Volume 281, number 1,2, 1–8 FEBS 09562 April 1991

ADONIS 001457939100271 1

Review Letter

Strategies for expression of foreign genes in plants

Potential use of engineered viruses*

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Received 14 January 1991; revised version received 8 February 1991

Advances in gene transfer techniques for higher plants have already permitted important achievements towards crop protection and improvement using recombinant DNA technology. Besides plant genetic engineering, the possible use of plant viruses to express foreign genes could be of considerable interest to plant biotechnology. However, insuring containment of engineered viruses for environmental use is an important safety issue that must be addressed.

Plant biotechnology; Recombinant DNA; Gene transfer; Gene expression; Plant viruses; Plant viral vectors

1. INTRODUCTION

One major focus of plant biotechnology is directed towards increasing plant productivity by reducing loss in crop yield due to weeds or pests such as viruses, insects, bacteria, fungi or nematodes. Another aim consists in improving crop resistance to stress conditions. It would also be highly desirable for example to modify crops in order to enlarge both spectrum and composition of plant products: i.e. starches with improved texture and storage properties, specific oils lacking particular fatty acids, proteins with a nutritionally balanced amino acid composition or plant polymers such as celluloses, rubber or waxes. Plants also offer the potential for production of foreign proteins relevant to human health: i.e. neuropeptides, growth hormones, blood factors or antibodies. The perspective of engineering nitrogen-fixing capacity into plants is another challenging issue that would be of great economic importance.

Towards crop modification using recombinant DNA technology to attain such goals, tremendous progress has been made in the development of gene transfer methods for higher plants. As an alternative to stable plant transformation, the use of plant viruses to express foreign genes could also greatly contribute to plant biotechnology.

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* Dedicated to Dr François Chapeville and Dr Anne-Lise Haenni with all our admiration and gratitude

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Abbreviations: *Ac*, activator; AlMV, alfalfa mosaic virus; BMV, brome mosaic virus; BNYVV, beet necrotic yellow vein virus; BSMV, barley stripe mosaic virus; CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; CCMV, cowpea chlorotic mottle virus; CHS, chalcone synthase; CLV, cassava latent virus; CMV, cucumber mosaic virus; CP, coat protein; CPMV, cowpea mosaic virus; CyRSV, cymbidium ringspot virus; DHFR, dihydrofolate reductase; *Ds*, dissociation; ds, double-stranded; GUS, β-glucuronidase; LUC, luciferase; MSV, maize streak virus; MTII, metallothionein II; NOS, nopaline synthase; NPTII, neomycin phosphotransferase; PAT, phosphinotricin acetyl transferase; PEBV, pea early browning virus; PG, polygalacturonidase; PVS, potato virus S; PPV, plum pox virus; PVX, potato virus X; PVY, potato virus Y; ss, single-stranded; TBSV, tomato bushy stunt virus; TCV, turnip crinkle virus; TEV, tobacco etch virus; TGMV, tomato golden mosaic virus; TMV, tobacco mosaic virus; TRV, tobacco rattle virus; TSV, tobacco streak virus; TVMV, tobacco vein mottling virus; TYMV, turnip yellow mosaic virus; WCIMV, white clover mosaic virus; WDV, wheat dwarf virus

2. GENE TRANSFER METHODS FOR HIGHER PLANTS

2.1. Agrobacterium-*mediated gene transfer*

The ability of *Agrobacterium tumefaciens* to transfer and integrate into the chromosomes of plants the genetic information present on the T-DNA of its Ti

plasmid in tumor development has been exploited to set up a simple and efficient gene transfer method for higher plants. Several detailed reviews on this technique have been published [1–4] and only a brief practical description is given here.

Usually, plant transformation vectors contain origins of replication functional in *Agrobacterium* and in *Escherichia coli* as well as an antibiotic resistance gene for selection in bacteria. Between the left and right borders of the T-DNA, a second selectable marker gene (i.e. encoding antibiotic or herbicide resistance) that will be functional only upon transfer into plant cells, and the foreign gene to be transferred are also present. After successful cloning in *E. coli*, the vector is introduced into an *Agrobacterium* strain harboring a disarmed Ti (D-Ti) plasmid (with T-DNA deleted) and that is then used to inoculate plant explants or tissues. The virulence gene products of the D-Ti allow transfer into plant cells of the DNA that lies between the T-DNA borders in the transformation vector and insert it into one of the plant chromosomes. The subsequent expression of the second selectable marker gene allows selection of transformed plant cells during plant regeneration.

