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20 Years of Transgenic Research in China for Resistance to *Papaya ringspot virus*

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ABSTRACT

Papaya is a favorite fruit and important industrial raw material in China. It is traditionally planted in southern China. Due to its wide uses and mystical health-related effects, papaya has been listed in fruits of high priority. *Papaya ringspot virus* (PRSV) is the main limitation of papaya production. PRSV infection rate reaches 100% in field in the late season of planting, which considerably reduces productivity and quality of papaya. Several research groups in China started around 1990 to develop transgenic papaya lines for resistance against PRSV, adopting pathogen-derived resistance strategy. The evaluation of resistance against PRSV and biosafety assessments with genetically modified (GM) papaya lines have been conducted in greenhouse and field. GM papaya with replicase gene from PRSV has been approved for commercial production in Guangdong province by Chinese government since 2006. In this review, we discuss 20 years research and regulatory management on transgenic papaya in China.

Keywords: coat protein, gene cloning, GMO, marker-free transformation, papaya transformation, regulatory research, replicase
Abbreviations: AFCD, Agriculture, Fisheries and Conservation Department; **bar**, bialaphos resistance gene; **BITC**, benzyl isothiocyanate; **Bt**, *Bacillus thuringiensis* endotoxin; **CHS**, chalcone synthase; **ESAT-6**, Early secretory antigenic target; **FAO**, Food and Agriculture Organization of the United Nations; **GMO**, genetically modified organism; **GUS**, β-D-glucuronidase; **nptII**, neomycin phosphotransferase II; **NSFC**, National Science Foundation China; **PDR**, pathogen derived resistance; **PTGS**, post-transcriptional gene silencing; **RdRP**, RNA-dependent RNA polymerase; **SBC**, State Biosafety Committee

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INTRODUCTION

Papaya (*Carica papaya* L.) is written as 番木瓜 in Chinese (pronouncing "Fan-mu-gua"), which means a tree melon originated from other countries. It is believed that papaya was introduced to China from India 300 years ago (Li *et al.* 2007). In recent years, papaya is extensively planted in six provinces or districts in the mainland of China. According to the statistical data from the Food and Agriculture Organization of the United Nations (FAO 2008), China cultivates 5,826 ha of papaya and produced 120,359 tons of fruits in 2008. In China, papaya has been used not only as favorite cooking dishes, dessert fruit and an important industrial raw material, but also as a famous herb medicine or healthy supplement. It is believed traditionally helpful for dozens of illnesses or health purposes. People in southern China like to make papaya fish soup to stimulate milk secretion for the mother who just gives birth of a baby. Many people, including the authors of this review, have experienced that eating fresh papaya can effectively reduce constipation problem.

Many diseases and pests limit papaya production. However, *Papaya ringspot virus* (PRSV) has developed to the most destructive threat since it was reported in China in 1959 (Li *et al*. 2007). Papaya is a perennial plant and can be found to grow over years in yards, roadsides and wild areas, which means PRSV sources and transmission vectors (aphids) are always available. Resistance to PRSV is not identified from cultured varieties. Therefore, PRSV is easily transmitted to field plants by aphids or human activities, and 100% farm plants are infected with the virus in late season. This kind of infection severity and percentage is not often seen on other plant diseases.

Chinese scientists have made great efforts for PRSV control, such as identifying papaya viral pathogens, trans-

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Invited Mini-Review

Table 1 Gene cloning and papaya transformation in China.

Resistance genes	Marker genes	PRSV strains or isolates	Research stages	References
Coat protein (*CP*)	nptII	Yellow spot (YS)	Greenhouse test	Ye *et al*. 1991; Yu *et al*. 2001
CP		Severe mosaic (SM)	Gene cloning	Liu *et al*. 1991
CP	*npt*II, GUS	Venial bleach (VB)	Greenhouse test	Rao *et al*. 2005, 2007
CP		Malformed leaf (ML)	Gene cloning	He *et al*. 2001
CP	*npt*II	Hainan	Field test	Gong *et al*. 1993; Zhou *et al*. 1993, 1996, 1997; Jiang *et al*. 2004
CP dsRNA	*npt*II, GUS	Conservative domain of multiple strains	Field test	Wei *et al*. 2007, 2008
CP hpRNA	GUS, bar	Conservative domain of multiple strains	Gene cloning	Wang *et al*. 2009; Yan *et al*. 2010
CP Inverted Repeat	*npt*II	Hainan	Gateway cloning	Jiang *et al*. 2008
CP ribozyme		Hainan	Greenhouse test	Zhao *et al*. 1998
The replicase (*TRP* or *RP*)	*npt*II	YS	Environmental release or Commercial use	Ye *et al*.1996; Chen *et al*. 2001; Ruan *et al*. 2001; Ye *et al*. 2003; Ruan *et al*. 2004; Wei *et al*. 2004; Rao *et al*. 2005; Li *et al*. 2007; Wei *et al*. 2007; Ruan *et al*. 2009; Jiao *et al*. 2010
RP		SM	Gene cloning	Liu *et al*. 1994
HC-Pro Inverted Repeat	*npt*II	Hainan	Gateway cloning	Gao *et al*. 2009
rhIFNa-2b	*npt*II	Human	Gene cloning	Zhou *et al*. 1998; Zhou *et al*. 2003
CP, *RP*, etc	*npt*II, GUS	SunUp, Huanong No.1, etc.	Inspection and quarantine	Yang *et al*. 2007; Chen *et al*. 2009; Tengs *et al*. 2009; Han *et al*. 2010; Zhao *et al*. 2010

