Improving and assessing viral vectors for recombinant protein production in plants

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Chapter 1

General introduction

Recombinant DNA technology has gained an enormous impact in both science and society leading to the generation of genetically modified organisms and opening new possibilities for agriculture and for the growing biotechnology industry. Plant biotechnology largely harnessed the rapid spread of transgenic technology (Castle et al., 2006; Christou and Whitelaw, 2000). Only a few decades has passed since the first transgenic plant was generated and currently in many countries the market is filled with transgenic plant-derived products, despite serious concern from several action groups and part of the general public (Castle et al., 2006; Raney, 2006; Stewart and Mclean, 2005).

Other areas also witnessed a rapid progress due to recombinant DNA technology, in particular the pharmaceutical industry, although less evidenced by the media and the public in general. Recombinant DNA technology allowed the ectopic production of proteins of different organisms leading to the production of recombinant proteins in large cultures of bacteria, yeast and mammalian cells. Before being produced as recombinant protein, these products were often obtained from their natural sources (Somerville and Bonetta, 2001), albeit at higher costs and with more effort. The contribution of using recombinant DNA technology was to make their production viable, either technically or economically.

Proteins currently produced by the biopharmaceutical industry include antigens, to be used as subunit vaccines; antibodies, for therapeutic and diagnostic application; hormones, cytokines and many other proteins of medical or industrial application (Ma et al., 2005b). Expression of recombinant proteins in plants is an attractive alternative, presenting a number of advantages over the commonly used expression systems based on animal cells, yeast or bacteria. Lower production costs, easy scale-up, and reduced risk of pathogen contamination are some of these advantages (Fischer et al., 2004; Hood et al., 2002; Ma et al., 2005a). Despite the economical advantages and the wide possibilities for plant-based products, there are some major limitations that still remain to be solved. One such limitation is the expression level of the foreign protein that frequently is too low to become viable for commercial productions or to be used as an oral vaccine. The other major problem is the post-translation modifications of plant produced proteins that can substantially differ from animal produced proteins.

This Chapter intends to give an overview on the use of plants as a source of recombinant proteins of medical, veterinary or industrial interest, which is the subject of this thesis. The plant transformation and gene expression techniques are described, as are the advantages and limitation of using plants as protein expression systems. Rather than describe each

particular case in detail, the basic principles that permeate this promising and exciting field are presented.

Plant transformation: transient and stable gene expression

The expression of a heterologous gene in a plant cell can be transient or stable. In transient expression the foreign gene is expressed only for a few days after being introduced into the cell, and does not get integrated in the genome. In stable expression the foreign gene is integrated in the genome and will be passed to the progeny (Sharma et al., 2005).

The choice for an expression system for recombinant protein production in plants depends largely on the aim and the scale of the project (Hansen and Wright, 1999; Sharma et al., 2005) (Figure 1). In that way, transient expression may be useful for research, for validating a technology or for small scale production of recombinant proteins. For large scale-production, however, a transgenic plant system may be more convenient, but will require longer periods for development and optimization of tissue culture, selection and transformation conditions (Horn et al., 2004; Twyman et al., 2003). Further aspects of stable and transient expression systems in the context of recombinant protein production are discussed below.

Stable expression of a foreign gene involves the production of transgenic plants or transgenic cell cultures. The method used for plant transformation will depend on the species, due mainly to technical aspects (Hansen and Wright, 1999; Sharma et al., 2005) Most transformation methods involve nuclear transformation, although the transformation of chloroplasts is also possible for some species.

Transformation methods can be classified in “indirect transformation”, where gene transfer is mediated by *Agrobacterium tumefaciens*, or “direct transformation”, where different techniques, such as particle bombardment or electroporation of protoplasts (Sharma et al., 2005) are used to physically introduced “naked” DNA into the plant cell (Figure1).

Agrobacterium-mediated transformation may be the choice for a large number of species for which well established protocols are available (Hansen and Wright, 1999). For some species, however, particularly important crops like maize and soybean, transformation through particle bombardment is usually employed (Aragão et al., 2000).

The gene delivery method can influence the integration pattern of the transgene in the nucleus, and higher number of copies and rearrangements are more frequently observed when a direct transformation method is used (Makarevitch et al., 2003). The integration pattern can affect expression level (generally referred to as “position effect”), as well as factors such as transcriptional gene silencing by DNA methylation or post-transcriptional gene silencing (Hansen and Wright, 1999; Sharma et al., 2005). As a consequence, regardless of the method of transformation employed, extensive analysis of the transgenic plants and their progeny are needed to select lines expressing high levels of the transgene (Hansen and Wright, 1999; Hood et al., 2002). The entire process may take many years and large investments. Despite all these hurdles, a successful transgenic line can produce

recombinant protein to levels that are commercially viable. The first plant-made recombinant proteins to reach commercial scale, avidin, β-glucuronidase (GUS) and trypsin, are produced in maize seeds to levels varying from 20% of the total soluble protein (TSP) for avidin, a diagnostic reagent, to 0.5% TSP for GUS and trypsin, which are enzymes for technical and industrial use (Evangelista et al., 1998; Hood et al., 2002).

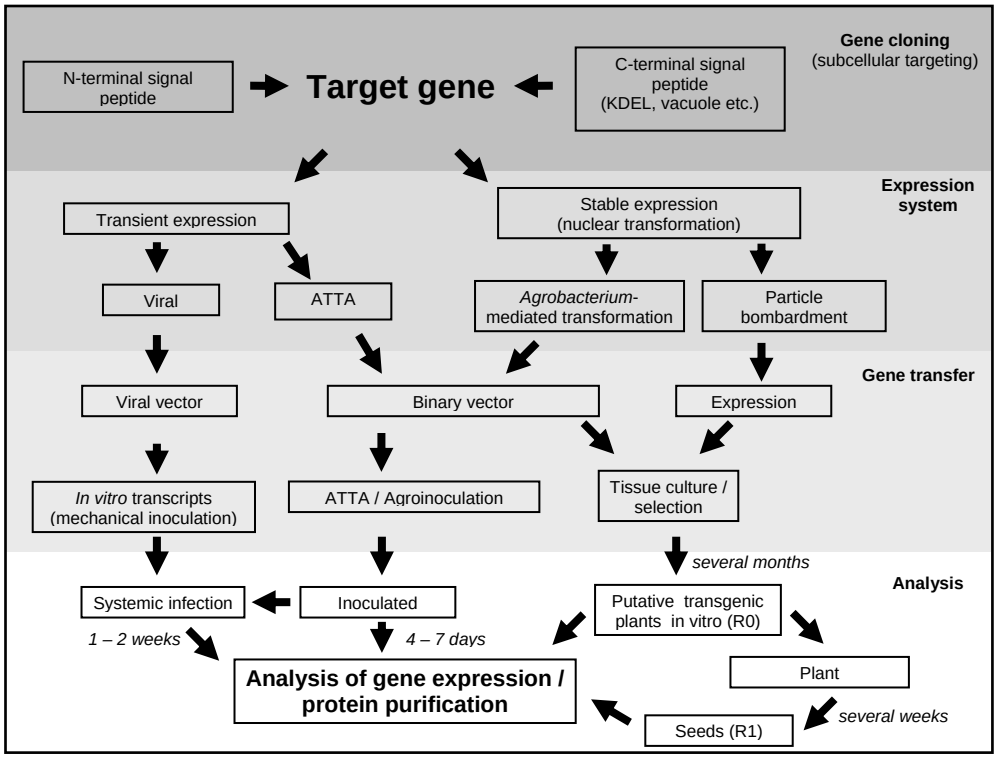

Figure 1. Flow chart for setting up the expression of a foreign gene for recombinant protein production in plants. Arrows indicate the choices and steps involved along the four main stages of the process (grey scale).

An alternative approach for high-level gene expression is the transformation of chloroplasts. This technology uses particle bombardment to deliver the foreign DNA containing flanking regions for homologous recombination within the chloroplast genome (Svab and Maliga, 1993). Because the chloroplast genome is present in high copy numbers per chloroplast, which in turn are present in large number per cell, transplastomic plants potentially can express very high levels of foreign protein (Daniell et al., 2005; Staub et al., 2000). This promising technology is being established as routine in tobacco and is being developed for other species (Daniell et al., 2002) Although it is a promising technology for high-level protein production, chloroplast expression is limited to proteins that do not require post-translational modification for their functionality (Sharma et al., 2005).

