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Stability of Transgenic Resistance Against Plant Viruses

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1. Introduction

Plant viruses constitute one of the main problems of the agricultural production worldwide (Kang et al., 2005). To date, there are not therapeutical measures available for the control of plant-virus diseases in the field and the main control strategy used in practice is based on prevention measures. Genetic resistance is by far the most effective way to control plant viruses. However, 'traditional' genetic sources of resistance to viruses are rare (Lecoq et al., 2004) and due to the high rate of mutation of the viral genomes this resistance even when applicable, is frequently broken under field conditions. The era of *Agrobacterium*-mediated genetic transformation of plants which started at the 80s (Thomashow et al., 1980; Zambryskiet al., 1980) offered new promising prospects for engineered genetic resistance to viruses with numerous following studies reporting a successful use of the transgenic technology against almost all genera of plant viruses or even viroids (Lin et al., 2007; Prins et al., 2008; Ritzenthaler, 2005; Schwind et al., 2009). However, mainly due to public concerns for the safety of using transgenic plants in agriculture only in a relatively small number of virus diseases transgenic technology has been used in the field and in these cases it was proved an efficient and safe way of control (Fuchs et al., 2007). The mechanism of resistance in the vast majority of the applications of transgenic-plant strategy is based on RNA-silencing. RNA-silencing is a sequence specific RNA degradation mechanism, highly conserved between kingdoms, which in plants, among other functions, operates as a natural antiviral defense system (Eamens et al., 2008). The role of RNA-silencing as an antiviral weapon has been further supported by the fact that almost every known plant virus species encodes for at least one protein with RNA-silencing suppression activity (Dıaz-Pendon & Ding, 2008). This knowledge raised the first concerns regarding the efficiency of RNA-silencing based resistance against viruses under field conditions. As silencing is sequence specific, the resistance of transgenic plants engineered to be resistant to typically one virus could be broken by a different, heterologous virus that could infect the plants in the field. The hypothesis was that the heterologous virus through its silencing suppressor protein(s) could repress the RNA silencing machinery of the plant as a whole, resulting in the loss of the initially engineered resistance. In addition, the extensive research on RNA-silencing that is going on for over a decade has revealed a number of environmental and plant physiological factors that can influence the silencing mechanism and consequently the effectiveness of RNA-silencing based transgenic resistance to viruses under field conditions.

This review summarizes a fair amount of data that have been produced during the last decade in studies that have examined the role of heterologous viruses, the effect of temperature, the influence of the developmental stage of the plants in the stability of the transgenic resistance to viruses as well as recent findings for a direct effect of light intensity on the RNA silencing machinery. Moreover, new approaches for the implementation of RNA silencing in transgenic plant virus resistance are discussed as possible ways to overcome constrains of the current applications.

2. Strategies for engineering resistance to plant viruses

After the revolutionary work that was carried on *Agrobacterium* as a vector for plant transformation, the breakthrough for the creation of transgenic resistance to plant viruses came by Beachy's group which showed that the expression of the coat protein gene of *Tobacco mosaic virus* (TMV) in transgenic plants is conferring resistance to TMV (Abel et al., 1986). This discovery led the way for the production of an enormous number of transgenic plants resistant to viruses, using most types of viral genes. This genetically engineered resistance, referred to as pathogen-derived resistance (PDR) (Sanford & Johnston, 1985), mechanistically was divided into two categories; protein mediated and RNA-mediated. In protein mediated resistance the transformation cassette is designed in such a manner that the introduced viral gene, most commonly either of the coat protein, the replicase or a defective movement protein gene, would be able to be translated and expressed into the plant and somehow interfere with the disassembly, the replication or the movement respectively, of the intruding virus. However, this division is rather simplistic as in most cases of resistance which were designed to be protein mediated, it was proved that multiple mechanisms were involved, most frequently the RNA-mediated one (Lin et al., 2007; Prins et al., 2008; Ritzenthaler, 2005). RNA-mediated resistance is related to RNA-silencing which is probably the most important and common strategy for engineered resistance to plant viruses and will be discussed more extensively below.

