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New strategies for virus control

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Abstract

New strategies of importance for the control of plant viruses in crops are discussed. The use of homologous recombination for precise transgene insertions via the use of the zinc-finger binding motif recombinase enzymes is possible, with advantages of public acceptance, precision and increased predictability. The use of genes from within the same species for the generation of cisgenic plants is a methodology aimed to improve public acceptance. Technology is available to produce transgenic plants bearing artificial miRNA genes to produce virus-resistant plants. There is a possibility to produce transgenic gene-silencing rootstocks which transmit virus resistance to the non-transgenic grafted scion. There are several useful methodologies for virus control not using transgenic plants, including induction of virus resistance by direct delivery of exogenously synthesized double stranded RNAs. Epidemiological advances in non-chemical control of vectors and the viruses they bear include the use of UV-absorbing covers (plastic sheeting or nets) and the use of a crop/host-free period to break a virus disease cycle. Deeper understanding of the molecular interactions between the host plant and virus is the prerequisite of the further developments.

1. Transgenic technologies

Transgenic technology based on protein expression and RNAi technology is widely used to achieve virus resistant or tolerant plants (see EG3 report for details). However, this technology is under the heavy pressure of safety issues and public fears (such as possible allergenic reactions, undesired recombination events, transgene escape, etc.). Nevertheless, production of genetically modified plants holds the promise of virus resistant plants against the constant appearance of new viruses. We can not afford to ignore this technological approach since we will lose an invaluable tool to cope with future challenges. Efforts must be made to make this technology safe and publicly acceptable. Regulatory costs imposed by the public acceptance issues are now a major problem in commercialization of transgenic plants.

One of the general challenges of plant transgenic technology is to control transgene insertion into defined genome sites. Such a technology would ease regulatory issues, increase efficiency, improve control of gene expression and increase predictability of responses. A developing technology based on the targeted introduction of transgene with the help of zinc-finger endonucleases could help to achieve this goal (Kumar et al., 2006). Coordinated zinc-finger endonuclease targeted double stranded breaks in genomic DNA facilitate homologous recombination, and thus provide a promising tool for precise transgene insertions. The zinc-finger binding motif can be modified and the usage of multiple zinc-fingers having different recognition specificities can allow the targeting of different genomic sites (Camenisch et al., 2008). Another approach which could make transgenic plants more acceptable for the public is the generation of cisgenic plants where the introduced gene or DNA fragment and its regulatory elements derive from sexually compatible relatives: thus the technology does not cross the species border (Jacobsen and Schouten, 2007). Such changes in the genome could be considered safer than the introduction of a transgene, since the modification does not alter the gene set of a particular species. However, plant viruses infect all crop species, and the consumption of plant viruses is not considered problematic. Therefore transgenic products bearing viral sequences have been de-regulated (permitted) for sale, and are now available on the US market (Fuchs and Gonsalves, 2007).

1.1 Artificial miRNA

Utilization of artificial miRNAs in transgenic plants can provide effective virus resistance. MiRNAs are short (21-25 nt long), non-translatable, RNA molecules which negatively regulate the expression of their target genes in plants. Recent studies demonstrated that miRNA precursor molecules can be modified to produce artificial miRNAs targeting viruses (Niu et al., 2006; Qu et al., 2007). The artificial miRNA-bearing virus resistant plant can be considered safer than the traditional transgenic plants since it expresses a slightly modified non-coding precursor molecule which produces the 21-25 nt long virus-targeting miRNA. The short size and non-translatable nature of the mature artificial miRNA eliminates the potential undesired recombination events and concerns associated with protein production in transgenic plants. It has been shown that infection of mutated Plum pox virus chimeras carrying recognition sites of endogenous miRNAs was inhibited, although mutant viruses evaded the inhibitory effect of miRNA by accumulating mutations in the miRNA recognition sites (Simon-Mateo and Garcia, 2006). Moreover, artificial miRNA-based technology is versatile, allowing the optimal design of miRNA recognition site on the virus genome. Targeting highly conserved region(s) of the virus genome characteristic for a virus family can inhibit virus multiplication and also confer broader resistance. The flexibility of the system also means that it can also be possible to engineer of multiple virus resistant plants by introducing artificial miRNA targeting more than one virus family. Another advantage of this technology is that it works effectively at low temperatures where production of the small interfering RNA (produced by the traditional dsRNA-based technology) can be severely inhibited (Szittyá et al., 2003). The potentially high level of miRNA production and probable lessened effect of viral suppressor proteins on the processing of the pre-miRNA may be an added advantage for this strategy. The clear advantages of artificial miRNA-mediated virus resistance make this technology a promising tool for future research.

