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Review

Strategies for antiviral resistance in transgenic plants

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SUMMARY

Genetic engineering offers a means of incorporating new virus resistance traits into existing desirable plant cultivars. The initial attempts to create transgenes conferring virus resistance were based on the pathogen-derived resistance concept. The expression of the viral coat protein gene in transgenic plants was shown to induce protective effects similar to classical cross protection, and was therefore distinguished as “coat-protein-mediated” protection. Since then, a large variety of viral sequences encoding structural and non-structural proteins were shown to confer resistance. Subsequently, non-coding viral RNA was shown to be a potential trigger for virus resistance in transgenic plants, which led to the discovery of a novel innate resistance in plants, RNA silencing. Apart from the majority of pathogen-derived resistance strategies, alternative strategies involving virus-specific antibodies have been successfully applied. In a separate section, efforts to combat viroids in transgenic plants are highlighted. In a final summarizing section, the potential risks involved in the introduction of transgenic crops and the specifics of the approaches used will be discussed.

INTRODUCTION

Since the dawning of the transgenesis era, some 25 years ago, the possibility of generating transgenic plants has been exploited to broaden the options for plant virus resistance. The number of viruses causing problems in plants is large, many viruses are capable of infecting a multitude of host plants, and moreover 'classical' genetic sources of resistance to viruses are scarce and, due to high plasticity of viral genomes, often not very durable in the field. The prospect of generating transgenic plants greatly increased the potential sources of resistance. And despite societal concerns –primarily in Europe- about the use of transgenic plants in agriculture, transgenic approaches have proven to be able to produce durable and safe virus resistance in the field, enabling the production of crops that would otherwise not have been possible (Fuchs and Gonsalves, 2007). Based on the pathogen-derived resistance (PDR) concept first proposed by Sanford and Johnston (1985) a variety of transgenic approaches based on viral genes and sequences was applied to many plant species.

COAT-PROTEIN-MEDIATED RESISTANCE

The archetypical transgene-induced virus resistance experiment involved the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) (Powell-Abel *et al.*, 1986). 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 (Beachy *et al.*, 1990, Lomonossoff, 1995).

Koo *et al.* (2004) noted several lines of evidence supporting the hypothesis that coat - protein-mediated resistance (CPMR) against TMV is a consequence of interaction between the transgenic CP and the CP of the challenging virus: a) transgenic plants expressing CP showed high resistance to challenge by virions, but not to inoculation with RNA or partially stripped virions (Powell-Abel *et al.*, 1986, Register and Beachy 1988); b) transgenic plants expressing TMV CP showed greater levels of CPMR against closely related viruses than to more distantly related viruses (Nejidat and Beachy 1990); c) transgenic plants expressing mutant CPs affecting electrostatic interactions between the subunits showed modified CPMR according to their self-assembly capacity (Bendahmane *et al.*, 1997a). This suggested that CPMR may be mediated by certain configurations of quaternary structures rather than by the subunit *per se*. 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. As an example, resistance to the donor virus mediated by expression of the nucleocapsid (N) gene of *Tomato spotted wilt virus* (TSWV) is commonly described as RNA-mediated (Goldbach *et al.*, 2003; and section below). Low levels of transgene transcripts are detected, resistance is limited to the donor TSWV strain, and lack of resistance to other tospoviruses is observed (Gielen *et al.*, 1991; De Haan *et al.*, 1992; MacKenzie and Ellis, 1992; Pang *et al.*, 1992, 1993, 1994; Prins *et al.*, 1995; Vaira *et*

al., 1995; Stoeva *et al.*, 1998). However, some plant lines expressing high amounts of TSWV N protein were protected against TSWV, as well as *Impatiens necrotic spot virus* (INSV) and partially *Groundnut ringspot virus* (GRSV) (Pang *et al.*, 1992; Vaira *et al.*, 1995). Using these lines it was indeed indicated that over expressing TSWV N protein can induce resistance against TSWV and GRSV by reducing systemic viral spread (Schwach *et al.*, 2004).

