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Genetic Engineering for Viral Disease Management in Plants

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Abstract

Viral diseases are a major threat to world agriculture and breeding resistant varieties against these viruses is one of the major challenge faced by plant virologists and biotechnologists. The development of the concept of pathogen derived resistance gave rise to strategies ranging from coat protein based interference of virus propagation to RNA mediated virus gene silencing. Much progress has been achieved in protecting plants against these RNA and DNA viruses. In this review, the most recent transgene based approaches for viral disease management in plants will be discussed.

Keywords: viral diseases, RNA viruses, DNA viruses, transgene, RNA interference, coat protein

Viruses are very widespread in nature, and they cause severe diseases and yield losses in economically important plants. Plant viruses can cause severe damage to crops by substantially reducing vigor, yield, and product quality. Losses of several billion dollars are reported annually in vegetable and fruit crops. Viruses that infect plants differ in particle morphology, type of genome and several other characteristics based on which they are classified. The most important characteristic feature of a virus is its genome. There are examples of viruses containing double stranded DNA (ds DNA, eg. *Caulimovirus*), single stranded DNA (ssDNA eg. *Geminivirus*) double stranded RNA (dsRNA eg. Reoviridae) and single stranded RNA (ssRNA) with positive sense or negative sense genome (eg. *Potyvirus*). However, majority of plant viruses have genome consisting of RNA (Brunt *et al.*, 1996). Management strategies for viral diseases are often directed essentially against vectors that spread viruses within and between fields. So far, the most effective approach to control viruses relies on the use of resistant cultivars and/or rootstocks. Host-resistance genes have been extensively exploited by traditional breeding techniques for the development of virus-resistant plants. However, host resistance has been identified for a few viruses only and a limited number of commercial elite crop cultivars and rootstocks exhibit useful resistance. Therefore, engineered resistance is attractive to complement conventional breeding approaches, in particular when resistant material with desired horticultural characteristics has not been developed successfully or when no host resistance sources are known. Actually, the deployment of virus-resistant transgenic plants has become an important strategy to implement effective and sustainable control measures against major virus diseases.

Generation of transgenic plants with virus resistance has been demonstrated as an effective strategy against virus infections through the expression of coat protein genes, viral replicase genes, or other viral sequences. This phenomenon is termed pathogen-derived resistance and includes protein-mediated resistance and RNA mediated resistance. The mechanism of RNA mediated resistance involves RNA silencing, in which sequence-specific RNA degradation occurs (Kawazu *et al.,* 2009). Here in this review, the coat protein and RNAi mediated resistance for viral disease management will be discussed.

Coat protein-mediated resistance (CP-MR)

The most important agricultural application of Pathogen Derived Resistance (PDR) is coat protein mediated protection (CPMP) against plant virus diseases. The viral coat-protein gene transferred into plants makes them resistant to virus from which the gene for the coat protein (CP) was derived. This was first demonstrated for Tobacco mosaic virus (TMV) in tobacco (Powel-Abel *et al.*, 1986). Coat Protein is required for systemic infection by monopartite geminiviruses (Rojas *et al.,* 2001), and tomato plants expressing the CP of the monopartite begomovirus tomato yellow leaf curl geminivirus (TYLCV) exhibited delayed symptom development that was dependent on the expression levels of transgenic CP (Kunik *et al.,* 1994).

In another attempt by Furutani *et al.* (2006), somatic embryos of *Glycine max* (L.) Merrill cultivar 'Jack' were co-transformed with coat protein (CP) gene of attenuated isolates of soybean mosaic virus (SMV) and hygromycin phosphotransferase (hpt) gene by means of microprojectile bombardment. These transformed embryogenic tissues were selected in hygromycin-containing liquid medium.

