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Safety of Virus-Resistant Transgenic Plants Two Decades After Their Introduction: Lessons from Realistic Field Risk Assessment Studies*

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Key Words

pathogen-derived resistance, virus transgenes, RNA silencing, perceived safety issues, real risks, benefits

Abstract

Potential safety issues have been raised with the development and release of virus-resistant transgenic plants. This review focuses on safety assessment with a special emphasis on crops that have been commercialized or extensively tested in the field such as squash, papaya, plum, grape, and sugar beet. We discuss topics commonly perceived to be of concern to the environment and to human health—heteroencapsidation, recombination, synergism, gene flow, impact on nontarget organisms, and food safety in terms of allergenicity. The wealth of field observations and experimental data is critically evaluated to draw inferences on the most relevant issues. We also express inside views on the safety and benefits of virus-resistant transgenic plants, and recommend realistic risk assessment approaches to assist their timely deregulation and release.

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TMV: *Tobacco mosaic virus*

INTRODUCTION

The successful development of virus-resistant transgenic plants in the mid-1980s (74) heralded a new era in the control of plant viruses. This novel way to develop virus-resistant plants proved particularly relevant in cases for which host resistance genes had not been successfully incorporated into susceptible cultivars through hybridization and introgression. Alternatively, virus resistance could be achieved by transferring a viral gene into the genome of a target plant. In 1991, however, DeZoeten published a timely and provocative editorial entitled "Risk assessment: do we let history repeat itself?" (17). The editorial challenged scientists to assess environmental risks associated with virus-resistant transgenic plants. In 1992, the United States Department of Agriculture (USDA) initiated a competitive research grants program on risk assessment of transgenic plants (5). A major goal of the funding program was to obtain research information that would help regulatory agencies make science-based decisions on the safe release of transgenic crops, including virus-resistant transgenic crops. This program, still ongoing, has supported numerous studies on risk assessment of virus-resistant transgenic plants (5).

This review aims to analyze science-based safety assessment studies of virus-resistant transgenic plants. After we describe the application of pathogen-derived resistance (PDR) to control plant viruses and analyze the benefits of virus-resistant transgenic crops, we evaluate the wealth of field observations and experiments to distinguish real from perceived risks. Potential safety issues associated with virus-resistant transgenic plants include heteroencapsidation, recombination, synergism, gene flow, effect on nontarget organisms, and food safety in terms of allergenicity. Although many studies have addressed these potential risks over the past 15 years, this review focuses only on those that have been carried out in the field and preferentially with crops that have been commercialized or extensively tested in the field. Why? We believe that field studies provide conditions for a realistic assessment of the environmental impact of virus-resistant transgenic plants. Our goal is to critically examine evidence from field safety assessment studies and determine the significance of potential risks to help decide whether virus-resistant transgenic crops will take their place in agriculture two decades after their field release. Are there sufficient data available to draw science-based conclusions on the real effects of any of the above-mentioned safety considerations? If not, what issues do we still need to address in order to assist regulatory officials? Can some requirements for virus-resistant transgenic plants be harmonized between countries in order to make the regulatory process less costly and approvals more timely? And, based on the extensive safety assessment data and history of safe commercial use of virus-resistant transgenic crops, is it time to focus more squarely on other factors that affect their deregulation and release?

PATHOGEN-DERIVED RESISTANCE AND PLANT VIRUSES

PDR (82) is a phenomenon whereby transgenic plants containing genes or sequences of a parasite are protected against detrimental effects of the cognate or related pathogens. The application of PDR to plant viruses was first demonstrated by Beachy's group who showed that tobacco (74) and tomato (69) plants expressing the coat protein (CP) gene of TMV (*Tobacco mosaic virus*) exhibited resistance or delayed infection when challenge-inoculated with TMV. The practical effectiveness of PDR for controlling plant viruses has been firmly established through numerous independent studies (9, 61, 65, 94, 101). In fact, resistance has been achieved against nearly all families of plant viruses in numerous crops by applying PDR.

Initially, the dogma was that a viral CP had to be expressed in order to provide

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resistance. Numerous studies showed that transgenic plants with viral CP indeed provided specific resistance against the viruses with identical or similar CPs. However, over the past decade, it has been shown that most examples of PDR for plant viruses is RNA-mediated and occurs through the mechanism of posttranscriptional gene silencing (PTGS), which is also now commonly referred to as the antiviral pathways of RNA silencing (61, 101–103). Basically, induction of RNA silencing using a viral transgene causes specific degradation of the genome of the invading cognate virus and those that have high sequence homology to the viral transgene, resulting in a resistance phenotype. Full-length and truncated viral gene constructs, i.e., CP, RNA-dependent RNA polymerase (RdRp), proteinase, movement protein, satellite RNA, defective interfering RNA, and noncoding regions, have been used to confer resistance. It has also been shown that certain viral genes can suppress RNA silencing, and thus lessen the effectiveness of transgenic resistance in plants via PDR (76, 102, 104). Notwithstanding, despite the tremendous advances on the mechanism of PDR, the approach for identifying resistant phenotypes is still empirical. Basically, following transformation, resulting plant transformants are screened for resistance to the target virus by inoculation. Selected advanced transgenic lines are then more fully characterized as they approach potential commercialization, including a thorough assessment of their potential risks to the environment and food safety.

BENEFITS OF VIRUS-RESISTANT TRANSGENIC PLANTS

Resistance is the most effective way of controlling plant viruses. In addition to conferring a resistance phenotype, several properties of the PDR approach are appealing. First, virus resistance can be incorporated into a plant without changing its intrinsic phenotypic properties, something that is virtually impossible to achieve with conventional breeding. Second, the same resistant gene can be incorporated into different plant genera and species that are affected by a given virus and are amenable to transformation and regeneration. Third, resistance can be incorporated into vegetatively propagated plants, some of which may be impossible to ameliorate through conventional breeding due to genetic incompatibility or linkage to undesired traits. In the next section, we analyze the benefits of virus-resistant transgenic crops that have been deregulated and commercialized in the United States, including squash and papaya.

ZYMV: *Zucchini yellow mosaic virus*

WMV: *Watermelon mosaic virus*

CMV: *Cucumber mosaic virus*

Transgenic Squash Resistant to *Cucumber mosaic virus*, *Zucchini yellow mosaic virus*, and *Watermelon mosaic virus*

Virus-resistant transgenic summer squash (*Cucurbita pepo* spp. *ovifera* var. *ovifera*) lines ZW-20 and CZW-3 have been successfully developed (29, 33, 87, 97) and received exemption status from regulation in 1994 (68) and 1996 (1), respectively. Plants of line ZW-20 express the CP gene of ZYMV (*Zucchini yellow mosaic virus*) and WMV (*Watermelon mosaic virus*) and are resistant to these two viruses (7, 15, 28, 56, 97). Plants of line CZW-3 express the CP genes of CMV (*Cucumber mosaic virus*), ZYMV, and WMV, and are resistant to these three viruses (31, 85, 97) (**Figure 1*a***). Transgenic lines ZW-20 and CZW-3 have also been successfully used as progenitors to develop numerous virus-resistant summer squash cultivars by conventional breeding (6). Pyramiding CP gene constructs derived from two or three viruses has been an effective approach to develop squash cultivars with resistance to multiple viruses. Similar degrees of resistance to several viruses have not been achieved in elite summer squash cultivars by conventional breeding.

The commercial release of squash cultivars derived from lines ZW-20 and CZW-3 has demonstrated the stability and durability

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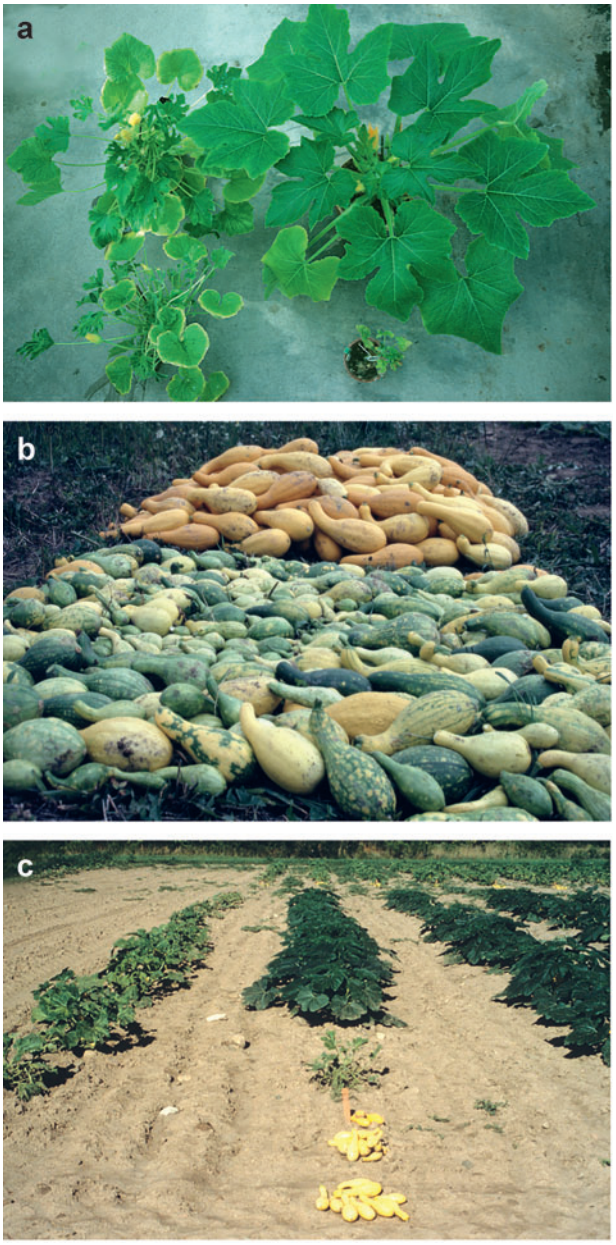

Figure 1

(*a*) Squash expressing the CP gene of ZYMV, WMV, and CMV is highly resistant to mechanical inoculation with a mixture of these three viruses (*upper right*), whereas control plants inoculated with only one of these three viruses are readily infected and exhibit severe symptoms (*left* and *lower right*). (*b*) Comparative fruit yield of healthy virus-resistant transgenic crookneck squash (*back*) and virus-infected nontransgenic squash (*front*). (*c*) Resistance of transgenic squash to aphid-mediated virus transmission (*center* and *right rows*) from border virus source plants (*left row*) under conditions where no insecticide was applied.

of the engineered resistance over more than a decade. In addition, virus-resistant transgenic squash allowed growers to restore their initial yields in the absence of viruses with a net benefit of $22 million in 2005 (87). A 50-fold increase in marketable yield was recorded for a crookneck cultivar derived from transgenic line CZW-3 in comparison to nontransgenic controls under conditions of severe disease pressure achieved by aphid-mediated virus transmission (31) (**Figure 1*b***). Virus-resistant transgenic cultivars are economically viable even if they do not display resistance to all the challenge viruses. For example, resistance to only ZYMV provided salable product in the presence of ZYMV, CMV, and WMV (31). A good adoption rate by growers mirrored the successful commercialization of virus-resistant transgenic summer squash cultivars. In 2005, transgenic squash accounted for 12% of the total acreage in the United States, with the highest adoption rate in New Jersey (25%), Florida (22%), Georgia (20%), South Carolina (20%) and Tennessee (20%) (87).

Control of aphid-borne viruses in squash is routinely achieved by cultural practices, including delayed transplanting relative to aphid vector flights, the use of film mulch to repel aphid vectors, and application of stylet oil in combination with insecticides to reduce aphid vector populations (73). In the state of Georgia, it is estimated that ten applications of stylet oil and insecticides are made routinely to control aphids and, hence, limit virus transmission (35). Restricting the reliance on chemicals directed to arthropod vectors is an important benefit of the commercial release of virus-resistant transgenic squash (**Figure 1*c***).

Transgenic squash resistant to ZYMV and WMV does not serve as virus source for secondary spread (56). It severely limits infection rates by restricting challenge viruses to inoculated tissues, reducing their titers, and inhibiting their replication and/or cell-to-cell or systemic movement (28, 31, 56, 97). Lower virus titers reduce the frequency of virus acquisition by vectors and subsequent transmission

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within and between fields; hence, virus epidemics are substantially limited (56).

