Transgenic plants for animal health: plant-made vaccine antigens for animal infectious disease control

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Abstract A variety of plant species have been genetically modified to accumulate vaccine antigens for human and animal health and the first vaccine candidates are approaching the market. The regulatory burden for animal vaccines is less than that for human use and this has attracted the attention of researchers and companies, and investment in plant-made vaccines for animal infectious disease control is increasing. The dosage cost of vaccines for animal infectious diseases must be kept to a minimum, especially for non-lethal diseases that diminish animal welfare and growth, so efficient and economic production, storage and delivery are critical for commercialization. It has become clear that transgenic plants are an economic and efficient alternative to fermentation for large-scale production of vaccine antigens. The oral delivery of plant-made vaccines is particularly attractive since the expensive purification step can be avoided further reducing the cost per dose. This review covers the current status of plant-produced vaccines for the prevention of disease in animals and focuses on barriers to the development of such products and methods to overcome them.

Abbreviations

AlMV	alfalfa mosaic virus
CaMV	cauliflover mosaic virus
CPMV	cowpea mosaic virus
CT	cholera toxin of *Vibrio cholerae*
CT-B	cholera toxin B subunit of *Vibrio cholerae*
EHEC	enterohemorrhagic *Escherichia coli*
ELP	elastin-like polypeptide
ER	endoplasmic reticulum
ETEC	enterotoxigenic *Escherichia coli*
FDA	Food and Drug Administration agency in United States
FMDV	foot and mouth disease virus
GFP	green fluorescence protein of *Aequorea Victoria*
GM	genetically modified
GRAS	generally recognized as safe
GUS	beta glucuronidase of *Escherichia coli*
HbsAg	hepatitis virus B surface antigen
IBDV	chicken infectious bursal disease virus
IBV	chicken infectious bronchitis virus
LT	heat-labile enterotoxin of *Escherichia coli*
LT-B	heat-labile enterotoxin subunit B of *Escherichia coli*
PEDV	porcine epidemic diarrhea virus

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PMP	plant-made pharmaceutical
PMV	plant-made vaccine
PPV	plum pox potyvirus
PVX	potato virus X
TEV	tobacco etch virus
TGEV	transmissible gastroenteritis virus
TMV	tobacco mosaic virus
TSP	total soluble protein
USDA	United States Department of Agriculture

Introduction

Vaccination continues to be the most important and cost-effective way to control animal and human infectious disease. No other means has had such an impact in increasing welfare by decreasing morbidity and mortality. Since the days of Jenner's discovery more than 200 years ago, successful vaccination strategies have been established against numerous animal and human diseases. Although vaccine technology has made substantial progress, the basic concept remains the same. The majority of licensed animal vaccines against virus or bacteria are either live-attenuated or killed. Live-attenuated vaccines are generally efficient and induce strong immune responses. However, their manufacture and use present potential risks, including contamination risk during the manufacturing process and the possibility to revert their virulence in vivo. While inactivated vaccines cannot replicate in vivo and are safer to use, they often have lower levels of immunogenicity and share the same risks during the manufacture.

Recombinant subunit vaccines can circumvent the risks associated with the production of whole cell vaccines and there is increasing regulatory demand for such products in animal infectious disease control. To conquer a disease with a subunit vaccine, identification of a suitable antigen with the ability to elicit a protective immune response is a critical early step. Known protective antigens can be expressed in transgenic plants. This could offer new vaccination strategies (e.g. oral applications vs. injections) due to the low costs of production and delivery. For new antigen discovery, plant transient expression systems are competitive to the other available production platforms in terms of time and scalability (Gleba et al. 2007).

Possibility to deliver plant-made vaccines (PMV) orally has been the focus of many studies. Oral delivery is attractive for its simplicity, and increases likelihood for local mucosal immune responses at sites of infection. However, the amount of antigen needed for oral delivery is high when compared to parenteral administration (Streatfield and Howard 2003b). A successful oral vaccine must survive the gastrointestinal conditions and must stimulate the mucosal immune system and provide protection against subsequent infections. The intestinal epithelium has a dual function. It allows penetration of nutrients and macromolecules important for growth and development, while providing a barrier to potentially harmful micro-organisms (Mestecky et al. 2005). The diet of all animals consists of a complex mixture of proteins and other macromolecules that are potentially antigenic. Since these food antigens usually do not elicit immune responses, the mucosal immune system apparently can distinguish between harmful pathogenic antigens and harmless food antigens (Holmgren et al. 2003). Pathogenic antigens tend to elicit immunosurveilance, leading to an active immune defense, whereas soluble food antigens usually activate an immunosuppressive state known as oral tolerance. However, certain soluble antigens, including cholera toxin (CT), heat-labile enterotoxin of *Escherichia coli* (LT), and some lectins that bind enterocytes efficiently, such as F4 fimbriae in pigs (Van den Broeck et al. 1999), can elicit immune responses upon oral administration (Strober et al. 1998). The exact mechanisms behind the induction of these opposite mucosal functions are not completely understood, but unresponsiveness is the default to soluble antigens (Holmgren et al. 2003). The mucosal immune response can be elicited either by transporting the antigen through specialized antigen sampling cells, designated M-cells, or by the receptor-mediated transport through epithelial enterocytes (Strober et al. 1998; Neutra and Kozlowski 2006). The transported antigens are captured by underlying antigen-presenting cells, which are activated by the co-stimulatory signals and can elicit the immune response at immunocompetent sites (Neutra and Kozlowski 2006). In addition, dendrite cells can capture micro-organisms or particulated antigens directly from the luminal content of the intestine (Rescigno et al. 2001; Rimoldi and Rescigno 2005).

The concept of oral vaccination by crudely processed plant material expressing antigens has been demonstrated on humans in several studies (Tacket et al. 1998, 2000, 2004; Kapusta et al. 1999; Thanavala et al. 2005). Direct guidelines for human medicine have hindered progress of these products behind the first preliminary clinical trials and the interest on human-use plant-made pharmaceuticals (PMP) has focused towards purified products.

The regulatory load for animal pharmaceuticals is less than that for human use, and for veterinary purposes the oral vaccination by feeding of transgenic plants remains very attractive. Efficacy of PMVs has been proved in target animals such as pigs and chicken (Lamphear et al. 2002; Zhou et al. 2004; Guerrero-Andrade et al. 2006; Joensuu et al. 2006b). Veterinary PMPs have been summarized in 2005 (Streatfield 2005; Rice et al. 2005) and 2007 (Floss et al. 2007). In this review, we reflect the current status of the advantages and challenges of vaccine antigen expression in plants and give an overview on applications designed to overcome animal infectious disease.

Expression of vaccine antigens in plants

Plant species for vaccine antigen expression

Leafy feed/food crops used for vaccine antigen production include alfalfa (Wigdorovitz et al. 1999a), lettuce (Kapusta et al. 1999), spinach (Modelska et al. 1998), and white clover (Lee et al. 2001). The legumes alfalfa and white clover are widely cultivated, high in protein and are capable of nitrogen fixation, which reduces both production costs and environmental load. Lettuce and spinach are regularly consumed by humans and thus, are potential delivery vehicles for human oral vaccines. The non-food model plant *Arabidopsis* has also been used for vaccine antigen production, but its small size probably limits its use to small-scale proof of principle studies (Gomez et al. 1998).

Using the logic that fruit and vegetable crops can be consumed raw or processed into various palatable forms without cooking, they have also been utilized in vaccine antigen production. Reported examples include bananas (Kumar et al. 2005), tomatoes (McGarvey et al. 1995), cherry tomatillos (Gao et al.

2003), potatoes (Haq et al. 1995), melons (Nagesha et al. 2007), and carrots (Bouche et al. 2003). Seed legumes, pigeon pea (Satyavathi et al. 2003), soybean (Piller et al. 2005), and peanut (Khandelwal et al. 2004) have been used to produce vaccine antigens. Maize (Streatfield et al. 2001), rice (Yang et al. 2007), and barley (Joensuu et al. 2006a) are the cereal crops that have been used for seed-based vaccine antigen production.