REPLICASE-MEDIATED RESISTANCE

Engineering virus resistance by using genes encoding viral RNA-dependent RNA-polymerases (RdRps) was first reported for TMV (Golemboski *et al.*, 1990). A notable inhibition of virus replication at the inoculation site and at the single-cell level in tobacco transformed with a modified RdRp was found. Resistance appeared to be strain-specific, against infection initiated by both TMV virions and RNA. However, although the 54 kDa protein itself was never detected in transgenic tissue, the finding that a mutant encoding only 20% of the protein was ineffective suggested at that time that the protein was indeed responsible for resistance (Carr *et al.*, 1992). In addition, expression of a modified TMV RdRp containing an unintended insertion of a bacterial transposon sequence conferred resistance against TMV and other tobamoviruses (Donson *et al.*, 1993). Differences between the replicase sequences in the transgene and those of the challenging viruses were not compatible with RNA-based mechanisms, favouring a role of the protein itself.

However, the picture of replicase-mediated resistance induced solely by the protein is certainly too simple. The full-length 54 kDa RdRp from another tobamovirus, *Pepper mild mottle virus* appeared dispensable for resistance induction and plants expressing a truncated construct (30% of the protein) were equally resistant. For this, the co-existence of dual protein- and RNA-mediated protection was suggested (Tenllado *et al.*, 1995 and 1996). A later case of TMV replicase-mediated resistance was definitely attributed to RNA-silencing (Marano and Baulcombe, 1998).

More recently, expression of nine distinct overlapping segments covering the full TMV 183 kDa RdRp generated resistance to TMV at low levels, and protein expression was not required. However, a higher protection was conferred by segments covering the polymerase domain of the protein, acting as dominant-negative mutants (Goregaoker *et al.*, 2000). A *par conditio* model was proposed, in which an initial RNA-based mechanism was responsible for low-level protection. This mechanism would be conferred by any sequence derived from the TMV genome, including the CP ORF. A more active protein-mediated resistance is then thought to intervene, possibly in conjunction with RNA-mediated mechanisms.

Replicase genes of other viruses, such as tobra- (MacFarlane and Davies, 1992), potex- (Braun and Hemenway, 1992), poty- (Audy *et al.*, 1994), alfamo- (Brederode *et al.*, 1995), and cucumoviruses (Anderson *et al.*, 1992; Carr *et al.*, 1994; Zaitlin *et al.*, 1994; Hellwald and Palukaitis, 1995; Wintermantel and Zaitlin, 2000), have also been described as resistance sources. For tobnaviruses, a 54 kDa portion of the full 201 kDa replicase of *Pea early browning virus* (PEBV) induced resistance to high doses of PEBV, as well as to *Broad bean yellow band virus*, but not to other tobnaviruses (MacFarlane and Davies, 1992). Since constructs with deletions or substitutions were ineffective, resistance was likely protein-

mediated, but as for TMV-resistant plants, protein has never been detected. Similarly, for potex- and potyviruses, resistance appeared protein-mediated, since again mutants of the 166 kDa protein of *Potato virus X* (PVX) or the NIb of *Potato virus Y* (PVY), carrying deletions or mutations in the conserved GDD domain were ineffective (Audy *et al.*, 1994). A reverse situation was described for alfamoviruses, where only plants expressing high doses of the *Alfalfa mosaic virus* P2 replicase carrying N-terminal deletions or mutations in the GDD motif, were resistant, as opposed to wild-type proteins (Brederode *et al.*, 1995). Resistance to CMV was obtained by engineering sequences from the 2a replicase (Anderson *et al.*, 1992; Carr *et al.*, 1994; Zaitlin *et al.*, 1994). Two different resistance mechanisms were described, one targeting virus replication at the single-cell level (Carr *et al.*, 1994; Hellwald and Palukaitis, 1995), the other limiting systemic (Wintermantel *et al.*, 1997) or cell-to-cell spread (Hellwald and Palukaitis, 1995; Nguyen *et al.*, 1996). Wintermantel and Zaitlin (2000). Later it was demonstrated that the translatability of the 2a replicase, full or C-terminally truncated, was indeed necessary to interfere with CMV infection.