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The hygromycin-resistant embryogenic tissues obtained through the selection were regenerated, and CP gene was detected in the 11 transgenic plants out of them. In order to assess their resistance to SMV, mechanical inoculation was performed in T_1 generation. The disease symptom was examined visually and confirmed by the enzyme-linked immunosorbent assay (ELISA). Finally, three independent lines highly resistant to SMV were obtained. This was the first report of the soybean plants that conferred a high resistance to SMV by the transformation with CP gene of the SMV attenuated isolates.

A transformation system of pepper was set up using *Agrobacterium* that had been transfected with the coat protein gene, CMVP0-CP, with the aim of developing a new CMVP1-resistant pepper line. A large number of transgenic peppers (T_1, T_2 and T_3) were screened for CMVP1 tolerance using CMVP1 inoculation. Transgenic peppers tolerant to CMVP1 were selected in a plastic house as well as in the field. Three independent T_3 pepper lines highly tolerant to the CMVP1 pathogen were found to also be tolerant to the CMVP0 pathogen. These selected T_3 pepper lines were phenotypically identical or close to the non-transformed lines. However, after CMVP1 infection, the height and fruit size of the non-transformed lines became shorter and smaller, respectively, while the T3 pepper lines maintained a normal phenotype (Lee *et al*., 2009)

The level of protection conferred by CP genes in transgenic plants varies from immunity to delay and attenuation of symptoms. In some cases protection is broad and effective against several strains of the virus from which the CP gene is derived, or even against closely related virus species (Lomonossoff, 1995). Molecular mechanisms that govern CP-mediated resistance (CPMR) are not fully understood, and furthermore, mechanisms of CPMR are different in different viruses (Bendahmane *et al*., 2007). Asurmendi *et al*. (2007) postulated that the state of aggregation of CPs is correlated with the level of CPMR. This suggested that CPMR may be mediated by certain configurations of quaternary structures rather than by the subunit *perse*. Bendahmane *et al*. (2007) further propose that the degree of regulation of replication by aggregates of CP determines the relative strength of CPMR. CPMR and other cases of PDR reviewed below are compatible with direct interference of these proteins with virus accumulation. However, the establishment of different levels of resistance indicates that multiple mechanisms could be involved. Furthermore, as will be discussed below, a transgene can confer both protein- and RNA-mediated protection. The attribution of resistance to expression of the viral protein or to its RNA is often posed as a 'dilemma'. Several explanations have been proposed to reconcile different and sometimes contrasting results. However, in spite of uncertainty about mechanisms, high levels or broad resistance may be attributed to co-existence of both protein- and RNA-mediated interferences.

A variety of yellow squash called 'Freedom 11' has been released in the USA. Several varieties of potato, cucumber and tomato, in which CP-MR has been used, are under field evaluation. Transgenic papaya resistant to papaya ring-spot virus (PRSV) has been developed and is being commercially grown in the USA. (Ferreira *et al*., 2002). Subsequently, virus-resistant transgenics have been developed in tomato, melon, rice, papaya, potato and sugarbeet (Tab. 1) (Fuchs and Gonsalves, 2007).