This method now makes it possible to genetically modify many herbacious and woody dicotyledonous plant species (reviewed in [5]).

2.2. *Direct gene transfer*

Direct gene transfer methods are being developed since difficulties have been encountered in applying *Agrobacterium*-mediated gene transfer to plants of major economic importance including cereals (reviewed in [6]). They consist in free DNA delivery into plant cells using techniques such as (i) fusion of protoplasts with liposomes containing DNA [7], (ii) microinjection [8], (iii) calcium phosphate and/or polyethylene glycol treatment of protoplasts in the presence of DNA [9], and (iv) electroporation [10]. Unfortunately, it appears that wide application of these techniques is limited by difficulties in regenerating plants from single transformed cells. In this respect, the latest technique consisting in free DNA delivery into plant cells by particle gun bombardment [11] looks very promising since it makes it possible to transform cells that are competent for regeneration within intact tissues.

Taking together all these developments in gene transfer techniques and the efforts devoted to regenerate plants from cells or tissues, it seems likely that major crops will be amenable to modifications in the near future ([12] and references therein). Finally, the possibility of gene targeting, via homologous recombination [13–15] or by site specific recombination using the bacteriophage P1-derived lox P-Cre system for example [16,17], would have an important impact in designing future strategies for plant genetic engineering.

3. APPLICATIONS OF *Agrobacterium*-MEDIATED GENE TRANSFER TO CROP PROTECTION OR IMPROVEMENT

3.1. *Herbicide tolerance*

Since herbicides applied against weeds often lack selectivity, limiting their use to preemergence applications, efforts have been devoted to modifying crop plants to become resistant to broad-spectrum herbicides. Toward this aim, three different approaches have been used: (i) overproduction of the target enzyme, (ii) expression of an altered form of the target enzyme that is less sensitive to the herbicides or (iii) introduction of a gene encoding an enzyme for the detoxification/degradation of the herbicide (reviewed in [18,19]). Using various genes isolated from both plants and microorganisms and either one of these strategies, plants resistant to herbicides such as atrazine [20], bromoxynil [21], 2,4-D [22,23], glyphosate [24,25], phosphinothricin [26,27] or sulfonylureas [28–30] have been obtained.

3.2. *Viral disease resistance*

Cross-protection is being practised to protect crops against viruses, satellites or viroids. Briefly, it consists in inoculating plants with mild strains of the pathogen to prevent subsequent more virulent strains of the same pathogen from infecting the plants and causing severe disease (reviewed in [31]). Since there are many disadvantages to the widespread use of cross-protection in agriculture, it was most interesting to observe that plants expressing the CP gene of TMV became resistant to TMV [32]. The excellent virus disease control obtained in field testing experiments [33] indicates that this strategy could have potential applications in agriculture. Resistance mediated by CP has subsequently been observed for many other viruses (reviewed in [34]) such as AIMV [35–37], CMV [38], PEBV [39], PVS [40], PVX [41–43], PVY [43,44], TEV [44], TRV [37,39] and TSV [45]. Limited resistance to CMV was also reported in plants expressing the antisense RNA corresponding to the CP gene [38]. More recently, plants were transformed with the 54 kDa nonstructural protein gene of TMV in order to assess its possible function. Strikingly, such plants showed complete resistance to TMV [46]. Attenuation of disease symptoms due to CMV and TRV was also observed in plants expressing satellite RNAs of these viruses [47,48]. However, potential application of this latter approach remains questionable since satellite RNAs might 'escape' during viral infection of such plants and infect other crops on which they might provoke necrotic diseases. Finally, some of the other strategies currently considered to engineer viral disease resistance in plants consist in the possible use of the promoters for replication of viral genomes [49,50] or engineered defective interfering RNAs [51] to interfere with

replication of the viral genome, or the use of ribozymes to selectively cleave viral RNAs at specific sites [52].

