sought. The cultivar H62-4671 that was readily transformed with insect and herbicide resistance genes previously, was transformed with constructs containing either promoter. Transformed plants will be tested for the presence and activities of transgenes.

*A. Dela Cruz, P. Moore (USDA/ARS), M. Fitch (USDA/ARS)*

## Hawaiian *Saccharum officinarum* Cultivars

Sugarcane is not indigenous to the Hawaiian islands, but was brought by the Polynesians. These were *Saccharum officinarum* cultivars selected for medicinal or religious purposes. They had colorful stalks of purple, red, yellow or green, often with stripes. They were given various Hawaiian names that described their colors or uses. The Hawaiians did not mill cane for sugar, but crushed it for juice or chewed on the stalks.

In the early 1900s, Edward L. Caum of the Hawaiian Sugar Planters' Association and W. W. G. Moir of American Factors began collecting and describing Hawaiian *S. officinarum* cultivars. Moir published descriptions of them along with what was known of their Hawaiian lore. Their article makes interesting reading and prompted a recent inventory of the remaining cultivars still in the Hawaii Agriculture Research Center's and other collections in the State. These consist of about 30 different cultivars with known Hawaiian names and a number of others that appear to belong to the same group or are mutations of original Hawaiian sugarcanes. Some of these may instead have been brought in by the early planters. It is possible that more may still be found. Caum and Moir placed the various selections in family groups based on pith color, bud position and internode shape. Until a genotyping study can be made, identification can only be based on general appearance, which is variable. *S. Schenck*

# Tropical Fruits

## Backcrossing Papaya Hybrids

Papaya ringspot virus (PRSV) -resistant UHRainbow and UHSunUp papayas have enabled Hawaii growers to produce fruit in areas infested with the virus. UHSunUp cultivar papayas were created by genetically transforming Sunset cultivar with PRSV resistance genes. UHRainbow was created by backcrossing UHSunUp with the untransformed Kapoho papaya. Kapoho has several outstanding characteristics such as fruit size suitable for export, firm flesh, a spicy, pungent flavor, and resistance to leafhoppers and powdery mildew. UHRainbow also has these characteristics and is resistant to PRSV. UHRainbow has now replaced Kapoho on many Big Island farms.

On Oahu, Kamiya papaya commands higher prices in the local market than most other cultivars. Kamiya papayas are large-fruited and flavorful, but are highly susceptible to PRSV. Consequently, a backcrossing project was initiated in 1997 by crossing Kapoho and Kamiya with UHRainbow F2 plants to transfer the PRSV resistance gene into the nonresistant inbred lines. Currently, Kapoho and Kamiya hybrids up to backcross 3 are being evaluated in the field. Average fruit size decreased with increasing percentages of Kapoho traits. Kamiya backcross 2 fruit resemble the original Kamiya cultivar, having glossy, waxy skin, deep orange flesh color, and the characteristic flavor. Intermediate hybrids of both inbred lines, that is, Kapoho backcross 1 and Kamiya F1, were named Poamoho Gold and Laie Gold. These cultivars have been well accepted by consumers. Laie Gold now occupies a sector of the Kamiya niche market on Oahu that still consistently commands a higher price compared to UHRainbow or other cultivars. Plant patent and plant variety protection certificate applications were submitted for both Laie Gold and Poamoho Gold in 1999-2000.

*M. Fitch (USDA/ARS), T. Leong, L. Akashi, L. Poland, S. Ferreira (University of Hawaii), A. Yeh, S. White, G. Yamamoto, N. Saito, P. Moore (USDA/ARS)*

## Transgenic Kamiya Papaya

In addition to backcrossing, virus-resistant Kamiya papayas were produced through transformation and are now being evaluated. Based on the results with Sunset papayas, Kamiya was similarly transformed with PRSV resistance genes. A Kamiya R0 line named UT9-8A was immune to virus and apparently contained two copies of the untranslatable PRSV coat protein gene. Unlike UHRainbow, the transgenic Kamiya line was never infected with PRSV despite multiple inoculations with the virus. The R1 progeny are being evaluated for fruit and tree quality. Fruit from the original R0 transgenic line were indistinguishable from Kamiya, with its deep orange flesh color, large, fragrant fruit, and tendency of producing



*Laie Gold Tree - a three way hybrid of (Kapoho X UH Sunup) X Kamiya*

carpellodic fruit during the cool winter months. Other transgenic lines were also immune to PRSV but many were aberrant and sterile, and appeared to be tetraploids or dwarfs.

