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10 **Antiviral strategies in plants based on RNA silencing**

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ABSTRACT

One of the challenges being faced in the twenty-first century is the biological control of plant viral infections. Among the different strategies to combat virus infections, those based on pathogen-derived resistance (PDR) are probably the most powerful approaches to confer virus resistance in plants. The application of the PDR concept not only revealed the existence of a previously unknown sequence-specific RNA-degradation mechanism in plants, but has also helped to design antiviral strategies to engineer viral resistant plants in the last 25 years. In this article, we review the different platforms related to RNA silencing that have been developed during this time to obtain plants resistant to viruses and illustrate examples of current applications of RNA silencing to protect crop plants against viral diseases of agronomic relevance.

1. Introduction

Plant viruses represent important threats to modern agriculture. Although accurate figures for crop losses due to viruses are not available, it is generally accepted that among the different plant pathogens, the economic relevance of viruses comes second to fungi. Until the emergence of genetic engineering technologies, plant viruses have been partially controlled using conventional cultivation techniques such as crop rotation, early detection and eradication of the diseased plants, cross protection, breeding for resistance, or chemical control of their vectors [1]. In the 1980s, the successful transfer of foreign DNA into the nuclear genome using *Agrobacterium* as a vector prompted the introduction of genetic engineering for crop improvement and the development of virus-resistant plants [2, 3]. Today, different antiviral strategies are being undertaken, either by exploiting natural plant defence mechanisms, or designing new tools, which in most cases are ultimately also based on natural defence mechanisms.

Most of the achievements obtained in plant biotechnology in the area of plant virus resistance are based on the principle of pathogen-derived resistance (PDR) [4]. The concept of PDR was proposed by Sanford and Johnston [5] twenty-five years ago using the bacteriophage Qß as a model, and considers that expression of pathogen genetic elements outside the context of infection may lead to resistance. This approach opened an interesting possibility for the practical control of diseases. For plant viruses, the concept of PDR was first validated with its use in tobacco plants transformed with the tobamovirus *Tobacco mosaic virus* (TMV) coat protein (CP) gene [6]. Soon this observation was validated using other viral CPs and other viral sequences that code for

proteins such as replicases, proteinases and movement proteins [for review, see 7-11]. CP is the most successful and widely applied viral protein for PDR. However, the protection conferred by CP-mediated resistance varies significantly from strong interference with virus multiplication to delay or attenuation of symptoms. The PDR based on the expression of viral proteins, with either the wild type or the mutated one, in transgenic plants has several general characteristics: i) it is not very specific, and protects against a broad range of viral strains; ii) it shows a positive correlation between the levels of accumulation of the viral product and the effectiveness in resistance; iii) it is usually overcome by high doses of inoculum. Despite extensive studies, the molecular mechanisms underlying protein-mediated resistance are not fully understood. What appears to be certain is that they are diverse, that they probably affect several steps of the infection process, and that each virus/transgenic plant combination has specific features. Moreover, it soon became apparent that many virus resistances initially envisaged as protein-mediated PDR did not rely on the expression of the corresponding viral proteins and that a majority of PDR phenomena seemed to work through RNA-mediated mechanisms [12].

2. RNA silencing and virus resistance

In the early nineties, two independent research groups found that the expression of a transgene mRNA with a high sequence similarity to an endogenous mRNA, led to specific degradation of both mRNAs through post-transcriptional gene silencing (PTGS), also known as "cosuppression" [13, 14]. Later, the W. Dougherty research group suggested that a similar mechanism might be involved in the resistance phenomena observed in transgenic plants transformed with viral genes. Some of the transgenic lines showed anomalous phenotypes; unexpectedly and unpredictably the highest level of resistance was observed in the transgenic lines showing very low levels of transgene mRNA accumulation, whereas plant lines expressing the same gene at high levels were fully susceptible. Interestingly, the virus resistant plants had actively transcribed genes but they had low steady-state levels of transgene mRNA. A breakthrough discovery, from transgenic lines included to serve as negative controls, showed that resistance occurred even with non-translatable versions of the viral genes, which demonstrated that the RNA itself was responsible for the virus resistance observed in the transgenic plants [15-17]. All the molecular analysis of these transgenic plants challenged the existing paradigm of genetic regulation and became the first

demonstration of an RNA-activated sequence-specific RNA degradation mechanism in a biological system, a phenomenon now referred to as RNA silencing or RNA interference (RNAi) [18, 19].