3.3. *Insect resistance*

Due to world-wide losses caused by insect damage and heavy costs of protective treatments, success in engineering insect resistance in plants represents another important achievement. Indeed, it was shown that plants transformed with the gene encoding *Bacillus thuringiensis* endotoxin (insect control protein) present insecticidal activities and acquire insect resistance [53–55]. The excellent insect control observed in initial field testing experiments indicates that this strategy could have potential applications in agriculture [56]. Other approaches have consisted in exploiting natural defense mechanisms that exist in plants against insect attack. One such mechanism involves trypsin inhibitors (from cowpea) and it was found that plants transformed with a gene encoding such an inhibitor become resistant to insect attack [57]. Growth of insect larvae feeding on leaves of transgenic plants expressing proteinase inhibitor II genes (from potato or tomato) was also retarded [58].

3.4. *Modulation of gene expression towards crop improvement*

With advances in identification and isolation of plant genes and characterization of the sequences required for subtle temporal and spatial gene regulation, it is becoming possible to precisely modulate gene expression in specific tissues to alter certain traits during plant growth and development. For instance, expression in tobacco and oilseed rape plants of chimeric RNase genes, under the control of the 5′ region of a tobacco tapetum organ-specific gene, has permitted selective destruction of the tapetum during anther development and prevented pollen formation thereby leading to male sterility [59]. These results represent the first important contribution for hybrid seed production towards crop improvement.

The antisense RNA approach was applied to repress the expression of introduced foreign genes encoding CAT [60,61], GUS [62], NOS [63,64] or PAT [65], as well as of endogenous plant genes coding for CHS [66,67] or the '10 kDa protein' of the photosystem II [68]. Initial efforts to apply this strategy to crop improvement have focussed on tomato fruit softening during ripening (reviewed in [69]). Since PG has been implicated as one of the key enzymes involved in this process, the antisense RNA approach was used to inhibit PG gene expression by as much as 90% at the level of both PG mRNA and its enzyme product. Unfortunately, it appears that even low levels of PG activity must be sufficient for fruit softening since it was not affected [70–72]. Finally, in addition to antisense RNAs, the use of ribozymes to specifically cleave target mRNAs [73,74] or the production of antibodies in plants [75,76] could also be exploited for plant biotechnology.

4. VIRAL GENOME-MEDIATED FOREIGN GENE EXPRESSION IN PLANTS

Plant virology has already made a major contribution to plant genetic engineering in providing tools derived from viral genomes, such as the 35 S promoter from CaMV, for gene constructs. Besides stable plant transformation, foreign gene expression in plants via engineered viruses would also be of considerable interest to biotechnology for the following attractive features. (i) Systemic infection by viruses of whole plants precludes the need for any difficult and time-consuming transformation and regeneration processes. (ii) Any foreign gene inserted into a viral genome would be amplified upon replication since viruses multiply as autonomous entities to high copy numbers within a plant cell. (iii) Foreign gene expression mediated by viral genomes would not encounter the 'position' effect often observed upon random integration of a gene into a plant chromosome. (iv) The use of engineered viruses could provide flexibility in rapidly changing the genes to be expressed and (v) would offer the possibility to decide when to perform virus inoculations so as to express the gene of interest at a given stage of plant growth and development. (vi) Finally, foreign gene expression mediated by viral genomes could provide a valuable means to rapidly (within a few days after plant inoculations) investigate targeting or functionality of engineered proteins. Thus, it could be very interesting to engineer plant viral genomes to serve as expression vectors (reviewed in [77–79]).

4.1. *DNA virus genome-derived expression vectors*

Much initial interest in construction of expression vectors based on the genome of plant viruses was focussed on CaMV since this virus is well characterized, possesses a single ds DNA genome that remains infectious by mechanical inoculation upon cloning. However, it appears that the complex mechanisms of viral replication and gene expression as well as packaging constraints impose severe restrictions on the insertion of foreign DNA into the genome of CaMV. Moreover, the host range of CaMV is limited to only a few dicotyledonous plant species. Nevertheless, vectors based on the CaMV genome were engineered by replacing open reading frame II, whose product is required for insect transmission but not for replication and systemic infection, with small genes encoding DHFR [80] and MTII [81]. The resulting vectors were indeed able to systemically infect turnip plants upon mechanical inoculation and express the foreign genes to high levels. The amounts of DHFR and MTII produced in infected plants were approximately 8 μg/g fresh

tissue and 0.5% of the soluble leaf protein, respectively.

