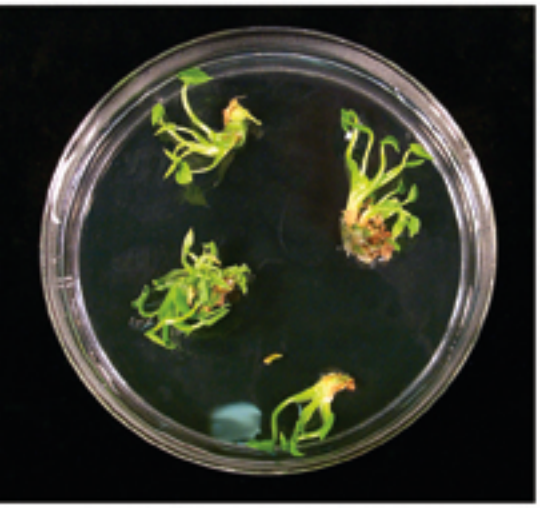

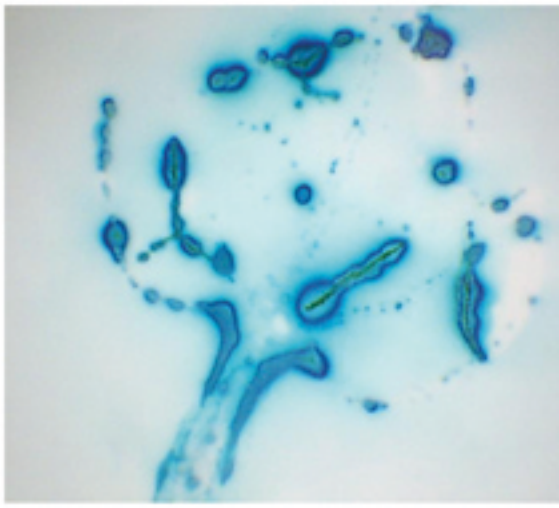

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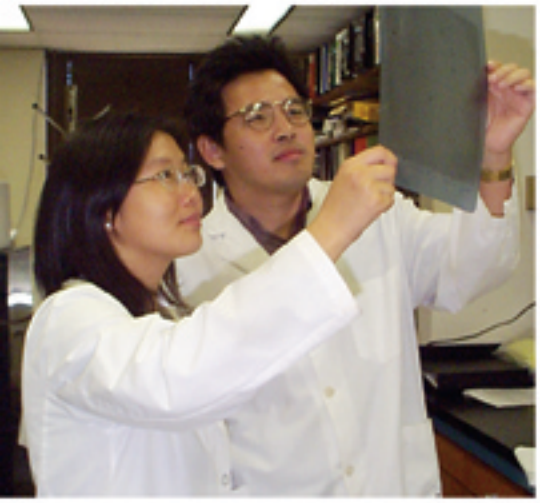

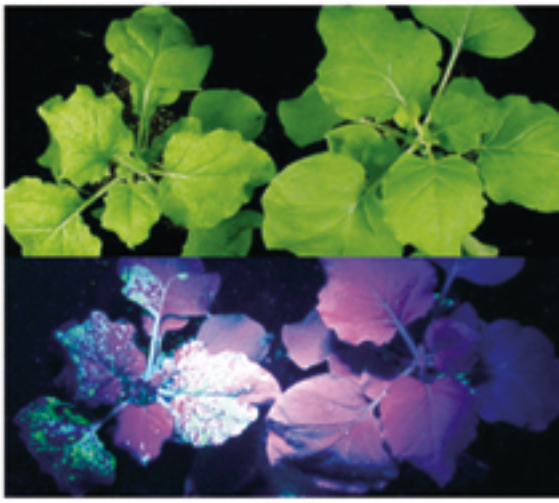

ANNUAL REPORT

On the Inside

Front cover images, clockwise, from top right corner:

(1) Leaf print of transgenic papaya, showing the expression of reporter protein
(2a) Tobacco plant inoculated with pPVX204 plasmid DNA under natural light at 9 DPI
(2b) Tobacco plant inoculated with pPVX204 plasmid DNA under UV at 9 DPI
(3) Apple snail eggs
(4) Papaya flowers
(5) Drs. Qingyi Yu and Ray Ming examining BAC Southern film
(6) Taro shoots

Background Image:

Cultivars of Hawaiian sugarcane (Saccharum spp.) - photo courtesy of Maui Nui Botanical Gardens, Kahului, Maui

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he year 2003 was the 109th year of continuous research and service performed by HARC for the agricultural sector in Hawaii. The sugarcane industry continues to lead Hawaii's agriculture in demonstrating its foresight in sponsoring research activities that contribute to its sustainability.

Harvested acres of 19,851 for 2003 produced 261,009 tons of raw sugar. The average tons sugar per acre was 13.15, a 3.79% increase from the 12.67 in 2002. By comparison, the average tons sugar per acre 10 years ago was 10.47 from the harvest of 64,705 acres. The 25.6% gain in yield for the industry reflects not only an increase in the r

Major efforts are focused on the molecular biology of papaya including the cloning and characterization of flower development, investigating systemic acquired resistance and disease and pest resistance through gene insertion, and discovering a primitive Y chromosome.

For coffee, a genetic map was constructed, transgenic nematode-resistant plants are being developed and improved quality is targeted both in the breeding and selection work as well as in shading effects.

A vascular wilt disease was identified as the cause of dieoff of koa trees. This could be of very significant concern as it has been found on all the islands and at many elevations. Our prior field trials with koa suggest that there is some resistance within the population. Before initiating future field trials, seedlings will be screened for resistance to support our goal to select the best seed sources for the expansion of this uniquely high-value Hawaiian crop.

The agronomic work in taro in the past years was followed this year with the first report of successfully inserting a useful gene into taro demonstrating that this technology can improve taro's resistance to diseases. Also in taro, a botanical extract was found to successfully control an extremely economically damaging pest, the apple snail.

Contract services have become a small but important part of our goal to develop new agricultural businesses or bring them to the state. We are proud that this year the potato certification business we have been building for 5 years was successful and passed on to farmers making room for us to develop and nurture another.

In this day and age when few people know where their food comes from, outreach is taken seriously by all the staff. They participate through assisting in the State Science Fair, the island farm fairs, visitor presentation and facility tours and schools and business meeting presentations. I am deeply appreciative of their volunteered time in this area and in their flexibility in the continuing transition both internally to this organization and externally in the agricultural community.

I also want to thank the sugarcane industry for its continuing support and commitment to research, to other commodity groups we are working with and to our clients on the mainland and other parts of the world.

Respectfully,

Stephanie A. Whalen
President and Director

100 Years Ago: 1903

The Hawaii Planters' Monthly reported that:
"By the end of 1903 we find that the station had increased its staff, which now included the director, Mr. Eckart, four chemists, Messrs. Peck, Werthmueller, Jordan, and Thompson, and with the field work still in the hands of Mr. Clarke. There had also been an addition to the laboratory buildings and the Experiment Station Committee for 1903 lament, 'It is unfortunate that the area of the Station grounds (Makiki) is so small, as the field experiments have to be restricted much more than is desirable, especially at such times when the necessity arises for fallowing portions of the land.'"

By 1903, the sugarcane leafhopper had spread to all the islands and caused such serious damage that the entire sugar industry was threatened with extinction. Parasites from Ohio were being propagated, but tangible results remained elusive.

Of the 55 plantations listed in the Plantation Directory (The Planters' Monthly, vol. XXII, 49 (Jan. 1903)), only Hawaiian Commercial & Sugar Co. and Gay & Robinson are still in operation.

Results of an irrigation experiment using salt water revealed that the yield of cane and sugar were reduced by half as the grains of salt per gallon was increased from 50 to 200. Other 1903 events included:
The 6-mile Waimea (Kikiaola) Ditch on Kauai and the Opaeula Ditch on Oahu were completed; the 12.5-mile Honokahau Ditch on Maui was under construction. These ditches were fundamental to irrigating sugarcane grown on the leeward sides of these islands.

On January 13th, 102 Koreans arrived in Hawaii to work at the Waialua Plantation. They were followed by nearly 500 more Koreans by that July who worked on Big Island, Kauai and Oahu plantations.

Fuel oil was first used for pumping at Kihei, Hawaiian Commercial & Sugar Co., Paia and Haiku plantations.

The first message was sent by telegraph cable from Hawaii to the mainland (San Francisco). This Pacific cable linked San Francisco, Hawaii, Guam and the Philippines.

Alexander Young Hotel opened in Honolulu on July 31.

The E. K. Fernandez Shows began entertaining the families of Hawaii with what was to become circus shows.

- Blake Vance

E. D. Tenny,
elected president of the Hawaiian Sugar Planters' Association on November 23

Sustainable Agriculture in Hawaii

S. Schenck

The Hawaiian sugarcane industry has a long history of sustainable agronomic, disease and pest control practices. As one of the first agriculture industries to adopt the practice of production and release of parasitoid insects to control sugarcane pest insects, it has not resorted to the use of insecticidal chemicals on Hawaiian plantations. Although numerous severe diseases have appeared over the years, control measures have followed Integrated Pest Management (IPM) practices by using good cultural practices, seed field disease surveys, breeding for resistance, quarantine regulations, and hot water seed treatment. Only a single, low toxicity fungicide, Tilt, is in use as a seed dip treatment to control Ceratocystis on planting material. Water use practices are carefully monitored and drip irrigation was installed to avoid overuse of water resources and to prevent runoff. Millwater from factory operations is recycled to fields. Mulching of fields helps cut down on weed problems, but herbicides are still necessary to a limited extent. Because of its sustainability, the Hawaiian sugarcane industry is still in business on the same fields after more than one hundred years.

