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REVIEW

Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview

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Abstract During the past two decades, antibodies, antibody derivatives and vaccines have been developed for therapeutic and diagnostic applications in human and veterinary medicine. Numerous species of dicot and monocot plants have been genetically modified to produce antibodies or vaccines, and a number of diverse transformation methods and strategies to enhance the accumulation of the pharmaceutical proteins are now available. Veterinary applications are the specific focus of this article, in particular for pathogenic viruses, bacteria and eukaryotic parasites. We focus on the advantages and remaining challenges of plant-based therapeutic proteins for veterinary applications with emphasis on expression platforms, technologies and economic considerations.

Keywords Molecular pharming · Plant-based vaccines · Therapeutic antibodies · Transgenic plants · Veterinary medicine

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Introduction

The discovery of the smallpox vaccine by Edward Jenner more than 200 years ago was the defining event for the development of vaccinology. Jenner found that immunisation with a less virulent, but antigenic related, Cowpox Virus protected against the more virulent Smallpox Virus. In the following one-and-a half-centuries vaccinology as a science became firmly established and its basic principles were developed. The world-wide eradication of smallpox, together with the remarkable reductions in other important infectious diseases of humans such as polio, diphtheria, tetanus, pertussis, measles and mumps underlined the feasibility and utility of vaccination and its economic benefits for the prevention, control and finally the extirpation of infectious diseases (Andre 2003). In veterinary medicine, vaccinology addresses a wider spectrum of challenges. These include the development of cost effective approaches to prevent and control infectious animal diseases, considering animal welfare and focusing on decreasing production costs of animals used as food (Shams 2005). In addition, mass vaccination programmes have helped to significantly lower the consumption of veterinary drugs, including antibiotics. Vaccines also led to reduced negative environmental impacts by eliminating chemical residues in products such as milk, eggs and meat. Production losses caused by illnesses can be avoided.

In 1890 Emil Behring and Shibasaburo Kitasato developed the principle of serum therapy. They found

that immunity against diphtheria and tetanus toxins resulted from the presence of substances in blood, they called antibodies. Behring and Kitasato were also able to transfer immunity to immunologically naïve animals by using the serum of animals treated with non-lethal doses of a crude toxin preparation (Behring and Kitasato 1890). In these early pioneering days Paul Ehrlich discovered that antibodies could act as ‘‘magic bullets’’ to target cancer cells (Ehrlich 1900). Later on, protein sequences and structures of antibodies as well structures and sequences of the genes coding for them were elucidated. The use of mouse hybridomas generated from stable fusions of immortalised myeloma cells with B cells of immunised mice provided highly specific monoclonal antibodies that could be used in therapy (Kohler and Milstein 1975). Mouse antibodies initially used in human therapy did not interact well enough with different receptor types resulting in inefficient effector functions and rather short terminal half-lives. Furthermore, the mouse antibodies were found to induce severe immune responses in humans (for rev. see Carter 2006). The development of innovative recombinant DNA technologies, including chimeras and humanisation of mouse antibody molecules, greatly enhanced the clinical efficiency and safety of murine-derived monoclonal antibodies. For almost 15 years, phage display has been used for the selection of specific antigen-binders from artificial libraries of single chain antibodies. Filamentous phages have been developed that carry the genetic information to express foreign proteins on their surface. This assures the coupling of phenotype and genotype during phage amplification and affinity selection. The ability to generate large antibody libraries, and the simplified antibody-backbone of a single chain antibody, has made antibody-phage display a powerful tool for the development of new therapeutic agents (for rev. see Hoogenboom et al. 1998; Kontermann and Duebel 2001; Winter et al. 1994). In parallel, new and evolving molecular strategies are helping to enhance affinity, stability and expression levels (Boder et al. 2000; Hanes et al. 2000; for rev. see Kurtzman et al. 2001; Low et al. 1996). The high cost of antibody production in mammalian systems has limited the wider use of antibody therapeutics (Scott 2005). These problems are essentially important for the application of antibody therapeutics in veterinary medicine. Production shortfalls and high costs are providing the impetus for further development of alternative antibody production technologies (Chadd and Chamow 2001; Fischer et al. 2004; Kipriyanov and Le Gall 2004). Infectious diseases are on the rise world-wide. It is estimated that 58% of the 1,407 recognised species of human pathogens are zoonotic, i.e. infect more than one host. Zoonotic pathogens represent the most likely source of emerging and re-emerging infectious diseases (Woolhouse and Gowtage-Sequeria 2005). Thus, providing solutions for the well-being of animals also impacts human health. The worldwide and dramatic increase of resistances against antibiotics, and the possible presence of residues in meat, milk, eggs and in the environment, has spurred the development of alternative products for the treatment of infectious diseases. Attempts are especially being made to reduce the quantities of antibiotics used for prophylaxis and growth promotional effects in animals. Active vaccination using live virulent or attenuated vaccines is widely used, but has its drawbacks in terms of low levels of immunogenicity, high production cost, antigenic variability between species and possible transfer of genetic material to wild-type strains. Vaccination is also not a feasible option for mammals post-weaning as well as in animals such as broilers, which have a short life-span. The production of vaccines for veterinary use also needs low cost systems combined with inexpensive application strategies. An ideal expression system for recombinant antibodies and antigens should therefore be amenable to genetic modification, inherently safe and economical. It should provide functional proteins of either high antibody affinity and/or neutralisation or vaccination capacity (Fischer and Schillberg 2004). Systems for the production of antigens and antibodies in transgenic plants have been under development during the last 15 years (for rev. see Giddings et al. 2000; Hood et al. 2002; Koprowski 2005; Ma et al. 2003, 2005b; Stoger et al. 2005a; Streatfield 2005b; Streatfield and Howard 2003; Warzecha and Mason 2003). Plants offer general advantages in terms of production scale and economy, product safety, and ease of storage and distribution (Ma et al. 2005a). Here we focus on the production of vaccines and therapeutic antibodies in transgenic plants for veterinary applications. Recent developments of plantibody and plantigen applications in veterinary medicine are

summarised. We discuss advantages and disadvantages of different plant expression systems for veterinary applications, also considering economic issues.

