Corporation obtaining approval, the name of its representative, and the address of its main office

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Papaya resistant to papaya ringspot virus - PRSV (Modified *PRSV CP*, *uidA*, *nptII*, *Carica papaya* L.) (55-1, OECD UI: CUH-CP551-8)
Content of the Type 1 Use of Living Modified Organism	Provision as food, cultivation, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	-

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effect on Biological Diversity

1. Information concerning a recipient organism or the species to which the recipient organism belongs

(1) Taxonomy and distribution in nature

1) English name and Scientific name

English name: Papaya
Scientific name: *Carica papaya* L.

2) Name of variety of the recipient organism or name of line

The recipient organism is the "Sunset" variety of Solo type papaya belonging to the genus *Carica*, Caricaceae.

3) Wild-growing areas under natural environment at home in Japan and abroad

Domesticated papaya originated from the species bearing small edible fruits in Central America (Badillo, 2000) and, today, papayas are cultivated in all tropical zones and many subtropical zones. On the other hand, wild papayas are present only in the Caribbean region and in Central America (from southern Mexico and the Yucatan on the north to Honduras on the south) and they are growing in man-made clearings of tropical rain forests (Paz and Vázquez-Yanes, 1998).

In Japan, there is a report that papayas have gone wild in the Ogasawara Islands and other areas (www2.kankyo.metro.tokyo.jp/sizen/isan/pdf/kentou-all.pdf), though specific growing areas have not been identified. However, literature from overseas revealed that the optimal growth temperature is between 21 and 33°C (Nakasone and Paull, 1998) and that the minimum ambient temperature for growth is 15°C (Samson, 1986). In addition, papayas are very sensitive to frost, and growth is severely affected when exposed to temperatures below 12 to 14°C, even for several hours at night. Furthermore, the minimum ambient temperature for survival is reportedly -1°C (Samson, 1986), though the plant reportedly withers and dies at 0°C due to freezing damage. Based on the above understanding, it is considered that the areas facilitating the growth of papayas in the Japanese Islands are limited to the islands south of the southern Amami Oshima Islands including the entire main Okinawa island, Ogasawara Islands and Minami-Torishima Island, where there is no "winter day" (during which the minimum temperature falls below 0°C) (Annex 1, p.18), and the monthly mean minimum temperature never drops below 12°C.

(2) History and present state of use

1) History of Type 1 Use both at home in Japan and abroad

Papaya was first domesticated in Central America (the region from southern Mexico to northern Honduras) (Manshardt and Zee, 1994). The region was once the center of the ancient Mayan civilization, and the domesticated papayas of today are considered to have developed during a long period of selection from wild papayas by the ancestors of the Mayans. The term "papaya" means sweet in the native language of Caribbean Indians, which indicates that papaya has a long history of use as a food (de Mello and Spruce, 1869).

Since the conquest of the American continents by Europeans, papaya was rapidly spread to the entire tropical regions through the channels of trade established by Spain and Portugal. Today, papaya is an essential food for diet in the tropical regions (especially serving as a source of vitamins A and C) (Nakasone and Paull, 1998). In lowland tropical areas, papaya is a common plant grown in gardens and/or entranceways, since it is well liked for its property to produce fruits constantly throughout the year (Gonsalves, 1998). In recent years, papaya has also become familiar to consumers in the temperate region as an article of international trade (Samson, 1986).

It is considered that papaya was first introduced to Okinawa in the same time period as the introduction into the Philippines and other countries in the mid-16th century (Yamazaki *et al*., 2004). Since then, in Japan, papaya was cultivated as a specimen in greenhouses in different areas across the country.

2) Main cultivating areas, cultivating methods, state of physical distribution and uses

Cultivation of papaya is centered in tropical and subtropical regions around the equator, serving as an important commercial crop plant. The world's leading papaya producers include Brazil (1.9 million tons), Mexico (800,000 tons), Nigeria (770,000 tons), India (700,000 tons) and Indonesia (650,000 tons), and the papaya production in these five (5) countries accounts for about 69% of the total production in the world (FAO, 2007). Major exporters include Mexico (95,000 tons), Malaysia (51,000 tons), Belize (34,000 tons) and Brazil (32,000 tons) (FAO, 2006).

Current domestic production of papaya primarily depends on Okinawa, where the production amounted to 1,420 tons in 2006, making the papaya an essential commercial tropical fruit in Okinawa Prefecture (Statistical data by Agriculture, Forestry and Fisheries Planning Division, Department of Agriculture, Forestry and Fisheries, Okinawa Prefecture, 2006). In addition to the domestic production, papaya is imported from overseas, recording about 3,817 tons in fiscal 2008, including about 2,918 tons, 76.5% of the total import, from the Philippines and about 889 tons, 23.3%, from the U. S. (Trade Statistics of Japan, 2008).

Cultivation of papaya in tropical and subtropical areas is based on large-scale cultivation in plantations or homegrown small-scale cultivation (Tabei, 2000).

Papaya is normally propagated by seed, and it takes 10 to 21 days for germination (OECD, 2005). Seedlings are transplanted into seedling containers at the two-leaf (cotyledonary leaves) stage and hardened in sunlight. One and a half to two months after germination when the plant height reaches 20 cm, the seedlings are field-transplanted (Nakasone and Paull, 1998). If conditions are right, the first flowers are open about 100 days after planting in the fields, allowing sexual reproduction (Marler and Discekici, 1996 adopted in Nakasone and Paull, 1998). In the tropical region, the fully-grown trees come into bloom throughout the year, allowing sexual reproduction, whereas in the subtropical region, papaya bears no fruit in the winter season (Nakasone and Paull, 1998). The first fully ripened fruits may be harvested in the seventh month or so after planting in the field in the shortest possible case (Marler and Discekici, 1996 adopted in Nakasone and Paull, 1998). Thereafter, papaya continues to flower and bear fruit, though the fruits become smaller and the yield drops in subsequent years. In addition, the plant grows too high for proper management, and then it is trimmed otherwise it is substituted by new seedlings (Samson, 1986).

In Japan, papaya is grown both outdoors and in greenhouses. However, papaya is traded at high prices, and consequently it is often cultivated in greenhouses for protection against damage from low temperatures in winter, rainfall in the rainy season, and typhoons.

Mature fruit good sweet, and it is rich in calcium and vitamins A and C, and it is utilized primarily for raw consumption. In addition, immature papaya (green papaya) is used as a vegetable for a salad in Asian countries, and "papain" (cysteine protease enzyme), a component of latex extracted from green papaya, has practical applications in the food and pharmaceutical industries as meat tenderizer and digestive medicine (Nakasone and Paull, 1998). Also, in Japan, papain is listed as a food additive and applied in a wide variety of uses (Tabei, 2000).

(3) Physiological and ecological properties

1) Basic properties

Papaya is an evergreen perennial herb-like tree, and it can be propagated both by seed and vegetatively. A single straight-standing stem becomes 2 to 10 m in height, forming a crown with large leaves (OECD, 2005). Usually, the stem grows without branching and becomes half-lignified and hollow. The bark is smooth and gray in color, with large, prominent leaf scars (marks left by falling leaves). Always new leaves emerge at the apex, and old leaves senesce and fall. Leaves are palmately lobed with prominent venation, and the diameters reach 40 to 50 cm (Nakasone and Paull, 1998).

The flowers appear in the axils of the leaves. Papaya inherently bears dioecious flowers, though many domesticated cultivars bear monoecious or hermaphrodite flowers (Kubo, 1987). Sunset, the recipient organism of papaya line 55-1, is gynodioecious, producing either female flowers or bisexual hermaphrodite flowers through segregation. (Hereinafter "papaya line 55-1" is referred to as "this recombinant papaya.") The progeny from the selfing of a hermaphrodite plant of Sunset were used for the development of this recombinant papaya, and the sex of

this R0 generation recombinant papaya was female. The female plant produces only female flowers, on short, 4- to 6-cm peduncles, with a pistil devoid of stamens. Five (5) petals are separated, though they are fused together at the base of the ovary. In addition, the R1 progeny of this recombinant papaya included hermaphrodite plants. The hermaphroditic form is between the two unisexual flower types, though it exhibits deviations. The basic form of flowers features an elongated pistils, five (5) stigmatic rays and five (5) petals that are fused for 2/3 of their length, forming a corolla tube. The flower has ten (10) stamens arranged in two whorls of 5 stamens. The pistil usually composed of five (5) carpels that vary in shape (Nakasone and Paull, 1998).

Hermaphrodite plants of the Sunset variety, the recipient organism of this recombinant papaya, bear pear-shaped fruits, that weigh 400g to 600g and have flesh thickness of 2 cm and sugar content of 12 to 17% (Hamilton *et al*., 1993).

2) Environmental conditions allowing inhabiting or growth

Papayas are cultivated in the regions between 30 degrees north latitude and 40 degrees south latitude, though the commercial cultivation is centered on the equatorial region between 25 degrees north and south latitudes (OECD, 2005). Papayas can grow in a variety of soil types, though the soils need to be well drained, since badly drained soils cause root rot. A soil pH of 5.0 to 7.0 is suitable for cultivation (Nakasone and Paull, 1998), with the pH of 6.5 to 7.0 considered best (Singh, 1990). A rainfall of at least 350 mm is necessary, while excess moisture can adversely affect the plant trunk and fruits. The maximum tolerable rainfall is about 2,500 mm (Singh, 1990). The relative humidity required for optimum growth is higher than 60% (FAO, 1986). The temperature for optimal growth is 21 to 33°C (Nakasone and Paull, 1998), and the minimum temperature for growth is 15°C (Samson, 1986). Papayas are very sensitive to frost, and the growth is severely affected when exposed to temperatures below 12 to 14°C even for several hours at night. In addition, the minimum temperature for survival is reported to be -1°C (Samson, 1986), though the plant reportedly withers and dies at 0°C due to freezing damage. Papayas need sunlight for growth and growth is inhibited when cultivated in the shade (Nakasone and Paull, 1998). In addition, the ambient temperature and direct sunlight are also critical for fruit ripening and in fact, it is generally known that lower temperatures cause delay in fruit ripening and also decrease sugar contents (Nakasone and Paull, 1998; Samson, 1986). There is no report that photoperiod can affect the growth of papayas (Lange, 1961b). Papaya trees are very susceptible to wind and they can be uprooted by winds of 18 m/s, especially if the soil is softened by rain. In addition, even though the trees may withstand uprooting, leaves are greatly damaged by wind, leading to flower and young fruit abscission (Nakasone and Paull, 1998).