Besides the PDR strategy, alternative biotechnological approaches for the manufacturing of plants resistant to viruses include the expression of plant virus-resistance genes in other plants than those from which they were isolated (Farnham, 2006; Seo et al., 2006; Spassova et al., 2001) and the expression of peptides (Lopez-Ochoa et al., 2006; Rudolph et al., 2003; Uhrig, 2003) or antibodies. After the first successful application of the later strategy in 1993 by Tavladoraki and co-workers, with antibodies that reduced the susceptibility to *Artichoke mottle crinkle virus* using a single-chain variable fragment (scFv) directed against the CP of the virus, technical difficulties hampered a wider application of this methodology. Nevertheless, several studies have reported the creation of plants resistant to viruses by expressing scFvs targeting structural as well as non-structural viral proteins (Binz & Plückthun, 2005; Prins et al., 1995; Prins et al., 2005; Ziegler & Torrance, 2002). The mechanisms of protein mediated resistance and of alternative methodologies are out of the scope of this review and will not be discussed further.

3. RNA-silencing based transgenic resistance against plant viruses

RNA silencing constitutes a vital element of the innate antiviral 'immune' response in plants. It uses cytoplasm-associated small interfering RNAs (siRNAs) to specifically target

and inactivate invading nucleic acids. Besides siRNAs, a vast population of small RNAs (sRNAs) accumulates in plant tissues, which includes microRNAs (miRNAs), *trans*-acting siRNAs (ta-siRNAs), heterochromatin-associated siRNAs (also referred to as *cis*-acting siRNAs that are linked to transcriptional gene silencing) and natural antisense transcript siRNAs. These sRNAs through RNA silencing mediate repressive gene regulation and play important role in developmental control, preservation of genome integrity and plant responses to adverse environmental conditions, including biotic stress (Brodersen & Voinnet, 2006; Chapman & Carrington, 2007; Pasquinelli et al., 2005; Ruiz-Ferrer & Voinnet, 2009; Vaucheret, 2006). To date, it has primarily been the cytoplasmic siRNA silencing pathway (also referred to as post transcriptional gene silencing, PTGS) that has been exploited by genetic engineering to confer resistance to plant viruses (Mlotshwa et al., 2008; Tenllado et al., 2004).

RNA silencing, is activated as a response to double-stranded RNA (dsRNA). Viruses, as well as transgenes, arranged as inverted repeats (IR), can directly produce dsRNA (which at a subsequent stage will give rise to primary siRNAs), whereas highly transcribed, sense orientated, single copy transgenes produce aberrant transcripts that serve as a substrate for producing dsRNA (subsequently processed to secondary siRNAs). In the latter case dsRNA is synthesized by one member of a family of cellular RNA-depended RNA polymerases (RdRPs) which counts six members in *Arabidopsis* (RDR1-6). Subsequently, the dsRNA can be targeted by a member of a group of Dicer-like ribonucleases (DCL1-4 in *Arabidopsis*) with each of them being involved in specific sRNA pathway(s) and generating specific size of sRNA duplexes (18-25nt in length). All four *Arabidopsis* DCL enzymes appear to be involved – directly or indirectly – in the production of siRNAs from DNA plant viruses, whereas the activities of DCL-4 and DCL-2 are mainly related to the production of siRNAs from single stranded RNA (ssRNA) viruses (Blevins et al., 2006; Ruiz-Ferrer & Voinnet, 2009; Vaucheret, 2006, and references therein). dsRNA cleavage is facilitated by another group of dsRNA-binding proteins (HYPONASTIC 1or HYL 1 and DRB2-5 in *Arabidopsis*). Then, siRNAs are stabilized by 2'O-methylation in their overhanging 3'ends and exported to cytoplasm for PTGS. One selected sRNA strand together with one member of the ARGONAUTE (AGO) family of proteins form the core of a nuclease complex (RNA induced silencing complex, RISC) that targets and cleaves sequence-specifically homologous ssRNA (Ronemus et al., 2006; Ruiz-Ferrer & Voinnet, 2009). The AGO family in *Arabidopsis* is predicted to contain ten members and for some of them a RNA slicer activity has been verified (Brodersen & Voinnet, 2006; Chapman & Carrington, 2007, and references therein). Many excellent reviews cover the functions of sRNAs and their role in RNA-silencing pathways in plants in great detail (Brodersen & Voinnet, 2006; Chapman & Carrington, 2007; Pasquinelli et al., 2005; Ruiz-Ferrer & Voinnet, 2009; Vaucheret, 2006; Mlotshwa et al., 2008).

RNA silencing impedes viral multiplication in plants by two major ways. First it degrades the dsRNA intermediates of virus replication themselves as well as the cognate mRNAs (referred to as cell-autonomous silencing), a procedure that leads to the increase of accumulation of the respective siRNAs. Second, it generates a mobile signal that triggers the degradation of homologous mRNAs in distant cells (systemic silencing). This systemic branch of antiviral RNA silencing is related to siRNA population or their dsRNA precursors that move between neighboring cells through plasmodesmata and over long distances through the phloem (Kalantidis et al., 2008).