Besides stable gene expression from transgenic or transplastomic plants, a foreign gene can also be expressed transiently. Gene transfer techniques are essentially the same as for obtaining transgenic plants, but in general transient gene expression peaks a few days after the target gene is introduced into a cell. Transient expression systems circumvent some limitations of stably transformed transgenic in relation to the variation in expression due to chromosomal position effect, and transcriptional gene silencing (Morrel et al., 2000). Transient expression systems are also typically fast and handy, as gene expression can be analyzed 2-4 days after DNA or RNA is transferred to the plant cell. Usually the gene constructs are small bacterial cloning vectors provided with eukaryotic transcription regulation signals, thereby making cloning less laborious. Techniques such as particle bombardment and transformation of protoplasts have been extensively used for studies on gene expression analysis, intracellular protein targeting, and many other aspects of plant biology (Hansen and Wright, 1999). Although high-level gene expression can be obtained, the relatively low number of cells that can be transformed does not make these methods suitable for the production of industrial scale recombinant proteins.

As noted above, *Agrobacterium tumefaciens* is largely used for stable transformation and generation of transgenic plants. In recent years, however, a transient assay based on *A. tumefaciens*-mediated gene transfer has been developed by infiltrating bacterial suspensions carrying binary vectors expressing the target gene into intact leaves of a plant. This system, known as the "*Agrobacterium tumefaciens* transient assay" (ATTA), combines the simplicity and high efficiency of gene transfer by *Agrobacterium* with the advantages of a transient assay (Voinnet et al., 2003; Yang et al., 2000). Whole leaves or even the whole aerial part of a plant can be infiltrated using a syringe or partial vacuum. The infiltrated tissue can be collected and analyzed after 3-6 days. As a large number of cells is transformed and may express the foreign gene, this method is considered to be viable for small to medium scale production of recombinant proteins (Fischer et al., 1999).

Yet another approach for gene expression in plants is to use plant viruses as episomal vectors that are able to amplify and spread through the inoculated plant (Awram et al., 2002; Scholthof et al., 1996). As for the other transient expression systems, a gene expressed from viral vectors is not heritable. In these systems, expression of the foreign gene is not restricted to a few cells, but may spread through extensive areas of an inoculated plant, making it an attractive approach for the production of recombinant proteins in plants (Awram et al., 2002; Canizares et al., 2005; Scholthof et al., 2002). Viral vectors are also

useful tools for gene function analysis, through a process known as “virus induced gene silencing” (VIGS) (Burch-Smith et al., 2004; Lu et al., 2003; Ratcliff et al., 2001; Watson et al., 2005). Further details of viral vector-based expression of heterologous proteins in plants are discussed in the next section.

Viral vectors

During their systemic infection of host plants, viruses often produce a large number of genome copies, as well as high titers of some of their encoded proteins. They do not integrate into the host genome and, therefore, can be considered as natural episomal vectors (Scholthof et al., 1996). The possibility of manipulating the viral genome *in vitro* allowed the exploitation of viruses as vectors for expressing foreign genes, taking advantage of the fact that a huge amplification of the inserted gene can be achieved (Scholthof et al., 1996).

The genome of most plant viruses consists of (one or more molecules of) positive-sense RNA. For *in vitro* manipulations, their genome must be cloned as cDNA (Ahlquist et al., 1984). DNA viruses (i.e. gemini- and caulimoviruses) can be cloned directly, also generating infectious clones. The conversion of the target virus into an expression vector can be done by replacing a viral gene that is not essential for replication or movement (e.g. a transmission factor) with the foreign gene. This strategy, resulting in replacement vectors, is usually required for viruses of which the genome size constrains viral encapsidation or viral movement. The most commonly used approach, however, is to insert the foreign gene into an intact viral genome (insertional vector). The foreign sequence is inserted after a duplicated coat protein promoter and expressed from a separate subgenomic RNA (sgRNA).

Infectious recombinant virus can be inoculated directly as DNA or as RNA transcript. An infectious cDNA copy can also be cloned in a binary vector for *Agrobacterium*-mediated transformation (agroinoculation) (Jones et al., 1999). In this scenario, the viral vector is cloned in a binary vector containing the required T-DNA left and right borders and a promoter sequence in front of the viral cDNA. Once inoculated, the recombinant virus carrying the foreign sequence will be expressed as positive strand RNA and replicated, transported and encapsidated using viral genes, essentially as the corresponding wilt type virus.

Most plant virus-derived vectors will rapidly replicate, move from cell-cell and spread systemically. Hence, foreign proteins can already be extracted after a few days from inoculated leaves, and after 1-2 weeks from systemically infected leaves. Compared to other transient gene expression methods, the expression level is maintained for longer periods, due to the elevated number of transcripts as the virus replicates and moves spreads throughout the plant (Chapman et al., 1992; Twyman et al., 2005).

Viral vectors also present several limitations and disadvantages. As a transient expression system, viral vectors are not integrated into the genome and must be repeatedly inoculated so that infected plants can express the desired protein. That implies extra work for inoculation and a need for a supply of plants suitable for being efficiently infected (Awram et al., 2002; Pogue et al., 2002; Scholthof et al., 2002). Some viral vectors, like the PVX

vector, have a limited host range. The choice for the host is an important aspect, as many *Solanaceae* species, commonly used as host for PVX and TMV, are rich in alkaloids that may hamper the use of the infected plants as raw material for feeding as an oral vaccine, for example (Awram et al., 2002). The use of viral vectors can also raise serious concerns for biosafety, for the risk of a spreading of the transgene to the environment (Greene and Allison, 1994; Pogue et al., 2002).

The main limitation for viral vectors, however, is the instability of the inserted sequence (Dawson et al., 1989; Donson et al., 1991; Pogue et al., 2002). During replication, deletion mutants may arise as a result of non homologous recombination within the inserted sequence. Moreover, as the cargo sequence is not essential for viral replication and movement, these mutants will replicate and move faster, standing higher chances of establishing systemic infection. The result is a plant systemically infected with virus that do not harbor the (intact) foreign sequence and, consequently, will not produce any recombinant protein (Pogue et al., 2002). The size of the inserted sequence has a direct relation to this instability, as longer inserts are more rapidly deleted. Although this instability is a major limitation for foreign protein production, it may also increase the biosafety as the recombinant virus is less fit than the wild type.

Several viral vectors have been developed over the past years, broadening the options for hosts and applications (Table 1) (Awram et al., 2002). For recombinant protein production vectors based on Potato virus X (PVX) and, particularly, Tobacco mosaic virus (TMV), have been the most commonly used (Table 1) (Awram et al., 2002; Pogue et al., 2002; Scholthof et al., 2002). Both are based on the insertion of a promoter sequence and restriction sites for the foreign sequence that is transcribed as an additional subgenomic promoter (Figure 2).

The use of TMV as a vector for foreign protein expression was first reported by Dawson et al. (1989). However, as this first designed vector contained a duplication of the TMV coat protein promoter it was unstable, readily losing the inserted foreign sequence, most probably by homologous recombination (Dawson et al., 1989). In a later version, the TMV coat protein gene and its promoter were substituted by those of Odontoglossum ringspot virus (ORSV) a related *tobamovirus*, the resulting hybrid vector (named TB-2) being significantly more stable coinciding with higher levels of foreign gene expression (Donson et al., 1991). Subsequently, promoter and coat protein sequences form other tobamoviruses were tested and a hybrid vector (named 30B) containing the coat protein from Tomato mild green mosaic virus (TMGMV) was found to be the most stable and efficient for foreign protein expression (Shivprasad et al., 1999). Further development of the TMV-based vectors followed different approaches, either to increase viral spreading through DNA shuffling of the movement protein, to improve cloning strategies or to increase viral containment for increased biosafety (Man and Epel, 2006; Marillonnet et al., 2004; Toth et al., 2002).