The Hawaii Agriculture Research Center also supports sustainable agriculture for all Hawaiian crops. Small farmers growing many different local crops need to learn IPM and sustainable practices in order to reduce the use of pesticides and bare-ground fallow. HARC is engaged in several projects to attain this goal. Sustainable agriculture is more complex than conventional farming and requires greater levels of effort and skills. The challenge now is to demonstrate its potential profitability, advantages to the environment and to increase its use in Hawaiian agriculture. HARC has carried out several research projects designed to increase and improve upon Hawaii agriculture's sustainability.

Adaptation of asparagus production to Hawaii's environment. Asparagus was evaluated for its production sustainability and marketability in Hawaii (HARC Annual Report 1999). California cultivars proved to have the highest yields. The crop can be adapted to continual year-round production by cutting the water supply periodically to allow the ferns to die back and restart new spears production. In temperate climates the dormant period always occurs over the winter months, but in Hawaii it can be made to occur at any time of year. Thus farms can stagger production in field sections to supply the market all year. Fertilizer rates and disease and pest control can also be managed in a sustainable production system. After the plants have established a good root mass, there is no need to replant for at least 15 years. Since the project was installed, acreage in asparagus has increased.

Use of molasses amendments to overcome nematode damage to papaya. The benefits of amending soil with molasses were recognized years ago in the sugarcane industry. During the process of decomposition, molasses appeared to reduce damage to roots caused by root parasites. Finding new uses for this sugar production by-product as a soil amendment would benefit sustainability of other crops as well as the sugar industry. It was applied to papaya trees that had ceased to be productive because they were heavily infested with reniform nematodes (HARC Vegetable Report 3, Oct. 2001). The molasses was injected into the irrigation tubing at a dilution rate of 1:20 once a week for 12 weeks. Counts of nematodes decreased somewhat. Trees regained their green color, leaves began to grow again and trees began to produce marketable fruits. The effect was probably due to a number of soil factors other than nematodes. Molasses also had slight, but positive effects on Maui onions and Chinese cabbage.

Wood ear mushroom production in forest understory. Timber production is a possible sustainable agricultural industry in Hawaii. Koa in particular would help to preserve Hawaii's natural ecosystem while producing high quality timber products. For generating additional revenue while the trees mature, production of edible wood ears (pepeiao) mushrooms was investigated (HARC Annual Report 1997). A simple production method was devised that takes advantage of fallen or cut tree branches. The mushroom spawn can be produced in bags of bagasse, a sugarcane milling byproduct, and inoculated into pieces of wood using a hand drill. The inoculated wood pieces are left on the forest floor and the harvestable wood ear fruiting bodies start to appear within about three months. Harvesting can continue for three months. The wood ears are then simply dried and sold for cooking in soups and sauces.

Natural farming methods in vegetable production. A project was installed to demonstrate a farming system using crop rotation, cover crops and green manures to maintain soil fertility and texture and manage diseases and pests. It was designed to demonstrate the sustainability of this method under Hawaiian growing conditions and to inform farmers and gardeners of its practical value. The field site was located at Waialua High School on Oahu and was supervised by S. Schenck and N. Kawachi, a high school teacher with a background in agriculture. Most of the hands-on work was performed by the high school students. A crop rotation schedule was planned and carried out for the duration of the three-year project. The produce was sold by the students to local markets or used to supply high school agricultural field days and sales. The principles of soil nutrient conservation and replenishment with alternating crops and intercrop covercrops are applicable all or in part to various small farms and gardening situations and, as it was designed to do, this project informed students and others about these methods.

Liquid Compost Factor (LCF) to improve tomato plant vigor and increase tolerance to nematodes. Liquid Compost Factor is a product made by ABR, Maui from molasses, pineapple mill waste and other natural products in which an edible fungus is cultured. The resultant liquid acts to increase growth rate, vigor and plant tolerance to various stress factors. Since it is composed of edible, non-toxic substances, it is usable in organic farming production and any other farming or gardening practice where pesticides are not warranted or desired. We tested the product in several different tomato trials, arugula, and turfgrass. Further trials are continuing. In field plots of tomato that were heavily infested with root-knot nematodes there was an increase in fruit number production when LCF was applied as a soil drench pre- and post-planting.

Evaluation of Paecilomyces lilacinus as a biocontrol agent of nematodes in tomato and cucumber. The nematode parasitic soil fungus Paecilomyces lilacinus has shown promise in controlling nematodes on a number of vegetable and fruit crops and one strain is certified for commercial sale in Europe. We are currently testing this soil fungus applied as a soil drench on tomato and cucumber in field plots infested with root-knot and reniform nematodes.

Cover crops to reduce the practice of bare-ground fallow. Certain cover crops such as Sunn hemp (Crotalaria juncea), are known to reduce levels of parasitic nematodes, keep weeds under control, reduce erosion between crop cycles, and add nutrients to soil. Several projects have been undertaken to quantify these effects and develop IPM systems using cover crops. Management of root-knot and reniform nematodes in tomato using Sunn hemp was successful (HARC Annual Reports 1998 and 1999). Sunn hemp proved less effective in controlling nematodes in ginger, although a growth response was observed, probably due to increased soil nitrogen (HARC Annual Report 2000). During 2003, a large cover crop project was initiated in several Oahu farms to demonstrate to farmers the advantages of cover cropping as compared to the

practice of bare-ground fallowing. Bare-ground fallow on Oahu's North Shore has resulted in much soil run-off into the ocean and threatens the offshore ecosystem with silting. The cover crops in the trial include Sunn hemp, oats, lana vetch, winter wheat and barley. The response of farmers and their willingness to participate in the project has been encouraging. The project is scheduled to continue through 2004.

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Sugarcane Research

Developing Transgenic Sugarcane for ScYLV Resistance

Sugarcane yellowleaf syndrome is caused by a luteovirus, sugarcane yellowleaf virus (ScYLV). There are reports of yield losses due to ScYLV infection. Yellow leaf virus infections resulted in yield losses in Hawaii in the 1990s when whole fields turned yellow. Virus infection is still present and widespread in all susceptible cultivars and has been shown to be spread by certain common aphid species. Infection within the plant spreads first to the juvenile, growing plant parts and young leaves and is eventually found throughout the entire plant. The virus also spreads by planting infected seed pieces. In the currently selected Hawaiian commercial cultivars, ScYLV leaf yellowing symptoms usually appear under certain environmental stress conditions, especially cool temperatures and drought. However, preliminary tests in Hawaii and Louisiana indicated that the infection is harmful to the plants' performance even when the plants are symptomless. The reduction in growth rate, tillering and sucker formation appears to be greater in young plants. Since all plants of the susceptible cultivars in plantation fields contain the virus, no clear-cut comparison of infected and virus-free plants of the same cultivar on the same location has so far been possible.

This project will undertake to create ScYLV-resistant sugarcane through transformation. Gene silencing seems to be a universal mechanism for plant resistance to viral infection and posttranscriptional gene silencing has already been achieved in sugarcane by transformation with an untranslatable piece of Sorghum Mosaic Virus. A similar approach was attempted in this project with ScYLV. The ScYLV genome and the functions of the viral proteins are known (F. Moonan, J. Molina and T. E. Mirkov (2000) Virology 269:156-171). Therefore, the strategy for production of a ScYLV-resistant cane can be straightforward. A H62-4671 variety has been transformed with a non-translatable DNA sequence piece of the viral coat protein, which will lead to gene silencing of viral RNA upon infection. Sugarcane seems to have a very powerful gene silencing system, which promises success of the project.

About twelve independent transgenic lines derived from H62-4671 were produced and PCR analysis has been carried out to verify the presence of coat protein gene and selectable marker gene, NPTII. A virus challenge assay to test the resistance level has been carried out on the greenhouse-grown transgenic lines. About 30 plants were inoculated with aphids feeding on the virus-infected cane, H73-6110 and plants were left in the greenhouse until new leaves were developed. Tissue blot was used to detect the virus in the inoculated plants. We are in the process of data collection. Southern blot analysis to verify the genome copy and insertion is also in process.

– Y. J. Zhu, G. Osterman, C. Moritomo, H. McCafferty, R. Agabayani, A. Lehrer, S. Schenck and P. Moore (USDA/ARS)

Greg Osterman *is inoculating sugarcane with virus transmission vector aphids*

Mechanisms of Transgene Silencing

Gene silencing, including post-transcriptional gene silencing (PTGS), is now recognized as an essential gene regulation mechanism which exists in plants, animals and all higher organisms. This process is important for regulation of endogenous genes, and in plants, PTGS is an important defense mechanism against viral infection. Plants engineered for virus resistance use PTGS of a virus gene, inserted in the plant genome, to protect against that virus. Although there are many useful applications of PTGS, this process can create a major problem when trying to engineer plants for high level expression of a transgene. We are studying the mechanisms of PTGS with one goal being to control PTGS. We are taking two general approaches: one is to control the number and pattern of transgene integrations so as to avoid configurations like inverted repeats which can trigger PTGS. The other approach is to identify viral genes that act to suppress PTGS, and determine their mode of action.

