

Review

Transcomplementation and synergism in plants: implications for viral transgenes?

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SUMMARY

In plants, viral synergisms occur when one virus enhances infection by a distinct or unrelated virus. Such synergisms may be unidirectional or mutualistic but, in either case, synergism implies that protein(s) from one virus can enhance infection by another. A mechanistically related phenomenon is transcomplementation, in which a viral protein, usually expressed from a transgene, enhances or supports the infection of a virus from a distinct species. To gain an insight into the characteristics and limitations of these helper functions of individual viral genes, and to assess their effects on the plant–pathogen relationship, reports of successful synergism and transcomplementation were compiled from the peer-reviewed literature and combined with data from successful viral gene exchange experiments. Results from these experiments were tabulated to highlight the phylogenetic relationship between the helper and dependent viruses and, where possible, to identify the protein responsible for the altered infection process. The analysis of more than 150 publications, each containing one or more reports of successful exchanges, transcomplementation or synergism, revealed the following: (i) diverse viral traits can be enhanced by synergism and transcomplementation; these include the expansion of host range, acquisition of mechanical transmission, enhanced specific infectivity, enhanced cell-to-cell and long-distance movement, elevated or novel vector transmission, elevated viral titre and enhanced seed transmission; (ii) transcomplementation and synergism are mediated by many viral proteins, including inhibitors of gene silencing, replicases, coat proteins and movement proteins; (iii) although more frequent between closely related viruses, transcomplementation and synergism can occur between viruses that are phylogenetically highly divergent. As indicators of the interoperability of viral genes, these results are of general interest, but they can

also be applied to the risk assessment of transgenic crops expressing viral proteins. In particular, they can contribute to the identification of potential hazards, and can be used to identify data gaps and limitations in predicting the likelihood of transgene-mediated transcomplementation.

INTRODUCTION

A synergism may be said to occur when, during the simultaneous infection of a plant by two distinct viruses, infection of one or both viruses is enhanced (Atabekov and Taliensky, 1990; Close, 1964; Falk *et al.*, 1995; Froissart *et al.*, 2002; Malyshenko *et al.*, 1989; Smith, 1945). When synergisms are asymmetric, the two viruses are often referred to as the 'helper' and the 'dependent' viruses (Malyshenko *et al.*, 1989). Viral synergisms are assumed, in this paper and elsewhere, to be protein-mediated and, in some cases, this assumption is supported, as the synergism can be mimicked in transgenic plants expressing single viral proteins (Giesman-Cookmeyer *et al.*, 1995; Vance, 1991; Vance *et al.*, 1995).

Transcomplementation (sometimes called heterologous complementation) is a related phenomenon, in which a viral protein, often expressed from an integrated transgene, supports or enhances infection by an invading 'dependent' virus. A well-known example of this is the enhancement of diverse plant viruses in tobacco by transgenes encompassing the *HC-Pro* region of potato virus Y (PVY) (e.g. Pruss *et al.*, 1997; Vance *et al.*, 1995).

Two additional experimental techniques can also demonstrate transcomplementation. The first includes experiments in which individual viral genes are successfully exchanged or replaced to produce functional hybrid viruses (e.g. Briddon *et al.*, 1990; Huppert *et al.*, 2002; de Jong and Ahlquist, 1992). The second includes transient assays in which a viral gene and a putative dependent virus are introduced simultaneously by co-bombardment (Agranovsky *et al.*, 1998; Morozov *et al.*, 1997).

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The purpose of this review is to update and extend our conceptual understanding of the extent to which infecting viruses may utilize proteins derived from distinct viruses. The data reviewed here are organized, in particular, to help to determine whether the expression of viral proteins in transgenic plants is likely to result in altered infection by non-target viruses.

This review is also the first to specifically and systematically address the principal questions relevant to the risk assessment of transcomplementation arising from viral protein expression in transgenic plants (although for coat proteins, see Falk *et al.*, 1995; de Zoeten, 1991). These questions are as follows: (i) how common are synergism and transcomplementation between plant viruses that are phylogenetically distinct (at the species level or above, see Table 1)?; (ii) which proteins and viruses can function as the 'helper' and which viruses as the 'dependent' partner (Table 1)?; (iii) what traits can synergisms confer on dependent viruses?; and (iv) what are the plausible negative outcomes (i.e. hazards) of plants expressing functional virus proteins?

AN OVERVIEW OF SYNERGISMS AND TRANSCOMPLEMENTATION

Synergisms and examples of transcomplementation discussed in this review are restricted to those in which there is a clear and measurable positive effect on the dependent virus (e.g. enhanced viral titre or a newly acquired ability to infect a non-host plant); they are documented in Table 1. The examples included in Table 1 are also restricted in requiring that the dependent virus comes from a distinct species. Thus, synergisms which result only in an enhancement of symptoms or which occur between viruses of the same species are not discussed here or included in Table 1. Also not discussed here are transcapsidation results obtained *in vitro*, and these are reviewed elsewhere (Dodds and Hamilton, 1976). In addition, although plant viruses have close associations with viroids, and viroids can utilize viruses for transmission and possibly other functions, we do not discuss the potential for transcomplementation to alter the infection of plants by viroids (Syller and Marczewski, 2001).

Table 1 documents the instances of synergism or transcomplementation reported in the scientific literature. These are displayed to highlight the phylogenetic relationship between the helper and dependent viruses involved. Where possible, the 'helper' protein is identified.

The findings summarized in Table 1 allow a broad set of generalizations to be made about synergisms and transcomplementation. Firstly, they are very common: 69 virus species from 35 genera have been shown to function as either a helper or a dependent virus, and most well-studied viruses appear in Table 1 on multiple occasions. As an example, the tobacco mosaic virus (TMV) can function as a helper to 17 viral species in 16 different

genera, and, as a dependent virus, TMV appears 20 times with dependence on 16 different genera (Table 1). Such promiscuity suggests that many of the empty boxes in Table 1 reflect data gaps rather than an underlying biological incompatibility. Nevertheless, synergism or transcomplementation is not universally observed, and there are many recorded instances of negative results (e.g. Hamilton and Nichols, 1977; Rao *et al.*, 1998), and also sometimes of interference between viruses (e.g. Mendez-Lozano *et al.*, 2003).