3) Predacity or parasitism

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4) Mode of propagation or reproduction

(a) Shedding habit, mode of dispersion, dormancy and longevity of the seed

The mature fruits have a round or star-shaped seed cavity (Nakasone and Paull, 1998), in which several hundred to a thousand seeds are formed (Tsuchihashi, 2003). The seeds are dark gray to black in color and enclosed in jellylike sarcotesta. Seedless fruit, or fruit with very few seeds, can be produced on female plants (Nakasone and Paull, 1998). Since the seeds are enclosed within the fleshy fruit that is several centimeters in thickness, they are extremely unlikely to be shed.

Because the seed sarcotesta inhibits germination, low germination rates would result compared to the case when seeds are sown after the sarcotesta is removed (Lange, 1961a). Seeds of papaya weigh about 50 mg each in the air-dried condition (Samson, 1986). Therefore, there is a low possibility that the seeds would be dispersed by wind for great distances, though dispersion of seeds may occur when birds and/or mammals eat the fruits.

There has been no report available for the germination rate of the seeds of the Sunset variety, though it is generally known that the germination rate of papaya seeds varies greatly (3 to 71%) with varieties (Bhattacharya and Khuspe, 2001).

For wild papayas, there are reports that the number of seeds germinating in a specified period of time is smaller and the number of days to seed germination is larger compared to that of domesticated papaya even if the germination inhibitor-containing sarcotesta is removed. This is due to the fact that wild papayas need light and other specific environmental conditions for breaking of dormancy and the timing of germination. Consequently, it is considered that wild papaya seeds could survive in the dormant condition for an extended period of time if buried in the soil. On the other hand, domesticated papaya seeds are not so highly sensitive to light and other environmental conditions as wild papayas and so, they do not have such dormancy as observed in wild papayas (Paz and Vázquez-Yanes, 1998).

Papaya seeds can be preserved for a long period of time at a relative humidity of 9 to 12% (Teng and Hor, 1976; Ellis *et al.*, 1991), and they can reportedly survive for three (3) years in a low-temperature and dry place (Malo and Campbell, 1994). On the other hand, there is a report that the preservation of seeds in dry conditions at room temperature or in the soil for three (3) years resulted in a germination rate of 0% (Orozco-Segovia and Vazquez-Yanes, 1990).

(b) Mode of vegetation propagation and the property of germination from any tissue or organ that could regenerate the plant body under natural conditions

Papaya accommodates both seed propagation and vegetative propagation through cutting, grafting, sprouts from the stump or other means. However, the vegetative propagation is not adopted by farmers due to low economical efficiency (Samson, 1986).

(c) The degree of autogamy and allogamy, presence or absence of self-incompatibility, possibility of crossing with wild relative, and the degree of apomixis causing characteristics, if present

The Sunset variety, the recipient organism of this recombinant papaya, is gynodioecious and thus, either a female plant or an hermaphrodite plant can arise in the progeny through segregation. When the Solo type papaya Sunrise, a close relative of Sunset variety, was grown adjacently to this recombinant papaya, the rate of cross pollination was found to be 70% for the female plant, but only 13% for the hermaphrodite plant, demonstrating that the hermaphrodite plant offers a higher rate of self pollination (Annex 2). This may be explained by the structure of the hermaphrodite flowers, that in general which allows spontaneous self-pollination. In addition, self-incompatibility is uncommon in hermaphrodite plants (Nakasone and Paull, 1998).

Domesticated papaya and its closely related wild relatives are found growing together in the tropical region in the Central and South America and Equatorial Africa, though it is generally known that crossing of papaya with these wild relatives is impossible without human intervention (Manshardt and Wenslaff, 1989a; Manshardt and Drew, 1998). In Japan, there are no wild relatives that can be crossed with papaya.

Previous studies have revealed that even unpollinated papaya ovules can occasionally produce seeds with the aid of embryo rescue (Tokumoto *et al.*, 2000a, b; Vegas *et al.*, 2003). This suggests that doubling of haploid egg cells generated apomictic embryos. However, ovules generated by apomixis lack endosperm, and consequently such seeds are considered not to germinate. There has been no report that papaya has produced apomictic seeds that can germinate.

(d) Production, fertility, shape, transmission method, dispersion distance and longevity of pollen

Papaya produces about 100,000 to 150,000 pollen grains per flower depending on the type of flower (male flower, hermaphrodite flower, etc.) and variety (Lassoudiere, 1968; Parés-Martínez *et al.*, 2004). However, there is a report that even for the same variety, the pollen production of the Yellow Cartagena variety produces is only about 20% (about 23,000 pollen grains per flower) of that of the Red Cartagena variety (Parés *et al.*, 2001). For the production of pollen per plant, the number of flowers set varies with different sexes. Consequently, based on the previously observed pollen production per flower, it is estimated that the hermaphrodite plant produces 100,000 to

750,000 pollens per day and the male plant produces several times as many pollen grains as of that of the former.

The Sunset variety, the recipient organism of this recombinant papaya, exhibited 95% pollen stainability (Lius, 1994). The production and fertility of pollen are both known to vary with season. The pollen viability which averaged 90% throughout most of the year was found to drop to 45% in the cold season in some varieties and as low as 4.5% in others (Garrett, 1995). In addition, the germination rate of pollen, which is typically between 40 and 80%, can drop to less than 10% at temperatures below 10°C (Cohen *et al.*, 1989).

For pollination of papaya, insects are considered to act as important pollinators, though wind pollination is considered to be also feasible. To date, a wide variety of insects (thrips, moth, fly, mosquito, etc.) have been identified to visit the papaya plants, though their role as pollinators has not yet been clarified definitely (OECD, 2005).

In Hawaii, examination was conducted for possible dispersion of pollen and transfer of genes from this recombinant papaya to non-recombinant female papayas cultivated in the same field (Annex 2). As a result, most outcrossings were found at sites within 9 m from this recombinant papaya, and there was a trend whereby the outcrossing rate decreased with increasing distance from this recombinant papaya (r=-0.32). In this examination, outcrossings were detected even at the 26 m-distant point, the longest distance in the test plot, and thus it is estimated that pollen can be dispersed up to that distance mediated by wind or insects. However, this examination was not on such an adequate scale for investigating the longest possible distance for pollen dispersion, and consequently, supplementary examination was later conducted in a commercial papaya field located about 400 m distant from the above-mentioned experimental plot. At this site, no transgene derived from this recombinant papaya was detected among the non-recombinant papayas cultivated in the field and therefore, it is considered that dispersion of pollen is unlikely to occur at the distance of 400 m or more, though this value is a rough estimate.

The longevity of pollen of papaya is relatively long, and there is a report that in petri dishes at room temperature, 16% of the total pollen tested remained fertile after 16 days (Sharma and Bajpai, 1969). In addition, at 5°C, pollen can be preserved for several months, and at -18°C, pollen can be preserved for 6 months or more with a high germination rate (Cohen *et al.*, 1989). To date, there has been no report for the longevity of papaya pollen under natural condition.

5) Pathogenicity

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6) Productivity of harmful substances

Extracts from papaya seeds contain benzyl isothiocyanate (BITC). Previous studies suggest that extracts from papaya seeds can cause functional aberrations of different mammalian tissues and organs, that are considered to result from the toxicity of BITC (Adebiyi *et al.*, 2003). However, in some areas in Asia and South America, extracts from papaya seeds containing BITC were traditionally used as vermifugal agent or abortifacients (Krishnakumari and Majumder, 1960; Quisumbing, 1951; Rao and Jamir, 1982). For example, Ayurvedic traditional medical treatment prescribes administration of 0.5 to 1 g powdered papaya seeds (Kapoor, 1990). In addition, in Cuba, it is recommended to take a maximum of 4.5 g papaya seeds per day, as a vermifugal agent (Roig y Mesa, 1974).

BITC is produced when benzylglucosinolate is hydrolyzed by the enzyme myrosinase. In actuality, however, benzylglucosinolate is primarily contained in the endosperm, and myrosinase is present in the sarcotesta covering the seeds (Tang, 1973) so, there is no chance that the substrate and enzyme will come into contact with each other and generate a large amount of BITC unless the seeds are crushed or damaged (Kermanshai *et al.*, 2001).

On the other hand, BITC belongs to the isothiocyanate (ITC) family, and consequently it is reported to have anticancer effects as similar as ITC family members. BITC and other various substances in the ITC family have been confirmed to be effective as chemo-protective agentsagent against chemical carcinogensis in experimental animals. Epidemiological researches have also demonstrated that the dietary intake of foods containing ITC negatively correlate with risks of lung cancer, breast cancer and/or colon cancer, thereby suggesting anticancer effects (Zhang *et al*., 2003; Basu and Haldar, 2008; Traka and Mithen, 2009). Furthermore, besides the anticancer effects, BITC is also reported to work effectively for protection against plant-parasitic nematodes that damage major crops of agricultural importance and cause huge losses every year throughout the world (Zasada *et al*., 2009).

The latex present in the leaves and immature fruits contains papain, a proteolytic enzyme, and it is known to play an important role in the defense of papaya plants against herbivorous insects (Konno *et al*., 2004). In tests by Konno *et al*. (2004), papaya leaves or artificial feed containing papain was given to lepidopteran larvae and as a result, it was confirmed that papain possesses toxicity and growth inhibition effects against the larvae.

In addition, there is a concern about possible effects on the fetus by intake of immature papaya during the period of pregnancy. However, as the fruits become ripe, the papain content decreases, and in the fully ripened fruits, it is extremely low (Thomas and Beckly, 1923 cited in Traub *et al*., 1935). In fact, the experiment using mice has reported that intake of mature fruits of papaya has no effect on the fetus (Adebiyi *et al.*, 2002).

Carpaine, an important alkaloid of papaya, is contained in the entire green portion and seeds of papaya plant (Burdick, 1971), and it is reported to produce bradycardiac symptoms and other physiological activity (Burdick, 1971; Hornick *et al.*, 1978).

7) Other information

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2. Information concerning preparation of living modified organisms

In the papaya producing areas in the world, papaya ringspot virus (hereinafter referred to as "PRSV") is defined as the most important pathogen to obstruct the production of papaya. Major symptoms appearing when infected with PRSV include distinct ringspots on the fruits, mosaic condition or chlorosis on the leaves, and suppressed growth or poor fruit development and decreased sugar content (Gonsalves, 1998; Tabei, 2000) (Annex 3). Transmission of virus occurs via aphids that have previously fed on virus-infected trees[1] (Gonsalves, 1998).

