Biosafety considerations relevant to virus-resistant transgenic plant, in particular to tomato resistant to CMV

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Abstract

The first plant resistant to the tobacco mosaic virus (TMV) was obtained by expressing a gene encoding the viral coat protein (CP); following this example, the vast majority of virus-resistant transgenic plants (VRTP) subsequently produced express viral sequences (and most of the time those encoding CP), since this strategy has proven to be the most adequate for conferring resistance in the field. The potential risks for the environment associated with VRTP are reviewed. An alternative strategy was explored in order to obtain VRTP, namely the use of satellite RNAs, a natural molecular parasite of the virus, associated with cucumber mosaic virus (CMV); however the risk of mutation into a necrogenic form was found to be excessive for considering any commercial uses of these constructs, even though the protection was effective.

Thus, the most widely used mechanism conferring resistance to viruses in plants remains the introduction of sequences of viral origin, in particular CP genes, which inhibit the development of the pathogens in the plant. However new viral strains can originate through recombination events such as transcapsidation, heterologous encapsidation, complementation. A potential risk associated with VRTP is the transmission by sexual outcrossing of viral resistance genes to plants related to transgenic crops, enhancing the fitness of the wild relative and thus perhaps increasing its weediness. Recombination plays a key role in viral evolution by increasing the variability of its genome. Identification of recombinational events between host-plant sequences and viral genomic RNA raised questions of possible generation of new viral strains bearing properties different from the original ones.

Riassunto

La prima pianta resistente al virus del mosaico del tabacco (TMV) fu ottenuta mediante espressione di un gene codificante per le proteine dell'involucro virale (CP, coat protein); la maggior parte delle piante transgeniche resistenti ai virus (VRTP) prodotte successivamente esprimono sequenze virali (e quasi tutte codificano per CP), essendo questa strategia la più adeguata a conferire resistenza in campo aperto. I rischi potenziali per l'ambiente associati all'uso di piante transgeniche virus-resistenti vengono qui esaminati.

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Una strategia alternativa per ottenere VRTP è stata l'uso di RNA satellite, un parassita molecolare naturale del virus, associato al virus del mosaico del cetriolo (CMV). Si è visto tuttavia che il rischio di mutazioni da una forma non necrogenica a una necrogenica è troppo alto per poter considerare la sua applicazione commerciale, sebbene la protezione sia efficace.

Quindi, il meccanismo di scelta per conferire resistenza ai virus nelle piante è l'introduzione di sequenze di origine virale, in particolare geni CP, capaci di inibire lo sviluppo del patogeno nella pianta. Nuovi ceppi virali si possono comunque originare attraverso eventi di ricombinazione quali transcapsidazione, encapsidazione eterologa e complementazione.

Un rischio potenziale associato alle VRTP è la trasmissione, attraverso incrocio sessuale, dei geni della resistenza al virus alle piante affini a quelle transgeniche, con conseguente aumento della loro capacità di adattamento e quindi anche delle loro proprietà infestanti.

La ricombinazione svolge un ruolo importante nell'evoluzione virale, aumentando la variabilità genomica. L'identificazione di eventi ricombinativi tra sequenze della pianta ospite e l'RNA genomico virale ha sollevato perplessità sulla possibile generazione di nuovi ceppi virali con proprietà diverse da quelli originali.

Strategies for creating virus-resistant transgenic plants

Viral diseases can have a dramatic negative impact on the quantity and quality of plant products, and hence one of the long-standing goals of plant breeding has been the incorporation of virus resistance genes in plant varieties by crosses between resistant and sensitive varieties or closely related sexually-compatible species. There are many cases however, where natural sources of resistance are not available. It is thus not surprising that the description of the first virus-resistant transgenic plants (VRTPs) fifteen years ago was greeted with enormous enthusiasm. These first plants were resistant to *Tobacco mosaic tobamovirus* (TMV) due to expression of a gene encoding the viral coat protein (CP), and since then the vast majority of VRTPs described are ones expressing viral sequences, most often ones encoding a CP (for review see Beachy, 1997). Although numerous strategies for creating virus resistance by expression of nonviral genes have also been described (for review see Robaglia and Tepfer 1996), none has been shown so far to confer adequate field resistance. Thus, here the focus will be on VRTPs expressing viral sequences, since these are the only ones that have been extensively field tested and released commercially. It should be noted however that one of the attractive features of resistance genes from nonviral sources is that they present fewer potential risks, and it should be hoped that truly effective genes of this type will be developed in the not too distant future.