English *et al*. provided an elegant approach to demonstrate the role played by RNA silencing in virus resistance in plants transformed with transgenes homologous to viral genome sequences [20]. These authors showed that while a recombinant *Potato virus X* (PVX) whose engineered genome contained coding sequences of GUS (PVX-GUS) was able to infect both wild type plants and plants actively expressing a GUS transgene, transgenic plants in which the GUS transgene was silenced, were resistant to PVX-GUS, but not to wild type PVX (Figure 1). These results provide an explanation for the negative correlation between accumulation levels of the transgene RNA and virus resistance that had been observed in plants transformed with virus-derived transgenes [21]. However, a transgenic plant actively expressing a virus-derived transgene is not always fully susceptible. Very often viral infection causes the silencing of a homologous transgene, which was initially active, thus leading to a phenomenon of delayed resistance referred to as "recovery" [17, 22] (Figure 2). Subsequent discoveries showed that RNA silencing naturally protects plants from viruses, indeed, recovery in tobacco plants infected with the nepovirus *Tobacco ringspot virus* was already documented as early as 1928 ([23], cited by [24]). Today, this phenotype has been shown to result from delayed resistance caused by virus-specific RNA silencing [25]. Even more importantly, later on, this RNA-mediated defence was shown to be a general response to viral infections that acts against the elicitor virus and can also cross-protect the infected plants against secondary infections [26, 27].

In response to this type of antiviral innate defence, it is not unexpected that viruses have devised counteracting mechanisms that interfere with it, mainly by means of factors that are able to suppress RNA silencing. Moreover, the ability of a virus to systemically infect a particular plant is greatly dependent on the effectiveness of these contra defence mechanisms [28-34] (Figure 2). Suppression of the antiviral silencing response of the plant by a virus can facilitate the replication of a second virus, giving rise to synergistic mixed infections [35]. In addition, the specific antiviral resistance conferred by silenced viral transgenes can be disturbed by the silencing suppression activity of heterologous viruses [36-39] (Figure 2). However, RNA silencing-based virus-immune transgenic plants do not always revert to a susceptible phenotype

119 following an infection by a heterologous virus [40], even in cases in which the
120 transgene silencing is suppressed [39].
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123 **3. RNA-mediated transgenic resistant plants**
124   The trigger of RNA silencing is a double-stranded RNA (dsRNA), which is
125 processed by a specific RNase III-type Dicer enzyme into 21- to 24-nt small molecules
126 (siRNA), then, the siRNAs are loaded into Argonaute protein-containing effector
127 complexes called RNA-induced silencing complexes (RISCs) to guide degradation or
128 translation repression of complementary RNA targets [41, 42]. By contrast, the first
129 examples of transgenic plants described to undergo RNA-mediated PTGS had been
130 transformed with transgenes designed to generate viral RNA fragments of positive
131 polarity. Although a single copy of the transgene was capable of inducing RNA
132 silencing [43], in general, induction of RNA silencing was enhanced by the existence of
133 multiple copies of the transgene [44], mainly when they were arranged in inverted
134 repeats able to form dsRNA [45]. Subsequent studies revealed the existence of two
135 branches of transgene-induced PTGS [46]. In the cases of transgenes transcribed as a
136 single strand RNA (S-PTGS), the dsRNA substrate cleaved by Dicer to produce the
137 siRNAs is generated by a host-encoded RNA-dependent RNA polymerase (RDR),
138 which can somehow recognize aberrant versions of highly abundant transgene RNAs
139 and copy them into dsRNA [47, 48]. Transgenes with inverted repeats producing long
140 double strand RNA regions do not depend on host RDRs to produce primary siRNAs
141 and efficient RNA silencing (IR-PTGS), but RDRs are involved in an amplification step
142 producing secondary siRNAs, which reinforces silencing and spreads it beyond the
143 initial trigger sequence (transitive RNA silencing) [49, 50]. In accordance with the key
144 role of dsRNA in the induction of RNA silencing, whereas transformation with
145 transgenes coding for single stranded viral RNAs gives rise to low and erratic numbers
146 of virus-resistant transgenic lines, most of the plants transformed with transgenes
147 producing viral dsRNA show a high level of virus resistance [51]. Transgenes encoding
148 intron-spliced hairpin RNAs are especially efficient as silencing triggers, and
149 consistently confer viral resistance when directed against virus genomes [52-54]. This
150 RNA silencing approach, known as hpRNAi, is now widely used in many plant species
151 and information for convenient generic plasmids for transgene generation is currently
152 available at http://www.pi.csiro.au/rnai/.

Nevertheless, the reasons why a viral transgene is silenced and confers resistance in some transgenic lines, whereas other lines actively express the same transgene and are fully susceptible to the homologous virus are still not completely understood [55]. As expected, in most cases, transgene silencing and virus resistance is associated with high accumulation of siRNAs specific to the viral transgene [56, 57]. Methylation of the transcribed region of the transgene DNA is also an usual hallmark of constitutive or virus-induced transgene silencing and virus resistance [58-60], but the cause-consequence relationship of transgene methylation with RNA silencing and virus resistance has not been unravelled yet.