RNA-silencing based resistance against viruses was first reported by Lindbo et al. (1993) and was shown to be related to the previously observed co-suppression mechanism (Napoli et al., 1990; Van der Krol et al., 1990). The following years, engineering of transgenic plants to harbor single-stranded sense and to a less extend antisense viral sequences became a common strategy to pre-activate the silencing machinery and obtain resistance against the homologous virus from which the introduced sequence has derived (Ritzenthaler, 2005). Further exploiting this knowledge led to constructing IR transgenes from which long double-stranded (ds) RNA precursors of siRNAs were directly generated. The utilization of such IR transgene constructs has become the method of choice for providing genetically engineered resistance to viruses because a single copy is sufficient to provide immunity, there is no expression of viral proteins, short genome incomplete sequences can be used and efficiencies of up to 90% of all transgenic plants produced to be resistant to the homologous virus were achieved (Lin et al., 2007; Tenllado et al., 2004; Ritzenthaler, 2005). In contrast to the situation with RNA viruses, the use of RNA silencing against DNA viruses most often resulted in delays in symptom development and did not always prevent virus replication (Lin et al., 2007). However, immune lines against *Tomato yellow leaf curl virus* (TYLCV) have been reported by Yang and co-workers (2004), and Fuentes and associates (2006).

In order to overcome the weakness of RNA-silencing based resistance [ineffective against viruses whose sequence differs from that of the transgene by more than 10% (Bau et al., 2003; Jones et al., 1998)], Bucher et al. (2006) fused 150-nt fragments of viral sequences of four tospoviruses in a single small chimeric IR construct. This strategy resulted in a high frequency of produced resistant plants. A most recent approach used modified plant miRNA cistrons to produce a range of antiviral artificial miRNAs (amiRNAs) (Niu et al., 2006; Qu et al., 2007; Schwab et al., 2006; Simon-Mateo & Antonio Garcıa, 2007; Zhang et al, 2011).

4. Factors that influence the RNA-silencing based transgenic resistance

4.1 Heterologous viruses

Since 1998 where the first viral suppressor of silencing was discovered it has been established that most known virus species carry at least one RNA silencing suppressor (Dıaz-Pendon & Ding, 2008; Ding & Voinnet, 2007). The awareness of this viral counter-defensive strategy against the innate antiviral defense system of plants guided several groups to investigate the effect that could invoke on transgenic resistance of plants that were immune to a virus, the infection with a different virus carrying a strong silencing suppressor.

The first studies were presented in 2001 by Savenkov and Valkonen, and Mitter and co-workers. Savenkov and Valkonen produced transgenic tobacco plants resistant to *Potato virus A* (PVA, genus *Potyvirus*) and examined whether the resistance to PVA was affected by infection of the transgenic plants with *Potato virus Y* (PVY), another potyvirus that was known to suppress RNA silencing through its HC-Pro protein (Dıaz-Pendon & Ding, 2008; Ding & Voinnet, 2007). The PVY infection resulted in increased steady-state levels of the transgene mRNA in the transgenic plants. PVA challenge was followed 15 days after inoculation with PVY. In contrast to healthy (non-PVY inoculated) transgenic plants, in

which no detectable infection with PVA was observed following challenge with PVA, all the PVY-infected transgenic plants were readily systemically infected by PVA. Moreover, in all PVA-infected plants, new leaves continued to display the severe symptoms, indicating no recovery from disease up to 90 days post inoculation. It was concluded that RNA-silencing mediated resistance in transgenic plants against viruses may be suppressed by infection of the plants with heterologous viruses that encode suppressors of gene silencing (Savenkov & Valkonen, 2001). Not equally definite was the outcome from the studies of Mitter et al. (2001; 2003) which showed that in transgenic tobacco plants, infection with *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) expressing the silencing suppressor 2b protein could transiently suppress the silencing mediated immunity to PVY but solely in new leaves that emerged after CMV inoculation and for a limited period of time. The experiments were carried out for six months and different time intervals were examined between the two virus inoculations. It was shown that longer periods of time between CMV inoculation and challenge of transgenic plants with PVY led to a larger proportion of PVY-susceptible plants. Nevertheless, in these plants the relative PVY titers tended to be lower as compared with untransformed control plants and the movement of PVY in the transgenic plants was restricted relatively to the controls. Most importantly, CMV infection supported only a transient PVY infection and did not prevent recovery of the transgenic plants. Moreover, re-inoculation with PVY of the recovered plants or of plants that had been infected with CMV nine weeks earlier, failed to establish a PVY infection. Finally, although CMV infection resulted in increased transgene-derived mRNA levels in the leaves where breakdown of immunity had been recorded, the transgene-specific siRNAs levels were left unaffected.

