Industry note of technological innovation for promoting global papaya production

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1 Introduction

Papaya (*Carica papaya L.*) is an economically important cash crop. Ripe papaya fruit, a rich source of vitamin A and C (Manshardt, 1992), is consumed as fresh fruit and also used to prepare several food products, while the unripe fruit is used as vegetable. The latex derived from unripe fruit and other parts of papaya plant is rich in cysteine proteases, such as papain, caricain, chymopapain and glycyl endopeptidase (Barrett and Rawling, 1998) that have food processing (Caygill, 1979), pharmaceutical (Salas et al., 2008), medical (Pendzhiev, 2002), cosmetic (Pendzhiev, 2002; Seki et al., 2007) and several other industrial (Caygill, 1979) applications. The presence of glucosinolates, cyanogenic glucosides and allosides (Bennett et al., 1997; Seigler et al., 2002) and α-tocopherol, ascorbic acid, flavonoids (Chan and Tang, 1978) appears to be basis of the anti-tumour and immuno-modulating properties of papaya (Osato et al., 1993; Otsuki et al., 2010).

Papaya crop, believed to be indigenous to the tropical regions of Central America, is now distributed widely throughout tropical and subtropical regions of the world. With a global productivity of 11.22 million metric tons, papaya is one of the ten most important fruit crops of the world, the others being apple, avocado, banana, grape, mango, pear, pineapple, strawberry and tomato. Papaya production in India, Brazil, Indonesia, Nigeria and Mexico accounts for more than 70% of the global production (Evans and Ballen, 2012).

Under normal conditions, papaya fruits can be harvested 8–10 months after transplanting in field and each tree can generate more than 100 kg fruit up to 2–3 years. Papaya is a climacteric fruit which undergoes striking colour changes and substantial

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pulp softening associated with a steep rise in ethylene production. In many papaya cultivating countries, considerable quantity of produced papaya fruits is wasted due to short shelf-life and fragile conditions of the fruits. Such post-harvest losses discourage large scale production of papaya for export. Of the global annual quantum of papaya fruit production, only a humble fraction of nearly 2% is being exported (Bapat et al., 2010).

Papaya cultivation is affected by a number of pests, including fruit flies, whiteflies, mites and nematodes; and diseases caused by various fungi, bacteria and viruses (Ventura et al., 2004; Teixeira da Silva et al., 2007). The aphid-borne potyvirus *Papaya ringspot virus* (PRSV) is a major obstacle to large-scale commercial production of papaya worldwide (Yeh and Gonsalves, 1984).

2 Worldwide threat on papaya production by PRSV

Because plantation is seriously affected by PRSV worldwide, papaya supply is not as abundant as banana and pineapple in international fruit markets. PRSV was first reported from Hawaii in the 1940s (Jensen, 1949), and subsequently its prevalence was recorded in all other papaya cultivating regions of the world (Yeh and Gonsalves, 1994). The virus is transmitted in nature by aphids and is a member of the genus *Potyvirus* of the family *Potyviridae*, one of the largest and most economically important plant virus groups (King et al., 2012). The flexuous rod-shape virus particles are in size of 780 × 12 nm containing a positive sense single-stranded RNA genome of 10.3 kb (Yeh et al., 1992). PRSV genome encodes a single polyprotein that is proteolytically processed by three virus-coded proteases into nine final proteins of P1, HC-Pro, P3, CI, 6K, NIa (processed further into VPg and NIa protease), NIb and CP (Yeh and Gonsalves, 1985; Yeh et al., 1992). Symptoms of PRSV-infected papaya plants include severe stunting in growth, mosaic and distortion on leaves, and malformed fruits with numerous ringspots on surface. The virus is transmitted by aphids non-persistently and can cause 100% disaster infection in a very short time and completely wipe whole orchards (Yeh and Gonsalves, 1984). This killer virus makes the large-scale plantation of papaya extremely difficult in all papaya-grown counties.