A second generalization is that synergisms and transcomplementation can occur between highly divergent viruses. Table 1 documents synergisms of both single-stranded and double-stranded DNA viruses with RNA viruses (e.g. Carr and Kim, 1983; Cooper *et al.*, 1995; Wege and Siegmund, 2007), of both ambisense and negative-stranded RNA viruses with positive-stranded RNA viruses (Huang *et al.*, 2005; Lewandowski and Adkins, 2005), and between viruses with diverse life histories, morphological structures and genome characteristics, perhaps the most notable of the latter being the extension of the host range of the insect virus flock house virus (FHV) to plants (Dasgupta *et al.*, 2001). Nevertheless, there are no instances in which ambisense viruses or negative sense RNA viruses are the dependent virus (Table 1). It is not known whether this lack of evidence reflects the relative lack of research on these viruses or an innate incompatibility.

A third generalization is that a very diverse set of viral proteins, including some of unknown function, can transcomplement (Hormuzdi and Bisaro, 1995; Teycheney *et al.*, 2000). Nevertheless, transcomplementation has most commonly been shown for viral proteins that are classed as movement proteins, inhibitors of gene silencing or coat proteins (see Table 1).

All the findings above are apparent from a study of Table 1. However, a more detailed examination of the papers referred to in Table 1 reveals additional important characteristics of transcomplementation and synergism.

Firstly, plant viral life cycles are highly complex and require the fulfilment of diverse functions by a limited set of often multifunctional viral proteins. Given this context, it is perhaps not surprising that the infection characteristics enhanced by transcomplementation and synergism are diverse. Synergisms or transcomplementation can confer, enhance or compensate for a lack of viral functions as different as mechanical transmission (Mayo *et al.*, 2000; Ryabov *et al.*, 2001), host range (e.g. Cohen *et al.*, 1988; Dasgupta *et al.*, 2001; Hacker and Fowler, 2000; Hamilton and Nichols, 1977; Spitsin *et al.*, 1999), seed transmission (Kuhn and Dawson, 1973), specific infectivity (Chiba *et al.*, 2006; Sunter *et al.*, 2001), cell-to-cell and long-distance movement (e.g. Carr and Kim, 1983; Yelina *et al.*, 2002), vector transmission (e.g. Briddon *et al.*, 1990; Lecoq *et al.*, 1993; Rochow, 1970), viral titre (e.g. Scheets, 1998; Valkonen, 1992), disease development (Cooper *et al.*, 1995) and genome activation (e.g.

Table 1 Transcomplementation and synergisms between plant viruses observed *in vivo*.

Table 1 records all known instances in which all or part of a virus has been observed to interact in a transcomplementation or synergistic manner with a distinct virus. Table 1 displays these data such that the genus of the helper virus (or gene) is shown in the far left-hand column and all viruses which have been shown experimentally to be transcomplemented by, or synergized with, this genus are to its right. Where the gene responsible for the helper function is known, the genus of the dependent virus is displayed in the column under that protein; otherwise, the dependent virus is recorded in the last column.

Helper virus*	Movement protein†	Replicase†	Coat protein†	Suppressor of gene silencing†	Other‡	Protein identity not known§
Begomovirus	Begomovirus (Hill <i>et al.</i> , 1998; Schaffer <i>et al.</i> , 1995)			Potexvirus (Voïnnet <i>et al.</i> , 1999)	Begomovirus (Morra and Petty, 2000; Qin and Petty, 2001; Sung and Coutts, 1995; Sunter <i>et al.</i> , 1994)	Begomovirus (Guevara-Gonzalez <i>et al.</i> , 1999; Mendez-Lozano <i>et al.</i> , 2003)
				Tobamovirus (Sunter <i>et al.</i> , 2001)		Nanovirus (Saunders <i>et al.</i> , 2002)
				Curtovirus (Sunter <i>et al.</i> , 2001)	Curtovirus (Hormuzdi and Bisaro, 1995)	
				Begomovirus (Vanitharani <i>et al.</i> , 2004)		
<hr/>						
Topocovirus						Begomovirus (Briddon and Markham, 2001)
Curtovirus			Begomovirus (Briddon <i>et al.</i> , 1990)	Tobamovirus (Sunter <i>et al.</i> , 2001)	Begomovirus (Sunter <i>et al.</i> , 1994)	Nanovirus (Guevara-Gonzalez <i>et al.</i> , 1999)
				Begomovirus (Sunter <i>et al.</i> , 2001)	Curtovirus (Hormuzdi and Bisaro, 1995)	Begomovirus (Briddon and Markham, 2001)
Caulimovirus					Caulimovirus (Edskes <i>et al.</i> , 1996; Markham and Hull, 1985)	Caulimovirus (Ducasse and Shepherd, 1995)
Luteovirus			Luteovirus (Creamer and Falk, 1990; Rochow, 1970; Wen and Lister, 1991)			Tobamovirus (Hii <i>et al.</i> , 2002)
						Luteovirus (Gill and Chong, 1981)
						Umbravirus (Hull and Adams, 1968; Okusanya and Watson, 1966; Smith, 1945, 1946; Waterhouse and Murrant, 1983; Watson <i>et al.</i> , 1964)
Polerovirus				Potexvirus (Pfeffer <i>et al.</i> , 2002)		Umbravirus (Falk <i>et al.</i> , 1979; Waterhouse and Murrant, 1983)
						Potyvirus (Wintermantel, 2005)
Enamovirus						Closterovirus (Wintermantel, 2005)
Tombusvirus	Cucumovirus (Huppert <i>et al.</i> , 2002)					Polerovirus (Mayo <i>et al.</i> , 2000)
				Potexvirus (Bayne <i>et al.</i> , 2005; Voïnnet <i>et al.</i> , 1999)		
				Closterovirus (Chiba <i>et al.</i> , 2006)		

Table 1 continued.

