Cauliflower Mosaic Viral Promoter - A Recipe for Disaster?

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Concerns have been raised over the spread of transgenic DNA by horizontal gene transfer. One main factor determining the success of horizontal gene transfer is its tendency to recombine. This paper examines the safety implication of recent revelations on the recombination hotspot of the cauliflower mosaic viral (CaMV) promoter, which is in practically all current transgenic crops released commercially or undergoing field trials. As a precautionary measure, we strongly recommend that all transgenic crops containing CaMV 35S or similar promoters which are recombinogenic should be immediately withdrawn from commercial production or open field trials. All products derived from such crops containing transgenic DNA should also be immediately withdrawn from sale and from use for human consumption or animal feed.

The release of transgenic crops into the environment has raised concerns over the spread of transgenic DNA, not only by cross-pollination to related species, but especially by horizontal gene transfer to unrelated species (reviewed by Ho et al (1) and Traavik (2)). On account of the persistence of DNA in all environments, and the ability of practically all cells to take up 'naked' or free DNA, the success of horizontal gene transfer may depend largely on the nature of the DNA itself. New revelations concerning the CaMV recombination hotspot (3) have prompted us to consider the safety implications of the CaMV promoter. That is all the more urgent as CaMV promoter is in practically all transgenic crops already released commercially or undergoing field trials.

Cauliflower Mosaic Virus (CaMV) is a pararetrovirus of crucifer plants. The genome is an 8-kbp double-stranded circular DNA with three single strand gaps. Two major RNA transcripts (19S and 35S) and six large open reading frames are encoded by the DNA. Transcription occurs from a nonintegrated, circular minichromosome in the nucleus of the plant cell, and virion DNA is synthesised in the cytoplasm by reverse transcription of the 35S RNA transcript (4, 5). Phylogenetically, CaMV belongs to a group of caulimoviruses most closely related to the hepadnaviruses of animals, which includes the human hepatitis B virus. The reverse transcriptase (RT) of CaMV, however, is most similar to that of retrotransposons belonging to the Gypsy group and also to that of retroviruses (6). This suggests that CaMV evolved subsequent to the horizontal transfer of a retrotransposon to the cruciferae, either as the result of capture of RTgene by a pre-existing virus or by the transposable element acquiring additional genes to become a virus.

The CaMV promoter is a sequence of about 350 basepairs upstream of the 35S transcript (-343 to $+8$, with Cap site at $+1$), about 250 basepairs of which overlap with the 3′ end of gene Vl, the last of the six large open reading frames. There are three domains in the promoter, the core promoter containing the TATA box (-46 to $+8$), and two other major domains with enhancer functions. Region A (-90 to -46) is mainly required for expression in roots, and region B (-343 to -90) for expression in leaves (see (7, 8) and references therein). Subdomains of the B region (B1 to B5) can be recognised based on differential interactions with various transcription factors. However, the complete B region allows a more general constitutive expression than expected from the combinations of subdomains. This suggests that important sequence elements are at the interfaces of the subdomains, or that the combination of subdomains is not simply additive but results in qualitatively novel specificities.

The roles of the different 35S promoter domains in pathogenesis of CaMV have only been studied fairly recently (4, 9). These studies show that the loss of up to 40 amino acids from the 3′ end of gene Vl (which overlaps with the 35S promoter) had no effect on pathogenesis whereas further truncation into a putative zinc finger region was fatal to the virus. Removing the TATA box

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also abolished infectivity. However, upstream deletions within the enhancer region between −207 and −56 were tolerated even though complete removal of this fragment caused loss of infectivity. Two separate enhancer domains for infectivity were identified, −207 to −150 and −95 to −56, only one of which is necessary. The enhancer region could even work in reverse orientation. Foreign gene sequences could be inserted into deletion mutants, which may alter the infectious characteristics of the virus.