Transgenic Papaya Resistant to *Papaya ringspot virus*

The development of transgenic papaya resistant to PRSV (*Papaya ringspot virus*) to the time of release in Hawaii has been thoroughly documented (38), and a number of reviews have been written since its commercialization in 1998 (39–42). The following section briefly summarizes the performance of PRSV-resistant transgenic papaya from its inception to the present.

Papaya production in Hawaii from 1940s to 1998. The term papaya ringspot virus was coined in 1948 by Jensen, who discovered the virus on Oahu island in Hawaii (54). PRSV is a potyvirus that is transmitted by several aphid species in a nonpersistent manner. Resistance genes to PRSV have not been identified in papaya (*Carica papaya*), but tolerance to PRSV is conferred in a quantitative manner (42). In the 1950s, Oahu island was the main papaya-growing area of Hawaii but PRSV forced the industry to relocate to the Puna district on Hawaii island, where the virus was not present, plentiful land was available, rainfall and sunshine were abundant, and volcanic rock substrates allowed for good drainage despite an annual average rain fall of 2,540 mm. Furthermore, the newly accepted Kapoho solo papaya cultivar was largely adapted to the Puna region. By the 1970s, Puna was growing 95% of Hawaii's papaya. However, the potential threat of PRSV remained because the virus was only 30 km away in Hilo.

Efforts to develop PRSV-resistant transgenic papaya were initiated in 1985 with the cloning and subsequent sequencing of the CP gene of PRSV strain HA 5-1, followed by the biolistic transformation of papaya embryos starting in 1989 and by the identification of transgenic papaya line 55-1 in 1991 with resistance to PRSV strain HA under greenhouse conditions (23). Line 55-1 was a transformant of Sunset, a commercial sibling selection of the red-fleshed Sunrise papaya cultivar. In April 1992, a small field trial of R0 plants of transgenic line 55-1 was established on Oahu island. By December 1992, experimental data convincingly showed that line 55-1 was fully resistant to PRSV under field conditions (62). This field trial proved to be pivotal because the current commercial cultivars SunUp and Rainbow were developed in this small plot (67). SunUp is a red-fleshed transgenic line 55-1 that is homozygous for the PRSV CP gene, whereas Rainbow is a yellow-flesh F1 hybrid of SunUp and nontransgenic Kapoho.

PRSV: *Papaya ringspot virus*

Coincidentally, in May 1992, PRSV was discovered in commercial papaya plantings in Puna on Hawaii island. Despite valiant efforts to suppress the spread of PRSV via scouting and rouging, more than half of Puna was severely infected with PRSV by 1994, and the Hawaii Department of Agriculture (HDOA) abandoned eradication procedures (**Figure 2*a***). By 1998, much of Puna was infected with PRSV and papaya production was reduced to about half of the 1992 level (12 vs 24 million kg of fruits) (**Table 1**), and much of the papaya fruits were harvested from infected fields. Hawaii's papaya industry was surely in crisis. In 1992, Puna had five packinghouses; by 1998, only two were left and they were not operating full time.

In October 1995, a field trial of SunUp and Rainbow along with nontransgenic papaya was initiated in Puna to determine the effectiveness of the engineered resistance to control PRSV under severe virus pressure and to assess the horticultural qualities of the transgenic cultivars. The field trial was very successful (22) and convinced researchers, public officials, and growers that the two transgenic cultivars, especially Rainbow, were acceptable for commercial production in Hawaii (**Figure 2*b*, *c***). In fact, the parent transgenic line 55-1 was deregulated by the USDA-Animal Plant Health Inspection Service (APHIS) in 1996, by the Environmental Protection Agency in 1997, and consultation with the Food and Drug Administration

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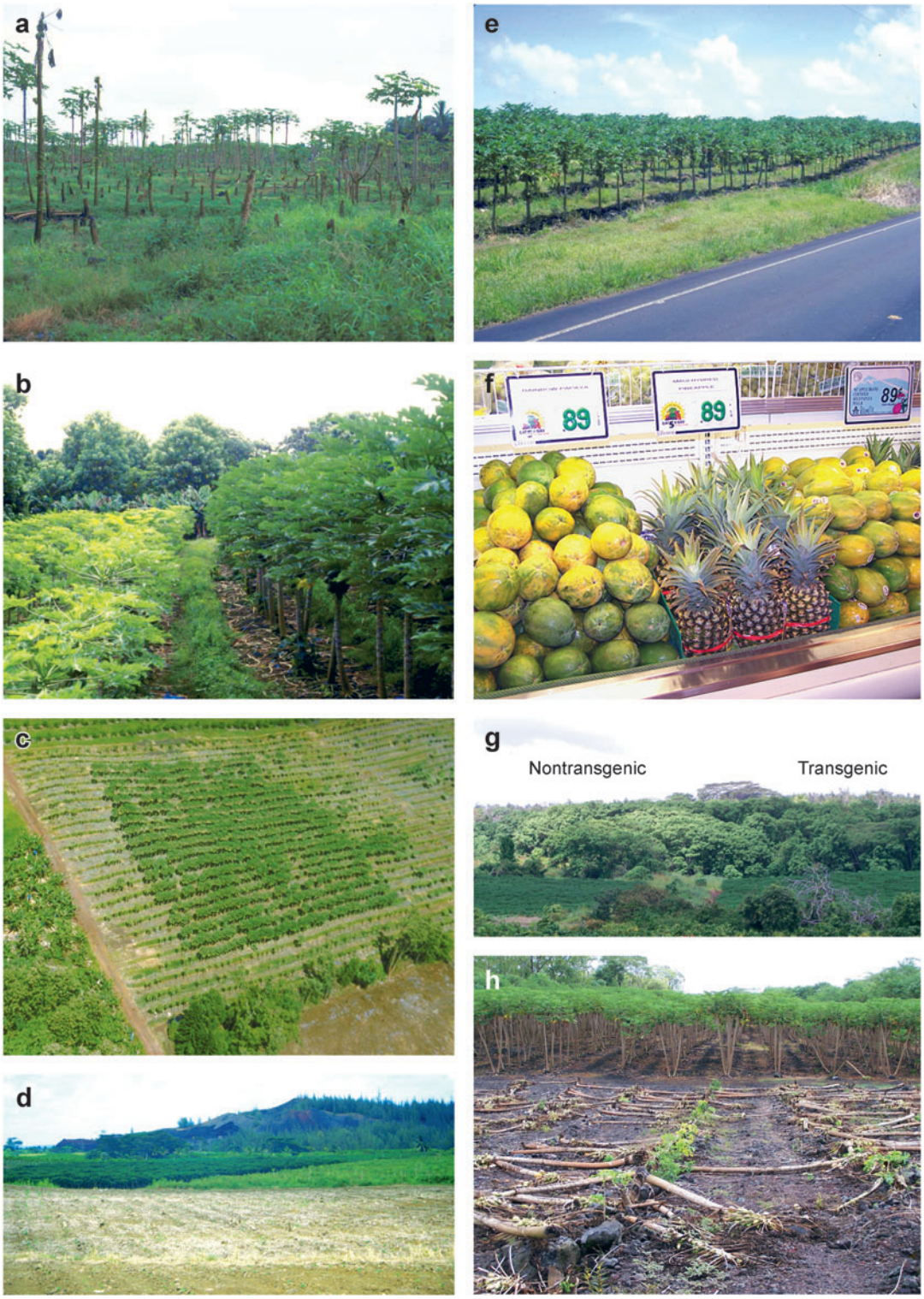

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was completed in 1997. Licenses to commercialize transgenic papaya were obtained by the Papaya Administrative Committee (PAC) in April 1998 (38). PAC contracted to produce seeds of SunUp and Rainbow, and subsequently started free distribution of seeds to growers under a lottery system based on need and the severity of PRSV in production fields (36).

Papaya production in Hawaii from 1998 to the present. Adoption of PRSV-resistant papaya by farmers was overwhelming and fast. The rather small size of the papaya industry in Hawaii, the confined area where the transgenic papaya would be released, and the close relationship between the team of researchers and papaya growers provided an unique opportunity to assess the adoption of the transgenic papaya before and soon after its release (36, 37).

In 1998, it was estimated that Hawaii had 256 papaya farmers, 171 of whom were in Puna. A survey of 54% (91 of 171) of the farmers in Puna indicated that (*a*) 90%–91% were of Filipino ethnicity, (*b*) many (46%) held off-farm jobs, and (*c*) 38% derived more than half of their income from raising papaya. These statistics showed that the Hawaii papaya industry mainly consisted of small family-oriented growers.

Of the 92 farmers who qualified to receive transgenic papaya seeds, 90% obtained them, 76% planted them, and 19% actually harvested fruits within a year after distribution.

Table 1 Fresh papaya fruit production (x 1,000 kg) in Hawaii and the Puna district from 1992 to 2004

Year	Hawaii production	Puna production	%
1992[a]	25,340	24,073	95
1993	26,430	25,108	95
1994	25,522	25,215	99
1995	19,028	17,808	94
1996	17,166	15,529	90
1997	16,212	12,629	78
1998[b]	16,167	12,148	75
1999	17,892	11,630	65
2000	22,820	15,417	68
2001	23,614	18,297	77
2002	19,391	16,294	84
2003	18,528	16,228	88
2004	15,533	13,737	88

[a]Outbreak of PRSV in Puna.
[b]Seed release of PRSV-resistant transgenic papaya.

Most farmers planted transgenic seeds soon after they received them: Of the 71 farmers surveyed, 38% planted less than a month after distribution, 42% planted after 1–3 months, and 20% planted between 4–9 months. The reason for adopting transgenic papaya seeds was overwhelmingly (96%) for resistance to PRSV. In summary, growers anxiously waited for seeds of transgenic papaya and planted them soon after distribution.

After the release of transgenic seeds, farmers mainly cleared infected fields and planted Rainbow papaya even next to abandoned infected fields (**Figure 2*d***). The performance under commercial settings was dramatic,

Figure 2

(*a*) Destruction caused by PRSV in a commercial papaya orchard in Puna in 1994. (*b*) PRSV-infected nontransgenic papaya (*left*) and healthy transgenic Rainbow papaya (*right*) in a field trial in Puna in 1995. (*c*) Aerial view of a solid block of healthy transgenic Rainbow papaya surrounded by rows of PRSV-infected nontransgenic papaya in a field trial in Puna in 1995. (*d*) Reclamation of land by transgenic papaya (*front*). Note the dark-green healthy Rainbow papaya (*back*) growing between abandoned PRSV-infected papaya orchards. (*e*) A field of healthy commercial transgenic Rainbow in 1999, only one year after releasing transgenic seeds to the Hawaii papaya industry. (*f*) Transgenic Rainbow papaya fruits sold in a supermarket. (*g*) Transgenic Rainbow papaya (*right*) growing next to nontrangenic Kapoho papaya (*left*) under an identity preservation protocol. Note the close proximity of nontransgenic and transgenic trees. (*h*) Healthy transgenic Rainbow papaya field (*back*) next to PRSV-infected nontransgenic Kapoho (*front*) that was cut down before harvest.

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and no breakdown of resistance was observed. By 1999, it was common to see many healthy commercial fields of transgenic papaya (**Figure 2*e***), a marked contrast from only a year before. The papaya production in Puna substantially increased and reached 88% of Hawaii's fresh fruit papaya production (**Table 1**). Resistance to PRSV has held up very well over time, and transgenic papaya fruits are commonly found in supermarkets in Hawaii (**Figure 2*f***).

Notwithstanding, Hawaii's papaya production has not yet reached the 1992 levels (**Table 1**). Several plausible reasons account for this situation: (*a*) soon after the release of transgenic seeds, many farmers planted Rainbow papaya and essentially flooded the market. Prices dropped and discouraged farmers from planting more papaya, (*b*) when the incidence of PRSV was high in 1992–1998, the papaya market expanded in the mainland United States through an increased importation of papaya from Mexico, while Hawaii's production decreased, and (*c*) a series of weather-related setbacks, such as drought or excess rain, and introduction of new fungal diseases (black spot) and pest (white peach scale), has put a damper on overall papaya production. The bottom line, however, is that papaya production in Puna was headed in a straight line decrease in production due to the incidence of PRSV (**Table 1**), and this production loss due to PRSV was stemmed by the release of the transgenic papaya. It is highly unlikely that Puna would be producing much, if any, papaya today without the transgenic papaya.