mission vectors, cross-protection, host plant resistance, papaya mutagenesis and cultivation modification. In the mid 1980s pathogen-derived resistance (PDR) brought up a promising approach to protect plants from viral infection (Abel *et al*. 1986). The primary success of developing virus-resistant transgenic papaya from Dr. Gonsalves and colleagues (Fitch *et al*. 1992) was encouraging to Chinese peers. Since late 1980s, several laboratories in China started employing PDR against major plant viral diseases including PRSV, and have achieved great progresses. A number of genes are cloned; papaya transformation and virus-resistant transgenic plants are reported. To date, two of the eight cases of commercialized genetically-modified (GM) crops in China are for virus-resistance (sweet pepper and papaya). The PRSV resistant transgenic papaya line Huanong No 1 was licensed for commercial production in 2006 by State Biosafety Committee (SBC) of Agricultural GM organisms (GMOs), Ministry of Agriculture, China (SBC 2006). This review will focus on transgenic papaya research for resistance against PRSV and the regulatory management in mainland China during the past 20 years.

TRANSGENIC PAPAYA RESEARCH

Resistance gene cloning

Inspired by the successes on PDR in transgenic plants against tobacco mosaic virus (Abel *et al.* 1986) and other viruses, Chinese scientists started cloning viral resistance genes and transforming plants in late 1980s, and made encouraging progresses for engineering resistance to *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) in China. I (Ye) was fortunate to participate in the earliest effort of gene cloning from PRSV and papaya transformation in 1990, when I went to South China Agricultural University (SCAU) to pursue a PhD degree under supervision of Professor Huaichong Faan. Professor Faan, Director of the Plant Virology Laboratory at SCAU, has led studies on papaya viruses since 1960s. He won a grant from the National Science Foundation China (NSFC, #38970037): gene cloning and development of transgenic papaya resistant against PRSV in 1989. At that time, there were few institutions in China possessing the technology and facility of molecular biology. In a whole year, Professor Faan could not find a proper person to set up the gene cloning conditions and to perform the experiment. In the fall of 1990, he decided to send me to Dr. Po Ten lab in Chinese Academy of Sciences (Beijing) to learn the gene cloning technique and to clone coat protein (CP) at same time. The outcome is the first paper of cloning CP gene from the PRSV dominate strain in south China, yellow spot, was reported (Ye *et al*. 1991). Later, more CP genes, CP sense/antisense RNA, CP dsRNA/hairpin RNA and CP ribozyme from other isolates (Gong *et al*. 1993; Zhou *et al*. 1993, 1996, 1997; Jiang *et al*. 2004), the replicase (TRP or RP) genes or fragments (Ye *et al*.1996; Chen *et al*. 2001; Ruan *et al*. 2001; Ye *et al*.2003; Ruan *et al*. 2004; Wei *et al*. 2004; 2007; Rao *et al*. 2005; Li *et al*. 2007; Ruan *et al*. 2009; Jiao *et al*. 2010) and HC-pro (Gao *et al*. 2009) were also cloned. A human interferon (rhINFa-2b) gene (Zhou *et al*. 1998, 2003), which is proved having broad antiviral ability in mammals, was also cloned to test its antiviral effect in papaya plant. Papaya transformation vectors were constructed, in which *npt*II, bar and/or GUS selective markers are adopted. Gene cloning and application details are listed in **Table 1**.