Various hybrid or combination promoters have been constructed from the CaMV 35S promoter which led to improved expression of transgenes: double 35S promoters (10), a hybrid containing the core 19S promoter from CaMV and the 35S upstream enhancers (11), and combination of CaMV 35S with mannopine synthase elements (12), or with *Adh1*- and *ocs*-promoter elements for expression in monocotyledons (13). These results emphasise the modularity and interchangeability of promoter elements (8), which have important implications for the safety of transgenic plants. It means, in effect, that recombination of the CaMV promoter elements with dormant, endogenous viruses may create new infectious viruses in all species to which the transgenic DNA is transferred.

Another factor which affects the safety of transgenic plants containing CaMV promoters and related constructs is that although CaMV itself infects only dicotyledons, its promoter is promiscuous; and functions efficiently in monocotyledons (14), in conifer cell lines (15), green algae (16), yeasts (17) and *E. coli* (18). The transfer of CaMV promoter to these other species could also give rise to unpredictable effects on gene expression, which may impact on the ecosystem as a whole.

It has been known for some time that recombination can occur between different CaMV viral strains in plants (19), between different homologous parts of an integrated CaMV viral sequence in transgenic plants (20) and between an integrated transgene and an infecting virus (21). Analysis of the junctions of recombination suggests that one of the recombination hotspots was at the 3′ end of the 35S promoter, and was thought to be due primarily to template switching during reverse transcription. This kind of recombination depends on sequence homology between the recombining partners as well as the action of virally encoded reverse transcriptase, and is expected to have little impact on nonhomologous DNA belonging to other organisms. However, it was suspected that recombination may also occur between double-stranded DNA, as recombination junctions were found away from the initiation site of DNA synthesis (where the 35S promoter is located).

Double-stranded DNA break repair (DSBR) is recognized to be involved in the illegitimate recombination which enables plasmid DNA to integrate into plant genomes following plant transformation (22, 23); and transgene rearrangements have been identified in both *Agrobacterium*-mediated transformation (24) and particle bombardment (25). Illegitimate recombination was also observed between a resident transgene in a transgenic tobacco plant and a newly delivered transgene (26). Illegitimate recombination involves sequences with either microhomology or no homology between the junctions, often resulting in filler DNA and deletions of nucleotides from one or both of the recombining ends (27).

Kohli *et al* (3) analysed 12 multicopy transgenic rice lines transformed with a co-integrate plasmid by means of particle bombardment in order to investigate the fate of exogenous transforming DNA. They not only discovered the same kind of illegitimate recombination between plasmids, but also that many of the illegitimate recombinations were located to the CaMV 35S promoter hotspot previously identified (19). Furthermore, recombination occurred at high frequency without the virally encoded reverse transcriptase or other enzymes, suggesting that plant factors can direct recombination events by recognising and using these highly recombinogenic viral sequences.

The hotspot located by Kohli *et al* (3) was an imperfect palindrome of 19 bp at the 3′ end of the CaMV 35S promoter containing the TATA box. The palindrome and surrounding DNA sequences were found to have a number of characteristics common to known recombination hotspots. One half of the 19 bp palindrome was purine rich, and it is known that recombinase proteins bind to such regions. Topoisomerase I cleavage sites, the trinucleotide AAG, are also found clustered around the recombining junctions in the hotspot, which is either part of the junction or present within 3 bp of it in 8 out of the 11 junctions analysed by Kohli *et al* (3). A 32 bp region with 90% AT content was found in the 35S promoter 28 bp upstream from the palindrome. AT-rich regions cause isotropic DNA bending and influence DNA melting. They contain matrix attachment region (MAR) motifs, which harbour intrinsically curved DNA, and have been found in the vicinity of other recombination hotspots. The 19 bp palindrome itself contains a short tract of alternating purine-pyrimidine (AT) residues situated 50 bp upstream from another alternating purine-pyrimidine sequence in the transgene. Such residues are known to adopt Z DNA conformation and have been shown to influence transcription and recombination, and are also binding sites for topoisomerase II, which is specifically involved in the resolution of recombination intermediates.