RNA interference mediated resistance

Homology-dependent selective degradation of RNA, RNA-interference (RNAi) or Post-transcriptional gene silencing (PTGS) is involved in several biological processes, including adaptive defence against viruses in plants (Herr 2005; Ratcliffe *et al.,* 1999; Vance and Vaucheret, 2001; Yu and Kumar, 2003). The first demonstration of RNAi-mediated virus resistance was shown by Waterhouse *et al.* (1998), against Potato virus Y (PVY) in transgenic tobacco plants. Resistance against PVY in transgenic tobacco plants expressing the PVY protease gene simultaneously in sense and anti-sense orientation was much higher than in lines expressing the same gene individually in either orientation. Antiviral RNAi technology has been used for viral disease management in human cell lines (Novina *et al.,* 2002). Such silencing mechanisms (RNAi) can also be exploited to protect and manage viral infections in plants (Ullu *et al.,* 2002; Waterhouse *et al.,* 2001). The P1/HC-Pro suppressors from the potyvirus inhabited silencing at a step down stream of dsRNA processing, possibly by preventing the unwinding of duplex siRNAs, or the incorporation into RISC or both (Chapman *et al.,* 2004). The utilization of RNAi technology has resulted in inducing immunity reaction against several other viruses in different plant-virus systems (Tab. 1). In phyto-pathogenic DNA viruses like geminiviruses non-coding intergenic region of Mungbean yellow mosaic India virus (MYMIV) was expressed as hairpin construct under the control of the 35S promoter and used as biolistically to inoculate MYMIV-infected black gram plants and showed a complete recovery from infection, which lasted until senescence (Pooggin *et al.,* 2003). RNAi mediated silencing of geminiviruses using transient protoplast assay where protoplasts were co-transferred with a siRNA designed to replicase (Rep)-coding sequence of African cassava mosaic virus (ACMV) and the genomic DNA of ACMV resulted in 99% reduction in Rep transcripts and 66% reduction in viral DNA (Vanitharani *et al.,* 2003). It was observed that siRNA was able to silence a closely related strain of ACMV but not a more distantly related virus.

About more than 40 viral suppressors have been identified in plant viruses (Ruiz and Voinnet, 2007). Results from some of the well-studied virus suppressors indicated that suppressors interfere with systemic signaling for silencing (Mlotshwas *et al*., 2002). During last few years, the *p69*

encoded by *Turnip yellow mosaic virus* has been identified as silencing suppressors that prevented host RDR-dependent secondary dsRNA synthesis (Chen *et al*., 2004). *P14* protein encoded by aureus viruses suppressed both virus and transgene-induced silencing by sequestering both long dsRNA and siRNA without size specificity (Merai *et al*., 2005). Multiple suppressors have been reported in *Citrus tristeza virus* where *p20* and coat protein (*CP*) play important role in suppression of silencing signal and *p23* inhibited intracellular silencing Multiple viral components, viral RNAs and putative RNA replicase proteins were reported for a silencing or suppression of *Red clover necrotic mosaic virus* (Takeda *et al*., 2005). In this case, the RNA silencing machinery deprived of DICER-like enzymes by the viral replication complexes appears to be the cause of the suppression. *Pns10* encoded by *Rice dwarf virus* suppressed local and systemic S-PTGS but not IR-PTGS suggesting that *Pns10* also targets an upstream step of dsRNA formation in the silencing pathway (Cao *et al*., 2005). Niu *et al*. (2006) used a 273-bp (base pair) sequence of the *Arabidopsis* miR159 a pre-miRNA transcript expressing amiRNAs against the viral suppressor genes *P69* and *HC-Pro* to provide resistance against *Turnip yellow mosaic virus* and *Turnip mosaic virus* infection, respectively. In addition, a dimeric construct harboring two unique amiRNAs against both viral suppressors conferred resistance against these two viruses in inoculated *Arabidopsis* plants. Similarly, Qu *et al*. (2007) used a different amiRNA vector to target the 2 b viral suppressor of the *Cucumber mosaic virus* (CMV), a suppressor that interacted with and blocked the slicer activity of *AGO1* had also shown to confer resistance to CMV infection in transgenic tobacco. A strong correlation between virus resistance and the expression level of the 2 b-specific amiRNA was shown for individual plant lines.

It is evident from above-mentioned reports that the RNA components, such as single strand template RNA, dsRNA and/or siRNA of the silencing pathways are the