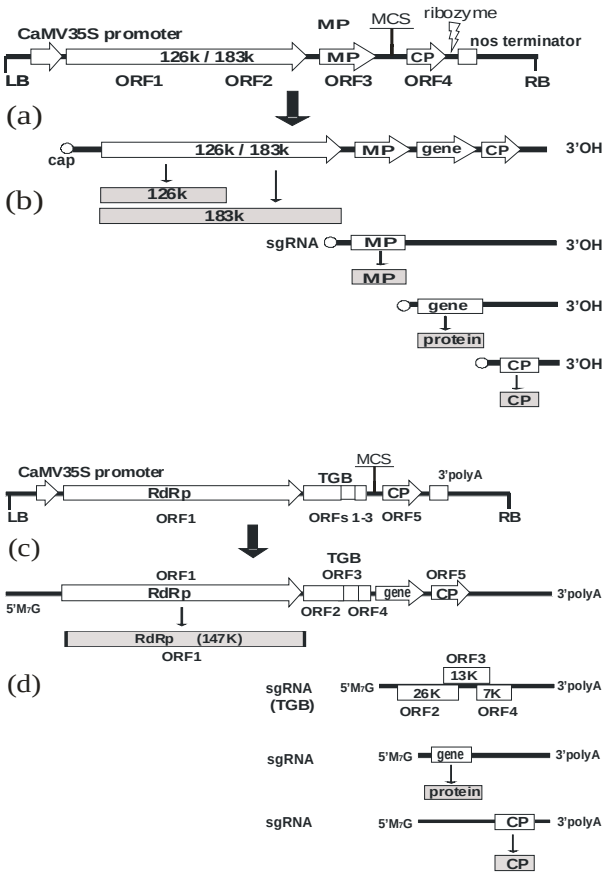

Figure 2. Schematic representation of viral inoculation by agroinfection and the genomic organization and expression of TMV (30B) and PVX (pGR107) - based vectors. (a and c) Upon *Agrobacterium*-mediated transformation the T-DNA containing the viral vector is transferred to the nucleus. After transcription the translation of the viral proteins takes place and the infectious virus replicates and moves from cell-to-cell and systemically. (b) The TMV vector is transcribed from the T-DNA and viral replicase proteins are translated directly from this transcript (126 and 183 kDa). The subgenomic promoters drive the synthesis of the mRNAs of the movement protein (MP), the inserted heterologous protein and the coat protein (CP). In this hybrid vector the inserted gene is transcribed from a TMV coat protein promoter, whereas the coat protein and the promoter sequences are from the related tobamovirus Tomato mild green mosaic virus. (c) The PVX vector transcribes the replicase (RdRp) directly from the genomic RNA transcript and the genes forming the "triple gene box" movement proteins (TGB), the heterologous gene and the coat protein (CP) are translated form subgenomic RNAs (d). The foreign gene is inserted gene in the multiple cloning site (MCS) and is regulated by a duplicated copy of the CP promoter.

Table 1. Examples of recombinant proteins expressed in plants via viral vectors

Virus – Genus **Protein expressed**	**Potential application**	**Expression level**	**References**
Tobacco mosaic viruss (**TMV**) – *Tobamovirus*			
Alpha trichosanthin	HIV therapies	2% TSP [a]	(Kumagai et al., 1993)
Idiotypic single-chain vaccine	B-cell lymphoma treatment	60 µg/ml (intercellular fluid)	(McCormick et al., 1999)
Human α-galactosidase A	Fabry disease	$5.5x10^6$ units/mg	(Turpen, 1999)
Foot and mouth disease virus (FMDV) VP1 protein	FMDV vaccine	50-150 µg/g fresh weight	(Wigdorovitz et al., 1999)
Birch major antigen Bet v1	Vaccine	200 µg/g fresh weight	(Krebitz et al., 2000)
Latex allergen; birch allergen, spina-bifida-associated allergens	Antigen		(Breiteneder et al., 2001)
Bovine follicle stimulating hormone	Superovulation induction	3% TSP	(Dirnberger et al., 2001)
Human papillomavirus (HPV) 16 L1 protein	HPV Vaccine	20-37 µ/kg	(Varsani et al., 2006)
Monoclonal antibody Co7-1A	Colon cancer treatment	Not reported	(Verch et al., 1998)
Bovine rotavirus VP8*	Rotavirus vaccine	5 µg/g fresh weight	(Perez Filgueira et al., 2004)
HIV-1 p24 protein	HIV diagnostic	100 µg/g fresh weight	(Perez-Filgueira et al., 2004)
Potato virus X* (PVX) – *Potexvirus			
Wasabi defensin(WT1)	Microbiocide	400 µg/g fresh weight	(Saitoh et al., 2001)
Single-chain antibody binding *Tomato spotted wilt virus*	Diagnostic, plant protection	Not reported	(Franconi et al., 1999)
Single-chain antibody binding *Potato virus Y*	Diagnostic, plant protection		(Hendy et al., 1999)
Single-chain antibody binding to granule-bound starch synthase	Modification of starch composition	1-3% TSP	(Ziegler et al., 2000)
Rotavirus VP6 protein	Rotavirus vaccine	50µg/g fresh weight	(O'Brien et al., 2000)
Proteinase inhibitor (WIN3), *Bacillus thuringiensis* (Bt) toxin (Cry 1 Ac)	Insecticidal proteins n	0.1-0.2%, not reported	(Lawrence and Novak, 2001)
Human lactoferrin	Microbiocide	0.6% TSP	(Li et al., 2004)

Virus – Genus **Protein expressed**	**Potential application**	**Expression level**	**References**
Plum Pox Virus* (PPV) – *Potyvirus			
VP60 Rabbit hemorrhagic disease virus; RHDV	Rabbies vaccine,	Not reported	(Fernandez-Fernandez et al., 2001)
Zucchini Yellow Mosaic Virus* (ZYMV) – *Potyvirus			
Delivery of *bar* gene to field crucifers 1	Herbicide tolerance	Not reported	(Shiboleth et al., 2001)
Clover Yellow Vein Virus* (ClYMV) – *Potexvirus			
Glutamine synthetase expression	Not reported		(Masuta et al., 2000)
Tobacco Etch Virus* (TEV) – *Potyvirus			
Expression of *bar* in *Arabidopsis*	Herbicide tolerance	Not reported	(Whitham et al., 1999)
Cauliflower mosaic virus* (CaMV) – *Caulimovirus			
Human interferon-α	Hepatitis B and C treatment,	196,830 IU/ml	(De Zoeten et al., 1989)

[a] TSP, total soluble protein.

The other widely used viral vector for foreign protein expression in plants is based on Potato virus X. This vector was initially tested by replacing the coat protein with the β-glucuronidase (GUS) gene as a marker (Chapman et al., 1992). The resulting virions could infect and express the GUS gene but were unable to move from cell to cell or systemically, being restricted to the inoculated focus of infection. A version containing a duplication of the coat protein was show to systemically infect the plants leading to a high level of the GUS expression in inoculated and systemically infected leaves (Chapman et al., 1992).

Virus induced gene silencing (VIGS)

In recent years, RNA silencing in plants has been intensively studied, unraveling a number of pathways involved in gene regulation and in the response to viral infection (Brodersen and Voinnet, 2006). The presence of double stranded RNA acts as a trigger for the silencing machinery, enabling the cell to recognize and degrade the imprinted target sequences. To assure successful infection, plant viruses express specific genes to suppress RNA silencing, as a counter defense to the plant RNA silencing machinery (Wang and Metzlaff, 2005). Many suppressors of gene silencing have been described and it is believed that most, if not all, plant viruses would exploit such a function (Qu and Morris, 2005). The balance between the antiviral plant response, the viral encoded suppression and the viral replication speed and movement through the plant assures infection is accomplished, without compromising the viability of the plant as a host (Qu and Morris, 2005; Voinnet, 2005).

Insertion of host sequences would trigger silencing of the corresponding host gene as the viral infection progresses, leading to partial or complete silencing of both the targeted viral and host sequences. The advantage of this system for gene function analysis is the simplicity of the assay and the convenience of observing the effect of the silenced gene only few weeks after plant inoculation (Angell and Baulcombe, 1999; Ratcliff et al., 2001). A number of studies have exploited that approach for silencing genes involved many aspects of plant biology, including pathogen resistance-related genes, hormone response and photosynthesis (Robertson, 2004; Voinnet, 2005). Therefore, viral vectors are valuable tools for gene function analysis, either by loss of function through VIGS or by over expression of a foreign gene (Burch-Smith et al., 2004; Horiguchi, 2004).

Epitope presentation

A different approach using plant viral vectors is to exploit their virus particle as epitope presenting system, i.e. by expressing small (poly)peptides as fusion to their coat proteins. Small peptides can be inserted in -or fused to- the coat protein of several viruses in such way that the virion will still assemble, thereby retaining its stability and infectivity (Canizares et al., 2005; Porta et al., 1996). After inoculation virus particles can be readily purified in large amounts and used as antigen for vaccine development. The stability and correct assembly of the modified virus will largely depend on the introduced sequence, and incorrect assembly has occasionally been observed. Particles that are correctly assembled, however, are stable and resistant to low pH (Liu et al., 2005; Porta et al., 2003).This strategy of epitope presentation has been shown to induce immune response and to confer protection in challenged animals. The virus most frequently exploited for this approach is Cowpea mosaic virus (CPMV) (Liu et al., 2005; Mechtcheriakova et al., 2006). Other viruses have also been tested, such as the TMV and PVX although for these viruses the compact structure of the assembled particle only allows small peptides (10-20 amino acids) to be added without interfering with the particle assemble (Canizares et al., 2005; Pogue et al., 2002).