*T. Leong, A. Dela Cruz, P. Moore (USDA/ARS),  
M. Fitch (USDA/ARS)*

## Clonally Propagated Transgenic Papayas

Papaya fields are currently established by planting five or more seeds or seedlings per hole. After waiting until the sex of the trees can be determined at flowering time, all seedlings except for a single hermaphrodite are removed from each bunch. Hermaphrodite plants are preferred because of their superior fruit shape, size and quality. Growers require about 6 hours to thin one acre of papayas. Clonal propagation of papaya hermaphrodites could save the time and costs associated with multiple plantings and thinning.

Three field tests on two islands were established in 1998 and 1999 to compare growth and yield of clonally propagated UHRainbow papayas. The data showed that clonally propagated UHRainbow, either rooted cuttings or micropropagated plants, bore fruit lower on the tree trunk and one to three months earlier than trees produced from seeds. This resulted in earlier harvesting on shorter trees over



a longer time interval with greater yields. Rooted cuttings and micropropagated UHRainbow and Laie Gold hermaphrodites were provided to growers on Oahu and the Big Island to establish demonstration fields of about one acre. Laie Gold clones were randomly micropropagated seedlings or were micropropagated lateral shoots from individuals selected for exceptionally good fruit shape and fruit columns of young, nine-month-old trees. The clonally propagated plants, especially the rooted cuttings, were evaluated in several locations to select for widely adapted superior lines that would then be micropropagated. Growers were encouraged to observe their plants over time and to select superior lines to be micropropagated as well.

*M. Fitch (USDA/ARS), T. Leong, L. Akashi, S. Ferreira (University of Hawaii), N. Saito, G. Yamamoto, A. Yeh, S. White, P. Moore (USDA/ARS)*

## Fungal Resistance in Papaya

Fungi represent the second most important papaya pathogens after viruses. The diseases *Phytophthora* root and fruit rot, powdery mildew and anthracnose require fungicide application on a regular basis, sometimes every two weeks under severe disease pressure. Genes for fungal resistance or tolerance were acquired from various sources. Papaya plants were transformed with anti-fungal genes from rice, grapevine or dahlia and appeared to have increased tolerance to powdery mildew. Numbers of powdery mildew pustules were more numerous on control leaves from untransformed plants compared to leaves transformed with rice chitinase, stilbene synthase and defensin genes. New transgenic lines containing two different chitinase genes from the bacterium *Streptomyces* are currently being tested for the presence of the resistance transgenes.

*L. Akashi, J. Zhu, A. Dela Cruz, T. Leong, P. Moore (USDA/ARS), M. Fitch (USDA/ARS)*

## Molecular Cloning of the Papaya NPR1 Gene and Overexpression of Arabidopsis NPR1 in Tobacco

Systemic Acquired Resistance (SAR) has been recognized as a plant response to pathogen attack displayed by many plant species. The SAR pathway is activated after formation of a small necrotic lesion, either as a part of the hypersensitive response (HR) or as a disease symptom. SAR provides resistance against a broad range of pathogens and the response is observed throughout the whole plant. In the SAR pathway, the NPR1 gene plays an important role in the plant's perception of attack by a pathogen and in turning on defensive genes. In *Arabidopsis thaliana*, a plant species often used as a model for studying disease responses of plants, plant lines were created to overexpress the NPR1 gene. When these overexpressing NPR1 plant lines were challenged by a

bacterial or fungal pathogen, a 1.5 to 3.0 fold increase in PR1 (Pathogenesis-Related) messenger RNA level was observed. Increased resistance to bacterial and fungal attack was also observed in *Arabidopsis* plants overexpressing the NPR1 gene.