Since the papaya species *C. papaya* lacks effective natural source of resistance to PRSV, conventional breeding of papaya for PRSV resistance cannot be conducted. The conventional control measures against PRSV, including pesticide application against aphids and different agricultural practices such as planting high-stem barrier crop, using UV-reflecting silver mulch, protection at the early stage with plastic shield to exclude aphid access are ineffective or only marginally beneficial (Yeh and Gonsalves, 1994). In Taiwan, most of papaya trees are planted under netting (32 mesh, a total of 2,500 Ha), which can effectively exclude aphid access and thus guarantees papaya production for 2–3 years if the net is not destroyed by storms. However, the quality of fruits from net-protected orchards is inferior because of reduced light. Moreover, the netting system is costly and not adapted to other country yet. In addition, the use of UV-resistant nylon material create hazard to the environment.

Since the virus disease cannot be cured by any chemicals, and conventional breeding and agricultural practices neither solve the PRSV problem, other approaches including control by cross protection or transgenic resistance have been developed to solve the noxious papaya ringspot disease.

3 Control of papaya virus by cross protection: an immunisation process with mild virus strain

Cross protection is a natural phenomenon in which an initial infection by a virus induces resistance in a host plant to subsequent infection by other strains of the same virus (McKinney, 1929). Hence, an initial infection by an attenuated (mild or asymptomatic) strain of a virus can cross protect plants against subsequent infection by the related severe strains (Ziebell and Carr, 2010). Attenuated strains with cross protection ability have been reported from many plant virus species of different genera. For example, protective strains of *Tobacco mosaic virus* (TMV) (Rast, 1972), *Citrus tristeza virus* (Costa and Müller, 1980), PRSV (Yeh et al., 1988), and *Zucchini yellow mosaic virus* (ZYMV) (Lecoq et al., 1991; Wang et al., 1991) have been used for several decades in large-scale applications for control of these destructive viruses (Ziebell and Carr, 2010; Ziebell and MacDiarmid, 2017). Thus, the non-reliance on recombinant DNA technology identifies cross-protection a conventionally and socially accepted crop protection strategy.

Protective strains of plant viruses may be isolated from nature (Costa and Müller, 1980; Kondo et al., 2015; Lecoq et al., 1991), induced by physical treatments such as UV irradiation or temperature (Oshima, 1975; Kosaka and Fukunishi, 1993) or chemical mutagens (Rast, 1972; Yeh and Gonsalves, 1984). However, in most of the cases appropriate naturally occurring or induced mutants for controlling severe strains of viruses may not be available. Such crisis can be solved by mapping genomic region harbouring major disease determinant of a severe target strain and replacing it by the corresponding genomic region from a mild strain (Albiach-Marti et al., 2010; Chiang et al., 2007). The resulting mild chimeric virus most likely cross-protect host plants against specific severe strains of the same virus.

4 Practical application of cross protection for control of PRSV in Taiwan and Hawaii

A mild strain HA 5-1 was derived through nitrous acid-induction from a Hawaii severe strain HA (Yeh and Gonsalves, 1984), causing infection on papaya without conspicuous symptoms and provides complete protection against infection by severe strains under greenhouse and field conditions (Wang et al., 1987). This mild strain has been used in Taiwan from 1984–1993 to maintain the production of papaya on the island. During this ten years, more than 400 million papaya seedlings were inoculated with the mild strain and released to 2,200 Ha orchards, representing the first large scale application of cross protection to control the noxious PRSV (Yeh and Gonsalves, 1994). After 1994, protection of papaya by the netting system became popular in Taiwan and the protection by the mild strain became not necessary. Moreover, although HA 5-1 provided high degrees of protection against PRSV in Hawaii, breakdown of protection provided in Taiwan did occur when some particular strains existed. The mild strain HA 5-1 is not effective in other countries, such as Thailand and Mexico, where variable strains can easily knock down the protection (Yeh and Gonsalves, 1994). Therefore, to solve the problem of this strain-specific protection, an ideal mild strain should be derived from a local severe strain to confer high degrees or complete protection against the homologous virus strain prevailing in that area.

