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Biotechnological Approaches for Plant Viruses Resistance: From General to the Modern RNA Silencing Pathway

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ABSTRACT

Virus diseases are significant threats to modern agriculture and their control remains a challenge to the management of cultivation. The main virus resistance strategies are based on either natural resistance or engineered virus-resistant plants. Recent progress in understanding the molecular mechanisms underlying the roles of resistance genes has promoted the development of new anti-virus strategies. Engineered plants, in particular plants expressing RNA-silencing nucleotides, are becoming increasingly important and are likely to provide more effective strategies in future. A general discussion on the biotechnology of plant responses to virus infection is followed by recent advances in engineered plant resistance.

Key words: Plant defense, Engineered resistance, Phytopathogens

INTRODUCTION

Plant viruses are among the most important of plant pathogens. Virus infestation of cultivated areas results in a range of effects, from reduced crop quality to complete plant devastation. Virus specificity varies greatly, with some viruses able to colonize different hosts, whereas others can only infect one defined species due to specific intricate interactions with the plant cell machinery. As a result of mutation in the viral genome, new virus varieties emerge, while others are excluded (Mangrauthia et al, 2008; Jones 2009). The appearance of pathogenic strains is especially important to agriculture. Disease management strategies need extensive knowledge of virus infection and its effect on host plants to allow the correct control procedures to be implemented. Reduction of crop loss is based on controlling the pathogen dissemination rather than the treatment of infected plants, as usually done with fungal or bacterial diseases (Ventura et al, 2004). Different approaches have been used to diminish the virus-spread throughout the plant, and/or the plantation. Results from epidemiological studies might indicate the main route by which the virus would reach its host and the mechanism(s) of inoculation (Gilligan and van den Bosch 2008, Rodrigues et al, 2009). Virus may be transmitted by contaminated seed, by vectors or during culture by normal agricultural practices (Fereres and Moreno 2009; Dieryck et al, 2009). The use of certified seeds may significantly reduce the occurrence of certain viruses (Novy et al, 2007). Furthermore, vector population control and the implementation of "clean" agricultural practices can considerably limit the virus spread (Fereresa and Moreno 2009; Castle et al, 2009). In general, damage to the

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barrier composed of the cell wall and plasma membrane allows virus delivery into a viable plant cell, a process known as inoculation (Rodrigues et al, 2009). Thereafter, should a compatible interaction occur between the virus and the plant cell, virus particles will replicate and spread within the host through plasmodesmata and vascular bundles (Taliansky et al, 2008). The intensity of these processes will depend on the relationship between the virus and the plant host. The set of plant resistance responses aims to reduce virus replication (Ascencio-Ibáñez et al, 2008). In some cases, breeding cultivars with elevated resistance levels represents a viable strategy to reduce the virus-induced crop loss (Ma et al, 2004). Another option is the use of attenuated virus strains to increase the resistance responses (Ichiki et al, 2005). Advances in the understanding the biochemistry of virus infection, such as RNA silencing, have resulted in potential new methods to efficiently limit the viral diseases (Tenllado et al, 2004). In this review, a general discussion on plant responses to virus infection is followed by an overview of recent advances in engineered plant resistance, the major antiviral strategy used for crop protection.

How do the plants defend themselves against viruses?

Viruses promote the infection of susceptible hosts by various strategies that involve well-documented modifications in host plant cells to enhance infection. Initially, replication complexes produce abundant amounts of viral genome followed by the formation new virus particles (Hills et al, 1987). At this stage, some viruses are able to suppress plant gene silencing strategies (Wang and Metzlaff, 2005; Ruiz-Ferrer and Voinnet, 2007). Interference with cell cycle regulation (Gutierrez, 2000) and cell-to-cell trafficking (Crawford and Zambryski, 1999) as well as loss of photosynthetic activity (Balachandran et al, 1994) may also occur. Virus spread within the plant body exploits cell-to-cell and long-distance pathways (Taliansky et al, 2008). Plasmodesmatas are used to allow the virus particles to move from the inoculation site to neighboring cells. Since plants control trafficking between the cells mainly by alteration of the plasmodesmata diameter, some viruses synthesize specialized movement proteins that overcome this barrier and enhance the pore diameter (Lucas 2006). Most viruses are loaded into phloem vessels in this manner, and transported with the photoassimilates to several plant organs (Kehr and Buhtz 2008). At this stage, many particles are available to be transmitted to another compatible plant, for example using an insect vector, as observed by *Cowpea severe mosaic comovirus* in soybean (Bertacini et al., 1998), thereby beginning a new life cycle. The host is not passive, however, during these processes. Plants can fight infection if the general resistance mechanisms are activated or if they possess resistance genes, the products of which are effective against the invading viruses (Baker et al, 1997). Such responses may be general or specific and detailed knowledge of these is valuable in implementing the appropriate preventative measures.