Simon-Meteo et al. (2003) performed similar experiments on *Nicotiana benthamiana* plants that displayed RNA-silencing based resistance and were regenerated from recovered tissue of plants which showed a delayed resistance to *Plum pox virus* (PPV, genus *Potyvirus*). They used two heterologous viruses with distinct silencing suppressors, CMV and *Tobacco vein mottling virus* (TVMV, genus *Potyvirus* carrying an HC-Pro silencing suppressor). Each heterologous virus and PPV were inoculated either simultaneously or sequentially with an interval of two to four weeks onto transgenic plants. Both viruses, when applied sequentially, were able to reactivate transgene expression, but surprisingly, only the silencing suppression caused by CMV and not that originating from TVMV, was able to revert the transgenic resistant plants to a PPV-susceptible phenotype.

Taking into consideration these first studies several of the numerous succeeding reports (Fuentes et al, 2006; Germundsson & Valkonen, 2006; Praveen et al, 2010; Kawazu et al, 2009; Yang et al, 2004) of engineered transgenic resistance to plant viruses have examined the possible effect of heterologous virus infection in the resistance. However, not always an influence on resistance was observed. Missiou et al. (2004) in transgenic potato plants resistant to PVY examined the effect on the resistance of *Potato virus X* (PVX, genus *Potexvirus*, carrying the P25 silencing suppressor) infection simultaneously with PVY or one week prior to the challenge with PVY. In either of the two variations, infections with PVX occurred without a PVY infection to be detected. Similarly, resistance of transgenic cucumbers incorporating the 54K polymerase domain of *Cucumber fruit mottle mosaic virus* (CFMMV) was not influenced by infection with the potyviruses *Zucchini green mottle mosaic virus* (ZYMV), *Zucchini fleck mosaic virus* (ZFMV), the ipomovirus *Cucumber vein yellowing*

virus (CVYV) or CMV (Gal-On et al., 2005). In a different work, Lennefors et al. (2007) tested whether the high levels of RNA silencing-based resistance to *Beet necrotic yellow vein virus* (BNYVV) in transgenic sugar beet roots could be reduced by co-infection with common soil-borne and aphid-borne beet viruses. The plants were first inoculated with the aphid transmitted *Beet mild yellowing virus* (BMYV), *Beet yellows virus* (BYV), or both viruses. Four weeks later, the plants were transplanted to soil infested with BNYVV, *Beet soil borne virus* (BSBV) and *Beet virus Q* (BVQ) and their fungal vector, *Polymyxa betae*. The effectiveness of the resistance was not detectably compromised even following co-infection with all five viruses. Most recently, transgenic tobacco plants were produced, transformed with an IR construct corresponding to sequences of the TMV movement protein gene and the exhibited resistance to TMV was not affected by infection with CMV regardless of the order that the latter was inoculated (prior to or simultaneously with TMV) (Hu et al., 2011). In a different approach, amiRNAs expressed in tomato plants against CMV coding sequences resulted in resistance against the virus which was not noticeably affected by infection with TMV or TYLCV (Zhang et al., 2011). Moreover, the stability of transgenic resistance of tobacco plants against *Tobacco rattle virus* (TRV) (Vassilakos et al., 2008) remained largely unaffected by infection with CMV, PVY or *Tomato spotted wilt virus* (TSWV) (Vassilakos, unpublished results).

In contrast, in *N. benthamiana* plants expressing a *Grapevine virus A* (GVA) minireplicon and displaying high resistance to GVA, infection with *Grapevine virus B* (GVB, genus *Vitivirus*, carrying a P10 silencing suppressor) or PVY resulted in suppression of the GVA-specific defense (Brumin et al., 2009). Interestingly, in these tests GVA and GVB or PVY inocula were applied simultaneously as a mixture of saps derived from plants infected with the respective viruses, unlike previous studies, in which only sequential inoculations with the heterologous viruses resulted in reduced resistance. Finally, sweetpotato transgenic plants transformed with an IR construct targeting the replicase encoding sequences of *Sweetpotato chlorotic stunt virus* (SPCSV, genus *Crinivirus*) and *Sweetpotato feathery mottle virus* (SPFMV, genus *Potyvirus*) exhibited mild or no symptoms and virus accumulation was significantly reduced following SPCSV infection. However, development of severe sweetpotato virus disease symptoms (attributed to infection by both viruses) occurred in transgenic plants infected with a SPFMV isolate with a limited sequence similarity to the sequence used in the transgene (Kreuze et al., 2008).