Non-food crop species like tobacco are an attractive option for recombinant protein production because they minimize regulatory barriers by eliminating the risk of entry into the food chain. The leaves are harvested before flowering, significantly reducing the potential for gene leakage into the environment through pollen or seed dispersal. Unlike seeds or tubers, tobacco leaves are perishable and will not persist in the environment. Therefore, tobacco is now recognized as the platform of choice for biopharmaceutical production and is the most common plant species used for the production of vaccine antigens (Table 1). There are well-established transformation and regeneration techniques available for tobacco and it is relatively easy to propagate and grow on a laboratory scale. Under field conditions, tobacco can produce over 50,000 kg/ha of fresh biomass in a single season (Woodlief et al. 1981). For oral administration of vaccine antigens in intact leaf tissue, certainly a low cost approach, the presence of nicotine alkaloids could limit the use of tobacco but low-nicotine tobacco platforms that are suitable for direct oral administration have been developed (Menassa et al. 2007).

Properties of host plant tissues for antigen production

Vaccine antigens have been expressed in fresh plant tissues such as leaves (Mason et al. 1992), fruits (McGarvey et al. 1995), tubers (Haq et al. 1995), and taproots (Bouche et al. 2003), or in mature seeds (Streatfield et al. 2001). Harvested fresh plant tissues usually need processing or freezing to preserve the antigen in a stable form, while mature seeds are desiccated and allow long-term storage at ambient temperatures. As well, fruits, tubers, and taproots can be stored for a limited time, especially in a chilled environment. Vaccine antigens have also been expressed in undifferentiated plant cell cultures such

Table 1 Subunit vaccine candidates for animal infectious disease control expressed by transgenic plants, plant cell cultures or plant viruses

Pathogen/Host	Antigen	Prod. system	Expression system[a]	Yield[b]	Immune response	References
Applications against animal virus pathogens						
Avian Rheo virus/Poultry	σC protein	Alfalfa leaves	p35S-3'nos[c] pAct-3'nos	0.008% TSP[c] 0.007% TSP	ND	Huang et al. (2006)
Bovine herpes virus/Cattle	Truncated glycoprotein D	TMV vectors in tobacco	Peptide expression under CP subgenomic promoter	20 μg/g FW	Immunogenic in mice and cows after parenteral administration. Reduced symptoms in cows challenged with the virus	Perez Filgueira et al. (2003)
Bovine rotavirus/Cattle	VP6 protein	Potato tubers	p35S-3'nos[d] 2x p35S-5'Ω-3'nos[c]	0.002% TSP[c]	Immunogenic[d] in mice after parenteral administration	Matsumura et al. (2002)
		Tobacco leaves (chloroplast)	prrn-5'rbcL-3'rrnB[c] ppsbA-5'psbA-3'rrnB ptrc-5'lacZ-3'rrnB	3% TSP[c]	ND	Birch-Machin et al. (2004)
	VP8* part of VP4	TMV vectors in tobacco	Peptide expression under CP subgenomic promoter	5 μg/g FW	Immunogenic and protective in mice after parenteral administration. Passive lactogenic protection of pups	Perez Filgueira et al. (2004)
	VP4 epitope BRV4 as fusion with GUS	Alfalfa leaves	p35S-3'nos	0.04% TSP	Immunogenic and protective in mice after oral or parenteral administration. Passive lactogenic protection of pups	Wigdorovitz et al. (2004)
Canine papilloma virus/Dogs	L2 epitope as fusion with streptavidin	TMV vectors in tobacco	Display on viral particles as part of CP	120 μg/g FW	Immunogenic in mice after parenteral administration	Smith et al. (2006)
Canine parvovirus/Dogs	VP2 epitope	PPV vectors in tobacco	Display on viral particles as part of CP	NR	Immunogenic in mice and rabbits after parenteral administration. Neutralizing antibodies in mice	Fernandez-Fernandez et al. (1998)
		CPMV vectors in cowpea	Display on viral particles as part of CP	NR	Protective against lethal challenge in dogs after parenteral administration	Langeveld et al. (2001)
		CPMV vectors in cowpea	Display on viral particles as part of CP	NR	Neutralizing antibodies in mice after parenteral or nasal administration.	Nicholas et al. (2002)
	VP2 epitope as fusion with GUS	*Arabidopsis* leaves	p35S-3'nos	3.3% TSP	Immunogenic in mice after parenteral or oral administration	Gil et al. (2001)
	VP2 2L21 epitope as fusion with cholera toxin B subunit, or with green fluorescent protein	Tobacco leaves (chloroplast)	prrn-5'psbA-3'psbA	22.6 and 33.1% TSP	Neutralizing antibodies in mice and rabbits after parenteral administration. Immunogenic but not neutralizing in mice after oral administration	Molina et al. (2004), (2005)

Table 1 continued

Pathogen/Host	Antigen	Prod. system	Expression system[a]	Yield[b]	Immune response	References
Classical swine fever virus/Swine	E2 glycoprotein as fusion with ubiquitin fragment	Lettuce and alfalfa leaves	p35S	160 and 10 µg/g DW	Immunogenic in mice after oral administration	Legocki et al. (2005)
	E2 epitopes	PVX vectors in tobacco	Display on viral particles as part of CP	NR	Immunogenic in rabbits after parenteral administration	Marconi et al. (2006)
Cottontail rabbit papillomavirus/Rabbits	L1 capsid protein	Tobacco leaves and TMV vectors in tobacco	p35S-3′ocs and display on viral particles as part of CP	1 and 0.6 µg/g FW	Immunogenic in rabbits after parenteral administration. Rabbits protected against viral challenge	Kohl et al. (2006)
Foot and mouth disease/Farmed and wild animals	VP1 epitope	CPMV vectors in cowpea	Display on viral particles as part of CP	NR	ND	Usha et al. (1993)
		Arabidopsis leaves	p35S-3′nos	NR	Immunogenic in mice after parenteral administration. Mice protected against viral challenge	Carrillo et al. (1998)
		TMV vectors in tobacco	Peptide expression under CP subgenomic promoter	150 µg/g FW	Immunogenic in mice after parenteral administration. Mice protected against viral challenge	Wigdorovitz et al. (1999b)
		Alfalfa leaves	p35S-3′nos	NR	Immunogenic in mice following parenteral or oral administration. Mice protected against viral challenge	Wigdorovitz et al. (1999a)
			p35S-3′nos	0.01% TSP	Immunogenic in mice following parenteral administration. Mice protected against viral challenge	Dus Santos and Wigdorovitz (2005)
		Potato leaves	p35S-3′nos[c] 2x p35S-3′nos	0.01% TSP[c]	Immunogenic and protective in mice after parenteral administration	Carrillo et al. (2001)
	VP1 protein	Tobacco leaves (chloroplast)	p16S-3′psbA	3% TSP	ND	Li et al. (2006)
	VP1 epitope as fusion with GUS	Alfalfa leaves	p35S-3′nos	0.1% TSP	Immunogenic in mice following parenteral administration. Mice protected against viral challenge	Dus Santos et al. (2002)
	VP1 epitope as fusion with hepatitis B core protein	Tobacco leaves	p35S-3′nos	0.05% TSP	Immunogenic in mice following parenteral administration. Mice protected against viral challenge	Huang et al. (2005)
Infectious bronchitis virus/Chicken	S1 glycoprotein	Potato tuber	p35S-3′nos	0.22% TSP, 2.53 µg/g FW	Immunogenic in chickens after parenteral or oral administration. Neutralizing antibodies protected chickens against viral challenge	Zhou et al. (2003), (2004)