We have cloned 75% of the coding region of the NPR1 gene from papaya. After we finish cloning the entire coding region for the NPR1 gene, we will overexpress it in papaya under the control of the cauliflower mosaic virus 35S promoter gene which allows over expression of the gene throughout the plant. We can then generate transgenic papaya and test disease resistance levels. In addition, we have produced transgenic tobacco expressing the *Arabidopsis* NPR1 gene at high levels. Analysis of the pathogenesis-related-1a gene induced by treatment with salicylic acid could answer whether or not the *Arabidopsis* NPR1 gene can function in a different plant species such as papaya.

*X. Qiu (University of Hawaii), H. Albert (USDA/ARS)*

## Transformation with a Pathogen-Inducible Stilbene Synthase Gene for Increased Fungal Resistance in Papaya

Papaya, an important tropical food crop is susceptible to a variety of pathogens including fungi, bacteria and viruses that reduce yields and marketability of fruit. Simultaneous control of both papaya ringspot virus (PRV) and fungal diseases would decrease dependence on fungicides and significantly improve pre- and post-harvest fruit quality.

Phytoalexins have been shown to be important natural components in the defense of plants against fungal infection. Several fruit crops, including grapevine and peanut synthesize the stilbene-type phytoalexin, resveratrol, (trans-3,4',5-trihydroxy-stilbene) when attacked by pathogens. Under a research material transfer agreement, we have obtained a transformation construct that contains the *stilbene synthase* gene (*Vst1*) from grapevine under control of its own inducible promoter and a hygromycin-resistance gene under the control of a CaMV35S-promoter. The beauty of this construct is that stilbene biosynthesis specifically depends on the product of the stilbene synthase gene since the precursor molecules for the formation of hydroxy-stilbenes, malonyl-CoA and *p*-coumaroyl-CoA, are both commonly present in plants. The end product of the action of the enzyme on these precursors is stilbene, a natural compound present in several consumed fruits and vegetables, which should be acceptable in papaya fruit. Furthermore, using a gene with a pathogen-inducible promoter means that stilbene synthase will be expressed only at a low basal level in transgenic plants unless there is a pathogen attack. Following a transitory rise in expression, it is expected to again return to a low level when the pathogen fails to establish.



Pathogen inhibition experiments proved that stilbene inhibits papaya fungi *in vitro*. In these assays, 1.0 mM stilbene in V8 agar culture medium inhibited mycelial growth of *Phytophthora palmivora* up to 50% of control. The compound was shown to be active against *P. palmivora* at concentrations as low as 0.1 mM. Stilbene was not as effective against the anthracnose pathogen, *Colletotrichum gloeosporioides*, which was inhibited by only 10%.

Embryogenic tissues of Kamiya and Kapoho papaya cultivars were transformed with the stilbene synthase gene, along with the kanamycin or hygromycin resistance selectable marker gene. The method used was particle bombardment optimized for papaya embryogenic callus. Several independent transgenic lines were selected by survivals on kanamycin or hygromycin selection medium. Transformation was confirmed by PCR and by Southern blot analysis to confirm proper integration of the transgene. The transformed plants are being rooted and micropropagated for evaluation for fungal resistance in greenhouse and field. Several transgenic plants were produced.

Twenty plants from the Vst7 line were challenged with a root drench inoculation of zoospores of *P. palmivora* and subsequently observed for disease development. The transformed Vst 7 plants were significantly more resistant to *P. palmivora* root rot than the untransformed controls. Transgenic lines Vst7, Vst 8, Vst11, Vst12 and Vst14 have been planted in the Kunia substation for sexual crossing with PRSV-resistant transgenic UHSunUp to produce hybrids with dual resistance for both fungal and viral diseases.