Damage by PRSV has been reported in papaya-cultivating areas all over the world and Hawaii has also being suffering devastating damage caused by PRSV (Gonsalves, 1998). To date, a number of control methods (extermination of transmitting insects, cross protection, variety improvement, etc.) have been attempted, though they are all found having limited efficacies and the wide spread of PRSV has been difficult to suppress. However, in 1986, recombinant tobacco resistant to TMV was successfully developed by introducing the coat protein (*TMV CP*) gene of tobacco mosaic virus into tobacco. By applying the same technology, development of PRSV-resistant papaya was launched in the same year.

This recombinant papaya was developed by introducing the coat protein (CP) gene derived from PRSV into the Solo type variety Sunset to confer the resistance to PRSV. The R0 generation of this recombinant papaya, a female plant and heterozygote for the transgene, was sib-crossed with a hermaphrodite plant of the non-recombinant variety Sunset to produce a recombinant hermaphrodite plant of papaya line 55-1. Then, self-pollination was repeated over several generations to develop homozygous plants for the target gene. This is the variety SunUp (Figure 2, p.19). Furthermore, SunUp and the non-recombinant variety Kapoho were crossed with each other to obtain the F1 hybrid variety Rainbow (Figure 3, p.20).

This recombinant papaya developed in Hawaii features higher yield and improved quality due to the resistance to PRSV. At present, this recombinant papaya Rainbow accounts for over half of the total papaya production in Hawaii.

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of the donor nucleic acid and the origins of component elements that was used for the development of this recombinant papaya are shown in Figure 1 (p.11) and Table 1 (pp.12-13).

[1] A possibility of virus transmission by aphids from papaya fruits was considered, though the fact could not identified based on the literature review.

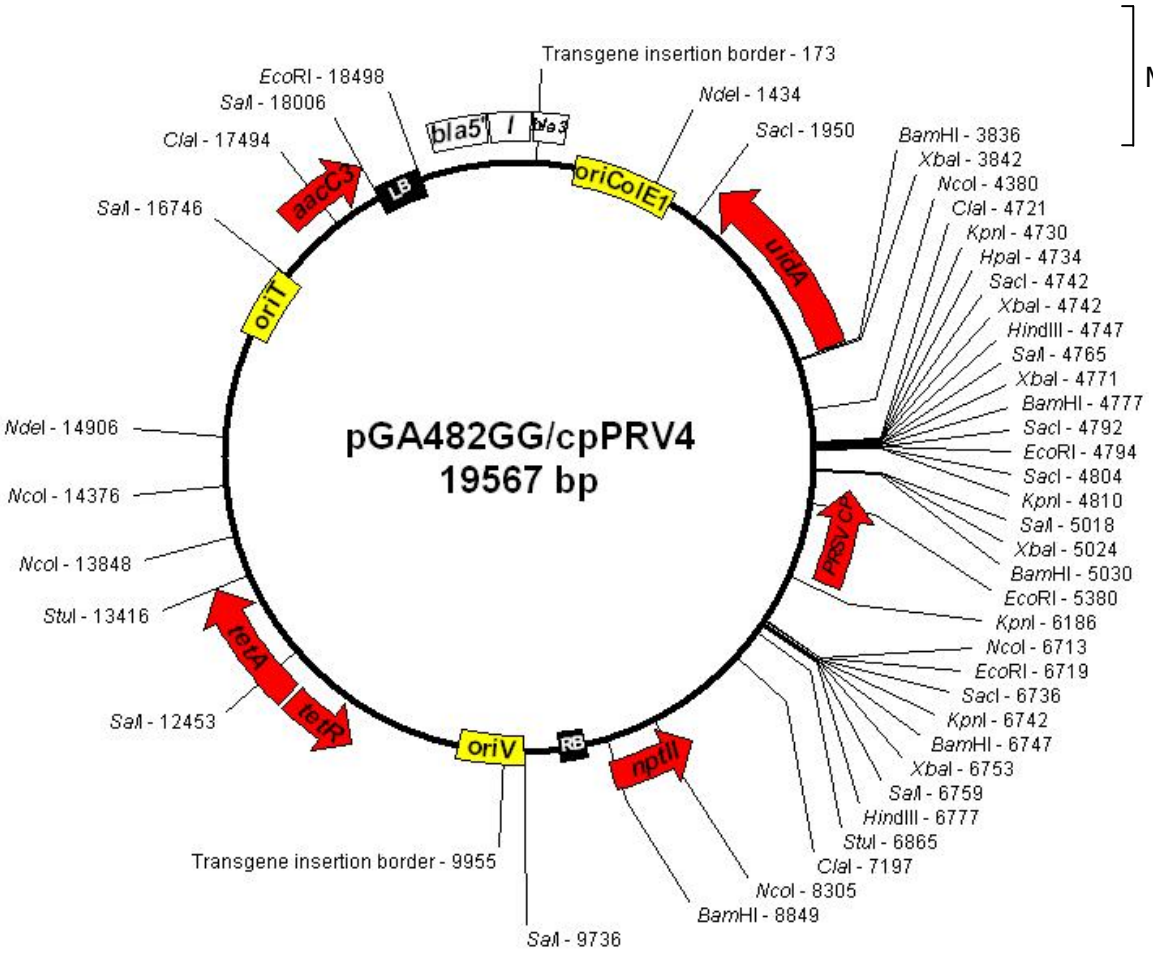

Figure 1 Map of plasmid vector pGA482GG/cpPRV-4

The red arrows represent relevant ORF (open reading frames) including, not only the target transgene, *uidA* gene (encoding the GUS protein) and the *nptII* gene (encoding the neomycin phosphotransferase) but also the *tetA* gene and *tetR* gene (conferring the resistance to tetracycline) and the *aacC3* gene (conferring the resistance to gentamicin) in the vector backbone sequence. The plasmid replication origin regions are shown in yellow, the T-DNA border regions are shown in black, and the truncated sequence (5' and 3' ends of *bla* gene and λ DNA including the *cos* sites) is shown in white. The *CP* gene expression cassette derived from PRSV HA5-1 strain was cloned in the single *Hind*III site present in the plasmid vector pGA482GG. This map provides nucleotide positions of restriction sites including those at and between restriction sites 4721 (*Cla*I) and 4747 (*Hind*III) of the multiple cloning site (MCS) and those found in the PRSV HA 5-1 CP gene cassette. The map also contains the *Stu*I site (the *Bgl*II site, another restriction enzyme used for the analysis is not present in the expression vector) used for analysis of the inserted gene in this recombinant papaya, and the *Nde*I and *Hpa*I sites used as the positive control for digestion of expression vectors during the Southern blot analysis.

Table 1 Origins and functions of the component elements of pGA482GG/cpPRV-4 used for the development of this recombinant papaya

Constitutive DNA	Size* (kb)	Origin and function
nptII gene expression cassette		
nos promoter	0.18	A promoter sequence of nopaline synthase gene derived from *Agrobacterium tumefaciens* Ti plasmid: It controls the expression of structural genes located adjacently or in the downstream (Depicker *et al*., 1982).
nos:nptII gene	0.82	A fused gene of a part of the *nos* gene derived from *A. tumefaciens* Ti plasmid and a gene region derived from *Escherichia coli* transposon Tn5. The *nptII* gene isolated from transposon Tn5 of prokaryote encodes the neomycin phosphotransferase II. This gene, when expressed in microorganisms, confers the resistance to kanamycin, acting as a selection marker during transformation (Beck *et al*., 1982).
nos terminator	0.25	A terminator sequence of the nopaline synthase gene from *A. tumefaciens* Ti plasmid: It terminates the transcription and initiates polyandelyation of structural genes located adjacently or in the upstream (Depicker *et al*., 1982).
Modified *PRSV CP* gene expression cassette		
CaMV 35S promoter	0.53	35S promoter sequence from cauliflower mosaic virus (CaMV): It controls the expression of structural genes located adjacently or in the downstream (Benfey and Chua, 1990; Franck *et al.*, 1980).
Modified *PRSV CP* gene	0.92	A coat protein gene obtained from the papaya ringspot virus (PRSV) HA 5-1 strain, which fused with coat protein sequence coding of the first 16 amino acids of cucumber mosaic virus (CMV) at N-terminus.
CaMV 35S terminator	0.20	35S terminator sequence from CaMV: It terminates the transcription of structural genes located adjacently or in the upstream (Franck *et al.*, 1980).
uidA gene expression cassette		
CaMV 35S promoter	0.83	35S promoter sequence from the cauliflower mosaic virus (CaMV), which controls the expression of structural genes located adjacently or in the downstream (Benfey and Chua, 1990; Franck *et al.*, 1980).
uidA gene	1.81	β-glucuronidase gene derived from *E.coli*, encoding the GUS protein (Jefferson *et al.*, 1986).
nos terminator	0.25	A terminator sequence of the nopaline synthase gene from *A. tumefaciens* Ti plasmid: It terminates the transcription and initiating polyadenylation of structural genes located adjacently or in the upstream (Depicker *et al*., 1982).

Constitutive DNA	Size* (kb)	Origin and function
Other component elements		
oriColE1	1.08	Plasmid replication origin region, pColE1, cloned from pBR322.
bla gene	0.55 & 0.32	A β-lactamse gene region conferring ampicillin resistance derived from pBR322 (Sutcliffe, 1978), though not functioning since it was divided by the *cos.*
λ insertion	0.40	A region containing the *cos* site of λ phage, facilitating the transfer of foreign DNA of larger size.
T-DNA border region (Left)	0.43	T-DNA left border region from *A. tumefaciens* Ti plasmid, containing the 25bp of termination point of T-DNA transfer (Zambryski *et al*., 1982).
aacC3 gene (*gent*)	0.86	Aminoglycoside N3'-acetyltransferase gene (*aacC3*) conferring the resistance to gentamicin, an aminoglycoisde antibiotic, derived form *E. coli* (Allmansberger *et al*., 1985).
oriT	0.10	Origin of replication region for conjugation and transmission of plasmid derived from broad host range plasmid RK2 (Guiney and Yakobson, 1983).
traJ gene	0.37	Relaxosome protein coding region involved in conjugation and transmission of plasmid derived from broad host range plasmid RK2, incorporated as part of *oriT* region (Fürste *et al.*, 1989).
tetR / tetA	0.65/1.20	Tetracycline-resistant gene region derived from broad host range plasmid RK2 (Schmidhauser *et al*., 1985), containing the *tet*R, transcription regulating region, and the tetracycline-resistant gene *tetA.*
oriV	0.71	Origin of replication region derived from broad host range plasmid RK2, conferring the autonomous replication ability in *Agrobacterium* to vectors (Stalker *et al.*, 1981; Rogers *et al.*, 1987).
T-DNA border region (Right)	0.28	T-DNA right border region from *A. tumefaciens* Ti plasmid, containing 25bp of initiation points for T-DNA transfer to plant genome. It initiates the transfer of T-DNA from *A. tumefaciens* to plant genome (Depicker *et al*., 1982; Zambryski *et al*., 1982).