Helper virus*	Movement protein†	Replicaset	Coat protein	Suppressor of gene silencing†	Other‡	Protein identity not known§
Dianthovirus	Tombusvirus (Qu and Morris, 2002; Reade <i>et al.</i> , 2001; Reade <i>et al.</i> , 2002)					
	Tobamovirus (Giesman-Cookmeyer <i>et al.</i> , 1995)					
	Cucumovirus (Rao <i>et al.</i> , 1998)					
	Bromovirus (Rao <i>et al.</i> , 1998)					
	Hordeivirus (Solovyev <i>et al.</i> , 1997)					
	Potexvirus (Morozov <i>et al.</i> , 1997)					
Machlomovirus						Rymovirus (Scheets, 1998)
Waikavirus						Badnavirus (Hibino and Cabauatan, 1987)
						Sequivirus (Elnagar and Murant, 1976)
Comovirus						Potyvirus (Lee and Ross, 1972)
						Tobamovirus (Malysenko <i>et al.</i> , 1989)
Nepovirus						Comovirus (Malysenko <i>et al.</i> , 1989)
						Tobamovirus (Malysenko <i>et al.</i> , 1989)
Potyvirus		Potyvirus (Teycheney <i>et al.</i> , 2000)	Potyvirus (Bourdin and Lecoq, 1991; Dolja <i>et al.</i> , 1994; Hammond and Dienelt, 1997; Lecoq <i>et al.</i> , 1993; Rojas <i>et al.</i> , 1997; Tobias <i>et al.</i> , 2001; Varrelmann <i>et al.</i> , 2000)	Potexvirus (Brigneti <i>et al.</i> , 1998; Li <i>et al.</i> , 2001; Shi <i>et al.</i> , 1997; Sonoda <i>et al.</i> , 2000; Vance <i>et al.</i> , 1995)	Potyvirus (Lecoq and Pitrat, 1985; Pirone, 1981; Sako and Ogata, 1981)	Luteovirus (Bourdin and Lecoq, 1994)
				Polerovirus (Savenkov and Valkonen, 2001b)		Polerovirus (Barker, 1987, 1989; Jayasinghe <i>et al.</i> , 1989; Wintermantel, 2005)
			Potexvirus (Fedorkin <i>et al.</i> , 2000, 2001)	Polerovirus (Savenkov and Valkonen, 2001b)		Machlomovirus (Goldberg and Brakke, 1987)
				Hordeivirus (Yelina <i>et al.</i> , 2002)		Comovirus (Anjos <i>et al.</i> , 1992; Calvert and Ghabrial, 1983)

Table 1 continued.

Helper virus*	Movement protein†	Replicaset	Coat protein	Suppressor of gene silencing†	Other‡	Protein identity not known§
				Potyvirus (<i>Atreya and Pirone, 1993</i> ; <i>Mlotshwa et al., 2002</i>)		Potyvirus (<i>Clinch et al., 1936</i> ; <i>Close, 1964</i> ; <i>Damirdagh and Ross, 1967</i> ; <i>Goodman and Ross, 1974</i> ; <i>Kassanis and Govier, 1971</i> ; <i>Manoussopoulos, 2000</i> ; <i>Rodhou and Ross, 1955</i>)
				Comovirus (<i>Mlotshwa et al., 2002</i>)		
				Closterovirus (<i>Chiba et al., 2006</i>)		Potyvirus (<i>Hobbs and McLaughlin, 1990</i> ; <i>Kassanis and Govier, 1971</i> ; <i>Wang et al., 1998</i>)
				Tobamovirus (<i>Pruss et al., 1997</i>)		
				Cucumovirus (<i>Pruss et al., 1997</i>)		Cucumovirus (<i>Anderson et al., 1996</i> ; <i>Cohen et al., 1988</i> ; <i>Ishimoto et al., 1990</i> ; <i>Poolpol and Inouye, 1986</i> ; <i>Sano and Kojima, 1989</i> ; <i>Wang et al., 2002, 2004</i>)
Sobemovirus	Tobamovirus (<i>Zhang et al., 2005</i>)		Potyvirus (<i>Fedorkin et al., 2001</i>) Dianthovirus (<i>Callaway et al., 2004</i>)	Potyvirus (<i>Voignet et al., 1999</i>)		Closterovirus (<i>Wintermantel, 2005</i>) Sobemovirus (<i>Hacker and Fowler, 2000</i>)
Umbravirus	Potyvirus (<i>Ryabov et al., 1998</i>) Polerovirus (<i>Ryabov et al., 2001a</i>) Tobamovirus (<i>Ryabov et al., 1999a</i>) Cucumovirus (<i>Ryabov et al., 1999b</i>)				Tobamovirus (<i>Ryabov et al., 2001b</i>)	Enamovirus (<i>Mayo et al., 2000</i>) Polerovirus (<i>Barker, 1989</i> ; <i>Mayo et al., 2000</i>)
Bromovirus	Bromovirus (<i>Mise et al., 1993</i>) Potyvirus (<i>Tamai et al., 2003</i>) Tobamovirus (<i>Tamai et al., 2003</i>)		Tobamovirus (<i>Choi and Rao, 2000</i>) Bromovirus (<i>Allison et al., 1988</i> ; <i>Osman et al., 1997, 1998</i>) Hordeivirus (<i>Peterson and Brakke, 1973</i>)			Potyvirus (<i>Malyshenko et al., 1989</i>) Sobemovirus (<i>Kuhn and Dawson, 1973</i>)

Table 1 continued.

Helper virus*	Movement protein†	Replicaset	Coat protein†	Suppressor of gene silencing†	Other‡	Protein identity not known§
Cucumovirus	Cucumovirus (Cooper <i>et al.</i> , 1996; Kaplan <i>et al.</i> , 1995) Bromovirus (Nagano <i>et al.</i> , 2001) Tobamovirus (Tamai <i>et al.</i> , 2003) Potexvirus (Tamai <i>et al.</i> , 2003)	Cucumovirus (Teycheney <i>et al.</i> , 2000)	Cucumovirus (Salanki <i>et al.</i> , 1997; Taliatsky and Garcia-Arenal, 1995)	Potexvirus (Brigneti <i>et al.</i> , 1998) Tobravirus (Liu <i>et al.</i> , 2002) Potyvirus (Ryang <i>et al.</i> , 2004) Closterovirus (Chiba <i>et al.</i> , 2006) Begomovirus (Wege and Siegmund, 2007)		Comovirus (Malysenko <i>et al.</i> , 1989) Potyvirus (Guerini and Murphy, 1999; Murphy and Kyle, 1995) Potexvirus (Close, 1964) Cucumovirus (Wang <i>et al.</i> , 1998)
Alfamovirus			Alfamovirus (Reusken <i>et al.</i> , 1995) Cucumovirus (Candellier-Harvey and Hull, 1993) Tobamovirus (Spitsin <i>et al.</i> , 1999) Iilarvirus (Sanchez-Navarro <i>et al.</i> , 1997; van Vloten-Doting, 1975)			Bromovirus (Malysenko <i>et al.</i> , 1989)
Iilarvirus			Alfamovirus (Sanchez-Navarro <i>et al.</i> , 1997; van Vloten-Doting, 1975)			
Tobamovirus	Potexvirus (Ajjikuttira <i>et al.</i> , 2005; Fedorkin <i>et al.</i> , 2001; Morozov <i>et al.</i> , 1997) Alfamovirus (Cooper <i>et al.</i> , 1995; Sanchez-Navarro <i>et al.</i> , 1997) Tobravirus (Cooper <i>et al.</i> , 1995; Ziegler-Graff <i>et al.</i> , 1991)	Bromovirus (Ishikawa <i>et al.</i> , 1991)	Tobamovirus (Donson <i>et al.</i> , 1991; Hilf and Dawson, 1993)	Potexvirus (Ajjikuttira <i>et al.</i> , 2005)		Comovirus (Malysenko <i>et al.</i> , 1988, 1989; Taliatsky <i>et al.</i> , 1993) Tobamovirus (Malysenko <i>et al.</i> , 1989) Hordeivirus (Malysenko <i>et al.</i> , 1989)