Recombinant protein expression level

One major limitation for plant made recombinant proteins is the low expression level. Most target proteins expressed in plants are of non-plant origin and there are no ways to anticipate the expression level of a given recombinant protein expressed in plants. However, knowledge accumulated from testing several potential candidates has pointed to many factors that can influence foreign protein expression and yield (Chargelegue et al., 2000; Clark and Cassidy-Hanley, 2005).

Expression of a foreign gene in plants is controlled at different levels. At the transcriptional level, the promoter sequence can greatly influence mRNA levels. In transgenic plants and in transient gene expression systems (other than viral vectors-based expression), high levels of gene expression are attempted by using gene constructs containing a constitutive promoter, such as the CaMV 35S promoter, including its variations as a duplicate form and with extra protein expression enhancing signals (Sharma et al., 2005). For monocotyledonous species improved expression levels have been obtained by using

promoters like Ubi-2 and Act-1, containing an intron for enhanced expression (Rishi et al., 2001). The choice of the promoter sequence must be suitable for assuring expression on a particular target tissue (e.g. seeds). Likewise, the gene sequence must contain the proper signal sequences for directing the proteins to the desired subcellular compartment (Tyagy, 2001).

Choosing the right gene construct and selecting the most promising transgenic plants lines can lead to high levels of gene expression in transgenic plants (Hansen and Wright, 1999). Still, frequently these levels are considerably lower than the levels observed for transient expression using the same gene construct. That points to several not quite well understood gene regulation mechanisms, including the position effect and the mechanisms underlying RNA silencing - both transcriptional gene silencing, involving methylation of the inserted gene(s), and post-transcriptional gene silencing (PTGS), where the mRNA is targeted and degraded (Baulcombe, 2005; Voinnet, 2005). The mRNA itself may contain sequences that are known to lead to increased instability (Gutierrez et al., 1999). Degradation of the mRNA may also result from cryptic introns, which may be present in heterologous genes, since the intron border sequences differ among animal and plant genes, and even between plant taxa. The codon usage of the targeted gene sequence may also influence mRNA level and gene expression, as frequently observed in bacteria, but also in plants (Marillonnet et al., 2004).

Following mRNA transcription, translation will take place and the polypeptide will be directed to the ER or the cytoplasm (Conrad and Fiedler, 1998). The nature of the recombinant protein may imply that the protein should be processed and/or secreted to other cellular compartments (Vitale and Denecke, 1999). In the ER and Golgi network glycoproteins may be processed, disulfide bonds can be formed, and proteins can be sent to different cellular compartments. The subcellular location of a recombinant protein can greatly influence its accumulation (Bosch et al., 1994; Chikwamba et al., 2003; Conrad and Fiedler, 1998; Drakakaki et al., 2006). In many cases, secretion to the apoplast or to storage plastids in seeds, or chloroplasts in leaves, are the main choice, but that depends largely on the expression system utilized and the target recombinant protein produced. For several proteins, the addition of motifs, such as H/KDEL has been demonstrated to increase their stability (Schouten et al., 1996). This has been observed in proteins to be retained in the ER, but also for cytosolic proteins (Schouten et al., 1997). Fusion to another (carrier) protein may also increase stability. This has been observed for fusions with GFP and GUS, and may be useful as a reporter for evaluating expression and further processing such as purification (Hondred et al., 1999).

Protein stability, in general, will also largely depend on its proper folding. A heterologous protein may be unstable due to improper folding, resulting from environmental conditions or lack of (proper interaction with) chaperones or proper post-translational modification. In any case, proteins recognized as defectives will be ubiquitinated and targeted for degradation by the proteosome (Doran, 2006; Vierstra, 2003). In that case, heterologous protein production will result is a very low yield or even remaining undetectable.

Potential application of recombinant protein produced in plants

Over the past few years an array of recombinant proteins has been expressed in plants that can be grouped into the following categories: antigens and vaccines, antibodies, therapeutic proteins (e.g. hormones, cytokines and blood-related proteins); and industrially used proteins. Some application, advantages and strategies developed for expressing these proteins in plants are briefly presented here below.

Antigens and vaccines

Proteins derived from pathogens, particularly structural and/or membrane proteins are good candidates to be used as antigens for recombinant subunit vaccines (Awram et al., 2002). The advantage, besides the lower cost of plant-based production is that such antigens derived from plants have a reduced risk of being contaminated with human or animal pathogens (Awram et al., 2002; Canizares et al., 2005; Streatfield et al., 2001). Another possibility is using plants for oral delivery of the expressed antigens (Haq et al., 1995; Mason et al., 2002; Walmsley and Arntzen, 2003). This concept of oral vaccination has important implications for the induction of mucosal immune response, which is the main barrier against pathogens present in food or water (Cripps et al., 2001; Streatfield, 2006). Plants containing the proper antigens could be delivered orally. If present in the seed, for example, these oral vaccines could be stored at ambient temperatures for several months, and may have a major impact for disease control in especially developing countries (Daniell et al., 2001; Ma et al., 2005a; Raney, 2006). The main limitation for such approach is that high antigen expression levels are essential. Moreover, the orally provided antigen must resist low pH and proteolysis in the digestive tract to eventually assure induction of a protective immune response. The most successful example of such an oral vaccine is based in the LT-B toxin from *Escherichia coli* (Mason et al., 1998; Walmsley et al., 2003). This toxin, which closely resembles the cholera toxin, is highly immunogenic and can be expressed in high levels in transgenic plants. Several trials are carried out for evaluating the efficacy and safety of this new plant-based potential oral vaccine. Taking advantage of the high immunogenic properties of the LT-B toxin, fusion proteins were also tested, and shown to successfully induce an immune response (Streatfield, 2006; Walmsley et al., 2003).

Antibodies

Antibodies are immunoglobulins which specifically recognize and bind to an antigen and have important therapeutic and diagnostic applications. Plants can produce human antibodies, as first demonstrated by Haq in 1990. Since then, a large array of antibodies have been produced in plants, including derivatives such as single chains variable fragments (Fv), full size variable fragments (Fab), *diabodies* and *minibodies*, among other fragments and combinations of immunoglobulins, often revered to as "*plantibodies)*". Plants can also produce and correctly assemble complex secretory IgA, which can have important applications as passive immunization. Antibodies produced in plants appear to have the same activity as the corresponding molecule produced in mammalian cell cultures.

However, since immunoglobulins are glycoproteins, concerns have been raised about possible adverse effects resulting from differences on glycosylation pattern between plant and animal cells. Nevertheless, some antibodies are already advanced on clinical trial as, for example, a full-length antibody against *Streptococcus aureus*, to be used to prevent tooth decay (Ma et al., 2005a; Walsh, 2005).

Table 2. Biopharmaceuticals produced in transgenic plants

Protein	**Potential application or indication**	**Plant host**	**Expression levels** [a]	**Reference**
Insulin	Diabetes (autoimmune)	Potato, *Arabidopsis*	0.1 % TSP [b]	(Arakawa et al., 1998; Nykiforuk et al., 2006)
Human protein C	Anticoagulant	Tobacco	<0.01% TSP	(Cramer et al., 1996)
Human hirudin	Thrombin inhibitor	Canola (Brassica napus)	0.3% TSP (seed)	(Cramer et al., 1999)
Human granulocyte-macrophage colony-stimulating factor	Neutropenia	Tobacco	Not reported	(Lee et al., 1997)
Human somatotropin,	Growth hormone	Tobacco (chloroplast)	7% TSP	(Staub et al., 2000)
		Tobacco (nuclear expression)	<0.01% TSP	(Staub et al., 2000)
Human erythropoietin	Anemia	Tobacco	<0.01 TSP	(Matsumoto et al., 1995)
Human enkephalins	Antihyperanalgesic by opiate activity	*Arabidopsis*	0.1% TSP (seed)	(Vandekerckhove et al., 1989)
Human epidermal growth	Wound repair and control of cell Proliferation	Tobacco	<0.01% TSP	(Cramer et al., 1996)
Human interferon-α	Hepatitis C and B treatment	Rice, turnip (*Brassica rapa*)	Not reported	(Zhu et al., 1994)
Human interferon-β		Tobacco	<0.01% fresh weight	(Eldelbaum et al., 1992)
Human serum albumin	Liver cirrhosis, burns, surgery	Tobacco	0.02% TSP	(Sijmons et al., 1990)
Human hemoglobin	Blood substitute	Tobacco	0.05% (seed)	(Giddings et al., 2000)
Human homotrimeric collagen	Collagen	Tobacco	<0.01% fresh weight	(Ruggiero et al., 2000)
Human α-1-antitrypsin	Cystic fibrosis, liver disease and hemorrhage	Rice	Not reported	(Terashima et al., 1999)
Human aprotinin	Trypsin inhibitor for transplantation surgery	Maize	<0.1% TSP (seed)	(Zhong et al., 1999)
Human lactoferrin	Antimicrobial	Potato	0.1% TSP (tuber)	(Chong and Langridge, 2000)
Angiotensin-converting enzyme	Hypertension	Tobacco, tomato	Not reported	(Hamamoto et al., 1993)
Glucocerebrosidase	Gaucher's disease	Tobacco	1-10% TSP	(Cramer et al., 1996)
Cyanovurin-N	HIV microbicide	Tobacco	0.86% TSP	(Sexton et al., 2006)