Transgenic papaya helps the production of nontransgenic papaya. Hawaii still needs to produce nontransgenic papaya in order to supply its very lucrative export market to Japan. Why? Japan has not yet deregulated the PRSV-resistant transgenic papaya. Thus, for Hawaii to keep its market share in Japan, it needs to produce nontransgenic papaya. Furthermore, the nontransgenic Kapoho cultivar has long been the dominant Hawaiian papaya in Japan but it is not adaptable outside of Puna. This perhaps is the reason why Kauai and Molokai islands, where there is no PRSV, have not increased their papaya production significantly when compared to statewide figures.

Ironically, the transgenic papaya actually made possible the production of nontransgenic papaya in Puna. This is a major benefit of PRSV-resistant transgenic papaya that is often overlooked. As noted above, in 1998, it was virtually impossible to grow nontransgenic papaya in much of Puna without severe infection with PRSV. Replacement of susceptible nontransgenic papaya orchards with resistant transgenic papaya drastically reduced the incidence of PRSV in many areas of Puna. This action along with the production of nontransgenic papaya in locations with low PRSV prevalence and strict rouging practices have helped with the economical, although risky, growing of nontransgenic papaya (**Figure 2*g***). Growing nontransgenic papaya among large plantings of transgenic papaya is possible most likely because transgenic papaya serve as barriers to cleanse viruliferous aphids of PRSV prior to their feeding on nontransgenic papaya.

Transgenic papaya promotes reduction in land used for papaya production. The transgenic papaya has helped reduce the extent of new marginal forestland that is cleared for growing papaya in Puna. In effect, PRSV-resistant transgenic papaya can be grown in existing papaya land in the presence of the virus, whereas isolation from virus sources is necessary to grow nontransgenic papaya. Therefore, growers often clear new areas that could be used for purposes other than raising papaya. This often overlooked environmental benefit of PRSV-resistant transgenic papaya is important because land in Puna and elsewhere in the state of Hawaii is limited and biodiversity is much treasured.

Transgenic papaya contributes to increased cultivar diversity. Transgenic papaya has increased the diversity of papaya

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cultivars in Hawaii and expanded the papaya market to Oahu island. In 1998, 95% of Hawaii's papaya industry was of the Kapoho cultivar and less than 5% of Sunrise and Kamiya cultivars. Today, Hawaii growers have access to PRSV-resistant SunUp, Rainbow, and a cultivar named Laie Gold. Rainbow and Laie Gold are grown on Oahu island where commercial production was largely abandoned due to PRSV. Whereas Oahu grew 2.5 hectares of papaya in 1960, it now grows 57 hectares of virus-resistant papaya (39). This would not have happened without the release and adoption of PRSV-resistant transgenic papaya.

Scientists in academia can commercialize a transgenic crop. With the exception of PRSV-resistant trangenic papaya, all commercial transgenic crops have been developed so far by large and medium-sized corporations. The fact that the transgenic papaya was produced and commercialized through the work of investigators from academia provides a clear example of how universities and small research institutions can be instrumental in developing transgenic crops to solve agricultural problems. Since resistance to viruses in minor commodity crops, such as papaya, is less lucrative relative to the economic value of major commodity crops, it is perhaps not too surprising that large companies would not pursue this transgenic arena. The commercial development of minor virus-resistant transgenic crops provides an attractive opportunity to small companies and scientists at public institutions.

POTENTIAL SAFETY ISSUES ASSOCIATED WITH VIRUS-RESISTANT TRANSGENIC PLANTS

Considerable attention has been paid to potential environmental risks associated with the release of virus-resistant transgenic crops over the past 15 years. Here, we review the major areas of potential concern with a special emphasis on crops that have been commercialized in the United States or have been extensively tested in the field over the past decade, such as squash, papaya, plum, grape, and sugar beet. However, safety issues are not specific to transgenic plants expressing viral genes. They also apply to conventional plants that are subjected to virus infection. It is the engineered trait, e.g., virus resistance, and the transgene, e.g., a virus-derived gene construct, that are the source of potential concern, not the methodology used to develop a virus-resistant plant. Therefore, it is critical to determine a baseline level of occurrence against which the impact of transgenic plants is compared.

PVY: *Potato virus Y*

Potential safety considerations relate directly to the fact that resistance to viruses in plants is achieved through the antiviral pathways of RNA silencing that are triggered by expressing constitutively viral sequences (17, 46, 64, 79, 80, 94). The expression of viral sequences does not commonly occur in conventional plants, except in a few infected by pararetroviruses (47, 48) or the one example of PVY (*Potato virus Y*) (91), for which part or the complete viral genome is inserted into the plant genome upon virus infection. Potential risks relate to heteroencapsidation, recombination, synergism, gene flow, effect on nontarget organisms, and food safety in terms of allergenicity (17, 46, 64, 79, 80, 94). However, it is not so much the occurrence of these issues but rather their consequences that need to be considered and addressed.

SAFETY ASSESSMENT OF VIRUS-RESISTANT TRANSGENIC PLANTS

Heteroencapsidation

Heteroencapsidation refers to the encapsidation of the genome of one virus by the coat protein of another virus, as sometimes occurs in plants infected by more than one virus. Heteroencapsidation also could result from the CP subunits expressed by the

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PLRV: *Potato leafroll virus*

transgenic plant rather than from the second viral genome (**Figure 3**). Because the CP can carry determinants for pathogenicity and vector specificity, among other key features (11), the properties of viruses in transgenic plants might change. For example, an otherwise vector-nontransmissible virus could become transmissible through heteroencapsidation in a transgenic plant. Also, a virus could infect an otherwise nonhost plant as a result of heteroencapsidation and subsequent vector-mediated transmission. Consequently, it is theoretically possible that new virus epidemics could result from heteroencapsidation.

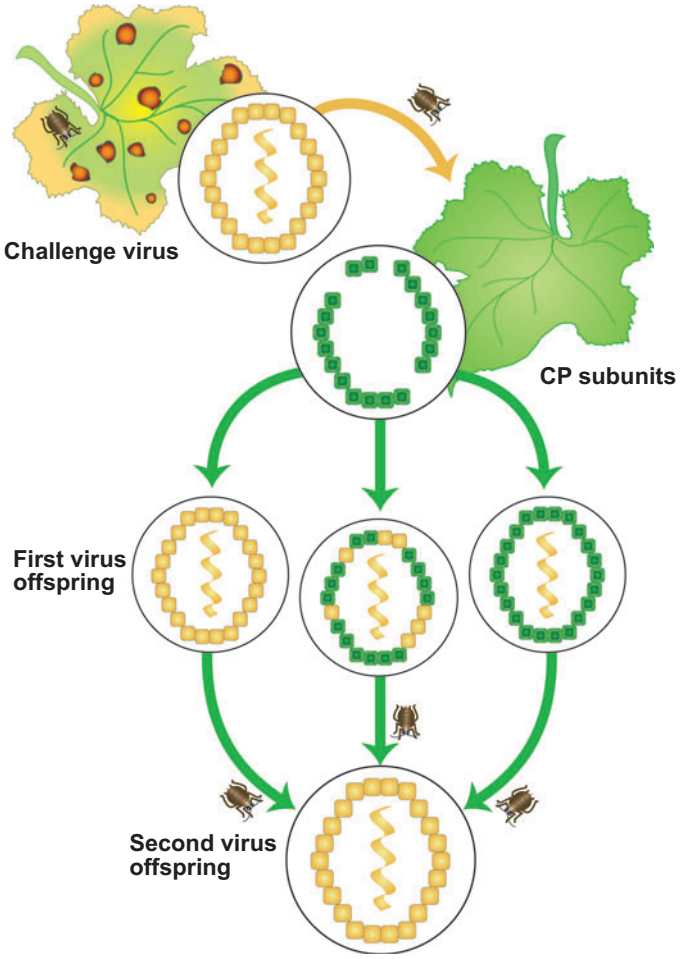

Figure 3

Schematic illustration of heteroencapdsidation. In nature, an insect vector can acquire a virus (*challenge virus*) from an infected plant and transmit it to a transgenic plant expressing a viral CP gene. Following particle disassembly, replication, and translation, the genome of the challenge virus can be encapsidated by its own CP subunits or those encoded by the transgene (*CP subunits*), either partially or fully (*first virus offspring*). Newly formed virions can be acquired by an insect vector and further transmitted. Note that the second virus progeny (*second virus offspring*) will be identical to the challenge virus.

Heteroencapsidation has been documented in transgenic herbaceous plants (13, 21, 45, 50, 59, 70). These studies showed that CP subunits expressed in transgenic plants are able to encapsidate the RNA genome of challenge viruses. Heteroencapsidation was also reported to assist the spread of an otherwise aphid-nontransmissible strain of ZYMV (59). However, it has not been found to occur in transgenic vegetable plants expressing viral CP gene constructs that were tested extensively in the field over several years at different locations. In particular, transgenic squash and melon expressing the CP gene of an aphid-transmissible strain of CMV have been tested for their capacity to trigger the transmission of an aphid-nontransmissible strain of CMV through heteroencapsidation (30). Spread of the aphid-nontransmissible strain did not occur to detectable level from 724 mechanically inoculated transgenic plants to 1,130 healthy susceptible nontransgenic plants. Aphid-mediated spread of CMV occurred but molecular characterization of the CP gene of challenge isolates and aphid-transmissibility assays indicated clearly that challenging CMV isolates did not result from heteroencapsidation but instead were introduced into the field sites from infected plants located outside the experimental area (30). Also, the characteristics of viruses challenging transgenic potato expressing the CP gene or RdRp gene of PLRV (*Potato leafroll virus*) were not altered to detectable level in terms of serological properties, transmission features, host range, and symptomatology, suggesting that heteroencapsidation did not occur (96). In addition, for transgenic papaya and squash, no unexpected emergence of virus species with undesired characteristics was reported 8–10 years postcommercialization.

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The only study that demonstrated the likely occurrence to date of heteroencapsidation in the field was with transgenic squash expressing the CP gene of WMV, for which a low rate of transmission of an aphid-nontransmissible strain of ZYMV (2%, 77 of 3,700) was documented (27). However, transmission of this ZYMV strain was restricted to individual plants that had a random distribution pattern and no spatial relationship. Therefore, spread of the aphid nontransmissible strain of ZYMV did not reach epidemic proportions (27).

Altogether, changes in vector specificity and host range are a single-generation, not a permanent, event because the viral genome is not affected (**Figure 3**). As a consequence, changes will not be perpetuated in the virus genome progeny. Therefore, heteroencapsidation in transgenic plants expressing virus CP genes has been of limited significance and would be expected to be negligible in regard to adverse environmental effects.

Recombination

Recombination refers to the exchange of genetic material between two distinct RNA molecules during virus replication. Recombination can also occur between transcripts of a viral transgene and the genome of a challenge virus during replication in a transgenic plant cell (**Figure 4**). Resulting recombinant viruses may have chimeric genomic molecules consisting of a segment from the challenge viral genome and another segment from viral transgene transcripts (**Figure 4**). Because recombination alters the genome of challenge viruses, new properties of chimeric viruses will be stably transmitted to and perpetuated within the virus progeny (**Figure 4**). Recombinant viruses may have identical biological properties as their parental lineages or new biological properties that could include properties that might negatively affect the environment such as changes in vector specificity, expanded host range, and increased pathogenicity. Numerous studies have documented the occurrence of recombination in transgenic herbaceous plants expressing viral genes (2, 3, 10, 24, 34, 43, 44, 53, 63, 84, 95, 98, 105). The stringency of selective pressure applied to the challenge virus is a critical factor in the recovery of recombinant viruses. Conditions of high selective pressure enhance the detection of recombinant viruses (10, 24, 34, 43, 63, 84). In contrast, limited, if any, recombinant viruses have been found under conditions of low or no selective pressure (2, 3, 44, 105). Also, the presence of a complete viral 3′ noncoding region in some transgene constructs favors recombination (3, 44, 98). This is likely explained by the fact that viral 3′ noncoding regions have sequence elements that are recognized by the RdRp for initiation of RNA synthesis; hence, the occurrence of template switching increases.