1.2 Possible adverse effects

The strategy of production of virus-resistant transgenic plants by expressing viral sequences as a Dicer-substrate via "hairpin RNA" (Smith et al., 2000) has now been used successfully many times (see Prins et al., 2008). However, the possibility of adverse effects in plants expressing a high level of siRNAs without the presence of a viral suppressor (as in natural virus infections) should be evaluated, since the viral siRNAs can possibly act, for example, as primers for DNA synthesis. Complementarity of viral to host sequences should be subject of careful selection and testing for side effects in each virus-host pair.

A detailed understanding of virus-host interaction is necessary. Identification of host factors interacting with viral proteins or the viral or viroid genome will help elucidate all aspects of the interaction, and such host genes are a potential source of resistance genes, as has already been shown for several translation initiation factors. Such genes can be identified in a mutant form using tilling approaches, and mutant plants evaluated for resistance.

1.3 Grafting

There is now an increasing use in grafting of vegetable crops (watermelon, melon, cucumber, tomato, eggplant, pepper) onto rootstocks resistant to soil diseases (Lee, 1994; Moffat, 2001; Lee and Oda, 2003), especially in the Mediterranean area. Developed industrially originally in Korea and Japan, grafting of herbaceous vegetable crops is now widespread in intensive agriculture; rootstock cultivars are bred and sold (Lee and Oda, 2003). Large investments have been made in the development of industrial grafting robot technologies (Kurata, 1994). Transgenic rootstocks can protect non-transgenic scions from infection by a soil-borne virus (Gal-On et al., 2005), although the resistance was not transmitted from stock to scion, probably as siRNA accumulation was not observed.

The ability of RNAi in plants to function non-cell autonomously may also hold promises in future applications (Ding and Voinnet, 2007). Local induction of double stranded RNA-mediated RNAi was shown to be associated with generation of mobile signals regulating target expression in distant region of the plant. Similarly, a recent

study describes the long distance movement and activity of a miRNA (Pant et al., 2008). Moreover, miRNAs have been identified in vascular system of the plants further supporting their possible function as mobile regulatory agents (Yoo et al., 2004; Valoczi et al., 2006), and are potential mobile virus resistance factors.

In several cases there have been successful silencing of endogenous genes or transgenes transmitted by a stock to a scion (Palauqui et al., 1997; see Kalantidis, 2004; Shaharuddin et al., 2006). Although the exact nature of the transmissible factor is unknown, it is assumed to include small interfering RNA. If such a transgenic virus resistance by siRNA (from "hairpin" constructs) or miRNA could be transmitted from the stock to the scion then the benefits would be great both for vegetable and tree crops. Such RNAi technology may allow the use of rootstock as the source of virus resistance in the future allowing the scion to be transgene-free. Moreover, as one conventional current resistant rootstock serves many different scion cultivars, so a single transgenic virus-resistant stock could protect many different scions. Such technology could be of great importance in any grafted tree crop (e.g. grape, citrus), and could be potentially used for virus eradication therapy in infected material.