In the first approach, we have used three methods. The first of these involves bombarding plant cells with linear DNA containing only the gene expression cassette(s), instead of using entire circular vector plasmids, as is done conventionally. This method has been used in rice to produce "predominantly single- or low" transgene copy lines. These low copy rice lines underwent transgene silencing at a much lower frequency than did lines produced by conventional bombardment. In sugarcane, we found this method did not produce a change in transgene copy number, with most lines still containing many copies. Even in those few lines recovered with low copy number, transgene expression was not enhanced.

The second method utilizes the Cre-lox recombination system to resolve multiple transgene copies to a single copy. This method did produce a high percentage of low copy lines; however, these lines did not show elevated gene expression.

The third method utilizes the Ac-Ds transposon system to insert transgenes by transposition. This method has been used in barley to produce single copy lines which underwent transgene silencing at a much lower frequency than did conventional multi-copy lines. This system can produce single copy insertions, but may also direct transgene insertions to actively transcribed regions of plant chromosomes, which would be an additional advantage. Over fifty transgenic sugarcane lines have been produced with this method. Transgene copy number and expression analysis of these plants is underway.

Our study of viral suppressors of PTGS is currently focused on the P0 gene of ScYLV. Transient expression experiments indicate this gene can suppress silencing in corn leaves. Experiments to determine which specific parts of the P0 gene product have the suppressor activity, and other experiments to determine at what step of the PTGS process P0 acts, are underway.

If either of these approaches to understanding PTGS leads to methods of controlling the process, this will provide a big boost to our efforts to use sugarcane as a biofactory to produce high-value proteins.

– T. Mangwende, M-L. Wang, S. Ancheta, J. Clayton, C. Goldstein, J. Carr (USDA/ARS) and H. Albert (USDA/ARS)

Evaluation of Genetic Diversity of Ustilago scitaminea Pathotypes

A new sugarcane smut pathovar that infected the previously resistant cultivar H78-7750 appeared in Hawaii in 2001. Field trials subsequently showed that many of the Hawaii sugarcane cultivars differed in susceptibility to the old and new Ustilago scitaminea isolates (HARC Annual Report 2001-2002). Molecular diversity of U. scitaminea and other fungal species was analyzed by amplified fragment length polymorphism (AFLP) markers. Thirty-seven U. scitaminea isolates and five isolates of other fungal species were fingerprinted with 310 AFLP markers.

Sugarcane smut fungal isolates were collected from Maui, Oahu and Kauai. In addition, U. hordei from barley, two isolates of U. maydis from corn, and two unrelated sugarcane pathogens were included in the trial. Fungal DNA was isolated by a method using EB buffer (0.1 M Tris, 0.05 M EDTA, 0.5 M NaCl, 1 g kg-1 NaHSO3, 1 g kg-1 sodium dodecyl sulfate, 2 g kg-1 CTAB) and agitation with glass beads. DNA digestion followed the protocol of Vos et al. (1995). Selective amplification was performed using labeled EcoR I and Pst I primers and followed the protocol of Qiagen (Valencia, CA). Eleven pairs of AFLP primers were used to fingerprint the genomic DNA. Polymorphic AFLP markers generated by each primer combination ranged from 21 to 27, producing an accumulated total of 310 markers.

The genetic similarity of the 37 U. scitaminea samples ranged from 0.85 to 1.0 and averaged 0.95. This suggested little genetic variation among the Hawaiian isolates or between the old and new pathotypes. However, there were dramatic differences in genomic composition between the three Ustilago species. Genomic DNA sequences are constantly evolving as shown by the polymorphisms of AFLP markers detected among isolates. The manner of fungal transmission by airborne teliospores, the ease of cross-fusing between haploid sporidia from different isolates, and the apparent mutation and recombination during dikaryon formation allow for continual change and recombination within the relatively small and isolated population of U. scitaminea in Hawaii. This may account for the appearance of new pathotypes and for their subsequent disappearance through hybridization.

A slight genetic mutation that is not detectable by AFLP analysis can result in U. scitaminea overcoming host resistance. Nonetheless, selection for resistance in the breeding program is still necessary in order to avoid releasing highly susceptible cultivars. But hot water treatment of planting material should not be neglected, even for resistant cultivars, because of the possibility of the appearance of new pathogen strains.

- S. Schenck, H. M. Pearl, Z. Liu, P. H. Moore (USDA/ARS) and R. Ming

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Production of a Biologically Active Pharmaceutical Protein in Sugarcane and Rice

Previously, we reported the production of transgenic sugarcane and rice plants expressing a high-value pharmaceutical protein, granulocyte macrophage-colony stimulating factor (GM-CSF, HARC Annual Report 2001-2002). In field trials, some sugarcane lines produced up to 0.03% of the total soluble protein (tsp) as GM-CSF. The transgenic lines differed for gene promoter, sub-cellular localization signals, and presence/absence of a peptide tag for affinity purification. All of the lines in the highest expression group (>0.01% tsp) contained expression constructs directing the protein to remain in the endoplasmic reticulum (ER). Protein processing and folding is carried on in the ER; these experiments show that extended time in the ER is essential for stable production of GM-CSF in sugarcane.

In July of 2002, approximately 50 selected transgenic sugarcane plants were transplanted to an experimental field at the Kunia substation for evaluation. The expression levels remained stable for at least 10 months after transplanting. We also observed relatively uniform expression throughout the plants. Within a single plant, small variations (less than 3X) were detected between leaves and internodes of different ages. Furthermore, addition of a costly cocktail of proteinase inhibitors and antioxidants was found to be essential for preventing degradation of GM-CSF when extracting the protein from sugarcane plants.

The GM-CSF produced in the sugarcane and rice showed native biological activity in a human cell proliferation assay. Extracts from sugarcane calli, sugarcane leaves, and rice grains stimulated the division of bone marrow (TF-1) cells in a manner essentially identical to purified, commercially produced GM-CSF, indicating the plant-produced GM-CSF had close to 100% native biological activity. In GM-CSF extracted from rice grains which had been stored at room temperature for over 12 months, concentrations remained similar to freshly harvested grain, and activity remained at the same high level, indicating that the GM-CSF is very stable when expressed in rice seeds. This stability provides storage and shipping options which would not be available for a protein expressed throughout the plant body.

Developing a crop plant as a biofactory to produce high-value proteins involves two major steps: engineering the plant to produce the desired protein with the correct folding and processing so the protein has biological activity, and engineering the plant to produce the protein at a level high enough to make extraction and purification economically feasible. The first of these hurdles has now been cleared for both sugarcane and rice. Current efforts are focused on achieving higher levels of accumulation for our model protein.

– M-L. Wang, S. Ancheta, J. Clayton, C. Goldstein and H. Albert (USDA/ARS)

Breeding and Selection

Sugarcane breeding season began on November 21, 2002 and was completed in the first week of January, 2003. Six biparental crosses were made between LCP85-384 as the sole pollen donor for six Hawaiian commercial cultivars. Polycrosses (732) were made from 2,500 tassels of 346 breeding clones. In 2003, we evaluated 464 seedling clones harvested from 23 yield tests (FT7s), of which 22% were selected for further yield trails. During the year, 23 FT7 tests were installed from seedling clones selected in and before 2002.

50,544 seedlings raised from true sugarcane seeds were transplanted in FT1 trial; 53,520 FT1 plants planted in 2002 were ratooned; 2,532 clones were planted in FT4; and 577 selected clones from FT4 (installed in 2002) will be advanced to FT5 in 2004. The top 4 commercial varieties ranked by occupied acreage at the end of 2003 were H78-7750, H77-4643, H65-7052 and H78-3567.

H78-7750 continued to be the leading variety for the fourth year. It occupied a total of 21,964 acres or 48.3% of total cane area. H77-4643, ranked number two since 2001, occupied 9,377 acres or 20.6% of total cane area. It has been the leading variety on Kauai since 1996. H65-7052 moved up to the third ranked variety, this year occupying 6,955 acres or 15.3% of total cane area. H78-3567 declined in acreage to the fourth position. It occupied 4,277 acres or about 9.4% of the sugarcane area.

New clones with commercial potential are H91-4392, H93-4068 and H93-4398 for Makaweli soil on Kauai; H90-5555, H90-7453 and H95-5783 for rocky and dry areas on Maui; H88-6401, H95-0181 and H95-1446 for sandy areas on Maui; H86-3792, H87-4394 and H90-0598 for windward Maui. H83-7061 and H87-5794 had the top FT7 records in the leeward region and H87-4319 is the best clone for mill water irrigated fields.

Sugarcane breeding continues to be one of HARC's most important activities. Changing plantation practices and new industry prerogatives make the development of new cultivars a critical concern. Changing weather patterns and the appearance of new pathogen strains require the plantations to have a number of different cultivars on hand. The Genetics and Pathology Department has always been able to take advantage of its international connections with other sugarcane breeders to import new breeding stock and to send Hawaiian clones to other locations for disease resistance screening for diseases not present in Hawaii.

