A Critical Appraisal of Non Conventional Resistance to Plant Viruses

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Abstract

Among natural resistance mechanisms to plant pathogens, cultivar resistance has been extensively used in plant breeding to introduce what can be defined as "conventional" resistance to a number of them, including viruses. The necessity of overcoming the constraints of genetic incompatibility, so as to widen the range of possibile use of genetic control of infectious agents, has propitiated the utilization of biotechnological procedures, whereby "non conventional" or transgenic resistance was developed. Transgenic resistance to plant viruses encompasses the identification, cloning and tranferring into the recipient host of single viral genes, which gives rise to what is known as "pathogen-derived resistance" (PDR). Of the hypothesized mechanisms underlying expression of PDR, post-transcriptional gene silencing has been most extensively investigated in recent years. Despite of the success that virus-resistant cropping of transgenic plants begins to enjoy, in Europe there is still a widespread sentiment against agricultural biotechnologies and the use of genetically modified plants in particular. Yet, experimental evidence is accumulating that, in the case of PDR, the feared risks associated with genetic trasformation are minimal, if not negligible.

Keywords: genetic engineering; transgenic resistance; biotechnology; virus; gene silencing

Notwithsdanding the tremenous efforts spent in the 20th century for combating diseases of plants, these are still a major source of crop losses. However, at the turn of the century plant disease control has undergone what appears to be a veritable revolution, consequent to the application of biotechnological techniques.

The most spectacular advances have been registered in the management of virus diseases against which no chemical control is possibile and preventive measures, such as the elimination of inoculum sources, production of sanitarily improved or virus-free propagative material, control of vectors, pre-immunization (cross-protection), and production of resistant varieties are only partially effective.

Breeding for resistance to viruses and/or to their vectors (JONES 1998) has long been used, but significant results have been obtained with a limited number of crops and viruses. In fact, genes utilized to date for introducing resistance to crop plants are less than 180, whilst cases of effective durable resistance are about a dozen, involving no more than ten viruses (FRASER 1990; KHETARPAL *et al*. 1998).

Major stumbling blocks to conventional breeding for resistance are genetic incompatibility barriers and unavailability of natural resistance genes. However, the use of recombinant DNA and the optimization of plant transformation systems, has opened new very promising ways for the obtention of crop plants that tolerate or resist viral attacks. This presentation will briefly review and discuss some of the aspects of transgenic resistance to plant viruses.

Genetic engineering and plant viruses

The resistance to virus infections obtained by genetic engineering (or GM technology), which is commonly called "non conventional" or "transgenic", is of two types: (i) parasite-derived resistance (PDR), i.e. that

Table 1. Some viral and non viral genes used for inducing transgenic resistance to plant viruses

A. VIRAL GENES
Coding sequences
Capsid protein (CP), movement protein (MP), replicase (POL), genome-linked protein (Vpg), protein 2b (cucumovirus), HC-Pro (potyvirus)
Non coding sequences
Viral sequences made deliberately non coding, antisense RNAs (-RNAs), satellite RNAs (satRNA), defective interfering RNAs (DI-RNA)
B. NON VIRAL GENES
Coding sequences
dsRNA-specific RNase (*pac1*), mammalian 2'–5' oligoadenylate system, plantibodies, ribosome-inhibiting proteins (RIP), RNA-dependent RNA polymerase (RdRp) of plant origin
Non coding sequences
Ribozymes, tRNA suppressors of amber stop codons

type of resistance conferred to a plant by genes isolated from the pathogen's genome, cloned, and engineered into the plant's genome (SANFORD & JOHNSTON 1985); (ii) resistance induced by other exogenous, generally non microbial, DNA sequences.

The most common methods for tranferring foreign genes to plants are biological (transformation mediated by the Ti plasmid of *Agrobacterium tumefaciens*) and physical (particle bombardment or biolistic method). The first technique induces stable and very efficient transformations, delivers intact transgenes in a reduced number of copies but is difficult to apply to some plants (e.g. gramineous). The biolistic method is less efficient, as transformed cells are few and scattered, but seems to operate successfully with all types of plants. The two systems have been combined in the "agrolistic" method, which combines the advantage of particle bombardment with the use of complex plasmids that improve transgene integration in the host genome (HANSEN & CHILTON 1996).