Papaya transformation

Although transgenic papaya plants were developed with microprojectile-mediated method (gene gun) and *Agrobacterium* approach by Fitch *et al*. (1992, 1993), Chinese scientists have experienced a hard time for papaya transformation. It is the transformation obstacles, plus the strict regulations on GMO, attribute to the situation in China that many genes are cloned, but only a few of successes on transgenic papaya development. The powerful Bio-Rad gene gun systems were not available in most institutions of China at that time, even now (2010). The good news is that the technique of papaya somatic embryogenesis was established by Professor Baojian Li's group at Sun Yat-sen University (SYSU) (Ye *et al*. 1991). His lab also self-made a gene gun system (quite different from Bio-Rad one). Unfortunately, we never regenerated papaya transformants from the system, neither other research teams utilizing other microprojectile systems in China.

Until 1994, by the means of *Agrobacterium* cocultivation with papaya cotyledons and hypocotyls, we managed to obtain the first group of papaya plantlets with kanamycin resistance and CP gene PCR positive. Later on, more efficient transformation via *Agrobacterium* was reported. It was demonstrated that 15 seconds of sonication during *Agrobacterium* cocultivation can increase mediated papaya transformation rate about 10 times (Jiang *et al*. 2004). Rao *et al*. (2007) transformed PRSV VB CP to papaya genome using somatic embryos as explants. It is exciting that pollen tube pathway was proved to be an effective and convenient measure to transform papaya, and 11 to 51% high transformation rates for PRSV *CP* or *CP* dsRNA genes were achieved (Wei *et al*. 2008; Cai *et al.* 2009). In this method, the binary plasmid DNA or *Agrobacterium* containing the binary plasmid was simply pipetted onto papaya flower stigmas before pollination, seeds were collected when the fruits mature,

germinated on selective medium and PCR was applied to screen transformed seedlings. The author (Ye) obtained transgenic papaya seedlings with a modified pollen tube pathway (un-published data), in which plasmid DNA or *Agrobacterium* is directly injected into young flower ovaries with 1ml syringe and 23-26 GX needle (BD Company). Remove all uninjected flowers in order to harvest seeds earlier. The apparent advantages of pollen tube pathway are that cell culture, regeneration and selection on culture media are avoided; and high transformation efficiency is reached.

Mechanisms of virus resistance in transgenic papaya

In most cases of transgenic papaya generated in China showed mild to extreme resistance to local PRSV strains. *CP* mediated complete protection to American papaya plants from PRSV (Gonsalves 2006), but the effect was not observed in several labs in China (Yu *et al*. 2001). Considering the perennial feature and long growing period in a single year, we anticipated a transgenic papaya line with high and persistent resistance. *RP* transgenic papaya lines exactly satisfy the purpose (Ye *et al*. 2003; Li *et al*. 2007). **Fig. 1** shows that in the October, RP+ papaya plants are highly productive, without any visible PRSV symptoms, while wild type papaya plants grow much fewer fruits and display severe PRSV symptoms. As a matter of fact, the high protection in T0 or later generations of RP+ transgenic papaya plants have persisted for more than 10 continuous years in greenhouse (data not shown).

A large number of studies have shown that post-transcriptional gene silencing (PTGS) or RNAi plays the determinant role in PDR against plant viruses. This mechanism is also proved transgenic papaya. Ruan *et al.* (2009) observed that RNA degradation happened dramatically when T4 RP+ papaya was inoculated with PRSV. Other studies applying dsRNA, hpRNA or inverted repeat RNA also evident the mechanism. We observed that the RP+ papaya plants with extreme resistance displayed mosaic symptoms in early spring of every year (unpublished data), which also supported the PTGS theory. It was reported that a crucial enzyme in PTGS pathway, RNA-dependent RNA polymerase (RdRP) is inhibited by low temperature (Qu *et al.* 2006), so that plants show severer viral diseases in colder environment (under 25°C).

REGULATORY MANAGEMENT

Since the beginning, transgenic plant research, commercialization, international collaboration or trade have drawn a great attention in China. Keeping along with the international practices on safety management of GMOs, Chinese authorities started legislation since late 1980s. In 1993-1997, individual ministries of the central government issued the biosafety measures or regulations. In 2001, State Council decreed the "Regulations on Administration of the Biosafety of Agricultural GMO", which has served as the highest rule to replace the old ministerial regulations. According to the rule, SBC for Agricultural GMO was established responsible for evaluation and issuance of research, test, production, importation and exportation of agricultural GMO (plant, animal and microorganism) in China. So far, permission for thousands of GMO cases has been granted by the SBC. To date, eight cases of commercial use of GM plants are licensed (SBC 2009). They are Bt cotton, storage-tolerance tomato, virus-resistant CP sweet pepper and chalcone synthase (CHS) gene petunia in 1997; Bt poplar in 2002; virus-resistant RP papaya in 2006; phytase corn and Bt rice in 2009.