Plant-derived vaccines for veterinary purposes

The development and improvement of vaccines are suitable ways to combat infectious diseases in wild and also in domesticated animals. Current strategies use intact or inactivated pathogen strains to induce immunity, as well as subunit vaccines, which are commercially produced in bacteria, yeast or mammalian cell cultures. In a key patent of 1990 Curtiss and Cardineau described the expression of the *Streptococcus mutans* surface protein antigen A (SpaA) in transgenic tobacco plants (Curtiss and Cardineau 1990). Subsequent to this key event Mason and co-workers succeeded in expressing the hepatitis B surface antigen in tobacco (Mason et al. 1992), and in 1993 Usha and co-workers expressed a peptide representing an epitope of the VP1 envelope protein of the Foot-and-Mouth-Disease Virus (FMDV) on the surface of a plant virus particle (Usha et al. 1993). Following this pioneering work various veterinary candidate vaccines have been produced using engineered plant viruses and transgenic plants. The overview presented here reflects the current situation on plant-based vaccines with a focus on veterinary applications. Summaries of plant-based vaccines for both, veterinary as well as human medicine, were published in 2003 (Streatfield and Howard 2003) and 2006 (Joensuu 2006). One major prerequisite for vaccine production *in planta* is the development of fast, reliable and safe systems for the generation of transgenic plants. Since the first report of the successful transformation of plant cells (Fraley et al. 1983), various plant expression systems have been established for molecular farming (for rev. see Fischer and Schillberg 2004; Stoger et al. 2005a, b). Four different methods are now generally used for the production of recombinant proteins (Horn et al. 2004): Generation of stable nuclear transgenic plants, transplastomic plants, transient expression using a plant virus and transient expression *via* Agrobacterium infiltration. The production of plants which release the vaccine into a hydroponic medium, as a component of the root exudates, is another possibility, but such production systems are impractical due to the high dilution of the vaccines and the technical facilities required for plant cultivation. Model plants such as *Arabidopsis thaliana* (Table 1, [15, 28, 51, 52, 65]) and tobacco (Table 1, [10, 12, 22–24, 29, 31, 44, 45, 48, 54, 56]) are strong candidates for initial studies to generate stable transgenic plants expressing proteins of interest. Species with edible leaves, e.g. lettuce (Table 1, [8, 59, 62]), alfalfa (Table 1, [4, 5, 8, 11, 36, 47, 62, 64]), white clover (Table 1, [63]); tubers/fruits, e.g. potato (Table 1, [13, 14, 25, 27, 35, 37, 38, 50, 61]), tomato (Table 1, [34, 42, 51]) or grains, e.g. maize (Table 1, [18, 26, 58]) and barley (Table 1, [46]) have also been used for the production of veterinary vaccines. Oral delivery offers ease of application and, *via* the induction of mucosal immunity, protection against pathogens interacting with host mucosal surfaces (for rev. see Streatfield 2005a). In contrast, the immunisation of animals *via* injection predominantly results in a systemic immune response. Transient expression methods using plant viruses have been established and this yields in high levels of protein accumulation in the host plants by the rapid amplification of an infectious plant viral genome (Table 1, [1, 3, 9, 10, 17, 19, 39, 40, 60]). In these reports antigenic peptides were fused to the coding sequence of the viral coat protein to obtain virus-like particles which present the desired peptide. The resulting virions can easily be purified by centrifugation. Transplastomic plants were developed to improve accumulation of recombinant proteins, as shown for the antigenic peptide 2L21 from the VP2 capsid protein of Canine Parvovirus (CPV). This peptide has been expressed as an N-terminal translational fusion with the GUS protein in nuclear-transformed Arabidopsis plants at a rather low level (Gil et al. 2001). The 2L21 peptide was then fused to GFP or the cholera toxin B subunit, and accumulated in tobacco chloroplasts to a significantly higher level compared with stable transformants (Table 1, [6]). Further examples of successful production in transplastomic plants are the heat-labile toxin B subunit (Table 1, [53, 55]), a candidate vaccine against enterotoxigenic *Escherichia coli* (ETEC), and a protective antigen for *Bacillus anthracis* (Table 1, [41–43]). The advantages of chloroplast transformation have been reviewed (Daniell et al. 2002; Maliga 2002, 2003).

In addition to the examples cited earlier, numerous veterinary candidate vaccines, mainly against virus infections, have been expressed in plants. Examples are Foot-and-Mouth-Disease Virus (Table 1, [11, 12]),