Quite an array of resistance-inducing genes (see reviews by JACQUEMOND & TEPFER 1998; KANIEWSKI & LAWSON 1998; MARTIN 1988) have been vehiculated into crop plants to control viruses (Table 1) and various hypotheses have been offered to explain their modes of action. These now appear to be more complex than originally thought but, for simplicity, can be reconducted to two models: protein-mediated resistance (P-MR) and RNA-mediated resistance (RNA-MR).

Capsid protein-derived resistance is best understood with *Tobacco mosaic virus* (TMV) and tobacco. This resistance is: (i) proportional to the amount of transgene protein expressed and accumulating in the cell; (ii) is more effective when CP is derived from a viral strain that naturally infects the recipient plant (e.g. tomato plants are better protected by *Tomato mosaic virus* CP than that of the very closely related TMV); (iii) can be overcome by inoculation with naked viral RNA. Thus, with TMV, CP-derived resistance may not involve induction of the plant's natural disease resistance system. It may operate in a manner similar to cross-protection, where CP accumulation is thought to interfere with uncoating of virus particles so as to to inhibit the establishment of infection, and with the spread of virus from cell to cell (LECOQ 1988).

With other viruses, CP-derived resistance may have different modes of action. For instance, high levels of resistance to *Potato virus Y* (PVY) and *Tobacco etch virus* (TEV) are shown by transgenic plants notwithstanding the fact that their cells do not accumulate viral CP. This has led to hypothesize that, in these cases, the resistance is due more to the RNA transcribed from the transgene than to its expression product (CP) and therefore, that the same construct can activate more than one mechanism of resistance. Further observations have confirmed the plurality of the type of resistance induced by single CP constructs and shown that the mechanism can be differentially activated in different plant lines (HAMMOND *et al*. 1999).

There are also indications that resistance induced by proteins other that CP may have multiple origins or, as with truncated disfunctional polymerases, an action similar to that of negative dominant mutations (PALUKAITIS & ZAITLIN 1997). The numerous ways

whereby the expression of CP genes *in planta* can interfere with the initial phases of infection, or with its local or systemic spread, or with viral replication, makes it plausible to conclude that any CP (or other viral protein) construct can elicit multiple resistance effects, and that the different mechanisms triggered by the same gene can be expressed differentially in the hosts, perhaps depending on the chromosomal localization of the transgene or on differences in virus-host interactions (HAMMOND *et al.* 1999).

As to RNA-MR, evidence is accumulating that this form of resistance is due essentially to post-transcriptional gene silencing (PTGS), a defence mechanism that appears to be conserved among eukaryotes and has two major traits in common with the immune system: (i) specificity against foreign elements; (ii) ability to amplify and raise a massive response against an invading nucleic acid.

PTGS is used by plants to defend themselves from viral infections, leading to the inactivation of the pathogen's RNA through a sequence-specific degradation process which spreads in the plant, perhaps through a mobile signal made up of small-sized nucleic acids with high sequence homology to the target of silencing (pathogen's RNA) (RATCLIFF *et al.* 1997; SMYTH 1999).

PTGS is thought to account for transgenic resistance to viruses in genetically modified plants (see reviews by MEINS 2000; WATERHOUSE *et al.* 2001; AHLQUIST 2002) thanks to the involvement of host factors such as a plant RNA-dependent RNA polymerase (RdRp) and a plant dsRNase (i.e. "dicer", a ribonuclease III-like nuclease) via two possible non mutually exclusive models referred to as "quantitative" and "qualitative"

The "threshold or quantitative model" is based on the observation that silenced genes have often a transcription level much higher than non silenced genes. In transgenic plants an increase above a certain concentration of cytoplasmic RNA due to the contemporary presence of transgene transcripts (mRNA) and of the RNA of the incoming virus from which the plant is to be protected, triggers the RNA degradation mechanism due to the production by a plant RNA-dependent RNA polymerase (RdRp) of short (-)RNA molecules with sequence highly homologous to that of target RNAs (transgenic mRNA and viral RNA). It ensues that dsRNA molecules are formed, which are processed by dicer into 21–25nt dsRNA fragments called "short interfering RNAs" (siRNAs). These associate with a nuclease complex called RISC (RNA-induced silencing complex) which is targeted to, and cleaves mRNAs (BAULCOMBE 1996; AHLQUIST 2002).