The natural plant immune system is based on dominant and recessive resistance genes. In this model, plant dominant resistance genes (*R*) interact with pathogen avirulence (*Avr*) genes in an allele-specific genetic relationship. A form of localized programmed cell death, termed as "hypersensitive response" (HR), is frequently observed in this type of interaction. Although it does not prevent the host invasion by the pathogen, a basal response conferred by the recessive resistance genes can also occur, thereby limiting the extent of invasion (Ritzenthaler, 2005; Iriti and Faoro 2007).

In general, all known dominant *R* genes have been grouped into eight classes based on their predicted protein structure. Only nine *R* genes have been isolated and sequenced: *N, Rx1, Rx2, Sw5, Tm2*^{2}, *HRT, RTM1, RTM2* and *RCY1*. Most of the proteins for which these genes code possess putative N-terminal leucine-zipper (LZ) or other coiled-coil (CC) amino acids sequences, a centrally located nucleotide-binding site (NBS) sequence and C-terminal leucine-rich repeats (LRR) of various lengths (Martin et al, 2003; Ellis et al, 2007). To-date, all *R* genes that confer resistance to viruses belong to the NBS-LRR class (Martin et al, 2003). *R* genes that confer HR, can recognize viral RNA polymerase subunits, movement proteins, coat proteins (CP) and genomic segments as avirulence factors.

A mechanism to explain the genetics of Avr-R genes disease resistance would be that *R* gene products serve as the direct receptors for pathogen-encoded *Avr* proteins (Ellis et al, 2007). An alternative mechanism would be that *R* proteins would form complexes that would recognize the pathogen molecules in the initial invasion stages. Binding of pathogen molecules would lead to a

sequence of cellular events that would constitute the defense response (Belkadir et al, 2004). Evidence in support of such a mechanism is that Hsp90 has been shown to be a critical component in immune responses triggered by the NBS-LRR proteins in plants against plant pathogens (Hubert et al, 2003; Takahashi et al, 2003). It specifically interacts with RAR1 (Required for MLA12 Resistance 1), a member of the CHORD (cysteine- and histidine-rich domain) protein family (Shirasu et al, 1999). Through its two zinc-coordinating domains, RAR1 can interact with Sgt1p, a component of the SCF (Skp1-Cullin-F-box) E3 ubiquitin complex (Azevedo et al, 2002). Both RAR1 and SGT1 are required for signal transduction mediated by most *R* genes. Thus Hsp90 can contribute to the signaling pathways performed by other proteins related to *R* gene products (Hubert et al, 2003). Furthermore, it has been suggested that the effect of Hsp90 in disease response could be through direct or indirect modulation of NBS-LRR protein levels (Hubert et al, 2003; Lu et al, 2003) and/or by suppressing viral resistance factors (Lu et al, 2003). HSP90 has been related as a general signaling factor in the pathogen-host interaction (Dangl and McDowell, 2006).

Less is known about the plant responses controlled by the recessive resistance genes. This resistance might be the result of a passive mechanism in which specific host factors required by the virus to complete its life-cycle are absent or present in a mutated form (Diaz-Pendon et al, 2004; Cavatorta et al, 2008). The translation initiation factor eIF4Ep was seemed essential to viral life cycle because its interaction with viral particle proteins (VPg) plays an important role in the regulation of translation initiation (Leonard et al., 2000). Contrarily, a mutation at eIF4Ep from pepper (Kang et al, 2005a), lettuce (Nicaise et al, 2003) and pea (Gao et al, 2004) impairs the potyvirus infection cycle. Other proteins, e.g. OLE1 and TOM1, are involved in membrane structure and distribution of virus proteins targeted to vacuolar membranes respectively (Lee et al, 2001; Hagiwara et al, 2003). OLE1 is a Δ9 fatty acid desaturase that converts saturated to unsaturated fatty acids and is a major determinant of membrane fluidity. In the case of tobamoviruses, TOM1 was shown to interact with both TOM2A (Tsujimoto et al, 2003) and a polypeptide with a helicase domain (Yamanaka et al, 2000), consistent with the idea that membranes are of universal importance for positive-strand RNA replication of viruses (Ritzenthaler, 2005).