Tab. 1 RNAi mediated approach for plant virus management

Target plant	Virus	Targeted region	References
Barley, wheat	Barley stripe mosaic virus	pds	Holzberg *et al*., 2002; Scofield *et al*., 2005
Barley, rice, maize	Brome mosaic virus	*pds*, *actin 1*, *rubisco activase*	Ding *et al.,* 2006
Rice	Rice yellow mottle virus (RYMV)	RYMV (wt.CP) and (ΔNLS.CP)	Kouassi *et al.,* 2006
Rice	Rice tungro bacilliform virus (RTBV)	*RTBV-Os*, *RTBV-O-Ds*	Tyagi *et al.,* 2008
Arabidopsis	Cabbage leaf curl virus	*gfp*, *CH42*, *pds*	Turnage *et al*., 2002
P. sativum	Pea early browning virus	*pspds*, *uni*, *kor*	Constantin *et al*., 2004
N. benthamiana, *M. esculenta*	African cassava mosaic virus	*pds*, *su*, *cyp79d2*	Fofana *et al*., 2004
N. benthamiana	Poplar mosaic virus	gfp	Naylor *et al*., 2005
N. benthamiana, *S. tuberosum*	Potato virus X	*pds*, *gfp*	Ruiz *et al*., 1998; Faivre-Rampant *et al*., 2004
N. benthamiana	Turnip crinkle virus	TCV-cp	Vasudevan *et al.,* 2008
Nicotiana tabacum	Satellite tobacco mosaic virus	Several genes	Gossele *et al*., 2002
N. benthamiana, *N. tabacum*	Tobacco mosaic virus	*pds*, *psy*	Kumagai *et al*., 1995
N. benthamiana, *Arabidopsis*, tomato, *Solanum* species, Chilli pepper, opium poppy, *Aquilegia*	Tobacco rattle virus	*Rar1*, *EDS1*, *NPR1/ NIM1*, *pds*, *rbcS*, *gfp*	Liu *et al*., 2002b; Ratcliff *et al*., 2001; Brigneti *et al*., 2004; Chung *et al*., 2004; Hileman *et al*., 2005; Gould and Kramer, 2007
N. benthamiana	Tomato bushy shunt virus	gfp	Hou and Qiu, 2003
N. benthamiana	Tomato golden mosaic virus	*su*, *luc*	Peele *et al*., 2001
N. benthamiana, *Lycopersicon esculentum* *N. glutinosa*, *N. tabacum*	Tomato yellow leaf curl China virus-associated b DNA satellite	*pcna*, *pds*, *su*, *gfp*	Tao and Zhou, 2004
N. benthamiana	Grapevine leafroll-associated virus-2 (GLRaV-2)	GLRaV-2 -cp	Ling *et al.,* 2008
Citrus	Citrus psorosis virus (CPsV)	CPsV-cp	Zenek *et al.,* 2008
N. benthamiana	Grape vine A	GVA-minireplicon	Brumin *et al.,* 2009
Potato	Potato virus X and Potato virus Y	PVX-cp , PVY-Nib	Bai *et al.,* 2009
Lettuce	Mirafiori lettuce virus MiLA	Ir- MiLA	Kawazu *et al.,* 2009
Pepper	Cucumber mosaic virus(CMV)	CMVP0-CP	Lee *et al*., 2009
Lycopersicon esculentum	Tomato leaf curl virus (ToLCV)	AC4	Praveen *et al.,* 2010

preferred targets of most viral suppressors. However, plant viruses are known to have evolved a counter-silencing mechanism by encoding proteins that can overcome such resistance (Díaz-Pendón and Ding, 2008; Li, 2006; Ding, 2006). These suppressors of gene silencing are often involved in viral pathogenicity, mediate synergism among plant viruses and result in the induction of more severe disease. Simultaneous silencing of such diverse plant viruses can be achieved by designing hairpin structures that can target a distinct virus in a single construct (Díaz-Pendón and Ding, 2008). Transgenic lettuce with resistance to big-vein disease was produced by introducing an antisense construct of the Lettuce big-vein associated virus LBVaV CP gene. The transgenic lettuce showed resistance not only to LBVaV but also to MiLV and big-vein symptoms expression (Kawazu *et al.,* 2006).