Consequently, attention was directed towards certain geminiviruses to serve as expression vectors since they infect a wider range of plants including monocotyledonous species (reviewed in [82]). The genome of these viruses is composed of either one (monopartite) or two (bipartite) circular ss DNA molecules. Generally, the geminiviruses transmitted by leaf-hoppers possess a monopartite genome (i.e. MSV, WDV) and infect both dicots and monocots whereas those transmitted by whiteflies have a bipartite genome composed of DNAs designated A and B (i.e. CLV, TGMV) and infect dicots. Although infection of host plants using cloned ds DNA copies of certain viral genomes can be obtained by mechanical inoculation, efficient infection is performed with a special technique designated agroinoculation (or agroinfection) [83–86]. It involves cloning DNA representing dimers of the viral genome within the T-DNA and inoculating host plants with the *Agrobacterium* containing such constructs. The viral genome somehow 'escapes' from the T-DNA and systemically infects the whole plant.

In the case of MSV and WDV, the CP gene is required for systemic infection whereas it is dispensible for CLV and TGMV. Recently, CP gene substitution vectors were constructed based on the genome of CLV, TGMV, MSV and WDV. The CP gene of CLV, present on DNA A, was replaced with the gene encoding CAT. Upon mechanical inoculation onto tobacco plants with the chimeric DNA A together with DNA B, systemic infection of whole plants and expression of the CAT gene was obtained [87]. Similar work was carried out with MSV [88]. However, expression of the CAT gene in this case was restricted to leaves agroinoculated using the chimeric MSV genome construct since the CP gene is required for systemic spread. The CP gene of WDV was also replaced with reporter genes coding for CAT, NPTII and GUS. The resulting vectors could mediate efficient expression of these genes in wheat, maize or rice protoplasts [89]. Recently, WDV genome-derived vectors containing the maize *Ac/Ds* transpons were constructed and introduced into wheat, maize and rice protoplasts. This resulted in rapid and efficient excision of these transposable elements [90].

In the case of TGMV, more sophisticated approaches were employed. It was known that DNA A carries all the elements required for replication as well as the CP gene (since freely replicating and encapsidated DNA A molecules accumulate in transgenic plants containing tandem repeats of DNA A integrated into the chromosome) and that DNA B is required for systemic spread. A partial dimer of a chimeric DNA A on which the CP gene was replaced with the NPTII gene was inserted within the T-DNA together with a dimer of DNA B and used to agroinoculate tobacco plants. This resulted in systemic infection and expression of the NPTII gene in whole plants. TGMV DNA A-mediated expression of NPTII was even greater when transgenic plants containing a dimer of DNA B integrated into chromosomal DNA were subsequently agroinoculated with constructs containing a partial dimer of DNA A with the NPTII gene replacing the CP gene [91]. Agroinoculation of DNA A-derived constructs in which the CP gene was replaced with the CAT and GUS genes was also performed using tobacco or petunia leaf explants and permitted expression of these genes [92].

The potential of geminivirus genome-derived vectors to express foreign genes in plants in the field could be limited however, since *Agrobacterium*-mediated inoculations that are required for efficient infections would probably not be acceptable.

All these attempts to engineer DNA virus genome-derived expression vectors are summarized in Table IA.

4.2. *RNA virus genome-derived expression vectors*

The vast majority of plant viruses consists of plus strand RNA viruses that perhaps offer a greater potential for developing more versatile expression vectors. These viruses have been studied in detail regarding their genome structure and the mechanisms of viral replication and gene expression. Their genome is composed of either one (monopartite), two (bipartite), three (tripartite) or four (tetrapartite) distinct RNA molecules. Besides the CP, the viral genome encodes nonstructural proteins that play a role in processes such as replication, cell-to-cell movement, systemic infection and insect transmission. Replication of these viruses is performed by an RNA-dependent RNA polymerase (replicase) that is partly encoded by the viral genome. Expression of viral genes involves various strategies such as suppression of termination codons, frameshifts and post-translational cleavages. The genome of viral RNAs being polygenic, 'internal' genes are often expressed via subgenomic RNAs synthesized during replication by the viral replicase by initiation at internal promoters on genomic RNAs of minus polarity. Although many of these viruses are naturally transmitted by insects, mechanical inoculation is possible and leads to efficient infections (reviewed in [93–97]). Success in cloning of the cDNAs corresponding to the genomes of numerous RNA viruses (i.e. BMV [98–100], BNYVV [101,102], BSMV [103,104], CCMV [105], CMV [106], CPMV [107,108], CyRSV [109], PPV [110], PVX [111], TBSV [112], TCV [113], TMV [114,115], TVMV [116], TYMV [117], WCIMV [118]) in plasmids from which infectious transcripts can be derived in vitro has opened the door for genetic modification of these viral genomes, and it is becoming possible to engineer RNA virus genome-derived expression vectors. Initial results have been obtained using viruses possessing a monopartite (TMV), a tripartite (BMV, BSMV) or a tetrapartite (BNYVV) genome.