* DNA sizes shown above refer to the size of individual constitutive DNAs, not containing any sequences between constitutive DNAs.

(2) Functions of component elements

1) Functions of individual component elements of donor nucleic acid, including target gene, expression regulatory region, localization signal, and selective marker

Functions of component elements of the donor nucleic acid used for the development of this recombinant papaya are shown in Table 1 (pp.12-13). Functions of the modified *PRSV CP* gene contained in the transgene region are detailed below.

[Modified *PRSV CP* gene]

The modified *PRSV CP* gene transferred in this recombinant papaya corresponds to the 9,254th to 10,120th bases in the PRSV genome, and it is considered to provide the PRSV resistance to the recipient organism through the post-transcriptional gene silencing (PTGS), a general function for controlling gene expression (Tennant *et al.*, 2001). The PTGS allows transcription to occur as usual, but any produced RNA will be broken down immediately. This causes the transgene in plant to inhibit or down-regulate the expression of homologous gene of virus and as a result, the resistance to virus infection is conferred.

2) Functions of proteins produced by the expression of target gene and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

[GUS protein (β-glucuronidase)]

The GUS protein (β-glucuronidase) expressed from the *uidA* gene is an enzyme that hydrolyzes the glucuronic acid and the β-glucuronide, a condensation product with various aglycons. Because this substrate produces the blue dye on hydrolysis by the GUS protein, it has been used as a visible quantitative maker during plant transformation.

[NPTII protein]

The NPTII protein expressed from the *nptII* gene is an enzyme that catalyzes the phosphorylation of kanamycin, neomycin and other antibiotics and, it was used as a selectable marker in the development of this recombinant papaya. Kanamycin and other antibiotics inhibit the protein synthesis in the eukaryotic cell by acting on the mitochondria and chloroplast ribosome subunits (Davis, 1988). Phosphorylation of kanamycin or neomycin by the NPTII protein helps eliminate the inhibitory action of these antibiotics on the ribosome subunits, thereby conferring the resistance to antibiotics (Dickie *et al.*, 1978).

A search for structural homology of the transgene region including the modified PRSV CP protein, the GUS protein and the NPTII protein with any known allergens was conducted with the ORF finder program on the NCBI Web site to check for formation of open reading frame (ORF). Then the detected ORF was examined for structural homology with any known allergens using the NCBI

BlastP program (Annex 4). As a result, there was no structural homology identified between the transgene region and any known allergen.

3) Contents of any change caused to the metabolic system of recipient organism

[Modified PRSV CP protein]

The virus CP protein is a structural protein to encapsulate and protect the viral RNA or DNA genome in the PRSV and other plant viruses (Hull, 2002; Dolja *et al*., 1994). This CP protein is considered to play an important role as a structural protein in the cell-to-cell or distant movement of viruses (Dolja *et al*., 1995, 1994; Andrejeva *et al*., 1999; Hong *et al*., 1995) in plants and insect-borne vector transmission (Hull, 2002; Briddon *et al*., 1990; Atreya *et al*., 1995). As a result of composition analysis of this recombinant papaya and the non-recombinant papaya, no significant difference was observed in any component examined (Annex 5 and Annex 6). In addition, there has been no report available to date that the CP protein possesses any enzymatic activity. Based on the above understanding, it is considered extremely low that the expression of the CP protein would affect the plant metabolism.

[GUS protein (β-glucuronidase)]

The glucuronide, the substrate of the GUS protein, is synthesized by the action of UDP glucuronosyltransferase. Plants are known to contain saponin-glucuronide (Yamaguchi *et al*., 1988), quercetin-glucuronide, flavonoid-glucuronide (Merfort and Wendisch, 1988) and others. Although the physiological role of the β-glucuronides in plants is still unclear, though it is generally known that the glucuronide is discharged to vacuoles or apoplasts in the form of readily water-soluble secondary metabolite and then eliminated from the primary metabolism (Luckner, 1977). Consequently, it is considered extremely low that this protein would affect the plant metabolism.

[NPTII protein]

The NPTII protein catalyzes the reaction of phosphorylation of the hydroxyl group in the aminoglycoside molecules of the antibiotic aminoglycosides with the ATP serving as a cofactor (Shaw *et al.*, 1993). The NPTII protein is reported to only take part in the phosphorylation of certain aminoglycoside antibiotics such as neomycin, kanamycin, paromomycin, ribostamycin and butirosin (Price *et al.*, 1974; Davies, 1980). As a result of examination for structure-function relationship of the aminoglycoside antibiotics, it is found that the aminoglycoside antibiotics cannot be a substrate of the NPTII protein simply by slightly changing the structure through removal of some specific hydroxyl group or changing amino group present in the aminoglycoside molecules in the aminoglycoside antibiotics (Price *et al.*, 1974), suggesting that the NPTII protein possesses very stringent substrate specificity. Since there is no report to date that papaya contains any structurally similar compounds to aminoglycoside antibiotics, it is concluded that the possibility that the NPTII protein would react with any compounds or molecules in this recombinant papaya is extremely low.

(2) Information concerning vector

1) Name and origin

The plasmid vector used for the development of this recombinant papaya is pGA482GG/cpPRV-4 which is composed of the modified *PRSV CP* gene expression cassette cloned at the *Hind*III site of plasmid pGA482GG derived from *A. tumefaciens* (Figure 1, p.11).

2) Properties

(a) The numbers of base pairs and nucleotide sequence of vector

The total number of base pairs in the plasmid vector pGA482GG/cpPRV-4 is 19,567 bp. The plasmid vector pGA482GG/cpPRV-4 contains the *nptII* gene expression cassette, the modified *PRSV CP* gene expression cassette and the *uidA* gene expression cassette (Figure 1, p.11). The entire nucleotide sequence of the region used for the transformation in the plasmid vector pGA482GG/cpPRV-4 is provided in Annex 7.

(b) Presence or absence of nucleotide sequence having specific functions, and the functions

The plasmid vector pGA482GG/cpPRV-4 also contains the tetracycline-resistant gene (*tetA*) and the gentamicin-resistant gene (*aacC3*), these antibiotic-resistant genes were used as selective markers in the construction of the vector, in addition to the above mentioned β-glucuronidase gene (*uidA*) and the *nptII* gene.

Southern blot analysis resulted in the evidence that a part of the *tetA* gene is inserted into the genome of this recombinant papaya (Annex 8), while Northern blot analysis confirmed that the *tetA* gene has not been expressed (Annex 9). In addition, it was confirmed as a result of Southern blot and Northern blot analyses that the gentamicin-resistant gene (*aacC3*) was not inserted into this recombinant papaya (Annex 8 and Annex 10).

In addition, the plasmid vector pGA482GG/cpPRV-4 contains the *bla* gene region which takes part in conferring the resistance to ampicillin, though it does not function in this plasmid vector since the sequence is divided by the sequence containing the *cos* site of λ phage.

(c) Presence or absence of infectivity of vector and, if present, the information concerning the host range

The infectivity of this vector is not known.

(3) Method of preparing living modified organisms

1) Structure of the entire nucleic acid inserted in the recipient organism

Component elements of the plasmid vector pGA482GG/cpPRV-4 transferred in the recipient organism are shown in Table 1 (pp. 12-13). In addition, the locations of component elements of the donor nucleic acid in the vector and the sites cleaved by the restriction enzyme are provided in Figure 1 (p. 11).

2) Method of transferring nucleic acid to the recipient organism

The plasmid vector pGA482GG/cpPRV-4 was inserted into the genome of non-recombinant papaya variety Sunset by the particle gun bombardment.

3) Processes of rearing living modified organisms

(a) Mode of selecting the cells containing the transferred nucleic acid

The plasmid vector pGA482GG/cpPRV-4 was inserted by the particle gun bombardment to the embryogenic callus from Sunset, the recipient organism. Then this recombinant papaya was selected from the transformed callus cells on the culture medium containing kanamycin. This recombinant papaya grown to the plant (female plant) exhibited the GUS activity and was immune to inoculation of PRSV. As a result, it was confirmed that the target gene was successfully transferred in this recombinant papaya (Annex 11).

(b) Presence or absence of remaining *Agrobacterium* in case of using *Agrobacterium* method for transferring nucleic acid

This does not apply since this recombinant papaya has the plasmid vector pGA482GG/cpPRV-4 transferred by the particle gun bombardment.

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line in which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effect on Biological Diversity

This recombinant papaya of R0 generation was female and heterozygous for the gene conferring the PRSV resistance, so it was sib-crossed with a hermaphrodite plant of the non-recombinant papaya variety Sunset to produce a recombinant hermaphrodite of papaya line 55-1. Subsequently, through self-pollination across several generations, a homozygous individual for the target gene was developed. This is referred to as the variety SunUp (Figure 2, p.19). The gynodioecious variety SunUp features red flesh, like the variety Sunset, from which it was derived, and red flesh is less liked by consumers in Hawaii. Consequently, SunUp was crossed with the yellow-fleshed non-recombinant papaya Kapoho to produce the F1 hybrid which features

yellow flesh and PRSV resistance. This is referred to as Rainbow (Figure 3, p.20), and this is the variety primarily produced in Hawaii.

In 1991, the license for cultivation was obtained from the Animal and Plant Health Inspection Service, US Department of Agriculture (USDA/APHIS) and cultivation of R0 generation was started in a greenhouse in the University of Hawaii Department of Horticulture. Then, environmental safety test was conducted on the scales listed below.

University of Hawaii Waimanalo Test Site:
20 clones of R0 generation from March 1992
23 plants of R1 generation from December 1992
145 plants of R2 generation from June 1994
Hawaii Island Volcano Isle Farm:
64 plants of R1 generation and 64 plants of R3 generation from August 1995

The approval status of this recombinant papaya in Japan are as shown below.

December, 2000: Based on the "Guideline for the Use of Recombinant in Agriculture, Forestry and Fisheries", the compatibility to the guideline regarding recombinant being imported was confirmed by the Ministry of Agriculture, Forestry and Fisheries.

February, 2006: An application was made to the Ministry of Health, Labor and Welfare for confirmation of safety for food use. In July 2009, safety assessment by the Food Safety Commission for food use was completed and the notification of the assessment result to the Ministry of Health, Labor and Welfare was completed.

The recombinant papaya 55-1 line referenced in this Assessment Report refers to the R0 generation to which the target gene was inserted as described in Figure 2 (p.19) and all of its progeny .