[a] Expression level in leaf tissue, unless otherwise stated.

[b] TSP, total soluble protein.

Biopharmaceutical proteins

Several proteins of medical or veterinary application have been produced in plants (Table 2), ranging from hormones and cytokines to blood-derived products (Table 2). Potential cost-effectiveness is a strong advantage of using plants as platform for the production of biopharmaceutical. As pointed out before, the reduced risk of containing human or animal pathogens, toxins or prions is an important trend for the industry toward an animal product-free production line (Hood et al., 2002). Some of these proteins are glycoproteins and that may represent some limitation for production or biological activity, as discussed later in this chapter.

Proteins for technical and industrial use

Plant-produced proteins can be an important alternative source of polymers to produce fibers. Fibers from spider or silk, for example, possess mechanical properties superior to those of synthetic fibers. Also biodegradable plastic might be produced in plants, thus generating environmental benefits (Conrad, 2005; Scheller and Conrad, 2005; Scheller et al., 2004). Other potential products are enzymes evolved in specific purification processes in the food, paper and brewing industries (Somerville and Bonetta, 2001). As mentioned above, enzymes for technical application such as trypsin and GUS have been produced in maize seeds and are already being commercialized (Evangelista et al., 1998; Hood et al., 2002; Woodard et al., 2003). Since these products are not for medical or veterinary use, the development of such plant-derived products are expected to demand lower investments and shorter timelines for reaching production scale, when compared to biopharmaceuticals (Ma et al., 2005b).

Post-translation modifications

Post-translation modification of protein in plant cells, as other eukaryote cells, involve the formation of disulfide bonds, the correct assemble of proteins assisted by chaperones, the assemble of the peptide chains into multimeric forms, and the addition of glycan groups to proteins as they enter the secretory pathways (Vitale and Denecke, 1999). The addition of glycan groups to the proteins are believed to aid folding and protect against proteolytic degradation. Nevertheless, the glycosylation profiles between different taxa can be quite diverse (Brooks, 2004). Plant glycans may contain xylose, rhamnose and arabinose residues, which are not found in human glycans, and α(1,3)fucose, that is present in animal cells as an α(1,6) linkage. Plant *N*-linked glycans also lack galactose and the terminal sialic acids (Gomord and Faye, 2004; Ma et al., 2005a).

Differences in the glycosylation pattern may represent a limitation for recombinant protein production. There are concerns that the extra *N*-linked residues may be immunogenic to humans and animals (Gomord et al., 2005). It has been shown that some plant-produced proteins induce an immune response to plant specific glycans, but the effect has not been fully evaluated. It is also possible that the effect of a heterologous glycosylation pattern may alter the conformation and properties of a recombinant protein, rendering it less stable

or altering its biological activity. For many glycoproteins, the sialic acid residues are important for avoiding the protein to be cleared from the blood stream, for example (Brooks, 2004). Still, for many recombinant glycoproteins already produced in plants, particularly antibodies, the differences in glycosylation pattern do not seem to alter their functionality, as they have equivalent activity to proteins produced in animal cells (Hood et al., 2002; Stoger et al., 2002).

Despite the differences in glycosylation patterns and their potential effects, plant and animal proteins entering the secretory pathways share many similar fates. At the ER, proteins find molecular chaperones that will help the folding of the protein and formation of sulphur bridges (Vitale and Denecke, 1999). Like in animal cells, *N*-linked glycosylation in plants occurs at the ER and Golgi apparatus, whereas *O*-linked glycosylation occurs exclusively in the Golgi apparatus (Brooks, 2004; Vitale and Denecke, 1999). Likewise, several N-terminal signal peptides from animal proteins were shown to be correctly processed in plants (Conrad and Fiedler, 1998; Vitale and Denecke, 1999), as for the H/KDEL N-terminal signal for retaining the protein at the ER (Schouten et al., 1996).

The production of "humanized" recombinant protein has been attempted by using transgenic plants expressing β(1,4)galactosyltransferase (Gomord et al., 2005) Inhibition endogenous glycosyltransferases in the plant ER, has also been proposed. Sialylation of proteins, however, would require the introduction of several genes into the plant cell ("humanization of plant cells"), which remains a challenge (Gomord et al., 2005; Ma et al., 2005a). Approaches such as the *in vitro* modification of purified plant-derived recombinant proteins could, therefore, still be required.

Thesis outline

The expression of recombinant proteins in plants represents an innovative approach for the production of proteins of pharmaceutical and industrial purposes, high-value products that are usually extracted from their natural sources or produced from cultured bacterial, yeast or animal cells. This alternative approach can potentially bring several direct and indirect benefits for society, mainly because of its expected economical advantages. However, as a new technology, there are still many hurdles to be taken. Keeping this view in mind, the research described in this thesis focuses on optimizing transient recombinant protein expression in plants using viral vectors and *Agrobacterium tumefaciens* transient assay in parallel.

As a first step to optimize transient protein expression protocols based on plant viral vectors, the widely used TMV and PVX-based vectors were modified to be compatible with the Gateway cloning technology in Chapter 2, thereby further extending the versatility of these expression vectors. The expression of Chicken anemia virus (CAV) proteins, in view of their potential application as oral vaccines against this important pathogen was evaluated in Chapter 3. In Chapter 4, the expression of the human erythropoietin (EPO) gene and analysis of its posttranslational modifications is described. Chapter 5 deals with the genetic instability of the inserted sequences in viral vectors. The approach of this investigation was not focused on the molecular aspects of the arising deletion mutants, but on the how these

mutants compete with the virions carrying a foreign gene and how they are distributed within an infected plant. In Chapter 6 the nucleoprotein N of *Tomato spotted wilt virus* is evaluated as a potential fusion protein to increase stability and facilitate purification of recombinant proteins produced in plants. Chapter 7, finally, summarizes the results and presents a discussion on the advantages and limitations of the expression systems tested.

Chapter 2

PVX and TMV-based viral vectors compatible with the Gateway™ cloning technology

Abstract

Virus-based expression vectors are important tools for high-level production of foreign proteins and for gene function analysis through virus induced gene silencing. To further exploit their advantages as fast, high yield replicons, a set of vectors was produced by converting and adapting Potato virus X (PVX) and Tobacco mosaic virus (TMV)-based vectors to allow easy cloning of foreign sequences through the Gateway™ recombination technology. Target genes were efficiently cloned by recombination and successfully expressed in *Nicotiana benthamiana* following inoculation by *Agrobacterium* (agroinoculation). Using green fluorescent protein (GFP) as marker, high level expression with both PVX-GW and TMV-GW vectors was confirmed. A Gateway inserted phytoene desaturase gene (*pds*) fragment in PVX-GW vector (PVX-GW-PDS), induced gene silencing of the *pds* gene in *N. benthamiana*. The PVX-GW vector was further adapted by cloning the GFP gene upstream of the Gateway sequences, allowing the easy production of GFP fusions after recombination of a target gene. Subcellular localization of resulting GFP fusion was validated by recombining and expressing the coat protein gene from *Tomato chlorotic mottle virus*, revealing its nuclear localization. A PVX-GW transient expression assay of a nucleocapsid protein gene fragment of Tomato spotted wilt virus (TSWV) and of a single chain antibody against this protein was shown to effectively confer resistance to TSWV infection.