Taken all together, it can be concluded that replicase-derived transgenes are a potent source of resistance, but interpretation of the role of (modified) replicase proteins or their transcripts is not always clear. Perhaps true replicase protein-mediated resistance can add to a basal level of RNA-mediated resistance.

REP PROTEIN-MEDIATED RESISTANCE TO SINGLE-STRANDED DNA VIRUSES

Unlike RNA viruses, the genomes of plant single-stranded DNA viruses do not encode polymerases. Instead, their replication requires interaction between a viral replication-associated protein (Rep) and host polymerases. Geminiviral Rep proteins have been widely exploited to generate resistance. The Rep gene of *African cassava mosaic virus* (ACMV) inhibited virus replication in protoplasts and induced virus resistance in plants, but, although a correlation between transcript level and resistance was reported, protein expression was not analysed (Hong and Stanley, 1996).

A protein-mediated resistance was described with a truncated *Tomato yellow leaf curl Sardinia virus* (TYLCSV) Rep protein (210 aa), that strongly inhibited virus replication in protoplasts and induced resistance when expressed at high levels (Noris *et al.*, 1996; Brunetti *et al.*, 1997). This dominant negative mutant, lacking a conserved NTP-binding domain, acts by inhibiting the expression of the viral Rep protein and by forming dysfunctional complexes with the viral Rep protein (Lucioli *et al.*, 2003). However, the resistance was in some cases unstable due to transgene silencing (Lucioli *et al.*, 2003). A similar construct, having a further deletion at the C-terminus and encoding the first 129 aa of the protein, induced resistance in a strictly sequence-specific manner in the closely related *Tomato yellow leaf curl virus* (TYLCV) (Antignus *et al.*, 2004) as well as in TYLCSV (Noris and Tavazza, *unpublished*).

Similar strategies with Rep proteins mutated in the ori- or NTP-binding sites were applied to generate plants resistant to other geminiviruses, such as *Bean golden mosaic virus* (BGMV) (Hanson and Maxwell, 1999) or ACMV (Sangaré *et al.*, 1999).

MOVEMENT-PROTEIN-MEDIATED RESISTANCE

Compared to CP- or replicase-mediated resistance strategies, the expression of dysfunctional or mutant movement proteins (MP) has been reported to confer broader resistance. Plants transgenic for the MP of TMV (p30), lacking three N-terminal amino acids (Lapidot *et al.*, 1993) or a temperature-sensitive version (Malysenko *et al.*, 1993) showed a delay in both symptom appearance and infection. The dysfunctional MP (dMP) was thought to act as a dominant negative mutant, interfering with local and systemic movement of the challenging virus. Resistance was also effective against taxonomically distant viruses, since a TMV dMP transgene interfered with systemic spread of the tobavirus *Tobacco rattle virus*, the caulimovirus *Peanut chlorotic streak virus* and the nepovirus *Tobacco ringspot virus*, but not the cucumovirus CMV. Little or no effect was observed in the inoculated leaves, indicating that replication and cell-to-cell movement were not impaired (Cooper *et al.*, 1995). Expression of wild-type MP, on the contrary, generally enhanced virus infection.

In contrast to tobamoviruses, which rely on a single protein for their movement, movement of potex- carla-, hordei- and some furoviruses is mediated by three overlapping ORFs composing the triple gene block (TGB). Expression of a 13 kDa protein of the potexvirus *White clover mosaic virus* disrupted in a highly conserved domain rendered plants resistant to the homologous virus, as well as to other TGB-containing viruses, but not to TMV (Beck *et al.*, 1994). These findings were substantiated by using mutant forms of the 12 kDa protein of the TGB of PVX, which gave resistance to PVX and other TGB viruses, but were inactive against PVY. Both cell-to-cell and systemic virus spread appeared to be blocked, in spite of the relatively low sequence similarity (30-50%) with the PVX TGB transgene (Seppanen *et al.*, 1997).