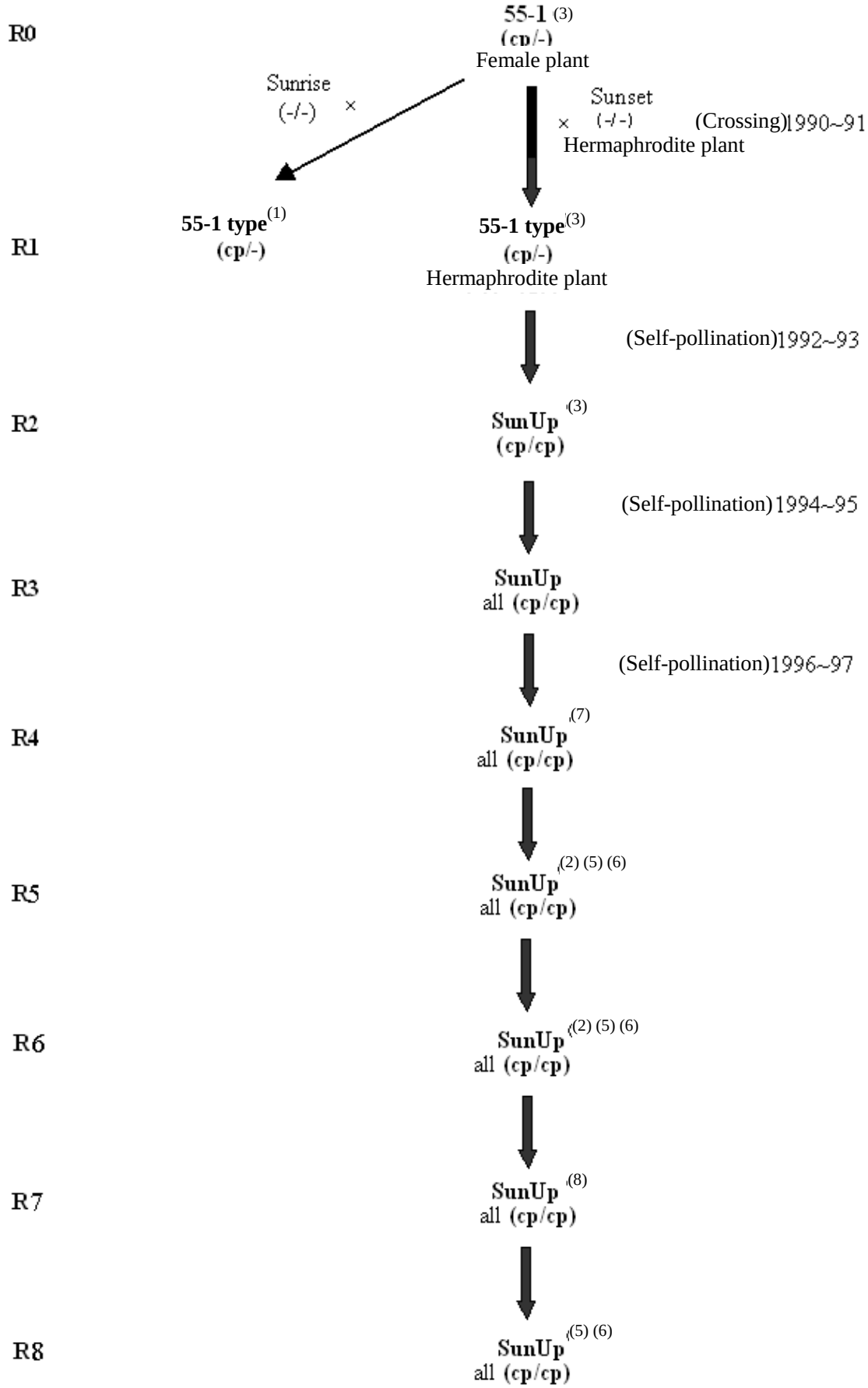

Figure 2 The process of rearing of this recombinant papaya

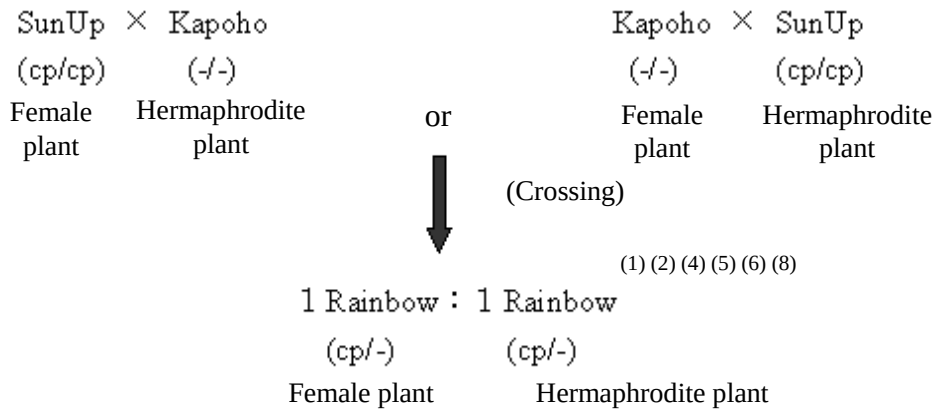

Figure 3 Process of rearing of this recombinant papaya (Production of "Rainbow")

(1) Generation subjected to the Southern blot analysis for transgene [For Rainbow, the individuals (=R5 or earlier generations) developed by crossing between R4 or earlier generation SunUp and Kapoho were subjected to tests.]
(2) Generation subjected to the PCR analysis for transgene and flanking sequences [For Rainbow, the individuals (=R6) produced by crossing between SunUp of R5 and Kapoho were subjected to tests.]
(3) Generation tested for the stability of protein expressed by the transgene
(4) Generation tested for analysis of DNA sequence of transgene [For Rainbow, the individuals (=R5 or earlier generations) produced by crossing between SunUp of R4 or earlier generation and Kapoho were tested. For SunUp, R5 or earlier generation (Annex 14) and R4 or subsequent generation (Annex 13) were tested.]
(5) Generation subjected to repeated Southern blot analysis [For Rainbow, the individuals (=R7) produced by crossing between SunUp of R6 and Kapoho were tested.]
(6) Generation examined for the stability of transgene [For Rainbow, the individuals (=R7) produced by crossing between SunUp of R6 and Kapoho were tested.]
(7) Generation subjected to isolated field tests conducted in Japan between 1999 and 2000 (Individuals =R4 obtained from the seeds of R3 generation were tested.)
(8) Generation subjected to the special screened greenhouse test in Japan in 2006 [For Rainbow, the individuals (=R7) produced by crossing between SunUp of R6 and Kapoho were tested.]

(Footnotes are common to Figure 2 and Figure 3.)

(4) State of existence of nucleic acid transferred in cells and stability of expression of traits caused by the nucleic acid

1) Place where the replication product of transferred nucleic acid exists

As mentioned before, since this recombinant papaya of R0 generation was female, the R1 generation was produced by cross with the non-recombinant papaya Sunset of hermaphrodite plant. Therefore, it was expected that in the R1 generation, heterozygous individuals for the transgene (GUS-NPTII-CP/ －) and the individuals containing no transgene (－/－) would appear at a ratio of 1 : 1. A total of 394 individuals of R1 generation were examined for the presence or absence of transgene and as a result, the heterozygous individuals for the transgene (GUS-NPTII-CP / -) and the individuals free from any transgene (- / -) were confirmed at the expected segregation ratio, so it was concluded that the transgene exists on the chromosome (Annex 12).

Table 2 Segregation ratio of expression of transgene in R1 generation of this recombinant papaya

R1 generation	GUS (+ : -)	NPTII (+ : -)	CP (+ :-)	Expected value	X^2 value
394	193 : 201	193 : 201	193 : 201	197 : 197	0.69

2) The number of copies of replication products of transferred nucleic acid and stability of its inheritance through multiple generations

In order to examine the structure of inserted gene in this recombinant papaya, Southern blot analysis, nucleotide sequence analysis, and PCR analysis for flanking sequence of transgene were conducted. As a result, it was confirmed that one copy of transgene region composed of the *bla* gene fragment, the *oriColE1*, the *uidA* gene expression cassette, the modified *PRSV CP* gene expression cassette, the *nptII* gene expression cassette and the *oriV* fragments (Annex 8), 290bp fragment derived from the *nptII* gene (*nptII* gene fragment) (Annex 13) and the 222bp of the 3' end of the *tetA* gene which was imbedded in the plasmid vector pGA482GG/cpPRV-4 sequence on either end (Annex 14) exists in this recombinant papaya.

In the *Hind* III fragment in the transgene region containing the modified *PRSV CP* gene expression cassette, 6 base deletions were detected. However these variations were found not occurring in the regions encoding the modified *PRSV CP* gene, and the modified PRSV CP protein is normally expressed, so it was confirmed that these variations did not affect the expression of the modified PRSV CP protein (Figure 2 in Annex 7, pp. 44-46).

In addition, it was also confirmed as a result of PCR analysis that the sequences adjacent to the transgene region, the *nptII* gene fragment and the *tetA* gene fragment in the right and left are derived from the non-recombinant papaya Sunset, the recipient organism of this recombinant papaya (Annex 15, Annex 16 and Annex 17). Furthermore, it was confirmed as a result of Southern blot analysis across multiple generations that the transgenes are stably inherited in the progeny (Annex 8).

Maps of transgene in this recombinant papaya are shown in Figure 4 to Figure 6 (pp. 23-25).