– K. K. Wu

Tropical Fruit Research

Genetic Transformation of Pineapple with Nematode Resistance and Flowering Control

Nematode damage and precocious flowering are two serious problems faced by the Hawaii pineapple industry. Since pineapple does not have natural resistance to nematodes, the growers have been relying on nematicides to reduce damage. Besides adding to the production cost, nematicides may not be available in the future due to environmental concerns. Spontaneous flowering of pineapple causes the fruit to ripen outside of the normal harvest period. This also adds to the production cost for the growers. In collaboration with the pineapple industry, University of Hawaii, and Leeds University (UK), a project is in progress to genetically engineer pineapple plants for nematode resistance and flowering control. Our strategy is to use a rice gene encoding a proteinase inhibitor, cystatin, to combat the nematodes. The anti-sense version of a pineapple gene encoding aminocyclopropane-1-carboxylic acid (ACC) synthase, a key enzyme in ethylene biosynthesis, is used to suppress the spontaneous flowering.

In early 2003, we established a fast and reliable method for screening the putative plants for the presence of transgene using polymerase chain reaction (PCR). However, we did not find any positive plants from the transformations performed before 2003. Since then, we adopted an improved plant selection procedure developed by Eden Perez (UH), in which transformed tissues were isolated by repeated cutting and regeneration of the plant materials with increasing levels of selection. A transgenic line (#51) carrying the anti-sense ACC synthase gene for flowering control was obtained using this method. Line 51 repeatedly tested positive using PCR and produced roots rapidly in media with a high level of selection (25 mg L-1 of hygromycin). Approximately 500 plantlets of Line 51 and 200 control plants are at the rooting stage in preparation for field tests.

From August to December 2003, we have transformed 9400 leaf bases using Agrobacterium carrying either pGU13 or pKLD121. Cystatin gene is under the control of a constitutive promoter in pGU13 or a pineapple root-specific promoter in pKLD121. We also infected 5400 leaf bases with Agrobacterium carrying pGU6, containing anti-sense ACC synthase under the control of a constitutive promoter. We are in the process of subjecting the plantlets regenerated from these transformations to the new selection procedure.

– M-L Wang, G. Uruu (UH), R. Paull (UH), J. Buenafe and C. Nagai

Discovery of a Primitive Y Chromosome in Papaya

Papaya, a polygamous angiosperm with male, female, and hermaphrodite forms, offers several advantages for genetic and evolutionary studies. These include a small genome, a short generation time, numerous flower types, a unique evolutionary process in female flowers, an intriguing sex determination system, and an established transformation system. Papaya sex determination has been a frequent subject of genetic analyses because it is directly related to efficient commercial fruit production.

Mechanisms of sex determination are diverse and have evolved independently in many groups of animals and plants. The most familiar system involves the structurally dis-

tinct (heteromorphic) sex chromosomes (X and Y, or Z and W) that are homozygous in one sex and heterozygous in the other. Male heterogamety has evolved more often than female heterogamety. Heteromorphic sex chromosomes are believed to have evolved from a homologous pair of autosomes. However, empirical data to support this hypothesis has been lacking. Sex chromosome evolution is associated with suppression of recombination by chromosomal rearrangement around the sex determination gene, and subsequent degeneration of the Y chromosome. In the most advanced systems, the sex chromosomes are morphologically distinctive, and there is no genetic exchange between them for part or all of their length. In humans, 95% of the Y chromosome is suppressed for recombination.

High-density genetic mapping, fine mapping, and physical mapping of the sex determination gene led to the discovery of a papaya primitive Y chromosome with a small male-specific region, the MSY, that is about 10% of the chromosome showing severe suppression of recombination and degeneration of DNA sequences. The MSY consists of a mosaic of conserved, X-degenerated, and ampliconic sequences. High frequencies of sequence duplications and transposable element insertions contributed to the degeneration of the MSY resulting in low-gene density. One unique feature of the incipient Y chromosome in papaya is the small physical size of the MSY region. The small size and the mosaic structure of sequence degradation in the MSY region suggest a recent origin of the papaya sex chromosomes. Hermaphrodite and male plants of papaya share identical DNA sequences in most parts of the MSY region, suggesting that divergence between male and hermaphrodite is a second step of sex chromosome evolution after recessive mutations resulted in producing the female sex. This finding provides direct evidence for the origin of sex chromosomes from autosomes.

Papaya male flowers

– Z. Liu, P. H. Moore (USDA/ARS), H. Ma, C. M. Ackerman, M. Ragiba, Q. Yu, H. M. Pearl, M. S. Kim, J. W. Charlton, J. I. Stiles (UH), F. T. Zee (USDA/ARS), A. H. Paterson (Univ. GA) and R. Ming

High-Density Linkage Mapping Revealed Suppression of Recombination at the Sex Determination Locus in Papaya

Papaya (Carica papaya L.) is a fruit crop cultivated worldwide in tropical and subtropical regions. It is believed to be native to tropical America where it has undergone a long period of selection. Papaya is polygamous with three basic sex types: female, male, and hermaphrodite. Hermaphrodite trees produce a pyriform-shaped fruit that is preferred in the market. However, seeds from hermaphrodite trees always segregate into hermaphrodites and females at the ratio of 2:1 and the sex types of the plants can be determined only by inspection of the flowers. Therefore, it is a general practice for farmers to plant three to five seedlings in one hill, allowing them to grow for four to six months until the sex types are identified, followed by removal

of the undesired plants leaving only hermaphrodite plants. Genetic mapping is the first step towards cloning the sex determination gene and ultimately to development of true hermaphrodite papaya varieties.

A high-density genetic map of papaya was constructed using 54 F2 plants derived from cultivars Kapoho and SunUp with 1501 markers, including 1498 amplified fragment length polymorphism (AFLP) markers, the papaya ringspot virus coat protein marker, morphological sex type, and fruit flesh color. These markers were mapped into 12 linkage groups covering a total length of 3294.2 cM, with an average distance of 2.2 cM between adjacent markers. This map revealed severe suppression of recombination around the sex determination locus with a total of 225 markers co-segregating with sex types. This high-density genetic map is essential for cloning of specific genes of interest such as the sex determination gene and for the integration of genetic and physical maps of papaya.

Segregating papaya F2 population used for genetic mapping

– H. Ma, P. H. Moore (USDA/ARS), Z. Liu, M. S. Kim, Q. Yu, M. M. M. Fitch (USDA/ARS), T. Sekioka (UH), A. H. Paterson (Univ. GA) and R. Ming

Cloning and Characterization of Flower Development Genes in Papaya

Instability of papaya flowers, revealed by environmentally influenced sex reversal and stamen carpellody, results in fruit malformation making it unmarketable. Hermaphrodite flowers may change toward maleness by carpel abortion or toward femaleness by stamen carpellody, (i.e., transforming stamens into carpels), whereas male and female flowers may revert toward hermaphrodism. Incomplete sex reversal results in a continuous graded series of flower types. For example, under natural conditions in Hawaii, hermaphrodite flowers can have from 0 to 10 stamens and 1 to 10 carpels, whereas the true female flowers have 0 stamens with 5 vascular traces and male flowers have 10 stamens and an aborted pistil. Although female flowers are fairly stable, stamens in female flowers were observed by Hofmeyr in 1939 and recently by Manshardt. Sex reversal in papaya flowers is controlled by genetic and environmental factors, including temperature, nutritional status and moisture.

Papaya is a good model system to study flower development in perfect flowers and dioecious flowers. Based on knowledge of flower development in the model plants Antirrhirum and Arabidopsis, we are cloning and characterizing homologous genes associated with carpel development in papaya. The Arabidopsis class C organ identity gene AGAMOUS has a papaya homolog named PAG that shares 85% identity with Arabidopsis AGAMOUS. Genomic Southern analysis showed that papaya has only one

copy of PAG that is expressed at a high level in carpels. The Arabidopsis gene LFY, a positive regulator of AGAMOUS, has a papaya homolog PFL that shares 65% identity with LFY. PFL encodes a protein sharing 71% identity with the LFY homologs of the tree species California sycamore (Platanus racemosa) and black cottonwood (Populus trichocarpal). Despite extensive sequence similarity in two conserved regions, the proline-rich and acidic motifs differ between PFL and its LFY counterparts from other plants. This difference may not affect the gene function as demonstrated by research on the Pinus radiata LFY homolog Needly. Genomic and BAC Southern analyses indicate that like PAG, PFL exists as a single copy in the papaya genome. In situ hybridization result showed that PFL is expressed at a relatively low level in the shoot apical meristem of very young seedlings but it is expressed at a high level in the floral meristem. Hua1, another regulator of stamen and carpel identities, has a papaya homolog named Phua1 that shares about 82% identity with the Arabidopsis Hua1 gene. Understanding the papaya floral development process could lead to strategies for controlling these problems of fruit production.

Papaya male flowers

– Q. Yu, P. H. Moore (USDA/ARS), C. Ackerman, H. H. Albert (USDA/ARS), R. E. Paull (UH) and R. Ming

Systemic Acquired Resistance in Papaya

Plants have natural defense responses against pathogens which can be induced by avirulent pathogens. An attack by one avirulent pathogen at one point on the plant can trigger enhanced resistance against a broad spectrum of pathogens throughout the plant body; this response is termed systemic acquired resistance (SAR). SAR has been studied extensively in model plant systems like arabidopsis, and it has been shown that SAR can be induced by application of salicylic acid or structurally similar chemicals like benzothiadiazole (BTH).