Most studies on RNA-silencing-mediated antiviral resistance have focussed on plus-stranded RNA viruses -the largest group of plant viruses- but RNA silencing of viral transgenes has been shown to be effective to protect plants against other viruses such as tospoviruses [61-63], with a minus-strand RNA genome, or geminiviruses, with a single-strand DNA genome [64-69]. Although DNA viruses appear to be less susceptible to transgene-derived RNA silencing than RNA viruses [70, 71], this antiviral strategy can sometimes be very effective against geminiviruses [72]. Interestingly, DNA virus infections induce not only postranscriptional gene silencing, but also transcriptional gene silencing [73-76], which can be used in biotechnological approaches to engineer viral resistance [77].

Transgenes expressing viral proteins can display protein-mediated and RNA-mediated overlapping resistance mechanisms, which can differ in intensity and broadness [78, 79]. Although these mechanisms can collaborate to protect plants against a range of viruses, it is also possible that a weak RNA silencing, unable to confer complete viral resistance, can suppress the expression of the transgene and thus inactivate the protein-mediated resistance [80].

The accumulation of large amounts of specific siRNAs in viroid infections demonstrates that the viroidal RNA is a substrate of Dicer-like enzymes [81-84]. Some reports suggest that, whereas these siRNAs are biologically active in guiding RISC-mediated cleavage, the secondary structure of the viroidal RNA protects it from RISC activity [85-87]. However, the fact that a transgenic tomato expressing a viroid hairpin transgene and accumulating high amounts of viroid-specific siRNAs, exhibits resistance to the homologous viroid, indicates that viroid RNA can be the target of RISC-mediated degradation [88].

Although effective RNA silencing can be induced by sequences as short as 23-60 nt [89], it appears that induction of RNA silencing-mediated antiviral resistance may need transgenes with regions of similarity to viral RNAs larger than 100 nt [90, 91]. However, transgenes with larger similarity regions, 300-800 nt, are usually preferred. In general, the effectiveness of the transgene RNA-mediated virus resistance is proportional to the sequence similarity between the transgene and the inoculated virus, however, there are exceptions that are not fully understood [92, 93]. Viruses whose sequence differs from that of the transgene by more than 10% usually escape RNA degradation [61]. To circumvent this limitation, different strategies to co-express several genetic fragments of different viruses, either as independent transcription units or as a single hairpin cassette have been explored [94, 95]. The transgenic expression of these types of constructs rendered a high proportion of transgenic lines heritably resistant against all or some of the source viruses, thus allowing broader virus resistance.

The methods used to engineer RNA silencing-mediated antiviral resistance in transgenic plants normally involve transgenes corresponding to a limited region of the viral genome. However, transgenic plants transformed with full-length copies of viral genomes, named amplicons, have also been constructed. They used to be silenced and resistant to exogenous infection with the virus from which the transgene was derived, however, amplicon lines showing transgene-derived virus infection have also been described [96-101]. In some cases, reactivation of a silenced amplicon and efficient replication of the resulting virus can be achieved by deliberate co-expression of a strong silencing suppressor [102, 103], but often this also occurs spontaneously, as a consequence of poorly characterized environmental or developmental signals [101, 104, 105].

4. RNA silencing-mediated resistance without transgenesis

Concerns regarding transgenic plants are quite strong in some places in the world, especially in Europe, thereby prompting increasing interest in approaches to generate viral resistance that do not rely on the use of genetically modified plants. Since dsRNA is a pivotal factor of RNA silencing processes, the most important efforts have been devoted to the exogenous delivery of this kind of molecules. Initial reports showed that dsRNA derived from viruses of three different families, and directly delivered to plant leaves either by mechanical inoculation of *in vitro*-synthesized molecules or via an

Agrobacterium-mediated transient expression system, interfered with virus infection in a sequence-specific manner [106]. Further research demonstrated that bacterial systems could be used to synthesize viral dsRNA able to promote specific antiviral interference at a very low cost [107-110]. These antiviral approaches could take advantage of recently-developed systems for large-scale production of dsRNA *in vitro* and in bacteria utilizing the RNA polymerase of phage ø6 [111].

Delivery of viral dsRNA cannot cure already infected plants and, in contrast with virus-resistant transgenic plants, it is not able to confer a permanent protection, however, research shows that spraying plants with an extract of bacteria expressing viral dsRNA confers specific antiviral protection for at least 5 days [107, 108].

Recent results demonstrate that the exogenous delivery of specific dsRNA can also protect plants against chloroplast- and nuclear-replicating viroids [112]. Moreover, they state that homologous viroid small RNAs co-delivered mechanically can interfere with one of the viroids examined. These results support the conclusion that the secondary structure of viroids does not provide them with complete protection against RISC activity.