Shortly after the first reports describing VRTPs, articles appeared raising questions of potential impacts that they could have on the environment (for example see Palukaitis 1991; Tepfer 1993; Hull 1994; Tepfer and Balázs 1997; Teycheney and Tepfer 1999). The current state of knowledge concerning these biosafety questions, as known at the time of writing (Oct. 2000), will be reviewed briefly here, with a particular mention of features that might be specifically pertinent to transgenic tomato resistant to *Cucumber mosaic cucumovirus* (CMV). A more complete and recent treatment of biosafety questions raised by VRTPs can be consulted for further information (Tepfer 2002).

Potential risks associated with virus-resistant transgenic plants

Satellite RNAs associated with CMV

Certain strains of CMV include an additional small RNA of 300-400 nt, termed satellite RNA, which is a natural molecular parasite of the virus. Satellite RNAs are replicated by the viral replicase, are encapsidated in CMV particles, and are transmitted by the virus' natural aphid vectors. CMV satellite RNAs are thus entirely dependent on the virus, but they are not necessary for any step in the viral cycle. In many cases, the presence of

a satellite RNA causes a notable decrease in CMV titer and greatly attenuates the gravity of infection. When transgenic plants expressing genes including satellite RNA sequences are infected with CMV, the satellite RNA is amplified to high levels, and the plants express only very mild symptoms. Such plants were the first CMV-resistant transgenic plants described. Nonetheless, this strategy has not been pursued further. The reason for this is that certain CMV satellite RNAs cause symptom worsening rather than attenuation. For instance, the catastrophic epidemics of lethal necrosis that devastated tomato fields in southern Italy and Spain in the late 1980s were due to CMV strains bearing necrogenic satellite RNAs. When the necrogenic satellite RNAs were carefully characterized, and the nucleotide positions responsible for necrosis identified, it was found that in some cases a single point mutation could be sufficient to transform a beneficial satellite RNA into a necrogenic one. It was concluded that the risk of mutation of a non-necrogenic satellite RNA into a necrogenic form was too great to consider commercial use of satellite RNA genes, even though the protection obtained was remarkably effective. It is worth noting that this is one of the very few cases where an interesting application of transgenic plants was abandoned because of a scientific consensus about potential risks. For a more complete, in-depth review of the use of CMV satellite RNAs in transgenic plants and of the associated biosafety issues, see Jacquemond and Tepfer, 1998.

Transgene complementation of a virus function, including heterologous encapsidation

Synthesis in a transgenic plant of any molecule of viral origin could in principle complement an essential function for a virus infecting the plant. For instance, it has been long known that there is an important synergy between *Potato potexvirus X* (PVX) and several potyviruses, when plants are simultaneously infected with both viruses. The underlying mechanism of this particular form of synergy has only recently been elucidated, when it was shown that the potyviral helper-component-protease (P1-HC-Pro) protein is responsible for the observed effect (Anandalakshmi et al., 1998), and indeed transgenic plants expressing P1-HC-Pro exhibit synergistic enhancement of PVX infection (Vance et al., 1995). This particular instance of synergy in transgenic plants is not important from a biosafety point of view, since P1-HC-Pro genes have not been used to confer resistance.

Concerning the widely used CP genes, the issue is much more complex. It has been shown that when plants expressing the *Plum pox potyvirus* (PPV) CP are infected with a second potyvirus, a strain of *Zucchini yellow mosaic potyvirus* (ZYMV) that is defective for transmission by aphids, the latter

virus can be transmitted by aphids to other plants (Lecoq et al., 1993). In this particular case, this complementation is of no particular epidemiological impact, since the ZYMV, once transmitted to plants that do no express CP is no longer aphid-transmissible, and in addition, nearly all wild-type strains of ZYMY are already normally transmissible by aphids. When a similar system of complementation for aphid transmission was examined under field conditions, very little ZYMV transmission was observed, and even among the transgenic plants expressing the CP gene there was very limited spread of the virus, confirming the low level of epidemiological impact of heterologous encapsidation in this biological system (Fuchs et al., 1999). However, although these results show that the epidemiological impact of heterologous encapsidation is slight in potyviruses, it should be noted that it is also possible to obtain resistance with CP genes encoding a protein that has been modified to prevent its interaction with aphids, thus eliminating this source of potential risk (Jacquet et al., 1998; Varrelmann and Maiss, 2000).

