96-051-01p

# PETITION FOR DETERMINATION OF REGULATORY STATUS

Transgenic Papaya lines 55-1 and 63-1 and their Derivatives

The undersigned persons submit this petition under 7 CFR 340.6 to request that the Director, BBEP, make a determination that the articles should not be regulated under 7 CFR 340.

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Contains No Confidential Business Information

Summary

Cornell University and University of Hawaii are submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for transgenic papaya (*Carica papaya*) 'Sunset' lines 55-1 and 63-1, and any progenies derived from crosses between these two lines, between these lines and any APHIS nonregulated transgenic papaya, and between these lines and any nontransgenic papaya or *Carica* species. Lines 55-1 and 63-1 are considered regulated articles because they contain sequences from the plant pests cauliflower mosaic virus (CaMV), papaya ringspot virus (PRV), and cucumber mosaic virus (CMV). Additionally, these transgenic plants have sequences from some marker genes used in laboratory screening and selection procedures (i.e., npt II, GUS, $TET^R$, and $GENT^R$).

Papaya ringspot virus is the most important plant pest of papaya on a worldwide basis. In the USA, PRV has been particularly damaging to Hawaii and Florida. Hawaii, in particular, is being devastated by PRV because the virus is now widespread in the Puna district of Hawaii Island where 95% of Hawaii's papaya is grown. Since the discovery of PRV in Puna in 1992, the Kapoho area of Puna (one third of the Puna acreage) has been virtually destroyed by PRV. Economically, papaya is the fourth most valuable agricultural commodity of Hawaii, only behind pineapple, sugarcane, and macadamia. Unfortunately, current control measures, such as mild strain cross protection, roguing, and breeding for resistance, have not been successful. Clearly, new control measures are needed.

We recently developed transgenic 'Sunset' papaya that express the coat protein gene of PRV HA 5-1, a mild mutant of a PRV strain which originated from Hawaii. Extensive greenhouse and field tests of line 55-1 showed that R0 plants and their progenies are highly resistant to PRV isolates from Hawaii. In a two year field trial in Hawaii, R0 plants remained healthy while all susceptible control plants became infected within 77 days. These data showed that 55-1 should be extremely valuable for controlling PRV in Hawaii. Interestingly, this line was susceptible to PRV isolates from other parts of the world, including Thailand.

Line 63-1 has been less thoroughly tested than 55-1 but it shows good promise. Greenhouse tests showed that it has resistance to PRV isolates from Hawaii, and also better resistance than 55-1 to isolates from Thailand. Thus, this line might provide better protection than 55-1 to the range of isolates in Hawaii and other isolates that might be introduced into Hawaii.

Transgenic papaya that are resistant to PRV should not pose abnormal risks to the environment. Papaya was not a weed in Hawaii even before PRV was introduced. Furthermore, other *Carica* species are not endemic in Hawaii and crosses between *Carica papaya* and other *Carica* species result in sterile

seeds. The chances of heteroencapsidation or recombination causing the spread of or the creation of new virulent PRV strains is nil; especially since PRV is the only potyvirus that infects papaya.

Transgenic lines 55-1 and 63-1 represent the most promising, and perhaps the only, control measure for PRV in Hawaii where the papaya industry is being severely affected. Cornell University and the University of Hawaii request a determination from APHIS that transgenic 'Sunset' lines 55-1 and 63-1, and their progeny will no longer be considered regulated articles under 7 CFR Part 340. The progeny that we petition to be no longer regulated could be derived from self pollinations of 55-1 or 63-1, crosses between 55-1 and 63-1, and crosses of 63-1 or 55-1 with nontransgenic papaya, other *Carica* species, and other transgenic papaya or *Carica* species that have been determined to be nonregulated.

Certification

The undersigned certify, that to the best of the knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes any relevant data and information known to the petitioner which are unfavorable to the petition.

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# Abbreviations used in Petition

| | |
|---|---|
| BR | T-DNA right border |
| BL | T-DNA left border |
| CaMV | Cauliflower mosaic virus |
| CMV | Cucumber mosaic virus |
| COS | Cohesive end site of bacteriophage lambda |
| CP | Coat protein |
| ELISA | Enzyme-linked immunosorbet assay |
| $GENT^R$ | Gentamycin resistance gene |
| GUS | ß-Glucuronidase |
| NOS | Nopaline synthase promoter |
| npt II | Neomycin phosphotransferase |
| PRV | Papaya ringspot virus |
| ORI | Col E1 origin of replication |
| ORI V | pRK2 origin of replication |
| ORI T | pRK2 origin of conjugative transfer |
| $TET^R$ | Tetracycline resistance gene |
| 35 S | 35 S-Cauliflower mosaic virus promoter |
| 5'UT | 5' untranslatable region (70 base pair nontranslatable intergenic region from cucumber mosaic virus RNA 3) |
| tet | Tetracycline resistance gene as labeled on pGA482GG |
| gent | Gentamycin resistance gene as labeled on pGA482GG |

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I. Transgenic Papaya Lines To Be Deregulated:

1) Line 55-1, a transgenic line of the gynodioecious cultivar Sunset, expressing the coat protein gene of papaya ringspot virus, strain HA 5-1.
2) Line 63-1, a transgenic line of the gynodioecious cultivar Sunset, expressing the coat protein gene of papaya ringspot virus, strain HA 5-1.

II. Rationale for the Development of Transgenic Papaya

Throughout the papaya-producing regions of the world, papaya ringspot virus (PRV) is the most important papaya pathogen and a major limiting factor in commercial papaya production (Gonsalves, 1994). All major production areas in the Western Hemisphere [Brazil, the Caribbean region, Mexico, and USA (Florida and Hawaii)] and Eastern Hemisphere (the Philippines, Taiwan, Thailand, and China) are affected, and the virus is still invading new areas (Hawaii, Israel, Malaysia and Australia). PRV belongs to the potyvirus group of plant viruses and is transmitted by aphids in a nonpersistent manner (Purcifull et al., 1984). Once introduced, PRV has never been successfully eradicated from any region.

Control measures, including use of insecticides against insect vectors, roguing of diseased plants and quarantine regulations restricting plant movement, have failed to eliminate the disease in infested regions (Purcifull et al., 1984; Shukla et al., 1994). Isolated regions can be temporarily protected from the virus through a combination of quarantine and roguing (Namba and Higa, 1977) but even isolated production areas are always vulnerable, as a disastrous outbreak of PRV in the major production areas of Hawaii has recently demonstrated (Isherwood, 1994). Furthermore, the quarantine approach has no merit in areas already infected with PRV.

Cross-protection (deliberate inoculation with mild forms of the virus to prevent economic damage by more virulent strains) and conventional breeding for genetic resistance and/or tolerance provide some control under certain conditions (Yeh and Gonsalves, 1994) but have definite limitations, as will be described below.

Cross-protection with a mild strain of PRV (HA 5-1) suffers several drawbacks. Each seedling generation must be inoculated with the protective strain, and although disease symptoms are relatively mild, there is an associated yield reduction of 10-20% (Mau et al., 1989). Protection by PRV HA 5-1 is effective only against PRV isolates from Hawaii (Yeh and Gonsalves, 1994).

Genetic tolerance to PRV exists in papaya germplasm, but it is only moderately effective and is polygenic in inheritance (Conover and Litz, 1978). The resistant germplasm is not suitable for commercial production in Hawaii,

so crosses with Hawaiian cultivars are necessary to improve fruit quality and other horticultural traits. Selection in segregating generations for individuals that combine acceptable quality and useful levels of resistance will require many years.

Genetically engineered resistance has distinct advantages over other methods of disease control. Relative to conventional breeding, 1) the level of resistance is potentially higher than any in existing papaya germplasm, 2) horticultural quality in the transgenic cultivar is not compromised, and 3) the development time is considerably less, perhaps 3-4 years. Relative to cross-protection, 1) stable incorporation of the coat protein (CP) gene in a papaya chromosome will result in Mendelian inheritance of virus resistance in the seedling progeny, eliminating the need to inoculate each generation and 2) the absence of a replicating, albeit mild, virus in the genetically engineered plants means that a) there should be no disease symptoms or yield reductions, and b) there will be no possibility of a mild virus escaping to infect other crops.

A recent outbreak of PRV in the Puna District of Hawaii Island, underscores the urgency to control this disease on papaya (Isherwood, 1994). Puna, where about 95% of Hawaiian production acreage is located, had been free of PRV until May, 1992, but the extent of the current outbreak makes it unlikely that it will be contained and eliminated. The consequence of uncontrolled spread of PRV can be seen on the neighboring Hawaiian island of Oahu. Backyard plants in Honolulu and other urban areas have provided a haven for PRV, and from these places the virus has spread into rural areas, so that papayas can now be grown only with the help of cross protection.

In a recent application of genetic engineering to crop improvement, the first papaya with a high level of resistance to PRV has been created by transforming the cultivar Sunset with the CP gene of the mild, cross-protective PRV strain, HA 5-1 (Fitch et al., 1992). This example of "coat protein-mediated resistance," funded partly by a USDA Section 406 grant ("Virus resistant papaya cultivars through genetically engineered cross-protection," PI's - Manshardt and Gonsalves, 1988-1992), is an important advancement, both in terms of biotechnological capability and PRV resistance. Under field conditions, the transgenic 'Sunset' clone 55-1 remained symptomless for 24 months following inoculations with PRV (Lius, 1994). These transgenic papaya lines represent the most effective means of controlling PRV in Hawaii.

## III. The Family Caricaceae

### A. *Carica papaya*

Papaya (*Carica papaya* L.) is the best known member of the Caricaceae, a small dicotyledonous family consisting of four genera (Badillo, 1971). With the exception of the genus, *Cylicomorpha*, which occurs in West Africa, the family has an entirely neotropical distribution. *Carica* is the largest genus with 23 described species, many of which have overlapping distributions in the foothills of the Andes in northwestern South America. However, members of the genus range from southern Brazil, Argentina and Chile to southern Mexico.

Papaya is a common plant in the gardens and doorvards of the lowland tropics, where its popularity is due to its tasty fruits, a continuous bearing habit, and the ease with which it can be propagated. It is consumed primarily as a fresh dessert fruit, and it provides a good source of vitamins A and C (Arriola et al., 1980). Fruits are also eaten as a cooked or raw vegetable, most notably in southeast Asia, where green fruits are grated to produce a salad. Because papaya latex contains the proteolytic enzyme papain, green fruits are frequently stewed with meat as a tenderizer. Papain itself is a commercial product extracted from the collected latex.

The plant growth habit is perennial, but the juvenile period is short, averaging about six months. The first fruits mature about one year after planting, and bearing thereafter is more or less continuous. Most commercial plantings follow a three or four year cycle, since yields decline and trees become too tall for efficient harvesting.