The results from the studies that examined the effect of heterologous virus infection on the silencing-based transgenic resistance indicated that this kind of resistance, despite the immunity that can confer to the plants against a specific virus, could be compromised to some degree if applied in the field where mixed virus infections occur frequently. However, it became evident that the outcome of the interference between the heterologous viruses and the silencing machinery of the plant is not so easily predictable (Table 1).

The reasons for the discrepancies are unclear, but could be related to the mode of action of the viral suppression proteins of the different virus tested. Viral silencing suppressors are highly diverse in sequence, structure and activity, and could target multiple points in RNA silencing pathways whereas viruses with large genomes may encode several functionally distinct proteins to achieve silencing suppression (Dıaz-Pendon & Ding, 2008; Ding &

Voinnet, 2007). It is considered that suppressor proteins interfere either with siRNAs biogenesis or siRNA function without a multifunctional nature to be excluded. For instance, most studies agree that the potyviral HC-Pro probably specifically blocks accumulation of secondary siRNAs and leaves primary siRNA accumulation unimpaired, whereas P25 blocks accumulation of primary siRNAs (Dıaz-Pendon & Ding, 2008). In contrast, the 2b protein of cucumoviruses directly sequestrate siRNAs duplexes using a pair of hook-like structures that interact more promiscuously with long and short dsRNA (Dıaz-Pendon & Ding, 2008; Ding & Voinnet, 2007; Ruiz-Ferrer & Voinnet, 2009). Additionally, it binds AGO1 and blocks slicing without interfering with sRNA loading *in vitro*. Although apparently contradictory, these two anti-silencing 2b activities are reconcilable, because 2b's affinity for dsRNA is weak and its interaction with AGO1 could increase 2b local concentrations and enhance specific binding to siRNAs (Ruiz-Ferrer & Voinnet, 2009). Besides, Buchmann et al. (2009) reported that geminivirus AL2 and L2 proteins act as inhibitors of transcriptional gene silencing, which is the branch of silencing that targets DNA viruses.

Additional antiviral plant defense pathways could also be involved in the interference between the heterologous virus infection and the transgenic resistance or as yet unknown factors involved in specific virus species interactions. Thus, the CMV 2b protein has been shown also to block silencing indirectly by interfering with the salicylic acid mediated defense pathway (Li & Ding, 2001). Moreover, *N. benthamiana* plants transformed with an IR construct containing partial *N* gene sequences from five tospoviruses [TSWV, *Groundnut ring spot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV), *Watermelon silver mottle virus* (WSMoV) *Tomato yellow ring virus* (TYRV-t)] displayed resistance against all five viruses. However, co-infection of one of the tospoviruses with a genetically distant strain of the same species (TYRV-s), resulted in specific intraspecies breakdown of resistance through a procedure that involved complementation of the silencing suppressors of the two viruses (Hassani-Mehraban et al., 2009) (Table 1).

4.2 Temperature

It has been well known to plant virologists that temperature strongly influences plant-virus interactions. In high temperature, symptoms are frequently attenuated and virus titers in infected plants are decreased. In contrast, outbreaks of virus diseases are frequently associated with low temperatures (Hull, 2002).

Kalantidis and co-workers (2002) examined the influence of elevated temperature on siRNAs in CMV-resistant transgenic tobacco plants. Two transgenic lines, one expressing very high and the other very low levels of siRNAs, were tested for siRNAs concentration at 25°C and 32°C and at two time points, 20 and 30 days post-germination. At the early time point, transgene derived siRNAs could be detected only in the first line at 25°C and in both lines at 32°C. However, in the first line transgene specific siRNAs were at 32°C in a significantly higher concentration compared to that of 25°C. The analysis of samples taken at the second time point revealed the presence of transgene derived siRNAs in both lines at 25°C. However, at 32°C, siRNAs were detected in both plant lines at a higher concentration. Apparently, in these experiments, except for temperature the developmental stage of the plants also influenced the siRNA concentration (discussed further below).