5. Antiviral resistance mediated by artificial miRNAs

RNA silencing regulates a large range of important processes by making use of different populations of small RNAs [113-117]. Among them, microRNAs (miRNAs) are known to play fundamental roles in organism development, and adaptation to environmental stresses [118-121]. These ~21-nt RNAs are the result of the processing of hairpin-like primary transcripts by specific RNAse III-type enzymes (Drosha plus Dicer in animals, and DCL1 in plants). MiRNAs negatively regulate endogenous target genes by cleavage or translational inhibition of their mRNAs. The miRNA primary transcript can be engineered to introduce several mutations within the miRNA 21-nt sequence without affecting its biogenesis [122]. Based on modified miRNAs, named "artificial miRNA" (amiRNA), a new RNA silencing technique has been developed. AmiRNAs were first generated and used in human cell lines and were shown to interfere with the expression of cognate mRNAs [123]. Later, amiRNA technology was also successfully used to direct endogenous gene silencing of individual genes or groups of endogenous genes in different organisms, including several plant species, mosses and unicellular algae [124-129].

253   Host- and virus-encoded miRNAs have been shown to participate in animal virus
254   infections, either by helping the virus or by contributing to host defence mechanisms
255   [130-135]. Moreover, although a role for miRNAs in natural plant virus infections has
256   not been demonstrated yet, endogenous miRNAs have been shown to interfere with
257   engineered plant viruses [136]. Thus, amiRNAs targeted to degrade the invading viral
258   RNA are suggestive candidates to be used in biotechnological approaches to fight plant
259   viral diseases. The first evidence of the effectiveness of this strategy came from the
260   demonstration that the stable expression of amiRNAs targeting RNA sequences that
261   encode the silencing suppressors of the tymovirus *Turnip yellow mosaic virus* (TYMV)
262   and the potyvirus *Turnip mosaic virus* (TuMV) confer specific virus resistance to
263   transgenic *Arabidopsis* plants [137]. Following this, other reports confirmed the validity
264   of this approach for other viral sequences, virus species and host plants [138-141].
265   Moreover, Niu *et al*. [137] explored the possibility of using a dimeric pre-amiRNA that
266   expressed two sequences from different viruses to confer resistance to both viruses on a
267   single transgenic plant. The combined production of multiple virus-specific amiRNAs
268   in plants allows increased virus resistance against a broad spectrum of virus.

269   Whereas efficient amiRNA-mediated resistance was observed against TYMV and
270   TuMV when stretches of the coding sequence of their silencing suppressors were
271   included in the amiRNA [137], when the coding sequence of the silencing suppressor
272   2b of the cucumovirus *Cucumber mosaic virus* (CMV) was targeted, the transgenic
273   plants showed various degrees of responses to CMV infection such as: full resistance,
274   delayed infection, recovery and susceptibility [140]. As previously reported, the
275   strength of the effect of siRNAs [93, 142, 143] and amiRNA [136] in their target
276   sequences not only depend on their own nature, but also on the position in which they
277   are included in the target transcript; this probably indicates either that some sites are
278   more accessible than others to the RNA silencing machinery or that processing is
279   somehow influenced by the flanking sequences rather than by the si/miRNA sequence
280   alone. To avoid amiRNA target positional defects, Duan *et al*. [139] have reported an
281   experimental approach to design miRNAs that target putative RISC accessible sites to
282   engineer effective RNA silencing and virus resistance in plants by amiRNAs.

283   The miRNA precursors produce miRNA-miRNA* duplexes with particular
284   structural features such as mismatches or bulges, and, in most cases, only the mature
285   miRNA associates preferentially with Argonautes [144, 145]. When the duplex region
286   in the miRNA precursor backbone is substituted by amiRNA and amiRNA* and the

mismatched positions are retained, the amiRNA strand will likely be accumulated and loaded in the correct effector RISC. An interesting possibility in the case of designing amiRNAs to produce virus-resistant transgenic plants is to replace the duplex by exact complementary sequences. There is evidence which shows that miRNA-directed RNA silencing targets both plus strand genomic RNA and those RNAs complementary to the viral genome synthesized during viral replication [136]. With constructs producing both amiRNA and amiRNA* complementary to the genomic RNA and the complementary strand respectively, that can be loaded in antiviral RISCs, two targets could be reached with a single amiRNA precursor.

One predicted drawback of amiRNA-mediated resistance is that the combination of the high specificity of miRNA cleavage and the high mutability of plant viruses make it possible for virus variants escaping resistance to emerge [146]. A study with recombinant PPV chimeras bearing miRNA target sequences provided the first evidence that viruses readily escape the negative pressure of miRNA activity through mutations within the miRNA target sequence [136]. The escape from the resistance was enhanced in a transgenic Arabidopsis line expressing the silencing suppressor P1/HCPro, which has been shown to inhibit miRNA activity [147, 148]. A frequent emergence of escape mutants was also observed in transgenic plants expressing an amiRNA that targeted non-essential sequences engineered in a recombinant TuMV [149]. Viruses escaping the miRNA-derived resistance showed deletions affecting the 21-nt target site or point mutations, which mainly affected nucleotides matching the 5' terminal region of the miRNA, thus, pointing out the relevance of this region in amiRNA-mediated cleavage activity. Recent results demonstrate that wild type viruses might also evolve to overcome amiRNA-mediated resistance through the selection of virus variants with point mutations in the amiRNA target sequence (Santiago Elena, personal communication).