The structure and sequence-specific properties of the 3′ end of the CaMV 35S promoter are similar to the petunia transformation booster-sequence which increased plant transformation efficiency, most probably by stimulating recombination (28). Similar structures and sequence-specific characteristics were identified for recombinogenic regions of SV40 DNA in Hela cells (29). The 25 bp border

repeats of the *Agrobacterium* T-DNA, the most commonly used vector for plant transformation, also show remarkable similarities to the recombination hotspot of the CaMV 35S promoter. There is an 11 bp imperfect plaindrome sequence with a TATAbox-like structure in the right border whereas the left border has a short purine-rich sequence in the centre. Kohli *et al* (3) predicts that these two regions of T-DNA could be involved in rearrangements which are often seen in T-DNA mediated plant transformations.

It is clear that the CaMV 35S promoter is well-endowed with motifs involved in recombination. An additional factor which may increase the instability of the plasmid is the junction between CaMV 35S promoter and foreign DNA. All these considerations make it highly likely that the CaMV 35S promoter will take part in horizontal gene transfer and recombination, and also cause largescale genomic rearrangements in the process.

Horizontal transfer of the CaMV promoter not only contributes to the known instability of transgenic lines (30), but has the potential to reactivate dormant viruses or creating new viruses in all species to which it is transferred, particularly in view of the modularity and interchangeability of promoter elements (8). In this regard, the close relationship of CaMV to hepadnaviruses such as the human hepatitis B is especially relevant. In addition, because the CaMV promoter is promiscuous in function (see above), it has the possibility of promoting inappropriate over-expression of genes in all species to which it happens to be transferred. One consequence of such inappropriate over-expression of genes may be cancer.

Our considerations should be seen in the light of the results of the first systematic safety testing of transgenic food backed up by histological studies, which was carried out by Pusztai and his collaborators. Ewen and Pusztai (31) conclude that a significant part of the toxic effects of transgenic potatoes with snowdrop lectin was due to the "construct or the genetic transformation (or both)". They further state, "The possibility that a plant vector in common use in some GM plants can affect the mucosa of the gastrointestinal tract and exert powerful biological effects may also apply to GM plants containing similar constructs…" The plant vector in common use is the T-DNA of *Agrobacterium*, and the construct in question is the CaMV 35S promoter, both of which are in the transgenic potatoes tested by Ewen and Pusztai (31).

As a precautionary measure, we strongly recommend that all transgenic crops containing CaMV 35S or similar promoters which are recombinogenic should be immediately withdrawn from commercial production or open field trials. All products derived from such crops containing transgenic DNA should also be immediately withdrawn from sale and from use for human consumption or animal feed.

REFERENCES

1. Ho MW, Traavik T, Olsvik R, Tappeser B, Howard V, von Weizsacker C, McGavin G. Gene Technology and Gene Ecology of Infectious Diseases. *Microbial Ecology in Health and Disease* 1998; 10: 33–59.
2. Traavik, T. (1999). *Too Early May Be Too Late. Ecological Risks Associated with the Use of Naked DNA as a Biological Tool for Research, Production and Therapy* (Norwegian), Report for the Directorate for Nature Research Tungasletta 2, 7005 Trondheim.
3. Kohli A, Griffiths S, Palacios N, Twyman RM, Vain P, Laurie DA, Christou P. Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. *The Plant Journal* 1999; 17: 591–601.
4. Turner DS, McCallum DG, Covey SN. Roles of the 35S promoter and multiple overlapping domains in the pathogenicity of the pararetrovirus cauliflower mosaic virus. J. Virol. 1996; 70: 5414–21.
5. Cann, A.J. (1997). *Principles of Molecular Virology*, 2nd ed., Academic Press, London.
6. Xiong Y, Eickbush TH. Origin and evolution of retroelements based upon their reverse transcriptase sequences. *EMBO J.* 1990; 9: 3353–62.
7. Benfy PN, Chua N-H. The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 1990; 250: 959–66.
8. Hohn T, Fütterer J. Transcriptional and translational control of gene expression in cauliflower mosaic virus. Curr. Op. Genet. Develop. 1992; 2: 90–6.
9. Noad RJ, Turner DS, Covey SN. Expression of functional elements inserted into the 35S promoter region of infectious cauliflower mosaic virus replicons. *Nucleic Acid Res* 1997; 25: 1123–9.
10. Fang R-X, Nagy F, Sivabramaniam S, Chua N-H. Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. *Plant Cell* 1989; 1: 141–50.
11. Fütterer, J. (1992) unpublished result, cited in [7].
12. Comai I, Moran P, Maslyar D. Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. *Plant Mol. Biol* 1990; 15: 373–81.
13. Last DI, Brettell DA, Chamberlain AM, Chaudhury AM, Larkin PJ, Marsh EL, Peacock WJ, Dennis ES. pEmu: an improved promoter for gene expression in cereal cells. *Theor. Appl. Genet* 1991; 81: 581–8.
14. Battraw MH, Hall TC. Histochemical analysis of CaMV 35S promoter-(-glucuronidase gene expression in transgenic rice plants. *Plant Mol. Biol* 1990; 15: 527–38.
15. Bekkaoui F, Datla RSS, Pilon M. The effects of promoters on transient expression in conifer cell lines. *Theor. Appl. Genet* 1990; 79: 353–9.
16. Jarvia EE and Brown LM, (1991). Transient expression of firefly luciferase in protoplasts of the green alga Chlorella
17. Hirt H, Kogl M, Murbacher T, Heberle–Bors E. Evolutionary conservation of transcriptional machinery between yeast and plants as shown by the efficient expression from CaMV 35S promoter and 35S terminator. *Curr. Genet* 1990; 17: 473–9.
18. Assad F, Signer ER. Cauliflower mosaic virus P35S promoter activity in *E. coli*. *Mol. Gen. Genet* 1990; 223: 517–20.
19. Vaden VR, Melcher U. Recombination sites in cauliflower mosaic virus DNAs: implications for mechanisms of recombination. *Virology* 1990; 177: 717–26.