Y. J. Zhu, C. S. Tang (University of Hawaii), M. Fitch (USDA/ARS), P. Moore (USDA/ARS)

## Chemical Induction of Broad Resistance in *Carica papaya* Against Fungal Diseases

Papaya, is susceptible to a root rot disease caused by a fungal pathogen, *Phytophthora palmivora*, which is currently controlled in the field by fungicide applications. This project, in collaboration with USDA/ARS and the University of Hawaii, tests the possibility of using a non-toxic chemical treatment to induce systemic acquired resistance (SAR) as an alternative control method for root rot disease. Several chemicals including salicylic acid (SA), 2, 6-dichloroisonicotinic acid (INA) and benzol (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) have been reported to induce SAR to a broad spectrum of diseases in both monocotyledonous and dicotyledonous plants such as tobacco, wheat and arabidopsis. Here we report that in *Carica papaya*, chemical treatments with SA and BTH can induce SAR against *Phytophthora palmivora*.

When papaya seedlings were pretreated with BTH and subsequently challenged by inoculating after one week with *Phytophthora* zoospores, the level of disease expressed depended on the concentration of BTH used in the pretreatment. Seedlings treated with 25 or 100  $\mu$ M BTH expressed very minor disease symptoms consisting of a little yellowing of the leaves. Disease development with these treatments was reduced to less than 20% of the levels exhibited by 1  $\mu$ M BTH or the water-treated controls. In the controls, the *Phytophthora* infection caused defoliation, stunted growth and wilting of the seedlings. Seedlings treated with 5 to 12.5  $\mu$ M BTH expressed an intermediate level of disease symptoms consisting of leaf yellowing. Ridomil® fungicide, the positive control, completely suppressed visible symptoms of *Phytophthora* disease.

The data show that BTH treatment one week prior to inoculation with *Phytophthora*, reduced root rot incidence and severity significantly in UHSunUp papaya cultivar. Although disease control was not as good as the Ridomil® fungicide treatment, root rot was significantly reduced by a single application of BTH. These results were similar to those of previous experiments and were consistent with results reported from other host-pathogen systems. BTH appears to induce a functional systemic acquired resistance response in papaya.

There was no significant effect of chemical treatment on either plant height or stem diameter of seedlings treated with up to 25- $\mu$ M BTH. Treatment with BTH 100  $\mu$ M or higher caused a transitory loss of chlorophyll in seedlings 2 weeks after application; however, 10 weeks after application, the seedlings were fully recovered and showed no significant effect (data not shown).

In July 1999, an experimental field that simulated the soil-borne phase of the *Phytophthora* root-rot pathogen was established at the University of Hawaii's Waimanalo Research Station. Papaya tree trunks were inoculated with agar plugs of a *P. palmivora* isolate by cutting wedges from the tree trunks, inserting an agar plug of the pathogen and subsequently burying the tree trunk in a furrow about 1.5 ft. deep. The area was irrigated for a four-month period, then the furrow was rotovated and a planting bed created. This was done to establish a planting bed with elevated soil chlamydospore inoculum of *P. palmivora*. This field will be used for root-rot experiments and to determine if BTH treatment for *Phytophthora* root-rot control in papaya is practical.

At the end of January, the first field experiment to assess the field efficacy of BTH for root-rot control was installed in the plant bed described above. BTH treatments were 50, 100 and 150  $\mu$ M with water and Ridomil (100 ppm soil drench) controls. Kamiya and Sunrise papaya seedlings were first established in the greenhouse,

treated with BTH and transplanted in the field a month later. Results of the trial are not yet complete, but observations suggest that BTH may offer some protection against *P. palmivora* root rot. We experienced some inconsistency between replications, indicating that soil inoculum levels will need to be augmented for future trials.