Table I

Engineering of plant viral genomes to mediate foreign gene expression

	Nature of viral genome	Viral gene/foreign gene inserted Expression in cells or tissues	Ref.
(A) DNA viruses			
Caulimoviruses			
CaMV	ds DNA	gene II/DHFR, MTII genes turnip plants	[80,81]
Geminiviruses			
MSV	ss DNA (monopartite)	CP gene/CAT gene maize leaves	[88]
WDV	ss DNA (monopartite)	CP gene/CAT, NPTII, GUS genes wheat, maize and rice protoplasts	[89]
CLV	ss DNA (bipartite)	CP gene/CAT gene tobacco plants	[87]
TGMV	ss DNA (bipartite)	CP gene/NPTII, CAT, GUS genes tobacco plants, tobacco and petunia explants	[91,92]
(B) RNA viruses			
Tobamoviruses			
TMV	ss RNA (monopartite)	CP gene/CAT gene tobacco leaves	[119,120]
Bromoviruses			
BMV	ss RNA (tripartite)	CP gene/CAT gene barley protoplasts	[121]
Hordeiviruses			
BSMV	ss RNA (tripartite)	58 kDa non-structural protein gene/LUC gene tobacco and maize protoplasts	[122]
Furoviruses			
BNYVV	ss RNA (tetrapartite)	25 kDa non-structural protein gene/GUS gene *Chenopodium* leaves	[123]

Towards engineering the TMV genome to serve as expression vector, the CP gene, which is 3'-proximal on the genome and is expressed via a subgenomic RNA, was replaced with the CAT gene. Expression of the CAT gene was observed in tobacco leaves inoculated with the derived chimeric TMV RNA [119]. The amount of CAT produced was estimated to be about 1 μg/g infected tissue. Attempts to insert the CAT gene into the complete genome of TMV were also made. However, in this instance the chimeric viral genome was unstable and the CAT gene was deleted during viral replication [120]. It would be important to further investigate the molecular basis of the possible unstability of a viral genome carrying a foreign gene. BMV possesses a genome composed of three RNAs designated 1, 2 and 3. The CP gene is 3'-proximal on RNA 3 and is expressed via a subgenomic RNA. The CP gene was replaced with the CAT gene and its expression was obtained in barley protoplasts inoculated with the chimeric RNA 3 together with wild-type RNAs 1 and 2 [121]. CAT gene expression in this instance was approximately 5–20-fold higher than that obtained in plant cells transformed with Ti plasmid-based vectors. The genome of BSMV is also composed of three RNAs designated α, β and γ. Open reading frame b in RNA β, encoding a 58 kDa non-structural protein that is essential for virus multiplication in whole plants, is again expressed via a subgenomic RNA and was replaced with the firefly LUC gene. The chimeric RNA β mediated efficient expression of the LUC gene upon transfection into tobacco and maize protoplasts in the presence of wild-type RNAs α and γ [122]. Finally, BNYVV contains a genome composed of four RNAs designated 1, 2, 3 and 4. The open reading frame in RNA 3, encoding a 25 kDa protein involved in natural transmission, was replaced with the GUS gene. Expression of the GUS gene was observed in *Chenopodium* leaves inoculated with the chimeric RNA 3 together with unmodified RNAs 1 and 2 [123].

All these attempts to engineer RNA virus genome-derived expression vectors are summarized in Table IB.

The stability of foreign genes inserted into the genome of RNA viruses has been questioned due to the high estimated mutation rates associated with replication of these viruses [78]. To further assess this question, it will be necessary to examine RNA virus genome-mediated expression of foreign genes in whole plants over several replication cycles. This potential problem would limit the usefulness of RNA virus genome-derived vectors for production of pharmaceuticals from infected plants since exceptional purity is required for polypeptide products destined for

human use. Nevertheless, these vectors would be useful for rapid investigation of the functionality of engineered proteins before stable plant transformation was undertaken.