Table 1 continued.

Helper virus*	Movement protein†	Replicaset	Coat protein†	Suppressor of gene silencing†	Other‡	Protein identity not known§
	<i>Dianthovirus</i> (Giesman-Cookmeyer <i>et al.</i> , 1995)					<i>Begomovirus</i> (Carr and Kim, 1983)
	<i>Cucumovirus</i> (Cooper <i>et al.</i> , 1995, 1996; Rao <i>et al.</i> , 1998)					<i>Sobemovirus</i> (Fuentes and Hamilton, 1991)
	<i>Hordeivirus</i> (Solovyev <i>et al.</i> , 1996)					<i>Bromovirus</i> (Taliensky <i>et al.</i> , 1982a)
	<i>Comovirus</i> (Taliensky <i>et al.</i> , 1992)					<i>Potexvirus</i> (Close, 1964)
	<i>Nepovirus</i> (Cooper <i>et al.</i> , 1995)					<i>Potyvirus</i> (Valkonen, 1992)
	<i>Caulimovirus</i> (Cooper <i>et al.</i> , 1995)					
	<i>Bromovirus</i> (de Jong and Ahlquist, 1992)					
	<i>Tobamovirus</i> (Deom <i>et al.</i> , 1994; Fenczik <i>et al.</i> , 1995; Nejdat <i>et al.</i> , 1991; Tamai and Meshi, 2001)					
	<i>Benyvirus</i> (Lauber <i>et al.</i> , 1998)					
<i>Hordeivirus</i>	<i>Hordeivirus</i> (Solovyev <i>et al.</i> , 1999)		<i>Tobamovirus</i> (Dodds and Hamilton, 1974)	<i>Tobravirus</i> (Liu <i>et al.</i> , 2002)		<i>Potexvirus</i> (Malysenko <i>et al.</i> , 1989)
				<i>Hordeivirus</i> (Yelina <i>et al.</i> , 2002)		<i>Bromovirus</i> (Hamilton and Dodds, 1970; Hamilton and Nichols, 1977; Taliensky <i>et al.</i> , 1982a)
				<i>Potexvirus</i> (Yelina <i>et al.</i> , 2002)		<i>Tobamovirus</i> (Taliensky <i>et al.</i> , 1982a)
<i>Furovirus</i>				<i>Tobravirus</i> (Liu <i>et al.</i> , 2002)		
<i>Tobravirus</i>			<i>Tobravirus</i> (MacFarlane <i>et al.</i> , 1994)	<i>Tobravirus</i> (Liu <i>et al.</i> , 2002)		<i>Tobamovirus</i> (Malysenko <i>et al.</i> , 1989)
<i>Pecluvirus</i>	<i>Benyvirus</i> (Lauber <i>et al.</i> , 1998)					<i>Polerovirus</i> (Barke, 1989)

Table 1 continued.

Helper virus*	Movement protein†	Replicase†	Coat protein†	Suppressor of gene silencing†	Other‡	Protein identity not known§
Potexvirus	Potexvirus (Morozov <i>et al.</i> , 1999) Tobamovirus (Ajjikuttira <i>et al.</i> , 2005)		Potexvirus (Baulcombe <i>et al.</i> , 1993)	Hordeivirus (Yelina <i>et al.</i> , 2002) Closterovirus (Chiba <i>et al.</i> , 2006)		Polerovirus (Barker, 1989; Jayasinghe <i>et al.</i> , 1989; Wilson and Jones, 1993) Comovirus (Malysenko <i>et al.</i> , 1989) Potexvirus (Taliensky <i>et al.</i> , 1982) Tobamovirus (Taliensky <i>et al.</i> , 1982b) Potyvirus (Wintermantel, 2005)
Closterovirus	Potexvirus (Agranovsky <i>et al.</i> , 1998) Hordeivirus (Agranovsky <i>et al.</i> , 1998)		Potexvirus (Fedorkin <i>et al.</i> , 2001)	Closterovirus (Chiba <i>et al.</i> , 2006; Reed <i>et al.</i> , 2003)		
Vitivirus				Closterovirus (Chiba <i>et al.</i> , 2006)		
Crinivirus						Potyvirus (Aritua <i>et al.</i> , 1998; Kanyeja <i>et al.</i> , 2000)
Carmovirus			Carmovirus (Kong <i>et al.</i> , 1997) Tombusvirus (Qu and Morris, 2002) Potexvirus (Thomas <i>et al.</i> , 2003)	Closterovirus (Chiba <i>et al.</i> , 2006)		
Rymovirus						
Tospovirus	Tobamovirus (Lewandowski and Adkins, 2005)					Machlomovirus (Scheets, 1998)
Rhabdovirus	Potexvirus (Huang <i>et al.</i> , 2005)					

*Nomenclature according to ICTV 2005.

†Protein function refers to the helper component supplied by the virus in the far left-hand column. Gene functions were assigned following the authors unless subsequent data clearly indicated otherwise.

‡Other means either viral proteins of unknown function or those with a function that is distinct from these other classes.

§In most cases, where the helper function is unknown, the synergism was between whole viruses in mixed infections.

van Vloten-Doting, 1975). Additionally, transcomplementation may even bypass the requirement for coat protein in systemic movement (Huppert *et al.*, 2002; Nagano *et al.*, 2001; Ryabov *et al.*, 1999).