Papaya is a polygamous species, having a mating system that is either dioecious (staminate and pistillate plants) or gynodioecious (hermaphrodite and pistillate plants). On a commercial scale, gynodioecious lines are generally preferred because of their potential for inbreeding and consequent uniformity. Sex inheritance is controlled by a single locus with multiple alleles (Hofmeyr, 1967; Horovitz and Jimenez, 1967; Storey, 1976). Staminate plants ($M_m/m$) and hermaphrodites ($M_h/m$) are heterozygous, while pistillate plants (m/m) are homozygous for the recessive allele. The homozygous condition involving either dominant allele is lethal.

Hermaphrodites can be self-pollinated to homozygosity, except for sex characters, yielding gynodioecious lines that segregate in a ratio of 2 hermaphrodites to 1 female. In most Hawaiian hermaphrodites, the position of the anthers relative to the stigma is such that self-pollination occurs at anthesis without manual assistance or bagging. A low out-crossing rate occurs because hermaphrodites produce copious pollen from staminate

flowers during most of the year. Pistillate plants never produce anthers, and are consequently obligate out-crossers. Each pollination produces hundreds of seeds, which are easily recovered from ripe fruits. In nature, pollination occurs through bees, butterflies and wind.

'Kapoho' and 'Sunset' are gynodioecious lines of commercial importance in Hawaii. 'Kapoho' is the major export papaya cultivar in the state. It has a yellow-fleshed fruit, and approximately 2,250 acres were being harvested in 1990. 'Sunset' is a sib line of the better known 'Sunrise', a red-fleshed fruit type that is planted on a much larger scale throughout the tropical world. The principal difference between 'Sunset' and 'Sunrise' is the longer shelf life of the former. All Hawaiian cultivars are highly susceptible to PRV. Thus, 'Kapoho' and 'Sunset' are especially useful for transgenic plant studies because of their commercial horticultural properties and susceptibility to PRV infection.

B. Interspecific Hybridization of Cultivated Papaya with Wild Relatives

Besides papaya, the genus *Carica* also includes 21 other species of herbaceous, tree-like dicots (Badillo, 1971). The latter are relatively unimportant economically, but several species have characteristics that are attractive from the standpoint of papaya improvement. Resistance to PRV has been reported in *C. candicans*, *C. cauliflora*, *C. pubescens*, *C. quercifolia*, *C. stipulata*, and *C.* x *heilbomii nm. pentagona* ('babaco') (Conover, 1964; Horovitz and Jimenez, 1967). 'Babaco' is a sterile, parthenocarpic, pistillate clone, which is vegetatively propagated in its native Ecuadoran range, and is reputed to have originated from natural hybridization of *C. pubescens* and *C. stipulata* (Horovitz and Jimenez, 1967). The pleasant fragrances of *C. stipulata* and 'babaco' fruits, the monoecious habit of *C. monoica*, the cold tolerance of *C. pubescens* and *C. stipulata*, and the ornamental qualities of pink-flowered *C. parviflora* are also traits of interest.

Early attempts to intercross papaya with other *Carica* species yielded only nonviable seeds, and these failures illustrate the reproductive isolation of *Carica papaya* from the rest of the genus (Mekako and Nakasone, 1975; Sawant, 1958). Endosperm failure occurs in interspecific crosses involving papaya, but some embryos continue to grow and develop (Manshardt and Wenslaff, 1989). Consequently, *in vitro* embryo culture techniques have been successfully employed to rescue hybrid embryos. Crosses between *C. papaya* and *C. cauliflora* have been reported most frequently. In most cases, this hybrid has proven to be of low vigor and viability (Horovitz and Jimenez, 1967; Litz and Conover, 1983; Manshardt and Wenslaff, 1989) although (Khuspe et al., 1980) reported vigorous, fertile hybrids in India. Also produced by embryo culture have been *C. papaya* x *C. stipulata* hybrids, which were apparently of low vigor and viability, dying upon transfer from sterile

medium, and *C. papaya* x *C. pubescens* hybrids, which were vigorous but reproductively sterile (Horovitz and Jimenez, 1967).

Because of the great difficulty in making hybrids, *C. papaya* is usually described as sexually incompatible with other members of the genus. In the past decade, protoplast fusion has been investigated as a means to circumvent sexual barriers between *Carica* species. Initial steps have been taken to develop methods for somatic hybridization of *C. papaya* with *C. stipulata* (Litz and Conover, 1979; Litz and Conover, 1980) and with *C. pubescens*, (Jordan et al., 1986) but no hybrid plants have been regenerated to date.

C. Papaya as a Crop

Papaya is a common plant in the gardens and dooryards of the lowland tropics because of its popular fruits and the ease with which it can be propagated. The 1994 FAO Production Yearbook ranks world papaya production in 1993 above grapefruit/pomelo and below plum, with more than 98% being grown in developing nations. In the subsistence diets of tropical developing countries, papaya is an important source of vitamins A and C, particularly because its continuous-bearing habit makes fruit available throughout the year. It is also becoming a valuable cash crop for domestic markets and for export to Europe, North America and Japan, where health-conscious consumers find the papaya an attractive breakfast fruit. In Hawaii, the $16-million papaya crop is the fourth most valuable agricultural commodity, after sugarcane, pineapple, and macadamia nuts. About 70% of Hawaiian production is exported, making a significant contribution to the state's economy.

D. Pollination in Papaya

Papaya is a polygamous species, individual plants being of pistillate, staminate, or hermaphrodite sex type. Pistillate plants produce ovaries, but never anthers. Staminate plants produce no fruit, but their long panicles bear many pollen-producing flowers. Hermaphrodite plants are actually andromonoecious, the terminal flowers in the inflorescence being hermaphrodite and those at the basal end of the inflorescence being staminate.

Pollination of papaya flowers seems relatively unspecialized and occurs through both insect and wind dispersal. Staminate plants produce masses of pollen-producing flowers, and although butterflies are attracted by nectar in staminate flowers in Latin America (Manshardt - personal observation), the light, powdery pollen is seemingly well adapted for wind dispersal.

Hermaphrodite plants have the capacity for self-pollination, especially when anthers are positioned so that they contact the stigmatic lobes at

anthesis. Anthesis generally occurs slightly before the flower opens, so that self-pollination is enhanced. The amount of pollen produced by hermaphrodite plants is much less than that released by staminate plants, and in commercial fields in Hawaii where gynodioecious papaya lines segregate for hermaphrodite and pistillate plants, it is common to find fruits on the pistillate plants that are smaller and contain fewer than normal seeds due to poor pollination. In Hawaii, honeybees and butterflies are occasionally seen on the flowers on hermaphrodite plants.

E. *Carica papaya* as a potential weed

Wild papaya plants with nearly inedible golf ball-sized fruits are found from southern Mexico to northern Honduras and throughout the Caribbean islands. The wild papayas are uniformly dioecious and have weedy characteristics (Barlett, 1936), such as prolific seed production, minimal edible flesh, and seed dormancy. Wild papayas are part of the secondary successional plant community in areas where the lowland tropical forest has been disturbed, occurring spontaneously in abandoned agricultural plots and along roadsides (Lundell, 1937). They do not persist long in the natural successional cycle and are typically overgrown by vines and forest vegetation in a few years (R. Manshardt, personal observation).

Domesticated papayas, with a higher ratio of edible flesh to seed and a lack of seed dormancy, have none of the weedy behavior of wild papayas. Although occasional escapes from cultivation may survive in waste spaces, they are not part of a successional community in the USA and they do not occur in large stands.

In Hawaii, wild papayas do not exist, and the cultivated papaya has never been listed among the state Department of Agriculture's noxious weeds. Records dating back to Territorial days in the 1940s, before PRV became a serious pathogen, contain no mention of papaya as a weed that threatened agricultural production. Casual observation bears this out, in that escapes from domestication are relatively rare, even in major production areas such as the Puna district on the island of Hawaii.

In Florida, wild papayas were probably introduced by indigenous peoples before Columbus' discovery of the New World, and they persist in the hammock areas around the everglades. However, according to Dr. Carl Campbell, Emeritus Extension Specialist, University of Florida, "Nobody considers papaya to be a weed nuisance in Florida agriculture in the usual sense of weediness" (Appendix A). However, the wild form may constitute a reservoir for PRV from which the disease may be carried to cultivated papayas.

In south Texas, low temperatures in winter and low rainfall are important limiting factors in the survival of papayas, either wild or domesticated. Because of these factors, Dr. Julian Sauls of the Texas Cooperative Extension Service states, "I cannot consider papaya as a weed under any circumstances in Texas, regardless of its potential freedom from virus diseases" (Appendix A). Similar environmental limits exclude weedy behavior in papayas in southern California, according to Dr. Mary Lu Arpaia, Extension Specialist at UC, Riverside (Appendix A).

From all available information, PRV resistance would be expected to have little impact on weediness of domesticated papaya in Hawaii.

IV. Description of regulated articles to be exempted:

The CP gene of PRV was derived from PRV HA 5-1, a mild mutant strain (Yeh and Gonsalves, 1994) that was produced by nitrous acid treatment of the severe PRV HA which is from Hawaii (Gonsalves and Ishii, 1980). The 3'- terminal region of PRV HA 5-1, including the CP gene, of PRV HA 5-1, has been sequenced (Quemada et al., 1990). The CP gene of PRV HA 5-1 was isolated and engineered into the transformation vector pGA482GG (Fig. 1) with the resulting plasmid designated as pGA482GG/PRV-4 (Fig. 2, Ling et al., 1991). The PRV CP gene construct in pGA482GG/PRV-4 is expressed as a chimeric protein because codons specifying the first 16 amino acids of CMV CP were fused to the CP gene of PRV HA 5-1 (Fig. 3; Ling et al., 1991). The construction of pGA482GG was described by Quemada et al. (1991). Transgenic 'Sunset' papaya plants were obtained by Fitch et al. (1992) following bombardment of embryogenic cultures of papaya with tungsten particles coated with pGA482GG/PRV-4 using the 'Biolistic' microprojectiles process (Sanford et al., 1992).

The genetic components in pGA482GG/PRV-4 are listed in Table A. Maps of pGA482GG and pGA482GG/PRV-4 are shown in Figures 1 and 2, respectively.