5 Genetic manipulation of viruses to generate useful mild strain for solving the problem of strain-specific protection

Potyviral HC-Pro is a multi-functional protein involved in many important functions of potyvirus (Maia et al., 1996; Urcuqui-Inchima et al., 2001; Valli et al., 2018), including aphid transmission (Govier and Kassanis, 1974; Maia et al., 1996), polyprotein processing (Carrington et al., 1989), gene silencing suppression (Anandalakshmi et al., 1998), viral pathogenicity (Pruss et al., 1997), long distance movement (Kasschau and Carrington, 2001), cell-to-cell movement, and virus amplification (Kasschau and Carrington, 1995). Among these functions, HC-Pro is important for antagonistic action against the host defensive response of specific RNA silencing and plays as the major pathogenicity factor for PRSV.

Upon the detection of viral genomic or messenger RNA after infection by a virus, host plants trigger a specific degradation mechanism on the invading foreign RNA referred to as post translational gene silencing (PTGS). This is the mechanism of plant host defence reaction (Baulcombe, 1999). Plant viruses have to evolve silencing suppressor genes to antagonise this defence mechanism of the host plant cell. Thus, the silencing suppressor is normally the key determinant for viral pathogenicity (Kasschau and Carrington, 1998). Manipulation of the silencing suppressor of a virus can thus affect its pathogenicity. Because RNA cannot be directly manipulated at the molecular level, useful mild strains are obtained through the cloning of an infectious cDNA into a plant expression cassette followed by the modification of the silencing suppressor through site-directed mutagenesis or DNA recombination. Strain-specific protection limits the application of a cross protection approach in different geographic regions where different strains of the same virus species exist (Yeh and Gonsalves, 1994). To overcome this problem, the genetic modification on the suppressor gene has to be conducted from an infectious clone of particular a local strain.

Cross-protective strains can be engineered from severe strains by precise manipulation of functional motifs of important viral proteins. For instance, arginine (R) 180 of the FRNK motif of ZYMV HC-Pro is essential for virus pathogenicity (Gal-On, 2000; Lin et al., 2007). $R^{180}I$ (arginine 180 to isoleucine) mutation of FRNK motif of HC-Pro of Israel (Gal-On, 2000) and Taiwan (Lin et al., 2007) strains of ZYMV generated attenuated variants which cross-protect zucchini squash plants against severe ZYMV strains. Similarly, $R^{182}L$ mutation of HC-Pro of *Turnip mosaic virus* (*TuMV*) generated an attenuated variant which cross protects *N. benthamiana* and *Arabidopsis thaliana* plants against severe TuMV strains (Kung et al., 2014).

Singly or in combinations, four conserved motifs of PRSV HC-Pro, including the N-terminal FWKG, highly conserved FRNK, and two amino residues at the central (F206) and C-terminal regions (D397), were modified by site-directed mutagenesis to generate attenuated mutants from the cDNA infectious clone of a Taiwan severe strain PRSV YK. Two useful attenuated mutants, YK F7I and YK F7I+F206L, were selected out based on the criteria of their mildness, stability, titer dynamic and cross-protection effectiveness. The two mild PRSV mutants provide complete cross protection (100%) on papaya plants against Taiwan severe strain YK, superior to that provided by HA-5-1 (10%), indicating that these mutants solve the problem of strain-specific protection and have great potential to be used in Taiwan for control of papaya ringspot disease. By this

approach, useful mild mutants for control of PRSV by cross protection in Thailand, Vietnam, Mainland China are being generated.

6 A prompt way to generate customised mild strains for different geographic regions

PRSV HA5-1 is a mild mutant of type P (papaya-infecting) Hawaii severe strain (PRSV P-HA, has been widely used for the control of PRSV type P strains in papaya, but does not provide practical protection against PRSV type W (watermelon-infecting) strains in cucurbits. In order to widen the protection effectiveness against W strains, chimeric mild strains were constructed from HA5-1 to carry the heterologous 3' genomic region of a type W strain W-CI. In horn melon and squash plants, the recombinant carrying both the heterologous coat protein (CP) coding region and the 3' untranslated region (3'UTR), significantly enhanced the protection against W-CI (You et al., 2005).

The same approach can be applied to different type P strains to generate recombinant mild strain against any geographically different strains. This provides a prompt method to engineer a useful mild strain at particular regions to solve the problem of strain-specific protection. Thus, HA 5-1 carrying a heterologous CP and 3 UTR from Taiwan, Thailand, Vietnam, Mainland China and Guatemala maintains its mildness and provides high degrees of protection against the severe PRSV strains prevailing in the corresponding countries. This customised production of a useful mild strain provides a quick and effective control measure in different geographic regions.