20. Gal S, Pisan B, Hohn T, Grimsley N, Hohn B. Genomic homologous recombination in planta. *EMBO J* 1991; 10: 1571–8.
21. Wintermantel WM, Schoelz JE. Isolation of recombinant viruses between cauliflower mosaic virus and a viral gene in transgenic plants under conditions of moderate selection pressure. *Virology* 1996; 223: 156–64.
22. Gheysen G, Villarroel R, van Montagu M. Illegitimate recombination in plants: a model for T-DNA integration. Genes Dev. 1991; 5: 287–97.
23. Salomon S, Puchta H. Capture of genomic and T-DNA sequences during double-stranded break repair in somatic plant cells. EMBO J. 1998; 17: 6086–95.
24. Deroles SC, Gardner RC. Analysis of the T-DNA structure in a large number of transgenic petunias generated by Agrobacterium-mediated transformation. Plant Mol. Biol. 1988; 11: 365–77.
25. Register JC, Peterson DJ, Bell PJ, et al. Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol. Biol. 1994; 25: 951–61.
26. De Groot MJ, Offringa R, Groet J, Does MJ, Van Hooykaas PJ, Danelzen PJ. Non-recombinant background in gene targetting. Illegitimate recombination between *htp* gene and defective 5′-deleted *nptII* gene can restore a Km^r phenotype in tobacco. Plant Mol. Biol. 1994; 25: 721–33.
27. Gorbunov V, Levy AA. Non-homologous DNA and joining in plants cells is associated with deletions and filler DNA insertions. Nucl. Acid Res. 1997; 25: 4650–7.
28. Puchta H and Meyer P. (1994). Substrate specificity of plant recombinases determined in extrachromosomal recombination systems. *In Homologous Recombination and Gene Silencing in Plants* (Paszkowski, J., ed.), Dorcrecht, Kluwer Academic Publishers, pp. 123–155.
29. Stary A, Sarasin A. Molecular analysis of DNA junctions produced by illegitimate recombination in human cells. Nucl. Acids Res. 1992; 20: 4269–74.
30. Ho MW, Steinbrecher R. Fatal flaws in food safety assessment. Environmental and Nutritional Interactions 1998; 2: 51–84.
31. Ewen SWB, Pusztai A. Effect of diets containing genetically modified potatoes expressing Galanthus nivalis lectin on rat small intestine. The Lancet 1999; 354: 1353–4.