Regulatory research of transgenic papaya

Following the regulations, the extensive studies of two to four different stage tests of biosafety assessments were individually performed on transgenic papaya with CP and RP genes. Three cases of GM papaya were approved in China for field test, two cases for environmental release (Zhongkang1 and 2 from SYSU and Huanong No.1 from SCAU). RP transgenic papaya, Huanong No. 1, developed by Li lab, was licensed for commercial use in Guangdong Province in 2006 (Li *et al*. 2007) (**Fig. 2**).

The RP+ papaya biosafety Assessments included four period tests of "Middle test", "Release of environment", "Pre-commercialization test" and "Release of biosafety licenses", concentrated on environment and food effects according to Regulation guidelines issued by SBC. Wei *et al*. (2006) investigated the soil microbial communities and enzyme activities. No fundamental changes were discovered, except that the kanamycin resistant bacteria colony forming units (CFU) in RP+ papaya planting soil increases 1.6-4.5-fold over the wild type papaya planting soil. This is understandable because the transgenic papaya contains kanamycin resistance gene. Fruit components were compared between RP+ papaya and wild type (Wei *et al*. 2007; Jiao *et al*. 2010). It is not surprising that, due to the viral resistance, RP+ papaya fruits certainly are bigger, their flesh is thicker, and contains more sugar and vitamins.

Fig. 1 Field test of RP+ transgenic papaya (left) comparing to the wild type papaya (right). Comparing to wild type papaya, RP+ papaya plants are more productive, only show light PRSV symproms in early spring. There are no symptoms in later seasons. They continue flowering and growing fruits to the end of year. The magnified RP+ fruit does not show any PRSV symptoms; While, the wild type papaya plants can be infected any time and develop typical symptoms such as mosaic, ringspot and abnormal leaves. Wild type plants are dying in late season. The magnified wild type fruit displays ringspot symptom.

Fig. 2 License of *RP* transgenic papaya commercial production issued by Ministry of Agriculture on July 20th, 2006. The license indicates that the transgenic papaya line Huanong No. 1 contains *RP* and *npt*II genes, it is categorized in biosafety class 1, and it is limited for commercial production in Guangdong province till July 20th, 2011.

There are no significant variances for two natural papaya toxicants, benzyl isothiocyanate (BITC) and carpaine (Jiao *et al*. 2010).

The series of research results on Huanong No.1 (Ruan *et al.* 2001; Feng *et al.* 2003; Ruan *et al.* 2004; Feng *et al.* 2005; Li *et al.* 2007; Ruan *et al.* 2009) showed that RP+ papaya did not change any papaya horticultural properties, and produced any influence on non-target organisms including other crops, animals, and microorganisms in plants and soil. The analysis of papaya food nutrients showed that papaya fruit was found to be equivalent to conventional varieties in contents of vitamins A and C, total soluble solids, and other components. The latex of green tissues and seeds in papaya contain a toxin called BITC, which has been linked to incidents of spontaneous abortions in pregnant women and with a higher incidence of prostate cancer in Japanese men over the age of 70. We found that the levels of BITC in both transgenic and conventional papaya cultivars were much lower in ripe fruit compared to immature fruit. There were no major differences between transgenic papaya and conventional papaya from either immature or ripe fruit. The PRSV replicase does not possess characteristics typical of known protein allergens or toxins, such as heat stability and resistance to digestion by simulated gastric fluids. Comparisons of the deduced amino acid sequence of the plant-expressed PRSV replicase did not reveal any homology to known protein allergens and toxins. Furthermore, PRSV-infected papaya fruit naturally did not contain any replicase protein, even any virus gene because of the action of gene silencing. All these results indicated that RP transgenic papaya did not produced any negative effects on both aspects of environment and food, and had substantial equivalence as conventional papaya, except virus resistance (Li *et al.* 2007).

Seven years of regulatory studies have shown that RP+ papaya did not produced any negative effects on both aspects of environment and food, and had substantial equivalence as traditional papaya, except virus resistance. Since the RP+ papaya lines were commercially planted in Guangdong province in 2006, its planting area has expanded dramatically year by year. In 2009, RP+ papaya lines is estimated standing for about 95% of total papaya area in Guangdong Province. Market and consumers responses are generally positive, although controversies are still heard from social activists, environmentalists, as well as common scientists.