7 Future perspectives for cross protection

The whole process of obtaining attenuated mild viral strains is laborious and time-consuming. The prerequisite to obtain a full-length infectious clone of the virus requires time and effort. The screen of useful mild variants or strains also takes time. Furthermore, field tests to verify the applicability of a useful candidate can take years. Importantly, the mild strain must be genetically stable, not cause adverse effects to the protected host and provide complete protection against the prevalent viral strain in a particular region.

By modifying the silencing suppressor *HC-Pro* of a potyvirus, our group has developed mild mutants from severe PRSV strains found in Taiwan and Thailand that provide complete protection against the virus in papaya crops. This would become a new role model for the control of plant viruses. Either through governmental agents or private sectors, we are confident that our cross-protection approach will change the production of papaya in different countries such as Vietnam, Thailand and Mainland China.

Because the approach is considered non-GMO, cross protection is subjected to a less strict regulation and can be applied after complying with the requirements for its registration as a plant vaccine after the evaluations of vaccine preparation and preservation, mass inoculation, greenhouse and field evaluations for cross protection, and animal toxicological tests are completed.

8 Control of PRSV by RNA silencing-based transgenic resistance

The defence reaction of a host plant against infection by a virus is underlying the mechanism of post-transcriptional gene silencing (PTGS), within which the alien invading RNAs of a virus are specifically degraded (Baulcombe, 1996). Thus, an appropriate segment (DNA or cDNA) of a virus genome can be constructed as a transgene and introduced into a plant host to trigger PTGS, a specific host defence response to degrade invading viral RNAs, thus generate resistance against the infection of the homologous virus (Baulcombe, 1999). In the case of the PTGS-mediated transgenic resistance, the translatable or untranslatable viral RNAs are destined to be degraded and viral proteins are not synthesised, thus most of the concerns about transgene-derived proteins do not arise. Since RNA silencing is a natural plant defence reaction during virus infection, this approach does not create extra non-natural events. Therefore, it has less bio-safety concerns and many crops have adopted this strategy for the control of infecting viruses (Powell-Abel et al., 1986; Beachy et al., 1990).

9 CP-transgenic papaya resistant to PRSV saves papaya industry in Hawaii

In late 1980s, to develop an effective solution to the severe loss caused by PRSV in Hawaii, Gonsalves team at Cornell University and the University of Hawaii initiated a program to generate PRSV CP transgenic papaya resistant to PRSV. The CP gene of PRSV HA 5-1 was constructed and delivered into the papaya genome via particle bombardment of embryogenic tissue derived from immature zygotic embryos of papaya. Transgenic papaya lines were obtained after regeneration from transformed cells (Fitch et al., 1990). Under greenhouse and field conditions, the transgenic line 55-1 was highly resistant to infection by PRSV Hawaii isolates (Fitch et al., 1992). The PRSV resistance is triggered by PTGS, a process of specific degradation of viral RNA against PRSV. The CP-transgenic lines of Rainbow and SunUp cultivars of papaya were deregulated for commercial applications in 1998, following approvals by the US APHIS, EPA and FDA. This represents the first successful case of a transgenic fruit tree being commercialised in the world (Gonsalves, 1998, 2002). After intensive studies to meet the strict biosafety regulations, the GM papaya of Hawaii was allowed to be marketed in Japan since 2012, representing the first case of GM fruit permitted for international trading (Yeh et al., 2014).

In Florida, Davis and coworkers at the University of Florida used untranslatable constructs of the CP gene sequence of Florida isolate H1K to create the transgenic lines. The selected line X17-2 has been granted deregulated status in the USA since 2009 (Yeh et al., 2014).

10 The development of CP-transgenic papaya with broad-spectrum resistance to different geographic PRSV strains

In Taiwan, the team of Yeh at National Chung Hsing University generated PRSV-resistant transgenic lines of papaya cultivar Tainung No. 2, using the CP gene of

an Taiwan strain PRSV YK by *Agrobacterium*-mediated transformation (Cheng et al., 1996). Several YK CP transgenic lines highly resistant to YK were obtained, which provide wide-spectrum resistance to exotic strains from three different geographic regions of Hawaii, Thailand and Mexico (Bau et al., 2003). During several field trials from 1996 to 1999, these transgenic papaya lines exhibited complete resistance against PRSV (Bau et al., 2004). After a long process of assessment, YK CP 16-0-1 has been formally approved for commercialisation in Mainland China in December 2018 and is expected to be approved by Taiwan government soon.