There are a few cases where it has long been known that CP complementation plays an essential role in the epidemiology of a virus disease, and where as a result complementation by a transgene-encoded CP could have a significant epidemiological effect. The best know of these concerns the umbraviruses, which can cause serious diseases, but which are totally dependent on an associated luteovirus for CP, and as a result also for aphid transmission (Robinson et al., 1999). As had been predicted (see for instance Tepfer, 1993), these authors have shown that indeed an umbravirus can be encapsidated in plants that are transgenic for a luteovirus CP. Fortunately, as is the case with potyvirus CP transgenes, here there is also a simple means of preventing transcapsidation from having an effect on virus transmission. It has been shown that the vector aphids only recognize a minor luteovirus CP component that has an additional readthrough domain (Gray and Bannerjee, 1998). The sequence encoding the readthrough domain can simply be deleted from the transgene in order to eliminate this potentially undesirable effect of heterologous encapsidation (Robinson et al., 1999).

Plant-to-plant gene flow

A point of great interest, and one where current scientific knowledge is particularly sparse, is that of potential effects of transmission by sexual outcrossing of virus resistance genes to plants related to the transgenic crop. This of course is not a problem with tomato or many other crops when cultivated in Europe, but there are several cases where this question merits consideration. For instance, oilseed rape (*Brassica napus*) is an

allotetraploid species whose genome is composed of that of two diploid species, cabbage (*B. oleracea*) and turnip (*B. rapa*). As a result, oilseed rape is sexually compatible with the other two species, including their wild forms. In this situation, the question that is of interest is whether transmission of a transgene conferring virus resistance from the crop plant to the wild relative could enhance the fitness of the latter, and thus perhaps increase its weediness. It has recently been show that virus infection has an effect on survival, growth and reproduction in wild cabbage (Maskell et al., 1999; Raybould et al., 2000). However, only further studies showing whether these aspects of the plants' life cycle are limiting in terms of population growth will show if a virus resistance gene could lead to ecological release of wild cabbage and thus increase its potential invasiveness. It will be even more interesting to carry out similar studies with wild *B. rapa* populations, since this species is much more widespread in its distribution, particularly in northern Europe. Similar questions are being studies regarding transmission of a transgene conferring resistance to *Beet necrotic yellow vein furovirus* (BNYVV) from sugar beet to wild beet (Pohl-Orf et al., 2000). In this case, the wild form seems to be partially resistant to the virus (and has been used in plant breeding as a source of resistance), and no virus was found in the wild populations, perhaps because of the absence of the vector organism. It will be of great interest to extend these studies to the weedy beets that are thought to derive from crosses between cultivated and wild forms of the species (Boudry et al., 1993), since they are already quite invasive in certain regions of Europe, and little or nothing is known about their virus resistance/susceptibility. If weed beets prove to be sensitive to BNYVV, then this raises the question of whether a BNYVV resistance trangene could increase the weediness of these already problematic plants.

Recombination

Virus recombination is universally considered to play a key role in virus evolution by increasing the variability of viral genomes. The favored hypothesis for the mechanism of virus recombination is by copy-choice, which would occur when the viral replicase changes templates during synthesis of the viral genome. As an increasing number of plant virus genomes have been analyzed, it became abundantly clear that indeed recombination has occurred frequently during virus evolution (for review see Aaziz and Tepfer, 1999a; Rubio et al., 1999). Among the recombinant genomes observed, there are also clear cases where host sequences have been inserted into the viral genome. These results clearly suggested that indeed recombination could be expected to occur upon virus infection of

transgenic plants expressing viral sequences. This has been confirmed experimentally in transgenic plants expressing sequences from several viruses: cauliflower mosaic caulimovirus (Gal et al. 1992), *Cowpea chlorotic mottle bromovirus* (Greene and Allison 1994), *African cassava mosaic begomovirus* (Frischmuth and Stanley 1998), *Tomato bushy stunt tombusvirus* (Borja et al 1999), and PPV (Varrelmann et al. 2000).

If we assume that recombination will occur in VRTPs, the critical question is that of the impact that this will have. In particular, will recombination in transgenic plants lead to the creation of viral genomes with novel biological properties, such as changes in host range or virulence? This is a particularly difficult question to answer at present, since recombination between viruses is a perfectly normal natural phenomenon, and thus the pertinent question is rather whether the recombinant viruses that could occur in transgenic plants are different from those that occur in non-transgenic plants when they are infected simultaneously by more than one virus. Only recently have experimental systems been developed for studying virus recombination in non-transgenic plants, with one study of recombination between complementary mutants of *Brome mosaic bromovirus* (Bruyère et al. 2000), and one of recombination between CMV and the related *Tomato aspermy cucumovirus* (TAV) (Aaziz and Tepfer 1999b). The latter study in particular showed that recombination between these two viral species was strongly limited to a hot spot corresponding to an area of particularly high sequence identity between CMV and TAV. The next step will be to compare the recombinant viruses observed in transgenic plants with those observed in non-transgenic ones, since only if there are differences would there be reason to believe that there are particular risks associated with recombination in VRTPs. Since the above studies have been carried out entirely in the greenhouse, it will also be important to carry out field studies, in order to validate the results obtained under confined conditions.