6. Agronomic applications of antiviral RNA silencing

Although other biotechnological strategies that interfere with virus infections in plants have been developed [4, 10, 150], PDR remains the most powerful approach to produce virus resistant plants, and RNA silencing appears to be the most promising PDR strategy, which potentially makes this technology of great agronomic relevance [9]. This could be specially applicable to developing countries, whose economy largely depends on agricultural activities, since they might use these relatively cheap tools to

solve specific local problems [151]. While the molecular processes and biological functions of RNA silencing are still not fully understood, our current knowledge of this RNA-mediated mechanism has enabled the development of new platforms for crop improvement. Nevertheless, despite the abundant scientific information obtained since the demonstration of the viability of the PDR concept 25 years ago, and although a large number of field trials have been conducted for diverse viruses and hosts species [152], not many crop plants expressing viral genetic elements and showing virus resistance have reached the commercialization stage [153]. The first virus-resistant cultivar for commercial application in the USA using RNA silencing for crop improvement was summer squash ZW-20 expressing the CP genes of the potyviruses *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV), which was developed by Asgrow Seed Co. [154, 155]. This line was later replaced by CZW-3, which also expresses the CP of the cucumovirus CMV [156, 157]. These plants were also used as parents to develop other cucurbit cultivars by conventional breeding [9]. In 1998 and 1999, Monsanto also commercialized the potato varieties NewLeaf Plus and NewLeaf Y, which were resistant to the polerovirus *Potato leafroll virus* (PLRV) and the potyvirus *Potato virus Y* (PVY), respectively (http://www.monsanto.com/newsviews/Pages/new-leaf-potato.aspx). However, these lines were withdrawn from the market in 2001 due to the reluctance of certain important food processors to use genetically modified potatoes [9, 158]. So far, the most prominent success of PDR against viruses have been the transgenic papayas Rainbow and SunUp, which are resistant against the potyvirus *Papaya ringspot virus* (PRSV) by virtue of expression of the viral CP gene, indeed, it has contributed to saving the papaya industry in Hawaii [159, 160]. Another virus-resistant papaya, X17-2, which is protected against a Florida isolate of PRSV is in an advanced stage towards commercialization in USA [9]. Very recently, the plum cultivar "HoneySweet", transformed with the CP gene of another potyvirus, *Plum pox virus*, [161] has been deregulated in USA. This cultivar has proven a highly effective and durable resistance to PPV in several field trials in different European countries [40, 162-164] (Figure 4). This plum variety has a great potential for fighting this worldwide-spread devastating disease, both as a high-quality commercial variety and as a progenitor in *Prunus* breeding.

The People's Republic of China is investing heavily in biotechnologies, and looking for a transgenic green revolution as a way to secure its food supply [165]. In

addition to PRSV-resistant papaya, both tomato and sweet pepper resistant to CMV have also been released in China [166]. However, the performance of these tomato and sweet pepper transgenic lines was apparently not very satisfactory, and investment in their commercial production was discontinued [151]. Another virus-resistant transgenic plant that is expected to be commercialized in China in the following years is wheat resistant to the bymovirus *Wheat yellow mosaic virus* [167, 168].

The interest in using PDR technology, mainly RNA silencing-mediated, is increasing worldwide [158]. Thus, the development of a number of virus-resistant transgenic plants appears to be close to commercial release in different countries. These include PVY-resistant potato in Argentina, rice resistant to the tungrovirus *Rice tungro bacilliform virus* in India, and bean resistant to the begomovirus *Bean golden mosaic virus* in Brazil [72, 169, 170].

There are three main factors that can determine the practical usefulness of antiviral strategies in plants: efficiency, durability and safety; and only further long-term research in the field of resistant varieties can provide us with definitive data on the stability of different forms of RNA silencing-based resistance. Unfortunately, social concerns, primarily in Europe, over the potential ecological impact of virus-resistant transgenic plants have so far significantly limited the use of virus-resistant crops. But the situation is changing since a significant increase worldwide in hectarage of Biotech/GM crops has been reported [153] and RNA silencing-based technologies will help, among other challenges faced by productive agriculture, to mitigate the impact of virus diseases in the twenty-first century.