The possibility to simultaneously obtain engineered resistance to viruses having a single MP or a TGB was pursued by Ares and co-workers (1998) by engineering both the p24 protein of PVX and the p30 protein of TMV. In spite of the lack of similarity between the two proteins, and of the different genome organization of the potex- and tobamoviruses, systemic resistance was indeed obtained in reciprocal challenges. The postulated mechanism relies on the existence of common functional domains shared by the two proteins, and is supported by the possibility to complement movement-defective PVX and TMV with heterologous MPs. These domains are thought to compete for common cellular factors required for movement and interfere with the movement of the challenging virus. Alternatively, a non-specific host defence response may be activated. In this case however, over-expression of MP should lead to a broader range of resistance, which has so far not been described.

RNA-MEDIATED RESISTANCE

RNA-mediated resistance against RNA viruses

As a spin-off of initially unexplained effects observed in protein-mediated resistance approaches, the role of RNA transcripts of viral transgenes made a major contribution to the discovery of an entirely new field in biology involving sequence-specific RNA breakdown. This post-transcriptional gene silencing (PTGS) process, occurring in plants but also in other eukaryotes, is also known as RNA interference (RNAi) or RNA silencing (Voinnet, 2005; Ding and Voinnet, 2007).

As mentioned above, expression levels of transgene-encoded viral proteins often did not correlate with the level of virus resistance. Indeed, plants expressing the lowest or even

undetectable levels of protein often displayed the highest resistance. Subsequent expression of untranslatable transgenes formally demonstrated the involvement of transgenic RNA in resistance. However, also in this case, the expression levels of transgenic RNA seemed inversely proportional to the resistance. Lindbo and coworkers (1993) were the first to connect this observation to the previously observed co-suppression phenomenon (Van der Krol *et al.*, 1990; Napoli *et al.*, 1990) involving post-transcriptional down-regulation of endogenous genes by transgenes of identical sequence. The model of Lindbo *et al.* (1993) is based on the idea that sequence-specific RNA degradation induced by transgenes will be targeting of all RNAs with sequence identity with the transgene RNA. In the case of viral transgenes, this process results in virus resistance. This model has been confirmed and expanded. The basis of sequence-specific recognition was found to be determined by the generation of small interfering RNA (siRNA) molecules derived from the transgene (Hamilton and Baulcombe, 1999). As these siRNA molecules were also observed in wild-type plants infected with viruses and viroids, it was concluded that the RNA-mediated transgenic approach pre-programmed an existing antiviral defence in plants (Baulcombe, 1996). Further exploiting this knowledge led to constructing inverted repeat (IR) transgenes from which long double-stranded RNA precursors of siRNAs were generated. Utilization of such IR transgene constructs yielded a marked increase in the efficiency of this approach. Where single-stranded sense or anti-sense approaches yielded resistance frequencies of in 5-20% of the transgenic plants, IR transgenes that produce dsRNAs proved to yield up to 90% of all transgenic plants resistant to the homologous virus (Waterhouse *et al.*, 1998; Smith *et al.*, 2000; Waterhouse and Helliwell, 2003). It is thought that this is due to the dsRNAs being fed into a later step in the silencing pathway, since the dsRNA itself is a substrate for the RNaseIII-like enzyme Dicer, without requiring the activity of plant-encoded RdRps to produce dsRNAs. Using IRs, it proved possible to produce effective RNA-mediated resistance to a wide range of RNA viruses, even ones for which sense-RNA-mediated silencing was not effective (Chen *et al.*, 2004; Kalantidis *et al.*, 2004; Nomura *et al.*, 2004; Hilly *et al.*, 2005). Thus, the siRNAs generated in transgenic plants charge RNA-induced silencing complexes (RISC) with sequence-specific antiviral recognition prior to infection. Upon inoculation with a limited number of viral RNA molecules these can be rapidly and effectively targeted and degraded, even before virus-encoded RNA silencing-suppressor proteins are produced to interfere. This contrasts normal infections, where the lag in induction of the RNA silencing response provides viruses the time to mount their suppressor-based counter defence.