Secondly, individual proteins may transcomplement multiple viruses. For instance, the red clover necrotic mottle virus (RCNMV) movement protein transcomplements viruses from seven distinct genera, the coat protein of alfalfa mosaic virus (AIMV) can transcomplement viruses from four distinct genera, and the movement protein of TMV can transcomplement members of 13 distinct genera (Table 1). Perhaps more unexpectedly, when single proteins transcomplement more than one virus, they may, even in a single host species, confer distinct attributes on each virus. Thus, TMV movement protein expressed from a transgene confers elevated titre on a caulimovirus and a nepovirus, accelerates disease development of cucumber mosaic virus (CMV) (without enhancing viral titre) and extends the host range of FHV (Cooper *et al.*, 1995; Dasgupta *et al.*, 2001). Whether these distinct manifestations of synergism stem from one single attribute of the helper protein, or reflect distinct protein functions, is not yet clear.

Lastly, to function in a synergism, the helper protein or virus must normally be host-adapted. However, there are exceptions to this rule, particularly amongst proteins that inactivate plant defences based on gene silencing (Voinnet *et al.*, 1999).

Taken as a whole, the data in Table 1 suggest that the ability to discriminate between viruses is not a dominant feature of viral protein function. Nevertheless, there is variation in the extent to which distinct classes of proteins seem able to discriminate, and these differences presumably reflect the mode of action of these proteins. Thus, proteins whose functions are known to require the recognition of specific viral genomic sequences or structures (e.g. coat proteins and replicases) are less likely to show transcomplementation of phylogenetically diverse viruses than proteins whose mode of action does not. However, in the case of replicase proteins, this rule has not been tested to any great extent, and for both coat proteins and replicases there are suggestions that these proteins can be multifunctional and may transcomplement using these 'secondary' functions. For example, some replicases appear to suppress host defences, and coat proteins can expand host range, inhibit gene silencing or show movement functions that may not require the recognition of viral sequences (Abbink *et al.*, 2002; Callaway *et al.*, 2001, 2004; Qu *et al.*, 2003; Spitsin *et al.*, 1999; Thomas *et al.*, 2003).

The above discussion summarizes some of the salient points that can be concluded from the evidence presently available. Nevertheless, in many respects, our understanding is based on a highly limited data set. For instance, synergisms may have diverse consequences, such as effects on infectivity (Chiba *et al.*, 2006; Sunter *et al.*, 2001), the speed with which infection proceeds (Cooper *et al.*, 1995), the efficiency of vector acquisition (Aritua *et al.*, 1998) or consequences for seed transmission (Kuhn and

Dawson, 1973), all of which are biologically very important. However, most investigations (especially of transcomplementation) report data on only a small subset of these potential consequences (for example, estimating changes in viral titre). Only relatively rarely do the subset of infection characteristics measured have unambiguous biological significance that would be useful for risk assessments. For instance, transcomplementation by viral suppressors of silencing is often reported to increase viral titre, but this may or may not have epidemiological importance. However, an impact of silencing suppressors that might be predicted and would almost certainly have epidemiological significance is the enhancement of specific infectivity; however, only two papers have reported testing a suppressor for this possibility and, in both cases, enhancement was observed (Chiba *et al.*, 2006; Sunter *et al.*, 2001). Our hope, therefore, is that one outcome of this review will be that, in future, reports of transcomplementation will provide data on a wider spectrum of infection characteristics, especially those with relevance to risk assessment. If this were to occur, it may well transpire that, as is the case with synergisms, the effects of transcomplementation, even by single proteins, will be found to be more complex and more diverse than the data at present imply.

VIRAL PROTEIN PRODUCTION IN VIRUS-RESISTANT PLANTS

As the relevance of transcomplementation and synergism to risk assessment is dependent on the extent to which transgenic virus-resistant plants express functional viral proteins, this section examines the evidence for protein expression and transcomplementation in transgenic virus-resistant plants, including those that have so far been approved for commercial release.

Transgenic crop plants coding for full-length proteins of viral origin represent a small but significant proportion of all genetically engineered crops approved worldwide. Listed in Table 2, they include NewLeaf[®] Y potato (potyvirus coat protein), SunUp Papaya (potyvirus coat protein), Newleaf Plus[®] potato (polverovirus replicase) and CZW-3[®] squash (two potyvirus coat proteins and a cucumovirus coat protein). All of these transgenic cultivars, as well as two pending US applications, one for a transgenic plum resistant to plum pox virus and one for a papaya ringspot-resistant papaya, are usually considered to resist viral infection by the mechanism of homology-dependent gene silencing, although this has not been formally proven (Beachy, 1997). Similar resistant cultivars containing diverse viral transgenes from a wide range of viruses have been approved for pre-commercial trials, primarily in the USA (www.nbiap.vt.edu/cfdocs/fieldtests1.cfm), and others are under development in various countries.

From the perspective of this review, the important question is whether the cultivars described in Table 2 are able to support

Table 2 Viral mRNAs and proteins in approved transgenic cultivars.

Line/event	Species	Transgene(s)	Full-length RNA	Protein present	Petition	Docket
RBMT21-129	Potato	PLRVrep	+	–	97-204-01p	97-094-1
RBMT21-152	Potato	PLRVrep	+	–	97-204-01p	97-094-1
RBMT21-350	Potato	PLRVrep	+	–	97-204-01p	97-094-1
RBMT22-82	Potato	PLRVrep	+	–	97-204-01p	97-094-1
RBMT22-186	Potato	PLRVrep	+	–	97-204-01p	97-094-1
RBMT22-238	Potato	PLRVrep	+	–	97-204-01p	97-094-1
RBMT22-262	Potato	PLRVrep	+	–	97-204-01p	97-094-1
RBMT15-101	Potato	PVYcp	+	+	97-339-01p	98-067-1
SEMT15-02	Potato	PVYcp	+	+	97-339-01p	98-067-1
SEMT15-15	Potato	PVYcp	+	+	97-339-01p	98-067-1
HLMT15-46	Potato	PVYcp	+	+	97-339-01p	98-067-1
55-1	Papaya	PRSVcp	N/A	+	96-051-01p	96-024-1
63-1	Papaya	PRSVcp	N/A	+	96-051-01p	96-024-1
ZW-20	Crookneck squash	WMV-2cp ZYMVcp	N/A	+	92-204-01p	92-127-1
			N/A	+		
CZW-3	Crookneck squash	Coat proteins of WMV-2, CMV, ZYMV	N/A	+	95-352-01p	96-002-1
			N/A	+		
			N/A	+		

+, present; –, none detected; CMV, cucumber mosaic virus; cp, coat protein; N/A, no data presented; PLRV, potato leaf roll virus; PRSV, papaya ringspot virus; PVY, potato virus Y; rep, replicase; WMV, wheat mosaic virus; ZYMV, zucchini yellow mosaic virus.