7. Open questions in RNA silencing-mediated virus resistance and concluding remarks

Since the first successful application in 1986 [6] of PDR used to confer virus resistance to transgenic plants, a range of powerful strategies using pathogen-derived sequences have been described. Initially main interest was focussed on the expression of wild type and mutated viral proteins, but RNA-mediated approaches based on natural antiviral RNA silencing have yielded the most promising results [150, 171, 172]. In these, specific resistance is the result of an accumulation of antiviral RISC complexes loaded with small RNAs derived from the viral transgene, which are ready to target and degrade the invading viral RNA before the virus has time to mount effective counter-defence mechanisms. The first transgenic lines resistant to viruses by an RNA-mediated

389 mechanism were found by chance as barely-characterized rare exceptions among a
390 majority of lines actively expressing sense viral RNA from the transgene and fully
391 resistant to the virus [12]. The huge progress in the understanding of RNA silencing
392 mechanisms, mainly the unravelling of the pivotal role played by dsRNA, allowed the
393 design of more rational strategies to achieve RNA-mediated viral resistance, such as
394 hpRNAi, which gave more consistent results [51, 53]. However, it is still not possible to
395 accurately predict the frequency of resistant lines and the level of resistance in plants
396 transformed with a particular viral transgene, even when the transgene is designed to
397 produce dsRNA. The silencing efficiency appears to depend, on specific features, still
398 not characterized, of the targeted sequences, as has been shown in a high throughput
399 analysis of the hpRNAi silencing of endogenous plant genes [173]. Further scientific
400 studies are required to understand the sequence and structure features affecting the
401 susceptibility of viral RNAs to antiviral silencing; this will allow us to design more
402 reliable strategies to construct proficient virus resistant transgenic plants.

403 An important value of RNA silencing-mediated resistance is the fact that it is
404 suitable for application on a very broad range of virus-host combinations. Although
405 viruses with plus stranded RNA genomes have been the main target of studies of
406 antiviral RNA silencing, RNA silencing-mediated resistance has been shown to be
407 effective against other viruses, including DNA viruses, such as geminiviruses [72], and
408 even against viroids [88]. By contrast, a limitation of RNA silencing-mediated
409 resistance is its high specificity, since it is only effective against virus isolates that are
410 very similar to the isolate from which the transgene derives. The relevance of this
411 problem will be different for each particular case, depending on the genetic diversity of
412 the virus populations challenging the resistant plant. The extent to which it may be
413 overcome by transforming plants with several viral transgenes or with chimeric
414 transgenes assembled with small genomic fragments derived from various viruses or
415 virus isolates is still unknown.

416 Since the application of transgene RNA silencing to produce virus resistant plants, a
417 number of different concerns have been raised. As most viruses produce silencing
418 suppressors, infection with a non-target virus could breakdown resistance [36-39].
419 Experimental tests have shown that this could happen in some cases, but not all, and it
420 seems to require a very precise coupling of the two viral inoculations [39, 40]. Thus,
421 although mixed infections by several viruses are abundant in nature, it is too early to
422 predict the effect they may have on the effectiveness of the RNA-mediated PDR in the

423   field. Reports show that RNA silencing can be disturbed at low temperatures [174], but

424   evidence that this fact could mean an important threat for the stability of virus resistance

425   in field conditions is still missing. There have also been no reports on ecological

426   problems derived from heteroencapsidation, RNA recombination between the transgene

427   and the viral RNA or emergence of more virulent resistance-breaking virus isolates, or

428   significant off-target effects caused by the transgene, in virus-resistant transgenic plants

429   [10, 152, 158]. However, the field experience is still too limited to make a confident

430   assessment on the relevance of these potential safety risks.

431   Direct administration of viral dsRNA cannot circumvent most of the potential risks

432   associated with RNA silencing-mediated virus resistance, but probably is less

433   concerned by the worry that genetically modified organisms pose in many people.

434   However, the short effect of dsRNA release, which needs to be closely coupled to the

435   viral challenge, limits the present utility of this technology. In this context, the COST

436   Action FA0806 of the EU is an important initiative that has as its main objective to

437   explore suitable, efficient and cost-effective non-transgenic gene silencing approaches

438   for managing plant viral diseases in Europe.

439   In contrast, the recently developed amiRNA technology, which depends on the

440   transgenic expression of a very short viral sequence, is not concerned with some

441   potential risks affecting plants expressing long viral transgenes, such as RNA

442   recombination or undesired off-target effects [146]. In addition, amiRNA-derived virus

443   resistance appears to be efficient even at low temperatures [137]. AmiRNA-derived

444   resistance can be as effective as virus resistance derived from long viral RNA hairpins

445   [137], but this is not the case for all viral amiRNAs [139, 140]. This can depend on the

446   accessibility to RISC of amiRNA targets in the viral RNA, but also on sequence and

447   structure features of the different pre-amiRNA constructs, which could condition their

448   exact processing sites, the levels of accumulation of amiRNA and amiRNA* strands,

449   and the ability of these strands to be loaded in effective antiviral RISCs. Much more

450   research on these topics is required to allow rational designs of efficient amiRNAs with

451   well-defined properties. Current information suggests that viruses can easily evolve to

452   escape amiRNA-derived resistance [136, 146, 149]. The expression of more than one

453   amiRNA targeting different sequences of the same virus or the use of highly conserved

454   regions on viral genomes is expected to mitigate the likelihood of resistance breakdown.