Table A. Components of plasmid used in the development of the transgenic papaya lines 55-1 and 63-1.

| Item | Brief description (Reference) |
|---|---|
| nos | nopaline synthase promoter (An, 1986; Bevan et al., 1983), orignally from *Agrobacterium tumefaciens* |
| npt II | neomycin phosphotransferase (Topfer et al., 1980), originally from *Escherichia coli* |
| 35S Pro | promoter from cauliflower mosaic virus (Odell et al., 1985; Pietrzak et al., 1986) |
| 5'-UT | 70 bp 5' untranslated region of cucumber mosaic virus RNA 3 (Quemada et al., 1991) |
| CMV-PRV Fusion Coat | coat protein gene of PRV HA 5-1 which has codons specifying the first 16 amino acids of CMV coat protein at its N-terminus (Ling et al., 1991) |
| 35S Poly (A) | poly (A) terminator from cauliflower mosaic virus (Odell et al., 1985; Pietrzak et al., 1986) |
| GUS | ß-glucuronidase (Jefferson, 1987), originally from *E. coli* |
| Gent | Gentamicin resistance gene (referred to as $GENT^R$ in list of abbreviations ) (Allmansberger et al., 1985), originally from *E. coli* |
| Tet | Tetracycline gene (referred to as $TET^R$ in list of abbreviations ) (An, 1986), originally from *E. coli* |

V. Analysis of Transgenic Papaya

The two transgenic papaya lines (55-1 and 63-1) that are put forth in this petition were derived from transformation experiments described by Fitch et al. (1992).

A. Southern blot analysis of genes integrated into 55-1 and 63-1.

Previous Southern blot analysis (Fitch et al., 1992) had clearly shown that R0 plants of 55-1 contained the npt II and CP genes, and assays showed that the GUS gene was functional in this line. Southern blot analyses are also presented in this petition for lines 55-1 and 63-1. Probes were prepared for

detecting genes contained within and outside the T-DNA border sequences (see Appendix B and Figures 1, 2 and 4 for details on how the probes were generated). Briefly, three probes inside the T-DNA borders were obtained after digestion of the pGA482GG plasmid: an npt II probe (1.9 kbp) obtained by *Bam* HI/*Hind* III digestion (Fig. 1); a GUS probe (2.8 kbp) obtained after *Sst* I digestion (Fig. 1), and a PRV CP probe (1.5 kbp) obtained by *Hin*d III digestion (Fig. 2). These three probes served to determine integration of npt II, GUS and PRV CP genes engineered inside the T-DNA borders of the pGA482GG/CP PRV-4 plasmid. In addition, Southern blot analysis was conducted to determine if any fragment outside the T-DNA borders was incorporated into the genome of the transgenic lines 55-1 and 63-1 during bombardment. The three probes that were used were obtained after complete digestion of the plasmid pGA482GG with *Sal* I (Fig 4). These probes were: a probe for the gentamycin resistance gene (1.1 kbp), a probe for the Ori V/Tet region (2.7 kbp) which includes about one-third of the tetracycline resistant gene, and a probe for the Ori T/Tet region which includes two thirds of the tetracycline resistance gene (4.1 kbp).

Southern blot analysis of a greenhouse-grown transgenic plant from a cross between R0 55-1 and nontransgenic 'Sunrise' indicated that the plant contained the npt II, PRV CP, and GUS genes inside the T-DNA borders (Fig. 5A-C). Genomic DNA did not hybridize with probes to the gentamycin resistance gene (1.1 probe) or the Ori V/Tet region (2.7 probe), but hybridized with a probe to the Ori T/Tet region (4.1 probe) (Fig. 5D). However, a complete tetracycline resistant gene is not present in this line, since the Ori V/Tet probe did not hybridize with the genomic DNA (Fig. 5D). Since the tetracycline resistance gene is under the control of a prokaryotic promoter (An, 1986), this gene is not expected to be expressed in plants. The results are also tabulated in Appendix B and Table B.

Southern blot analysis of a greenhouse-grown R0 clone of line 63-1 gave a different pattern from that of line 55-1 (Fig. 5A-C). It appears that the npt II and PRV CP genes are integrated into the plant DNA and are functional since genomic DNA hybridized with probes corresponding to these genes (Fig. 5A-C) and assays for expression of these genes were positive (e.g. Table C). However, genomic DNA did not hybridize with the probe for the GUS gene, which is also confirmed by the lack of positive GUS assays in plant tissues (e.g. Table C). Genomic DNA hybridized with probes to the gentamycin resistance gene (1.1 probe), Ori V/Tet region (2.7 probe) and Ori T/Tet region (4.1 probe) followng *Sal* I digests (Fig. 5D). However, it is not likely that the Tet and Gent genes are functional since their prokaryotic promoters do not drive expression of these genes *in planta* (Allmansberger et al., 1985; An, 1986). Some rearrangements of genes may have occurred during integration since multiple bands are observed on this line following *Hin*d III/*Bam* HI digests, and the hybridization bands are located at a much higher molecular

weight than expected. The results are also tabulated in Appendix B and Table B.

Table B. Summary of Southern blot analysis results.

| Probes | Control | 55-1 | 63-1 |
|---|---|---|---|
| Inside T-DNA borders | | | |
| Coat protein (CP) | - | + | + |
| ß-glucuronidase (GUS) | - | + | - |
| Neomycinphosphotransferase (npt II) | - | + | + |
| Outside T-DNA borders | | | |
| Gentamycin (Gen) | - | - | + |
| Ori T/Tet | - | + | + |
| Ori V/Tet | - | - | + |

B. Expression of Transgenes in 55-1 and 63-1 and Mendelian Inheritance

Transformed plants were assayed for GUS expression using the histological and fluorimetric assays (Jefferson, 1987) and for npt II expression using the enzyme linked immunosorbent assay (ELISA) and commercial γ-globulins (5' Prime 3' Prime). Coat protein expression was determined by direct double-antibody sandwich ELISA with polyclonal coating antibodies and conjugated monoclonal antibodies against PRV produced in Gonsalves' laboratory (Gonsalves and Ishii, 1980; Tennant et al., 1994). Extraction buffer, healthy papaya tissue, PRV-infected tissue and/or purified virus were included as controls in each ELISA plate (Immulon 2, Dynatech Co., Chantilly, Va). Replicated wells were loaded for each plant sample and optical density was read at 405 nm.

Expression of npt II, GUS, and CP genes in R0 plants of 55-1 and 63-1 revealed that the 55-1 expressed all three transgenes, while 63-1 expressed only npt II and CP genes. These results confirmed the Southern blot analyses.

Inheritance of transgenes was examined in the Rl generation by analysing segregation ratios in 1- to 2-month-old seedlings resulting from crosses of R0 transgenic plants. Crosses of R0 55-1 with nontransgenic 'Sunset' produced progenies that conformed well to a ratio of 1 transgenic: 1 nontransgenic plant (Table C) for all three transgenes, indicating a single transgene insertion site.

A transgenic 46-1 R0 plant, which expressed only the npt II gene, was crossed as female parent to a 63-1 R0 plant to produce 46-1 x 63-1 Rl progenies.

The observed segregation ratio for CP was a poor fit to the expected 1 CP+: 1 CP- ratio, but the npt II segregation was very close to the expected 3 npt II+: 1 npt II- (Table C).

Table C. Inheritance of transgenes (+:-) in seedlings of the Rl generation.

| | | Expected | Observed Ratio | | | |
|---|---|---|---|---|---|---|
| Rl Line | n | Ratio | GUS | npt II | CP | $X^2$ |
| 55-1 | 394 | 197: 197 | 193: 201 | 193: 201 | 193: 201 | 0.16 |
| 63-1 | 60 | 30: 30 | | | 39: 21 | 5.40 |
| 63-1 | 60 | 45: 15 | | 46: 14 | | 0.09 |

n= numbers tested, $X^2$= chi square

The transgene expression of 55-1 was also analyzed in another set of R1 plants. These results were previously reported by (Tennant et al., 1994). Seedling progenies from a cross of transgenic R0 55-1 and nontransgenic 'Surise' were tested for the expression of the npt II gene. Out of 2,318 seedlings tested, 52% were npt II-positive, which is very close to the expected 50%. The level of CP expression was also measured for 698 npt II positive seedlings. Thirty-two percent had ELISA absorbance values between 0.05-0.1, 43% between 0.1-0.2, and 25% between 0.2-1.1. The negative controls (nontransgenic 'Sunrise') gave average absorbance values of 0.02, while a purified PRV preparation containing 100 nanogram of virus had an absorbance value of 0.19.

Similarly, transgene expression was analyzed in another set of greenhouse grown 63-1 R1 plants. R1 plants were obtained from seeds of a self pollinated hermaphrodite 63-1 R0 plant. Out of 81 seedlings analyzed, 63 gave positive npt II ELISA reactions. Negative controls were nontransgenic 'Sunrise' and positive controls were transgenic 55-1 R1 plants. These same seedlings were tested for CP expression. Positive CP ELISA reactions were detected in 73 of the 81 plants tested. Of the 73 ELISA positive plants, 1 had absorbance between 0.08-0.10, 5 between 0.11-0.20, and 67 between 0.21-0.60. Negative controls (nontransgenic 'Sunrise') averaged 0.00 absorbance while positive controls (PRV infected 'Sunrise') averaged 0.187. The positive threshold ELISA value was 0.060.

Expression of transgenes was measured in R2 and R3 generations of 55-1. Segregation of the CP gene in leaves of 2-month-old R2 seedlings from self-pollinated 55-1 R1 plants followed the 3 CP+: 1 CP- ratio expected from a single transgene insertion site, but GUS expression was found to occur less frequently than expected (Table D). GUS expression in R3 seed (embryo and endosperm) from mature R2 plants was found to be normal, yielding all GUS+ seed from homozygotes (GUS/GUS), and either 1 GUS+: 1 GUS- or 3 GUS+: 1 GUS- from heterozygotes (+/GUS) when crossed with non-transgenic (+/+) plants or self-pollinated, respectively (Table D).

Table D. Inheritance of transgenes from R2 and R3 generations of 55-1.

| | Expected | | Observed Ratio | | | |
|---|---|---|---|---|---|---|
| Cross | n | Ratio | GUS | npt II | CP | $X^2$ |
| Leaves of R2 plants | | | | | | |
| 55-1 (+/CP) selfed | 323 | 242:81 | | | 237:86 | 0.45 |
| 55-1 (+/CP) selfed | 279 | 209:70 | 157:122 | | | 52:19 |
| R3 seed (embryo & endosperm) | | | | | | |
| 55-1 (GUS/GUS) selfed | 1410 | 1410:0 | 1410:0 | | | 0.0 |
| 55-1 (+/gGUS x (+/+) | 1290 | 645:645 | 625:665 | | | 1.24 |
| 55-1 (+/GUS) selfed | 285 | 214:71 | 224:61 | | | 1.88 |

n= number of seeds tested, $X^2$= chi square

Expression levels of CP in mesocarp tissue of ripe R2 fruit of 55-1 were also much reduced relative to levels in mature leaf tissues of either homozygous (CP/CP) or heterozygous (+/CP) R2 plants (Table E). In fact the level of CP expression in ripe fruit was not significantly different from that in nontransgenic fruit.