GFLV: *Grapevine fanleaf virus*

So far, no recombination event has been found in CP gene-expressing transgenic plants in the field. In particular, recombination has not been detected in transgenic grapevines expressing the CP gene of GFLV (*Grapevine fanleaf virus*) that were tested in two GFLV-infected vineyards sites in France (99, 100). Test plants consisted of conventional scions grafted onto transgenic rootstocks. Analysis of the CP gene of 347 challenge GFLV isolates by immunocapture-reverse transcription-polymerase chain reaction-restriction fragment length polymorphism indicated no emergence of recombinants with transgene sequences (100). Sequence analysis further showed that GFLV recombinants developed in conventional but not in transgenic plants (99). Also, a comprehensive analysis of the nucleotide diversity among GFLV isolate populations further indicated a lack of genetic differentiation according to the host (transgenic vs conventional) or field site for the majority of haplotypes (99). Therefore, there was no evidence that transgenic grapevines assisted the creation of viable GFLV recombinants nor affected the molecular diversity of GFLV populations during a three-year trial period (99, 100).

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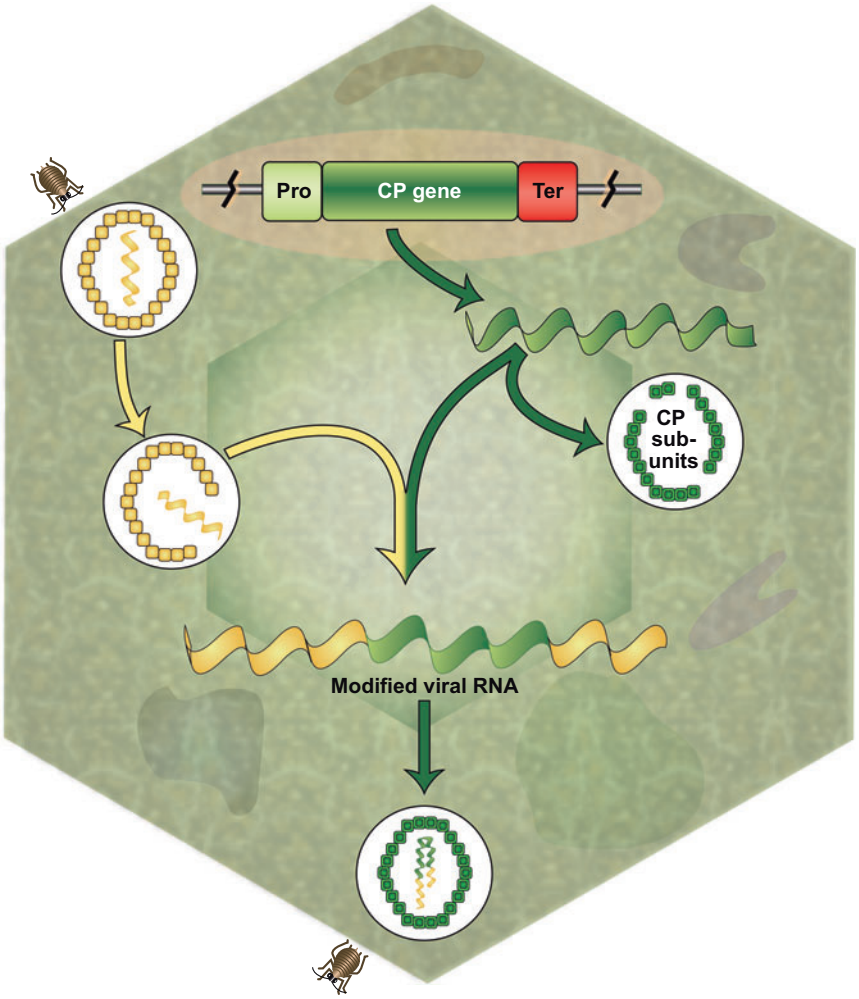

Figure 4

Schematic illustration of recombination. In nature, an insect vector can transmit a virus to a transgenic plant expressing a viral CP gene; such expression is driven by a promoter (Pro) and a terminator (Ter). Following particle disassembly, the viral genome can replicate. If template switching occurs during replication between transcripts of the transgene and the viral RNA, a chimeric RNA molecule (*modified viral RNA*) can form and be encapsidated for subsequent vector acquisition and transmission.

Similarly, recombination has not been detected in transgenic plums expressing the CP gene of PPV (*Plum pox virus*) that have been tested in experimental orchards over 8–10 years in Spain (14) and Romania (I. Zagrai, unpublished observations). No significant difference in nucleotide variability was found between PPV isolates from transgenic (37 isolates) and conventional (109 isolates) plum trees (14; I. Zagrai, unpublished observations). A few PPV recombinant isolates were detected but they did not emerge in transgenic plum trees because recombination events mapped to the RdRp, not to the CP gene (I. Zagrai, unpublished observations). Also, no recombination was detected in CMV isolates from transgenic squash, and no correlation was found between the variability of the CMV CP gene within isolates from transgenic and conventional squash (60). Furthermore, growers, extension educators, and scientists have not detected the emergence of viruses with undesired properties in transgenic squash, papaya, and plum over 8–10 years of commercial release or extensive experimental testing.

PPV: *Plum pox virus*

Altogether, the significance of recombination between transgenes and viruses appears to be very limited in regard to adverse environmental effects. Similar conclusions

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were reached with different viruses (CMV, GFLV, PPV, PRSV, ZYMV, and WMV) and different transgenic crops (squash, papaya, grape, and plum) in different environments in Europe and the United States.

Transgene Movement by Pollen Flow

Gene flow from cultivated crops to compatible wild relatives is another environmental issue. In the case of virus-resistant transgenic crops, wild relatives that acquire host genes and/or transgenes through pollen flow, and their progeny could express viral transgenes (**Figure 5**), resist the corresponding virus(es), exhibit increased fitness, and be eventually more competitive if transferred genes provide them with a selective advantage (19, 88, 90). Questions have been raised on (*a*) the development and evolution of weed species that can overrun and disrupt natural ecosystems, and (*b*) the potential threat to the genetic diversity in wild populations and increased risks of extinction of wild relatives. Gene flow is well documented for major conventional crop species (18, 19, 90). Therefore, understanding the effects of transgene introgression requires an understanding of the transgene effect on wild populations in addition to an understanding of transgene movement per se (19, 90).

The movement of transgenes from virus-resistant transgenic squash CZW-3 (31, 97) to its wild relative, *Cucurbita pepo* spp. *ovifera* var. *texana*, which is referred to here as *C. texana*, was recently documented in experimental field settings (25). The rate of hybridization increased with high ratios of donors over recipients of transgenic pollen and with overlapping flowering patterns. More important, movement of transgenes occurred over three generations under conditions of low disease pressure whereas it was

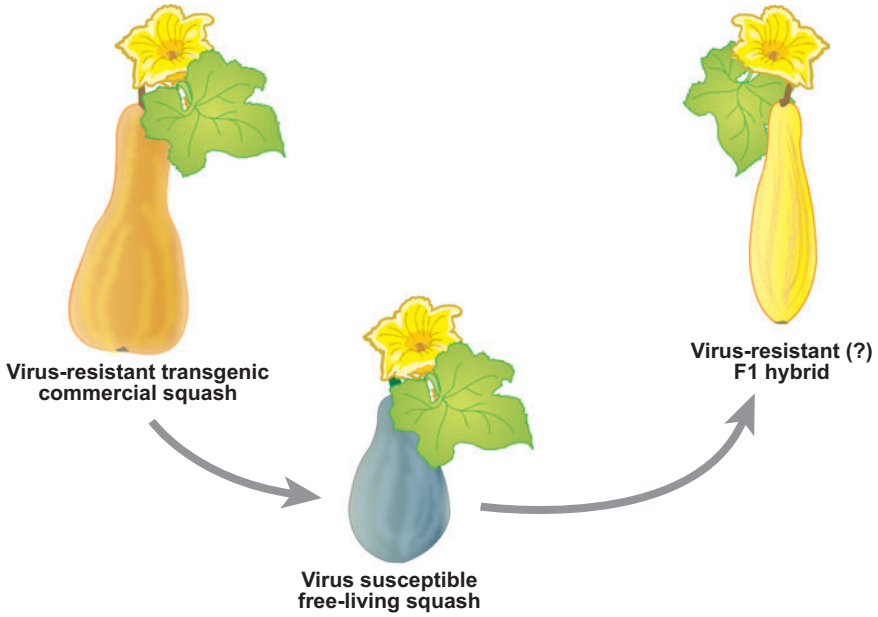

Figure 5

Schematic illustration of transgene movement through hybridization. Transfer of transgenes can occur from a virus-resistant transgenic crop, for example, a commercial squash, into a compatible virus-susceptible wild relative via pollen flow. Resulting hybrids can acquire the transgenes and have a fitness advantage if virus resistance provides them with a selective advantage.

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BNYVV: *Beet necrotic yellow vein virus*

not sustained beyond the first generation under conditions of high disease pressure (25). This differential is explained by the severe effect of viruses on plant growth and reproductive potential at an early development stage under conditions of high disease pressure. As expected, progeny of *C. texana* that acquired the CP transgenes through gene flow exhibited resistance to the three target viruses, e.g., CMV, ZYMV, and WMV. Also, progeny of *C. texana* expressing transgenes exhibited increased fitness not only by displaying resistance against CMV, ZYMV, and WMV, but also by growing more vigorously, and producing significantly more fertile fruits and setting more viable seeds than *C. texana* and their nontransgenic counterparts under conditions of high disease pressure (26). In contrast, under conditions of low disease pressure, *C. texana* outperformed all the other genotypes tested whether expressing the transgenes or not (26).

Our findings indicated that the fitness and competitiveness of *C. texana* could be affected in natural ecosystems if selective virus pressure is high. Therefore, knowledge on virus incidence in natural habitats is important to anticipate the ecological fitness of *C. texana* and the dynamics of their population structure. Surveys of wild squash populations, including *C. texana*, for virus incidence have been conducted in natural habitats (77, 78). Across most populations studied, virus incidence was extremely low, although it was found to vary over time and space. The majority of wild squash showed no virus infection; only a limited number were symptomatic and some were infected by CMV, ZYMV, and/or WMV. Therefore, it appears that viruses have a limited effect on the dynamics of wild squash populations (78).

Similarly, movement of transgenes was also documented from transgenic sugar beet expressing the CP gene of BNYVV (*Beet necrotic yellow vein virus*) to wild beet (*Beta vulgaris* ssp. *maritima*). Transgenic beet did not show any significant increase in competitiveness when compared to classically bred BNYVV-tolerant sugar beet (8). In addition, a survey of wild beet populations in northeastern Italy showed that none were infected by BNYVV (8). These results suggested that BNYVV-resistant transgenic sugar beets are unlikely to become more competitive in natural habitats upon hybridization and introgression.

Another means of gene flow is from a transgenic crop into a conventional crop (52). People reluctant to adopt virus-resistant transgenic plants often refer to this movement of transgenes as contamination or genetic pollution. This subject is discussed below under coexistence of transgenic and nontransgenic crops since it does not pertain to environmental risk assessment per se.

Synergism

Synergism refers to the interaction of a viral protein product with another challenge virus that can result in an aggravation of host symptom severity and an increase in virus titer that neither virus can cause independently. In a transgenic plant, expression of viral genes can protect against infection by a homologous virus but can also increase the susceptibility to a synergistic heterologous virus and affect the rate of disease spread. Synergism may result from the inhibition of the plant's PTGS defense response to virus infection (75, 81, 83). However, it does not modify existing viruses nor create novel viruses with new characteristics; hence, it is not deemed to cause any environmental hazard. Therefore, the significance of synergism is limited and is not discussed further here.