We have initiated a study of SAR in papaya to build the base for development of improved papaya cultivars or protective treatments. Root drench of papaya seedlings with BTH resulted in increased tolerance to the virulent pathogen Phytophthora palmivora, increased ß-1,3-glucanase and chitinase activities (both are defense related enzymes), and increased accumulation of a PR1 (a gene widely used as a marker for SAR) mRNA. All these indicate that papaya has a SAR response which can be induced by BTH, making this chemical a valuable research tool and possible future field treatment.

Four members of the papaya PR-1 gene family were cloned. BTH reduced mRNA accumulation for two of them and increased it in the other two. One of these, PR-1d, was induced over twentyfold higher

than its normal level. Accumulation of the mRNA for this gene increased for at least 14 days after BTH treatment. In contrast, both chitinase and ß-1,3-glucanase activities peaked after 1 to 2 days then returned to base levels at approx. 10 days.

The arabidopsis NPR1 gene plays an essential regulatory role in SAR. An NPR1 gene was cloned from papaya; it contains structural domains similar to the arabidopsis gene. These domains are involved in protein-protein interactions and nuclear localization, which are essential for function in SAR of arabidopsis. The papaya NPR1 gene is expressed constitutively and is slightly induced by BTH treatment. Overall, these findings indicate that the basic elements of papaya SAR resemble the pathway as described in arabidopsis so the knowledge and resources available for this model system can be widely applied in our study of papaya.

- Y. J. Zhu, X. Qiu (UH), M-L. Wang, P. Moore (USDA/ARS) and H. Albert (USDA/ARS)

Improved Disease Resistance in Transgenic Papaya with a Grapevine Vst1 Gene

Papaya, one of the most important fruit crops in the tropics, is susceptible to a variety of pathogens including fungi, bacteria and viruses (Nishijima, 1994) 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 to increase productivity.

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 from Bayer AG 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 (Hain et al., 1993). 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, the expression is expected to return to a low level when the pathogen fails to establish.

A stilbene synthase gene (Vst1) has been isolated from grapevine (Vitis vinifera L.) and transformed into a range of species to increase resistance of host to pathogens to which they were originally susceptible. Since resveratrol at 1.0 mM inhibited in vitro mycelium growth of P. palmivora, we hypothesized that papaya resistance to this pathogen might be increased by transformation with the grapevine stilbene synthase construct pVst1, containing the Vst1 gene and its pathogen inducible promoter. Multiple transformed lines were produced, clonally propagated, and evaluated with a leaf-disk bioassay and whole plant response to inoculation with P. palmivora. We found RNA transcripts of stilbene synthase were induced in plant lines transformed with the grapevine pVst1 construct shortly after pathogen inoculation and that the transformed papaya lines exhibited increased resistance to P. palmivora. Five

independent transgenic lines, Vst7, Vst8, Vst11, Vst12 and Vst14, along with non-transformed control plants were selected for leaf-disk assay to assess tolerance of plants to P. palmivora. Twenty microliters of spore suspension (1 x 106 spores mL-1) were pipetted onto the center of leaf-disks cultured on MS medium in the Petri plate. Plates were placed in the growth chamber maintained at 24 oC with 12 h light and 100% relative humidity. Water soaked spots were observed on the non-transformed controls within 24 hrs; necrotic lesions appeared later and proceeded to expand. Necrotic lesions were observed on all treated leaves except those inoculated with a water extract from culture medium without the fungus. Diameters of lesions measured 3 days after inoculation were significantly smaller ($P<0.05$) in all five Vst1-transformed lines than in the non-transformed controls. On the average, the lesions of the transgenic plants were reduced about 25 to 30% in diameter and about 40 to 50% in infection area. The immature transformed plants appeared normal and will be advanced to field trials to evaluate their utility.

– Y. J. Zhu, R. Agabayani, M. Jackson, C. S. Tang (UH) and P. Moore (USDA/ARS)

Phosphomannose-Isomerase as an Efficient Selectable Marker in Genetically Engineered Papaya

In traditional selection systems, the most widely used selectable markers are the antibiotic resistance genes such as neomycin phosphotransferase gene. Although proven successful for the production of transgenic plants, the presence of such genes may be undesirable. Because of this, the use of mannose, which cannot be metabolized by many plant species, was developed as a new selection strategy.

In this study, mannose was used as a selectable agent and the phosphomannose isomerase (PMI) gene as the selectable marker. Transformed cells are able to utilize mannose as a carbon source and grow in the absence of other carbon sources such as glucose and sucrose. Cells genetically transformed to express PMI acquire a growth advantage (positive selection) on mannose-containing media, which makes mannose a useful selection agent for the generation of transgenic plants.

Transgenic shoot regenerated from Mannose-resistance calli

Transgenic plant about 4 weeks after transplanting

We evaluated the use of PMI as a selectable marker for the recovery of transgenic papaya plants following biolistic bombardment of papaya embryogenic callus. In this study, we concluded that papaya embryogenic callus cannot utilize mannose as the sole carbon source and PMI can be used as a selectable marker for transformation of papaya. Transformation efficiency was higher than with an antibiotic selectable marker. Transformed papaya embryogenic calli with PMI can utilize mannose as efficiently as sucrose. A new technology was developed using alternative selectable marker genes for genetic engineering which should improve consumer acceptance of "genetically modified" agricultural products.

– R. Agbayani, H. Albert (USDA/ARS), P. Moore (USDA/ARS) and Y. J. Zhu

Engineering Papaya for Improved Pest Resistance

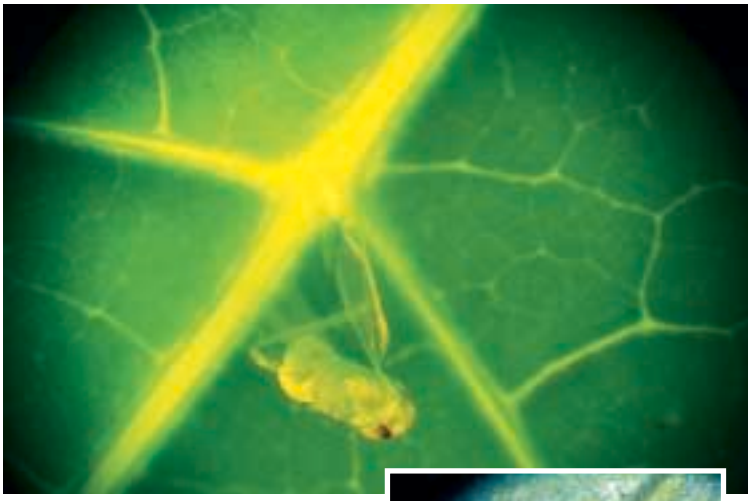
The productivity of papaya (Carica papaya L.) is often limited by its susceptibility to a number of natural enemies. Papaya pests include nematodes, aphids, leafhoppers and mites.

The aim of this study is to generate papaya plants with improved pest resistance. For plant transformation, a biolistic gene gun procedure was used. A gene encoding a protein with known entomotoxic properties was introduced into a commercial papaya cultivar. The protein selected was a lectin, termed GNA, from the snowdrop lily, Galanthus nivalis.

Lectins are naturally-occurring proteins which possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide. They are ubiquitous not only in plants but also in animals and microorganisms. In animals and microorganisms, their main function is cell recognition. In plants, their role is still something of an enigma. Some have been shown to be toxic to insects. Feeding studies have shown that the GNA protein has an insecticidal nature but is non-toxic to higher animals.

It is hoped that papaya plants expressing GNA may have improved resistance to defoliating pests. Also, plants may have some resistance to nematodes which can be detrimental to the growth and development of papaya. GNA was recently shown to have potential as an anti root-knot nematode protein when expressed in Arabidopsis thaliana plants.

We have generated a number of independent papaya lines expressing the GNA protein. Lines were first identified by molecular analysis. Protein expression was then examined. It has been determined that the recombinant GNA protein is biologically active. Future experiments will investigate the resistance of the transgenic papaya plants to pest attack.



Two common pests of papaya:
Above: Stevens leafhopper (Empoasca stevensii)

Right: Carmine spider mite (Tetranychus cinnabarinus)

– H. McCafferty, P. Moore (USDA/ARS) and Y. J. Zhu

Coffee Research

Cloning of Arabica Coffee

Arabica coffee is traditionally seed propagated, since the majority of arabica cultivars are inbred and growers can easily obtain enough seedlings of their cultivars. However, hybrid arabica coffee cultivars need to be cloned (vegetatively propagated) in order to preserve their superior traits for commercial cultivation. A protocol was developed at HARC (HARC Annual Report 1997, p. 22) to vegetatively propagate arabica coffee by rooted cuttings. Efficiency of rooting was 28% and varied among cultivars tested. The protocol is useful only for small-scale propagation. Cloning via somatic embryos is the method of choice for production of large quantities of propagules (HSPA Annual Report 1991, p. 51-52). Induction of coffee somatic embryos takes 6-8 months from initiation.