We also conducted studies to better understand the underlying molecular mechanism of BTH-induced resistance. The expression of PR-2 ( $\beta$ -1,3-glucanase) was characterized as well as the PR-3 (chitinase) genes associated with BTH treatment. BTH at 100  $\mu$ M was applied to the roots of four-week old papaya seedlings, and plants were assayed for enzyme activity 24 hours later. The results showed that the activity of both PR genes (glucanase and chitinase) increased 3-6 fold over water-treated, and pathogen-inoculated controls. As expected of SAR responses, enzyme activity was elevated in both the treated root tissues and in the leaf tissues, indicating that the BTH response was systemic.

Activities of the SAR-induced PR proteins,  $\beta$ -1,3-glucanases and chitinases, were followed from 1 h up to 20 days after seedlings were treated with 100- $\mu$ M BTH. Both PR-2 and PR-3 genes were induced within 1 h of BTH treatment and reached a maximum at 3 h and 6 h, respectively. The elevated activities lasted up to 10 days and then returned to basal levels.

Taken together, these results suggest that BTH treatment induced an SAR response in papaya and could explain the resistance response of papaya to *Phytophthora* root rot. The underlying mechanism of action may involve at least 2 different PR genes operating systemically in papaya.

Y. J. Zhu, S. Ferreira (University of Hawaii), P. Moore (USDA/ARS)

## Evaluation of Green-Fluorescent Protein as a Selectable Marker in Transformed Papaya

Concerns are often expressed about risks that may be involved in the use of antibiotic-resistance genes. The main concern is that the presence of antibiotic-resistance genes in transgenic plants might affect the therapeutic efficacy of antibiotics that are used in human medicine. The fear is that the gene might be transferred to pathogenic bacteria, which would then become resistant to the antibiotic and thus pose a danger to humans. This might occur either through humans eating transgenic plants in which the gene has been incorporated or through animals eating the plants and subsequently transferring the resistant bacteria to humans.

To address this problem, other markers are now used, such as beta-glucuronidase (GUS), luciferase (LUC), and mannose selections etc. These reporter systems have been instrumental in many studies but neither allows for convenient non-invasive *in vivo* analysis. Green fluorescent protein (GFP) isolated from *Aequorea victoria*, has been widely used in transgenic plants. Plants in which the gene has been incorporated can be easily detected when illuminated with an ultraviolet light. The intrinsic fluorescence of GFP allows for non-invasive analysis that can be monitored without the destruction of the biological sample. With the use of GFP in plants, fluorescent imaging microscopy can be used to track the expression and location of proteins and other microstructures within organisms as diverse as viruses, nematodes and fungi.

This project evaluates the effectiveness of GFP as a selectable marker in papaya. This will be especially important for research with papaya and the study of its fungal parasites. By using papaya plants that produce GFP in certain areas that are attacked by fungus, researchers will be able to find out exactly from which cells the fungus feeds and to follow up the fate of these cells throughout the life cycle of the parasite. By finding out how the fungus invades the plant, future researchers would be able to create new forms of resistance to the pathogen.

We have transformed GFP constructs into papaya embryogenic callus using a biolistic method. Two GFP constructs were used in this experiment. The first one is GFP plus antibiotic selection, NPTII, and the second one is GFP in pCambia vector without NPTII. Visual selection was carried out once a week starting from 15 days after bombardment. For the construct containing GFP and NPTII genes, the calli were split into two parts, with one selected on antibiotics G418 selection medium and the second selected based on the GFP expression. The transformation efficiency of two selection systems will be compared. Selected GFP calli will be regenerated into plantlets for PCR and Southern blot analysis to confirm the transgene integration.

Y. J. Zhu, R. Agbayani, H. Albert (USDA/ARS),

P. Moore (USDA/ARS)

## Field Residue Trials for Provado Registration in Papaya

GLP field residue trials for the EPA registration of Imidacloprid (Provado) on papaya were completed in November 1999 (HARC 1999 Annual Report, p.16). The Field Data Books were inspected in April 2000, and submitted to the study director at IR-4 Headquarters in August 2000. EPA registration is expected in 2002. H. Chen