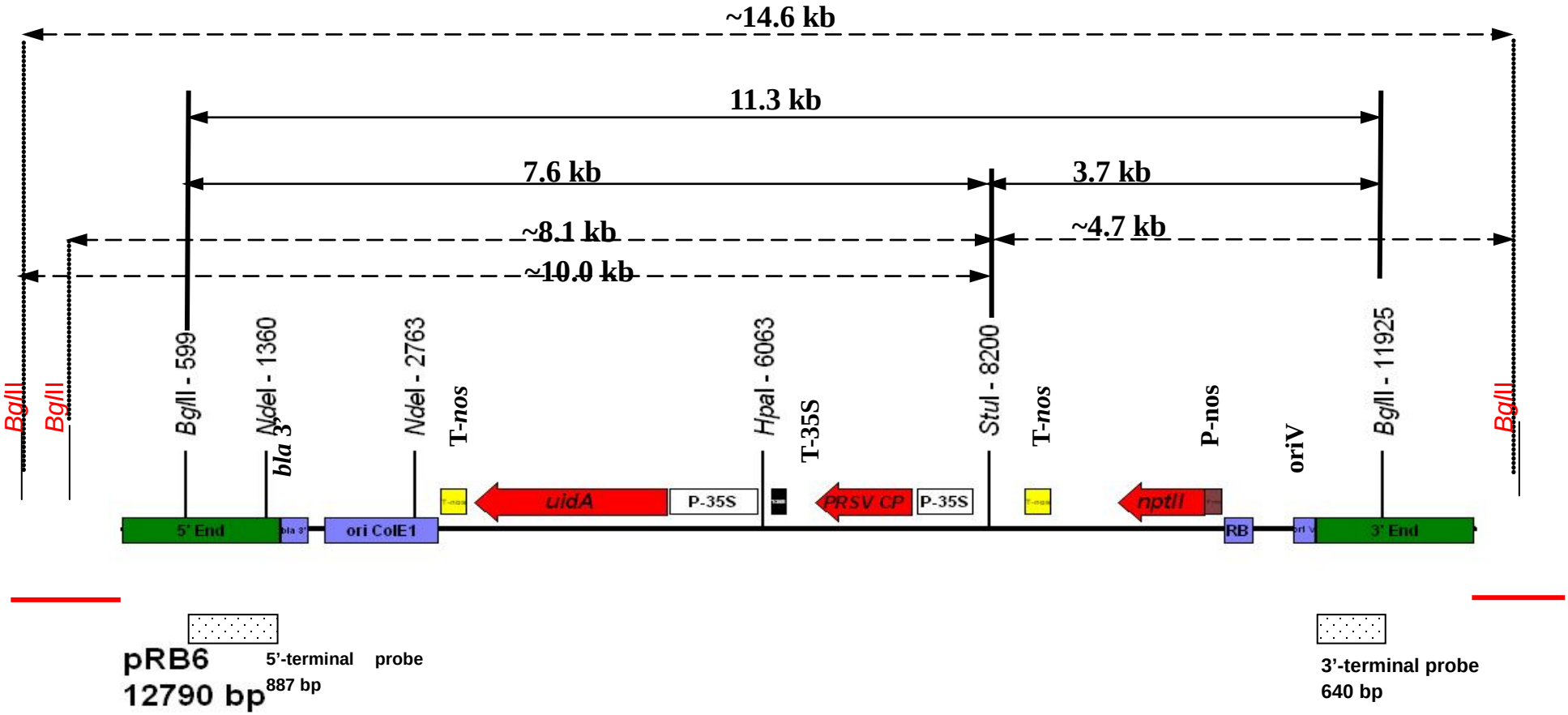

Figure 4 Restriction map of transgene and flanking sequences digested by restriction enzymes *Bgl*II and *Bgl*II/*Stu*I

For the sites cleaved by *Bgl*II, the solid line represents the inherent restriction sites and the dotted line represents the restriction sites produced by the partial digestion (red letters). In addition, the dotted line arrows indicate the fragments produced by the partial digestion.

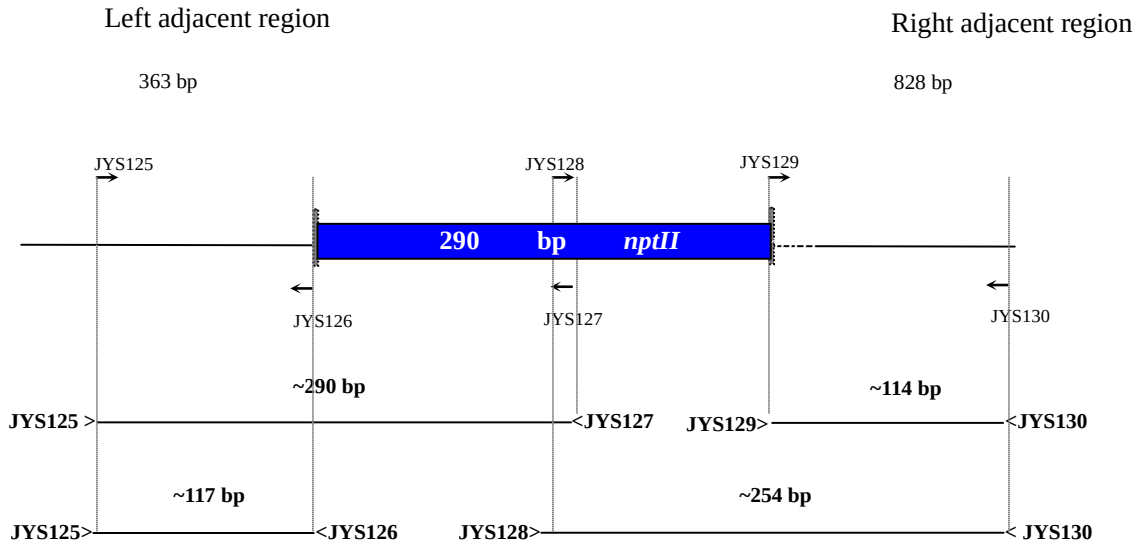

Figure 5 Map of the inserted *npt*II gene fragment

5

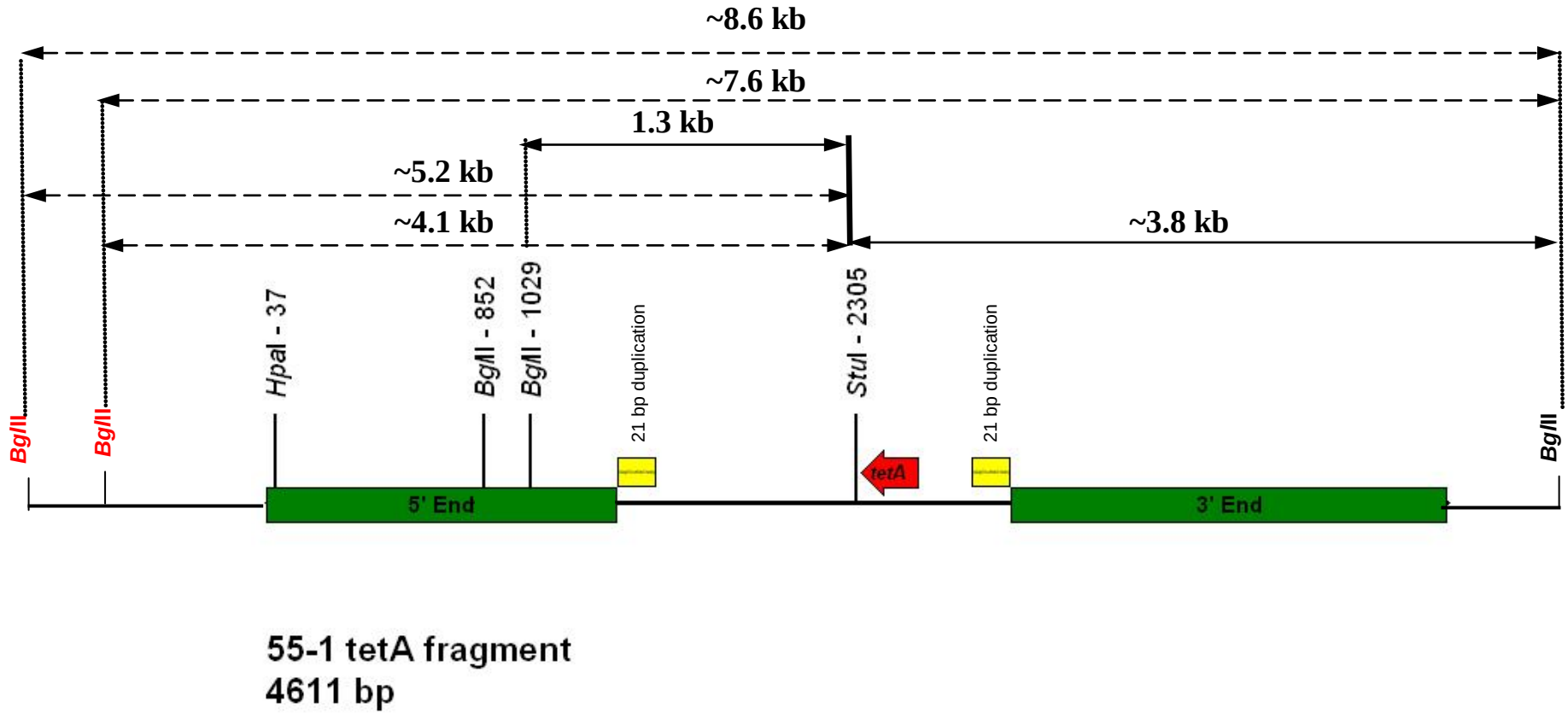

Figure 6 Restriction map of the *tetA* gene fragment and flanking sequences digested by restriction enzymes *Bgl*II and *Bgl*II/*Stu*I

For the sites cleaved by *Bgl*II, the solid line represents the inherent restriction sites and the dotted line represents the restriction sites produced by the partial digestion (red letters). In addition, the dotted line arrows indicate the fragments produced by the partial digestion.

3) The position relationship in the case of multiple copies existing in the chromosome

As discussed in (4)-2), one copy of the transgene region containing the modified *PRSV CP* gene is inserted at one site in the genome DNA of this recombinant papaya, so this is not applicable.

4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

For determination of expression level of the modified PRSV CP protein in the fruits, the recombinant papayas Rainbow and SunUp, and the non-recombinant papaya Sunset, and the Kamiya infected with PRSV were examined. For determination of expression level of the modified PRSV CP protein in the leaves, the recombinant papayas Rainbow and SunUp, and the non-recombinant papaya Kapoho (leaves infected and not infected with PRSV) were examined. Expression level of the modified PRSV CP protein in the fruits and leaves were determined based on the ELISA method (Annex 18).

As a result, the level of the modified PRSV CP protein in the fruits and the standard deviation were found 6.3±2.1μg CP/g fresh weight on average for the recombinant papaya Rainbow, and 48.5±28.3μg CP/g fresh weight for the PRSV-infected non-recombinant papaya Kamiya, showing eight times as large as difference in the expression level of the modified PRSV CP protein. In addition, the expression level for this recombinant papaya SunUp and the non-recombinant papaya Sunset not infected with PRSV was below the limit of detection of 0.25μg CP/g fresh weight (Table 3, p.28). Similarly, the level of the modified PRSV CP protein in this recombinant papaya leaves was found significantly lower than that in the leaves naturally infected with PRSV. For comparison of the level of protein in fruits and leaves, the level of protein in fruits was much lower for both this recombinant papaya and the non-recombinant papaya.