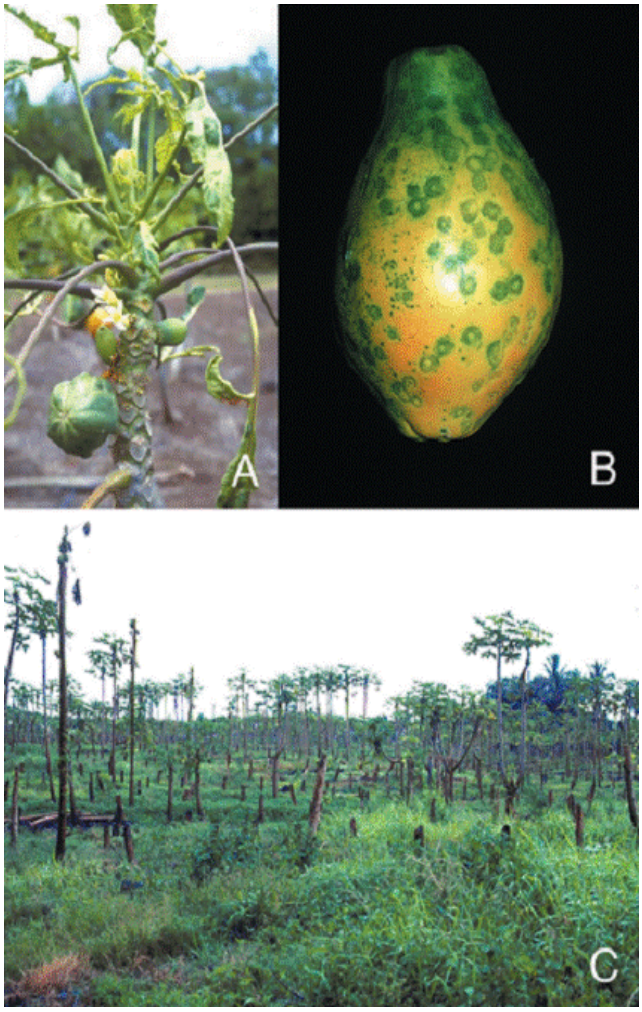

Fig. 1. Fig. 1. Symptoms of PRSV on papaya: PRSV-infected papaya tree; (B) ringspot symptoms on fruit; (C) PRSV-infected papaya orchard in Puna area of Hawaii in 1994 (Tripathi *et al.*, 2008)

Papaya Ringspot Virus resistance (coat protein and gene silencing approach)

Papaya is one of the most important cash crops in the tropics and subtropics. However, the production of this economically important fruit crop is limited by the destructive disease caused by the Papaya ringspot virus (PRSV) (Bau *et al.*, 2008) and the fragile and perishable fruit traits unfavorable for large-scale exportation make papaya lag far behind banana and pineapple in the world market.

PRSV is a member of the genus Potyvirus (Murphy *et al.,* 1995), is transmitted nonpersistently by aphid, and is sap-transmissible in nature. PRSV genome contains a single-stranded positive sense RNA of about 40 S (Yeh and Gonsalves, 1985). Strains of PRSV from Hawaii (Yeh *et al.,* 1992) and Taiwan (Wang and Yeh, 1997) have been completely sequenced, both containing 10.326 nucleotides in length. In papaya, PRSV causes severe mosaic and distortion on leaves, ringspots on fruits, and water-soaking oily streaks on upper stems and petioles (Fig. 1). It stunts the plant and drastically reduces the size and the quality of the fruit. In order to solve the problems caused by PRSV, in the late 1980s the group of Gonsalves at Cornell University and Hawaii started a research project to develop transgenic papaya. Ling *et al.* (1991) first demonstrated that the expression of the PRSV HA 5-1 CP gene in tobacco affords a broad-spectrum protection against different potyviruses.

However, effective gene transfer systems require reliable and efficient procedures for plant regeneration from cells. Fitch and Manshardt (1990) reported that somatic embryogenesis from immature zygotic embryos of papaya can be integrated into a useful gene transfer technology. By the same year, Fitch *et al.* (1990) successfully incorporated the CP gene of HA 5-1 into papaya via microprojectile bombardment and obtained plants resistant to infection by the severe Hawaii HA strain. Among their transgenic papaya lines, line 55-1 was virtually immune to infection by HA.