One of the drawbacks of RNA-mediated resistance is that it is ineffective against viruses whose sequence differs from that of the transgene by more than 10% (De Haan *et al.*, 1992). In order to create broader resistance, Bucher *et al.* (2006) therefore 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 multiply resistant plants. It is envisaged that by extending the transgene construct with additional viral sequences, resistance could be broadened even further. In addition, since this multiple virus resistance was observed in a high proportion of transgenic lines, this approach can also be applied to plant species for which large numbers of transgenic lines are difficult to obtain.

The most recent addition to the palette of options was provided by the Chua lab. Virus resistance was produced by modifying plant microRNA (miRNA) cistrons to produce a range of antiviral artificial miRNAs (Niu *et al.*, 2006; Qu *et al.*, 2007). The durability of this novel approach, producing relatively few antiviral small RNAs compared to the long dsRNA approach, needs to be demonstrated (Garcia and Simón-Mateo, 2006)

RNA-mediated resistance against DNA viruses

As mentioned above, plant cells infected with RNA viruses produce virus-specific siRNAs, which are thought to originate from the breakdown of dsRNA replicative forms or from secondary structures of the viral RNA (Hamilton and Baulcombe, 1999; Molnar *et al.*, 2005). Interestingly, plant pararetro- and DNA viruses like caulimoviruses and geminiviruses are also targets of RNA silencing (Al-Khaff *et al.*, 1998; Lucioli *et al.*, 2003; Chellapan *et al.*, 2004a and 2004b). In some cases, this response can lead to recovery of the plants from virus symptoms (Covey *et al.*, 1997; Al-Khaff *et al.*, 1998; Chellapan *et al.*, 2005), suggesting that RNA silencing is a remarkably broad natural defense mechanism that protects plants from viral invasion (Covey *et al.*, 1997; Ratcliff *et al.*, 1997).

Biotechnological approaches expressing sense and anti-sense RNA in transgenic plants have been employed successfully against *Tomato golden mosaic virus* (TGMV) (Day *et al.*, 1991), TYLCSV (Bendhamane and Gronenborn, 1997) and TYLCV (Yang *et al.*, 2004), confirming the suggestion that RNA silencing can be harnessed for antiviral defence (Lapidot and Friedman, 2002). In attempts to further improve transgenic resistance, Pooggin *et al.* (2003) obtained recovery from virus infection in a transient assay using IR constructs containing the common region of the begomovirus *Vigna mungo yellow mosaic virus* (VMYMV). Gafni and colleagues obtained plants resistant to TYLCV by targeting the CP gene with an IR construct (Zrachya *et al.*, 2006). Similarly, Noris (2004) and Ribeiro (2007) with their respective co-workers produced transgenic plants expressing siRNAs from IR constructs against TYLCSV and *Tomato chlorotic mottle virus* (ToCMoV), respectively. Though these plants often showed significant delays in symptom development, particularly at low inoculum dosage. In sharp contrast to the situation with RNA viruses, completely immune lines were not observed. This may suggest that viral mRNAs are targets of RNA silencing, and that the success of the strategy depends on the relevance of the targeted gene product in the systemic spread of the virus.