Presence or absence of viral mRNA and protein in transgenic cultivars subsequently approved for unrestricted commercial use in the USA. Data were obtained from petitions submitted to USDA. Petitions are available from http://www.aphis.usda.gov/brs/not_reg.html

transcomplementation. Unfortunately, this question cannot be answered directly, because on only one occasion has any direct test for transcomplementation been performed as part of a formal risk assessment. In this experiment, four plants of CZW-3 squash were infected with papaya ringspot virus (PRV-FI) (USDA docket 96-002-1). Levels of PRV-FI were measured and found to be unaltered. No other viruses were tested and, apart from virus concentration, no other infection characteristic was assessed. However, it is known that all transgenic virus-resistant cultivars commercialized so far produce detectable quantities of either full-length viral mRNAs or full-length viral proteins (Table 2). In all cases in which protein was found (all were coat proteins), these levels were lower than in (non-transgenic) virus-infected plants.

The detection of full-length proteins and mRNAs in commercialized plants has, nevertheless, not been sufficient to convince regulators in the US that transcomplementation is a possibility (e.g. USDA 97-204-01p; see Table 2). They, and others (e.g. Goldbach *et al.*, 2003), have tended to assume that gene silencing prevents sufficient (or, depending on the authors, any) protein expression, and thus transcomplementation, in virus-resistant plants. The limited evidence available, however, suggests that this conclusion may be premature. Although gene silencing does reduce protein levels, silenced transgenes can constitutively produce protein (Longstaff *et al.*, 1993). More importantly, transgenes that have been shown to be silenced in the absence of viral

infection can nevertheless transcomplement when challenged by non-target viruses (Farinelli *et al.*, 1992; Hammond and Dienelt, 1997; Mlotshwa *et al.*, 2002).

These observations of transcomplementation by apparently silenced transgenes may be accounted for by two alternative mechanisms. The first possibility is that a minority of transcripts evade silencing, and these transcripts produce sufficient quantities of viral protein to allow transcomplementation. A second possibility is that infecting non-target viruses inhibit gene silencing and thus permit transcomplementation. Support for this second possibility is provided by three lines of evidence: many plant viruses can inhibit gene silencing (e.g. Anandalakshmi *et al.*, 1998; Beclin *et al.*, 1998; Mitter *et al.*, 2003; Pfeffer *et al.*, 2002; Qu *et al.*, 2003; Voinnet *et al.*, 1999); infection by non-target viruses can relieve silencing-based resistance directed against target viruses (e.g. Mitter *et al.*, 2003; Savenkov and Valkonen, 2001a); and non-target viruses can rapidly induce protein expression from silenced transgenes, and this induction is the basis of an assay used to identify viral proteins that inhibit gene silencing (e.g. Voinnet *et al.*, 1999).

Experiments that might distinguish between these two alternative mechanisms have yet to be performed, but what seems to be clear is that the justifications noted above for discounting transcomplementation in transgenic virus-resistant plants are contradicted by the available evidence. Instead, non-target viruses infecting a commercial virus-resistant plant, either as

productive infections or as subliminal (non-productive) infections, may well encounter transgenic viral protein, either immediately, or shortly after, the initiation of infection.

Various authors have previously expressed concern that virus-resistant transgenic plants that carry viral transgenes may transcomplement non-target viruses. Some have expressed this concern for plant viral proteins in general (Power, 2002), and others for specific classes of viral proteins, including viral replicases (Miller *et al.*, 1997), movement proteins (Beachy, 1995), coat proteins (Falk *et al.*, 1995; Hull, 1994; Tepfer, 2002; de Zoeten 1991) and viral inhibitors of plant defences (Hammond *et al.*, 1999; Tepfer, 2002). Other authors, citing the possibility of transcomplementation, have created experimental resistant lines that cannot produce proteins (e.g. Higgins *et al.*, 2004; Masmoudi *et al.*, 2002). Nevertheless, developers of commercial transgenic virus-resistant cultivars and those responsible for crop approvals have consistently downplayed the biosafety risk arising from transcomplementation (e.g. USDA 97-204-01p), and continue to approve cultivars encoding full-length viral open reading frames (ORFs) for commercial use. Indeed, the US Environmental Protection Agency is currently proposing the extension of this policy to automatically deregulate (i.e. approve) any crop plant containing transgenic coat protein genes derived from plant viruses found in the USA (Federal Register Vol. 72, No. 74, 18 April 2007).

TRANSCOMPLEMENTATION AS A HAZARD

In any risk assessment, it is necessary to hypothesize direct or indirect negative outcomes (hazards) whose probability of occurring is then estimated. In the case of transcomplementation occurring in field-grown crops, four clear hazards can be identified.

1 Failure of the transgenic crop is perhaps the most clearcut hazard. Crop failure as a result of transcomplementation may follow from either enhanced infection by an established viral pathogen (e.g. Barker, 1989; Guerini and Murphy, 1999; Jayasinghe *et al.*, 1989; Valkonen, 1992; Wang *et al.*, 2004) or infection by a novel virus, i.e. one that is normally non-infectious (e.g. Cohen *et al.*, 1988; Hacker and Fowler, 2000; Hamilton and Dodds, 1970; Malysenko *et al.*, 1989; Sonoda *et al.*, 2000). Such an effect may result not only when a transgene disables host resistance or when it enhances viral spread within or between individual plants, but also when transcomplementation elevates virus titre, accelerates disease development or enhances symptoms.