There are also strategies that have been shown to be useful for limiting the frequency of recombination in plants expressing viral sequences. For instance, it has been shown that the site of initiation of RNA synthesis located at the 3' end of the viral genome can be recognized by the viral replicase within the transcript of a viral transgene (Teycheney et al. 2000), and that deletion of this region from the transgene can indeed reduce the frequency of appearance of recombinant viruses (Greene and Allison, 1996). Presumably, if the hot spot for recombination identified by Aaziz and Tepfer (1999b) were removed, this would also reduce the frequency of recombination in this region. However, complex recombination products, which must have required more than one template switch, have also been

observed in transgenic plants (Borja et al 1999), showing the insufficiency of strategies that simply attempt to reduce the frequency of recombination.

There are two principle mechanisms of resistance in VRTPs. In certain cases, resistance is due to the transgene-encoded protein interfering with some essential viral function, while in other cases, expression of viral sequences from the transgene induces sequence-specific degradation of the transgene RNA and any other RNA with identical sequences, such as those of the corresponding viral genome (Beachy, 1997). In the latter case, resistance is based on a mechanism similar to that of post-transcriptional gene silencing (PTGS). In this case one would expect that there would be very little opportunity for recombination between the transgene-derived RNA and that of a viral genome, since the former accumulates to extremely low levels. The idea that this type of RNA-mediated resistance is an interesting one that merits further exploration. There are however some possible difficulties that need evaluation. One important point is that certain plant viruses can suppress PTGS, including CMV and the potyviruses (Voinnet et al., 1999), many of which can infect tomato. This may in part explain why among the numerous publications describing CMV-resistant VRTPs, there is only one where the resistance appears to be of the RNA-mediated type (Carr, et al., 1994). In addition, there is a considerable risk that the resistance could be lost if the plants were infected with another virus that suppresses PTGS. For instance, one might hypothesize that tomato plants that are resistant to CMV by an RNA-mediated mechanism would lose this resistance if they were infected with any of the potyviruses that frequently infect tomato, such as *Potato potyvirus Y* (PVY). In this regard, it is of great interest to note that when plants resistant to PVY^{O} via a PTGS-mediated mechanism are infected with PVY^{N} strains, they are sensitive to the latter, and reversion of the PTGS of the viral transgene was observed (Mäki-Valkama et al., 2000).

The particular case of CMV-resistant transgenic tomato

As mentioned above, tomato crops are often strongly affected by serious epidemics of CMV infection. For this reason, there has been considerable interest in creating transgenic CMV-resistant lines, particularly since until very recently there were no known sources of resistance to CMV in plants compatible with tomato. Although it was reported this year for the first time that a gene conferring resistance to certain CMV strains of could be introgressed into tomato from a related species, *Lycopersicon chilense* (Stamova and Chetelat, 2000), the resistance is not effective against all CMV strains, and in any case, years of plant breeding would be required to

introduce this gene into commercial tomato varieties.
Some of the potential risks described above do not present serious problems with regard to CMV-resistant transgenic tomato. There is no evidence that a CMV CP gene would cause problems of synergy, or that heterologous encapsidation with CMV CP could have epidemiological effects. For tomato, plant-to-plant gene flow will not be a problem, except in parts of South America, since there are no plant species compatible with tomato elsewhere, and gene flow between tomato plants is unlikely, since this species is strongly autogamous. In contrast, the questions concerning recombination are certainly pertinent for CMV and tomato, but it should be hoped that the potential effects of recombination in plants expressing CMV CP genes will be adequately evaluated in the near future. In the longer term, the critical determinant question that will arise once the potential ecological risks have been evaluated will be that of how this evaluation fits into a larger one including economic, political, and social issues.

Areas recommended for further research

From the above discussion it is clear that there are still areas where futher research would be desireable. This is the case for the evaluation of the potential impact of recombination in VRTPs, and also for that of plant-to-plant gene flow, in the cases where wild plants that are sexually compatible with the VRTPs are present. Another area that merits greater attention that has not been discussed above is that of the durability of the resistance that is obtained with VRTPs. It is essential to test whether a given VRTP is effectively resistant to the range of strains of the target virus present in the area where the crop is tested. In addition, since they have attractive features from a biosafety perspective, it would be particularly interesting to explore the degree to which infection by PTGS-breaking viruses could interfere with RNA-mediated resistance.

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