Effect on Nontarget Organisms

Virus-resistant transgenic crops can potentially influence the diversity and dynamics of nontarget organisms, including insect vectors. Also, viral genes that confer resistance in transgenic crops could provide soil microorganisms such as bacteria or fungi with a selective advantage upon horizontal gene transfer (79). These perceived risks have been

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addressed and shown to have limited, if any, adverse repercussions. For example, no significant difference was found in the diversity and dynamics of arthropods, including viruliferous aphid vectors, that visited nontransgenic and transgenic plum trees expressing a PPV CP gene construct (14; M. Cambra, unpublished observations). Also, PRSV-resistant transgenic papaya does not have any significant effect on the total count and diversity of actinomycetes in different soil layers (51). Altogether, virus-resistant transgenic crops have not been found to pose a risk to nontarget organisms.

Food Safety and Allergenicity

Human health effects in terms of allergenicity refer to potential allergenic properties of proteins encoded by viral sequences that are expressed in transgenic plants. Virus-derived transgene protein products can have stretches of amino acid sequences that are identical to potential immunoglobulin-E-binding linear epitopes of allergen proteins, and hence, could cause new food, contact, or inhalant allergies, or modify the level or nature of intrinsic allergens.

Numerous observations suggest that a viral protein in transgenic plants does not pose a threat to allergenic safety. Most notable is that virus-infected crops have been consumed since plants have been food with no apparent ill effects known to be due to virus components. Also, the deliberate inoculation of millions of citrus trees to control *Citrus tristeza virus* through mild strain cross protection has been practiced for many years in Brazil (16), and no adverse outcome on human health has been reported. Similarly, no ill effect has been reported from the consumption of papaya fruits harvested from thousands of trees that were deliberately inoculated with a mild strain of PRSV (106). Also, to the best of our knowledge, there is no scientific report documenting any plant viral CP as allergen. Nevertheless, it is prudent to investigate food safety aspects of virus-resistant transgenic plants.

At present, only three virus-resistant transgenic crops have been deregulated in the United States, and squash and papaya are in commercial production. Transgenic potato resistant to PVY and PLRV was deregulated but withdrawn from the market (55), and a transgenic plum expressing the CP gene of PPV is under consideration for deregulation by USDA-APHIS (86). None of the virus-derived products expressed by these transgenic crops can be considered a potential allergen if one uses the suggested criteria of minimum sequence relatedness (35%) and a continuous stretch of eight identical amino acids to known allergens (49). However, using six contiguous identical amino acids as criterion, the CP of PRSV strain HA 5-1, which is expressed in transgenic SunUp and Rainbow papaya, matches with the putative ABA-1 amino acid allergen determinants of roundworms (57). Nevertheless, a 2002 report showed that the ABA-1 protein is not an allergen by itself (72), indicating that a stretch of six identical amino acids was not a valid approach for judging potential allergens. Other criteria for protein allergenicity are their stability in simulated gastric juices and stability to heat. Studies on transgenic papaya showed that the CP of PRSV strain HA 5-1 is digested in simulated gastric juices in less than 5 seconds after exposure, and much of the protein is degraded by heat (D. Gonsalves, unpublished observations). Altogether, allergenicity appears not to be a significant risk for PRSV-resistant transgenic papaya.

ARE PERCEIVED RISKS REAL AND SIGNIFICANT?

Many studies have been conducted to address safety issues of virus-resistant transgenic crops, in particular heteroencapsidation and recombination. However, only a limited number have a real significance for risk assessment as most deal more with virus-host interactions rather than with safety. Deciphering virus-host interactions is a valuable approach

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to identify potential risks (2, 10, 13, 21, 24, 34, 43–45, 50, 53, 59, 63, 70, 84, 95, 98, 105). For example, examining the occurrence of recombination and determining factors that can affect the extent of its occurrence is important. However, virus-host interactions have limited relevance to environmental safety because they do not evaluate the consequence(s) of the occurrence of recombination. As discussed previously, it is not so much the occurrence but rather the consequences that are critical to assess the impact of virus-resistant transgenic plants because risks are not fundamentally different in transgenic and conventional crops.

It is important to discriminate perceived and real risks associated with virus-resistant transgenic plants for the deregulation process. Field environmental safety assessment studies have provided strong evidence of limited, if any, environmental risks, beyond background events (14, 27, 30, 56, 60, 96, 99, 100; I. Zagrai, unpublished observations). These findings indicate that serious negative environmental impacts associated with virus-resistant transgenic plants are substantially less significant than initially predicted (17, 46, 79, 94). To fully grasp the significance of environmental risks, the occurrence of heteroencapsidation and recombination in the absence of transgenic plants needs to be taken into account and considered as baseline information. So far, there is no compelling evidence to indicate that transgenic plants expressing viral genes increase the frequency of heterologous encapsidation or recombination beyond background events. Similarly, there is little evidence, if any, to infer that transgenic plants expressing viral genes alter the properties of existing virus populations or create new viruses that otherwise could not emerge in conventional plants subjected to multiple virus infection (20).

For heteroencapsidation or recombination to occur and become significant, a sequence of low probability events needs to be fulfilled successfully. Viruliferous vectors need to land on or be in contact with susceptible transgenic host plants, then probe or feed, and transmit virus particles. Virions need to disassemble, and the genome of challenge virus isolates needs to replicate and interact with transgene-derived products for heteroencapsidation or template switching to occur in infected cells. Heteroencapsidated RNA molecules need to assemble and recombinant RNA molecules need to be encapsidated. Subsequently, heteroencapsidated and recombinant virions need to move from cell-to-cell and through the vascular system to cause systemic infection. Finally, virions need to be acquired by vectors and transferred onto new host plants. Each step of this cascade of events requires a relatively reasonable probability of occurrence in order for a viable heteroencapsidated virus to develop or a viable recombinant virus to emerge and start an outbreak. Several constraints associated with each of these steps will reduce the success of the final outcome.

On the other hand, transgene introgression from virus-resistant transgenic crops to wild relatives is a dynamic process to be assessed on a case-by-case basis. Based on outcrossing potential and empirical evidence of crop-to-wild introgression, among other factors such as spatial proximity, overlapping flowering phenology, and virus incidence in natural habitats, risk categories can be defined (19, 90). Although many virus-resistant transgenic crops should be safe to release, others should be approached with caution to avoid the creation of weeds with enhanced fitness and competitiveness.

OTHER ISSUES OF VIRUS-RESISTANT TRANSGENIC PLANTS

Although not directly related to safety issues, resistance breakdown and coexistence of transgenic and nontransgenic crops should be considered when addressing the commercialization of virus-resistant transgenic crops. We discuss these in relation to the PRSV-resistant transgenic papaya.

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Breakdown of Resistance

The question of resistance breakdown was addressed during the development of the transgenic papaya. A number of PRSV isolates were collected throughout Hawaii and tested against the transgenic papaya. Resistance held up and no breakdown was observed in commercial orchards on Oahu and Hawaii islands (S.A. Ferreira, unpublished observations). Thus, eight years after its commercial release, the transgenic Rainbow papaya continues to perform well in Hawaii.

Early on, greenhouse inoculations of transgenic Rainbow showed resistance to PRSV strains from Mexico but not to strains from Thailand, Australia, and Brazil (93). Later studies showed that resistance could be broadened through increased transgene dosage (89, 92), and SunUp, which is homozygous for the CP gene, showed resistance to a number of, but not all, PRSV strains outside of Hawaii (92).

Although breakdown of resistance would not pose an environmental or a food safety risk, it is critical to constantly and proactively monitor the introduction and emergence of new viral strains from the standpoint of disease management. This is important because it takes a long time to develop resistant plants, and one wants to maximize the effectiveness of the engineered resistance overtime. Additionally, developing new resistant transgenic plants is prudent so that solutions can be obtained in a timely manner, should there be a breakdown of resistance. In regard to papaya, transgenic plants that are resistant to PRSV strains of Hawaii and outside of Hawaii have been developed (D. Gonsalves & S.A. Ferreira, unpublished observations).

Coexistence of Transgenic and Nontransgenic Crops

Our definition of coexistence is the growing of transgenic and nontransgenic crops in practical spatio-temporal proximity such that they can be raised with minimal transfer of genetic characteristics from transgenic to nontransgenic, and vice versa.

In the United States, transgenic crops that have been deregulated, like any other nontransgenic crop, are not bound by law to be grown in restricted locations. However, there are circumstances for which coexistence is essential. The two most common examples are for (*a*) growing a crop organically, and (*b*) shipping nontransgenic products to countries that have not deregulated the transgenic version of that crop. In relation to virus-resistant transgenic papaya, coexistence is necessary in that Japan represents a significant share of Hawaii's export market for papaya; and Japan has not yet deregulated the transgenic papaya. Therefore, transgenic and nontransgenic papaya have to exist in close spatial proximity, including in Puna where 88% of Hawaii's papaya are produced.

An identity preservation protocol for the papaya market in Japan. This section was largely taken from a recent review by one of the authors (42). Japan and Canada are large markets for the Hawaii papaya industry (20% and 11%, respectively). Canada approved the import of transgenic SunUp and Rainbow papaya in January 2003. Therefore, shipments of transgenic papaya fruits are continuing to Canada. However, as discussed above, the sale of transgenic papaya in Japan has not yet been approved, and thus it is critical that papaya shipments to Japan do not contain transgenic fruits. Several safeguards are being implemented to minimize the presence of transgenic papaya fruits in shipments destined for export to Japan.

At the request of Japanese importers, HDOA adopted an Identity Preservation Protocol (IPP) that growers and shippers must adhere to in order to received an IPP certification (12, 42). This is a voluntary program. Papaya fruit shipments with this certification can be distributed in Japan without delay while Japanese officials conduct spot testing to detect the fortuitous presence of transgenic papaya fruits. In contrast, papaya shipments

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without this certificate must remain in custody at the port of entry until Japanese officials complete their spot checks for transgenic papaya fruits. The tests may take several days or a week to complete, during which time fruits lose quality and marketability.

A significant feature of the IPP is that nontransgenic papaya fruits must be harvested from orchards approved by HDOA. To get approval, every tree in the proposed field must be tested for the expression of the β-D glucuronidase (*gus*) reporter gene that is linked to the virus-resistance trait (23), and be found negative. Nontransgenic trees must be separated by at least a 4.5 m papaya-free buffer zone, and new fields to be certified must be planted with papaya seeds harvested from trees grown in approved nontransgenic fields. Tests are monitored by HDOA and conducted by the applicant who must submit detailed records to HDOA. Before final approval of a field, HDOA will randomly test one fruit from 1% of papaya trees in the orchard. If approved by HDOA, fruits from these fields can be harvested. Additionally, the applicant must submit detailed protocols that will be followed to minimize the presence of transgenic papaya fruits within fields of nontransgenic papaya trees. This includes a random testing of papaya before they are packed for shipment. If the procedures are followed and tests are negative, a letter from HDOA will accompany the shipment of papaya fruits to Japan, stating that the shipment is in compliance with a properly conducted IPP (12, 42). The scheme of IPP has proved workable and economical.

The above procedure represents a good faith effort by HDOA and applicants to prevent transgenic papaya fruits in shipments of nontransgenic papaya fruits to Japan. It also illustrates a productive collaboration between Japan and HDOA, resulting in continued shipments of nontransgenic papaya fruits with a minimal delay once they arrive in Japan. These safeguards, along with the effectiveness of the transgenic papaya in boosting production of nontransgenic papaya, have allowed Hawaii to maintain significant shipments to Japan without evidence of fortuitous presence of transgenic fruits.

Transgene flow and organically grown papaya. The IPP process has worked remarkably well to prevent the shipment of transgenic papaya fruits to Japan. This suggests that gene flow is extremely low among papaya, which should lessen the concern of organic growers. Note that nearly all papaya plants in Hawaiian commercial orchards are hermaphrodites, which are largely self-pollinated. Preliminary studies in the 1995 transgenic field trial in Puna, which consisted of a large solid block of Rainbow surrounded by six outside rows of nontrangenic papaya (**Figure 2*c***), showed transgenic seeds in 7% of the nontransgenic hermaphrodites and 43% of the females plants (66). The nearest row of nontransgenic papaya tree was about 3 m away from rows of transgenic trees. Transgenic seeds were not recovered from a PRSV-infected nontrangenic papaya orchard located 400 m away from the solid Rainbow block.