Crop protection based on natural resistance

The use of virus-resistant cultivars is a cheap and effective approach to reduce the economic loss caused by the plant viruses (Cerqueira-Silva et al, 2008). In contrast, breeding for resistance is a long and costly process (Borém and Milach, 1998) as the selected variants must have durable resistance to the target virus(es), at least throughout the life of the cultivar. Virus resistance can be either specific or non-specific. Specific resistance occurs when only one virus isolate from the group sampled from different hosts and geographical regions is able to infect the resistant cultivars. Non-specific resistance occurs when the resistance is effective against all the virus population. As a consequence of their error prone polymerases and the lack of a proof-reading mechanism during replication, viral pathogens have a high mutation rate (Drake and Holland, 1999). The stability of host resistance depends on whether a new virus mutant emerges and overcomes the artificially selected or naturally achieved resistance (Lecoq et al, 2004). The durability of host resistance will, therefore, be determined by both the virus and host factors. In general, it will depend on the ability of the host to resist new virulent strains from the virus population.

Breeding resistant plant cultivars based on the recessive genes may show more durable resistance than those based on dominant genes since recessive resistance is due to the loss of factor(s) essential for virus multiplication in the host cells (Cavatorta et al, 2008). The virus, therefore, needs to overcome the function of this missing factor to defeat the host resistance (Lecoq et al, 2004). Dominant resistance is generally less durable as virus mutations more easily suppress the interaction between the plant resistance factors and virus avirulance factors (Lecoq et al, 2004), although in some cases, the resistance remains useful for many years (Kang et al, 2005). For example, the dominant *I* gene that protects *Phaseolus vulgaris* against BCMV and a number of others viruses has been used in Snap Bean breeding for decades (Keller et al, 1996). Dominant resistance is preferred in breeding programs because it targets the precise pairs of host genes (Ritzenthaler, 2005) facilitating plant selection.

Crop protection based on engineered resistance

The majority of virus-resistant transgenic plants can be considered to be the result of pathogen-derived resistance (PDR) brought about the expression of viral sequences in plant cells leading to plant protection (Prins et al, 2008). A pre-requisite for the use of PDR is that no interference with essential host functions should occur. PDR can be separated into protein-mediated resistance and nucleic acid-mediated resistance. Among the viral proteins used for PDR are replicases, movement proteins, proteases and, most often, coat protein(s) (CP) (Tepfer, 2002). The observation that transgenic RNA, rather than the expressed viral proteins, was responsible for the observed resistance, created new opportunities based on RNA-mediated resistance (Tenllado et al, 2004). An overview of the main mechanisms and applications related to these two types of engineered resistance are presented in the following sections.

Protein mediated resistance (PMR)

The initial report on PMR used *Tobacco mosaic virus* (TMV) CP gene expression to produce the resistance in tobacco plants (Powell et al, 1986). Since then, a number of studies have used PMR to confer plant resistance to a variety of viruses (Miller and Hemenway, 1998; Tepfer, 2002; Gharsallah Chouchane et al, 2008). Viral coat protein-mediated resistance can provide either broad or narrow protection (Tepfer, 2002). Thus, the CP gene of *Potato mosaic virus* (PMV) strain N605 provides resistance in transgenic potato plants against this virus strain and also to the related strain 0803 (Malnoe et al, 1994). Similarly, transgenic tobacco plants expressing a TMV CP gene are resistant to TMV and other closely related TMVs (Beachy, 1999). In contrast, the CP gene of *Papaya ringspot virus* (PRSV) strain HA provided resistance in papaya only against this HA strain (Tennant et al, 1994). CPs have roles additional to acting as CPs during the life cycle of a virus. Thus TMV CP was shown to enhance the production of movement proteins and coordinate the formation and size of virus replication complexes (Asurmendi et al, 2004). Transgenically expressed CP genes have been reported to interfere with this process leading to virus resistance as well as to reducing the production of movement proteins thereby limiting the spread of cell-to-cell infection (Bendahmane et al, 2002). It has also been shown that the production of TMV CP in engineered plants interferes with TMV assembly (Assurmedi et al, 2004). This mechanism is able to confer resistance to a number of viruses including PVX, AIMV, CMV and TRV (Michael and Wilson, 1993; Beachy, 1994; Baulcombe, 1996).