GENERATION OF RESISTANCE BY MEANS OF PLANTIBODIES

As an alternative to protein-mediated PDR or pre-activation of intrinsic virus resistance mechanisms such as RNA silencing, biotechnological approaches offer the opportunity to exploit approaches that are entirely novel to plants. One such approach is the expression of antibodies, commonly used in animals to recognize pathogens. Although the immune system linked to these proteins in animals is not present in plants, affinities of selected antibodies can be high enough to disrupt essential functions of a viral protein in plants. Though technically complex, the generation of single-chain variable fragment (scFv) antibodies, the development of the phage display approach, and the generation of synthetic scFv libraries, have greatly improved the applicability of this strategy (Ziegler and Torrance, 2002).

The first successful use of plantibodies described reduced susceptibility to *Artichoke mottle crinkle virus* by means of an scFv directed against the CP of the virus (Tavladoraki *et al.*, 1993). Subsequently, only little progress was made for many years. It became clear that the main problem for a broad application of this technique was the instability of many scFvs in plant cells. As most viruses replicate in the cytoplasm, it is necessary to direct scFvs into this cell compartment, which presents problems for the correct folding of scFvs. In some cases, it was demonstrated that fusion to the endoplasmic reticulum (ER) retention signal

"KDEL" stabilises them (Schouten *et al.*, 1996; Conrad and Fiedler, 1998). Fecker *et al.*, (1996) demonstrated that scFvs directed against the CP and the non-structural protein p25 of *Beet necrotic yellow vein virus* could be expressed in *Nicotiana benthamiana* plants. However, expression was only possible if the scFvs were targeted to the ER, which seems in contrast with their place of function in the cytosol. TMV infection of transgenic tobacco plants expressing scFvs against the intact CP of TMV can be reduced, even with low expression levels of the scFv (Zimmermann *et al.*, 1998), and lead to immunity upon stabilized expression (Bajrovic *et al.*, 2001). Xiao *et al.* (2000) generated an scFv from an existing broad range monoclonal antibody (MAb) obtained against *Johnsongrass mosaic virus*, which reacts with several potyviruses. Transformed tobacco plants had lower susceptibility to two potyviruses, PVY and *Clover yellow vein virus*.

A different approach is based on scFvs directed against non-structural proteins that play an important role in virus replication, such as the viral replicase. Using scFvs generated against the RdRp of *Tomato bushy stunt virus* (TBSV), Boonrod *et al.* (2005) obtained *N. benthamiana* plants with high levels of resistance not only to TBSV, but also to other members of the family *Tombusviridae*: *Red clover necrotic mosaic virus*, *Cucumber necrotic virus* and *Turnip crinkle virus*. Gargouri-Bouزيد *et al.* (2006) demonstrated that high levels of expression of an scFv directed against the NIa of PVY led to complete protection against PVY. Apart from a structural protein associating with the viral RNA, the nucleocapsid proteins of tospoviruses are known to regulate viral transcription/replication and cell-to-cell movement, and thus might be particularly sensitive to inactivation by an scFv. Indeed it was demonstrated that specific scFvs can interrupt virus replication at an early stage of infection (Prins *et al.*, 2005).

For the future it is expected that increasing knowledge of the structure of antibodies will provide the opportunity to improve their stability (Ewert *et al.*, 2004). In addition, new protein scaffolds will be developed that can be used as protein-binding alternatives to antibodies, which may circumvent the shortcomings of scFvs (Binz and Plückthun, 2005).