The second mechanism, referred to as "aberrant mRNA or qualitative model" is based on the presence in transgenic plants of aberrant RNAs originated by transgene methylation, or depurination, or other undetermined causes. These RNAs become the preferential template for the synthesis of (–)RNA by the host's RdRp, thus generating dsRNAs that, in turn will stimulate the degradation activity of the plant's dsRNase (BAULCOMBE & ENGLISH 1996).

In transgenic plants, PTGS can be induced by sense-oriented transgenes (S-PTGS), antisense-oriented transgenes (ASGS), transgenes containing inverted repeats (IR-PTGS), and by replicating viruses (VIGS). The resulting dsRNAs can be generated by virus RdRp (VIGS), by transgene transcription (IR-PTGS), or by host RdRp (S-PTGS and ASGS).

RNA-MR mechanisms are also triggered by defective interfering RNAs (DI-RNAs) and satellite RNAs (sat RNAs). These, however, operate via the preferential replication of these small RNA molecules, resulting in a decreased synthesis (down regulation) of genomic RNA, thus in the attenuation of symptoms. With certain virus-satRNAs combinations (e.g. *Cucumber mosaic virus* and its satRNA) transgenic resistance operates also via PTGS (CILLO *et al.* 2001).

Transgenic virus resistance in practice

GM technology for virus resistance makes it possible: (i) selecting the gene to be introduced, in function of the target pathogen and of the type of resistance mechanism wanted; (ii) breaking genetic incompatibility barriers, thus overcoming one of the unsurmountable stumbling blocks to genetic improvement via traditional breeding; (iii) pyramidizing single resistance to diverse disease agents and pests (viruses, fungi, bacteria, insects, nematodes) and combining it with superior qualitative and commercial traits; (iv) cropping under high disease pressure, thus reclaiming to high-value agricultural crops areas that had forcibly been abandoned because of recurrent destructive virus outbreaks; (v) increasing yield and food quality of staple, pulse, and other crops under conditions of medium to high disease pressure. No wonder, then, that much attention has been paid to this technology for practical applications.

To date, P-MR (mostly CP-mediated) has been successfully used against about 50 viruses belonging in no less that 15 different taxa (KANIEWSKI & LAWSON 1998). Commercial crops of papaya, squash, potato, tomato, and tobacco resistant to *Papaya ringspot virus* (PRSV), *Cucumber mosaic virus* (CMV), *Potato*

virus Y (PVY), *Watermelon mosaic virus* (WMV-2), *Zucchini yellow mosaic virus* (ZYMV), *Potato leafroll virus* (PLRV) and *Tobacco mosaic virus* (TMV) are being grown in the USA and China (KANIEWSKI & LAWSON 1998; YIE & TIEN 1998).

A striking example of succesful transgenic field control of the tropical virus PRSV is given by the cultivation of genetically modified papayas under the prohibitive disease pressure conditions that had almost wiped out the industry in Haway and Taiwan (GONSALVES 1998). Much is expected in Africa from rice transgenically resistant to RYMV (PINTO *et al.* 1999), in Italy, from tomatoes engineered for CMV resistance (TOMASSOLI *et al.* 1999), and in Europe from plums and apricots engineered for PPV resistance (RAVELONANDRO *et al.* 2000). Highly encouraging is also the outcome of the first commercial release and cropping of potatoes transgenically resistant to PLRV and PVY in the USA (KANIEWSKI *et al.* 1999).

Viruses fight back

It was recently shown that certain virus-encoded proteins (e.g. potyvirus HC-Pro, cucumovirus protein 2b, potexvirus protein p25, tombusvirus protein p19, tospovirus protein NSs) inhibit host-activated PTGS (ANANDALAKSHIMI *et al.* 1998; BECLIN *et al.* 1998; VOINNET *et a*l. 2000; CARRINGTON *et al.* 2001).

Some of the known PTGS suppressor viruses are CMV, PVY, TMV, TEV, *African cassava mosaic virus (*ACMV) *Narcissus mosaic virus* (NMV), *Nandina virus X* (NVX), *Viola mosaic virus* (VMV), *Tomato bushy stunt virus* (TBSV), *Cymbidium ringspot virus* (CymRSV), and *Tomato spotted wilt virus* (TSWV), but the list is due to grow rapidly.