Table E. Differential CP expression in 55-1 R2 leaf and fruit tissues.

| | | ELISA (absorbance + Std. Dev.) | |
|---|---|---|---|
| CP dosage | n | Leaf | Fruit |
| negative control (+/+) | 2 | 0.035 + 0.003 | -0.011 + 0.002 |
| infected control | 5 | 0.853 + 0.090 | 0.064 + 0.039 |
| 55-1 R2 (+/CP) | 10 | 0.124 + 0.033 | -0.004 + 0.010 |
| 55-1 R2 (CP/CP) | 9 | 0.061 + 0.016 | -0.002 + 0.006 |

n= number of samples tested

C. Resistance of Transgenic Papaya 55-1 and 63-1 to Papaya Ringspot Virus

Our initial tests of PRV resistance in the transgenic materials were conducted in the greenhouse on clones of four R0 plants carrying the CP gene. The results showed that only clone 55-1 remained uniformly disease-free during the experiments, which lasted for up to six months following single or repeated mechanical inoculations with a virulent Hawaii isolate of PRV (Fitch et al., 1992). Still in question, however, was the level of resistance

under field conditions, especially when plants were exposed to viruliferous aphid vectors over long periods of time. Consequently, a two-year field trial was conducted in an area with a well developed PRV epiphytotic to determine the degree of PRV resistance in transgenic 55-1 R0 plants relative to control plants lacking the CP gene.

A split-plot experimental design was used in which main plots contained inoculation methods (mechanical versus aphid-vectored) and subplots contained genotype differences [CP-positive (CP+) transgenic R0 clone 55-1, CP-negative (CP-) transgenic clone 62-1 (Fitch et al., 1992) and CP-nontransgenic 'Sunset' seedlings]. The ten replicates were embedded in border rows of non-transgenic papayas, which were mechanically inoculated during July 1992, four months after planting in the field, to insure adequate disease pressure for the experiment. Half of the main plots were also mechanically inoculated at the same time, while the other half were left to be inoculated by naturally occuring aphid vectors. Twenty plants of each of the genotypes constituted the test population.

All CP- controls and border rows that were mechanically inoculated in the field developed PRV symptoms within 20 days after inoculation (Fig. 6). CP- controls that were left for inoculation by aphids took longer to become infected, but all developed symptoms within 2 to 4 months after the other plants in the experiment had been manually inoculated. While the inoculation method clearly affected the rate of disease symptom development among CP- controls, it had no significant effect on the severity of symptom expression at any evaluation date during the experiment (data not shown).

The CP gene had a highly significant effect on expression of PRV symptoms among the test plants. CP- control plants, whether transgenic or nontransgenic, were equally susceptible to viral infection, developing mild to moderate PRV symptoms over the first several months of the experiment. By the second year of the trial, the PRV-weakened CP- plants began to die of fungal root infections, reflecting a reduced ability to remove soil moisture from the root zone through transpiration. Two years after the first manual inoculation, none of the CP- controls was still alive, all having succumbed to a combination of PRV and root rot.

In marked contrast to the CP- plants, the CP+ 55-1 R0 cloned plants performed exceptionally well in the field, showing virtual immunity to PRV infection throughout the 2-year course of the experiment. In fact, the distinction between CP+ and CP- plants with respect to virus reaction was so obvious that even though all plants were rated for disease symptoms during the experiment, no quantitative assessment of disease severity was necessary or reported here. At the end of the trial, 12 of the 20 CP+ plants were still alive and without PRV symptoms, eight plants having died during the last six months of the experiment due to root rot.

ELISA tests conducted on each plant in the test population at each evaluation date confirmed the resistant character of CP+ clone 55-1. Over the course of the experiment, clone 55-1 had ELISA readings ranging from 0.000 to 0.084, in contrast to CP- controls, which had ELISA readings ranging from 0.157 to 2.138.

The PRV resistance provided by the CP gene positively influenced other characteristics of line 55-1 in the presence of the virus. In the above field trial, CP+ 55-1 R0 plants grew normally throughout the experiment, in contrast to infected CP- controls. There was a highly significant effect of the CP gene on tree vigor, as measured by stem diameter at 45 cm above ground level (Fig. 7). Trunk girth of 55-1 R0 plants continued to increase throughout the trial, whereas in CP- controls, it stopped enlarging after the first six months.

In Geneva, NY, R1 seedlings from a cross of R0 55-1 and 'Sunrise' were mechanically inoculated with PRV isolates from Hawaii and other parts of the world. These results were reported by Tennant et al. (1994). Transgenic 55-1 plants showed excellent resistance against three PRV isolates from Hawaii; only 5% of 128 inoculated plants became infected. However, 55-1 was not resistant to PRV isolates collected outside of Hawaii. For example, all 38 transgenic 55-1 plants became infected after being mechanically inoculated with PRV from Thailand.

In Hawaii, inoculation of CP+ R1 seedlings of 55-1 with a virulent Hawaiian strain of PRV resulted in 50% (11/23) becoming infected from two to five-months after inoculation in the greenhouse, while CP- controls were all infected at two months. After transplanting into the field, these plants showed relatively mild symptoms, although they had ELISA A405 values greater than 2.0. Recent data (not shown), however, show that R1 and R2 plants have good resistance in the field in Hawaii.

In Geneva, R1 seedlings of 63-1 line were mechanically inoculated with three PRV isolates from Hawaii (designated HA, Oahu, and Panaewa). Ten of 29 inoculated seedlings showed infection by HA, 9 of 22 by Oahu, and 2 of 22 by Panaewa. These results indicate that 63-1 could also provide effective resistance to PRV isolates from Hawaii. Furthermore, in contrast to line 55-1, only 9 of 19 transgenic seedlings inoculated with PRV from Thailand became infected. All inoculated control plants became infected.

D. Disease and Pest Characteristics of Transgenic Papaya

The most important disease (other than PRV) and pest problems observed during the field trials described above involved fungal root rot pathogens, presumed to be *Phytophthora* and/or *Pythium*, and "hopper

burn" caused by feeding of the leafhopper *Empoasca stevensii* in the papaya canopy. During the field trial of the R0 55-1 plants, root rot was much more evident among plants lacking the PRV CP gene, since these plants invariably became infected with PRV, which reduced the canopy area, the growth rate, and presumably the transpiration rate. The lower transpiration rate probably resulted in wetter conditions in the root zones of control plants, which favored the development of root rot.

*Empoasca stevensii* and symptoms of hopper burn were noted on all plants at various times during the three field trials involving transgenic papayas. Control was achieved by periodic spraying with Malathion as symptoms became apparent. No differences in susceptibility or symptom expression were noted among transgenic plants as opposed to controls.

Other minor disease and pest problems included the occurrence of powdery mildew *(Oidium caricae),* broadmites, and nematodes. Again, no differences in susceptibility or symptom expression were noted among transgenic plants as opposed to controls.

E. Nutritional Composition

Total soluble solids (sugar) content was compared in the field trial of line 55-1 R0 plants and CP-negative controls, using a refractometer to measure % brix. Sugar content in 65 fruits from 55-1 plants averaged significantly higher than the mean of six CP-negative controls (Table F). The control plants were infected with PRV at the time of the refractometer analyses. Similar tests were also performed on fruits on Rl trees of 55-1. These results are also given in Table F.

Table F. Total soluble solids (TSS) in transgenic lines relative to nontransgenic controls

| Line | n | Mean TSS (range) | |
|---|---|---|---|
| R0 generation | | | |
| 55-1 | 65 | 13.0 (9.6-15.4) | a[1] |
| CP- control | 6 | 11.3 (10.2-12.8) | b |
| Rl generation | | | |
| 55-1 | 130 | 13.3 (6.6-17.6) | a |

1= Values with different letters are significantly different at the 5% level of confidence.

Lipid-phase micronutrient analyses of fruits from eight papaya lines, including transgenic lines 55-1 (R2) and 46-1 x 63-1 (Fl), were performed by Dr.

Adrian Franke's lab at the Cancer Research Center of Hawaii, Molecular Carcinogenesis unit, at the University of Hawaii. These analyses were performed on pureed samples consisting of three fruits of each papaya line or cultivar. Total carotenoids in the two transgenic lines fell between the extremes of the range of carotenoid contents among the eight lines/cultivars, with line 55-1 being near the top of the range and F1 46-1 x 63-1 being near the bottom (Table G). See Appendix C for more information.

Table G. Total carotenoid content (µg/100g) of transgenic lines 55-1 and Fl 46-1 x 63-1 relative to other papaya cultivars.

| Cultivar/line | Total carotenoids |
|---|---|
| Kamiya | 4211 |
| line 55-1 (R2) | 3369 |
| line 655 | 3365 |
| Kuala Lumpur Yellow | 2972 |
| line 40 | 2795 |
| Kapoho | 2735 |
| line 46-1 x line 63-1 (Fl) | 2725 |
| Saipan Red | 2484 |

## VI. Environmental Consequences of the Introduction of the Transgenic Papaya

### A. Weediness

The Hawaii Department of Agriculture has not listed papaya among noxious weeds in the state, either currently or in Territorial days before PRV was widespread in the Islands. Since papaya was not considered a weed before PRV became an important limitation to commercial production, it seems logical that resistance to PRV will not contribute importantly to the weediness of cultivated papayas in Hawaii. Expert testimony from the mainland states of Florida, Texas, and California indicate that papaya is not considered a weed in any of these areas (see Appendix A). Furthermore, in Texas and California the factors limiting papaya weediness are environmental, including cold temperatures and lack of water, not PRV (see Appendix A).

### B. Transcapsidation and Heterologous Recombination

The likelihood of effective transcapsidation or heterologous recombination occurring between products of the CP gene and the genomes of infective viruses is greater if the invading virus is a related potyvirus. In Hawaii and elsewhere around the world, there are no other potyviruses,

besides PRV, that infect papaya. Since our work has established that the level of CP (and presumably m-RNA) produced in the leaves and fruits of transgenic papayas is lower than in naturally infected non-transgenic plants (see Table E), the likelihood of these events occurring in transgenic plants is less than currently exists in the Kapoho production area on the Big Island, where most of the commercial acreage is naturally infected with PRV. With regard to transcapsidation and heterologous recombination in Hawaii, transgenic plants are not likely to pose a greater threat than presently exists in nontransgenic commercial cultivars.