Newcastle Disease Virus (Table 1, [16–20]), Rinderpest Virus (Table 1, [30–33]) and Rotavirus (Table 1, [2–4, 34–38]). Complete antigenic proteins, as well as peptides representing major antigenic determinants, have been produced in plant systems. In the case of vaccination against viruses, proteins located at the virion surface are generally the most suitable targets for an efficient immune response. Vaccines against the Newcastle Disease Virus (NDV) have been generated based on plant expression systems. The fusion protein (F) and the hemagglutinin-neuraminidase (HN) of NDV initiate the infection process, and are essential targets for the host immune response. Neutralising epitopes from these proteins were selected and expressed using a plant virus system (Table 1, [17, 19]). The complete fusion protein (F) can also be formed in transgenic maize plants (Table 1, [18]). This strategy of expressing virus proteins was used to produce different targets for the induction of an adequate host immune response (Table 1, [1, 8, 10, 11, 13–16, 18, 26–29, 31–38]), whereas certain epitopes of viral proteins with antigenic properties were selected in other cases (Table 1, [3, 4, 6, 7, 9, 12, 22]). In addition, bacterial infectors have also been chosen as targets for recombinant vaccines, e.g. *B. anthracis* (Table 1, [39–43]), *E. coli* (Table 1, [44–61]) or *M. tuberculosis* (Table 1, [65, 66]). Several bacterial pathogens listed in Table 1 could also infect humans and therefore the described veterinary plant-derived vaccines are useful tools for human medicine (*B. anthracis, E. coli, M. tuberculosis*). Further examples of animal pathogens, which are also infective to humans, are e.g. Salmonella or Avian flu. Especially the latter will attract notice to plant-based expression platforms in the near future. Antigens with immunological potential have been identified as vaccines against bacterial diseases, e.g. ESAT-6 (6 kDa early secreted antigen target) in the case of *M. tuberculosis* (Table 1, [65, 66]) or the fimbrial antigens of enterotoxigenic *E. coli* strains (Table 1, [44–50]). For vaccination against ETEC the heat-labile toxin subunit B (LTB), also known as a carrier molecule, acts as an immunogen and has been produced in plants (Table 1, [51–59]). Another interesting approach to obtain a plant-based vaccine against coliform mastitis, caused by *E. coli*, is the expression of a bovine CD14 receptor (Table 1, [60]). This receptor occurs in a membrane-bound and in a soluble form. The latter binds to lipopolysaccharides (LPS) in the outer membrane of e.g. *E. coli*, and induces the secretion of cytokines followed by host innate immune responses (Table 1, [60]). The pathogen *B. anthracis* primarily infects animals, but humans are also susceptible. This bacterium is a candidate for the development of biological weapons, and was identified as a category A agent for bioterrorism. The protective antigen (PA) of *B. anthracis* is useful for the immunisation of animals and humans, and this candidate vaccine has been expressed using different plant systems (Table 1, [39–43]).

Expression levels of veterinary vaccines in plants are crucial for the economic success of such strategies, since costs for production and application have to be low. In this respect, expression levels of the candidate vaccines summarised in Table 1 are generally not adequate. The only exception is the expression of the 2L21 peptide fused to GFP or LTB in tobacco chloroplasts (Table 1, [6]). In this case, the expression level was calculated to be approx. 23% of total soluble protein for GFP-2L21 and approx. 31% for the LTP-2L21 fusion. Other translational fusions have been reported (e.g. Table 1, [28]). The structural protein VP60 of RHDV was fused to ubiquitin or rbcS, but unfortunately the expression levels were rather low, 0.8% of total soluble protein for VP60 without fusion and 0.1% for the ubiquitin VP60 fusion. Low costs for application of the plant-based vaccines were achieved either by oral delivery of crude plant material through ‘‘edible vaccines’’ or by the development of simple purification methods. Purification details for plant-based veterinary vaccines on a large scale have not been published yet. There are only few hints for the purification of plant virus particles expressing antigenic epitopes using centrifugation (Table 1, [9, 17, 19, 34]) and of veterinary subunit vaccines using either affinity chromatography (Table 1, [3, 43, 51, 60]) or anion exchange chromatography (Table 1, [29]) on a small scale. Most of the plant-derived veterinary vaccines shown in Table 1 have been tested in laboratory animals, either by injection (Table 1, [1, 3, 6, 9–12, 29, 31, 34, 40, 42–44, 46, 49, 67]), oral delivery (Table 1, [8, 13, 18, 26, 27, 32, 35–38, 48, 50, 61, 62]) or both (Table 1, [4, 7, 14–16, 22, 28, 33, 58]) to determine their ability to provoke humoral or mucosal immune responses. As mentioned above the induction of mucosal antibodies against epitopes of certain pathogens is especially favoured by the oral delivery of the plant-derived recombinant vaccines. Most pathogens enter or colonise their host *via*

Table 1 Transgenic plant-based candidate vaccines for veterinary purposes

Pathogen/host	Antigen	Production system	Expression level	Efficacy	References
Bovine Herpes Virus (BHV) Cattle	(1) Truncated glycoprotein D (gDc)	Tobacco Mosaic Virus (*Nicotiana benthamiana*)	20 μg/g FW	Immunogenic by parenteral delivery to mice and cows, reduced symptoms in cows after virus challenge	Pérez Filgueira et al. (2003)
Bovine Rotavirus Cattle	(2) Inner coat protein VP6	Tobacco chloroplasts (3 different promoters, P*rrn*; P*psbA*; P*trc*)	3% TSP (P*rrn*), 0.6% TSP (P*psbA*)	No data published	Birch-Machin et al. (2004)
	(3) VP8 fragment of the VP4 capsid protein	Tobacco Mosaic Virus (N*icotiana benthamiana*)	5 μg/g FW	Immunogenic by intraperitoneal delivery to mice, protective to the offspring of immunised mice	Pérez Filgueira et al. (2004)
	(4) Capsid protein VP4 (eBRV4a peptide) fused to β-glucuronidase	Alfalfa	0.4–0.9 mg/g TSP (based on GUS activity)	Immunogenic by oral and intraperitoneal delivery to mice, protective to the offspring of immunised mice	Wigdorovitz et al. (2004)
Bovine Viral Diarrhoea Virus (BVDV) Cattle	(5) Glycoprotein E2	Alfalfa	0.05–0.5 mg/g TSP	No data published	Dus Santos and Wigdorovitz (2005)
Canine Parvovirus (CPV) Dogs	(6) Capsid protein VP2 (2L21 peptide) Capsid protein VP2 (2L21 peptide) fused to GFP Capsid protein VP2 (2L21 peptide) fused to cholera toxin B subunit	Tobacco chloroplasts	No expression (2L21), 22.6% TSP (GFP-2L21), 31.1% TSP (CTB-2L21)	Immunogenic by intraperitoneal delivery to mice (CTB-2L21), marginal response (GFP-2L21)	Molina et al. (2004)
	(7) Capsid protein VP2 (2L21 peptide) fused to cholera toxin B subunit or GFP	Tobacco chloroplasts	See Molina et al. (2004)	Immunogenic by oral delivery to mice (CTB-2L21), intraperitoneal to mice (CTB-2L21) and intradermic to rabbits (CTB-2L21)	Molina et al. (2005)
Classical Swine Fever Virus (CSFV) Wild boars, domestic pigs	(8) Glycoprotein E2 fused to ubiquitin fragment	Lettuce Alfalfa	160 μg/g DW 10 μg/g lyophilised leaves	Immunogenic by oral delivery to mice	Legocki et al. (2005)
	(9) Glycoprotein E2 peptides ($E2^1$, $E2^2$) fused to PVX coat protein via 2A from FMDV	Potato Virus X (*Nicotiana benthamiana*)	No data published	Immunogenic by subcutaneous delivery to rabbits ($E2^1$)	Marconi et al. (2006)