Experiments were conducted for in vitro propagation of arabica coffee using in vitro shoot multiplication for an alternative to cuttings and somatic embryo derived propagules. Vertical shoots of matured catuai and typica trees were used as explants. Surface sterilization of shoot tips was optimized using various combinations of sodium hypochlorite concentrations (0.6-1.8%) and treatment durations (10-30 min). Fifty percent of shoots treated with 1.2% hypochlorite for 20 min kept tips green during the first 28 days of culture, whereas no significant difference was found among treatments to establish clean shoot cultures. Effects of sucrose (0-40%), cytokinins including BA (6-benzylaminopurine, 0-10 mg L-1), kinetin (1.5 mg L-1) and 2iP (2.5 mg L-1) were also tested on new shoot growth in culture. Addition of NAA (0.02 mg L-1) did not help increase initial shoot tip growth. In 43 days, we obtained 18 in vitro propagules (37.5%). All the propagules had 1-2 shoots. We were not successful at root induction from these shoots using growth regulators such as IBA (indole-3-butyric acid). These preliminary results indicated that in vitro shoot tip culture is not as efficient a propagation method as cloning via somatic embryos.

– L. Fournier (E.S.I.T.P.A. Rouen, France), J. Clayton and C. Nagai

Progress of Coffee Breeding and Selection Program

Our coffee breeding and selection program, to develop a uniquely Hawaiian coffee, is in its 7th year and over 100 hybrid families have been produced. The program's objectives are to produce coffee cultivars adapted to Hawaii's specific growing environments with desirable characteristics, such as, enhanced flavor, excellent cupping quality, large bean size, increased yield and disease resistance.

In 1999, a large scale coffee crossing program was undertaken (HARC Annual Report 1999, p. 19) using potentially elite trees with desirable characteristics selected from five coffee growing areas in Hawaii. By mid-2000, approximately 1500 progeny trees from 165 crosses were under careful field cultivation at Kunia (HARC Annual Report 2000, p. 21). Tree morphology, yield, cherry/bean characteristics and cupping quality of the original selected trees were evaluated by the fall of 2001 and fruit (cherry)/seed (bean) characteristics were evaluated in the fall of 2002.

Twelve superior hybrid families were selected from the original 120 crosses made in 1999. Selection was based on tree morphology

and height, cherry size and yield potential. These promising coffees included larger-bean Mokka hybrids and higher yielding Margogipe (average taste, large-bean, low-yield) hybrids. In December 2003, a cupping test of 2 new hybrids of Margogipe (H99-34: Yellow Catuai x Margogipe, H99-36: MA1-12-mokka x Margogipe) was conducted at Cathy Cavaletto's laboratory at UH. H97-1700 (KA17C-Yellow Catuai) was used as a control. A seven-judge panel found no significant difference in cupping including dry and wet aroma, acidity, flavor and body. This preliminary cupping test indicated that size and yield of coffee were modified without changing cupping quality. Seeds of these trees were grown in the HARC Maunawili greenhouse and then shipped to Kauai in October for field evaluation at Kauai Coffee Co. The first field evaluation at a commercial coffee field will be initiated in early 2004.

Coffee hybrid families (H99- series) at Kunia Substation

- C. Nagai, R. V. Osgood, J. Clayton, C. Cavaletto (UH) and S. Bittenbender (UH)

Genetic Transformation of Coffee for Nematode Resistance Using Cysteine and Serine Proteinase Inhibitors

Root-knot nematodes, Meloidogyne spp., adversely affect coffee production in many coffee-growing regions. Meloidogyne konaensis causes severe damage to Coffea arabica cv. Typica ('Guatemala') grown on the Island of Hawaii. A weakened root system and overall decline of the tree occur when a nematode infestation is severe. Grafting Typica on C. liberica rootstocks is currently providing growers with partial resistance to M. konaensis. In an effort to increase resistance levels, genetic engineering a Typica rootstock is being explored.

Cystatin, a cysteine proteinase inhibitor from rice, and a dual construct, cystatin with a cowpea trypsin inhibitor were chosen for their success in reducing nematode levels in other crops. The cystatin gene was driven by the CaMV35S promoter or tubulin (root specific) promoter with the NPTII gene for selection. The dual construct was driven by the 35S promoter (HARC Annual Report 2001-2002, p. 26-27). Transformation was performed using both Agrobacterium tumefaciens and particle bombardment.

Plants were produced from selected somatic embryos (SE) in culture media with an antibiotic, G418. Plants were regenerated from 29 of the 1,100 selected somatic embryo lines inoculated with A. tumefaciens. Fifteen plant lines were produced from the 1,200 selected SE lines using the particle bombardment method.

PCR analysis confirmed the presence of the cystatin gene in 12 lines of plants (Figure). Multiple plants were obtained in several SE lines. Eleven PCR-positive plants were obtained from the SE line B17-3 using two different primer sets. These multiple plants will be used for the nematode bioassay in the University of Hawaii greenhouse next year. Further molecular analyses, Western and Southern blottings will also be performed.

– R. Myers-Cabos (UH), C. Nagai, B. Sipes (UH), D. Schmitt (UH), H. Atkinson (Leeds Univ.)

Transgenic coffee with nematode resistance genes, cystatin and trypsin inhibitors

Construction of a Genetic Map for Arabica Coffee

The coffee breeding and selection program initiated in 1997 has as its objective the development of new cultivars adapted to Hawaiian growing conditions and having desirable characteristics. It is made more difficult by the limited genetic diversity detected among arabica coffee cultivars. This lack of genetic diversity in the gene pool of arabica coffee limits the potential for germplasm improvement. The occasionally occurring spontaneous interspecific hybrids have been widely used for improving disease and pest resistance in arabica coffee. One extensively used source is the Timor hybrid (2n = 44), discovered on the island of Timor, that was derived from a spontaneous interspecific cross between C. arabica and C. canephora. One example of the use of the Timor hybrid in coffee breeding programs is the development of the coffee leaf rust-resistant Catimor cultivar. This was derived from a cross between the Caturra cultivar and the Timor hybrid. Another spontaneous interspecific hybridization between C. arabica and C. liberica increased the genetic diversity among C. liberica introgressed lines and was used as the main source of rust resistance in coffee breeding programs in India.

Molecular marker linkage maps are being used successfully in many crop species for directed germplasm improvement. Marker assisted selection allows for screening of large numbers of trees for a gene of interest at an early stage of growth and reduces the number of backcrosses required to obtain quality traits. As reported in the HARC 2001-2002 Annual Report, we used amplified fragment length polymorphisms (AFLPs) to construct a genetic linkage map on a pseudo F2 population of arabica coffee (Coffea arabica L.) derived from a cross between the cultivars Mokka hybrid and Catimor. Sixty trees from this population were selected on the basis of plant height distribution to construct a linkage map. A total of 456 dominant markers and eight co-dominant markers were generated from 288 AFLP primer combinations.

The results to date determined that, of the total number of markers generated, 68% were from Catimor, 30% from Mokka hybrid

and 2% co-dominant. This distribution suggests that the heterozygosity within the Catimor sub-genomes was twice that within the Mokka hybrid sub-genomes. Linkage groups were constructed resulting in 16 major linkage groups containing 4 to 21 markers, and 15 small linkage groups consisting of 2 to 3 linked markers each. The total length of the map was 1802.8 cM with an average distance of 10.2 cM between adjacent markers. This genetic map will serve as the framework for mapping quantitative trait loci (QTL) controlling source-sink traits in the same population.

Jamie Clayton and Heather Pearl collecting leaf samples in coffee field.

– H. M. Pearl, C. Nagai, P. H. Moore (USDA/ARS), D. L. Steiger, R. V. Osgood and R. Ming

Quality Aspects of Shade-Grown Coffee

Traditionally, coffee was grown as a shade crop and this is still the case in some parts of the coffee-growing world. Shade-grown coffee has often been associated with higher quality at cupping. Therefore, a collaborative effort is underway between HARC and the University of Hawaii to determine what level of shade might be suitable for growing high quality coffee in Hawaii. Trials are going to be undertaken at a number of sites around the state, including HARC's Kunia substation, where extensive coffee plantings have been made for breeding purposes and are available for this kind of study. Different kinds of shading will be studied, including spraying a kaolinite mixture directly onto the leaves of the plant. HARC's Analytical Chemistry Laboratory will be undertaking analyses of a number of coffee components in green and roasted coffee harvested from this study.

Currently, the project is in its early stages, and pruning of coffee trees to be included in the study has just been completed. A graduate student will soon be working at HARC to develop methods for roasted coffee analysis. Green coffee methods were developed in a previous study. When the study trees are ready for harvest, all analytical work associated with this project will be undertaken at HARC.

– M. C. Jackson, T. Idol (UH), S. Steiman (UH) and H. C. Bittenbender (UH)

Forestry Research

Koa Research at HARC

Koa (Acacia koa) is the dominant tree species in much of the remaining native Hawaiian forests and provides important habitat for Hawaiian plants and animals, many of which are endangered. Native Hawaiian culture places a high importance on koa for uses such as canoe building and carving. Koa is the basis of an estimated $50 million hardwood furniture and crafts industry in Hawaii yet little is known about the suitability of diverse island seed sources for sustainable planting at commercial sites. Information is also lacking on the management of koa for commercial, sustainable operations on former agriculture, ranch and degraded native forest lands. At present, koa is an endemic tree of great value but little is known about management. Protecting and restoring koa forests, both natural and plantation, is therefore a high priority among land managers and commercial foresters in the state.