Table 1 continued

Pathogen/Host	Antigen	Prod. system	Expression system[a]	Yield[b]	Immune response	References
Infectious bursal disease virus/Chicken	VP2 protein	*Arabidopsis* leaves	NR	4.8% TSP	Immunogenic and protective in chickens after oral administration	Wu et al. (2004)
Mink enteritis virus/Minks	VP2 epitope	CPMV vectors in cowpea	Display on viral particles as part of CP	1,200 µg/g FW	Immunogenic and protective in minks following parenteral administration	Dalsgaard et al. (1997)
Murine hepatitis virus/Mice	Glycoprotein S 5B19 epitope	TMV vectors in tobacco	Display on viral particles as part of CP	NR	Immunogenic and protective in mice following parenteral or nasal administration	Koo et al. (1999)
Newcastle disease virus/Poultry	F and HN surface glycoproteins	Potato leaves	p35S-5′Ω-3′nos	0.06% TSP	Immunogenic in mice following parenteral or oral administration	Berinstein et al. (2005)
	F and HN epitopes	CMV vectors in tobacco	Display on viral particles as part of CP	430µg/g FW	ND	Zhao and Hammond (2005)
	F and HN epitopes	PVX vectors in tobacco	Display on viral particles as part of CMV CP	NR	ND	Natilla et al. (2006)
	F surface glycoprotein	Maize seeds	pUbi-3′35S	3% TSP	Immunogenic and protective in chickens following oral delivery	Guerrero-Andrade et al. (2006)
		Rice leaves and seeds	pUbi-3′nos pGT1-3′nos	0.55% TSP	Immunogenic in mice after parenteral delivery	Yang et al. (2007)
	HN surface glycoprotein	Tobacco leaves	p35S-5′TEV-3′35S	0.069% TSP	Immunogenic in chickens following oral delivery	Hahn et al. (2007)
Peste des petits ruminant virus/Farmed and wild animals	Hemaglutinin-neuramidase	Pigeon pea leaves	p35S-3′nos	NR	ND	Prasad et al. (2004)
Porcine epidemic diarrhea virus/Swine	Spike protein	Tobacco leaves	NR	20 µg/g FW	Systemic and mucosal antibodies in mice after oral administration	Bae et al. (2003)
		Tobacco leaves	2xp35S-5′Ω-3′nos	2.1% TSP	ND	Kang et al. (2005a, b)
		Potato tubers	p35S-5′Ω-SEKDEL-3′nos	0.1% TSP	ND	Kim et al. (2005)
	COE epitope as fusion with LTB	Rice seeds	pUbi-3′nos	1.3% TSP	ND	Oszvald et al. (2007)
Porcine parvovirus/Swine	VP2 capsid protein	Tobacco leaves	2x35S-5′AlMV-3′nos	0.3% TSP	Neutralizing antibodies in mice after parenteral administration	Rymerson et al. (2003)

Table 1 continued

Pathogen/Host	Antigen	Prod. system	Expression system[a]	Yield[b]	Immune response	References
Rabbit hemorrhagic disease virus/ Rabbits	VP60 protein	Potato leaves	p35S-3'nos	0.16% TSP	Immunogenic in rabbits after parenteral administration. Neutralizing antibodies protected rabbits against lethal challenge	Castanon et al. (1999)
		PPV vectors in tobacco	Protein expression as part of viral polyprotein (protein released by viral protease)	NR	Immunogenic in mice and rabbits after parenteral administration. Neutralizing antibodies protected rabbits against lethal challenge	Fernandez-Fernandez et al. (2001)
		Potato tubers	ppatatin-SP(T)-3'pin2	0.35% TSP	Immunogenic and partially protective in rabbits after oral delivery	Castanon et al. (2002); Martin-Alonso et al. (2003)
		Arabidopsis leaves	p35S-5'TEV-3'nos	0.8% TSP	Immunogenic in mice after parenteral/oral administration + parenteral boosting	Gil et al. (2006)
Rabies virus/ Farmed and wild animals, humans	Glycoprotein	Tomato leaves and fruit	p35S-3'nos	0.001% TSP	ND	McGarvey et al. (1995)
		Tobacco leaves	p35S-SP(Pr1a)-SEKDEL-3'nos	0.38% TSP	Protective antibodies in mice after parenteral administration	Ashraf et al. (2005)
		Cantaloupe melon fruits	p35S-KDEL-3'nos	1.2% TSP	Immunogenic and protective in mice after parenteral administration	Nagesha et al. (2007)
	Glycoprotein and nucleoprotein epitopes	AlMV vectors in tobacco	Display on viral particles as part of CP	NR	Neutralizing antibodies in mice after parenteral administration	Yusibov et al. (1997)
		AlMV vectors in tobacco and spinach	Display on viral particles as part of CP	30 µg/g FW	Protective antibodies in mice after parenteral and oral administration. Immunogenic in humans after oral administration	Modelska et al. (1998), (2002)
Rinderpest virus/ Cattle	Hemagglutinin protein	Pigeon pea leaves	p35S-3'nos	0.49% TSP	ND	Satyavathi et al. (2003)
		Tobacco leaves	p35S-3'nos	0.75% TSP	Immunogenic in mice after parenteral administration	Khandelwal et al. (2003b)
		Peanut leaves	p35S-3'nos	1.3% TSP	Neutralizing antibodies in mice after oral and parenteral administration. Immunogenic in cattle after oral administration. Serum neutralized virus in vitro	Khandelwal et al. (2004), (2003a, c)

Table 1 continued

Pathogen/Host	Antigen	Prod. system	Expression system[a]	Yield[b]	Immune response	References
Transmissible gastroenteritis coronavirus/Swine	Spike (S) glycoprotein	*Arabidopsis* leaves	p35S-3′nos	0.06% TSP	Neutralizing antibodies in mice after parenteral administration	Gomez et al. (1998)
		Potato tubers	p35S-3′nos	0.07% TSP	Immunogenic in mice after parenteral or oral administration	Gomez et al. (2000)
		Tobacco leaves	pSUPER-SP(Pr1a)-3′nos pSUPER-3′nos[c]	0.2%TSP[c]	Neutralizing antibodies in pigs after parenteral administration	Tuboly et al. (2000)
		Maize seeds	NR	2%TSP	Immunogenic and protective in piglets after oral administration	Lamphear et al. (2002); Streatfield et al. (2001); Streatfield and Howard (2003b)
Applications against bacterial pathogens infecting animals						
Actinobacillus pleuropneumoniae/ Swine	ApxIIA exotoxin	Tobacco leaves	2x35S-3′nos	0.1% TSP	Immunogenic and protective in mice after oral administration	Lee et al. (2006)
Escherichia coli, enterohemorrhageic/ Cattle, humans	intimin of O157:H7	Tobacco leaves	p35S-5′TEV-3′vsp[d] p35S-5′TEV-SP(vspαS)-3′vsp[c]	3 μg/g FW[c]	Immunogenic[d] and partially protective in parenterally primed mice after oral administration	Judge et al. (2004)
	Stx2 toxin A and B subunits	Tobacco cell culture	p35S-5′Ω-3′vsp p35S-5′TEV-3′vsp	8.2 μg/g FW	Immunogenic and protective in mice after oral administration	Wen et al. (2006)
Escherichia coli, enterotoxigenic, Farmed animals, humans	Heat labile toxin, B subunit	Potato tubers	p35S-5′TEV-3′vsp p35S-5′TEV-SEKDEL-3′vsp[c]	0.01% TSP[c]	Neutralizing serum and mucosal antibodies in mice after oral administration	Haq et al. (1995)
			p35S-5′TEV-3′vsp	17.2 μg/g FW	Immunogenic and partially protective in mice after oral administration	Mason et al. (1998)
			p35S-5′TEV-3′vsp	15.7 μg/g FW	Specific serum and mucosal antibodies in humans after oral administration	Tacket et al. (1998)