Table 1 continued

Pathogen/host	Antigen	Production system	Expression level	Efficacy	References
Cottontail Rabbit Papillomavirus (CRPV) Rabbits	(10) Capsid protein L1	Tobacco	0.4–1.0 mg/kg FW	Immunogenic and protective by subcutaneous and intramuscular injection to rabbits, no prevention of papilloma growth	Kohl et al. (2006)
		Tobacco Mosaic Virus (*Nicotiana benthamiana*)	0.15–0.6 mg/kg FW		
Foot-and-Mouth-Disease Virus (FMDV)	(11) Polyprotein P1 and protease 3C (P1–3C)	Alfalfa	0.005–0.01% TSP	Immunogenic and protective by intraperitoneal delivery to mice	Dus Santos et al. (2005)
Cloven-hoofed animals	(12) Structural protein VP1 (VP21epitope) fused to hepatitis B core protein (HBcAg)	Tobacco	0.05% TSP	Immunogenic (HBcAg antibody, anti-FMDV) and protective (FDMV) by intraperitoneal delivery to mice	Huang et al. (2005)
Infectious Bronchitis Virus (IBV) Poultry	(13) Glycoprotein S1	Potato tubers	0.07–0.22% TSP	Immunogenic and protective by oral delivery to chicken, immunogenic by gastric delivery to mice	Zhou et al. (2003)
	(14) Glycoprotein S1	Potato tubers	2.39–2.53 µg/g FW	Immunogenic and protective by oral and intramuscular delivery to chicken	Zhou et al. (2004)
Infectious Bursal Disease Virus (IBDV) Poultry	(15) Viral protein VP2	*Arabidopsis thaliana*	0.5–4.8% TSP	Immunogenic and protective by oral and subcutaneous delivery to chicken	Wu et al. (2004b) Wu et al. (2004a)
Newcastle Disease Virus (NDV) Poultry, wild birds	(16) Fusion protein (F) and hemagglutinin-neuraminidase protein (HN)	Potato leaves	0.3–0.6 µg/mg of total leaf protein	Immunogenic by oral and intraperitoneal delivery to mice	Berinstein et al. (2005)
	(17) Fusion protein (F) and hemagglutinin-neuraminidase protein (HN) (epitopes fused to CMV coat protein)	Cucumber Mosaic Virus (*Nicotiana benthamiana, Nicotiana tabacum*)	F epitope not stable, 1.2–1.5 mg purified HN-CMV particles in 3.5 g (*N. benthamiana*) or 7 g (*N. tabacum*) leaf material	No data published	Zhao and Hammond (2005)
	(18) Fusion protein (F)	Maize seeds	0.9–3.0% TSP	Immunogenic and protective by oral delivery to chicken	Guerrero-Andrade et al. (2006)
	(19) Fusion protein (F) and hemagglutinin-neuraminidase protein (HN) (epitopes and tandem F/HN epitope fused to CMV coat protein)	Potato Virus X (*Nicotiana benthamiana*)	No data published	No data published	Natilla et al. (2006)
	(20) ''Vaccine''	Tobacco cell culture	No data published	No data published	Vermij and Waltz (2006)

Table 1 continued

Pathogen/host	Antigen	Production system	Expression level	Efficacy	References
Peste des Petits Ruminants Virus (PRPV) Domestic and wild animals	[21] Hemagglutinin-neuraminidase protein (HN)	Pigeon pea leaves	No data published	No data published	Prasad et al. (2004)
Porcine Epidemic Diarrhoea Virus (PEDV) Pigs	[22] Epitope COE (CO-26K equivalent) of the spike protein	Tobacco	8–20 μg/g FW	Immunogenic by oral and subcutaneous delivery to mice	Bae et al. (2003)
	[23] Synthetic epitope S-COE of the spike protein	Tobacco	1.4–2.1% TSP (50–60 μg/g FW)	No data published	Kang et al. (2005b)
	[24] Epitope K-COE of the spike protein (Korean strain)	Tobacco	0.1% TSP	No data published	Kang et al. (2005c)
	[25] Epitope COE (CO-26K equivalent) of the spike protein	Potato tubers	0.1% TSP	No data published	Kim et al. (2005)
Porcine Transmissible Gastroenteritis Virus (PTGEV) Pigs	[26] Glycoprotein S	Maize seed	13 mg/kg	Immunogenic by oral delivery to gilts, induction of lactogenic immunity	Lamphear et al. (2004)
Rabbit Hemorrhagic Disease Virus (RHDV) Rabbits	[27] Structural protein VP60	Potato tubers	6–18 μg/g FW (individual tubers) 3.5 μg/mg TSP (maximum)	Immunogenic and partially protective by oral delivery to rabbits	Martin-Alonso et al. (2003)
	[28] Structural protein VP60 (different fusion strategies)	*Arabidopsis thaliana*	0.3–0.8% TSP (VP60) Lower than 0.1% TSP (VP60 fused to ubiquitin) No data published (VP60 fused *to A. thaliana* rbcS)	Immunogenic by intraperitoneal and oral delivery to mice	Gil et al. (2006)
Rabies Virus Domestic and wildlife animals, humans	[29] Surface glycoprotein G	Tobacco	0.001–0.38% TSP	Immunogenic and protective by intraperitoneal delivery to mice	Ashraf et al. (2005)
Rinderpest Virus (RPV) Domestic and wild ruminants	[30] Hemagglutinin protein (H)	Pigeon pea leaves	0.12–0.49% TSP	No data published	Satyavathi et al. (2003)
	[31] Hemagglutinin protein (H)	Tobacco	Up to 0.75% TSP	Immunogenic by intraperitoneal delivery to mice	Khandelwal et al. (2003a)
	[32] Hemagglutinin protein (H)	Peanut leaves	0.2–1.3% TSP	Immunogenic by oral delivery to cattle	Khandelwal et al. (2003b) Khandelwal et al. (2003c)