Complete or partial viral replicase genes have been shown to confer immunity to infection. This is generally limited to the virus strain used to provide the replicase gene (Beachy, 1997). Thus, mutant replicases from *Cucumber mosaic virus* (CMV) subgroup I conferred high levels of resistance in tobacco plants to all subgroup I CMV strains but not to subgroup II strains or other viruses (Zaitlin et al, 1994; Morroni et al, 2008). Similarly, a mutant, but not a wild type replicase, conferred resistance to infection against PMV (Audy et al, 1994) and AIMV (Brederode et al, 1995). It has been proposed that this replicase-mediated resistance is brought about by the repression of replication due to the transgene protein interfering with the virus replicase, possibly by binding to host factors or virus proteins that regulate the replication and virus gene expression (Beachy, 1997).

Viral movement proteins (MPs) allow infection to spread between the adjacent cells (cell to cell) as well as systemically (long distance). Transgenic plants that contain mutant MPs from PMV show resistance to several TMVs as well as to AIMV, *Cauliflower mosaic virus* (CaMV) and other viruses (Cooper et al, 1995). Similar results were found for *Nicotiana occidentalis* plants expressing a movement protein (P50) and partially functional deletion mutants (DeltaA and DeltaC) of the *Apple chlorotic leaf spot virus* (ACLSV) showed resistance to *Grapevine berry inner necrosis virus* (GINV) (Yoshikawa et al, 2006). The use of mutated MPs could, therefore, lead to transgenic plants that efficiently inhibit the local and systemic spread of many different viruses.

The evaluation of mutant genes coding for CP and other viral genes used to confer PMR is of special interest for the commercial release of transgenic plants. It has been shown that the molecular interaction between the challenging viruses and the transgenic plants can lead to heterologous encapsidation, complementation, and recombination (Varrelmann and Maiss, 2000). This has raised concerns on the potential biological and environmental risks associated with virus-resistant transgenic plants. Heterologous encapsidation occurs when closely related viruses

use the functional viral CPs expressed in transgenic plant cells (Varrelmann and Maiss, 2000). Transgenic CPs can transfer functions such as vector and host specificity. Similarly, complementation occurs in transgenic plants if the transgenically expressed protein complements a mutant virus, which is defective in one or more genes. One method to prevent this phenomenon would be to abolish, by mutation of specific amino acids, the ability of transgenic CPs to form virus particles or the specific function of complemented proteins (Varrelmann and Maiss, 2000).

Nucleic acid-mediated resistance (NAMR)

Pathogen-derived resistance has also been achieved through the expression of virus sequences, the acquisition of resistance being dependent on the transcribed RNA. This RNA-mediated virus resistance can be considered to be an example of post-transcriptional gene silencing (PTGS) in plants (Prins et al, 2008). Napoli et al (1990) firstly reported PTGS in *Petunia hybrida* transgenically expressing the chalcone synthase gene. They observed a co-ordinated and reciprocal inactivation of the host gene and the transgene encoding the same RNA. This process has been called RNA silencing or RNA interference (RNAi) and occurs in a variety of eukaryotic organisms (Mlotshwa et al, 2008). The silencing process involves the cleavage of a dsRNA precursor into short (21-26 nucleotides) (nt) RNAs by an enzyme, Dicer, that has RNase III domains. These RNAs are known as short interfering RNAs (siRNA) and microRNAs (miRNAs). Both siRNA and miRNA are able to guide an RNA-induced silencing complex (RISC) to destroy single-strand cognate RNA ((Naqvi et al, 2009). In addition, longer siRNAs (24-26 nt) have been shown to result in methylation of homologous DNA causing chromatin remodeling and transcriptional gene silencing (TGS). In contrast, it was shown that the introduction of a part of the pMADS3 genomic sequence in *P. hybrida* induces ectopic expression of endogenous pMADS3 (Shibuya1 et al, 2009).