5. SAFETY ISSUES FOR ENVIRONMENTAL RELEASE OF ENGINEERED VIRUSES

5.1. *Possible uncontrolled spread of engineered viruses: a major concern*

In considering environmental use of engineered viruses to express foreign genes in plants, possible uncontrolled spread of such viruses outside of the target area could constitute a potential danger for agriculture. Thus, engineered viruses could be used to express foreign genes in plants in the field only if it were possible to restrict multiplication of such viruses to the target area.

5.2. *Search for a strategy permitting virus containment*

One possible strategy to reach this goal could consist in genetically engineering both the viral genome and the host plant in such a way that the engineered viruses would be able to multiply and express the foreign gene they carry solely in the modified host plants. The modification of the viral genome would consist in deleting a gene that is essential for virus multiplication and replacing it with the foreign gene to be expressed. This should preclude infection of normal host plants with such a modified viral genome. On the other hand, host plants could be transformed using conventional gene transfer techniques (sections 2.1 and 2.2) with the viral gene that would have been deleted from the viral genome. Transgenic plants expressing the viral gene at high levels could be selected and those plants could support virus multiplication using a modified viral genome by complementation. Thus in this framework, there would be an obligate association between such 'disarmed' viral vectors and 'helper' transgenic host plants, and this could constitute an expression 'system' for foreign genes.

Although such a strategy could in principle be applicable to DNA viruses, there would be a potential risk of 'escape' of fully infectious virus resulting from recombination between a disarmed viral genome and a viral gene integrated into the chromosome of helper host plant. However, this kind of approach could be envisaged with RNA viruses. Host plants could be transformed with viral genes encoding non-structural proteins that are absolutely required for processes such as replication, cell-to-cell movement, systemic spread. Precedence for transgenic plants complementing a mutation of the viral genome already exists. Indeed, it has been shown that tobacco plants transformed with the TMV 30 kDa protein gene, involved in cell-to-cell movement of this virus, can potentiate the systemic spread at the non-permissive temperature of a TMV mutant (LS1) that is temperature sensitive for virus spread [124]. More recently, in the case of AIMV that possesses a tripartite genome composed of RNAs 1, 2 and 3, tobacco plants were transformed with the viral genes involved in replication and encoded by RNAs 1 and 2. Interestingly, such plants were able to replicate RNA 3 upon inoculation with RNA 3 alone [125].

The use of the viral CP gene per se as a complementing gene would meet with the difficulty that transgenic plants expressing the viral CP become resistant to virus infection (section 3.2). To circumvent this problem, one could attempt to express in transgenic host plants part of the minus strand of the viral genome that could serve as template for the synthesis of the CP mRNA by the replicase upon infection with a disarmed viral genome. Alternatively, plants could be transformed with a mutated CP gene to provide a functional CP that is, however, unable to mediate cross-protection as recently shown with AIMV [126]. Even if the coat protein were not involved in systemic spread of certain viruses, its expression during virus multiplication would nevertheless be necessary to obtain an inoculum of stable virus particles.

Finally, the possibility of RNA recombination in the expression system described above must be addressed. Since a complementating viral RNA present in the helper host plant would lack the signals for replication at both the 3′ and 5′ ends, recombination would require template switching by the viral replicase first from a replicative intermediate of a disarmed viral genome to the complementating RNA and then back to a replicative intermediate of the disarmed viral genome and would seem very unlikely. In line with this conclusion, it can be pointed out that in transgenic plants complementing the TMV movement function, no appearance of wild-type TMV has been reported upon inoculation with the mutant TMV (LS1) [124]. Another concern could be that virus 'escape' might occur if the complementing RNA were encapsidated. However, in this situation infection of any non-helper plants would be aborted since the complementating RNA would not be able to replicate.

6. CONCLUDING REMARKS

Non-scientific issues such as regulatory approval, proprietory rights and public perception will be decisive in future field-release of plants genetically engineered using recombinant DNA technology. Such issues might be most controversial for environmental use of viruses expressing foreign genes, particularly if 'containment' of engineered viruses is not assured.

Acknowledgements: We thank Drs John F. Bol, Francine Casse-Delbart, Ben J.C. Cornelissen, Chantal David, Bruno Gronenborn, Isabelle Jupin and Andrzej B. Legocki for useful discussions and Mrs Madeleine Garafoli for her constant help.

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