C. Native Floral and Faunal Communities

None of the transgene products has toxic qualities, so the effect on pollinators, animals that may consume the fruit or seeds, aquatic communities, or plant communities growing in the decaying remains of these plants, is expected to be insignificant. Since there are no wild papayas or related species in Hawaii, the possiblity of transfering transgenes inadvertantly to important sources of germplasm does not exist.

D. Statement of Grounds Unfavorable

To the extent that the transgenic papaya cultivars with the important attribute of PRV resistance contribute to narrowing of the papaya genotypes that are cultivated, this will be detrimental. One objective of our breeding program is to avoid this by introducing the CP gene into cultivars other than 'Sunset', both by transformation and by conventional breeding methods, to provide growers with a choice of plant materials.

The wide deployment of a single resistance gene, whether introduced into a crop via transformation or by conventional breeding, could result in the selection of PRV populations that can overcome the resistance. Ideally, it would be best to employ additional resistant genes, such as the replicase gene of PRV or genes from papaya that confer tolerance to PRV (Conover and Litz, 1978). In reality, the economic need for this PRV resistance trait is so great in Hawaii at the moment that transgenic lines 55-1 and 63-1 will distributed as rapidy as possible should these lines become deregulated.

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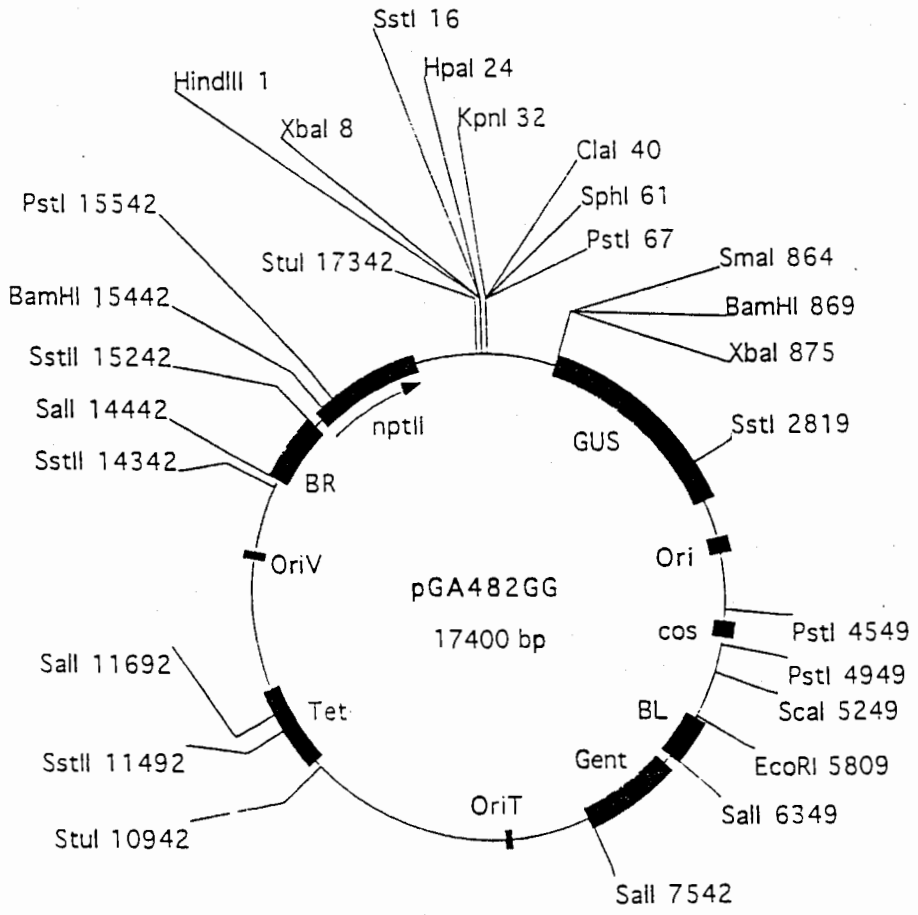



Fig. 1. Restriction enzyme sites on the binary vector pGA482GG (Quemada et al., 1991). The plant expression cassette containing the CMV-PRV coat protein gene was inserted into the *HindIII* site of pGA482GG to produce pGA482GG/cpPRV-4 (see Fig. 2; Ling et al., 1991).

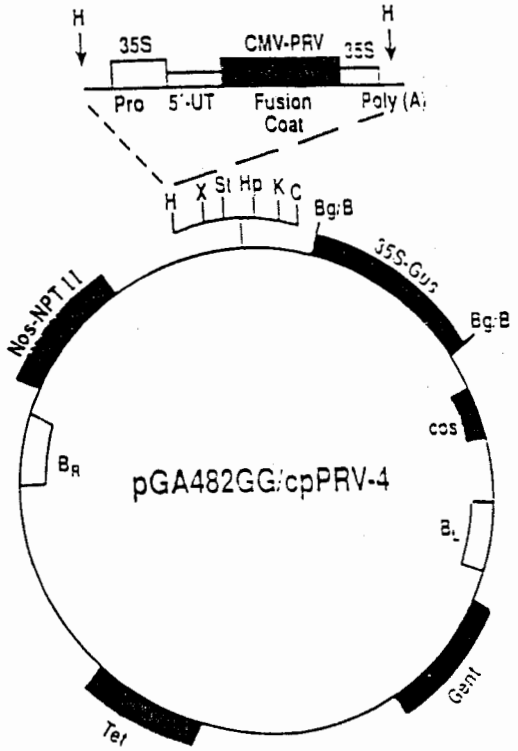



Fig. 2. Genetic map of the transformation vector pGA482GG/cpPRV-4 (Ling et al., 1991) that was used to coat tungsten particles for subsequent transformation of papaya via particle bombardment Fitch, et al. (1992).


Fig. 3. Nucleotide and amino acid sequence of the CMV-PRV fusion coat protein gene construct (Ling et al., 1991) that was used in transformation of transgenic 55-1 and 63-1 papaya (Fitch et al., 1992). The first 16 amino acids of the CMV-PRV protein are from the CMV coat protein gene. PRV is the intact coat protein gene using the cleavage site proposed by Quemada, et al. (1990).

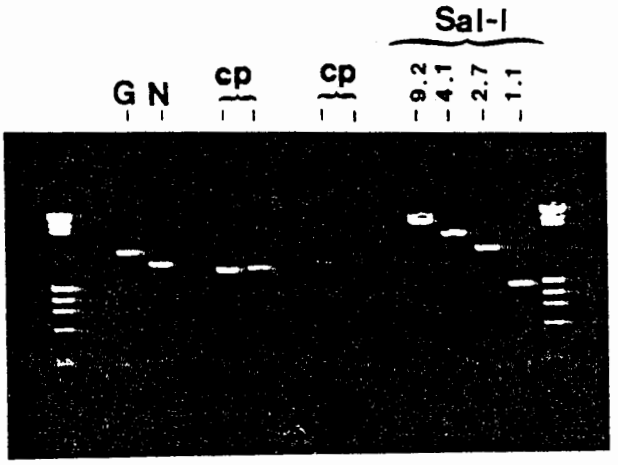
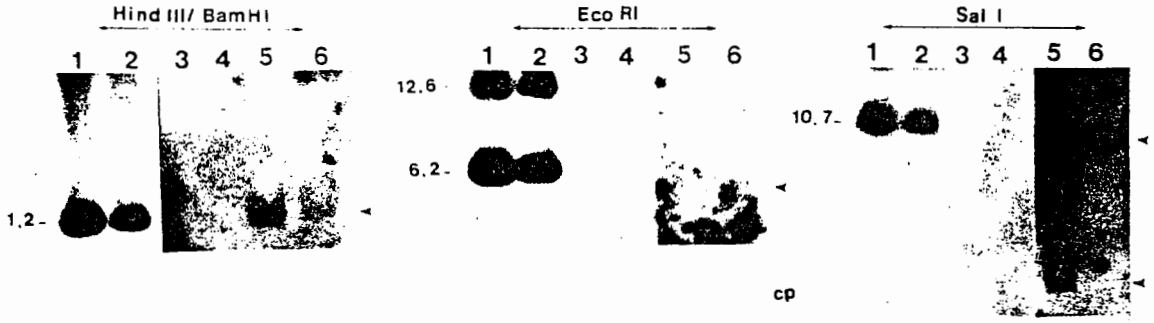



Fig 4. Agarose gel separating the DNA fragments used as probes for Southern blot analysis to determine integration of fragments inside and outside the T-DNA borders in the transgenic papaya lines. The probes used were obtained after *Sal* I digestion of the plasmid pGA482GG and included: a gentamycin gene probe (1.1 kbp), an OriT/tetracycline resistance gene probe (4.1 kb), an Ori V/tetracycline resistance gene probe (2.7 kb), and a T-DNA probe (9.2 kb). These probes represent 6.8%, 23.9%, 15.8% and 53.3% of the pGA482GG plasmid (17.3 kb), respectively. In addition, probes for the npt II (N), GUS (G) and PRV CP gene were also used to characterize the transgenic lines. Outside wells were loaded with the molecular size standards λ-*Hin*d III and ϕ-*Hae* III.

Fig 5. Southern blot analysis. Lanes 1 and 2 were loaded with 30 or 5 pg of the plasmid pGA482GG/cpPRV-4, respectively. Lanes 4 to 6 were loaded with 10 µg of plant DNA. Lane 3 was not loaded, lane 4 corresponds to DNA from a non-transgenic papaya 'Sunrise' plant , lane 5 to R1 transgenic 55-1, and lane 6 to R0 transgenic 63-1. Plasmid and plant DNA were digested with either *Hind* III/*Bam* HI, *Eco* RI or *Sal* I. Blots shown were hybridized with: **A.** the CP gene probe, **B.** the GUS gene probe (pG), **C.** the npt II gene probe (pN), or **D.** the OriT/Tet (4.1 kb), OriV/Tet (2.7 kb), and gentamycin resistance gene (1.1 kb) probes. Positive hybridization for genomic DNA of transgenic lines 55-1 (lane 5) and 63-1 (lane 6) indicates that the probed DNA fragment was integrated during bombardment. DNA from line 55-1 hybridized positive with the PRV CP, GUS, npt II, and OriT/Tet probes, while DNA from line 63-1 hybridized positive with the PRV CP, npt II, OriT/Tet, OriV/Tet, and gentamycin resistance gene probes, but did not hybridize positive with the GUS probe.