RNA silencing was first recognized as an antiviral mechanism that protected organisms against RNA viruses (Waterhouse et al, 2001; Pins et al, 2008) or the random integration of transposable elements. However a general role for RNA silencing in the regulation of gene expression only became evident after it had been demonstrated that specific short miRNAs precursor molecules (fold-back dsRNA) were actively involved in RNA silencing in plants and animals (Bartel, 2004; Naqvi et al, 2009). Several miRNA genes are evolutionarily conserved. Their function in plants is mainly to cleave the sequence-complementary mRNA, whereas in animals such as *Caenohabditis elegans*, they appear to predominantly inhibit the translation by targeting the partially complementary sequences located within the 3´ untranslated region of mRNA (Meister and Tuschi, 2004; Naqvi et al, 2009).

Plant RNA silencing appears to be more diverse in comparison with other organisms. Some aspects of silencing are common for all eukaryotic organisms (e.g. the requirement of Dicer and Argonaute proteins, see below). Sequence-specific DNA methylation (RNA-directed DNA methylation – RdDM) can be induced by dsRNA molecules in various plant systems and in response to various dsRNA inducers (Cao et al, 2003). It has been suggested that RdDM also occurs in mammals (Kawasaki and Taira, 2004) but not in fungi (Freitag et al, 2004). Silencing in plants is systemically transmissible within the plant body and can spread from the initial genomic target region to adjacent 5´ and 3´ non-target sequences (Himber et al, 2003; Vaistji et al, 2002). A similar process appears to be absent in mammals and insects but occurs in *C. elegans* (Baulcombe, 2004). Furthermore, the size of siRNAs can vary from 21 to 25 nt in different species. In plants, siRNAs with 21-nt and 24-nt are found (Tang et al, 2003) but only two sizes, 21 nt and 25 nt, are present in the fungus *Mucor circinelloides* (Hamilton, 2002), whilst only a ~21 nt species of small RNAs appears to be present in animals (Wang and Metzlaff, 2005). In all organisms diverse proteins interact among themselves and with nucleic acids leading to different RNA silencing pathways.

Proteins involved in plant RNA silencing

Several silencing-associated protein factors have been identified in the plants. To-date, Dicer-like (DCL) proteins, Argonaute (AGO) proteins and RNA-dependent RNA polymerases (RdRP) have been reported to play key roles in RNA silencing (Xi and Qi, 2008). However, RNA helicase (Kobayashi et al, 2007) and other proteins such as HEN1 (Lózsa et al, 2008) and HYL1 (Baulcombe, 2004; Dong et al, 2008) are also involved. RdRPs are particularly important in plant silencing in that they copy target RNA sequences to generate dsRNA and that they are also required for RNA-

directed DNA methylation (He et al, 2009). Until present, six RdRPs were reported in Arabdopsis (Brodersen and Voinnet, 2006).

Arabidopsis thaliana and rice encode for four DCL (DCL1, DCL2, DCL3 and DCL4) proteins with distinct functions. Although DCL1, together with HEN1 (Xie et al, 2004) and HYL1 has been previously shown to be involved in miRNA biogenesis (Han et al, 2004), the protein represses antiviral RNA silencing through negatively regulating the expression of DCL4 and DCL3 (Qu et al, 2008). It appears to function in the nucleus, processing both miRNA primary transcripts and precursors (Papp et al, 2003). Purified DCL1 from *A. thaliana* extracts was shown to be involved in the production of 21 nt siRNAs (Qi et al, 2005a). This enzyme is structurally and functionally similar to *Drosophila* Dicer-1 and human Dicer and is composed of two RNaseIII domains and a dsRNA-binding domain, a RNA helicase domain and a PAZ domain (Finnegan et al, 2003). The PAZ domain, characteristic of enzymes that process small dsRNA, binds to the 2 nt 3´-overhang of dsRNA termini (Ma et al, 2004). Its absence seems to be a typical aspect of long-dsRNA-processing enzymes (Carmell et al, 2004). DCL2 has been implicated in viral siRNA and the loss of function of this enzyme leads to reduced siRNA levels and increased virus susceptibility (Xie et al, 2004; Qu et al, 2008). In *A. thaliana*, DCL3 is required for chromatin silencing through DNA methylation, and is also required for the production of endogenous (transposons) siRNA (Xie et al, 2004). DCL3 has a minor role in antiviral RNA silencing than DCL 2 and DCL4 (Qu et al, 2008). DCL4 is the only one that lacks a PAZ domain (Finnegan et al, 2003). Recently, *A. thaliana* mutant in DCL4 was identified and analyzed (Xie et al, 2005). This mutant lacks each of three families of 21-nt trans-acting siRNA (ta-siRNA) and possesses elevated levels of ta-siRNA target transcripts. Likely mi-RNA, ta-siRNAs acts to guide target mRNAs cleavage.