Another ongoing study is monitoring evidence of movement of transgenes by pollen flow in commercial orchards in Puna. Seeds were sampled from border or close to border papaya trees in nontransgenic orchards growing adjacent to Rainbow orchards (**Figure 2*g***). So far, expression of the *gus* gene has not been detected in 447 nontransgenic trees that have been sampled (C. Gonsalves, unpublished observations). Although not yet complete, this study suggests that transgene flow is minimal in nontransgenic papaya orchards growing in close proximity to transgenic papaya under commercial conditions in Puna. In summary, coexistence is being routinely and successfully practiced in Hawaii.

Perhaps a larger challenge in growing nontransgenic and organic papaya in Puna is that PRSV is still present. Risks to growing susceptible trees are real (**Figure 2*h***). In relation to growing organic papaya, fungi and insects create additional problems. With 2,540 mm of rain per year, *Phytophthora* and other

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fungal problems are severe if not controlled by fungicides, many of which are not certified for organic production. In fact, we are not aware of any significant planting of organic papaya in Puna. On the other hand, on Molokai island, where PRSV is not present and rainfall is considerably less than in Puna, a small organic papaya industry has just started. Currently, there are about 20 hectares of organically grown papaya. The state of Hawaii grows roughly 800–1,000 hectares of papaya.

Various groups have complained about transgenic papaya being widespread in the Hawaii islands. As noted above, the deregulated transgenic papaya can be grown anywhere, as can any nontransgenic papaya. The option to grow transgenic or nontransgenic papaya, or both, in back yards is eminently feasible. As noted above, coexistence is routine at the commercial level (**Figure 2*g***), and a single grower will grow transgenic and nontransgenic papaya depending on market preferences, among other factors.

Presence of Plasmid Backbone Sequences and Marker Genes

An ideal virus-resistant transgenic plant would contain only the gene(s) conferring resistance, without any plasmid backbone sequences and scorable marker genes. However, virus-resistant transgenic plants typically contain marker genes that facilitate their selection and identification during the transformation process. The neomycin phosphotransferase II (*npt II*) gene, which imparts kanamycin resistance, is commonly used in transgenic plants, and less commonly used is the *gus* gene. The *npt II* and *gus* genes have been thoroughly tested and found to be safe for use in transgenic crops (4, 32). Plants transformed with *Agrobacterium tumefaciens* largely do not contain plasmid backbone sequences outside of the T-DNA, but plants transformed via the biolistic approach will often have plasmid backbone sequences.

Should there be any hesitation over the use of the *npt II* and *gus* genes, and presence of plasmid backbone sequences? The wide and safe use of these genes and sequence elements in transgenic crops has not given any reason for concern. PRSV-resistant transgenic papaya contain both the *npt II* and *gus* genes and the consumption of million of kilograms of transgenic papaya fruits over the past eight years has not provided any evidence of compromising safety. It seems that the usefulness of the *npt II* gene far outweighs any risks that might evolve from its presence in transgenic plants (32).

SUMMARY OF NEARLY TWO DECADES OF SAFE RELEASE OF VIRUS-RESISTANT TRANSGENIC PLANTS

The concept of PDR has been successfully applied to develop virus-resistant transgenic crops over the past two decades. Many crop plants have been tested under field conditions, and a few have been commercialized (**Table 2**). Deciphering the mechanisms underlying resistance has shed new light on the antiviral pathways of RNA silencing as potent defense mechanisms against viruses in transgenic plants. There is no doubt that the technology is effective. However, only a very limited number of virus-resistant transgenic crops have been made available to growers. Why? Several factors and impediments can account for the limited success stories to date. Regulatory requirements may be complex, time-consuming, impractical, and too costly. Thus, potential applicants who seek to deregulate a virus-resistant transgenic crop may be discouraged. The hurdles facing claims to intellectual property can be overwhelming. A lack of strong commitment to deliver a product to end-users, despite the benefits of the technology, is another key factor. Also, the political pressure exerted by non-governmental organizations to the development and release of virus-resistant transgenic plants is another important factor in many countries.

The safety of virus-resistant transgenic plants has been extensively addressed over

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Table 2 Virus-resistant transgenic crops that have been tested in the field or commercially released

Crop		
Category/Common name	**Scientific name**	**Resistance to**
Cereals		
Barley	*Hordeum vulgare*	*Barley yellow dwarf virus*
Canola	*Brassica napus*	*Turnip mosaic virus*
Corn	*Zea mays*	*Maize dwarf mosaic virus*,
		Maize chlorotic dwarf virus,
		Maize chlorotic mottle virus,
		Sugarcane mosaic virus
Oat	*Avena sativa*	*Barley yellow dwarf virus*
Rice	*Oryza sativa*	*Rice stripe virus*,
		Rice hoja blanca virus
Wheat	*Triticum aestivum*	*Barley yellow dwarf virus*,
		Wheat streak mosaic virus
Flowers		
Chrysanthemum	*Chrysanthemum indicum*	*Tomato spotted wilt virus*
Dendrobium	*Encyclia cochleata*	*Cymbidium mosaic virus*
Gladiolus	*Gladiolus* sp.	*Bean yellow mosaic virus*
Fruits		
Grapefruit	*Citrus paradisi*	*Citrus tristeza virus*
Grapevine	*Vitis* sp.	*Grapevine fanleaf virus*
Lime	*Citrus aurantifolia*	*Citrus tristeza virus*
Melon	*Cucumis melo*	*Cucumber mosaic virus*,
		Papaya ringspot virus,
		Squash mosaic virus,
		Watermelon mosaic virus,
		Zucchini yellow mosaic virus
Papaya[a]	*Carica papaya*	*Papaya ringspot virus*[a]
Pineapple	*Ananas comosus*	*Pineapple wilt-associated virus*
Plum	*Prunus domestica*	*Plum pox virus*
Raspberry	*Rubus idaeus*	*Raspberry bushy dwarf virus*,
		Tomato ringspot virus
Strawberry	*Fragaria* sp.	*Strawberry mild yellow edge virus*
Tamarillo	*Cyphomandra betacea*	*Tamarillo mosaic virus*
Walnut	*Juglans regia*	*Cherry leafroll virus*
Watermelon	*Citrullus lanatus*	*Cucumber mosaic virus*,
		Watermelon mosaic virus,
		Zucchini yellow mosaic virus,
		Papaya ringspot virus
Forage		
Alfalfa	*Medicago sativa*	*Alfalfa mosaic virus*

(*Continued*)

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Table 2 (*Continued*)

Crop		
Category/Common name	**Scientific name**	**Resistance to**
Grass		
Sugarcane	*Saccharum* sp.	*Sugarcane mosaic virus*,
		Sugarcane yellow leaf virus,
		Sorghum mosaic virus
Legumes		
Bean	*Phaseolus vulgaris*	*Bean golden mosaic virus*
Clover	*Trifolium repens*	*Alfalfa mosaic virus*
Groundnut	*Arachis hypogaea*	*Peanut clump virus*,
		Groundnut rosette virus
Pea	*Pisum sativum*	*Alfalfa mosaic virus*,
		Bean leafroll virus,
		Bean yellow mosaic virus,
		Pea enation mosaic virus,
		Pea seed-borne mosaic virus,
		Pea streak virus
Peanut	*Arachis hypogaea*	*Tomato spotted wilt virus*,
		Groundnut rosette assistor virus,
		Peanut stripe virus
Soybean	*Glycine max*	*Soybean mosaic virus*,
		Bean pod mottle virus,
		Southern bean mosaic virus
Vegetables		
Cucumber	*Cucumis sativus*	*Cucumber mosaic virus*,
		Papaya ringspot virus,
		Squash mosaic virus,
		Watermelon mosaic virus,
		Zucchini yellow mosaic virus
Lettuce	*Lactuca sativa*	*Lettuce mosaic virus*,
		Lettuce necrotic yellows virus
Pepper[b]	*Capsicum*	*Cucumber mosaic virus*,[b]
		Tobacco etch virus,
		Potato virus Y
Potato	*Solanum tuberosum*	*Potato virus A*,
		Potato virus X,
		Potato virus Y,
		Potato leafroll virus,
		Tobacco rattle virus,
		Tobacco vein mottling virus
Squash[a]	*Cucurbita pepo*	*Cucumber mosaic virus*,[a]
		Papaya ringspot virus,
		Squash mosaic virus,
		Watermelon mosaic virus,[a]
		Zucchini yellow mosaic virus[a]

(*Continued*)

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Table 2 (*Continued*)

Crop		
Category/Common name	**Scientific name**	**Resistance to**
Sugar beet	*Beta vulgaris*	*Beet necrotic yellow vein virus*,
		Beet western yellows virus
Sweet potato	*Ipomea batatas*	*Sweet potato feathery mottle virus*
Tomato[b]	*Solanum lycopersicum*	*Beet curly top virus*,
		Cucumber mosaic virus,[b].
		Tobacco mosaic virus,
		Tomato mosaic virus,
		Tomato spotted wilt virus,
		Tomato yellow leaf curl virus

[a]Commercially released in the United States.
[b]Commercially released in the People's Republic of China.

the past 15 years. Elucidating key areas of environmental concern is an enticing research subject. However, risk assessment studies need to be realistic to provide useful information that enables us to distinguish perceived and real risks. Risk assessment studies, if focused exclusively on virus-host interactions, for example, will remain little more than excellent academic exercises with scant relevance to risk assessment and a timely release of virus-resistant transgenic plants. A major impetus of our work was to provide a realistic evaluation of virus-resistant transgenic crops. For this reason, worst case scenarios were imagined and experiments designed accordingly. For example, the interaction between an aphid-transmissible strain of WMV and an aphid-nontransmissible strain of ZYMV was selected to assess heteroencapsidation in squash based on previous evidence on the specific relationship between the CP and helper component (HC) of these two viruses to enhance aphid-mediated spread (58). A 2% heteroencapsidation rate was obtained with this model system. This rate would have likely been lower with another system, for example, PRSV and ZYMV, based on the less stringent specificity of their CP and HC (58). Also, we mainly designed experiments to approach commercial field settings under natural conditions of virus infection and spread via arthropod vectors. Another major driving force of our work was to focus more on the consequences than the occurrence of a specific potential risk. For example, we not only monitored the occurrence of gene flow between virus-resistant transgenic squash and a wild relative in experimental field settings but also examined factors that influence the rate of occurrence, and more important, the consequences of transgene movement in hybrids in terms of increased fitness.

Knowledge on the real effects of virus-resistant transgenic plants has expanded as more studies have been completed under realistic open environments in distinct locations. Extensive research has provided a reasonable certainty of limited, if any, consequences beyond natural background events. Two decades after their introduction, no scientific study has documented any detriment to the environment attributable to virus-resistant transgenic plants. Also, there is a documented history of safe commercial use of virus-resistant transgenic squash and papaya in the United States. Lessons from field experiments and commercial releases have provided overwhelming evidence that benefits largely outweigh risks and that virus-resistant transgenic plants are safe for the environment and consumers. Based on existing scientific evidence, is a case-by-case approach still justified to make

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decisions on the release of virus-resistant transgenic crops? Or, can broader conclusions be drawn, especially regarding those plants that express CP genes, without compromising safety on the environment and human health?

SOME SUGGESTIONS

A major reason for funding risk assessment research is to gather information that help government officials make decisions on setting up regulatory frameworks for transgenic plants. It is reasoned that the research will help authorities determine the potential safety issues that need to be minimized, disregarded, or emphasized.

Evidence suggests that heteroencapsidation and recombination between viral transgene products and challenge viruses are not real risks and can be minimized or disregarded when assessing virus-resistant transgenic plants for deregulation. Likewise, the case of allergenicity of CPs can be minimized. Why? People have been consuming virus-infected fruits and vegetables for extended time with no ill effects caused by the plant virus components, such as the CP and promoter sequence elements. Furthermore, because the mechanism of virus resistance using CP and other viral genes is based on PTGS, resistant plants will almost always produce undetectable or low concentrations of proteins and transgene transcripts as compared to virus-infected plants. Thus, we suggest that heteroencapsidation and recombination can be broadly minimized and do not have to be considered on a case-by-case basis. This could also apply to the allergenicity of CP. However, given that a viral CP sequence can be easily analyzed for its potential allergenicity using accepted bioinformatics and other criteria, this question can be addressed early on. If the transgene sequence does not have significant amino acid sequence homology with known allergens, then allergenicity would not need to be considered.