A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1,2-
Eco RI
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12.6
6.2
cp
Sal I
1 2 3 4 5 6
10.7-

B

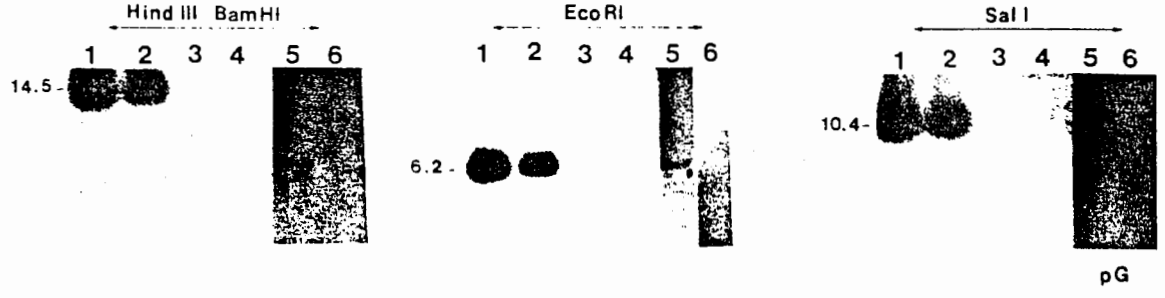
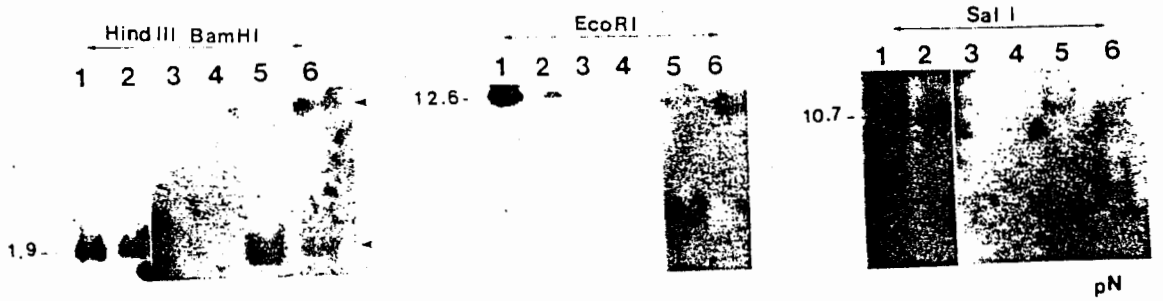
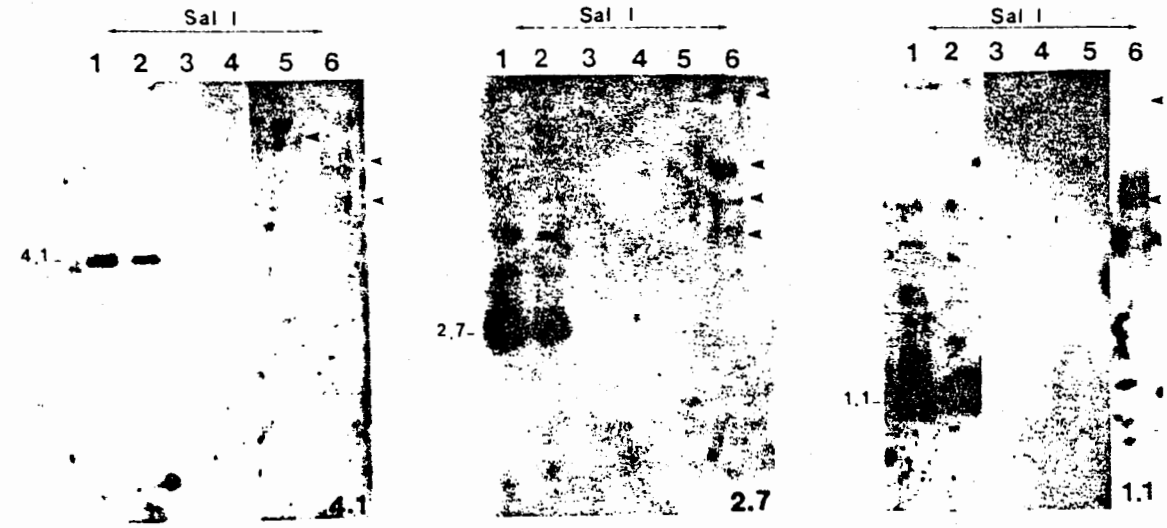

Hind III BamHI
1 2 3 4 5 6
14.5-
Eco RI
1 2 3 4 5 6
6.2-
Sal I
1 2 3 4 5 6
10.4-
pG

Figure 5 C-D

C




D



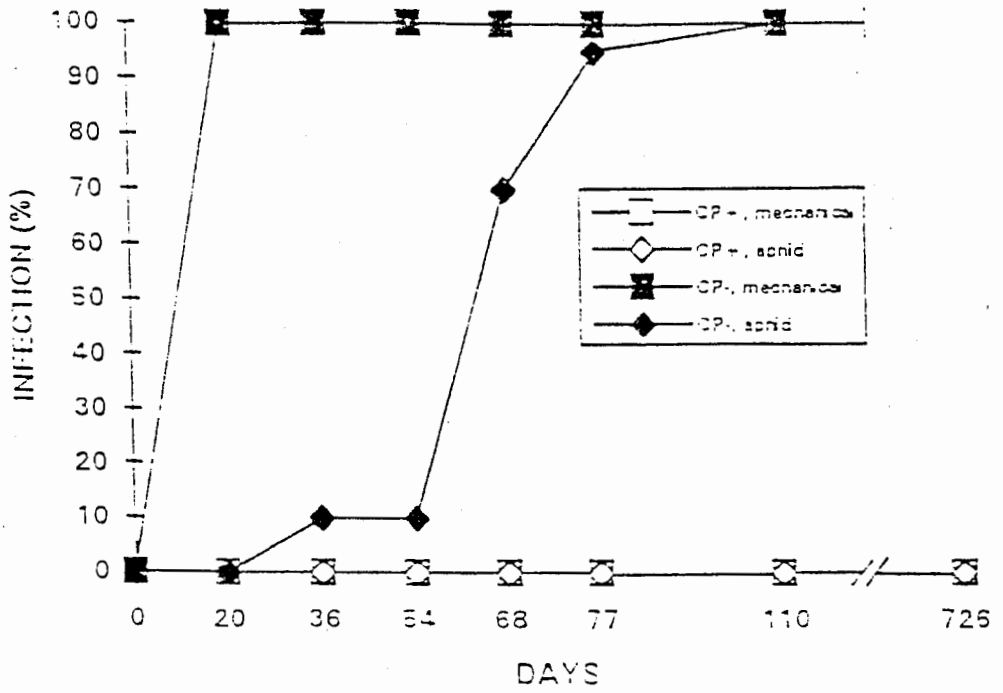



Fig. 6. Resistance of 55-1 R0 cloned plants (CP+) under field conditions in Hawaii. CP- plants were R0 transgenic plants that did not contain the PRV CP gene. Ten plants were in each category (aphid or mechanical inoculated)

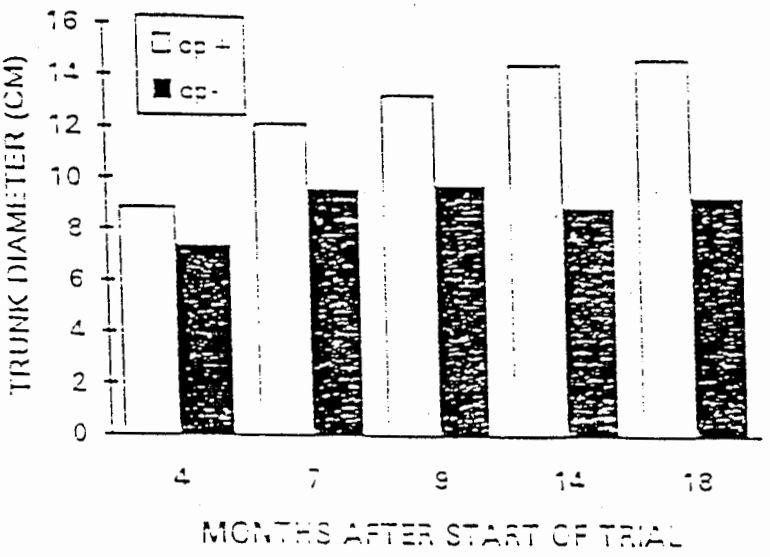



Fig. 7. Relative trunk diameter of transgenic 55-1 R0 cloned papaya 'Sunset' and RO cloned transgenic papaya 'Sunset' without the CP gene of PRV grown under field conditions in Hawaii under severe PRV disease pressure. Measurements are averaged from 18-20 test plants.

Appendix A-1 CARL W. CAMPBELL ~~Appendix 1~~

FLORIDA COOPERATIVE EXTENSION SERVICE
UNIVERSITY OF FLORIDA
INSTITUTE OF FOOD AND AGRICULTURAL SCIENCES

COOPERATIVE EXTENSION SERVICE — AGRICULTURAL EXPERIMENT STATION
SCHOOL OF FOREST RESOURCES AND CONSERVATION — COLLEGE OF AGRICULTURE

21 July 1995

Dr. Richard Manshardt
Dept. of Horticulture
Univ. of Hawaii at Manoa
Fax 808-956-3894

REPLY TO: Carl W. Campbell
University of Florida
Tropical Res. + Edn. Center
Homestead, Florida
33032
Fax 305-246-7003

Dear Richard:

As you undoubtedly know, "wild" papaya have been growing in Florida for centuries. Botanists generally do not consider papaya do being native to Florida, but they have been here at least since the first European settlement (and in my opinion were probably brought before that by Caribe people from the West Indies).

Nobody considers papaya to be a weed nuisance in Florida agriculture in the usual sense of weediness. In one way, however, some would consider that wild papaya plants are undesirable, because they apparently can carry the PRV pathogen without showing symptoms of the disease, and thus serve as a reservoir for future re-infection of cultivated papayas.

Dr. Bob McMillan has worked with papaya diseases and could enlarge upon what I have said (fax number above). Dr. Julian Saucedo works for the Texas Cooperative Extension Service at Weslaco, Texas, and he knows papayas well (mostly from his days in Florida). His fax number is 210-969-5639. I hope this info helps

Appendix A-2

Richard Manshardt

Fax 808/956-3894

I cannot consider papaya as a weed under any circumstances in Texas, regardless of its potential freedom from virus diseases.

Irrespective of any other aspect of papaya, cold intolerance would still limit papaya longevity in South Texas to that of the next frost or severe freeze.

Moreover, without cultivation, papaya does not survive in South Texas because of inadequate rainfall — i.e., without supplemental irrigation, papaya cannot survive in the wild under our weather conditions of $\leq$ 25 inches annual precipitation.

Hope this information helps.