Table 1 continued

Pathogen/Host	Antigen	Prod. system	Expression system[a]	Yield[b]	Immune response	References
			ppatatin-3′nos	13 µg/g FW	Immunogenic in primed mice after oral administration	Lauterslager et al. (2001)
		Maize seeds	NR	NR	Protective antibodies in mice after oral administration	Streatfield et al. (2001)
			NR	10% TSP	Serum and mucosal antibodies in mice after oral administration	Lamphear et al. (2002); Streatfield et al. (2003); Streatfield and Howard (2003b)
			p)′-zein-5′TEV-3′vsp p)′-zein-5′TEV-SEKDEL-3′vsp³ p35S-5′TEV-3′vsp p35S-5′TEV-SEKDEL-3′vsp	3.7% TSP[c]	Immunogenic in mice following oral and parenteral administration	Chikwamba et al. (2002); Karaman et al. (2006)
			NR	NR	Mucosal and serum antibodies in humans after oral administration	Tacket et al. (2004)
		Tobacco leaves	p35S-3′nos pUBI-3′nos[c]	3.3% TSP[c]	GM1 ganglioside binding in vitro	Kang et al. (2005c)
		Ginseng embryos	pUBI-SP-3′nos[c]	0.36% TSP	GM1 ganglioside binding in vitro	Kang et al. (2006)
		Lettuce leaves	p35S-SP-SEKDEL-3′nos	2% TSP	GM1 ganglioside binding in vitro	Kim et al. (2007)
		Soybean seeds	pGly-SP(bchit)-3′Gly	2.5% TSP	Immunogenic and protective in mice after oral administration	Moravec et al. (2007)
	LT-B and a non-toxic mutant of L-B	Tobacco leaves (chloroplast)	prm-3′psbA	3.7% TSP	GM1 ganglioside binding in vitro	Kang et al. (2004b), (2003)
	LT-B expressed as fusion with immunocontraceptive antigen	Tomato fruit and leaves	p35S-5′TEV-3′vsp	65 µg/g DW, 10 µg/g FW	ND	Walmsley et al. (2003)
	LT-B expressed as fusion tuberculosis antigen ESAT-6	Arabidopsis leaves	p35S-5′TEV-3′vsp	24.5 µg/g FW	GM1 ganglioside binding in vitro	Rigano et al. (2004)
Escherichia coli, enterotoxigenic/ Cattle, Poultry, Swine	F5 fimbrial adhesin FanC	Soybean leaves	p35S-5′TEV-3′35S[c] p35S-5′TEV-TP (rbcS)-3′35S	0.5%TSP[c]	Immunogenic in mice after parenteral administration	Piller et al. (2005), Garg et al. (2007)

Table 1 continued

Pathogen/Host	Antigen	Prod. system	Expression system[a]	Yield[b]	Immune response	References
Escherichia coli, enterotoxigenic/ Swine	F4 fimbrial adhesin FaeG	Tobacco leaves	p35S-3'nos	0.15% TSP	Immunogenic in mice after parenteral and oral administration	Huang et al. (2003), Liang et al. (2006)
			p35S-TP(rbcS)-3'nos	1%TSP	Receptor binding *in vitro*	Joensuu et al. (2004)
		Alfalfa leaves	p35S-TP(rbcS)-3'nos	1%TSP	Immunogenic and partially protective in pigs after oral administration	Joensuu et al. (2006b)
		Barley seeds	pTI-SP(TI)-SEKDEL-3'nos	1%TSP	Neutralizing antibodies in mice after parenteral administration	Joensuu et al. (2006a)
Fasciola hepatica/ Farmed animals, humans	Cysteine protease as fusion with ubiquitin fragment	Lettuce and alfalfa leaver	p35S	160 and 10 µg/g DW	Immunogenic in mice after oral administration	Legocki et al. (2005)
Mannheimia haemolytica/ Cattle	A1 leucotoxin 50 as a fusion with GFP	White clover leaves	p35S-SP-3'nos (origin of SP NR)	1%TSP	Neutralizing antibodies in rabbits following parenteral administration	Lee et al. (2001)
		Alfalfa leaves	p35S-SP-HDEL-3'nos prbcS-SP-HDEL-3' nos (origin of SP NR)	NR	ND	Ziauddin et al. (2004)
Mycobacterium/ Farmed animals, humans	ESAT-6 as fusion with LT-B	*Arabidopsis* leaves	p35S-5'TEV-SP-3'vsp	24.5 µg/g FW (LT-B)	ND	Rigano et al. (2004)
	ESAT-6 as fusion with CP	PVX vectors in tobacco	Transient *Agrobacterium*-mediated expression	1% TSP	ND	Zelada et al. (2006)
Toxoplasma gondii/ Farmed animals/ humans	Surface antigen 1		Transient *Agrobacterium*-mediated expression	0.1% TSP	Immunogenic and partially protective in mice after parenteral administration	Clemente et al. (2005)

[a] Only heterologous signal peptides are reported; [b] maximum accumulation level as reported in the literature; [c] reported yield obtained with this expression system; [d] reported immune response obtained with antigen derived from this expression system; 2x p35S, 35S promoter with double enhancer region; 3', polyadenylation signals and site; 5', 5' untranslated region; aad, aminoglycoside 3'-adenylyltransferase; Act, rice actin; AlMV, alfalfa mosaic virus; atpB, ATP synthase subunit B; Bchi, *Arabidopsis* basic chitinase; CMV, cucumber mosaic virus; CP, coat protein; CPMV cowpea mosaic virus; CsVMV, cassava vein mosaic virus; DW, dry weight; g4, *Agrobacterium* gene 4; g7, *Agrobacterium* gene 7; Gly, soybean glycinin; GT1, rice glutelin 1; GT3, rice glutelin 3; γ-zein, maize storage protein; E8, tomato ethylene synthesis regulatory protein; EFE, banana ethylene forming enzyme; FW, fresh weight; lacZ, *E. coli* β-galactosidase; m Ig, mouse immunoglobulin; mas, *Agrobacterium* mannopine synthase; ND, not detected; nos, *Agrobacterium* nopaline synthase; NR, not reported; Ω, untranslated 5' leader from TMV; ocs, *Agrobacterium* octopine synthase; p, promoter; patatin, potato storage protein; pin2, potato proteinase inhibitor 2; PPV plum pox virus; Pr1a, tobacco pathogenesis related protein 1a; psbA, photosystem II subunit B; PVX potato virus x; rbcL, rubisco large subunit; rbcS, rubisco small subunit; rrn, 16S rRNA; (SE)K/HDEL, endoplasmic reticulum retain signal; SP, signal peptide for secreted proteins; SUPER, synthetic promoter based on ocs and mas promoters; T7gene10, bacteriophage T7 gene 10; TI, barley trypsin inhibitor; trc, *E. coli* tryptophan operon; TEV, untranslated 5' leader from tobacco etch virus; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus; ToEV, untranslated 5' leader from tomato etch virus; TP, chloroplast transit peptide; TSP, total soluble protein; 35S, Cauliflower mosaic virus 35S; UBQ3, *Arabidopsis* ubiquitin 3; vsp, soybean vegetative storage protein; vspzL, full vsp signal peptide (vacuole targeting); vspzS, truncated vsp signal peptide (ER targeting)

as, cell suspensions (Smith et al. 2002), somatic embryos (Kang et al. 2006), and callus cultures (Kapusta et al. 1999).

High overall protein content in the target plant tissue is beneficial for high-level accumulation of recombinant proteins. This demand is fulfilled by leafy crops, and in applications were the antigen is targeted to seeds. However, only a minor proportion of the fruit and tuber tissues is protein, limiting the amount of antigen which can be expressed and delivered in these tissues. Antigen expression levels of individual potato tubers (Tacket et al. 2000) and tomato fruits (Sandhu et al. 2000) have been reported to vary. To increase long-term storage and to concentrate the antigen, these tissues may need to be processed with such techniques as freeze-drying, followed by grinding or powdering. Batch processing homogenizes the raw material and allows products with more uniform antigen dosage to be manufactured. Similarly, leafy crops have to be processed to homogenize the starting material. If the vaccine antigen is produced in the grains, the batch-to-batch variation can be monitored directly from the harvested seeds, and the material can be delivered in an unprocessed form to animals. However, carefully selected processing techniques can be used to increase the palatability and antigen concentration (Streatfield et al. 2003).