In contrast, gene flow and all its ramifications need to be considered on a case-by-case basis. The fact that a viral CP gene could be used to confer resistance to viruses in many different plant species that may have different outcrossing potential, weediness tendencies, and distinct habitats provides strong reasons for a case-by-case approach to determine if there is a serious potential environmental risk. An accumulation of objective case-by-case analyses will likely provide a solid framework to determine the amount of information necessary to make realistic regulatory decisions.

Although each country ultimately determines its own regulatory framework and requirements for granting exemption status to transgenic crops, it seems that the risks of heteroencapsidation and recombination can be eliminated or minimized in regulations across countries. This streamlining would have the merit of reducing expenditure in the time and resources involved in deregulating virus-resistant transgenic crops. Furthermore, allergenicity of viral CPs does not need to be investigated if there is evidence for routine consumption of plant products already infected with the target virus(es), and if the sequence of the CP transgene does not have significant homology with potential allergens according to accepted bioinformatics and other criteria.

Finally, viruses continue to play a major role in limiting the production of many crops. Research is ongoing in numerous institutions worldwide to develop innovative and sustainable control strategies needed to mitigate the losses to agriculture from viruses. As such, virus-resistant transgenic plants are in the interest of stakeholders, including growers and consumers. Virus-resistant transgenic crops, which offer numerous benefits to growers and consumers, need to be deployed safely after due assessment of safety considerations. However, risk assessment studies need to be realistic to provide valuable assistance to regulatory authorities for their safe and timely release.

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SUMMARY POINTS

1. Numerous virus-resistant transgenic crops have been successfully developed over the past two decades, and a few of them have been commercially released.
2. Virus-resistant transgenic crops offer many benefits, of economical, horticultural, epidemiological, environmental, and social importance, to agriculture and society.
3. Questions regarding the potential safety ramifications to the environment and human health have been raised over virus-resistant transgenic crops.
4. Extensive safety assessment studies have been carried out over the past 15 years. They provided new insights into the real effect of virus-resistant transgenic plants and demonstrated a limited, if any, significance beyond background events of issues such as heteroencapsidation, recombination, synergism, impact on nontarget organisms, and food safety in terms of allergenicity.
5. Realistic risk assessment is recommended to assist regulatory authorities in making decisions for the safe and timely release of virus-resistant transgenic crops.

FUTURE ISSUES

1. Taking into account the wealth of field observations and experimental data on the limited, if any, adverse effects of heteroencapsidation, recombination, synergism, impact on nontarget organisms, and food safety in terms of allergenicity should be an important step towards reaching a consensus and simplifying regulatory requirements for the release of virus-resistant transgenic crops.
2. Addressing the consequences of gene flow from virus-resistant transgenic crops to wild relatives should remain a major research priority for risk assessment grant programs.
3. Harmonizing regulatory frameworks between countries should be a priority to facilitate technology transfer efforts and timely releases of virus-resistant transgenic crops.

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LITERATURE CITED

1. Acord BD. 1996. Availability of determination of nonregulated status for a squash line genetically engineered for virus resistance. *Fed. Regist.* 61:33484–85
2. Adair TL, Kearney CM. 2000. Recombination between a 3-kilobase tobacco mosaic virus transgene and a homologous viral construct in the restoration of viral and nonviral genes. *Arch. Virol.* 145:1867–83

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3. Allison RF, Schneider WL, Greene AE. 1996. Recombination in plants expressing viral transgenes. *Semin. Virol.* 7:417–22
4. Anonymous. 2003. *Food Produced from Glyphosate-Tolerant Sugar Beet Line 77. Saf. Assess. Rep. Tech. Rep. Ser. No. 24*, Canberra, Aust.: Food Standards Aust. NZ
5. Anonymous. 2006. *Program synopsis. USDA Biotechnol. Risk Assess. Grants (BRAG) Program*. **http://www.csrees.usda.gov/funding/brag/brag_synopsis.html**
6. Anonymous. 2006. *Summer Squash. Seminis Vegetable Seeds*. **http://us.seminis.com/pc/downloadDocument.do?id=5721**
7. Arce-Ochoa JP, Dainello F, Pike LM, Drews D. 1995. Field performance comparison of two transgenic summer squash hybrids to their parental hybrid lineage. *HortScience* 30:492–93
8. Bartsch D, Schmidt M, Pohl-Orf M, Haag C, Schuphan I. 1996. Competitiveness of transgenic sugar beet resistant to beet necrotic yellow vein virus and potential impact on wild beet populations. *Mol. Ecol.* 5:199–205
9. Baulcombe D. 2004. RNA silencing in plants. *Nature* 431:356–63
10. Borja M, Rubio T, Scholthof HB, Jackson AO. 1999. Restoration of wild-type virus by double recombination of tombusvirus mutants with a host transgene. *Mol. Plant-Microbe Interact.* 12:153–62
11. Callaway A, Giesman-Cookmeyer D, Gillock ET, Sit TL, Lommel SA. 2001. The multifunctional capsid proteins of plant RNA viruses. *Annu. Rev. Phytopathol.* 39:419–60
12. Camp SG III. 2003. Identity preservation protocol for non-GMO papayas. *Proc. Virus Resistant Transgenic Papaya in Hawaii: A Case for Technology Transfer to Lesser Developed Countries.* OECD/USAID/ARS Conf., pp. 95–100. Oct. 22–24, Hilo, HI
13. Candelier-Harvey P, Hull R. 1993. Cucumber mosaic virus genome is encapsidated in alfalfa mosaic virus coat protein expressed in transgenic plants. *Trans. Res.* 2:277–85
14. Capote N, Pérez-Panadés J, Monzó C, Carbonell EA, Urbaneja A, et al. 2007. Risk assessment of the field release of transgenic European plums carrying the coat protein gene of *Plum pox virus* under Mediterranean conditions. *Trans. Res.* In press
15. Clough GH, Hamm PB. 1995. Coat protein transgenic resistance to *Watermelon mosaic* and *Zucchini yellow mosaic virus* in squash and cantaloupe. *Plant Dis.* 79:1107–9
16. Costa AS, Muller GW. 1980. Tristeza control by cross protection: a U.S.-Brazil cooperative success. *Plant Dis.* 64:538–41
17. DeZoeten GA. 1991. Risk assessment: Do we let history repeat itself? *Phytopathology* 81:585–86
18. Ellstrand NC. 2003. Current knowledge of gene flow in plants: implications for transgene flow. *Philos. Trans. R. Soc. London Ser. B* 358:1163–70
19. Ellstrand N, Prentice HC, Hancok JF. 1999. Gene flow and introgression from domesticated plants into their wild relatives. *Annu. Rev. Ecol. Syst.* 30:539–63
20. Falk BW, Bruening G. 1994. Will transgenic crops generate new viruses and new diseases? *Science* 163:1395–96
21. Farinelli L, Malnöe P, Collet GF. 1992. Heterologous encapsidation of potato virus Y strain P (PVY^O) with the transgenic coat protein of PVY strain N (PVY^N) in *Solanum tuberosum* cv. Bintje. *Bio/Technology* 10:1020–25
22. Ferreira SA, Pitz KY, Manshardt R, Zee F, Fitch M, et al. 2002. Virus coat protein transgenic papaya provides practical control of papaya ringspot virus in Hawaii. *Plant Dis.* 86:101–5
23. Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC. 1992. Virus resistant papaya derived from tissues bombarded with the coat protein gene of papaya ringspot virus. *Bio/Technology* 10:1466–72

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24. Frischmuth T, Stanley J. 1998. Recombination between viral DNA and the transgenic coat protein gene of African cassava mosaic geminivirus. *J. Gen. Virol.* 79:1265–71
25. Fuchs M, Chirco EM, Gonsalves D. 2004. Movement of coat protein genes from a virus-resistant transgenic squash into a free-living relative. *Environ. Biosaf. Res.* 3:5–16
26. Fuchs M, Chirco EM, McFerson J, Gonsalves D. 2004. Comparative fitness of a free-living squash species and free-living x virus-resistant transgenic squash hybrids. *Environ. Biosaf. Res.* 3:17–28
27. Fuchs M, Gal-On A, Raccah B, Gonsalves D. 1999. Epidemiology of an aphid nontransmissible potyvirus in fields of nontransgenic and coat protein transgenic squash. *Trans. Res.* 99:429–39
28. Fuchs M, Gonsalves D. 1995. Resistance of transgenic squash Pavo ZW-20 expressing the coat protein genes of zucchini yellow mosaic virus and watermelon mosaic virus 2 to mixed infections by both potyviruses. *Bio/Technology* 13:1466–73
29. Fuchs M, Gonsalves D. 2002. Genetic Engineering and Resistance to Viruses. In *Transgenic Plants and Crops*, ed. G Khatachtourians, A McHughlen, R Scorza, WK Nip, YH Hui. 1:217–31. New York: Marcel Dekker. 876 pp.
30. Fuchs M, Klas FE, McFerson JR, Gonsalves D. 1998. Transgenic melon and squash expressing coat protein genes of aphid-borne viruses do not assist the spread of an aphid non-transmissible strain of cucumber mosaic virus in the field. *Trans. Res.* 7:1–14
31. Fuchs M, Tricoli DM, McMaster JR, Carney KJ, Schesser M, et al. 1998. Comparative virus resistance and fruit yield of transgenic squash with single and multiple coat protein genes. *Plant Dis.* 82:1350–56
32. Fuchs RL, Ream JE, Hammond BG, Naylor MW, Leimgruber RM, et al. 1993. Safety assessment of the neomycin phosphotransferase-ii (nptII) protein. *Bio/Technology* 11:1543–47
33. Gaba V, Zelcer A, Gal-On A. 2004. Cucurbit biotechnology—the importance of virus resistance. *In vitro Cell Dev. Biol. Plant* 40:346–58
34. Gal S, Pisan B, Hohn T, Grimsley N, Hohn B. 1992. Agroinfection of transgenic plants leads to viable *Cauliflower mosaic virus* by intermolecular recombination. *Virology* 187:525–33
35. Gianessi LP, Silvers CS, Sankula S, Carpenter JE. 2002. Virus resistant squash. In *Plant Biotechnology: Current and Potential Impact for Improving Pest Management in U.S. Agriculture. An Analysis of 40 Case Studies*. Washington, DC: Natl. Cent. Food Agric. Policy. 75 pp.
36. Gonsalves C, Lee D, Gonsalves D. 2004. *Transgenic virus resistant papaya: The Hawaiian 'Rainbow' was rapidly adopted by farmers and is of major importance in Hawaii today.* **http://www.apsnet.org/online/feature/rainbow**
37. Gonsalves CV. 2001. *Transgenic virus-resistant papaya: farmer adoption and impact in the Puna area of Hawaii*. MA thesis. Albany: State Univ. NY. 170 pp.
38. Gonsalves D. 1998. Control of papaya ringspot virus in papaya: a case study. *Annu. Rev. Phytopathol.* 36:415–37
39. Gonsalves D. 2006. Transgenic papaya: development, release, impact, and challenges. *Adv. Virus Res.* 67:317–54
40. Gonsalves D, Ferreira S, Manshardt R, Fitch M, Slightom J. 1998. *Transgenic virus resistant papaya: new hope for control of papaya ringspot virus in Hawaii.* **http://www.apsnet.org/education/feature/papaya/Top.htm**
41. Gonsalves D, Gonsalves C, Ferreira S, Pitz K, Fitch M, et al. 2004. *Transgenic virus resistant papaya: from hope to reality for controlling of papaya ringspot virus in Hawaii.* **http://www.apsnet.org/online/feature/ringspot**