Richard D Sands

Appendix 4-3

33

# COOPERATIVE EXTENSION

UNIVERSITY OF CALIFORNIA
Riverside, California 92521-0124

Department of
Botany and Plant Sciences

REPLY TO:
Batchelor Hall Extension
Telephone: (909) 787-3335
FAX: (909) 787-5717

August 15, 1995

Richard Manshardt
Department of Horticulture
University of Hawaii at Manoa
Honolulu, HI 96822

Dear Dr. Manshardt:

I am writing in response to your inquiry regarding the weed status of papaya in Southern California. Papaya, is not widely established as either a commercial crop or backyard fruit. This is primarily due to the papaya's sensitivity in low but non-freezing temperatures which are commonly encountered during winter months.

It is my opinion that papaya poses a minimal risk for establishing itself as a weed pest in Southern California.

Sincerely,

Mary Lu Arpaia
Extension Subtropical Horticulturist

MLA:sbd

Appendix B

Papaya Southern blot results

| probe | sample | *Hind* III /*Bam* H1 | *Eco* RI | *Sal* I |
|---|---|---|---|---|
| 1.1 Kb ($GENT^R$) | plasmid | 14,573 | 12,630 | 1,193 (Gent gene) |
| | control - | - | - | - |
| | 55-1 | - | - | - |
| | 63-1 | + | + | + (Gent gene) |
| 2.7 Kb (OriV/Tet) | plasmid | 14,573 | 12,630 | 2,750 (Ori V/Tet) |
| | control - | - | - | - |
| | 55-1 | - | - | - |
| | 63-1 | + | + | + (Ori V/Tet) |
| 4.1 Kb (OriT/Tet) | plasmid | 14,573 | 12,630 | 4,150 (Ori T/Tet) |
| | control - | - | - | - |
| | 55-1 | - | - | + (Ori T/Tet) |
| | 63-1 | + | + | + (Ori T/Tet) |
| N (NPTII gene) | plasmid | 1,900 (NPTII gene) | 12,630 | 10,774 |
| | control - | - | - | - |
| | 55-1 | + (NPTII gene) | + | + |
| | 63-1 | + (NPTII gene) | + | + |
| PRVcp (CP gene) | plasmid | 1,235 (cp cassette) | 6,237<br>12,630 | 10,774 |
| | control - | - | - | - |
| | 55-1 | + (cp cassette) | + | + |
| | 63-1 | + (cp cassette) | + | + |
| G (GUS gene) | plasmid | 14,573 | 6,237 (GUS gene) | 10,774 |
| | control - | - | - | - |
| | 55-1 | + | + (GUS gene) | + |
| | 63-1 | - | - | - |

**Results:**

Line 55-1 does not contain the gentamycin resistance gene (1.1 probe) or the Ori V/Tet region (2.7 probe), but it contains the Ori T/Tet region (4.1 probe)

outside the T-DNA borders. The NPTII, PRV CP and GUS genes inside the T-DNA borders are all present in line 55-1.

Line 63-1 contains the gentamycin resistance gene (1.1 probe), Ori V Tet region (2.7 probe) and Ori T Tet region (4.1 probe). Some rearrangements of inserted genes may have occurred during integration since multiple bands are observed on these line on the *Hind* III/*Bam* H1 digests, and the 1.1 band is located at a much higher molecular weight than expected.

Appendix C-1

Carot's Papaya7/95-short

~~Appendix B~~

**These are the carotenoid levels in papaya samples from Richard Manshardt-UH/J. Bertram-CRCH. Extracted and run 18-20 July 95 by Laurie Custer+Adrian Franke**

**Protocol Apr-July1994**

Internal standards= Tocol (100%-20 uL in 1mL); BACOX

NON-ESTERIFIED LEVELS

| Food Item | | tr-LUT [ug/100g] | tr-ZEA [ug/100g] | tr-LUT/ZEA [ug/100g] | cis-LUT/ZEA [ug/100g] | AH-LUT [ug/100g] | cis AH-LUT [ug/100g] | a-CRX [ug/100g] | b-CRX [ug/100g] | cis b-CRX [ug/100g] | LYC [ug/100g] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ln655 | 6 nsp | 19.8 | 0.0 | 19.8 | 42.1 | 25.9 | 23.0 | 0.0 | 117.9 | 18.4 | 1609.5 |
| Km3(1) | 2a nsp | 16.7 | 0.0 | 16.7 | 63.0 | 43.5 | 73.4 | 0.0 | 209.2 | 40.9 | 0.0 |
| 55-1(1) | 2b nsp | 12.4 | 0.0 | 12.4 | 48.4 | 7.8 | 22.0 | 0.0 | 117.3 | 20.1 | 945.7 |
| BrR(8) | 3a nsp | 18.3 | 0.0 | 18.3 | 93.1 | 71.8 | 55.8 | 0.0 | 205.4 | 6.9 | 557.0 |
| 46-1x63-1 | 3b nsp | 22.2 | 0.0 | 22.2 | 5.6 | 19.1 | 20.3 | 0.0 | 88.1 | 12.0 | 848.4 |
| Ln40 | 4a nsp | 16.9 | 0.0 | 16.9 | 25.3 | 15.8 | 36.9 | 0.0 | 202.7 | 22.0 | 0.0 |
| KLyellow | 4b nsp | 22.9 | 0.0 | 22.9 | 35.0 | 8.9 | 38.5 | 0.0 | 163.8 | 20.5 | 0.0 |
| KaNumo(4) | 5a nsp | 16.1 | 0.0 | 16.1 | 38.1 | 9.3 | 26.1 | 0.0 | 130.1 | 25.8 | 0.0 |

ESTERIFIED LEVELS

| Food Item | tr-LUT [ug/100g] | tr-ZEA [ug/100g] | tr-LUT/ZEA [ug/100g] | cis-LUT/ZEA [ug/100g] | AH-LUT [ug/100g] | cis AH-LUT [ug/100g] | a-CRX [ug/100g] | b-CRX [ug/100g] | cis b-CRX [ug/100g] | LYC [ug/100g] |
|---|---|---|---|---|---|---|---|---|---|---|
| Ln655 | 120.4 | 104.4 | 224.8 | 40.0 | 44.8 | 7.8 | 0.0 | 631.9 | 13.9 | 0.0 |
| Km3(1) | 399.6 | 132.4 | 532.0 | 195.0 | 410.6 | 88.3 | 0.0 | 2219.6 | 27.7 | 0.0 |
| 55-1(1) | 175.6 | 100.3 | 275.9 | 71.7 | 135.2 | 42.8 | 0.0 | 925.9 | 11.7 | 0.0 |
| BrR(8) | 171.5 | 17.8 | 189.3 | 79.8 | 105.3 | 28.0 | 0.0 | 480.4 | 21.6 | 0.0 |
| 46-1x63-1 | 147.9 | 113.0 | 260.9 | 108.8 | 56.6 | 27.2 | 0.0 | 682.5 | 26.0 | 0.0 |
| Ln40 | 314.5 | 142.0 | 456.5 | 116.4 | 128.3 | 76.8 | 0.0 | 1413.6 | 47.2 | 0.0 |
| KLyellow | 310.4 | 234.2 | 544.5 | 45.7 | 74.0 | 50.0 | 0.0 | 1483.6 | 49.6 | 0.0 |
| KaNumo(4) | 418.0 | 37.0 | 455.1 | 108.6 | 216.5 | 102.2 | 0.0 | 1426.2 | 37.1 | 0.0 |

TOTAL LEVELS

| Food Item | | tr-LUT [ug/100g] | tr-ZEA [ug/100g] | tr-LUT/ZEA [ug/100g] | cis-LUT/ZEA [ug/100g] | AH-LUT [ug/100g] | cis AH-LUT [ug/100g] | a-CRX [ug/100g] | b-CRX [ug/100g] | cis b-CRX [ug/100g] | LYC [ug/100g] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ln655 | 6 sp | 140.2 | 104.4 | 244.5 | 82.1 | 70.7 | 30.8 | 0.0 | 749.8 | 32.4 | 1609.5 |
| Km3(1) | 2a sp | 416.3 | 132.4 | 548.7 | 258.0 | 454.1 | 161.7 | 0.0 | 2428.7 | 68.6 | 0.0 |
| 55-1(1) | 2b sp | 188.0 | 100.3 | 288.2 | 120.1 | 143.0 | 64.8 | 0.0 | 1043.2 | 31.8 | 945.7 |
| BrR(8) | 3a sp | 189.8 | 17.8 | 207.7 | 172.8 | 177.1 | 83.9 | 0.0 | 685.8 | 28.6 | 557.0 |
| 46-1x63-1 | 3b sp | 170.2 | 113.0 | 283.1 | 114.4 | 75.8 | 47.5 | 0.0 | 770.6 | 38.0 | 848.4 |
| Ln40 | 4a sp | 331.3 | 142.0 | 473.4 | 141.7 | 144.1 | 113.7 | 0.0 | 1616.3 | 69.3 | 0.0 |
| KLyellow | 4b sp | 333.3 | 234.2 | 567.5 | 80.8 | 82.9 | 88.5 | 0.0 | 1647.4 | 70.0 | 0.0 |
| KaNumo(4) | 5a sp | 434.1 | 37.0 | 471.1 | 146.7 | 225.7 | 128.4 | 0.0 | 1556.4 | 62.9 | 0.0 |

0=absent/below detection limit

37 Appendix B-2



NON-ESTERIFIED LEVELS

| DH-LYC [ug/100g] | tot. LYC [ug/100g] | aCAR [ug/100g] | bCAR [ug/100g] | cis-bCAR [ug/100g] | TOTAL tot. b.-CAR [ug/100g] | CAROT's [ug/100g] | RET [ug/100g] | dTOC [ug/100g] | gTOC [ug/100g] | aTOC [ug/100g] | RETPALM [ug/100g] | Food Item |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 233.7 | 1843.2 | 64.7 | 246.8 | 0.0 | 246.8 | 2401.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Ln655 |
| 0.0 | 0.0 | 0.0 | 291.2 | 0.0 | 291.2 | 737.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Km3(1) |
| 344.2 | 1289.9 | 63.2 | 325.2 | 0.0 | 325.2 | 1906.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 55-1(1) |
| 229.1 | 786.1 | 35.7 | 306.6 | 0.0 | 306.6 | 1579.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | SrR(8) |
| 239.2 | 1087.7 | 66.2 | 241.4 | 0.0 | 241.4 | 1562.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 46-1x63-1 |
| 0.0 | 0.0 | 0.0 | 236.3 | 0.0 | 236.3 | 555.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Ln40 |
| 0.0 | 0.0 | 0.0 | 434.5 | 0.0 | 434.5 | 724.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | KLyellow |
| 0.0 | 0.0 | 0.0 | 143.6 | 0.0 | 143.6 | 389.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | KaNumo(4) |