Plant vaccine expression systems

In the context of using plants as antigen production and delivery systems, there are three main options for engineering a plant to produce immunologically active peptides. The first is to integrate the DNA encoding the gene of interest into the nuclear or organelle genome of the plant to generate stable transgenic plants that express the antigen either constitutively or in specific tissues. Stable transgenic plants are most commonly obtained by *Agrobacterium*-mediated gene transfer or by bombardment with DNA-coated high-velocity gold/tungsten particles, both followed by appropriate tissue culture procedures. In *Arabidopsis* and some other species the laborious tissue culture steps can be avoided by *Agrobacterium* floral dip and vacuum infiltration methods. (for reviews see: Clough 2005, Curtis 2005). The second option is to integrate the genetic

material encoding the immunologically active protein or peptide into the genome of a plant virus and to use that to infect the plants. This transient expression system is initiated by virus inoculation of plants. The protein or peptide is then expressed either on the surface of the virus particle as a fusion epitope with the viral coat protein or as an autonomous protein produced as a by-product of the virus infection. The third option is a transient gene expression system known as agroinfiltration that uses infiltration with engineered *Agrobacterium*. The main characteristics, along with the major advantages and disadvantages of these expression systems are discussed below.

Transgenic plants

The major advantage of stable transgenic plants over transient protein production systems is that the protein production trait is heritable and is therefore transmitted over multiple generations, making scale-up simple, and allowing the establishment of seed stock to ensure long-term availability of starting material (Kirk and Webb 2005). The production of antigenic proteins in tissues of transgenic plants that are applicable for consumption presents the possibility that these tissues can be consumed directly, providing an "edible vaccine" which obviates the need to purify the vaccine protein. As purification of pharmaceutical proteins often account for more than 50% of the final cost (Streatfield and Howard 2003a), it is often the limiting step in commercialization of such products and is particularly important in the production of vaccines for livestock where profit margins can be very narrow. Oral delivery of vaccine antigens within plants following limited processing (e.g. grinding) may be an attractive low-cost way of improving animal health.

Drawbacks of using transgenic plants as a production platform include the time required to regenerate and analyze the transformants, the unpredictability of expression and accumulation and the scale-up time needed for seed production. The generation of sufficient transgenic plant material for protein analysis may take several months, and despite advancements in the field some plant species are recalcitrant with respect to transformation. In addition, low yield of the antigenic protein can also be a significant barrier. However, recent progress in plastid transformation has enabled very high-level

expression of foreign proteins in transgenic plants. These transplastomic plants have been reported to accumulate immunologically active peptides in amounts of up to 33.1% of total soluble protein (Molina et al. 2004). Plastid transformation currently works efficiently only in solanaceous plants, such as tobacco (Svab and Maliga 1993), tomato (Ruf et al. 2001), and potato (Sidorov et al. 1999), but it has been introduced into other crop species, including carrot (Kumar et al. 2004a), cotton (Kumar et al. 2004b), soybean (Dufourmantel et al. 2004) and oilseed rape (Hou et al. 2003).

Transient expression with viral vectors

Plant viral expression systems have generally been more efficient than transgenic plant-based production systems in terms of the yield of foreign protein that can be expressed per gram of plant tissue. When virus-based production systems are used for antigen production, the chimeric virus particles or free foreign protein must usually be purified from infected leaf tissues. If the immunogenic peptide is expressed in the form of an epitope fused to the plant viral coat protein, the recombinant viral particles can be simply purified from infected tissues. In addition, virions are often remarkably stable structures. However, strict limitations are present in the size of the insert that can be introduced into the virus genome and successfully assembled into virions (Yusibov et al. 1997). This is usually not the case with peptides or proteins expressed as an autonomous by-product of the virus infection or in transgenic plants. In contrast to transgenic plant material (particularly seeds, tubers, fruits), storing leaf tissue of virus-infected plants is not very practical. However, purified plant virus particles can be stored for long periods of time under appropriate conditions. Further advantages of plant virus platform expression include savings in time and labor. Various genetic constructs can be tested and scaled up in a matter of weeks. This is an important feature for a vaccine production system against emerging new pathogenic variants. However, the bulk-scale production of antigens is inconvenient since the recombinant plant viruses need to be generated in vitro and inoculated into individual plants, and genetic stability of the virus must be monitored through multiple passages.

While it is possible to inoculate field-grown plants with the viral vectors to transiently express the vaccine antigen, the risk of spread of the modified plant viruses would be very high, and the use of this system must therefore be limited to contained environments such as greenhouses. Examples in which epitopes of vaccine antigens have been successfully displayed as a part of chimeric plant viruses include cowpea mosaic virus (CPMV) (Dalsgaard et al. 1997), tobacco mosaic virus (TMV) (Koo et al. 1999), alfalfa mosaic virus (AlMV) (Yusibov et al. 2005), plum pox potyvirus (PPV) (Fernandez-Fernandez et al. 1998), and potato virus X (PVX) (Marusic et al. 2001). Successful co-expression of antigens has been employed with TMV (Wigdorovitz et al. 1999b), PVX (Franconi et al. 2002), and PPV (Fernandez-Fernandez et al. 2001).

Transient expression with agroinfiltration

In agroinfiltration, the *Agrobacterium* culture is forced into intact or harvested plant leaf tissues by pressure, the transgene being expressed in plant cells for few days and subsequently harvested for the foreign protein (Kapila et al. 1997). Inhibition of gene silencing in agroinfiltrated leaves was shown to increase recombinant protein yields up to 50-fold (Voinnet et al. 2003). Agroinfiltration is very convenient for preliminary laboratory-scale testing for transformation vector capacity, and for the production of small amounts of purified recombinant proteins, and is now being scaled up, thus becoming an efficient production platform. Large-scale agroinfiltration is currently being utilized for antigen and antibody production by the Canadian biotechnology company Medicaco (www.medicaco.com) that is processing alfalfa and tobacco plants (D'Aoust et al. 2005). The German company Icon Genetics (www.icongenetics.com) has established an agroinfiltration-based application called magnifection, which combines the transfection efficiency of *Agrobacterium* and the high expression yield of viral vectors (Marillonnet et al. 2005). Recently, magnifection has shown a great promise for rapid and up to gram-magnitude scalable production system for antigens and antibodies (Giritch et al. 2006; Santi et al. 2006). Similarly to transient viral expression, agroinfiltration-based production needs contained environment and requires purification of recombinant protein after the harvest, and has limitations in scalability compared to expression in transgenic plants.

Plant-made vaccine antigens for animal infectious disease control

Published studies of vaccine candidates expressed in plants for animal infectious disease control are summarized in Table 1. The majority of these applications are against viral pathogens. The likely reason for this is not a lack of potential microbial pathogens but a lack of cost-effective means to treat animal virus infections, whereas microbe infections can usually be controlled by antibiotics. However, the recent and forthcoming bans on non-therapeutic use of antibiotics will increase the demand for new treatment options to promote animal health. European Union banned the use of all antibiotics for growth promotion purposes in January 2006 (Anonymous 2005) and similar bills to preserve antibiotics for medical treatment have been proposed in US government in February 2007 (Anonymous 2007b). Most of the plant-produced vaccines have only preliminary been tested in laboratory animals (mainly mice), but the most advantageous examples show protection in the target species following oral delivery (Lamphear et al. 2002; Khandelwal et al. 2003a; Zhou et al. 2004; Guerrero-Andrade et al. 2006). In some instances, vaccination of animals also serves to protect human health. Rabies (McGarvey et al. 1995; Yusibov et al. 2002; Ashraf et al. 2005), O157:H7 EHEC (Judge et al. 2004), and avian influenza (Mihaliak and Webb 2005) are examples of target diseases for which plant-produced vaccines are under development and that would offer protection to both animals and humans.

Applications for poultry

A chinese research group has successfully developed an edible potato-based vaccine against chicken infectious bronchitis virus (IBV) (Zhou et al. 2003, 2004). Sliced tubers expressing viral S1 glycoprotein were administered in three doses over two weeks, and a week after the last administration the chickens were challenged with IBV. Orally immunized chickens developed a virus-specific antibody response and were protected against IBV. Wu et al. (2004) succeeded in vaccinating chickens against infectious bursal disease virus (IBDV). Chickens orally immunized with *Arabidopsis* crude extracts were protected in a manner similar to animals, who had received a commercial injectable vaccine. The efficacy of this vaccine was verified in three replicate experiments (10 chickens per group) with identical results (Wu et al. 2004).