In animals, siRNAs generated by Dicer enzymes associate with RISCs, which recognize the target RNA. The enzymatic activity (Slicer) of the RISC is responsible for the cleavage of homologous viral RNA or mRNA (Pantaleo et al, 2007). AGO proteins are the main responsible for this activity (Qu et al, 2008). They possess two conserved domains: PAZ and PIWI (Carmell et al, 2002). The PIWI domain has been implicated in interacting with Dicer in complex formation. In mammals, it was shown that AGO2 contains the catalytic activity (Slicer) of the RISC and is directly responsible for mRNA cleavage (Liu et al, 2004). AGO1 was previously supposed to be a Slicer candidate since accumulation of miRNAs is decreased in *ago1* mutants, this being accompanied by increased levels of mRNA from target genes (Vaucheret et al, 2004). Furthermore, it has been shown that AGO1, miRNAs and trans-acting siRNA may associate *in vivo*, with the complexes formed able to cleave the target mRNAs *in vitro* (Qi et al, 2005a). Recently, Qu et al (2008) have shown that AGO1 ensures efficient clearance of viral RNAs. Nowadays, ten *A. thaliana* AGOs are reported (Brodersen and Voinnet, 2006). This suggests the existence of multiple other Slicers in *A. thaliana* besides AGO1 (Rivas et al, 2005). In fact, AGO7 from Arabdopsis was shown to clivage viral RNA (Qu et al, 2008). This large number of Slicers also suggests that different AGOs might regulate gene expression in specialized tissues or at particular developmental stages (Qi et al, 2005).

RNA silencing pathways

Three RNA silencing pathways have been described in plants (Baulcombe, 2004). These are cytoplasmic siRNA silencing, important in virus-infected cells (Mlotshwa et al, 2008), the silencing of endogenous mRNAs by miRNAs and a third pathway associated with DNA methylation and the suppression of transcription (Xie and Qi, 2008). In general, these pathways begin with the production of RNA transcripts of the organism genome with complementary or near-complementary 20 to 50 bp inverted repeats that can form dsRNA hairpins (Meister and Tuschi, 2004). Such transcripts are considered to be miRNA precursors. Maturation involves Dicer-like proteins that possess dsRNA-specific RNase III-type endonuclease activity and dsRNA binding domains. Initial processing by Dicer occurs in the nucleus of the cell and the miRNA precursor is then exported to cytoplasm by means of nuclear export receptors such as the exportin 5 protein (Lund et al, 2004). Once in the cytoplasm, the miRNA precursor is further processed by Dicer, yielding miRNA duplexes of ~21 nt length (Elbashir et al, 2001; Xie and Qi, 2008). Other sources of these dsRNA molecules are RNA template derived RNA polymerization, e.g. from viruses, or hybridization of overlapping transcripts from repetitive sequences such as transgene arrays or transposons (Meister and

Tuschi, 2004). Furthermore, they can be artificially introduced in the plant tissues (Johansen and Carrington, 2001; Tenllado et al, 2003; Duan et al, 2008). Such dsRNAs lead to siRNAs production, which generally guide mRNA degradation and chromatin modification. As discussed above, four different Dicer genes are reported in plants, with each Dicer preferentially processing dsRNA from a specific source. For example, DCL1 and DCL4 process miRNA precursors, whereas DCL2 and DCL3 are involved in the production of siRNAs from plant viruses and from repeated sequences respectively (Meister and Tuschi, 2004).