2 Transcomplementation may lead to the enhanced infection of nearby crops or wild species by non-target viruses (Fuchs *et al.*, 2000; Lecoq *et al.*, 1993). A number of the outcomes of transcomplementation documented here have the potential for consequences that are observable partially or even only in neighbouring (i.e. non-transgenic) plants, either of the same or distinct species. This hazard can be divided into several components,

including: (i) transcomplementation may qualitatively expand opportunities for plant-to-plant transmission (by extending the range of vector species or subspecies that are able to transmit the non-target virus); (ii) transcomplementation may lead to quantitatively enhanced acquisition and transmission of a non-target virus by the vectors that normally transmit that virus; for example, the acquisition of a non-target virus from the transgenic crop may be enhanced by increased susceptibility of the transgenic crop to viral infection, by elevated viral titre, increased speed of infection or expanded tissue distribution within the transgenic crop; (iii) transcomplementation may lead to infection of the transgenic crop by viruses that are new to the crop (e.g. resulting from a loss of resistance), and this may, in turn, affect neighbouring crops (see examples below). It is worth noting that the effects outlined above are, in principle at least, independent of any direct effect on the transgenic crop itself. Thus, they can occur in the absence of any visible effect on the transgenic crop itself (Fuchs *et al.*, 2000; Lecoq *et al.*, 1993).

Such indirect effects, in which the crop functions essentially as a new or enhanced viral reservoir, are well known to have epidemiological importance (Hooks and Fereres, 2006; Malmstrom *et al.*, 2005). They can be illustrated by two hypothetical examples that are discussed briefly below. The purpose of these examples, which focus on the event of a crop becoming susceptible to a new viral species, is to show that the necessary preconditions for this hazard can be commonly found in agriculture.

In the USA, soybean commonly hosts *Myzus persicae* (an insect vector of PVY), but soybean is not itself a host for PVY (Schultz *et al.*, 1985). If transgenic soybean were to become able to support infection by PVY (as a result of transcomplementation), it would become a reservoir (rather than a sink) for PVY, allowing PVY to become more prevalent on its usual solanaceous host plants. Such hazards would not necessarily be restricted to the immediate geographical area of the susceptible crop, as many insect vectors migrate over large distances and (unlike *M. persicae* for PVY) retain infectivity for long periods. As a second example, cucurbit yellow stunting disorder (CYSDV) is caused by a cucurbit-infecting closterovirus transmitted semipersistently by the whitefly *Bemisia tabaci* (Celix *et al.*, 1996). Whiteflies feed on tomatoes, but tomatoes are resistant to CYSDV. Should their resistance to CYSDV be abolished, CYSDV would probably become more prevalent on cucurbits.

3 A usual response of farmers to virus infection is to deploy insecticides against their insect vectors (Lapidot and Friedmann, 2002). Increased pesticide use can be predicted if hazards 1 or 2 occur.

4 In supporting transmission by new insects and infection of new plant hosts, transcomplementation may bring together viruses that normally are separated in space or time. If so, transcomplementation may increase opportunities for recombination to generate novel viruses (Roosinck, 1997).

LIMITATIONS IN PREDICTING TRANSCOMPLEMENTATION

Predicting the likelihood (preferably quantitatively) of carefully defined hazards is necessary to complete the task of risk assessment. Table 1 is intended to serve as a basic guide to reported synergisms and transcomplementation. It provides a starting point for a case-by-case type assessment of any virus-resistant cultivar using data from peer-reviewed publications, and, importantly, it indicates potential data gaps. However, in addition to the gaps, it is possible to identify, from the publications noted in Table 1, other limitations to the usefulness of the strategy of predictive risk assessment. Some of these limitations are considered below.

One of the most important of these limitations arises from the evidence, from both viral synergism and transcomplementation, that a previously resistant crop plant may become susceptible to a wider than usual range of viruses (Cohen *et al.*, 1988; Dasgupta *et al.*, 2001; Hacker and Fowler, 2000; Hamilton and Nichols, 1977; Malyshenko *et al.*, 1989; Sonoda *et al.*, 2000; Spitsin *et al.*, 1999). Effective risk assessment for this possibility does not require the testing of all known viruses, but it does require specific testing of all those viruses that are carried by insect vectors that normally visit the crop without causing productive infections (Hooks and Fereres, 2006). Especially in countries in which local knowledge of virus diseases is poor, the identification of candidate viruses for testing will constitute a considerable challenge and may, in practice, prove impossible, particularly as these will vary regionally and even locally.

A second limitation is that synergisms can be affected by the specific strain of the dependent virus, the host species or cultivar and, probably, the virus strain used to make the transgene (Cooper *et al.*, 1995; Hii *et al.*, 2002; Mendez-Lozano *et al.*, 2003; Rao *et al.*, 1998; Voinnet *et al.*, 1999; Wang *et al.*, 2004). Thus both positive and, perhaps more importantly, negative results cannot confidently be extrapolated to agricultural situations in which the relevant components are not identical. Similarly, interactions between stacked transgenes may also influence the risk. As an example, the movement of brome mosaic virus (BMV) by the CMV movement protein also requires the presence of the CMV coat protein (Nagano *et al.*, 1999).

A third limitation is illustrated by risk assessments which have historically made presumptions about the biological function of the virus-derived sequence. One such assumption, that the transgene contains no unidentified functional ORFs, has been shown to be incorrect in the case of NewLeaf[®] Plus potatoes. NewLeaf[®] Plus potatoes express not only the P1 and P2 ORFs of potato leaf roll virus (PLRV), but also 229 of the 273 amino acids of the overlapping P0 ORF, which was identified as a suppressor of host defences only subsequent to risk assessment and commercial release (Pfeffer *et al.*, 2002).

A related limitation is incomplete current knowledge of viral protein function, which can be inferred from the fact that new functions of both plant viruses and their proteins are continually being discovered (Abbink *et al.*, 2002; Belliure *et al.*, 2005). Some of these, such as the recent discovery that the coat protein of turnip crinkle virus also inhibits host defence mechanisms, have potential implications for transcomplementation (Qu *et al.*, 2003; Thomas *et al.*, 2003). This latter example illustrates the difficulty in assuming that assigned classes of protein (movement, replicase, coat protein, etc.) constrain the consequences of transcomplementation. Coat proteins, for example, as well as being capable of transcapsidation, have also been shown to expand host range (Spitsin *et al.*, 1999), inhibit gene silencing (Qu *et al.*, 2003; Thomas *et al.*, 2003) and transcomplement defects in movement (Fedorkin *et al.*, 2000; Taliansky and Garcia-Arenal, 1995). Replicase proteins can inhibit host defences (Abbink *et al.*, 2002), and movement proteins can confer mechanical transmission (Ryabov *et al.*, 2001), expand host range (Dasgupta *et al.*, 2001; Fenczik *et al.*, 1995) and increase virulence (Cooper *et al.*, 1995; Schaffer *et al.*, 1995). These findings reinforce the theory that viral genes are frequently multifunctional and that commonly applied labels, although useful in other contexts, are nevertheless simplistic descriptors of gene functions and are not appropriate in risk assessment. Thus, in the risk assessment of any particular transgenic plant, each and every endpoint that might be a hazard, or lead to one, needs to be tested for specifically and regardless of the protein transferred.