455   Although it may be anticipated that amiRNA technology could be applied to any crop

plant, as has been shown in the tomato, the general effectiveness of this approach needs to be studied further.

An interesting RNA silencing-related technology to be explored for virus resistance is the use of artificial trans-acting (ta) siRNAs (atasiRNAs). Like miRNAs, tasiRNAs are also negative regulators of gene expression that belong to a plant-specific class of endogenous small RNAs whose biogenesis requires an initial miRNA-mediated cleavage of its precursors [175-178]. Engineered atasiRNAs have been used successfully for RNA silencing of endogenous genes in Arabidopsis [179], and can be envisaged as promising antiviral tools.

The development and application of different approaches to achieve resistance to viruses based on PDR have certainly reached a remarkable maturity and there is increasing evidence supporting their effectiveness. But there is no doubt that the outlook is even better and in the course of this century an explosion in the use of RNA silencing to obtain plant cultivars "à la carte" that are resistant to a particular virus or have some other improved agronomic traits will be witnessed.

7. Acknowledgements

482 **Legends to figures**

483

484 Figure 1. RNA silencing of a nuclear gene in a transgenic plant can suppress the

485 accumulation of a cytoplasmic virus and confer virus resistance. Modified from [20].

486

487 Figure 2. Schematic representation of natural and artificial RNA silencing based

488 antiviral resistance. Depending on the final outcome of the confrontation between

489 defence/contradefence mechanisms, different results of resistance, recovery or

490 susceptibility after virus infection can be obtained.

491

492 Figure 3. Schematic representation of antiviral activity conferred by transgenic

493 expression of an artificial miRNA in a plant.

494

495 Figure 4. Leaf symptoms caused by *Plum pox virus* in a susceptible cultivar.

496 Asymptomatic leaf and fruits from the resistance cultivar HoneySweet. Courtesy of R.

497 Scorza and M. Cambra.

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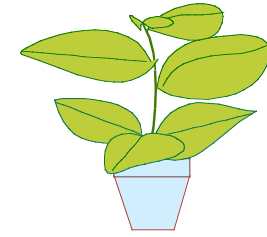1026   2368-2379.

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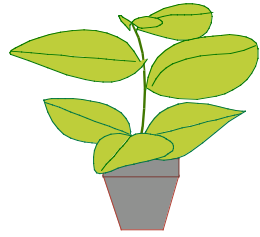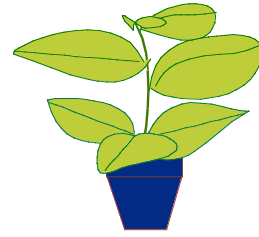
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PVX	Infection	Infection	Infection
PVX-GUS	Infection	Infection	RESISTANCE
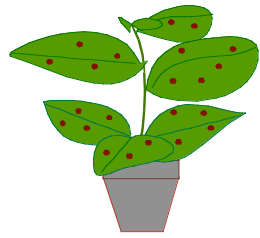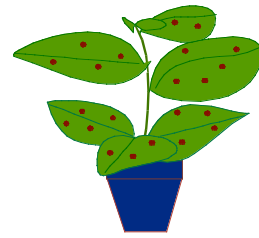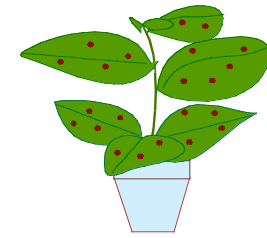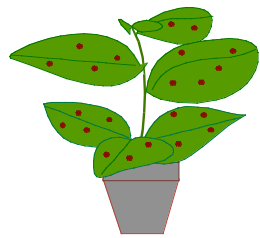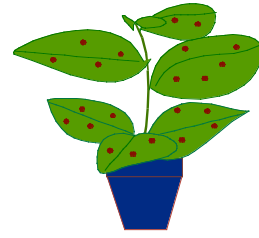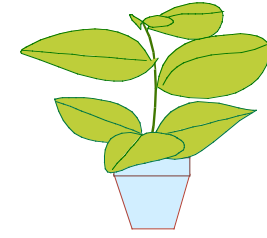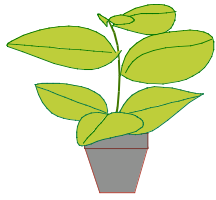
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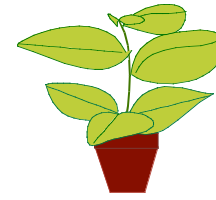
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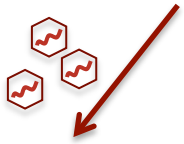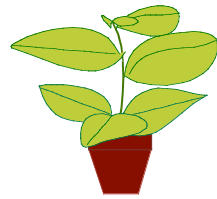
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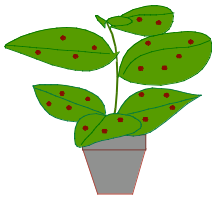
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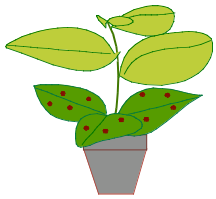
dsRNA treated plant