However, during the 4-yr field trials, unexpected severe symptoms were noticed in several plants of the YK CP-transgenic lines. The causal agent was identified as a P type strain of *Papaya leaf-distortion mosaic virus* (PLDMV) (Tripathi et al., 2004; Bau et al., 2008). Since all YK CP-transgenic papaya lines were susceptible to PLDMV, it becomes an emerging threat for the application of the PRSV CP transgenic papaya in Taiwan and other regions.

11 Generation of double resistance against PRSV and PLDMV

The breakdown of PRSV YK CP transgenic papaya to PLDMV infection exposed the urgent necessity for controlling the newly emerging PLDMV along with the predominant PRSV in Taiwan and elsewhere. For generating transgenic papaya lines with resistance to both viruses, an untranslatable chimera construct comprised of untranslatable segments of PRSV YK CP and PLDMV P-TW-WF CP coding sequences was constructed and transferred into papaya cv 'Thailand' by *Agrobacterium*-mediated transformation to induce PTGS based resistance. Three selected lines showed high degrees of resistance not only to the transgene donors of PLDMV and PRSV, but also to the heterologous strains of PRSV MX, TH, and HA, originated respectively from Mexico, Thailand and Hawaii (Kung et al., 2009). These transgenic papaya lines with resistance to both PRSV and PLDMV are considered having a great potential for the control of PRSV and PLDMV in Taiwan and elsewhere.

12 Threats by PRSV super virulent strains selected by CP-transgenic resistance and the solution

In Taiwan, extremely virulent PRSV strains infecting YK-CP lines in the test fields were also found. The isolated strain 5-19 was able to infect not only the PRSV YK CP-transgenic papaya lines, but also the PRSV-PLDMV double resistance lines (Kung et al., 2015). The recombination analysis between 5-19 and YK revealed that 5-19 possesses a stronger gene silencing suppressor HC-Pro which can suppress the PTGS and thus break down the CP-transgenic resistance in a transgene sequence homology-independent manner (Kung et al., 2015). These super virulent strains infect non-transgenic papaya plant more severely and cause more severe damage than the common strains. Thus, the selection and emergence of the supper virulent strains is a dangerous threat for the application of CP-transgenic crops for virus control.

In order to disarm the silencing suppression ability of PRSV super virulent strains, new transgenic lines of the papaya cultivar Sunrise carrying the untranslatable sequence of suppressor HC-Pro were developed by *Agrobacterium*-mediated transformation of somatic embryos derived from selected hermaphrodite individuals of elite varieties

(Kung et al., 2010, 2015). Several HC-Pro transgenic lines confer high levels of resistance to both super virulent strain PRSV 5-19 and other severe strains from Taiwan, Hawaii, Thailand and Mexico (Kung et al., 2015). Thus, the transgenic approach targeting the virus suppressor can solve the threat of super virulent strains selected by CP-transgenic lines.

13 Future perspectives on transgenic resistance

The recent advances in transformation of somatic embryos derived from the adventitious roots of elite papaya cultivars provide a prompt way to generate transgenic papaya lines with desired horticultural properties and hermaphrodite sex, and can avoid the time-consuming process of breeding. The developed transgenic lines can be directly applied in the field through tissue-culture micro-propagation which has been widely used in Taiwan.

The molecular markers derived from the flanking sequences of the transgene integration significantly fasten the breeding process for pyramiding of single, double and super transgenic resistance into a super hybrid variety (Fan et al., 2009). The pyramided resistance in a super hybrid is expected to confer more durable and wide-spectrum resistance to different geographic strains of PRSV and PLDMV, and avoid the selection for super virulent virus strains. Currently, the single, double, and supper transgenic resistances have been pyramided in a single papaya hybrid which is expected having a great potential for application in different geographic regions to promote global papaya production.

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