ESTERIFIED LEVELS

| DH-LYC [ug/100g] | tot. LYC [ug/100g] | aCAR [ug/100g] | bCAR [ug/100g] | cis-bCAR [ug/100g] | TOTAL tot. b.-CAR [ug/100g] | CAROT's [ug/100g] | RET [ug/100g] | dTOC [ug/100g] | gTOC [ug/100g] | aTOC [ug/100g] | RETPALM [ug/100g] | Food Item |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 963.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Ln655 |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3473.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Km3(1) |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1463.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 55-1(1) |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 904.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | SrR(8) |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1162.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 46-1x63-1 |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2238.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Ln40 |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2247.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | KLyellow |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2345.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | KaNumo(4) |

TOTAL LEVELS

| DH-LYC [ug/100g] | tot. LYC [ug/100g] | aCAR [ug/100g] | bCAR [ug/100g] | cis-bCAR [ug/100g] | TOTAL tot. b.-CAR [ug/100g] | CAROT's [ug/100g] | RET [ug/100g] | dTOC [ug/100g] | gTOC [ug/100g] | aTOC [ug/100g] | RETPALM [ug/100g] | Food Item |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 233.7 | 1843.2 | 64.7 | 246.8 | 0.0 | 246.8 | 3364.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Ln655 |
| 0.0 | 0.0 | 0.0 | 291.2 | 0.0 | 291.2 | 4211.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Km3(1) |
| 344.2 | 1289.9 | 63.2 | 325.2 | 0.0 | 325.2 | 3369.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 55-1(1) |
| 229.1 | 786.1 | 35.7 | 306.6 | 0.0 | 306.6 | 2484.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | SrR(8) |
| 239.2 | 1087.7 | 66.2 | 241.4 | 0.0 | 241.4 | 2724.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 46-1x63-1 |
| 0.0 | 0.0 | 0.0 | 236.3 | 0.0 | 236.3 | 2794.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | Ln40 |
| 0.0 | 0.0 | 0.0 | 434.5 | 0.0 | 434.5 | 2971.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | KLyellow |
| 0.0 | 0.0 | 0.0 | 143.6 | 0.0 | 143.6 | 2734.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | KaNumo(4) |

0=absent/below detection limit

38 Appendix E-3



| | | |
|---|---|---|
| Tot.t-LUT/ZEA | = | Total (trans-Lutein+trans-Zeaxanthin) |
| t-AH-LUT | = | trans-Anhydrolutein |
| cis-AH-LUT | = | cis-Anhydrolutein |
| a-CRX | = | alpha- Cryptoxanthin |
| b-CRX | = | beta- Cryptoxanthin |
| cis-CRX | = | cis-beta- Cryptoxanthin |
| LYC | = | Lycopene |
| DH-LYC | = | Dihydrolycopene |
| a-CAR | = | alpha-Carotene |
| tot. b-CAR | = | tr-beta-Carotene+cis-beta-Carotene |
| TOT.CAR | = | ALL CAROTENOIDS |
| Retinol | = | Total Retinol |
| g-Toc. | = | gamma-Tocopherol |
| a-Toc. | = | alpha-Tocopherol |
| ZEA | = | trans-Zeaxanthin |
| LUT | = | trans-Lutein |
| Ret.Palm | = | Retinol-Palmitate |

| | LUT/ZEA [ug/100g] | b-CRX [ug/100g] | LYC [ug/100g] | aCAR [ug/100g] | bCAR [ug/100g] | |
|---|---|---|---|---|---|---|
| [1] | n/a | 470 | 0 | 0 | 38-160;median=99 | [1] |
| [2] | n/a | 470 | n/a | n/a | 99 | [2] |
| [3] | n/a | 665 | n/a | n/a | 409 | [3] |


[1] Mangels et al. 1993: J. Am. Diet. Assoc. 93, 284-296 reviewing the literature from 1971-1991 and incorporating reports from:
a) Speek et al. 1988: Food Chem. 27, 245-257, and
b) Popping et al. 1988: J. Sci. Food Agric. 45, 359-371.

[2] USDA-NCI Carotenoid Food Composition Database, Version1-1993.
*This is more or less based on data in [1]!*

[3] Gross, J. 1987: Pigments in Fruits; Academic Press, London, including data from:
a) Subbarayan & Cama 1964: Ind. J. Chem. 2, 451-454
b) Yamamoto 1964: Nature 201, 1049-1050.
*These data are old...*

# Notices

Federal Register
Vol. 61, No. 87
Friday, May 3, 1996

## Animal and Plant Health Inspection Service

[Docket No. 96-024-1]

## Cornell University and University of Hawaii; Receipt of Petition for Determination of Nonregulated Status for Papaya Lines Genetically Engineered for Virus Resistance

**AGENCY:** Animal and Plant Health Inspection Service, USDA.

**ACTION:** Notice.

**SUMMARY:** We are advising the public that the Animal and Plant Health Inspection Service has received a petition from Cornell University and the University of Hawaii seeking a determination of nonregulated status for papaya lines designated as 55-1 and 63-1 that have been genetically engineered for virus resistance. The petition has been submitted in accordance with our regulations concerning the introduction of certain genetically engineered organisms and products. In accordance with those regulations, we are soliciting public comments on whether these papaya lines present a plant pest risk.

**DATES:** Written comments must be received on or before July 2, 1996.

**ADDRESSES:** Please send an original and three copies of your comments to Docket No. 96-024-1, Regulatory Analysis and Development, PPD, APHIS, Suite 3C03, 4700 River Road Unit 118, Riverdale, MD 20737-1238. Please state that your comments refer to Docket No. 96-024-1. A copy of the petition and any comments received may be inspected at USDA, room 1141, South Building, 14th Street and Independence Avenue SW., Washington, DC, between 8 a.m. and 4:30 p.m., Monday through Friday, except holidays. Persons wishing access to that room to inspect the petition or comments are asked to call in advance of visiting at (202) 690-2817.

**FOR FURTHER INFORMATION CONTACT:** Dr. Keith Reding, Biotechnology Permits, BBEP, APHIS, Suite 5B05, 4700 River Road Unit 147, Riverdale, MD 20737-1237; (301) 734-7612. To obtain a copy of the petition, contact Ms. Kay Peterson at (301) 734-7612; e-mail: mkpeterson@aphis.usda.gov.

**SUPPLEMENTARY INFORMATION:** The regulations in 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason to Believe Are Plant Pests," regulate, among other things, the introduction (importation, interstate movement, or release into the environment) of organisms and products altered or produced through genetic engineering that are plant pests or that there is reason to believe are plant pests. Such genetically engineered organisms and products are considered "regulated articles."

The regulations in § 340.6(a) provide that any person may submit a petition to the Animal and Plant Health Inspection Service (APHIS) seeking a determination that an article should not be regulated under 7 CFR part 340. Paragraphs (b) and (c) of § 340.6 describe the form that a petition for determination of nonregulated status must take and the information that must be included in the petition.

On February 20, 1996, APHIS received a petition (APHIS Petition No. 96-051-01p) from Cornell University, Geneva, NY, and the University of Hawaii, Honolulu, HI (Cornell/Hawaii), requesting a determination of

nonregulated status under 7 CFR part 340 for papaya lines designated as 55–1 and 63–1 that have been genetically engineered to contain genes that confer virus resistance. The Cornell/Hawaii petition states that papaya lines 55–1 and 63–1 should not be regulated by APHIS because they do not present a plant pest risk.

As described in the petition, papaya (*Carica papaya*) lines 55–1 and 63–1 have been genetically engineered to express the coat protein gene of papaya ringspot virus (PRV), strain HA5–1, which confers resistance to PRV. Both the subject papaya lines also contain the selectable marker gene *nptII*, and line 55–1 contains the *gus* selectable marker gene. In addition, Expression of the added genes is controlled by the untranslated 3' region of the nopaline synthase gene from *Agrobacterium tumefaciens* and the 35S promoter and 35S terminator from the plant pathogen cauliflower mosaic virus (CAMV). In developing lines 55–1 and 63–1, the microprojectile process was used to transfer the introduced gene sequences into the gynodioecious cultivar Sunset. The Sunset cultivar is of commercial importance in Hawaii, where PRV is a serious plant pest of papaya.

The subject papaya lines have been considered regulated articles under the regulations in 7 CFR part 340 because they contain gene sequences from the plant pathogens mentioned above. The subject papaya lines have been evaluated in field trials conducted under APHIS permits. In the process of reviewing the applications for field trials of lines 55–1 and 63–1, APHIS determined that the vectors and other elements were disarmed and that the trials, which were conducted under conditions of reproductive and physical containment or isolation, would not present a risk of plant pest introduction or dissemination.

In the Federal Plant Pest Act, as amended (7 U.S.C. 150aa *et seq.*), "plant pest" is defined as "any living stage of: Any insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof, viruses, or any organisms similar to or allied with any of the foregoing, or any infectious substances, which can directly or indirectly injure or cause disease or damage in any plants or parts thereof, or any processed, manufactured or other products of plants." APHIS views this definition very broadly. The definition covers direct or indirect injury, disease, or damage not just to agricultural crops, but also to plants in general, for example, native species, as well as to organisms that may be beneficial to plants, for example, honeybees, rhizobia, etc.

The Food and Drug Administration (FDA) published a statement of policy on foods derived from new plant varieties in the Federal Register on May 29, 1992 (57 FR 22984–23005). The FDA statement of policy includes a discussion of FDA's authority for ensuring food safety under the Federal Food, Drug, and Cosmetic Act, and provides guidance to industry on the scientific considerations associated with the development of foods derived from new plant varieties, including those plants developed through the techniques of genetic engineering.

In accordance with § 340.6(d) of the regulations, we are publishing this notice to inform the public that APHIS will accept written comments regarding the Petition for Determination of Nonregulated Status from any interested person for a period of 60 days from the date of this notice. The petition and any comments received are available for public review, and copies of the petition may be ordered (see the **ADDRESSES** section of this notice).

After the comment period closes, APHIS will review the data submitted by the petitioner, all written comments received during the comment period, and any other relevant information. Based on the available information, APHIS will furnish a response to the petitioner, either approving the petition in whole or in part, or denying the petition. APHIS will then publish a notice in the Federal Register announcing the regulatory status of the Cornell/Hawaii papaya lines 55–1 and 63–1 and the availability of APHIS' written decision.

Authority: 7 U.S.C. 150aa-150jj, 151–167, and 1622n; 31 U.S.C. 9701; 7 CFR 2.22, 2.80, and 371.2(c).

Done in Washington, DC, this 29th day of April 1996.

Lonnie J. King,

*Administrator, Animal and Plant Health Inspection Service.*

[FR Doc. 96–11016 Filed 5–2–96; 8:45 am]

BILLING CODE 3410–34–P