TRANSGENE-MEDIATED VIROID RESISTANCE

Despite the fact that viroids - small (246-401 nt) single-stranded circular RNA pathogens - do not code for any protein, viroid infection resembles virus infection in many respects (Tabler and Tsagris, 2004; Flores *et al.*, 2005). Upon mechanical inoculation or by vector transmission, viroids enter the plant cell, replicate autonomously, move from cell to cell through the plasmodesmata, become systemic by long-distance movement via the vascular system, and cause disease symptoms. Most of the (>30) known viroid species including *Potato spindle tuber viroid* (PSTVd; *Pospiviroidae*) replicate in the nucleus. The four members of the *Avsunviroidae* family, of which *Avocado sunblotch viroid* (ASBVd) is the type species, replicate in the chloroplasts. Importantly, PSTVd and ASBVd infection is associated with the activation of the cytoplasmic RNA-mediated plant defence mechanism, which is manifested by the generation of viroid-specific siRNAs of both orientations (Itaya *et al.*, 2001; Papaefthimiou *et al.*, 2001; Martínez de Alba *et al.*, 2002; Markarian *et al.*, 2004). Nonetheless, viroids evade the silencing machinery to maintain infection, even though their mature RNA genomes move from the nucleus or chloroplast into neighbouring cells. Most plant viruses express silencing suppressor proteins that impede cleavage of their genomes. Since viroids do not encode proteins, they cannot adopt this strategy, but must have evolved a still unknown mechanism to combat RNA silencing.

Previous attempts to create viroid resistance were based on antisense RNA, ribozyme and RNase-mediated approaches. The antisense strategy revealed a high degree of variability among different transgenic potato lines, and did not result in immunity against the targeted PSTVd (Matousek *et al.*, 1994). Antisense RNA targeting of the *Citrus exocortis viroid* (CEVd) also failed to produce fully resistant tomato plants (Atkins *et al.*, 1995). Highly resistant potato plants were generated by the expression of hammerhead ribozymes that were directed against the PSTVd minus-strand RNA, but not against the PSTVd positive-strand RNA (Yang *et al.*, 1997). However, introduction of ribozymes that were functional in potato failed to mediate PSTVd resistance in tomato. The inefficiency of the anti-PSTVd ribozymes in transgenic tomato may be the result of the ability of PSTVd to replicate much faster in tomato than in potato.

So far, one of the most efficient transgenic approaches to achieve viroid resistance is based on the expression of the yeast dsRNA-specific ribonuclease, PAC-1 (Sano *et al.*, 1997; Ishida *et al.*, 2002). In transgenic potato and chrysanthemum plants, PAC-1 production conferred resistance against PSTVd and *Chrysanthemum stunt viroid* (CSVd), respectively. Moreover, PAC-1 not only degraded the dsRNA structures of different viroids, but also those of numerous viruses (Watanabe *et al.*, 1995). Thus, production of transgenic plants expressing recombinant dsRNA-specific RNases could be a promising approach to generate future crop plants with resistance to multiple plant RNA pathogens. However, it should be noted that some plants expressing PAC-1 (Berthomé *et al.*, 2000) or a bacterial RNase III (Langenberg *et al.*, 1997) displayed abnormal developmental phenotypes, and in the former case, this was shown to be due to induction of PTGS of plant genes.

In plants, RNAi is a major molecular defence mechanism against viruses (this review; Voinnet, 2005; Ding and Voinnet, 2007). Although primarily initiated by dsRNA in the cytoplasm, RNAi is also activated in viroid-infected plants. Double-stranded replicative intermediates of viroids are generated in the nucleus or in chloroplasts (Tabler and Tsagris, 2004) where they can hardly trigger RNAi. It appeared that the rod-like-structured mature *Pospiviroidae* RNA is processed into siRNAs. Nonetheless, these siRNAs do not prevent viroid infection. The viroid RNA structure, and probably association of the RNA with host proteins, may account for resistance of viroids against RNAi. However, recently, transgenic tomato plants expressing a PSTVd-specific inverted repeat construct (Wang *et al.*, 2004) and accumulating high concentrations of PSTVd-specific siRNAs prior to PSTVd inoculation were found to be PSTVd resistant (Wassenegger, *unpublished data*). This finding suggests that the RNAi machinery can be charged for increased cleavage of PSTVd and resistance.

POTENTIAL RISKS ASSOCIATED WITH VIRUS-RESISTANT TRANSGENIC PLANTS

Since the early 1990s, there have been a fair number of articles regarding the potential impacts of virus resistance transgenes on the environment. Since this subject has been reviewed from various points of view with some regularity (e.g. Tepfer, 2002; Fuchs and Gonsalves, 2007), we do not aim at presenting details here. A few points, however, still warrant discussion.