Table 1 continued

Pathogen/host	Antigen	Production system	Expression level	Efficacy	References
	(33) Hemagglutinin protein (H)	Peanut leaves	See Khandelwal et al. (2003b)	Immunogenic by oral and intraperitoneal delivery to mice	Khandelwal et al. (2004)
Rotavirus Animals, humans	(34) Capsid proteins VP2 and VP6	Tomato	1% TSP	Immunogenic by intraperitoneal delivery to mice	Saldana et al. (2006)
	(35) Capsid protein VP6	Potato tubers	0.01% TSP	Immunogenic by oral delivery to mice	Yu and Langridge (2003)
	(36) Capsid protein VP6	Alfalfa	0.06–0.28% TSP	Immunogenic by oral delivery to mice, protective to the offspring of immunised mice	Dong et al. (2005)
	(37) Capsid protein VP7	xPotato tubers	0.18–3.84 µg/mg TSP (leaves), no data published (tubers)	Immunogenic by oral delivery to mice	Wu et al. (2003)
	(38) Capsid protein VP7	Potato tubers	3.6–4.0 µg/mg TSP (leaves), 40 µg/g (tubers)	Immunogenic by oral delivery to mice	Li et al. (2006)
Bacillus anthracis Animals, humans	(39) Fragment of protective antigen (PA) fused to capsid protein of Tobacco Mosaic Virus	Tobacco Mosaic Virus (spinach)	No data published	No data published	Sussman (2003)
	(40) Pa-D4s epitope of protective antigen (PA) fused to Alfalfa Mosaic Virus coat protein	Alfalfa Mosaic Virus (*Nicotiana tabacum*)	0.3 mg/g FW	Immunogenic by intraperitoneal delivery to mice	Brodzik et al. (2005)
	(41) Protective antigen (PA)	Tobacco chloroplasts	2.56 mg/g FW (mature leaves)	No data published	Watson et al. (2004)
	(42) Protective antigen (PA)	Tomato Tobacco chloroplasts	No data published 8% TSP	Immunogenic by injection to mice (tomato-derived PA)	Aziz et al. (2005)
	(43) Protective antigen (PA)	Tobacco chloroplasts	4.5–14.2% TSP (mature leaves, illumination dependent)	Immunogenic and protective by subcutaneous delivery to mice	Koya et al. (2005)
Enterotoxigenic *Escherichia coli* (ETEC) Animals, humans	(44) Fimbriae K88 (major subunit FaeG fragment)	Tobacco	0.15% TSP	Immunogenic by intraperitoneal delivery to mice, sera of immunised mice neutralise K88ad fimbriae-expressing ETEC	Huang et al. (2003)
	(45) Fimbriae K88 (major subunit FaeG fragment)	Tobacco (targeting to chloroplasts)	1% TSP	Receptor binding *in vitro*	Joensuu et al. (2004)
	(46) Fimbriae K88 (major subunit FaeG)	Barley grains	0.04–1.0% TSP	Immunogenic by subcutaneous delivery to mice, sera of immunised mice inhibit adhesion of ETEC bacteria to piglet brush borders	Joensuu et al. (2006a)