The small RNA molecules RNAs (siRNA and miRNA) poroduced are next incorporated into ribonucleoprotein particles, which are subsequently rearranged into RISCs (Hammond et al, 2000; Xie and Qi, 2008). At least one member of the AGO protein family is present in the RISC, probably interacting directly with the target RNA in the complex. The AGO PAZ domain specifically recognizes the terminus of the base-paired helix of siRNA and miRNA duplexes (Vaucheret, 2008) although the functional form of the RISC contains only single-stranded small RNAs. AGO proteins either bind preferentially to small RNAs of a specific sequence or use specific adaptor proteins that were associated with dsRNA during its production (Meister and Tuschi, 2004; Vaucheret, 2008). The described interaction with PAZ ensures the safe transitioning of small RNAs into the RISC by minimizing the possibility of unrelated RNA-processing or RNA turnover products entering the RNA silencing pathway. The small RNAs in the RISC guide a sequence specific degradation of complementary or near-complementary target mRNAs. Using a *Drosophila in vitro* system, it was shown that the target mRNA is cleaved in the middle of complementary region, ten nucleotides upstream of the nucleotide paired with the 5´ end of the guide siRNA (Elbaschir et al, 2001).

The first evidence for miRNA-guided translational regulation was that miRNA targeted to a specific *C. elegans* gene reduced protein synthesis without affecting mRNA levels (Bartel et al, 2004). The translational repression of gene expression by miRNA may occur through prior- or post-translation initiation (Cannell et al, 2008). Similar processes also occur in plants. Although the mechanisms of translational repression are poorly understood, miRNAs appear to block translation elongation or termination rather than translational initiation (Garcial, 2008).

RNAi can also induce gene repression at the transcriptional level through chromatin remodeling (Xie and Qi, 2008). Some regions of the chromosome structure are more loosely packaged (transcriptionally active euchromatin) whereas other regions are more tightly packaged (transcriptionally silent heterochromatin) (Elgin and Grewal, 2003). Heterochromatin formation in plants and animals is associated with cytosine methylation (Mathieu and Bender, 2004) and this covalent DNA modification can be induced by plant or viral RNA. Thus RNA viruses have been shown to trigger methylation of identical DNA sequences present in the host genome (Jones et al, 1998; Wang et al, 2001). Cytosine methylation in plants is brought about by CG methyltransferases (Saze et al, 2003) and cytosine methyltransferases (Cao et al, 2000). A dense methylation pattern was observed in a RNA virion-infected tobacco system, with almost every available cytosine in the target transgene sequence methylated (Pélissier et al, 1999). This suggested that trigger RNAs efficiently recruit methyltransferases to establish and maintain methylation of target DNA sequences. Interestingly, in *A. thaliana* some cytosine methyltransferases are dependent on the H3 K9 methyltransferase KHP/SUVH4 (Jackson et al, 2002; Lippman et al, 2003) suggesting that histone methylation might be a prerequisite for DNA methylation. Alternatively, DNA methylation might trigger transcriptional silencing thereby causing enrichment of H3 K9 mRNA, which would then recruit other methyltransferases possibly to maintain the silent state (Mathieu and Bender, 2004).

Use of RNA silencing to biotechnological control of virus disease

Enhanced resistance of transgenic plants to viruses has been shown to have been brought about by expression of sequences able to trigger RNA silencing (Pruss et al, 2004; Andika et al, 2005). However, the possible environmental risks (see below) and the difficulties of transforming some species are obstacles to the application of this technology. Strategies that confer RNA silencing, such as dsRNA molecules of viral origin, could result in undesired consequences in hosts with unmodified genomes. Thus RNAi was synthesized in *C. elegans* when incubated together with *E. coli* expressing a dsRNA corresponding to a specific

gene (Timmons and Fire, 1998). An alternative method for the production of resistance in transgenic plants is the use of *Agrobacterium tumefaciens* to express dsRNA molecules (Johansen and Carrington, 2001). Thus expression of a dsRNA coding for green fluorescent protein (GFP) in *N. benthamiana* tissues that also had the GFP gene present resulted in inhibition of GFP production. GFP synthesis was not inhibited when the *N. benthamiana* strains used either carried plasmids coding for GFP-specific dsRNA molecules or for viral suppressors of RNA silencing.

Strategies using exogenously supplied dsRNA have already been use to combat virus infestation in plants. *E. coli* was used to produce large amounts of dsRNA coding for partial sequences of two different viruses, *Pepper mild mottle virus* (PMMoV) and *Plum pox virus* (PPV) (Tenllado et al, 2003). Simultaneous injection of dsRNA together with purified virus particles resulted in the inhibition of both viruses. Interestingly, resistance to infection was also observed when the crude bacterial preparations were sprayed onto the *N. benthamiana* leaves. These data suggest a simple, economic and effective application of RNA silencing technology. In the near future, we believe other such simple approaches to induce and enhance the efficiency of RNA silencing will emerge, leading to large scale applications of this sophisticated molecular pathway.