Factor		Transgenic Plant	Engineered resistance against	Effect on the resistance	Reference
Heterologous viruses	PVY	*N. tabacum*	PVA	Suppressed	Savenkov & Valkonen, 2001
	CMV	*N. tabacum*	PVY	Reduced	Mitter et al., 2001; 2003
	CMV TVMV	*N. benthamiana*	PPV	Suppressed Unaltered	Simón-Mateo et al., 2003
	PVX	Potato	PVY	Unaltered	Missiou et al., 2004
	ZYMV ZFMV CVYV CMV	Cucumber	CFMMV	Unaltered	Gal-On et al., 2005
	BMYV BYV BSBV BVQ	Sugar beet	BNYVV	Unaltered	Lennefors et al., 2007
	SPFMV-C	Sweetpotato	SPCSV SPFMV-Uganda	Suppressed	Kreuze et al., 2008
	GVB, PVY	*N. benthamiana*	GVA	Suppressed	Brumin et al., 2009
	TYRV-s	*N. benthamiana*	TSWV GRSV TCSV WSMoV TYRV-t	Suppressed	Hassani-Mehraban et al., 2009
	CMV	*N. tabacum*	TMV	Unaltered	Hu et al, 2011
	TMV TYLCV	Tomato	CMV	Unaltered	Zhang et al, 2011
	CMV, PVY TSWV	*N. tabacum*	TRV	Unaltered	Vassilakos (unpublished)
Temperature	32^{0}C	*N. tabacum*	CMV	n/t	Kalantidis et al., 2002
	15^{0}C	*N. benthamiana*	CymRSV	Suppressed	Szittya et al., 2003
		N. tabacum	TMV CMV	Unaltered	Hu et al, 2011
		N. tabacum	TRV	Suppressed locally	Vassilakos (unpublished)
Light	High/Low Intensity	*N. benthamiana*	PPV	n/t	Kotakis et al., 2010
Early developmental stage		*N. benthamiana*	PMMoV	Reduced	Tenllado & Dıaz-Ruız, 1999
		Squash	SqMV	Suppressed	Jan at al., 2000
		Papaya	PRSV	Suppressed	Tennant at al., 2001
		N. tabacum	CMV	n/t	Kalantidis et al., 2002
		N. tabacum	TRV	Reduced	Vassilakos et al., 2008

Table 1. Synopsis of the studies described in the text that involved experiments with transgenic plants resistant to viruses and the influence to the resistance of the various factors examined; n/t, not tested.

Szittya and associates (2003) provided further insight into the mechanism that is involved in these observations. Through a set of delicate experiments they demonstrated that RNA silencing induced by viruses or transgenes is inhibited at low temperatures and enhanced with rising temperatures. They used wild type *Cymbidium ringspot virus* (CymRSV) encoding a p19 viral suppressor and a mutated one unable to express p19 (Cym19stop). In virus transfected *N. benthamiana* protoplasts, virus derived siRNA were undetectable at 15°C and gradually increased with temperature from 21 to 27°C indicating that virus-induced cell-autonomous silencing is temperature dependent. The effect of temperature on virus-induced systemic RNA silencing was also examined. *N.benthamiana* plants were inoculated with CymRSV and Cym19stop and grown at different temperatures. CymRSV infected plants died within 2 weeks at 15, 21 and 24°C whereas CymRSV symptoms were attenuated at 27°C and associated with reduced virus level. Confirming the role of p19 as a suppressor of systemic silencing, plants infected with the Cym19stop showed a recovery phenotype at 21 and 24°C. At 27°C, the mutant virus was unable to infect the plants, while at 15°C, Cym19stop-infected plants displayed strong viral symptoms demonstrating that at low temperature, RNA silencing failed to protect the plants even when the virus lacked the silencing suppressor. In addition, using a strain of *Agrobacterium tumefaciens* carrying a green fluorescent protein (GFP) gene construct which was infiltrated sole or together with p19, to wt *N.benthamiana* or *N.benthamiana* plants expressing GFP, it was shown that transgene-induced silencing is also temperature dependent. The stability of RNA silencing mediated transgenic virus resistance at different temperatures was examined using transgenic *N.benthamiana* plants expressing a CymRSV-derived RNA. After inoculation with CymRSV the plants displayed strong resistance at 24°C whereas at 15°C, severe symptoms were developed and CymRSV RNA accumulated to a high level demonstrating that the transgene-mediated virus resistance was broken at low temperature. A temperature effect was also observed on the antisense-mediated endogen gene inactivation of *Arabidopsis* and potato plants, in which antisense inhibition of genes involved in carbohydrate metabolism is broadly used. Interestingly, in contrast to siRNA production, miR157, miR169 and miR171 RNAs accumulated to equal levels at 15, 21 and 24°C in arabidopsis suggesting that accumulation of miRNAs is not affected by temperature.