The vascular wilt disease Fusarium oxysporum f.sp. koae, commonly known as koa wilt, was recently identified as a disease causing dieoff of koa trees in Hawaii. Little is known of its ecology or origin, but it is found on all of the main islands of the Hawaiian chain. It infects trees through their roots and attacks the vascular system causing eventual death. Koa plantings at lower elevation have especially been impacted by this pathogen, limiting managers' abilities to use koa in forestry and restoration projects. At Maunawili, Oahu, Acacia koa families' survival percent ranged from a low of 4.0% to a high of 91.6%. The average family survival percent was 35.4%. However, the two best families had survival percentages of 91.6% and 75%, respectively. Koa wilt in natural forests at high elevations has also been found on the islands of Maui and Hawaii. On Maui, 4200 acres are affected by the disease in Haleakala National Park. Koa survival was reported to be 50% in areas affected by koa wilt on Maui. Recently, there have been outbreaks of koa wilt reported on the island of Hawaii in Volcano National Park.

HARC's forestry program is primarily aimed at selecting the best suited seed sources of koa for specific sites in Hawaii. Koa family variation in HARC field trials strongly suggests the presence of resistance to koa wilt disease. Work continued on screening of koa seedling families for genetic resistance to koa wilt (Table 1.)

– N. Dudley

Table 1. HARC Koa Seedling Families Trial Network

Site	Island
Maunawili (HARC)	Oahu
Kapa'a (Midler Trust)	Kauai
Honolua Plantation (Maui Pineapple Company)	Maui
Kula (L. Dorcay Trust)	Maui
Humu'ula (Parker Ranch/DHHL)	Big Island

Miscellaneous Crops

Development of a Transformation and Regeneration System for Taro

Taro had a market value of 2.7 million dollars in Hawaii in 2003 (Hawaii Agricultural Statistics Services) and is an important segment of Hawaii's agriculture. However, due to its minor crop status, there are few pesticides registered for this crop and none for fungal pests commonly occurring on taro (Ooka, 1994). Thus, there is a need to develop biologically-based management strategies for economically significant pest/disease problems on Hawaii's important crops. Genetic transformation of crop plants can result in increased resistance to pests. Such transgenic crops provide biologically based pest management with lowered production costs and a cleaner environment.

HARC, UH and USDA are collaborating to develop a taro transformation system. The specific objectives are: a) to develop a regeneration system for transformed taro to produce fertile plants; b) to develop a transformation system for taro, using selection genes, a reporter gene and the putative fungal resistance gene, rice chitinase gene (CHI111); and c) to test for increased fungal resistance of transgenic taro.

We have established tissue cultures of taro using meristem tip culture. Shoot tips of two commercial taro cultivars, 'Bun Long' and 'Maui Lehua', were explanted using the method of Hartman (1974) to avoid dasheen mosaic virus. An ELISA test (Agdia Kit for Dasheen Mosaic virus of PathoScreen) was performed on tissue-cultured taro to confirm the absence of virus.

More than 20 tissue culture media with varying phytohormones were tested for callus and shoot initiation for two taro cultivars. Only two media were found to induce embryogenic calli, with most of the media inducing shoot growth.

To select transformed tissue in an antibiotic medium, the appropriate level of an antibiotic needs to be established. Kill curves for taro tissue culture were carried out in the MS+BA (4.0 mg L-1) media with varying concentrations of the antibiotic, G418. A concentration of 50 mg G418 L-1 inhibited growth of taro and will be used in future selection media.

Five plasmids (pHP12679, pHP5897, pBI121, pBISN1 and pAHC27), all containing the ß-glucoronidase (GUS) reporter gene, were tested for GUS transient expression after being delivered by biolistic bombardment to taro shoots. Two plasmids (PHP12679 and PHI 121) that gave the highest expression will be used for later transformations of taro.

A rice chitinase gene has been successfully inserted into taro using particle bombardment with 1% transformation efficiency. The transgenic plantlets have been multiplied for further fungal resistance tests. We also have successfully transformed a rice chitinase gene into taro calli using an efficient Agrobacterium-mediated transformation method. This is the first report of transformation of taro with a useful gene and showed that the genetic transformation technique can be successfully applied to the improvement of taro resistance to diseases.

– J. Zhu, M. M. M. Fitch (USDA/ARS), P. H. Moore (USDA/ARS), L. He (UH), S. C. Miyasaka (UH), M. Tanabe (UH) and J. Cho (UH)

Determination of Environmental Factors for Increased Kavalactones in 'Awa

Hawaiian `awa, although originating in the South Pacific, now comprises approximately 13 cultivars that are morphologically and chemically distinct from their South Pacific counterparts. Because of 'awa's propensity to spontaneously mutate, generations of astute growers were able to carefully select mutant plants with the most pleasing and useful psychoactive effects. The result is that Hawaiian `awa today represents some of the best to be found anywhere.

With the gradual diversification of agriculture in Hawaii, the Hawaii Agriculture Research Center's Analytical Chemistry Laboratory has moved to develop research projects that service the needs of this fast growing agricultural sector. HARC now analyzes the bulk of the `awa commercially grown and sold throughout the Hawaiian islands, and also services the needs of growers and distributors in other regions. Clients need accurate, dependable information on overall kavalactone content and the relative concentrations of each of the six major kavalactones, the active ingredients.

Traditional cultural practices indicate that `awa should be grown in partial shade. No clear practices for fertilization rate and degree of pruning have ever been established. Therefore, HARC, together with collaborators at the University of Hawaii, College of Tropical Agriculture and Human Resources (Dr. H. C. Bittenbender, Dr. C. S. Tang) are evaluating the effects of parameters such as light, pruning and fertilization rate on overall kavalactone content in the root. The work began in September 2000 and is still continuing. The project is based at the UH Magoon horticultural facility and utilizes a novel basket cultivation system based upon experimentation by Big Island `awa growers. This system allows for the close monitoring of irrigation and fertilization rates, and also the amount of sunlight. Its greatest advantage is that it facilitates the sampling of root pieces. The basket is simply opened, root pieces excised and then the basket re-sealed. In addition, soil-borne pests such as the root knot nematode are eliminated. By 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 is providing analytical support to this effort.

Recently, there has been evidence suggesting that solvent extracts of `awa are responsible for liver disease in a relatively small number of people regularly taking this form of supplement. In collaboration with Dr. C. S. Tang's group at the University of Hawaii, HARC is currently working on defining methodology and plant physiological processes involved in natural production of alkaloids found in `awa stems that have been shown to be toxic to human liver cells grown in culture.

In addition, a HARC/UH collaboration resulted in the development of a very rapid and inexpensive method for determining individual kavalactone content using near infrared reflectance spectroscopy (NIRS).

– M. C. Jackson, H. C. Bittenbender (UH) and C. S. Tang (UH)

Developing Highly Efficient Transient Expression System with Plant Viral Vector

This project is aimed at developing a system to transiently express a high level of foreign protein in plants without tissue-culture procedures. Plants offer several advantages over other, more traditional expression systems, for the production of high-value products. It has been demonstrated that plants can express, fold, assemble and process complex foreign proteins. There may be significant economic benefits in the production of bulk quantities of valuable pharmaceutical products in plants compared with animal cell lines or transgenic animals, and there may be fewer safety concerns associated with use of plant expression systems.

Several studies have demonstrated that plant viruses can be used as vehicles to introduce and express foreign proteins in plants. Many plant viruses multiply to high levels in plants, leading to concomitantly high levels of foreign protein expression when a foreign protein gene is incorporated in the viral construct. Virus delivery does not lead to permanent incorporation of the transgene into plants. Nevertheless, depending on which virus is used, virus multiplication and gene expression can continue for long periods (weeks or months). Plant virus expression vectors have several other potential advantages over the more commonly used transgenic plant technology. In this study, we used two model genes to evaluate expression and function of foreign genes in plants. One of the genes coded for human granulocyte-macrophage colony-stimulating factor (GM-CSF) and the other for Citrus AP24 (CsAP24), an antifungal protein.

GM-CSF has important clinical applications in the treatment of neutropenia and aplastic anemia and reduction of infections associated with bone-marrow transplants. In this study, potato virus X (PVX) viral vector system was evaluated for efficient introduction and expression of the gene for GM-CSF protein. Nicotiana benthamiana plants were inoculated with the plasmid DNA of PVX vector containing GM-CSF gene driven by CaMV35S promoter. The expression level and size of recombinant GM-CSF protein were determined with ELISA and Western blot analysis. The results showed that leaf age significantly affected GM-CSF protein productivity with younger leaves expressing a higher level of recombinant protein. Protein expression levels declined slightly over several days following induction. The two leaves above the inoculated leaves also produced significant amounts of systemic GM-CSF. Protein extracts of the Nicotiana tobacco leaves contained up to 2% recombinant GM-CSF protein. The protein extract actively stimulated the growth of human TF-1 cells, suggesting that GM-CSF expressed via the PVX viral vector was biologically active.

PVX viral vector was also used to identify the functional role of AP24 proteins in plant disease resistance. PVX viral vectors were constructed for CsAP24 and for CsAP24 gene without a start codon. PVX vector containing the CsAP24 gene without the start codon caused virus disease symptoms in the inoculated plants. The reason may be due to surplus or untranslatable CsAP24 RNA interference. When Phytophthora parasitica was plated on V8 agar medium containing CsAP24 protein, fungal spore germination was inhibited, but not mycelial growth. Nicotiana benthamiana plants infected with PVX vector without the CsAP24 gene developed severe disease symptoms when challenged with the fungus. The plants inoculated with PVX viral vectors containing the CsAP24 gene showed more resistance to P. parasitica. It is proposed that endogenous CsAP24 protein promoted fungal resistance in N. benthamiana plants.