2. Technologies not using transgenic plants

2.1 Induction of virus resistance by direct delivery of exogenously synthesized double stranded RNAs

An alternative method to utilize the power of RNA interference and evade the use of transgenic plants is the direct delivery of RNA interference inducer long dsRNAs on the surface and cells of the leaves (Tenllado and Diaz-Ruiz, 2001; Tenllado et al., 2004). It has been showed that rub inoculation of leaves with a mixture containing infectious viral RNA and virus specific dsRNA resulted in the inhibition of viral replication. The inhibitory effect of the exogenously added double stranded RNA has been demonstrated to be efficient against different viruses close to the site of the

inoculation. To reduce the cost of this methodology the inducer double stranded RNA can be produced in bacterial expression system (Tenllado et al., 2003). Crude extract prepared from dsRNA-expressing bacteria was demonstrated to inhibit virus infection when sprayed onto the surface of the leaves. A major problem with this approach is to maintain the effectiveness of the signal. The suitability of this approach to be delivered with spraying may open the way of large-scale industrial application in future. This method is in an early phase of development and needs further efforts to prove effectiveness and safety.

2.2 Epidemiology

We feel that despite the great progress made in the production of transgenic virus-resistant crops that more conventional methods of plant protection also need to be the focus of constant research. Additionally, such methods do not have the public acceptance issues that are the current bugbear for GMO crops in Europe. Moreover, the implementation of epidemiological results may be much faster than the development of new transgenic crops. One has also to be aware of the need for reduction in pesticide use. The use of epidemiological knowledge may enable crop production in difficult circumstances. We wish to highlight two interesting cases of the application of epidemiology in the aid of virus-free crop production, in the absence of pesticide use against the insect vectors.

2.3 UV-absorbing covers

There is increasing use of protected environments for the production of vegetable crops. The use of UV-absorbing covers (on greenhouses or netting) has a dramatic impact on both the penetration and movement of the insect vectors inside the covered area, and greatly reduces viral transmission (Moffat, 2001; Antignus et al., 1996, 1998, 2001; Chyzik et al., 2001; Antignus 2001, 2007). The UV-A region (320-400 nm) is of critical importance for the orientation of many agriculturally important insects. The use of 50-mesh nets supplemented with UV-absorbing material provides enhanced protection against insect and virus (Chyzik et al., 2001; Antignus 2001, 2007). In Israel, a whitefly/geminivirus plagued area, such plastic covers have been

of great assistance to the farmers, enabling crop production where previously this was very difficult. This is a revolutionary change in agriculture (Moffat, 2001).

2.4 Crop/host-free period

It is common practice in Mediterranean and similar warm climates for crops to be grown in a patchwork, so that there are host plants present year round in a given area, acting as a reservoir of virus and vector. Even in temperate climates there may be two plantings a year, so that infection of a new crop may proceed from the older. The concept behind the crop/host-free period is to break the virus disease cycle in vegetable crops, with a period of several weeks when there are no crop/host plants for the insect vectors (or sources of inoculum), resulting in a drastic reduction in the vector population and virus inoculum. The concept was pioneered in the 1950s in the Salinas Valley of California, where one of the recommended control measures for Lettuce mosaic virus in lettuce was a lettuce-free period of several weeks before planting (Wisler and Duffus, 2000), continuing to the present. A similar control measure was effective for sugar beet against the sugar beet virus yellows disease throughout California (Wisler and Duffus 2000). The process was also effective in the isolated Arava desert in southern Israel, and broke the cycle of virus epidemics in cucurbit, pepper and tomato crops (Ucko et al., 1998). With enforcement by regulation this provided a 12-year period without virus epidemics (Ucko et al., 1998), continuing to the present day. In the Dominican Republic epidemics of tomato yellow leaf curl virus resulted in catastrophic losses to industrial tomato production. Enforcing a mandatory 3-month whitefly host-free period (tomato, common bean, cucurbits, eggplant, pepper) greatly reduced subsequent epidemics (Salati et al., 2002). This approach is epidemiology-based to define both disease sources in crop and wild plants, as well as vector host plants (Ucko et al., 1998; Wisler and Duffus, 2000; Salati et al., 2002). Such a concept could be applied to wide areas in southern Europe and the Mediterranean region.

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