Table 1 continued

Pathogen/host	Antigen	Production system	Expression level	Efficacy	References
	(47) Fimbriae K88 (major subunit FaeG)	Alfalfa	Up to 1% TSP	Immunogenic and protective by intragastric delivery to piglets	Joensuu et al. (2006b)
	(48) Fimbriae K88 (major subunit FaeG)	Tobacco	See Huang et al. (2003)	Immunogenic by oral delivery to mice, sera of immunised mice inhibit adhesion of ETEC bacteria to piglet intestinal villi	Liang et al. (2006)
	(49) Fimbriae K99 (major subunit FanC)	Soybean leaves	0.4% TSP	Immunogenic by intraperitoneal delivery to mice, $CD4^{+}$ T-lymphocyte response	Piller et al. (2005)
	(50) CFA/I fimbrial antigen combined with cholera toxin A2 (fragment) and B subunits and Rotavirus enterotoxin epitope	Potato tubers	0.0006–0.002% ~ TSP	Immunogenic (CFA/I) by oral delivery to mice, sera of immunised mice inhibit ETEC binding to human colon carcinoma cells	Lee et al. (2004)
	(51) Heat-labile toxin B subunit (LTB)	Tomato	37.8 μg/g DW (T1), 354.7 μg/g DW (T2)	No data published	Walmsley et al. (2003)
	fused to epitope of mouse zona pellucida 3 glycoprotein (immunocontraceptive epitope)	*Arabidopsis thaliana*	No data published		
	(52) ESAT-6 fused to heat-labile toxin B subunit (LTB)	*Arabidopsis thaliana*	11–24.5 μg/g FW (LTB)	Antigenicity of both components of the fusion protein	Rigano et al. (2004)
	(53) Heat-labile toxin B subunit (LTB)	Tobacco chloroplasts	2.3–2.5% TSP	Binding to the intestinal membrane GM1 ganglioside receptor *in vitro*	Kang et al. (2003)
	(54) Heat-labile toxin B subunit (LTB)	Tobacco	2.2% TSP	Binding to the intestinal membrane GM1 ganglioside receptor *in vitro*	Kang et al. (2004a)
	(55) Heat-labile toxin B subunit (LTB) (LTK63 mutant)	Tobacco chloroplasts	3.7% TSP	Binding to the intestinal membrane GM1 ganglioside receptor *in vitro*	Kang et al. (2004b)
	(56) Heat-labile toxin B subunit (LTB)	Tobacco	3.3% TSP	Binding to the intestinal membrane GM1 ganglioside receptor *in vitro*	Kang et al. (2005a)
	(57) Heat-labile toxin B subunit (LTB)	Ginseng (somatic embryos)	0.36% TSP	Binding to the intestinal membrane GM1 ganglioside receptor *in vitro*	Kang et al. (2006)
	(58) Heat-labile toxin B subunit (LTB)	Maize	0.01–0.07% (Chikwamba et al. 2002)	Immunogenic by oral and intraperitoneal delivery to mice	Karaman et al. (2006)
	(59) Heat-labile toxin B subunit (LTB)	Lettuce	1–2% TSP	Binding to the intestinal membrane GM1 ganglioside receptor *in vitro*	Kim et al. (2007)

Table 1 continued

Pathogen/host	Antigen	Production system	Expression level	Efficacy	References
Escherichia coli Cattle (coliform mastitis)	[60] Bovine CD14 protein (truncated version)	Potato Virus X (*Nicotiana benthamiana)*	1.25–1.5% TSP	Ability to promote LPS-induced IL-8 production and LPS-induced apoptosis and to enhance LPS responses and bacterial clearance	Nemchinov et al. (2006)
Escherichia coli Aquaculture	[61] Gut adhesion molecule (LTP) fused to GFP, VP2 peptide of CPV or H peptide of Influenza Virus	Potato tubers	No data published	Immunogenic by anal (LTP-CPV) and oral (LTP-GFP) delivery to carps	Companjen et al. (2006)
Fasciola hepatica Domestic animals (morbidity and mortalit)	[62] Cysteine protease (leader or catalytic domain) fused to ubiquitin fragment of Hepatitis B Virus protein	Lettuce	100 μg/g DW (catalytic domain 10–12 μg/g DW (leader domain)	Immunogenic by oral delivery to mice	Legocki et al. (2005)
		Alfalfa	10–12 μg/g DW		
Mannheimia haemolytical	[63] A1 leukotoxin 50 fused to GFP	White clover	1% TSP (Lee et al. 2001)	See Lee et al. (2001)	Lee et al. (2003)
Cattle	[64] A1 leukotoxin 50 fused to GFP	Alfalfa	No data published	No data published	Ziauddin et al. (2004)
Mycobacterium tuberculosis	[65] ESAT-6 fused to heat-labile toxin B subunit (LTB)	*Arabidopsis thaliana*	11–24.5 μg/g FW (LTB)	Antigenicity of both components of the fusion protein	Rigano et al. (2004)
Animals, humans	[66] ESAT-6 and ESAT-6 - 2A peptide of FMDV (fusions with PVX coat protein)	Agrobacterium-mediated transient expression *(Nicotiana tabacum)*	0.5–1% TSP (ESAT-6)	No data published	Zelada et al. (2006)
Toxoplasma gondii Domestic animals, humans	[67] Surface antigen 1 (SAG1) (constructs based in PVX amplicons)	Agrobacterium-mediated transient expression *(Nicotiana tabacum)*	0.06–0.1% TSP	Immunogenic and protective by subcutaneous delivery to mice	Clemente et al. (2005)

FW, fresh weight; P*rrn*, plastid *rrn* promoter; P*psbA*, plastid *psbA* promoter; P*trc*, *Escherichia coli trc* promoter; TSP, total soluble protein; GUS, *β*-glucuronidase; GFP, green fluorescent protein; CTB, cholera toxin B subunit; DW, dry weight; PVX, Potato Virus X; CMV, Cucumber Mosaic Virus; rbcS, small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase; GM1, monosialotetrahexosylganglioside; LPS, lipopolysaccharide; Il-8, interleukin-8; Esat-6, 6 kDa early secreted antigen target

mucosal surfaces of the gastrointestinal, respiratory or genital tract. To combat these infectors a mucosal vaccine, which induces the generation of serum (IgG) and mucosal antibodies (IgA), is needed. This was achieved by oral delivery of e.g. transgenic peanut leaves expressing the Rinderpest Virus hemagglutinin protein (H) to mice (Table 1, [33]). Splenocytes were collected 13 weeks post-oral immunisation and were proliferated in the presence of the specific antigen. Immune response to the H protein has further been studied in cattle (Table 1, [32]). Also here, systemic immune response was induced upon oral delivery. Oral immunisation with plant-derived Rotavirus capsid proteins induced both serum IgG and mucosal IgA in mice (Table 1, [34–38]). Functionally active anti-Rotavirus intestinal antibodies could be detected in the faeces of mice immunised with transgenic potato tubers expressing the capsid protein VP7 (Table 1, [38]). The oral immunisation of female mice with Rotavirus capsid protein VP6 resulted in reduced symptoms in their offspring after virus challenge (Table 1, [36]). Passive transfer of anti-Rotavirus antibodies from immunised dams to their offspring, and protection of the pups after virus challenge, were observed (Table 1, [4]). A corn-based vaccine against Porcine Transmissible Gastroenteritis Virus (PTGEV) boosts the antibody levels in serum, colostrum and milk of immunised gilts, indicating that immunity can be passively transferred to suckling piglets (Table 1, [26]). Detection of an immune response to the delivered plant-based candidate vaccine was observed in every case (Table 1) when the recombinant protein was given to the animal. Virus challenge experiments showed protection of the immunised animals (Table 1, [1, 10–15, 18, 27, 29]). However, the vaccination of rabbits with plant-derived CRPV L1 protein did not completely prevent papilloma growth (Table 1, [10]) . Clinical symptoms of BHV appeared later and were milder in cows vaccinated with the plant-produced truncated glycoprotein D (Table 1, [1]). It was demonstrated that mice immunised with the plant-derived protective antigen of *B. anthracis* survived challenge with a lethal dose of toxin (Table 1, [43]).