Risks related to genetically engineered plants

The main risks associated with genetically engineered plants (Tepfer, 2002; Keese, 2008) are the transgenic expression of viral genes in a compatible host, which can directly interfere with the life cycle of other viruses. A normal transgenic protein, for example those related to cell-to-cell and long-distance movement proteins, may complement defective viral proteins. Similarly, heterologous encapsidation using viral coat proteins expressed in the host represent a possible alteration in the process of transmission and host specificity that can contribute to infection. The natural process of gene flow between crop plants and their wild relatives can potentially alter the plant genome. Two possible problems are the fixation of crop genes in small populations of wild plants leading to a loss of biodiversity and consequent population extinction, and increased "weediness" of wild relatives of the crop plant brought about by gene introgression resulting in plant growth in undesirable locations. This, however, would only occur if the transgene conferred an advantage that overcame a population size limiting factor, which would result in increased gene prevalence in the wild population. If the transgene were to confer resistance to conditions established by human activities, resultant problems could be controlled. If the transgene were to confer resistance to viruses, other pathogens or climatic conditions, the problems are far more complex as the selection pressure cannot be controlled.

Recombination, a covalent joining of nucleic acids that were not previously adjacent (Keese, 2008) might also allow the flow of plant genes to the virus genome. Recombination is seen to occur by a copy-choice mechanism during virus replication, involving one or more changes of template while the replicase complex synthesizes RNA complementary to the template molecule. Different types of recombination occur in the viral RNA genome, i.e. between identical sequences at equivalent sites (homologous recombination) or between unrelated sites that lack appreciable sequence identity (nonhomologous recombination). Reports have identified the incorporation of chloroplast tRNA and cellular mRNA coding for an hsp70 homolog in the virus genome (Mayo and Jolly, 1991; Masuta et al, 1992). The advantages of recombination to the virus include elimination of deleterious alleles and creation of new variants. Indeed, sequence comparison has suggested that recombination might play a key role in viral evolution (Miller et al, 1997). The susceptibility of virus resistant transgenic plants to recombination and the resultant emergence of new virus diseases is therefore of particular importance to the genetic engineer. It must be pointed out that recombination can also introduce point mutations and others errors into the viral genome, leading to a loss of viral fitness.

CONCLUSIONS

Plant virus diseases are critical problems in agriculture. Virus occurrence may be completely excluded if preventive strategies are established. Plant variants that possess increased natural resistance could substitute for susceptible cultivars. Although this "classical breeding" is a powerful method to produce the resistant plants, it

is usually costly and time consuming work. In addition, features such as crop quality and quantity may be compromised by breeding for resistance. In contrast, genetic manipulation is a relatively rapid method to introduce the virus resistance. This is especially advantageous for virus diseases that suddenly emerge. Transgenic plants expressing the RNA-silencing pathway have been shown to efficiently resist viral infection. This pathway perhaps represents the most specialized molecular strategy that plants use to combat viruses. Thus, RNA-silencing based approaches might be an effective way of reducing crop loss caused by viruses. More important are the methods that induce viral RNA-silencing without altering the plant genome since these methods overcome risks associated with transgenic plants. In the near future, knowledge of changes in the mRNA, protein and cellular metabolites after virus infestation will lead to a greater understanding of the plant:virus interaction. This will in turn enhance the efficiency of the current approaches and allow the development of new strategies.

RESUMO

As viroses são problemas importantes para a agricultura moderna e o seu controle representa um desafio para o manejo de áreas cultivadas. As principais estratégias de resistência a vírus se baseiam em mecanismos naturais ou em engenharia genética. Recentemente, a maior compreensão dos mecanismos moleculares envolvidos na função de genes de resistência da planta facilitou o desenvolvimento de novas estratégias antivirais. Plantas modificadas geneticamente, em particular aquelas expressando a via de silenciamento de RNA, são alvo de interesse crescente e representam a possibilidade de estratégias futuras mais efetivas. Neste trabalho são discutidos diferentes aspectos relacionados à resistência a viroses em plantas. Adicionalmente, a perspectiva de aplicação biotecnológica das diferentes vias de resistência é apresentada.

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