Additional limitations to risk assessment may also result from the changing and/or diverse effects of cropping systems, geographic location, vector type and abundance, availability of alternative hosts and even temperature, all of which can alter either the results or the implications of synergism (Close, 1964; Falk *et al.*, 1995).

Lastly, viruses may in time adapt to transgenic hosts. For example, cowpea chlorotic mottle virus (CCMV), whose own movement protein was replaced with that of BMV, was not infectious on cowpeas (Mise *et al.*, 1993). However, four of 42 inoculations of the hybrid virus generated infectious host-adapted mutants. The authors suggested that the number of mutations required to adapt the hybrid CCMV to the host was small. Thus, transcomplementation modifies the selective environment and, by lowering host barriers to infection, may create opportunities for pathogen evolution.

These confounding factors place severe constraints on the likelihood that published results, or even any conceivable risk assessment process, will accurately predict the hazards noted above for commercial transgenic plants. It will perhaps be argued that plant breeders will detect the negative consequences of transcomplementation and discontinue development of the transgenic cultivar. It is perfectly possible that they may notice susceptibility to novel pathogens, but it should be noted that the difficulties for

breeders will not be less than those mentioned above. It should also be recognized that commercial breeders have released both transgenic and conventional cultivars that have subsequently turned out to be unexpectedly susceptible, even to well-known pathogens (Brodie, 2003; Colyer *et al.*, 2000; Tomlinson, 1987).

CONCLUSIONS AND RECOMMENDATIONS

This review has established that viral transgenes, even those that are normally silenced, may produce viral proteins and may transcomplement non-target viruses (Farinelli *et al.*, 1992; Hammond and Dienelt, 1997; Mlotshwa *et al.*, 2002). Transcomplementation, although not inevitably observed, can be caused by genes from many viruses, and typically leads to the enhanced replication and spread of non-target viruses within or between plants, and sometimes causes plants to become susceptible to viruses against which they are normally resistant. Importantly, a single viral transgene may transcomplement multiple virus species.

Viral proteins are therefore often indiscriminate facilitators of viral infection. The exceptions to this rule appear to be coat proteins, which, at least in their role as transcapsidators, show some degree of species specificity, as do replicase proteins in their role as polymerases. One explanation for this variability in discrimination is likely to be that many viral proteins interact directly with the plant to disable host defences, thus allowing any virus present to benefit. Nevertheless, significant questions of specificity remain to be answered. Perhaps the most important of these is the extent to which the proteins of DNA viruses can transcomplement RNA viruses, and vice versa. Transcomplementation of a caulimovirus by the movement protein of TMV is the single example of transcomplementation of a DNA virus by an RNA viral protein that cannot at present be explained by the inhibition of host defences (Cooper *et al.*, 1995). This intriguing observation, which has not been followed up, may indicate a peculiarity of caulimoviruses or of the TMV movement protein, or may represent a general, but so far unexplored, phenomenon.

A further important conclusion of this review is the difficulty of excluding empirically the possibility that transcomplementation will occur in agricultural situations. One response to the possibility of transcomplementation, and which has been specifically accepted by US regulators, is to rely on market disapproval as a mechanism to withdraw any transcomplementing transgenic cultivars (e.g. USDA 97-204-01p). The effectiveness of this option, however, is open to question. Experience with Starlink[®] maize suggests that, even under highly favourable conditions, eradication of a transgene from an agricultural system may take many years (UCS, 2004). The time taken will vary and will be dependent on ecological variables, such as seed bank survival and the extent of gene flow to other cultivars and wild relatives, as well as social factors, such as speed of discovery and communication, the ability to identify the transgene and levels of seed saving. For many

nations and agro-ecosystems, these parameters are unfavourably aligned, and therefore reliance on withdrawal is probably an inappropriate strategy. A second problem is that crop failure, such as might result from the loss of virus resistance, is sometimes not an acceptable outcome. This is particularly true for staple crops anywhere, but especially in regions in which food security and farm incomes are low. A third problem is that it is far from clear whether a virus that takes advantage of transcomplementation will necessarily revert to its original host range. A fourth is that, as described above, the effects of transcomplementation may not be limited to, or even found at all in, the transgenic crop itself.

Viral protein expression appears to be an unnecessary consequence of engineering virus resistance (Higgins *et al.*, 2004; Masmoudi *et al.*, 2002; Niu *et al.*, 2006; Waterhouse *et al.*, 1998). A straightforward and technically simple solution is therefore to ensure that the transgene contains a series of termination codons or frame shift mutations that prevent or disrupt protein production. This preventative measure has been proposed or specifically recommended by almost all authors of papers reviewing the risks of transgenic virus-resistant plants, and yet it has not been adopted by commercial producers and it is still not required by regulators (Beachy, 1995; Hammond *et al.*, 1999; Miller *et al.*, 1997; Tepfer, 1993, 2002). Disruption should be applied to all potential viral ORFs (in case functional proteins have been overlooked). It should also be applied regardless of any presumed protein function, and should be performed using multiple dispersed termination codons, because any single termination codon may be fully or partially ineffective. These precautions are also necessary because even truncated viral proteins may support synergisms (Sunter *et al.*, 2001). Indeed, there are even reports in which a truncated protein demonstrated a transcomplementation function lacking in the full-length protein (e.g. Nagano *et al.*, 2001). The final recommendation is that viral sequences should be as short as possible, and that applicants should demonstrate this fact experimentally as a condition of approval. An alternative approach that has also shown promise for conferring virus resistance is the use of transgenes containing inverted repeats of short viral sequences (Waterhouse *et al.*, 1998). Precautions such as those listed above should nevertheless still be taken to ensure that viral protein expression is avoided.

Disabling protein expression has two significant additional benefits. Firstly, it will greatly reduce any risks from viral/transgene recombination. Secondly, viral proteins are derived from pathogens. Unexpected and undetected negative effects of viral proteins on plant health or even human health might occur, and would be prevented by avoiding protein expression. Taken together, these recommendations are in line with an important but widely underestimated aspect of safe technologies: that safety is established not only by risk assessment but by safeguards incorporated in good design (Kapuscinski *et al.*, 2003).

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