Thanks to improved scientific understanding of the underlying phenomena, some of the potential risks thought to be serious in the 1990s are now considered to be relatively unimportant. This is the case of possible negative effects that could be caused by synergy between the transgene and an infecting non-target virus, or by its encapsidation in particles

composed in part of transgene-encoded CP. These risks can simply be avoided and/or attenuated, even in plants that accumulate viral transgene RNA and protein (Tepfer, 2002). In addition, if a problem might arise through synergy or heterologous encapsidation, they would nearly always be reversible; i.e. they would cease to be problems if the plants in question were no longer cultivated.

It has been generally recognized that a more serious potential risk could result from recombination between a viral transgene mRNA and the genomic RNA of a non-target virus. However, it was recently shown in cucumoviruses that similar populations of recombinant viruses appear in transgenic plants expressing a CMV CP gene infected by another cucumovirus and equivalent non-transgenic ones infected simultaneously with two cucumoviruses (Tepfer, *unpublished data*), thus indicating that transgenic plants do not contribute to the generation of recombinant viruses that would not have been generated in natural double infections. These results, particularly if similar ones can be obtained with other virus groups, should alleviate fears that recombination in transgenic plants expressing viral sequences could be a significant source of emergence of novel viruses.

A second of the original concerns that has remained quite intractable is whether sexual outcrossing between the transgenic virus-resistant plant and a wild relative could have a significant effect on the wild plant's fitness and invasiveness/weediness. Although it would appear that many wild plant species are not strongly influenced by virus infection, there are quite a few examples where virus infection has a strong impact on plant fitness. It is important to point out that increased fitness does not necessarily lead to increased population size, which is the appropriate proxy for invasiveness/weediness. This has been very nicely demonstrated for Brassicas in the UK, since it was shown that although virus infection can have a strong negative impact on their fitness, if wild Brassica species receive a virus resistance gene, this is unlikely to have an impact on the size of their populations (Raybould and Cooper, 2005). This is quite reassuring, and may represent the most widespread case, but there is at least one historic example where introduction of a new plant virus into an ecosystem has had a major impact on ecosystem structure; the introduction of cereal yellow dwarf viruses has led to large-scale replacement in California of native perennial grasses by non-native annual species (e.g. Borer *et al.*, 2007; Malmstrom *et al.*, 2005; Malmstrom *et al.*, 2006). Another recent study predicts that introduction of a potyvirus resistance gene into clover could also have an effect on invasiveness (Godfree *et al.*, 2007). Clearly, this does not mean that this type of phenomenon should be expected to be general, but it does suggest that some consideration should be given to the potential impact of all novel types of pathogen resistance (transgene-mediated or not) on the receiving ecosystem.

CONCLUSIONS: EFFICACY, DURABILITY, SAFETY

Considering the number of strategies that have been developed for creating virus and viroid resistance, this area must certainly be considered one of the major success stories in plant biotechnology. However, nearly all of these strategies have never been taken past the stage of proof of principle in the lab or at best small-scale field trials. Indeed it is striking that the only plants that have been grown on a large (i.e. commercial) scale were transformed with complete viral transgenes, and that it appears that the resistance is in all cases of the RNA-mediated type. As a result, our understanding of the field durability of the diverse resistance strategies is truly quite limited. This suggests that the choice of a resistance strategy should take into consideration not only the likelihood of obtaining effective resistance in the greenhouse and small-scale field trials, but also the breadth of sequence diversity of the

target virus, since this would be expected to have an important impact on the probability of resistance breakdown. This also means that it is premature to reject outright resistance strategies based on expression of viral proteins on the grounds that they present a greater potential risk of recombination; in some cases, these may be the best choice in terms of efficacy and durability of resistance, and it is certainly possible to evaluate the potential risks in a given virus/host system.

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