Annu. Rev. Phytopathol. 2007.45. Downloaded from arjournals.annualreviews.org
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42. Gonsalves D, Vegas A, Prasartsee V, Drew R, Suzuki J, et al. 2006. Developing papaya to control papaya ringspot virus by transgenic resistance, intergeneric hybridization, and tolerance breeding. *Plant Breed. Rev.* 26:35–78
43. Greene AE, Allison RF. 1994. Recombination between viral RNA and transgenic plant transcripts. *Science* 263:1423–25
44. Greene AE, Allison RF. 1996. Deletions in the 3′ untranslated region of cowpea chlorotic mottle virus transgene reduce recovery of recombinant viruses in transgenic plants. *Virology* 225:231–34
45. Hammond J, Dienelt MM. 1997. Encapsidation of potyviral RNA in various forms of transgene coat protein is not correlated with resistance in transgenic plants. *Mol. Plant-Microbe Interact.* 10:1023–27
46. Hammond J, Lecoq H, Raccah B. 1999. Epidemiological risks from mixed virus infections and transgenic plants expressing viral genes. *Adv. Virus Res.* 54:189–314
47. Hansen CN, Harper G, Heslop-Harrison JS. 2005. Characterisation of pararetrovirus-like sequences in the genome of potato (*Solanum tuberosum*). *Cytogenet. Genome Res.* 110:1–4
48. Harper G, Hull R, Lockhart B, Olszewski N. 2002. Viral sequences integrated into plant genomes. *Annu. Rev. Phytopathol.* 40:119–36
49. Hileman RE, Silvanovich A, Goodman RE, Rice EA, Holleschak G, et al. 2002. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. *Int. Arch. Allergy Immunol.* 128:280–91
50. Holt CA, Beachy RN. 1991. In vivo complementation of infectious transcripts from mutant tobacco mosaic virus cDNAs in transgenic plants. *Virology* 181:109–17
51. Hsieh YT, Pan TM. 2006. Influence of planting papaya ringspot virus resistant transgenic papaya on soil microbial biodiversity. *J. Agric. Food Chem.* 54:130–37
52. Ilardi V, Barba M. 2001. Assessment of functional transgene flow in tomato fields. *Mol. Breed.* 8:311–15
53. Jakab G, Vaistij FE, Droz E, Manoë P. 1997. Transgenic plants expressing viral sequences create a favourable environmental for recombination between viral sequences. In *Virus-Resistant Transgenic Plants: Potential Ecological Impact*, ed. M Tepfer, E Balazs, pp. 45–51. Heidelberg: INRA Ed.-Springer
54. Jensen DD. 1949. Papaya virus diseases with special reference to papaya ringspot. *Phytopathology* 39:191–11
55. Kaniweski WK, Thomas PE. 2004. The potato story. *Agric. Biol. Forum* 7:41–46
56. Klas FE, Fuchs M, Gonsalves D. 2006. Comparative spatial spread overtime of *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV) in fields of transgenic squash expressing the coat protein genes of ZYMV and WMV, and in fields of nontransgenic squash. *Trans. Res.* 15:527–41
57. Kleter GA, Peijnenburg AA. 2002. Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential, IgE-binding linear epitopes of allergens. *BMC Struct. Biol.* 2:8
58. Lecoq H, Pitrat M. 1985. Specificity of the helper-component-mediated aphid transmission of three potyviruses infecting muskmelon. *Phytopathology* 75:890–93
59. Lecoq H, Ravelonandro M, Wipf-Scheibel C, Mansion M, Raccah B, Dunez J. 1993. Aphid transmission of a non-aphid transmissible strain of zucchini yellow mosaic virus from transgenic plants expressing the capsid protein of plum pox potyvirus. *Mol. Plant-Microbe Interact.* 6:403–6
60. Lin HX, Rubio L, Smythe A, Jiminez M, Falk BW. 2001. Genetic diversity and biological variation among California isolates of *Cucumber mosaic virus*. *J. Gen. Virol.* 84:249–58

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61. Lindbo JA, Dougherty WG. 2005. Plant pathology and RNAi: a brief history. *Annu. Rev. Phytopathol.* 43:191–204
62. Lius S, Manshardt RM, Fitch MMM, Slightom JL, Sanford JC, et al. 1997. Pathogen-derived resistance provides papaya with effective protection against papaya ringspot virus. *Mol. Breed.* 3:161–68
63. Lommel SA, Xiong Z. 1991. Reconstitution of a functional red clover necrotic mosaic virus by recombinational rescue of the cell-to-cell movement gene expressed in a transgenic plant. *J. Cell Biochem.* 15A:151
64. Martelli GP. 2001. Transgenic resistance to plant pathogens: benefits and risks. *J. Plant Pathol.* 83:37–46
65. MacDiarmid R. 2005. RNA silencing in productive virus infections. *Annu. Rev. Phytopathol.* 43:523–44
66. Manshardt R. 2002. Is organic papaya production in Hawaii threatened by cross-pollination with genetically engineered varieties? *Univ. Hawaii Coll. Trop. Agric. Hum. Res. Biol.*-1. 2 pp.
67. Manshardt RM. 1998. 'UH Rainbow' papaya. *Univ. Hawaii Coll. Trop. Agric. Hum. Res. Germpl.* G-1. 2 pp.
68. Medley TL. 1994. Availability of determination of nonregulated status for virus resistant squash. *Fed. Regist.* 59:64187–89
69. Nelson R, McCormick SM, Delannay X, Dube P, Layton J, et al. 1988. Virus tolerance, plant growth and field performance of transgenic tomato plants expressing the coat protein from tobacco mosaic virus. *Bio/Technology* 6:403–9
70. Osburn JK, Sarkar S, Wilson TMA. 1990. Complementation of coat protein-defective TMV mutants in transgenic tobacco plants expressing TMV coat protein. *Virology* 179:921–25
71. Deleted in proof
72. Paterson JCM, Garside P, Kennedy MW, Lawrence CE. 2002. Modulation of a heterologous immune response by the products of *Ascaris suum*. *Infect. Immun.* 70:6058–67
73. Perring TM, Gruenhagen NM, Farrar CA. 1999. Management of plant viral disease through chemical control of insect vectors. *Annu. Rev. Entomol.* 44:457–81
74. Powell-Abel P, Nelson RS, De B, Hoffmann N, Rogers SG, et al. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232:738–43
75. Pruss G, Ge X, Shi XM, Carrington JC, Vance VB. 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* 9:859–68
76. Qu F, Morris TJ. 2005. Suppressors of RNA silencing encoded by plant viruses and their role in viral infections. *FEBS Lett.* 579:5958–64
77. Quemada H. 1998. The use of coat protein technology to develop virus-resistant cucurbits. In *Agricultural Biotechnology in International Development*, ed. CL Ives, BM Bedford, pp. 147–60. Wallingford, UK: CAB Int.
78. Quemada H, Strehlow L, Decker-Walters D, Staub J. 2002. Case study: gene flow from commercial transgenic *Cucurbita pepo* to "wild" *C. pepo* populations. *Proc. Sci. Methods Workshop Ecol. Agron. Conseq. Gene Flow from Transgenic Crops to Wild Relatives*, pp. 65–70, March 5–6, Columbus, OH
79. Rissler J, Mellon M. 1996. *The Ecological Risks of Engineered Crops*. Cambridge: MIT Press. 168 pp.
80. Robinson DJ. 1996. Environmental risk assessment of releases of transgenic plants containing virus-derived inserts. *Trans. Res.* 5:359–62

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81. Ryang BS, Kobori T, Matsumoto T, Kosaka Y, Ohki ST. 2004. *Cucumber mosaic virus* 2b protein compensates for restricted systemic squash of Potato virus Y in doubly infected tobacco. *J. Gen. Virol.* 85:3405–14
82. Sanford JC, Johnston SA. 1985. The concept of parasite-derived resistance—deriving resistance genes from the parasite's own genome. *J. Theor. Biol.* 113:395–405
83. Savenkov EI, Valkonen JP. 2001. Potyviral helper-component proteinase expressed in transgenic plants enhances titers of *Potato leafroll virus* but does not alleviate its phloem limitation. *Virology* 10:285–93
84. Schoelz JE, Wintermantel WM. 1993. Expansion of viral host range through complementation and recombination in transgenic plants. *Plant Cell* 5:1669–79
85. Schultheis JR, Walters SA. 1998. Yield and virus resistance of summer squash cultivars and breeding lines in North Carolina. *HortScience* 8:31–39
86. Scorza R, Hily JM, Callahan A, Malinowski T, Cambra M, et al. 2007. Deregulation of plum pox resistant transgenic plum 'HoneySweet'. *Acta Hortic.* In press
87. Shankula S. 2006. Quantification of the impacts on US agriculture of biotechnology-derived crops planted in 2005. **http://www.ncfap.org**
88. Snow AA, Palma PM. 1997. Commercialization of transgenic plants: potential ecological risks. *BioScience* 276:932–34
89. Souza JMT, Tennant PF, Gonsalves D. 2005. Influence of coat protein transgene copy number on resistance in transgenic line 63-1 against papaya ringspot virus isolates. *HortScience* 40:2083–87
90. Stewart CN, Halfhill MD, Warwick SI. 2003. Transgene introgression from genetically modified crops to their wild relatives. *Nat. Rev. Genet.* 4:806–17
91. Tanne E, Sela I. 2005. Occurrence of a DNA sequence of a non-retro RNA virus in a host plant genome and its expression: evidence for recombination between viral and host RNAs. *Virology* 332:614–22
92. Tennant P, Fermin G, Fitch MM, Manshardt RM, Slightom JL, et al. 2001. Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. *Eur. J. Plant Pathol.* 107:645–53
93. Tennant PF, Gonsalves C, Ling KS, Fitch M, Manshardt R, et al. 1994. Differential protection against papaya ringspot virus isolates in coat protein gene transgenic papaya and classically cross-protected papaya. *Phytopathology* 84:1359–66
94. Tepfer M. 2002. Risk assessment of virus-resistant transgenic plants. *Annu. Rev. Phytopathol.* 40:467–91
95. Teycheney PY, Aaziz R, Dinant S, Salanki K, Tourneur C, et al. 2000. Synthesis of (-) strand RNA from the 3′ untranslated region of plant viral genome expressed in transgenic plants upon infection with related viruses. *J. Gen. Virol.* 81:1121–26
96. Thomas PE, Hassan S, Kaniewski WK, Lawson EC, Zalewski JC. 1998. A search for evidence of virus/transgene interactions in potatoes transformed with the potato leafroll virus replicase and coat protein genes. *Mol. Breed.* 4:407–17
97. Tricoli DM, Carney KJ, Russell PF, McMaster JR, Groff DW, et al. 1995. Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to *Cucumber mosaic virus*, *Watermelon mosaic virus 2*, and *Zucchini yellow mosaic virus*. *Bio/Technology* 13:1458–65
98. Varrelmann M, Palkovics L, Maiss E. 2000. Transgenic or plant expressing vector-mediated recombination of *Plum pox virus*. *J. Virol.* 74:7462–69
99. Vigne E, Bergdoll M, Guyader S, Fuchs M. 2004. Population structure and genetic diversity within *Grapevine fanleaf virus* isolates from a naturally infected vineyard: evidence for mixed infection and recombination. *J. Gen. Virol.* 85:2435–45

100. Vigne E, Komar V, Fuchs M. 2004. Field safety assessment of recombination in transgenic grapevines expressing the coat protein gene of *Grapevine fanleaf virus*. *Trans. Res.* 13:165–79
101. Voinnet O. 2001. RNA silencing as a plant immune system against viruses. *Trends Genet.* 17:449–59
102. Voinnet O. 2005. Induction and suppression of RNA silencing: insights from viral infections. *Nat. Rev. Genet.* 6:206–21
103. Waterhouse PM, Wang MB, Lough T. 2001. Gene silencing as an adaptative defense against viruses. *Nature* 411:834–42
104. Waterhouse PM, Fusaro AF. 2006. Viruses face a double defense by plant small RNAs. *Science* 313:54–55
105. Wintermantel WM, Schoelz JE. 1996. Isolation of recombinant viruses between *Cauliflower mosaic virus* and a viral gene in transgenic plants under conditions of moderate selection pressure. *Virology* 223:156–64
106. Yeh SD, Gonsalves D. 1994. Practices and perspectives of control of papaya ringspot virus by cross protection. In *Advances in Disease Vector Research*, ed. KF Harris, pp. 237–57. New York: Spinger-Verlag

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