Chellappan and co-workers (2005) expanding the above findings quantified gemini virus-derived siRNAs at different temperatures and evaluated their distribution along the virus genome for isolates of five species of cassava geminiviruses, consisting of recovery and non-recovery types. In cassava plants, geminivirus-induced RNA silencing increased by raising the temperature from 25°C to 30°C and the appearance of symptoms in newly developed leaves was reduced, irrespectively of the nature of the virus. Consequently, high temperature rendered non-recovery type geminiviruses to recovery-type viruses. The distribution of virus derived siRNAs on the respective virus genome at three temperatures (25°C, 25°C-30°C and 30°C) remained unaltered only for recovery-type viruses. siRNAs derived from recovery-type viruses accumulated at moderately higher levels during virus-induced silencing at higher temperatures. However, siRNAs from non-recovery-type viruses accumulated six times higher than those observed for infections with recovery-type viruses at high temperature. Thus, the decreased symptom severity and virus concentration that were recorded at higher temperature indicate a similar effect of temperature on ssDNA and RNA viruses although there was a differential effect of temperature on the level of virus-derived siRNAs between recovery and non-recovery types of ssDNA viruses.

As with the effect of heterologous viruses, inhibition of RNA silencing or decreasing of siRNAs concentration in low temperature has not always been observed. Thus, transgene anti-sense induced RNA silencing was not inhibited in potato plants at low temperature (Sos-Hegedus et al., 2005). Moreover, tomato plants carrying an IR construct derived from *Potato spindle tuber viroid* (PSTVd) sequences and exhibiting resistance to PSTVd infection, did not show an elevated IR-siRNA accumulation at 31°C in comparison to 21°C (Schwind et al., 2009). In a more recent study, transgenic tobacco plants transformed separately with IR constructs corresponding to sequences of TMV movement protein gene or CMV replication protein gene, exhibited at both 15°C and 24°C similar high levels of resistance to TMV or CMV, respectively (Hu et al, 2011). In addition, the resistance against TRV of transgenic tobacco plants (Vassilakos et al., 2008) grown at 15°C was influenced only in the inoculated leaves but not systemically (Vassilakos, unpublished results).

In summary (Table 1), the well-known temperature effect on the development of viral diseases is closely associated to the RNA silencing antiviral pathway and consequently influences the efficiency of silencing-based transgenic resistance. However, it appears that the low temperature effect on the transgenic resistance depends on additional factors that remain to be identified, fact supported by inconsistencies in the results of the diverse studies described here. Importantly, although at low temperature the siRNA-based silencing machinery is partially inactivated as an adaptive response of plants to adverse conditions, the miRNA-mediated, which is essential for regulatory functions, continues to operate ensuring plant growth (Szittya et al., 2003).

4.3 Light

Studies on the effect of light on transgenic resistance to viruses are not available, however light has been implicated as one of the factors that affect RNA silencing initiation and maintenance in several studies. Although in most of them light effect on silencing was not clearly isolated from that of temperature (Nethra et al., 2006; Vaucheret et al., 1997) recently, Kotakis et al. (2010) investigated solely the role of light intensity in physiological ranges on RNA silencing. They used as a system *N. benthamiana* transgenic lines engineered to express GFP, which exhibited spontaneously silencing at different frequencies and of different spreading intensities. The authors demonstrated that high light intensity increased the frequency of plants displaying both short range and systemic silencing. In contrast, plants grown under low light conditions, showed lower silencing frequencies. In addition, increased light intensity positively affected siRNA levels corresponding to the GFP transgene (sense) transcript. In a different set of experiments, *N. benthamiana* plants were used, incorporating an IR structure derived from the NIb gene of *Plum pox virus* (PPV) and it was shown that levels of all distinguishable siRNA classes corresponding to the IR transcript were also positively affected by high light intensity (Table 1). Although in the latter case, the effect of light intensity on virus resistance was not tested, the authors proposed that light conditions comprise an additional environmental factor that should be taken under consideration when transgenic technology against viral infections applies on the field.