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Introduction

Plant viral vectors represent a promising strategy for heterologous protein production in plants (Canizares et al., 2005; Pogue et al., 2002; Scholthof et al., 2002). This transient expression system presents advantages over transgenic plants, particularly the fast response, typically days or a few weeks after inoculation, and the high level of the heterologous protein production that can be achieved (Gleba et al., 2004). An array of foreign proteins has been successfully produced using plant viral vectors, including proteins of pharmaceutical interest (e.g. hormones, antigens and antibodies), as well as enzymes and other proteins of industrial use (Awram et al., 2002; Canizares et al., 2005; Ma et al., 2003). Over the years viral vectors have also been used as an important tool to help elucidating gene function through reverse genetics in a process known as "virus induced gene silencing" (VIGS) (Burch-Smith et al., 2004; Watson et al., 2005). In this manner, viral vectors have become useful tools to study gene function allowing high-throughput screening of candidate genes by loss of function or by over expression of the gene product.

Plant viral vectors based on different viruses have been developed and tested with varying results. Among these vectors, Tobacco mosaic virus (TMV) (Shivprasad et al., 1999), Potato virus X (PVX) and Tobacco rattle virus (TRV) (Liu et al., 2002; Ratcliff et al., 2001) have been successfully used in different experimental approaches. These include their use as a replicon for transient gene expression, for VIGS and to study host-pathogen interaction and subcellular protein localization (Burch-Smith et al., 2004; Escobar et al., 2003; Takken et al., 2000).

Despite the multiple applications and advantages of the PVX-based vector, cloning and analyzing a large number of genes can be time consuming and cumbersome. An alternative approach is the use of the Gateway technology, which does not rely on restriction/ligation but on *in vitro* site-specific recombination through the bacteriophage λ recombination sites (*att*) and the λ integrase (Hartley et al., 2000). The Gateway technology has proven to be very efficient for high-throughput cloning for both prokaryotic and eukaryotic expression systems. For plants, a set of binary vectors containing an array of plant selectable markers, promoters, fusions to green fluorescent protein (GFP) or β-Glucuronidase (GUS), and hairpin generating constructs, all compatible with the Gateway cloning technology are available (Curtis and Grossniklaus, 2003; Karimi et al., 2002; Wesley et al., 2001). For viral vectors, a Tobacco rattle virus (TRV) viral vector has also been successfully tested (Liu et al., 2002).

In this report, the construction of TMV and PVX derived vectors compatible with the Gateway cloning technology is explored. These vectors were tested for protein expression and VIGS. Also, a set of PVX-based vectors containing the green fluorescent protein (GFP) were developed to obtain recombinant tagged proteins allowing subcellular localization of the target protein.

Materials and Methods

Bacteria strains and culture conditions

Escherichia coli strains DH5α and DB3.1 were grown on LB medium at 37°C. *Agrobacterium tumefaciens* strains GV3101 and LBA404 were grown on LB medium at 28ºC. Plasmids were electroporated into bacteria using a BioRad (Hercules, USA) electroporator, according to the manufacturer's instructions.

Plant material and inoculation conditions

Nicotiana benthamiana plants, 4-week-old (four leave stage), were used for inoculation. Leaves were agroinfiltrated at the abaxial side with bacterial suspension using a 5-ml needle-less syringe. Bacterial suspensions were obtained by centrifuging 2 ml of an LB overnight culture and resuspending the cells on 5 ml of Murashige-Skoog medium (MS) (Murashige and Skoog, 1962) with 10 mM morpholineethanesulfonic acid (MES), pH 5.6, and 150 µM acetosyringone. The final volume was adjusted to an OD_{600} of 0.5 by adding MS medium. Alternatively, leaves were inoculated by using a sterile toothpick to transfer bacterial colonies from an agar plate and puncture the leaves close to the midvein. TSWV was mechanically inoculated by rubbing the leaves with extract from infected plant and carborundum powder. Inoculated plants were maintained in a growth chamber at 25 ± 2°C and a 12 hrs photoperiod.

PVX and TMV-based destination vectors

The Gateway™ conversion cassette (frame A) (Invitrogen, Carlsbad, USA) was cloned into the *Sma*I site of vector pGR107 (Figure 1a) (Jones et al., 1999). Ligation reaction was electroporated into DB3.1 cells and selected on LB agar plates containing 50 µg/ml kanamycin and 20 µg/ml chloramphenicol. This pGR107 Gateway-compatible destination vector, hereafter referred to as PVX-GW vector (Figure 1b), was further modified by cloning the green fluorescent protein gene (GFP), upstream of the *att*1 sequence. The sGFP gene (Chiu et al., 1996) was amplified by PCR, using primers containing the *Cla*I site as extension, as well as extra nucleotides, creating all three reading frames for the GFP and the *att*R sequence. These vectors are referred to as PVX-GW-GFP: (Figure 1c, d).
The TMV-based vector 30B (Shivprasad et al., 1999) was cloned into the *Not*I site of a pBin19 binary vector, containing the CaMV 35S promoter and a nos terminator. A *Pac*I-*Sun*I fragment containing the Gateway cassette was cloned into the *Pac*I-*Sun*I site of the pBinTMV (Figure 1e). Transformed DB3.1 cells were selected on LB agar plates containing 100 µg/ml kanamycin and 20 µg/ml chloramphenicol, resulting in vector pBinTMV-GW, hereafter referred to as TMV-GW (Figure 1f). Clones of PVX and TMV-based vectors containing the Gateway *att*R sequences were verified by sequence analysis. DNA restriction and ligation were done according to standards methods (Sambrook et al., 1989).

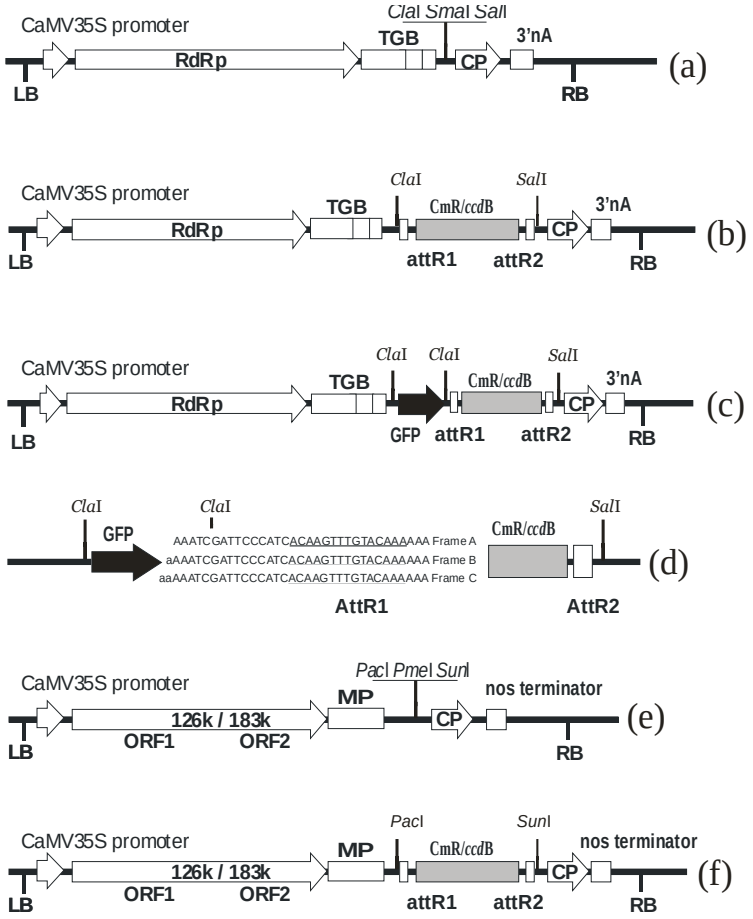

Figure 1. Schematic representation of PVX and TMV-based vectors. (a) Genomic organization of pGR107, a PVX-based viral vectors. RNA dependent RNA polymerase (RdRp), RNA dependent RNA polymerase (120 k and 64 k); TGB, triple gene box; CP, coat protein. (b) PVX-GW: Gateway compatible PVX vector, converted from pGR107. (c, d) PVX-GFP:GW: a PVX-GW for GFP N-terminal fusion. (d) Detail of PVX-GFP:GW showing the sequences at the 3`end of the GFP gene corresponding to the three possible reading frames for translational fusions. (e) Genomic organization of a TMV-based vector in a binary vector. ORF1/ORF2, corresponding to the 126k and 183k proteins that show regions similar to methyltransferase, helicase, and RdRp; MP, movement protein; CP, coat protein from TMGMV. (f) TMV-GW: Gateway compatible TMV-based vector (f). LB and RP, left and right border of T-DNA; *att*R1 and *att*R2, Gateway recombination sites; CmR/*ccd*B, selection markers for Gateway recombinational cloning (chloramphenicol resistance gene and the *ccd*B gene that inhibits growth of *E. coli*).