As it was experimentally ascertained, one of the implications of these findings is that a plant transgenically resistant to any given virus can undergo loss of PDR resistance following infection with one of the PTGS suppressor viruses, e.g. CMV (MITTER *et al.* 2001), PVY (SAVENKOV & VALKONEN 2001), or CymRSV (DI SERIO *et al.* 2002). However, whether and to what extent this mechanism operates under field conditions, thus constituting a real threat, remains to be established.

Public perception of GM technology

In 2001, over 52 million hectares were planted to GM plant in the world. More than one-third of all USA corn land was planted to GM maize, and about three-fourths of land planted with soybean and cotton are GM. As compared to 2001, GM corn acreage has increased by 8%, GM soybean acreage by 7%, and GM cotton acreage by 2%. In the USA, such continued increase in GM crops acreage since the first release in 1996, has been attributed to better public acceptance of biotech products coupled with the continued benefits for the farmers who plant them. By contrast, in the European Union, notwithstanding the undisputable benefits of GM technology, especially when targeted to virus resistance, its use has raised a number of questions on the possible detrimental impact on the environment, agriculture, and human health. Misinformation and counterinformation has generated in the public a widespread "contra" sentiment which has led to a severe penalization of research and may have significant economic consequences in terms of levels of income, wealth, and employment generation (BROOKES 2002).

Taking in account available experimental evidence, the biological risk for the environment, agriculture and health associated with the use of GM plants expressing PDR was critically analysed in a number of papers by individual scientists (see among the others MILLER *et al.* 1997; HAMMOND *et al.* 1999; GALLITELLI & ACCOTTO 2001; MARTELLI 2001 and references therein), Scientific Academies (ANONYMOUS 2000), and Professional Associations (ANONYMOUS 2001). The conclusion was that risks are minimal.

In addition, a recent report by The Royal Society (ANONYMOUS 2002) concludes that: (i) in principle, the allergenic risks posed by GM plants are no greater that those posed by conventionally derived crops; (ii) the risk to human health associated with the use of specific viral DNA sequences in GM plants are negligible; (iii) it is unlikely that transgenic DNA consumption poses any significant risk to human health given the very long history of DNA consumption from a wide variety of sources; (iv) the expression products of viral transgenes are commonly found in infected plants and most ingested DNA is rapidly broken down in the intestinal tract. The small amount that can enter the so-called M-cells is degraded by these cells.

Finally, a review of results from field trials carried out in the framework of research projects financed by the EU since 1984 (KESSLER & ECONOMIDIS 2001) shows that no particular safety or environmental problems are associated with the use of GM plants, that these plants and non GM plants are not inherently risky nor inherently safe, and that GM plants pose no risk beyond the usual uncertainty of conventional breeding.

Notwithstanding the above largely reassuring statements, it seems that a good proportion of the public,

stakeholders, and political decision makers, is still very much against GM technology. In the EU, among other things, difficulties arise from the interpretation and application in a questionable manner of a couple of key principles:

(a) **Precautionary principle**. This principle was born as "precautionary approach" in 1992 at Rio de Janeiro during the United Nation Conference on Environment and Development. An article of the "Rio declaration" states that: "In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation".

In the EU, however, the "precautionary approach" became "precautionary principle", i.e. a principle that can be applied whenever: "based on incomplete or weak scientific evidence or information, it is reasonable to fear the possibile development of potentially dangerous effects for the environment, or for human, animal, or plant health". The EU document uses the precutionary principle as a guide for risk management, separating it from the scientific evaluation of the risk (TORGESEN 2001). No wonder then, if, for the EU, the evaluation of the risk connected with the use of GMOs is a subjective concept, not necessarily based on scientific assessments. This has generated the request for total safety, hence for the utopian "zero risk".

(b) **Substantial equivalence**. Substantial equivalence of edible products from GM and non GM plants refers to composition, nutritional value, level of unwanted components, and type of utilization of the food. The recognition of substantial equivalence is a most controversial issue. Products from plants expressing transgenic virus proteins do not differ in their composition from comparable products from naturally infected plants. Thus, they are substantially equivalent, but, as yet, this is not being recognized.

In conclusion, benefits and risks of GM plants are not certain nor universal, as they can vary over time, with different geographical and environmental situations, and case-by-case. In the evaluation of benefits and risks, the case-by-case approach is essential if any progress is to be made towards the acceptance of GM technology in agriculture by the public and political decision makers. Specific analysis of transgenic resistance to plant viruses would in fact show that, among genetic engineering applications, this is one of the least hazardous for agriculture, environment, and health.

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