Including the first FDA-approved plant-produced vaccine made in tobacco cell culture (Vermij and Waltz 2006), Newcastle disease virus has been the target of many recent studies for a plant-made vaccine. Virus surface glycoprotein F and HN epitopes have been displayed on the surface of Cucumber mosaic virus particles (Zhao and Hammond 2005; Natilla et al. 2006). Chimeric virus particles were assembled in tobacco leaves, but their immunogenicity was not determined. Full length glycoproteins were expressed in transgenic potato (Berinstein et al. 2005) and tobacco (Hahn et al. 2007) leaves, and maize (Guerrero-Andrade et al. 2006) and rice seeds (Yang et al. 2007). The antigens were shown to be immunogenic and protective in chickens after oral delivery.

Applications for cattle

Transgenic peanut plants expressing bovine rinderpest virus hemaglutinin were reported to raise immune responses in cattle (Khandelwal et al. 2003a). Cows were fed three times with 5–7.5 g of transgenic leaf tissue. This oral vaccine was able to raise virus-specific antibodies, which also neutralized the virus in vitro. Immunogenicity of a TMV-based vaccine against bovine herpes virus (BHV) was studied in cattle (Perez Filgueira et al. 2003). Immunogenic glycoprotein D was produced as a by-product in TMV-inoculated tobacco plants, and the crude plant extract emulsified in oil and subsequently injected into cows was able to raise specific humoral and cellular immune responses. Most importantly, these animals were protected against BHV to a similar level as cows vaccinated with the commercial vaccine.

Foot and mouth disease virus (FMDV) infects many meat- and milk-producing domestic animals, including cows. In an Argentinean laboratory, a vaccine against FMDV has been extensively developed. This vaccine is based on the viral structural VP1 protein, and expression has been reported in *Arabidopsis* (Carrillo et al. 1998), potato tubers (Carrillo et al. 2001), and alfalfa leaves (Wigdorovitz et al. 1999a; Dus Santos et al. 2002; Dus Santos and Wigdorovitz 2005). Alfalfa was chosen as a platform

for oral delivery and protective immune response was reported in mice (Wigdorovitz et al. 1999a).

The same authors have also developed a plant-produced vaccine against bovine rotavirus infections. Epitopes of rotavirus VP4 protein were expressed with a TMV-based transient system (Perez Filgueira et al. 2004) and in transgenic alfalfa plants (Wigdorovitz et al. 2004). Immunogenicity was again determined in a mouse model. Most importantly, alfalfa-fed mice developed a virus-specific antibody response, with pups subsequently being protected against viral challenge by passive lactogenic immunity (Wigdorovitz et al. 2004). Furthermore, the expression of bovine rotavirus VP6 protein has been reported in transplastomic tobacco plants (Birch-Machin et al. 2004) and potato tubers (Matsumura et al. 2002).

A Canadian group has investigated a plant-produced vaccine against bovine pneumonic pasteurellosis, "shipping fever" caused by *Mannheimia haemolytica*. Transgenic white clover (Lee et al. 2001) and alfalfa (Ziauddin et al. 2004) plants expressing a fragment of leucotoxin fused with green fluorescent protein (GFP) were generated, and the immunogenicity of this fusion protein was established in rabbits after intramuscular injection. The generated antibodies also neutralized a related leucotoxin *in vitro*. Recently, Dow AgroSciences launched license agreement to produce the leucotoxin antigen it their plant-cell-based platform (Anonymous 2007a).

The colonization factor of O157:H7 bovine diarrhea-causing enterohemorraghic *E. coli* (EHEC) was expressed in tobacco plants (Judge et al. 2004). His-tagged intimin was purified from plant extracts and injected intraperitoneally in mice. Alternatively, mice were fed the transgenic plant material. In the following EHEC challenge, reduced *E. coli* shedding was observed in the parenterally immunized group as well as in mice that had received an oral boost after being intraperitoneally primed.

Applications for swine

Development of a plant-produced vaccine against porcine transmissible gastroenteritis virus (TGEV) has been carried out by several research groups. Neutralizing virus spike protein antigens have been expressed in the leaf tissue of transgenic tobacco (Tuboly et al. 2000) and *Arabidopsis* plants (Gomez et al. 1998), in potato tubers (Gomez et al. 2000), and in maize seeds (Streatfield et al. 2001). Plant-produced spike protein antigens were immunogenic in mice following parenteral or oral administration (Gomez et al. 1998, 2000). Tuboly et al. (2000) immunized weaned piglets intraperitoneally with crude tobacco extracts and detected virus-specific neutralizing antibodies after three injections. The use of maize seeds as an edible delivery vehicle against TGEV has been studied by Streatfied and colleagues. The efficacy of this plant-made vaccine has been presented in multiple experiments with piglets (Streatfield et al. 2001; Lamphear et al. 2002). In addition, it was found that the antigen was stable during storage in various conditions and authors were able to concentrate the antigen with milling techniques (Lamphear et al. 2002).

Development against a plant-produced vaccine against porcine epidemic diarrhea virus (PEDV) has been established by a Korean research group. PEDV-neutralizing epitope has been expressed in tobacco (Bae et al. 2003; Kang et al. 2005b), potato (Kim et al. 2005) and in rice seeds (Oszvald et al. 2007). The tobacco-derived protein was also reported to raise virus-specific antibodies in mice after an oral delivery (Bae et al. 2003).

Enterotoxigenic *E. coli* (ETEC) expressing F5 fimbriae causes diarrhea in various farm animals, including pigs, chickens, and cows, whereas $F4^+$ ETEC is pathogenic only for pigs (Van den Broeck et al. et al. 2000). The major subunit protein of F4 fimbriae have been expressed in the leaves of tobacco (Huang et al. 2003; Joensuu et al. 2004; Liang et al. 2006), alfalfa (Joensuu et al. 2006b) and in seeds of barley (Joensuu et al. 2006a) while the F5 fimbrial subunit was expressed in leaves of soybean plants (Piller et al. 2005; Garg et al. 2007).

F4 subunit vaccine was shown to be immunogenic and partially protective after oral delivery to weaned piglets (Joensuu et al. 2006b). The immunogenicity of F5 ETEC vaccine was confirmed by vaccinating mice parenterally with crude leaf extracts (Piller et al. 2005). In addition to these candidate vaccines based on colonization factors, the expression of ETEC heat-labile toxin subunit B in plants has been widely studied. LT-B forms homopentamers to mediate the binding of the toxin to enterocytes. The autoassembly of these pentamers has been observed when the LT-B encoding gene was

expressed in transgenic plants. Successful examples include tobacco and (Kang et al. 2005c), lettuce leaves (Kim et al. 2007), potato tubers (Haq et al. 1995; Mason et al. 1998; Lauterslager et al. 2001), and maize (Streatfield et al. 2001; Chikwamba et al. 2002) and soybean seeds (Moravec et al. 2007). Orally administered plant-made LT-B was able to protect mice against subsequent challenge with the LT-holotoxin and the immunogenicity of this antigen was also shown in humans (Tacket et al. 1998, 2004).

Optimizing antigen yield in transgenic plants

Oral immunization of herds of farm animals with subunit vaccines requires bulk-scale production of the antigens and low expression levels still remains to be one of the major hurdles for vaccine production in transgenic plants (Table 1). High expression levels are equally important for adequate-sized oral doses and cost-effective purification of vaccine antigens. The overall biomass yield of the crop species and the intrinsic protein content of the plant tissue define the capacity of the chosen production system. However, multiple factors determine the final antigen content of the production platform. To achieve high yields, the expression construct design must consider all stages of gene expression, from transcription to protein stability.