Although expression of veterinary vaccine candidates in different plant species has been well studied, and their immunogenicity evaluated, challenges for plant-derived veterinary vaccines still remain. Other than barley (Table 1, [46]), maize (Table 1, [18, 26, 58]), white clover (Table 1, [63]) and alfalfa (Table 1, [4, 5, 8, 11, 36, 47, 62, 64]), which constitute the main components of animal feed, model plants are routinely used as expression systems; the use of crop plants for the production of veterinary vaccines therefore needs to be developed further. Purification technology is essential if plant-derived vaccines are to be administered by injection, and the development of low cost purification methods is important for commercial success. For oral delivery of vaccines very large amounts of the recombinant protein are required, and increases in expression levels remain a major challenge, especially for edible vaccines. Multi-component vaccines, e.g. *E. coli* fimbrial antigen combined with subunits of cholera toxin and an epitope of Rotavirus (Table 1, [50]), protect animals much the same way as they do humans against multiple infectious diseases. However, these also require further development of reliable production systems, well defined dosing regimens and ultimately a marketable product. One major step towards a marketable plant-derived vaccine was made by Dow AgroScience which, at the beginning of 2006 (Table 1, [20]), obtained federal approval in the US for a vaccine against the Newcastle Disease Virus, produced in tobacco cell culture. Regrettably the chicken vaccine has not been introduced into the market yet.(http://www.news.dow.com/dow_news/feature/2006/05_22_06/index.htm).

Therapeutic antibodies for veterinary use from transgenic plants

In view of the spread of microbial resistance to antibiotics and the emergence of new pathogens, passive immunisation by recombinant antibodies is viewed as one of the most promising alternatives to combat infectious diseases (Casadevall 1998). The market for human therapeutic monoclonal antibodies is growing at a forecast compound annual growth rate of 21%, to reach $16.7 billion by 2008 (Pavlou and Belsey 2005). This market is heavily focused on oncology and arthritis, and immune and inflammatory diseases. The role of antibodies for mitigation and therapy of infectious diseases is only slowly emerging, but is impeded by high Cost of Goods. High Cost of Goods for recombinant antibodies has so far also prevented their successful introduction into the

animal health market. Plant-based production provides a solution to these cost problems. In addition, plants provide an adequate system for oral delivery of recombinant biomolecules as part of the diet. Infrastructure and costs for downstream processing can thus be avoided, as well as production losses which are often significant. Proof-of-concept for the expression of recombinant antibodies and antibody fragments in plants was demonstrated in the late 80s (Hiatt et al. 1989). Since then, different moieties have been generated ranging from single chain molecules (scFvs) to Fab fragments, small immune proteins (SIP), IgGs and chimeric secretory IgA (for rev. see Ma et al. 2005a). Despite progress in the production of antibodies in plants for human health, their application to the veterinary field is rather limited with most potential product developers focusing on vaccines (see earlier). However, recent encouraging developments have been reported in the field of passive immunisation. Focus has been on the generation and development of products for oral application in production animals, for prevention and/or therapy of some of the major commercially relevant infectious diseases. In most studies, the goal has been to apply the antibody molecule orally with no or limited purification thus making the product compatible with the already in place cost structures in the market for animal production. One major hurdle is the low concentration of the heterologous protein in the plant tissue. While efforts to overcome this limitation are being addressed in the literature, little attention has so far been given to issues such as final product formulation and efficacy, and long-term stability under farming conditions. So far, scFv, scFv fusion proteins, IgA and IgA fusion proteins have been expressed in transgenic tobacco and cowpea as well as using transient viral systems (for rev. see Fischer et al. 2004). Several examples are presented below in more detail.

Working on the borderline of animal and human health and biodefense, Almquist and co-workers transformed tobacco with a synthetically optimised gene for a scFv against Botulinum neurotoxin A (Almquist et al. 2006). A single chain antibody that binds to the lipopolysaccharide (LPS) of *Salmonella enterica* Paratyphi B was expressed in tobacco (Makvandi-Nejad et al. 2005). This scFv was developed for higher affinity by introducing two point mutations resulting in the formation of dimers and multimers (Deng et al. 1995). These earlier findings were confirmed for tobacco. In different T1 lines different functional and structural properties of the scFv were observed and will have to be further investigated. Transmissible Gastroenteritis Virus (TGEV) is a coronavirus that causes near 100% mortality in newborn piglets (Enjuanes and van der Zeijst 1995). A TGEV-specific small immune protein (SIP) was expressed in *Nicotiana clevelandii* and cowpea (*Vigna undulata*) for oral application (Monger et al. 2006). The SIP was a dimeric fusion of the ε-CH4 domain of human IgE with scFv antibodies specific for TGEV, stabilised by a C-terminal cysteine residue. Expression was achieved by Agrobacteria inoculation with two different viral vectors based on Potato Virus X (PVX) and Cowpea Mosaic Virus (CPMV). Effective dimerisation of the εSIP and its capacity to bind and to neutralise TGEV *in vitro* was demonstrated. Crude plant extract containing εSIP was orally applied to-two-day old piglets together with a TGEV challenge. As a result, reduction of virus titres in gut and lung were observed, although to a lower extent than with the full-length mammalian produced parental monoclonal antibody.