4.4 Plant developmental stage

Quite a few studies with plants carrying sense transgenes and displaying RNA-silencing mediated resistance have suggested an influence of plant developmental stage on the degree

of the expressed resistance. Tenllado and Diaz-Ruiz (1999) reported that a higher percentage of transgenic *N. benthamiana* plants, transformed with the 54K read-through domain of the replicase gene of *Pepper mild mottle virus* (PMMoV), displayed complete virus resistance at maturity than at an earlier stage of development. Subsequently, Jan et al (2000) demonstrated that a recovery type of resistance, in squash genetically transformed with the coat protein genes of *Squash mosaic virus* (SqMV), was due to RNA silencing that was activated at a later developmental stage, independently of virus infection. However, a different phenotype of complete resistance was not altered after SqMV inoculation at early developmental stages. Moreover, analysis of crosses between lines exhibiting complete resistance, recovery and susceptible phenotypes revealed that the time of activation of silencing, besides the developmental stage, is affected by the interaction of transgene inserts. Similarly, transgenic papaya plants were susceptible to *Papaya ringspot virus* (PRSV) at a younger stage but resistant when inoculated at an older stage (Tennant et al., 2001).

As mentioned already, Kalantidis and associates (2002) showed that siRNA accumulation in transgenic tobacco, incorporating an IR construct carrying CMV sequences, was higher at later developmental stages. No significant differences in the siRNA concentration were observed between leaves of different age from a single plant or from the seven-leaf stage on, while the siRNA concentration reached a plateau that remained stable in the course of further development.

In a more recent work, *N. tabacum* plants were transformed with the 57-kDa read-through domain of the replicase gene of TRV and were highly resistant to homologous (to the transgene sequence) TRV isolates and moderately resistant to the genetically distinct TRV-GR. Very young transgenic plants with detectable levels of transgene transcript were resistant only systemically to homologous isolates and were susceptible to TRV-GR. Conversely, older plants (at a five-leaf stage) containing a low steady state level of transcripts were immune to homologous isolates and displayed moderate resistance against TRV-GR (Vassilakos et al., 2008).

In conclusion (Table 1), most studies agree that younger transgenic plants accumulate reduced amounts of transgene specific siRNAs compared to older ones, or correspondingly accumulate higher amount of transgene specific transcripts suggesting a reduced efficiency of transgenic resistance against plant viruses. However, the resistance phenotype was not always affected in younger plants, possibly due to reasons associated with the type of the transgene construct used, its integration into the plant genome or the viral sequences that are targeted.

5. Conclusion

A great deal of progress has been made towards comprehension of plant virus biology and the ways in which plants defend themselves against these pathogens. RNA silencing has provided a promising potential for generating virus-resistant transgenic plants and this potential is certainly not cancelled by the awareness of factors that may affect under specific conditions the acquired resistance. However, as with any other pathogen control strategy, RNA silencing does not constitute a panacea and a number of issues should be taken into consideration before being applied in the field. Noticeably, silencing based transgenic

resistance is not influenced solely by the factors that were presented in this review. However, planting into areas where endemic virus diseases occur and mixed virus infections are expected especially during early stages of the vegetation period, time intervals of low air temperature and greenhouse or open field cultivation practices could affect the stability of transgenic resistance against plant viruses.

Further exploitation of our knowledge on RNA-silencing pathways is essential to improve the efficiency of the existing strategies or for the development of potential new strategies which will hopefully lead to a better reception by the public. Recent advances like the construction of chimeric IR constructs incorporating sequences derived from different virus species if combined with epidemiological data and pest risk analyses could reduce the effect of mixed virus infections on the resistance (Bucher et al., 2006; Dafny-Yelin & Tzfira, 2007; Kung et al., 2009). Recently, virus resistance was achieved through expression of amiRNAS against viral coding sequences (Ding & Voinnet, 2007; Duan et al., 2008; Niu et al., 2006; Qu et al., 2007; Simon-Mateo & Antonio Garcia, 2006; Zhang et al, 2011). Although there was evidence that amiRNA-mediated virus resistance may not be inhibited by low temperature (Niu et al., 2006) this possibly depends on the plant species examined (Qu et al., 2007). Moreover, the durability of this approach, which resulted in relatively few antiviral small RNAs compared with those of the long dsRNA approach, needs to be further demonstrated (Duan et al., 2008; Simon-Mateo & Antonio Garcia, 2006).

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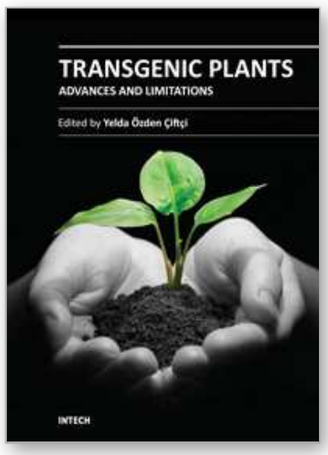

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Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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