Inspection and quarantine of transgenic events

As a part of transgenic papaya research and the regulatory requirement, transgenic events from the domestic or imported papaya and its derived products are continuously monitored. Techniques of PCR, RT-PCR, real-time PCR regarding to a specific transgenic event or multiple targets are developed (Yang *et al.* 2007; Chen *et al.* 2009; Han *et al*. 2010; Zhao *et al*. 2010). Recently, researchers in Shanghai, China, collaborating with a Norwegian team, successfully characterized GM Arabidopsis, rice and papaya by the means of the high throughput sequencing and computational approach (Tengs *et al.* 2009). This and other new high throughput technologies are absolutely needed as more and more GM crops and products are developed and enter the market.

Hong Kong GM papaya incident

Hong Kong (HK) only has a small area of papaya plantation. Notably, HK imports 28,000 tons of papaya fruit annually, ranking as the second largest papaya market after USA in the world. Since returned to China in 1997, HK has implemented independent law system, so that the GMO regulations issued in mainland have no effect in HK. In 2005, the Agriculture, Fisheries and Conservation Department (AFCD) of the HK government provided papaya seeds to local farmers. Unfortunately, 13 papaya farms were detected by Greenpeace with the contamination of GM papaya. Under the tremendous social pressure, in the spring of 2006, AFCD eradicated all 300 GM papaya trees, and advised farmers not to plant papaya any longer. **Fig. 3** shows Greenpeace patrols are removing papaya plants. The good news is the incident speeded up legislation of GMO regulation. In January 2010, the "Genetically Modified Organisms Bill" was drafted in HK (AFCD 2010).

Fig. 3 Greenpeace patrols are helping AFCD, Hong Kong, remove all papaya trees from GM contaminated farms. Permission to use from Greenpeace China.

It is necessary to point out that the Greenpeace patrols might over-responded to the GM papaya contamination. As we know, there have never been GM plants developed which should be treated as contagious pathogens or other horrible organisms. As a matter of fact, all of the scientists, Greenpeace or common citizens share the same core value to produce safe, high quality and environment-friendly food. However, the GM papaya incident is not the only one event in the world. It reminds that governments, companies and scientists have to pay a great attention to GMO safety, to implement highly strict regulations and longtime studies to ensure that GM foods have no negligible effects on human health and environment. We believe that through efforts from all aspects, we can develop absolutely healthy and safe GM papaya for virus resistance.

Marker-free transformation

Controversies surrounding GMOs have been continued since it early stage of the transgenic technology in the 1980s. Apart from political and religion reasons, people often worry more about the transgenes expressed in transgenic plants, the selective marker genes, such as antibiotics resistance, herbicide tolerance and β-D-glucuronidase (GUS). In fact, marker genes have been located at the central dispute. Therefore, marker-free transformation has been proposed as early 1980s and many techniques have been established. In 2001, the European Union proposed the "identification and phasing out antibiotic resistance marker genes in GMOs which may have adverse effects on human health and the environment." (The Directive 2001/18/EC).

A pertinent question is, regarding GM crops that are being massively planted and contain antibiotic or herbicide resistance genes, how can these genes be removed? Recently, two revolutionary techniques were developed to target in vivo specific genes including transgenes. One is chimeric RNA/DNA oligonucleotide mediated gene targeting (Suzuki 2008), and the other is zinc-finger nuclease mediated gene modification (Carroll 2008). Details of the procedures can be found in recent publications.

FINAL COMMENTS

A compelling trend occurring in China is that Chnese scien-

tists are participating in more and more international collaborations in science and technology. One of the signature achievements is the draft genome of transgenic papaya, SunUp, accomplished by a group scientists led by Dr. Wang in Tianjin, China, and American colleagues (Ming *et al*. 2008). Among many genetic information uncovered, three foreign gene integration locations were identified in the transgenic papaya genome.

Besides resistance to PRSV, some other purposes are pursued with transgenic papaya. With the advantages of high yield and favorite fresh food, papaya has a great potential to be applied for the edible vaccine or for expression of valuable proteins. Zhang *et al.* (2003) cloned the Early secretory antigenic target (ESAT-6) gene from *Tuberculosis mycobacterium* and transformed it to papaya for oral vaccine against tuberculosis. We see this is a beneficial exploration. Certainly much more research has to be done in future. Reducing damage in transportation and extending storage time crucial for many vegetables and fruits, which can be realized by shutting down cell wall degradation enzymes. He *et al*. (2009) made a preliminary progress towards to goal for papaya by constructing binary vector of β-galactosidase RNAi gene. It is expected that a wider field of GM papaya is opening in future.

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