Transcription and mRNA stability

The promoter selected to control the transcription of the transgene is perhaps the most important component of the expression construct. Several promoters have been identified to provide high levels of gene expression in plants. The most commonly used promoter to control antigen expression in dicotyledonous plant species is the strong and constitutive cauliflower mosaic virus 35S promoter (CaMV 35S). A version of CaMV 35S with a duplicated transcriptional enhancer region is also used to increase gene expression (Smith et al. 2002; Warzecha et al. 2003; Dong et al. 2005). Another strong candidate promoter is the synthetic Super promoter (Ni et al. 1995), which has been successfully used to drive vaccine antigen expression in plants (Tuboly et al. 2000; Pogrebnyak et al. 2005). For expression of vaccine antigens in the

potato, the auxin-inducible mannopine synthase (mas) P1, P2 dual promoter system has been widely used by Langridge and colleagues (Arakawa et al. 1997, 1998, 2001; Yu and Langridge 2001), while other groups have preferred the tuber-specific patatin promoter (Mason et al. 1996; Lauterslager et al. 2001). In maize, the CaMV 35S promoter can be used to accumulate vaccine antigens in seeds (Chikwamba et al. 2002). However, more efficient antigen production has been achieved with maize seed-specific globulin-1 and γ-zein promoters (Chikwamba et al. 2002; Streatfield and Howard 2003a). In general, the most widely used promoter to control transgene expression in monocotyledonous plants has been the constitutive maize ubiquitin-1 promoter (Christensen and Quail 1996). For seed-specific gene expression in dicotyledonous plants, phaseolin and arcelin promoters derived from common bean (*Phaseolus vulgaris*) have shown high accumulation of recombinant antibodies (De Jaeger et al. 2002; Van Droogenbroeck et al. 2007).

After transcription, the mature transcript has to be protected from premature degradation and transported efficiently to the cytoplasmic translation machinery. As a result, post-transcriptional processing events like capping, splicing, polyadenylation can have a major effect on the levels of protein produced in the plant cell (Gutierrez et al. 1999). Introduction of introns into the expression construct has been shown to increase the stability of mRNA (Topfer et al. 1993). Particularly in monocot plants, the introduction of introns elevates the expression levels of marker genes (Callis et al. 1987; Maas et al. 1991). Polyadenylation signals also strongly influence the stability of mRNA and the level of gene expression in plant cells (Ingelbrecht et al. 1989; Hunt 1994). The most widely used polyadenylation signals in vaccine applications include those from the CaMV 35S transcript (Huang et al. 2001; Piller et al. 2005), the soybean vegetative storage protein (vsp) (Richter et al. 2000; Vieira da Silva et al. 2002), and the *Agrobacterium* nopaline synthase (nos) and octopine synthase (ocs) genes (Mason et al. 1992; Aziz et al. 2002). Richter et al. (2000) did a direct comparison of three different polyadenylation signals in potato and found that plants with vsp and potato proteinase inhibitor 2 (pin2) polyadenylation signals accumulated hepatitis virus B surface antigen (HBsAg) in quantities several times higher than plants transformed with expression

vectors with the nos-terminator, indicating that post-transcriptional effects contributed strongly to the enhanced accumulation hepatitis B surface antigen. Generation of synthetic antigen-encoding genes enables the elimination of potential internal methylation and polyadenylation sites, mRNA secondary structure hairpins, and premature transcriptional termination sites to ensure efficient transcription and stability of the generated transcript (Dong et al. 2005).

Translation

Initiation of mRNA translation is often a rate-limiting step in polypeptide synthesis in plants (Kawaguchi and Bailey-Serres 2002). Translation initiation can be optimized by matching the translational start site to the Kozak consensus for plants (Joshi et al. 1997). After initiation, the rate of translation may become limited by a lack of suitable tRNAs. Indeed, genes of foreign origin may have sub-optimal codon composition for the plant translational machinery, and increased levels of protein expression have been reported in plants after modification of the gene-coding sequences of bacterial origin (Perlak et al. 1991; Adang et al. 1993; Horvath et al. 2000). Similarly, optimization of gene sequences for plant codon usage has enhanced the expression of various vaccine antigens such as *E. coli* heat labile enterotoxin subunit B (LT-B) (Mason et al. 1998) and cholera toxin subunit B (CT-B) (Kang et al. 2004a). For example, LT-B accumulation was enhanced 5- to 40-fold in potato tubers when a codon-optimized gene was used instead of an unmodified LT-B gene (Mason et al. 1998) However, reports of the effectiveness of codon optimization of genes of viral origin are limited, perhaps viral gene expression is already optimized for expression in eukaryotes and codon optimization has limited impact.

RNA leader sequences of plant viral origin have been identified to boost antigen expression in plants (Dowson Day et al. 1993). The most widely used 5′ untranslated regions used in antigen applications include 5′ leader sequences from tobacco etch virus (TEV) (Thanavala et al. 1995; Mason et al. 1996; Richter et al. 2000; Dong et al. 2005) and the TMV Ω element (Richter et al. 2000; Matsumura et al. 2002; Biemelt et al. 2003).

Subcellular targeting: optimal yield and glycosylation

The subcellular environment in which the recombinant protein accumulates will influence its folding, assembly, and post-translational modification. Factors such as surrounding pH and presence of chaperones or proteases can affect antigen stability and therefore accumulation. Recombinant proteins can be directed to the secretory pathway by a N-terminal signal peptide, and both plant or native signals appear to work equally well. Secreted proteins are co-translationally synthesized in the endoplasmic reticulum (ER) and transported by default through the Golgi network to the apoplast, or in the presence of a suitable signal directed to the vacuole (Matsuoka and Nakamura 1991). In the apoplast, depending on the protein's size and structure, it can be retained in the cell wall matrix or secreted from the cell. The ER is an oxidizing environment with an abundance of molecular chaperones and very few proteases. Comparative analysis with recombinant antibodies has shown that they accumulate more efficiently when targeted to the secretory pathway than to the cell cytoplasm (Schillberg et al. 1999). This has also been shown with vaccine antigens; Richter et al. (2000) targeted the synthesis of HBsAg to the apoplast or vacuoles and found 2- to 7-fold accumulation level enhancements compared with cytoplasm-targeted potato plants. Streatfield et al. (2003) studied the subcellular targeting of LT-B in maize seeds, and reported that targeting to the apoplast and vacuoles increased the expression level 3080-fold and 20,000-fold, respectively, compared to the cytoplasm. Secreted proteins can be retained in the ER though the use of a C-terminal (SE)H/KDEL peptide (Munro and Pelham 1987). This further enhanced the accumulation of recombinant antibodies than the targeting into the secretory pathway (Conrad and Fiedler 1998). Similarly, a 4-fold increase in LT-B accumulation was shown in tobacco and potato plants when the protein was retained in the ER (Haq et al. 1995). In contrast accumulation LT-B retained in the ER of maize seeds was one-tenth of that when the protein was secreted (Streatfield et al. 2003).

Recombinant proteins can also be targeted to organelles like the mitochondria and plastids. This can be done by adding N-terminal transit peptides, which are recognized by the organelle transport

machinery delivering the proteins to organelles (Glaser and Soll 2004). Richter et al. (2000) were unable to detect viral protein HBsAg when it was targeted to chloroplasts. In maize seeds, targeting bacterial LT-B to the plastids led to a 7-fold increase compared with cytosolic targeting, but did not reach the levels obtained with apoplast, ER, or vacuolar targeting (Streatfield et al. 2003). In contrary, FaeG, the major subunit of ETEC F4 fimbria accumulated higher levels in chloroplasts than cytosol, ER or apoplast (Huang et al. 2003; Van Molle et al. 2007).

Candidate vaccine antigens include proteins or peptides of viral or bacterial origin as well as tumor and autoantigens. Proteins of bacterial origin are not glycosylated, but other antigens including viral surface proteins are often heavily glycosylated by the host cell. Appropriate glycosylation of vaccine antigens can be achieved by appropriate subcellular targeting. When glycosylation is desired, the antigen should be directed to the ER. The level of glycosylation can be affected by retaining the antigen peptide in the ER instead of being secreted through the Golgi apparatus, where further carbohydrate groups will be added (Faye et al. 2005). Correct glycosylation can be a prerequisite (Yelverton et al. 1983) or alter (Judge et al. 2004) the immunogenicity of vaccine antigens. Glycosylation can be avoided by targeting the accumulation of the antigen to the cytoplasm or to intracellular plant organelles. Alternatively, the addition of carbohydrates can be prevented by mutating the putative glycosylation sites on the antigen peptide. Mammals and plants have a similar structure of core high-mannose glycans, but some differences in glycosylation do exist. Plant glycans use a α-1,3 fucose linkage rather than the α-1,6 fucose linkage found in mammals, have additional β-1,2 xylose linkages, and lack the sialic acid moieties typical of mammalian glycosylation (Faye et al. 2005). Completely mammalized plant glycosylation has not been reported to date, but has been of a considerable interest (For review see Saint-Jore-Dupas et al. 2007).