Silenced transgene

Active transgene

Heterologous viral infection
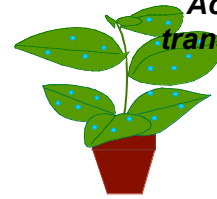
Active transgene

Silenced transgene

Infection

RECOVERY

RESISTANCE

RESISTANCE

Silencing suppression

RECOVERY

Infection

Plant virus

Viral sequence

amiRNA precursor

amiRNA duplex

RISC

Viral RNA cleavage

Translation inhibition
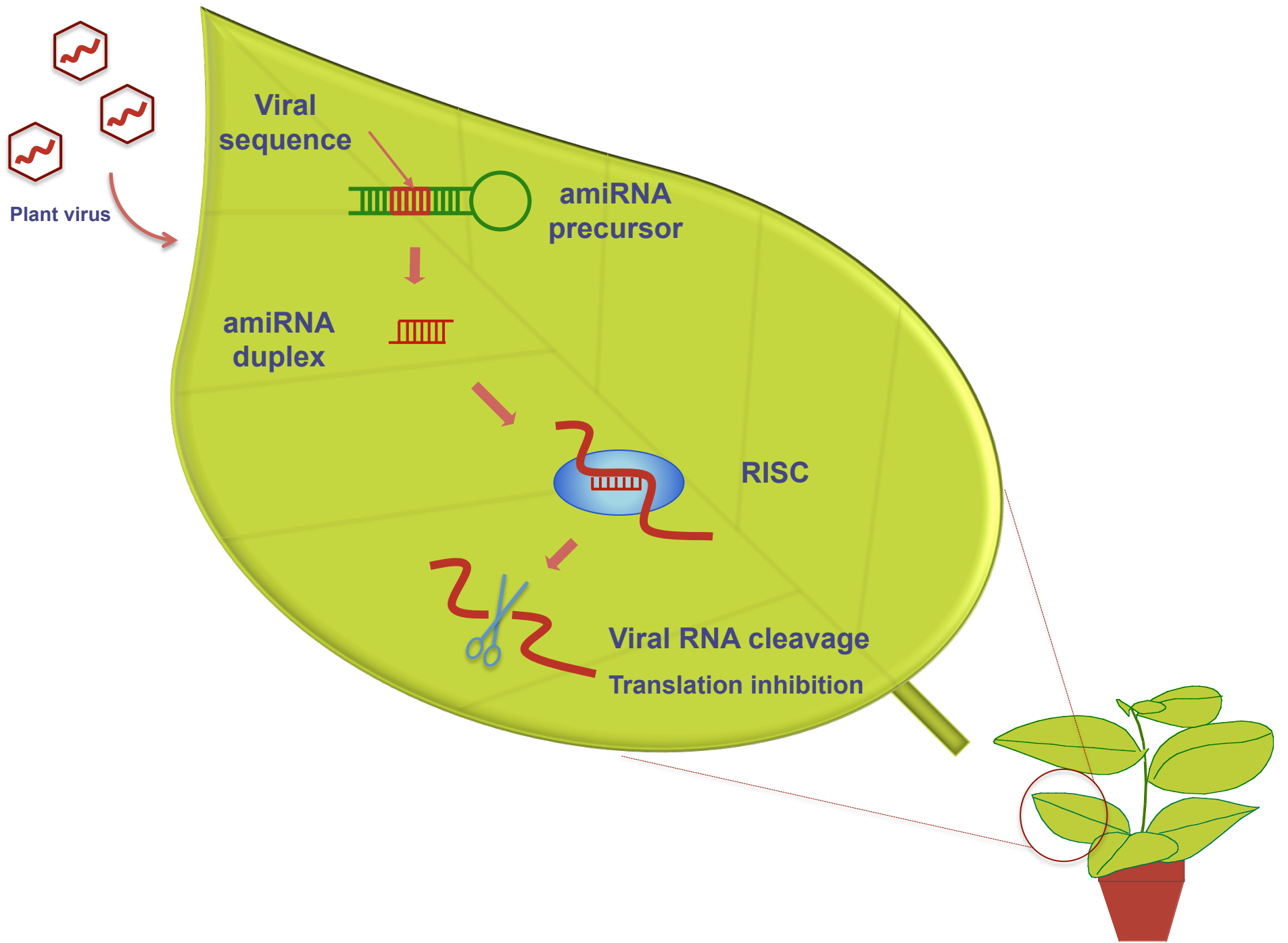
Nontransgenic *Prunus domestica* cv. Stanley

Transgenic *Prunus domestica* cv. HoneySweet