In this recombinant papaya, in addition to the modified PRSV CP protein, β-glucuronidase (GUS protein), a hydrolytic enzyme, and the NPTII protein which confers the resistance to the antibiotics such as kanamycin are also expressed.

For the β-glucuronidase (GUS protein), the expression level in the fruits from this recombinant papaya Rainbow was determined based on the Indirect

HRP-Sandwich ELISA method (Annex 19). As a result, the expression level of the GUS protein in the fruit samples of R7 generation and R8 generation of Rainbow and the standard deviation were found 159.39±200.763ng/g fresh weight on average and 64.74±58.497ng/g fresh weight, respectively. The expression of the GUS protein in Rainbow varied greatly, ranging from 8.43 to 890.30 ng/g fresh weight (Table 4, p.28).

In addition, for the NPTII protein, expression level in the fruits of the recombinant papayas (SunUp and Rainbow) was determined using the NPTII ELISA kit (Agdia PSP 73000, Elkhart, IN) (Annex 20 and Annex 21). As a result, the expression level for SunUp was found 396 ng/g fresh weight for fully ripened fruits and 1,836 ng/g fresh weight for immature fruits. The expression level for Rainbow was 72 ng/g fresh weight for fully-ripened fruits and 273 ng/g fresh weight for immature fruits (Table 5, p.28). In addition, the expression level of the NPTII protein in leaves of SunUp has been found 938 ng/g fresh weight at the maximum (Table 5, p.28).

In order to confirm whether the trait of PRSV resistance acquired by the transgene is stably inherited in the progeny, the recombinant papayas of R0 generation (heterozygote), SunUp (homozygote) and Rainbow (heterozygote), were subjected to the PRSV inoculation tests. As a result, all the generations tested exhibited the resistance to PRSV (Annex 11; Annex 22).

In addition, the stability of the modified PRSV CP protein, the GUS protein and the NPTII protein expressed in this recombinant papaya was examined based on the ELISA method and color reaction and as a result, it was confirmed that the proteins are stably expressed through multiple generations (Table 6, p.30; Annex 12).

Table 3 Expression level of the modified PRSV CP protein in the recombinant papayas and the non-recombinant papayas

Site tested	Cultivar and treatment	No. of samples	CP (μg/g fresh weight)	SD
Fruit	Recombinant papayas			
	Rainbow	5	6.3	2.1
	SunUp	5	ND[a]	-
	Non-recombinant papayas			
	Sunset	5	ND	-
	Kamiya (PRSV-infected)	5	48.5	28.3
Leaf	Recombinant papayas			
	Rainbow	1	257.6	
	SunUp	1	137.0	
	Non-recombinant papayas			
	Kapoho (PRSV-infected)	1	3,580.6	
	Kapoho	1	ND	

ND[a] Measured values found equal to or less than the limit of detection (0.25 μg CP/g fresh weight)

5

Table 4 Expression level of β-glucuronidase (GUS protein) in the recombinant papayas

	GUS expression level (ng)/g fresh weight	
	Rainbow-1 (GM)	Rainbow-2 (GM)
Generation	R7	R8
Cultivating field	Diamond Head Farm (Kapoho)	Ruby's Farm (Hamakua Coast)
No. of samples	22	22
Mean value	159.39	64.74
S.D.	200.763	58.497
Range	13.69-890.30	8.43-236.16

10

Table 5 Expression level of the NPTII protein in the recombinant papayas

	NTPII Protein
	Expression level (ng/g fresh weight)
SunUp (R8 generation)	
Fully ripened fruit	396

	Immature fruit [*1]	1836
Rainbow (R8 generation)		
	Fully ripened fruit	72
	Immature fruit [*1]	273
SunUp (R0 generation)		
	Leaf	938[*2]

[*1] Data for the fully ripened fruits refers to an average of 9 individuals, and data for the immature fruits refers to a single individual.

[*2] Highest expression level detected in the tests

5

Table 6 Stability of expression of the modified PRSV CP protein, GUS protein and NPTII protein

		CP	GUS	NPTII
Test method		ELISA method	Color reaction	ELISA method
Generation	R0	+	+	+
	R1	+	+	+
	R2	+	+	Not tested
	R4	Not tested	+	Not tested

The positive sign ＋ indicates the expression was verified.

5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

Regarding the plasmid vector pGA482GG/cpPRV-4 used for the development of this recombinant papaya, the range of host organism, which allows autonomous replication, is limited to gram-negative bacteria such as *E.coli* and *A.tumefaciens*. However, the plasmid vector pGA482GG and the plasmid vector pGA482GG/cpPRV-4 containing the modified *PRSV CP* gene cloned in the *Hind*III site of the former plasmid vector do not contain *trans*, *mob* and other conjugating factors that allow conjugation and transmission. Therefore, it is considered that these plasmid vectors are least likely to offer virus transmissibility to wild animals and wild plants by themselves.

(5) Methods of detection and identification of living modified organisms and their sensitivity and reliability

Detection methods of this recombinant papaya are posted on the Web site of Ministry of Health, Labor and Welfare (http://www.mhlw.go.jp/topics/idenshi/kensa/tuuchi2.html) under the heading of "Detection methods of Foods Produced by Recombinant DNA Technique." Among the methods posted, the color reaction, which utilizes the expression of the *uidA* gene transferred in this recombinant papaya, is free from any possibility of misidentifying negative subjects, and it allows easy and fast detection only with reagents, using such instruments and equipment as available in ordinary laboratories (Wakui *et al.*, 2004).

(6) Difference between the modified organism and the recipient organism or the species to which the recipient organism belongs

1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of replication products of transferred nucleic acid

This recombinant papaya is given the traits to be resistant to PRSV by the transferring of the coat protein gene (modified *PRSV CP* gene) of papaya ringspot virus (PRSV HA 5-1 strain). However, the resistance is selective and, according to the results of isolated field tests, the recombinant papaya exhibited the resistance to Hawaii PRSV strain (HA) and Taiwan PRSV strain (R175P), though it was found susceptible to Japanese PRSV strain (J126P), Thailand PRSV strain (T164P) and Malaysian PRSV strain (M185P) (Annex 1, pp. 6-7). In addition, this recombinant papaya was also found susceptible to the Japanese strain of papaya leaf-distortion mosaic virus (J56P) which is found in Japan (Annex 1, pp. 6-7).

In addition, this recombinant papaya is also given the traits to be resistant to kanamycin due to the *nptII* gene transferred as a selection marker and to exhibit the β-glucuronidase activity due to the *uidA* gene.

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

Differences between this recombinant papaya and the non-recombinant control papaya, the recipient organism, have been examined based on the results of isolated field tests conducted from May 19, 1999 to March 31, 2000 in the isolated fields in the Okinawa Branch Office of the Japan International Research Center for Agricultural Sciences (JIRCAS). In addition, using the results of field tests conducted from August 2004 to May 2005 in Kapoho and Waimanalo in Hawaii for reference, the differences have been comprehensively examined. Furthermore, for soil microflora test, plow-in test and succeeding crop test, additional tests were conducted in 2006 in special screened greenhouse and based on the test results, examination was carried out.

(a) Morphological and growth characteristics

Plant height, trunk circumference, number of nodes, leaf length, leaf width, leafstalk length, leafstalk erect angle, nodal position of the first flower, Initial?? flowering date, number of fruits set, number of emergences of female and hermaphrodite flower plants, and fruit characteristics were evaluated.

General characteristics: Evaluation was conducted four (4) times on the general characteristics (plant height, trunk circumference, number of nodes, leaf length, leaf width, leafstalk length, and leafstalk erect angle) of hermaphrodite plant and female plant during the growing period. As a result, on all of the four days examined (Sept. 17, Oct. 18, Dec. 24, and Feb. 10), no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya for the items examined (Table 3 of Annex 1, p.9).

Growth characteristics: As a result of examination on the growth characteristics (nodal position of the first flower, number of days to initial?? flowering, and number of fruits set) of hermaphrodite plant and female plant, no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya (Table 4 and Table 5 of Annex 1, p.10).

Sex segregation: The ratio of emergence of hermaphrodite plant to female plant was found 24:6 for this recombinant papaya and 11:6 for the non-recombinant control papaya. To check for any difference in the emergence of hermaphrodite plant and female plant between the two (2) samples, this recombinant papaya and the non-recombinant control papaya, χ^2 test was conducted. As a result, it was found that there is no statistically significant difference in the number of emergences of hermaphrodite plants and female plants between this recombinant papaya and the non-recombinant control papaya (Table 6 of Annex 1, p.10).

Fruit characteristics: Twenty one (21) fruits from hermaphrodite plant and 9 fruits from female plant of this recombinant papaya, and 12 fruits from hermaphrodite plant and 4 fruits from female plant of the non-recombinant control papaya were obtained. Using the fruits, comparison was conducted for the fruit characteristics (fruit weight, fruit length, fruit diameter, shape

index of fruit [fruit length/fruit diameter, Note: This is the simplest index to refer to the shape by variety of fruit.], sugar content, acid content, and the number of seeds). As a result, for the fruits from hermaphrodite plants, statistically significant difference were observed in the sugar content and acid content between this recombinant papaya and the non-recombinant control papaya, though no difference was observed in the other items (Table 7 of Annex 1, p.11). The mean value of sugar contents where the statistically significant difference was observed were found 13.9% and 15.0% for the fruits from hermaphrodite plants of this recombinant papaya and the non-recombinant control papaya, respectively (Table 7 of Annex 1, p.11). In addition, the mean value of acid contents where the statistically significant difference was observed were 0.92% and 1.05% for the fruits from hermaphrodite plants of this recombinant papaya and the non-recombinant control papaya, respectively (Table 7 of Annex 1, p.11).

(b) Cold-tolerance and heat-tolerance at the early stage of growth

This recombinant papaya and the non-recombinant control papaya raised at 24°C for 71 days were transferred into the environment chambers at 4°C and 15°C to examine the cold-tolerance. As a result, the seedling plants of this recombinant papaya and the non-recombinant control papaya all fell to the ground at the roots and wilted after one month elapsed in the condition at 4°C, while in the condition at 15°C, many individuals of the both plants were survived. Although it was difficult to identify whether a plant wilted or died and the number of days until the plant wilted or died could not be quantified, it was considered that there is no difference in cold-sensitivity between this recombinant papaya and the non-recombinant papaya (Figure 6 of Annex 1, p.19).

(c) Wintering ability and summer survival of the mature plant

Based on the findings that as a result of examination on the cold-tolerance at the early stage of growth discussed above, no difference was observed between this recombinant papaya and the non-recombinant control papaya and that papayas are tropical plants and they are known to die at temperatures below 0°C, it was judged as follows: In Japan, except the subtropical climate areas such as the islands south of southern Amami Oshima Islands including the entire Okinawa Prefecture, Ogasawara Islands and Minami-Torishima Island,

this recombinant papaya will wither and die at the stage of seedling plants similarly as typical non-recombinant papaya and fail to grow up to adult.