– F. Zhou, P. Moore (USDA/ARS) and Y. J. Zhu

Snails

The Golden Apple Snail (Pomacea canaliculata) is a major pest in wetland taro patches in Hawaii, causing major crop losses. Hawaii taro production in 2001 was estimated at 6.4 million pounds, with a sales value of about $3.4 million, a 9% decrease from 2000 (State of Hawaii, Dept. of Agriculture Agricultural Statistics Service Report, March 22, 2002). This was in large part due to the effects of the Golden Apple snail. Golden Apple snail was introduced into Hawaii, Japan and many other countries in Southeast Asia from South America as a source of food in the early 1980s. However, after its commercial markets had failed, discarded and escaped snails invaded taro and rice ecosystems and have been causing significant economic damage. In Hawaii, these snails were also purposely introduced into taro paddies (lo`i), the reasoning being that they could be harvested for food. However, the consequences of this action were not fully understood at the time. The snails are voracious, fast growing and with a huge reproductive potential. A single female can produce as many as 15,000 offspring per year, and can thrive in water at a density of 1,000 snails per square meter. They mature within 60 to 85 days and spawn at weekly intervals and have been described as the most damaging pest ever to hit neotropical areas. The snails very quickly spread throughout taro lo`i, via the extensive and interconnected irrigation system. In 1996, the Hawaii State Department of Agriculture statistical services recorded that in 1992, approximately 60,000 lb. of fresh taro was marketed from the islands of Oahu, Molokai and Maui, yet in 1996, only approximately 10,000 lb. was marketed; an 84% decline, largely due to apple snails. This picture of rapid and overwhelming infestation is reflected elsewhere in the world. For example, $1 million has been spent annually to control the snails in rice paddies in Taiwan since 1982. In addition, an estimated 20% of farm income was spent on apple snail control in the Philippines in 1993. Since taro grows in water where there are aquatic animals sharing the same ecosystems, chemicals are not allowed for pest control.

In 2003, HARC conducted limited field studies of two botanical extracts. Extracts were made from plants that had shown promise in prior toxicology studies. The field trial was initiated in December 2003 and is scheduled to be completed in May of 2004. Of the two extracts tested, application of one of the extracts has resulted in an average snail mortality rate of greater than 90% compared with the untreated control plots, 66 days after the initiation of the trial. The majority of the snail deaths were observed within 15 days after initiation of the test.

HARC will use the data gained from the trial to to move toward an EPA registration of the extract for use as a molluscicide in wetland taro.

Apple snail eggs on taro leaf stems

Apple snail setup

– M. C. Jackson

Degradation of Chlorinated Organic Compounds Using Saprophytic Fungi from Hawaii's Forests

Since the mid-1950s, agriculture and the military have employed many compounds for pest control and other uses. Some of these are extremely long lived, including DDT, heptachlor, PCP, PCBs and PAHs. There are a variety of means by which soils could be treated to remove persistent chemicals, for instance, removal of the contaminated soil and incineration or landfill. Considering the extremely large quantities of soil involved and the lack of a suitable landfill or a facility large enough to incinerate the volumes that would be required, these alternatives are not suitable.

The research undertaken in this project sought to address the problem in a different manner; by isolating strains of saprophytic, wood-rotting fungi present in Hawaii that can efficiently degrade chemicals such as those described above, in soils, in situ. Several wood-rotting basidiomycetes have been reported to be extremely active degraders. The results from this project can be used as the basis for further studies of field delivery systems and evaluation of effectiveness, ultimately resulting in an efficient, environmentally friendly means of bioremediating contaminated soils.

Certain saprophytic fungi have already been shown to efficiently degrade persistent contaminants in soils. However, the reported strains were isolated in regions other than Hawaii and consequently they may be poorly adapted to Hawaii's environment. In addition, there is difficulty in legally importing these microorganisms. There is therefore a need to find effective fungal species growing within the state. The varied microclimates and ecosystems within the islands provide a rich repository of fungi that may have unique properties with respect to their ability to degrade persistent compounds. The U.S. military in Hawaii and other Pacific basin regions will benefit from a technology developed in an area and climate that is similar to theirs.

Wood-degrading fungi growing in forests on Oahu and Hawaii were collected, isolated in pure culture in vitro and identified. Fungi were grown in liquid culture and test compounds added at 1 µg mL-1. After seven days, certain species of fungi were able to significantly degrade some chlorinated pesticides and PCBs and PAHs. Radio-labeled chlorinated test compounds, used in some experiments, demonstrated that the radio-label used was released as a gas, probably CO2. The extensive degradation indicated little possibility of the formation of potentially toxic intermediates in significant quantities. Expanding the work by mixing selected fungi with soils proved difficult. Some results were encouraging but were inconclusive that degradation was occurring.

– M. C. Jackson, J. Pitz, S. Schenck and D. Hemmes (UH)

Services

Environment

In keeping abreast of the latest environmental regulatory developments, HARC assists Hawaii's farmers in correctly applying the rules to everyday farming activities. HARC continues to provide training for members and others who need to know and understand these specialized and often highly technical requirements. HARC worked this year with the Hawaii state legislature, regulatory agencies, and various stakeholder groups to help develop appropriate environmental policies relating to nonpoint source pollution, surface and coastal water quality, agricultural air quality issues, crop protection chemicals, and endangered species habitat, among others.

– J. Ashman

Quality Assurance Unit

HARC continued to participate in the pesticide registration process under the Environmental Protection Agency's Federal Insecticide Fungicide, and Rodenticide Act. An independent Quality Assurance Unit (QAU) is maintained to inspect and audit related studies. The QAU participated in a sugarcane magnitude of residue study. HARC's facilities were inspected for compliance with the EPA's Good Laboratory Practice standards by one of our sponsors. As a member of the Hawaii Pesticide Advisory Committee, input from the sugarcane industry was provided to the State Department of Agriculture.

— B. Vance

Computer System Administration

New users to the local area network (LAN) were provided access to HARC's LAN, an e-mail account if appropriate and an introduction to the network and use policies. HARC's computer inventory grew by 5 computers, 1 printer, 1 hub and 1 switch. The LAN at the Experiment Station is still tightly integrated with the USDA/ARS and their users are provided technical support. Continued assistance was provided to users of Sun Microsystem's office software suite, StarOffice, which underwent 2 upgrades. Limited testing of the Linux (vs. Windows) operating system on the desktop was begun. To complement our disaster recovery plan, Standard Operating Procedures for the major procedures were written or revised. Users were reminded of the necessity to keep their PCs updated with the latest antivirus signatures and security patches. Anti-SPAM software at the user level was promoted. The firewall's software and hardware was upgraded and maintained. The file server was replaced as was its network operating system and relocated to a more secure location; RAID was deployed. The dial-up e-mail system was replaced by a faster POP3/SMTP system that further allowed users to access their e-mail off-site. Lower rates were secured for our telephone system.

– B. Vance

Laboratory Services

Analytical Chemistry Services

Our contract laboratory provided analytical chemistry services to Hawaii's agricultural community, industries, government agencies and to U.S. mainland and international companies. We analyzed for natural and synthetic compounds in plants, soil, water and air using gas chromatography and high-performance liquid chromatography. Our analytical residue determinations ranged from quick screens for general information to detailed research studies including pesticide registration projects for the United States Environmental Protection Agency (USEPA).

Pesticide registration for use of imidacloprid in papaya was completed this year (ref., Federal Register, 68 (114) June 13, 2003). HARC's work began in 1998 and provided field phase and analytical residue phase data for imidacloprid in papaya. Funding was provided by Hawaii's Department of Agriculture and the Papaya Action Committee, PAC. Minor Crop Pest Management, IR-4, a national agricultural program to clear pest control agents for minor crops, prepared the final imidacloprid registration report for USEPA. This registration of imidacloprid not only allows use on papaya, but also on a crop grouping including star apple, black sapote, sapodilla, mango, canistel and mamey sapote. The tolerance for imidacloprid is 1 ppm. Labels have been updated for Provado® and Admire®. Imidacloprid provides protection against sucking insects such as leafhoppers and aphids on many crops. Without treatment, heavy infestations of leafhoppers have destroyed papaya fields.

Agricultural commodities and products that are exported from the United States and other countries must meet the pesticide residue tolerance standards of the import country. Analytical chemistry determinations were performed on pineapple, papaya, Kona coffee, stevia, and wood products to show that these products comply with Japan's import tolerance regulations.

– J. Pitz

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Sugar Production

COMPANY	ACRES HARVESTED	TONS RAW SUGAR (96•)	TONS SUGAR PER ACRE
2001			
Gay & Robinson, Inc.	4,193	54,691	13.04*
Hawaiian Commercial & Sugar Co.	15,101	191,512	12.68
Totals & average	19,294	246,203	12.76**
2002			
Gay & Robinson, Inc.	4,754	54,196	11.40*
Hawaiian Commercial & Sugar Co.	16,557	215,888	13.04
Totals & average	21,311	270,084	12.67**
2003			
Gay & Robinson, Inc.	4,191	55,267	13.19
Hawaiian Commercial & Sugar Co.	15,660	205,742	13.14
Totals & average	19,851	261,009	13.15**

* Includes Kekaha salvage cane
** Weighted average

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