Fusion proteins

Fusing peptides or whole proteins of poor stability to other known stable proteins can improve the stability of the selected antigen in the plant tissues and during the subsequent vaccine delivery. This approach is commonly used for peptides produced with the plant virus expression system, and has also been used to optimize the antigen expression in stably transformed plants. Such vaccine antigen fusion partners include green fluorescence protein (GFP) (Molina et al. 2004; Ziauddin et al. 2004), CT-B (Arakawa et al. 2001; Yu and Langridge 2001; Kim et al. 2004; Molina et al. 2004, 2005), CT-A (Yu and Langridge 2001), and β-glucuronidase (GUS) (Gil et al. 2001; Dus Santos et al. 2002). Antigen fusion with marker genes also allows antigen production to be screened conveniently. The Canadian company Sembiosys Genetics Inc. (www.sembiosys.ca) has established a production system in which recombinant proteins are expressed in oilseed crops as a fusion with oleosin (Parmenter et al. 1995). Fusion proteins accumulate in oil bodies and can be extracted using a simple extraction procedure and the recombinant protein can be released from its fusion partner by proteolytic cleavage. Another application based on protein fusions has been introduced by the Spanish company ERA Biotech (www.erabiotech.com). It has introduced a production system in which recombinant proteins are fused with peptides derived from storage proteins and accumulate host-independently in ER-derived protein bodies that can be separated by their high density. Similarly, fusions with elastin-like polypeptide (ELP) can simplify purification procedure of recombinant proteins. ELP can undergo reversible thermal denaturation and can be used for temperature based non-chromatographic separation (Meyer and Chilkoti 1999). ELP-fusions have been also reported to enhance expression levels of recombinant proteins in plants (Scheller et al. 2006; Patel et al. 2007).

Transplastomy

Chloroplast transformation can provide better gene expression capacity than nuclear genetic engineering. This is mainly because of the high transgene copy number; a single cell can possess up to 10 000 copies of the plastid genome (Daniell et al. 2004). Transgenes are introduced into the chloroplast genome by site-specific integration, and despite the high accumulation level of transcripts, transgene silencing has not been reported in transplastomic plants (Lee et al. 2003; Dhingra et al. 2004). Similarly, lack of post-transcriptional gene silencing was evident with the accumulation of bacterial Cry toxins in over 46% of

total soluble protein (TSP) in transplastomic tobacco lines (De Cosa et al. 2001). In addition, chloroplasts offer a contained environment within a plant cell where potentially plant-toxic compounds have been successfully expressed (Lee et al. 2003; Leelavathi et al. 2003). As a drawback, chloroplasts cannot complete all protein post-transcriptional modifications and are best suited for expression of proteins that do not require complicated assembly or glycosylation. Successful examples of vaccine antigen expression in tobacco chloroplasts include CT-B (Daniell et al. 2001), the TetC fragment of the tetanus toxoid (Tregoning et al. 2003) and an anthrax protective antigen (Aziz et al. 2005).

Risks and regulatory issues associated with the use of plants for the production of vaccine antigens

Kirk et al. (2005) have examined vaccine antigen production in plants and listed gene transfer, allergenicity, oral tolerance, inconsistent dosage, worker exposure, and detrimental effects to the environment as the six main risks. They suggest several ways to mitigate risk including stewardship, active risk management and production quality standards (Kirk et al. 2005). Pharma Planta, an European consortium for biopharmaceuticals produced in plants has looked at the situation and showed that the complexities of the regulation of genetically modified crops intertwined with the regulation of pharmaceutical products creates a challenging environment for approvals (Sparrow et al. 2007). What is clear from both of these papers is that regulatory issues at the transgenic plant level, and the product safety and efficacy level are as important as the technical challenges associated with engineering plants to produce vaccines antigens. While there have long been efforts to produce vaccine antigens in whole plants and although the work has matured to the point of field testing and clinical evaluation, the first plant based veterinary vaccine to be approved by any regulatory authority in the world was produced in a sealed and sterile plant cell culture (Vermij and Waltz 2006). The oral vaccine for Newcastle disease of chickens was approved by the United States Department of Agriculture and registered in January of 2006 by Dow AgroScience. The fact that this vaccine and not one from either field or greenhouse production

went forward first might speak for the importance of pharmaceutical regulations that require product uniformity, safety and efficacy at their core and that avoiding regulatory barriers though the judioscious choice of production platform might be the best way to advance vaccine antigens produced in plants.

Although all manner of crop platforms have been considered for the production of vaccine antigens, the reasons for the choice of a particular platform were not always clear but appeared to be more about easy access to enabling technologies like expression vectors and transformation protocols than production capability or biosafety. Driven by interest in oral vaccines and the GRAS (Generally Recognized As Safe) status of many crops the prevailing belief was that food crops like maize or rice were the ideal platforms. Beginning in the late 1990s our laboratory recognized that food crops would be a regulatory barrier and focused our efforts on developing a non-food platform (Brandle et al. 1998). In early 2000, the Canadian regulatory agencies also began to understand the risk associated with the use of food crops and actually issued a one year moratorium on field testing in 2001 to allow regulation to catch up to technology development (Canadian Food Inspection Agency 2000). The outcome was the recommendation that food or feed crops not be used as platforms for recombinant pharmaceutical production. Although there was not much reaction from the industry at that point, what followed was a strong catalyst for change. In what became known as the Prodigene affair, a Texas based plant recombinant protein company failed in its responsibility for post-harvest monitoring and contaminated a soybean crop with maize producing a vaccine (Hileman 2003). The company was fined and eventually ceased operations. In the years that followed that event the number of molecular farming field tests on maize conducted in the US dropped from 13 in 2001 to two in 2003, most likely the result of the clear understanding of the liability associated with the use of food crops (Fox 2006). Since that event the calls for the exclusive use of non-food crops have been quite loud and many investigators switched to non-food or contained systems (Anonymous 2004; Fox 2006; Murphy 2007). Constrained by their intellectual property portfolios and technological capabilities, those research programs that could not change suffered the consequences of the unwillingness of the regulatory system to accommodate their

systems along with a great deal of public resistance (Fox 2005, 2006; Waltz 2006). Given all of the product safety and efficacy challenges that are already associated with bringing a recombinant vaccine antigen to market, the best platform must be the one that does not add to the regulatory burden. In fact Kirk et al. (2005) speak to the fact that edible "plant made vaccines" should no longer be thought of as food at all, but instead as PMVs that are regulated by the USDA or FDA and prescribed by a physician or veterinarian. The use of non-food crops as production platforms would go a long way to completing that shift in thinking.

Conclusion

The first PMVs to reach the market will most likely be for veterinary use since their regulatory processes are less stringent than for human vaccines. To prove their full potential as a production platform, transgenic plants expressing vaccine antigens must follow the foot steps of plant cell culture based vaccines approved by the FDA. Oral delivery is the most economical and convenient way to administer these veterinary vaccines. Only a limited amount of studies have addressed the efficacy of PMVs in the final host and more efforts have to be pointed to optimize the antigen dosage and administration schedule to prove the full value of oral delivery of PMVs.

The bans on prophylactic use for livestock antibiotics drive demand towards new products to promote animal health. Due to recent advances on transient plant expression systems they can now compete with current vaccine production platforms in terms of yield and speed. The economical feasibility of many veterinary applications still relies on the open-field cultivation and oral administration of transgenic plants. Many vaccine antigens are highly host specific and pharmacologically inactive and non-toxic in secondary hosts. Safety of each application needs to be evaluated separately, but the health or environmental effects of most veterinary vaccine-producing plants might not significantly differ from the existing approved GM crop cultivars. Additionally, several strategies have been developed to improve containment and safety of plant biopharming. One of the major issues confronting the public perception of biopharming in food crops is the potential to contaminate the human food chain. Production of veterinary vaccines in non-food or dedicated feed crops might be the needed step towards the acceptance and approval for open-field production of PMVs.

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