(d) Fertility and size of the pollen

One flower, seemingly at the time of just before opening, was selected from each of four plants of this recombinant papaya and the non-recombinant control papaya and then the pollens taken from the flowers were stained with acetocarmine to examine the fertility. As a result, no statistically significant difference was observed in the pollen fertility between this recombinant papaya and the non-recombinant control papaya (Table 9 of Annex 1, p.12).

Examination on the size of pollen was not conducted in the isolated field test. However, in the field in Hawaii, pollens were taken from this recombinant papaya and the non-recombinant control papaya and compared for the size with a graduated ocular micrometer. As a result, the pollen size was found 41.8 μ and 42.0 μ on average, respectively, and no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya (Table 1 of Annex 23, p.1).

(e) Production, shedding habit, dormancy and germination rate of the seed

Examination on the seed production was not conducted in the isolated field test because a sufficient amount of seeds could not be harvested. However, in the field in Hawaii, ten (10) fruits were taken from each of this recombinant papaya and the non-recombinant control papaya and the obtained seeds were compared for average weight. As a result, no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya (Table 4 of Annex 23, p.3).

Dispersion of papaya seeds does not depend on fruit cracking but it occurs due to the transmission by mammals and/or birds, so shedding habit of the seeds is considered least possible. Consequently, shedding habit of the seeds was not examined.

Comparison of dormancy and germination rate was planned using the seeds obtained from fruits of this recombinant papaya and the non-recombinant control papaya harvested in the isolated field test, though it was impossible to

obtain a sufficient amount of seeds for the examination on germination rate. Then germination test was conducted with the seeds taken from the mature fruits harvested in Hawaii and sent to Japan. The growth temperature was set at 4, 15, 23 (or 24), and 35°C. As a result, regarding the germination characteristics of seed, germination started about one month later in the condition at 24°C, and after the second and subsequent months, no new germination was observed. The number of seeds germinated on the 56th day was 32.25/40 (80.63%) for this recombinant papaya and 29.25/40 (73.13%) for the non-recombinant papaya (Table 11-1 of Annex 1, p.14). In addition, in the condition at 35°C, germination was observed earlier and on the 31st day, the number of germinating seeds was 6.25/40 (15.63%) and 3.25/40 (8.13%) for this recombinant papaya and the non-recombinant papaya, respectively, and no increase was observed later in the number of germinating seeds (Table 11-2 of Annex 1, p.14). Regarding the number of germinating seeds, no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya in the both conditions at 24°C and 30°C (Tables 11-1 and 11-2 of Annex 1, p.14).

Furthermore, the seeds taken from this recombinant papaya and the non-recombinant control papaya harvested in Hawaii was left to stand for 2 months in the conditions at 4°C and 15°C and then transferred to the conditions at 25°C and 35°C to examine the number of germinating seeds. As a result, regarding the seeds left in the conditions at 4°C then transferred to the condition at 25°C, no germination was observed for this recombinant papaya, though one of a total of 120 seeds was found germinating for the non-recombinant control papaya. In addition, regarding the seeds left in the conditions at 4°C then transferred to the condition at 35°C, no germination was observed for the both papayas (Tables 12-1 and 12-2 of Annex 1, p.17). On the other hand, regarding the seeds left in the condition at 15°C then transferred to the conditions at 25°C and 35°C, no statistically significant difference was observed in the germination rate of seeds between this recombinant papaya and the non-recombinant control papaya (Table 13-1 and Table 13-2 of Annex 1, p.18).

(f) Crossability

As mentioned earlier, no wild relatives that can be crossed with papaya are growing naturally in Japan and thus, a crossability test of this recombinant

papaya was not performed.

(g) Productivity of harmful substances

In 2006, soil microflora test, plow-in test and succeeding crop test were conducted in special screened greenhouse. For the tests, the seeds of the recombinant papayas (SunUp and Rainbow) and the seeds of the non-recombinant control papaya (Sunset) sent from Hawaii were used. As a result, regarding all the items for which statistical analysis was performed (the number of bacteria in soil microorganisms, the number of germinating seedlings, plant height, fresh weight and dry weight of radish), no significant difference was observed between this recombinant papaya and the non-recombinant control papaya. Also for the other items for which statistical treatment was not performed (germination rate of radish), no difference was observed between this recombinant papaya and the non-recombinant control papaya (Annex 24).

In addition, also in Hawaii, succeeding crop test and soil microflora test were conducted for reference. In the soil microflora test, regarding the number of bacteria and fungi in the soil in which papayas were cultivated for 9 months, no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya (Table 10 and Table 11 of Annex 23, pp.11-12). In addition, also in the succeeding crop test using cucumber and corn, no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya regarding the germination rate and growth of cucumber and corn (Figures 2 to 5 and Tables 6 to 9 of Annex 23, pp.7-9).

II. Review by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity

1. Item-by-item assessment of Adverse Effect on Biological Diversity

(1) Competitiveness

In Japan, papaya has not been listed as invasive alien species that can specifically affect

the ecosystem like the introduced dandelion species group and tall goldenrod. Therefore, there is a possibility that papaya can grow in some limited areas of subtropical climate in Japan, though it is considered extremely low that papaya is highly invasive plant. In addition, papaya is not listed in the Noxious Weed List by Hawaii Department of Agriculture, and there has been no report available to date from the papaya-cultivating areas in the continental United States that papaya is hazardous weed. Furthermore, this recombinant papaya has been commercially cultivated in Hawaii since 1998, though there is no report that the papaya has become a weed under natural environment.

In the isolated field tests conducted from 1999 to 2000 in the isolated field in the Okinawa Branch of Japan International Research Center for Agricultural Sciences (JIRCAS), comparison was conducted between this recombinant papaya and the non-recombinant control papaya on the characteristics relating to the competitiveness (morphological and growth characteristics, cold-tolerance at the early stage of growth, fertility and size of pollen, seed production, shedding habit, dormancy and germination rate),. As a result, , statistically significant differences were observed in the sugar content and acid content between this recombinant papaya and the non-recombinant control papaya, though no statistically significant difference was observed in the other items between this recombinant papaya and the non-recombinant control papaya. The statistically significant differences observed in the sugar content and acid content are estimated to result from the difference in the degree of maturity of samples tested and therefore, it is considered unlikely that the difference would increase the competitiveness of this recombinant papaya.

This recombinant papaya possesses the resistance to Hawaii PRSV strain (HA) and Taiwan PRSV strain (R175P) due to the expression of the modified *PRSV CP* gene, though these virus strains are not distributed in Japan. In addition, this recombinant papaya exhibits susceptibility to the major pathogenic viruses PRSV (J126P) and PLDMV (J56P), which are distributed in the Southwest Islands in Japan, so it is considered extremely low that the competitiveness of this recombinant papaya could exceed the competitiveness of conventional papayas.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant papaya poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

(2) Productivity of harmful substances

Since the benzyl isothiocyanate (BITC), papain and carpaine are listed as the harmful substances contained in papaya, analysis was conducted for the BITC, papain and carpaine in this recombinant papaya. As a result, no difference due to genetic modification was observed in these substances when compared with non-recombinant control papaya otherwise below the limit of detection.

In 2006 in the special screened greenhouse in Japan, comparison was conducted on the productivity of harmful substances (substances secreted from roots to affect the other plants and microorganisms in soil, substances possessed in the plant to affect the other plants after dying) between this recombinant papaya and the non-recombinant control papaya based on the soil microflora test, plowing-in test and succeeding crop test. As a result, in all the tests conducted, no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya regarding the items for which statistical analysis was performed. In addition, also in Hawaii, soil microflora test and succeeding crop test were conducted and as a result, in the both tests, no statistically significant difference was observed between this recombinant papaya and the non-recombinant control papaya. Furthermore, in Taiwan and Thailand, environmental impact assessment of the PRSV-resistant papaya containing the coat protein has been performed and as a result, in all the field tests conducted, it has been confirmed that cultivation of the recombinant papayas has no any adverse effects on the microorganisms in soil, insects, etc .

This recombinant papaya contains the modified PRSV coat protein (CP) conferring the PRSV resistance and the NPTII protein and the GUS protein functioning as selection markers, though there is no report that these proteins become harmful substances. The virus coat protein is a structural protein, which acts to encapsulate and protect the virus RNA or DNA genome in the plant virus. For the functions of the coat protein, the coat protein is considered unlikely to affect the metabolic pathway in plants. In fact, as a result of compositional analysis during the food safety assessment of this recombinant papaya, it was confirmed that there is no significant difference from the non-recombinant control papaya.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant papaya poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

(3) Crossability

In the Japanese natural environment, there are no wild plants which can cross with papaya. Therefore, it was judged that there are no specific wild plants that are possibly affected by this recombinant papaya, and that the use of such papaya poses no risk of Adverse Effect on Biological Diversity that is attributable to crossability is reasonable.

(4) Other characteristics

The modified *PRSV CP* gene transferred in this recombinant papaya is derived from virus and thus there is a possibility that recombination occurs between the RNA of virus, which lives in Japan and makes the papayas as a host, and the RNA produced from the modified *PRSV CP* gene and resultantly a new recombinant virus appears. Then investigation was carried out.

As a result, it was found that the possibility that any virus living in Japan and making the papayas as host infects this recombinant papaya is limited to the areas of subtropical climate, and should recombination occur between the RNA produced from the transgene in this recombinant papaya and the RNA of the infected virus, the frequency is considered to be no different from that of recombination taking place in the nature. Even if recombination occurs between the infected virus and the transgene and a new recombinant virus appears, it is considered unlikely that the recombinant virus can survive and reproduce itself under the natural environment in which there is no conditions to provide selective advantage to the recombinant virus. In addition, although the possibility that the coat protein of PRSV takes part in the determination of pathogenicity or host cannot be negated, there has been no report that the PRSV coat protein has been acting as an independent determinant of the pathogenicity or host of the virus. Consequently, it was considered that even if any recombinant virus occurs, possible effects on the environment or ecosystem are no different from the effects of the original virus.

Based on the above understanding, it was judged that the conclusion by the applicant that this recombinant papaya poses no risk of Adverse Effect on Biological Diversity attributable to the recombination between the RNA of the virus living in Japan and making the papaya as host and the RNA produced from the modified *PRSV CP* gene transferred in this recombinant papaya is reasonable.

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant papaya in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.