Transgenic papaya in Hawaii

The plants of transgenic papaya line 55-1 are highly resistant to Hawaiian PRSV isolates under greenhouse and field conditions (Lius *et al*., 1997). The resistance is triggered by the posttranscriptional gene silencing (PTGS) -an RNA-mediated specific degradation process of innate nature of plants against pathogens (Gonsalves 2002; Hamilton and Baulcombe, 1999). However, the characteristic of sequence homology-dependent resistance limits the application of CP-transgenic papaya for controlling PRSV in other geographic regions other than Hawaii (Gonsalves, 2002). The field trial of the homozygous line SunUp and hemizygous line Rainbow indicates that both of them offer a good solution to the PRSV problem in Hawaii (Ferreira *et al*., 2002). This was the first successful case

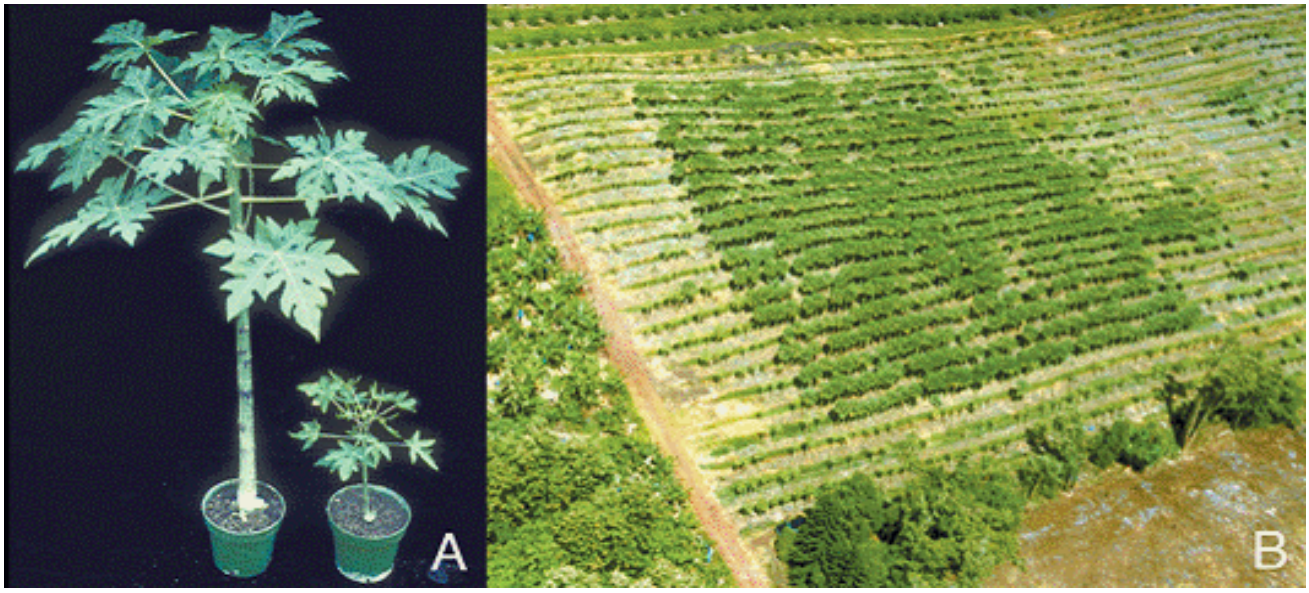

Fig. 2. Evaluation of transgenic papaya for PRSV resistance. (A) R0-transgenic papaya line 55-1 (left) and nontransgenic control (right) six months after inoculation with PRSV HA in the greenhouse. (B) Aerial photograph of the 1-acre plot of Rainbow papaya in Puna (Hawaii) 28 months after transplanting. The Rainbow block was surrounded by non-transgenic susceptible Sunrise plants, which are severely infected by PRSV (Tripathi *et al.*, 2008)