Entry vectors and LR/BP recombinational cloning

The sGFP gene, the nucleoprotein N from TSWV (Gielen et al., 1991) and the single chain antibody (scFv) N56 (Prins et al., 2005) were cloned into the *Nco*I/*Not*I sites of pEntr11™ (Invitrogen). The phytoene desaturase (*pds*) gene from *N. benthamiana* (Ratcliff et al., 2001), mGFP and the coat protein gene of Tomato chlorotic mottle virus (ToCMoV) (Ribeiro et al., 2003) were amplified by PCR using specific primers containing the *att*B1 and *att*B2 modifications (Invitrogen) for the forward and reverse primers, respectively. The PCR product was purified and recombined with pDonr207™ (Invitrogen) using the PB reaction mix (Invitrogen). LR and BP reactions were done essentially as described in the manufacturer's manual, except that the reaction volume was scaled down to 5 µl. Reactions were electroporated into *E. coli* DH5α cells and selected on LB agar plates containing the proper antibiotic selection. Prior to the LR reaction, pEntr11-derived entry vectors were digested with *Nhe*I, to linearize the plasmid and allow selection of PVX-GW or TMV-GW derived clones. Plasmid DNA from selected clones was transferred to the *Agrobacterium tumefaciens* strain GV3101 and used for agroinfiltration. For the PVX-based constructs, the strain GV3101 also carried the pSoup helper plasmid (Hellens et al., 2000).

Protein extraction and analysis

Protein was extracted from leaf samples obtained by piercing the leaf with a cork borer. Four leaf discs (approximately 10 mm diameter and 100 mg fresh weigh) were macerated in 100 µl of PBS (50 mM sodium phosphate buffer, 10 mM NaCl), using a plastic pestle. For Western blot analysis, 20 µl samples were loaded on 12% SDS-PAGE, subjected to electrophoresis at 100-120 volts and electroblotted (BioRad, USA) to a PVDF membrane (Millipore, USA). Blotted membranes were blocked with PBS containing 0.1% Tween (PBST) and 5% non-fat milk, for 1 hour at room temperature. After blocking, the membranes were incubated in PBST with 1:2000 diluted anti-GFP or anti-N polyclonal antibodies. After washing three times with PBST, secondary antibodies were added and incubated for 1 hour at room temperature. Blots were again washed and incubated in Tris-HCl 10mM, pH 9.5, for 10 min, in a plastic bag. Ready-to-use CSPD (Roche) was added and incubated for 5 min at room temperature and 10 min at 37°C. Blots were exposed to X-ray film for 1-3 hours.

UV photography and imaging

Pictures of whole plants were made with a Nikon camera with a 400 ASA Kodak film and a yellow filter. UV illumination was provided by two UV lamps (365 nm). Exposure times ranged from 10 to 60 sec were used, depending on the intensity of the fluorescence. Close-up UV pictures were made with a digital camera (CoolSNAP™) using a binocular stereomicroscope (M3Z; Leica). Laser-scanning microscope (LSM) images were obtained using a Zeiss LSM510 microscope and the software Zeiss LSM Data Server. GFP fluorescence was detected by excitation with a blue laser light at 488 nm and emission through a 505-530 nm bandpass filter.

Results

Heterologous protein expression from PVX-GW and TMV-GW vectors

Existing TMV and PVX-based vectors were modified to be compatible with the Gateway cloning technology (Figure 1). To validate their use as expression vectors, an entry vector containing the mGFP gene (pDonor-mGFP) was used for recombination. Recombination with entry vectors was highly efficient resulting in hundreds of positive clones after *E. coli* transformation. These expression vectors were transferred to *Agrobacterium* and inoculated on *N. benthamiana* plants by infiltrating *Agrobacterium* suspension in the abaxial side of the leaves. For PVX-GW-GFP, after 3-4 days intense GFP fluorescence was observed under UV light. For TMV-GW-GFP the number of initially transformed cells was much lower than observed for PVX-GW-GFP, and fluorescence after 3-4 days could only be detected with a stereo microscope. After 7-10 days post inoculation (d.p.i.), systemic infection revealed intense fluorescent areas for both PVX-GW-GFP and TMV-GW-GFP (Figure 2). Inoculation by piercing the leaves with a toothpick carrying respective appropriate *Agrobacterium* colonies was successful for both vectors, although in this case systemic expression of GFP could be observed only 15-20 d.p.i. Extracts from systemically infected leaves were passage-inoculated and systemic GFP expression was also observed in this second passage. No difference in GFP expression was noticed for the PVX-GW and pGR208, an established GFP-containing PVX vector (Peart et al., 2002). Plants inoculated with TMV-GW-GFP, however, took longer to establish a systemic infection than the vector containing mGFP inserted by using restriction sites (data not shown).

PVX-GW and TMV-GW-vectors are functional in inducing VIGS

To test the Gateway-compatible PVX and TMV vectors to induce gene silencing, an internal fragment of approximately 500 bp of the *N. benthamiana pds* gene (Ratcliff et al., 2001) was amplified by PCR. This fragment was cloned into an entry vector (pEntr11) and selected clones containing the fragment in a sense and anti-sense orientations were recombined with pPVX-GW. Inoculated *N. benthamiana* leaves started showing chlorotic areas 15-20 d.p.i. as systemic infection progressed (Figure 3). No apparent difference on the extent of silenced areas could be observed between plants inoculated with a PVX containing the same *pds* fragment either in the sense or antisense orientation. Plants inoculated with the TMV-GW-PDS vector, containing the ~500 nt internal sequence of the *pds* gene showed only small chlorotic areas after 20-30 d.p.i. (data not shown).

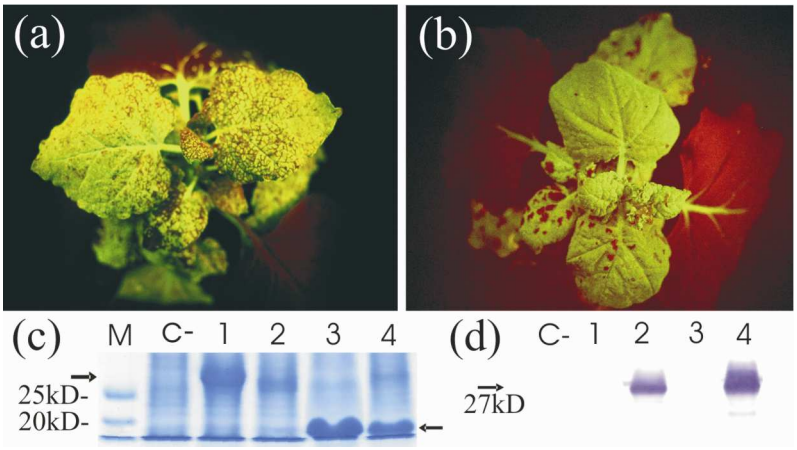

Figure 2. GFP fluorescence in *N. benthamiana* plants and Western blot detection. (a, b) Systemic infection with PVX-GW-GFP (a) and TMV-GW-GFP (b). (c) Coomassie brilliant blue-stained protein gel (c). Arrows indicate the expected sizes for TMV coat protein (30 kDa) and PVX coat protein (17 kDa). (d) Western blot of samples from systemically infected plants. The arrow indicates the position of GFP. M: molecular weight marker; C-: non inoculated control; 1: PVX (pGR107 - no insert); 2: PVX-GW-GFP; 3: TMV 30B (no insert) 4: TMV-GW-GFP.

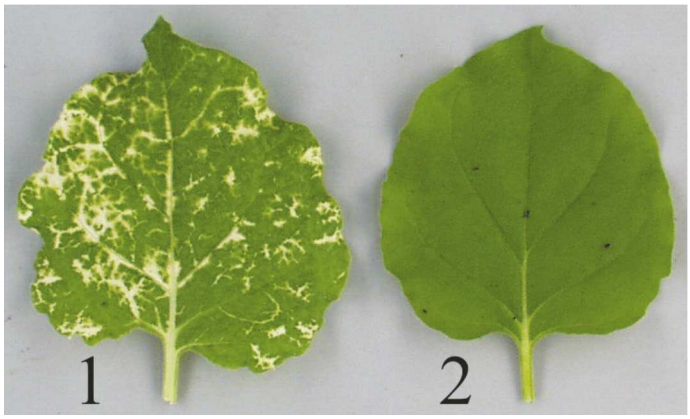

Figure 3. Virus induced gene silencing (VIGS) of the *pds* gene in leaves of *N. benthamiana*, 15 d.p.i. 1: Leaf infected with PVX-GW-PDS; 2: wt PVX infected leaf.