In a subsequent report by the same group, an IgA derived SIP, containing the CH3 domain of IgA lacking a stabilising C-terminal cysteine, was expressed in plants, together with the full-length recombinant IgA (Alamillo et al. 2006). CPMV and PVX inoculation was used for expression of the SIP, whereas the recombinant IgA was expressed using the PVX system. Effective dimerisation of both αSIP and recombinant IgA was demonstrated. Expression levels for αSIPs were generally low, and a difference in vector efficiency for αSIP and εSIP was observed: εSIP expression was 20 times higher in the CPMV system, whereas αSIP expression was higher using the PVX vector. Crude plant extracts containing either αSIP or recombinant IgA were administered orally to newborn piglets after TGEV challenge. A notable reduction of virus titres was observed both for αSIP and full-length recombinant IgA: αSIP reduced virus titres in the lung by more than 10,000-fold and in the gut by more than 100-fold. In contrast, recombinant IgA was almost ineffective in the lung, but highly effective in the gut, although activity was generally lower than with the parental monoclonal antibody. These differences

in tissue specific activity can be explained by the smaller size of the αSIP, which allows for higher tissue penetration. However, an adjuvant effect of the plant extract cannot be ruled out.

Coccidiosis is the most commercially relevant infectious disease in chickens, and is caused by intracellular protozoan parasites belonging to the genus *Eimeria*. Recombinant chicken IgA has been proposed as a potential means for passive immunisation against this disease (Wieland et al. 2006). IgA was chosen as it is assumed to have a role in the protection of mucosal surfaces, similar to mammalian IgA. A set of ten full-length chicken IgA cDNAs was cloned into Agrobacterium vectors for transient expression. Tobacco (*N. benthamiana*) was co-infiltrated with two different vectors containing the genes coding for the IgL and the IgH_{alpha} chains. Functionality of the full-size antibodies was proven in ELISA assays against Eimeria antigens. Large differences were found in the production levels of the different immunoglobulins. Plants with poor or without expression were shown to have low or non-detectable IgL levels. Clones with a degraded IgH_{alpha} chain showed low light chain expression or did not even express the light chain. It is likely that the light chain stabilises the full-length heavy chain and prevents its degradation. Thus, expression of the light chain might be a limiting factor in the assembly and stability of the plant-made chicken IgA. Further co-infiltration experiments demonstrated the capacity of the plant cells to assemble chicken secretory IgA complexes, a dimeric IgA (dIgA) complex including the J chain and also associations of dIgA and the chicken secretory component.

Although results discussed here are rather preliminary, there is increasing evidence that orally applied recombinant antibodies have the capacity to reduce the infectious load in animals following oral administration. Issues such as stability in the gut, tissue penetration, clearance and general immunogenic effects have to be addressed, as well as technical issues and commercial applicability. Current results indicate that there is no generally applicable ''ideal'' plant system for the expression of antibodies and antibody fragments, but that such systems must be carefully chosen and tailored to the specific type, and even to the specific sequence, of the antibody under study.

Production of therapeutic proteins for veterinary purposes in transgenic plants - advantages and remaining challenges

Efficient transformation and regeneration of plants are major prerequisites for the development of suitable expression systems for vaccines and therapeutic antibodies. Such systems are not only available for model plants such as tobacco and Arabidopsis, but have also been developed for crops such as maize, rice, barley, pea, potato, tomato, alfalfa and lettuce. As outlined in sections ''Plant-derived vaccines for veterinary purposes'' and ''Therapeutic antibodies for veterinary use from transgenic plants'', many different transformation systems have been successfully applied for veterinary purposes, including viral systems. Further developments also need to consider product safety. Plants do not contain human pathogens, oncogenic DNA or microbial endotoxins, but may contain pesticide residues as possible contaminants. Specific plants may comprise several toxic secondary metabolites and toxins derived from plant pathogens. Removal of these substances during the purification process, including final proof of absence, will inevitably increase costs. Crops currently used as animal feed provide an already proven safe alternative (equivalent to the GRAS status of food crops). The development of rather simple procedures for downstream processing and formulation that can be scaled up is essential for veterinary applications. Methods such as ''inverse transition cycling'' using elastin-like-peptide fusions are examples of such low cost large scale purification schemes (Scheller et al. 2004).

A major advantage of crop plants is easy upscaling through field cultivation and established harvesting and processing technologies. However, increased costs due to quality control, quality assurance and regulatory surveillance also have to be taken into account. Easy storage and distribution, key advantages of molecular farming, can only be achieved by seed-specific expression. High-level production and long-term storage of antibodies in seeds was shown more then 10 years ago (Fiedler and Conrad 1995; Stoger et al. 2005b). Vaccine production in seeds is also outlined in several examples in Table 1. In our view, the major issue in terms of reducing costs and maximising economic value is an adequate production level *in planta*. Increase in expression level,

particularly in seeds, directly improves the economic value of any veterinary product in molecular pharming applications. This leads to decreasing costs for planting, quality control, harvest and storage. New promoters and regulatory sequences, as well as fusions to specific peptides, have improved the accumulation of transgenic proteins in seeds (De Jaeger et al. 2002; Scheller et al. 2006). These techniques can now be applied for the development of new products, for vaccines as well as therapeutic antibodies. A high demand for new, specific products, spurred by the ban of persistent drugs, especially antibiotics, now catalyses the development of therapeutic protein-based treatments in veterinary medicine, and this is where molecular pharming can provide better solutions.

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