Fig. 3. View of PRSV disease progress in the field test in Puna (Hawaii) at 18 and 23 months (top and bottom, respectively). In each view the susceptible Sunrise variety is shown on the left and resistant transgenic Rainbow on the right (Tripathi *et al.,* 2008)

of transgenic fruit tree being commercialized in the world (Fig. 2, 3)

Transgenic papaya in Taiwan

Other than Hawaii, a CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium* mediated transformation (Cheng *et al.,* 1996). The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity (Bau *et al.,* 2003). Several lines highly resistant to the homologous strain (PRSV YK) provide wide-spectrum resistance to three different geographic strains from Hawaii, Thailand, and Mexico (Bau *et al.*, 2003). During four repeats of field trials from 1996 to 1999, the transgenic papaya exhibited high degrees of protection against PRSV in Taiwan (Bau *et al.,* 2004). Unfortunately, 18 months after plantation in the fourth field trial, unexpected symptoms of severe distortion on fully expended leaves, stunning on apex, water-soaking on petioles and stem, and yellow ringspot on fruit were noticed on PRSV CP-transgenic papaya plants. The causal agent was distinguished from PRSV by host reactions and serological properties (Bau, 2000) and later identified as Papaya leafdistortion mosaic virus (PLDMV), a potyvirus which originated from Okinawa, Japan, in 1954 (Maoka *et al.,* 1996). All of the PRSV CP transgenic papaya lines were susceptible to PLDMV infection when evaluated under greenhouse conditions. Therefore, in Taiwan, PLDMV is considered as a serious threat to papaya production once PRSV CP-transgenic papaya is widely applied for the control of PRSV.

Development of PRSV-resistant transgenic plants faces a major hurdle in achieving resistance against geographically distinct isolates. One of the major reasons of failing to achieve the broad-spectrum PRSV resistance is the involvement of silencing suppressor proteins of viral origin (Ruanjan *et al*., 2007). For the effective control of PRSV and Papaya leaf-distortion mosaic virus (PLDMV), an untranslatable chimeric construct containing truncated PRSV YK CP and PLDMV P-TW-WF CP genes has been transferred into papaya (*Carica papaya* cv. 'Thailand') by *Agrobacterium*-mediated transformation via embryogenic tissues derived from immature zygotic embryos of papaya (Kung *et al.,* 2009). Based on sequence profile of silencing suppressor protein, HcPro, it was that PRSV-HcPro, acts as a suppressor of RNA silencing through micro RNA binding in a dose- dependent manner. In planta expression of PRSV-HcPro affects developmental biology of plants, suggesting the interference of suppressor protein in micro RNA-directed regulatory pathways of plants. Besides facilitating the establishment of PRSV, it showed strong positive synergism with other heterologous viruses as well (Mangrauthia *et al.,* 2010). Therefore, resistance in transgenic papaya can be overcome by PRSV with distant homology to the transgene, or by PRSV strains with HC-Pro that can sufficiently suppress the silencing mechanism of transgenic papaya. It would therefore be important to develop transgenic papaya that could avoid the impact of these PRSV strains (Tripathi *et al*., 2008).

Conclusions

Genetic engineering remains an alternative and rapid method to transfer resistance genes into traditional cultivars, bypassing the long procedure of introgression and the appearance of undesired traits usually associated with it. When traditional breeding can offer cultivars with a medium level of resistance, genetic engineering could provide additional tools to implement virus resistance. In this respect, genetic engineering and traditional breeding are complementary. The availability of protocols for genetic transformation of various important plants like cereals and vegetables has simplified the application of genetic engineering in those crops. Developing broad and durable resistance is the main objective in producing virus resistant transgenic plants. Therefore, as discussed above RNA mediated resistance is more durable and the method of choice for producing virus resistant plants for resistance against new strains of a virus.

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