Expression of tagged proteins from PVX-GFP:GW vectors

To analyze the subcellular localization of a vector-expressed candidate gene, a set of vectors was prepared, containing the GFP gene immediately upstream of the *att*R sequence of the Gateway cassette, with the three possible frames (Figure 1d). To validate the use of these vectors, the coat protein (CP) gene of ToCMoV was recombined into PVX from an entry vector and the recombinant clones were selected after *Agrobacterium* transformation. Leaves infiltrated with the construct containing the GFP:ToCMoV CP fusion was analyzed at 4-5 d.p.i., under UV light, by epifluorescence microscopy and LSM. The GFP fluorescence was only observed in nuclei, showing both nucleoplasmic and nucleolar labeling (Figure 4a)., The intensity of the fluorescence, however, was much lower than a PVX-GW-sGFP control, which showed a distinct cytoplasmic and nuclear labeling (Figure 4b).

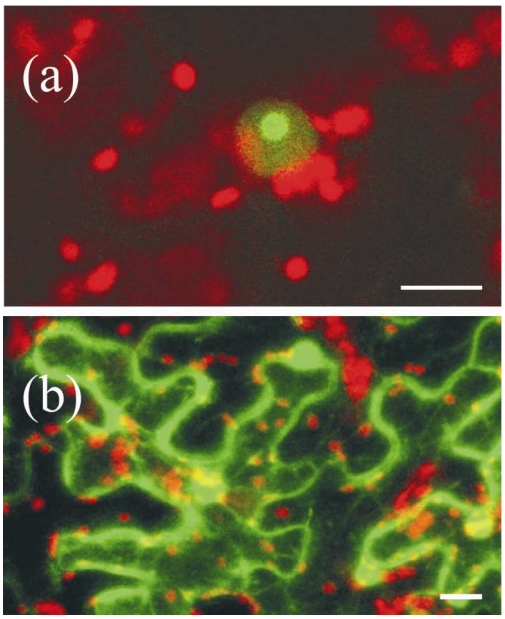

Figure 4. LSM image of *N. benthamiana* leaf cells infected with different PVX constructs. (a) GFP fluorescence in cells infected with PVX-GFP:ToCMoV CP, showing the restricted nuclear localization of the GFP fusion to the coat protein (CP) of TCMV. (b) PVX-GFP infected cells showing fluorescence in the cytoplasm and nucleus. Bar=10 µm.

PVX transient expression system for evaluating virus resistance strategies

The gene for single chain antibody (scFv) N56, directed against the TSWV nucleocapsid protein N (Prins et al., 2005) was recombined into PVX-GW and a selected *Agrobacterium* clone was grown and infiltrated in *N. benthamiana* leaves. After 3 days, the same leaves were mechanically inoculated with TSWV. After 30 days, plants that were infiltrated with the N56 single chain, displayed resistance to TSWV infection whereas control plants were severely infected or died (Figure 5a). No synergistic effect positive or negative on the infection of either virus was observed in plants co-infected with wt PVX and TSWV. Western blot analysis showed the presence of TSWV in both inoculated and systemically infected leaves, indicating that the transient expression of the single-chain in the inoculated leaves did not fully abrogate TSWV infection like in the N56 transgenic plants (Prins et al., 2005), although symptoms were clearly reduced and delayed when compared to control plants (Figure 5c). Though readily detectable in the locally infected leaves, the N56 scFv could not be detected in systemic leaves, indicating that the PVX-GW-N56 may have lost the insert (data not shown). Another transient resistance approach was tested by expressing the TSWV N gene from a PVV-GW-N. Plants transiently expressing the N gene sequence at the TSWV inoculated leaves showed only mild systemic symptoms typical of a PVX infection. After 30 days from TSWV inoculation, the nucleocapsid N protein could not be detected in Western blot assay, indicating that plants were not infected by TSWV and that the N gene was also not being expressed by the PVX-GW construct, most likely having lost this foreign sequence (Figure 5c).

Discussion

Here we have shown the generation and successful testing of a set of TMV and PVX-based vectors which were adapted to allow efficient cloning of foreign sequences using the Gateway technology. Both TMV and PVX-based vectors containing the Gateway sequences were efficiently recombined with entry vectors, and the resulting expression vectors successfully infected *N. benthamiana* plants, expressing the target genes. GFP expression was observed in the inoculated leaves and systemically infected tissue with both PVX-GW-GFP and TMV-GW-GFP.

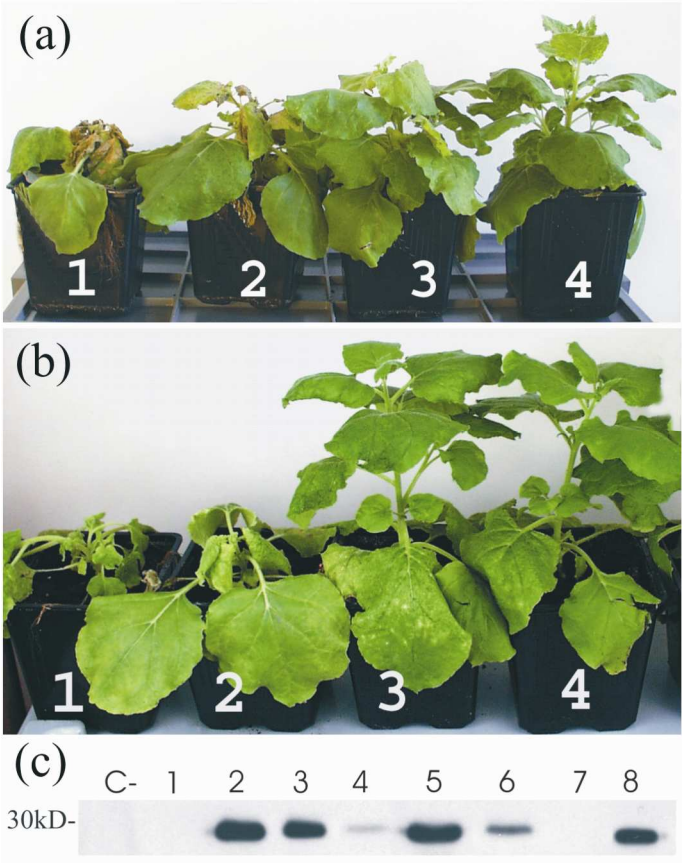

Figure 5. Evaluation of virus resistance strategies using transient gene expression from a PVX-based vector. (a, b) *N. benthamiana* plants infected with PVX-GW expressing either the N56 single chain (a) or the nucleocapsid gene (b). a1, b1: TSWV infected plant; a2, b2: PVX (inoculated from pGR107) and TSWV co-infected plant; a3, b3: PVX-GW expressing either the N56 single chain (a3) or the nucleocapsid N protein (b3) and inoculated with TSWV. a4, b4: Non infected plants. Plants were mechanically inoculated with TSWV 3 days after PVX agroinoculation. (c) Western blot detection of TSWV in leaf extracts from plants co-inoculated with PVX and TSWV. C-: Non inoculated (control) plant; 1: PVX infected plant (inoculated with pGR107); 2: TSWV infected plant; 3: wt PVX and TSWV co-infected plant systemically infected leaf; 4 and 5: PVX-GW-N56 and TSWV inoculated leaf (4) and systemically infected leaf (5); 6 and 7: PVX-GW-N and TSWV inoculated leaf (6) and: systemically infected leaf (7); 8: purified nucleoprotein N.

Viral vectors based on TMV and PVX have been efficiently used for a number of purposes, including high level expression of heterologous proteins, virus induced gene silencing, suppression of gene silencing, isolation of hypersensitive reaction (HR) related genes, and several aspects of virus biology and host interaction (Angell and Baulcombe, 1999; Bendahmane et al., 2000; Chapman et al., 1992; Lacomme et al., 2003; Takken et al., 2000). The set of vectors described here were efficiently used for gene function analysis, either by VIGS or by the subcellular localization of GFP-fusions, and for heterologous protein production, all obtainable by a single cloning step through the Gateway technology. The Gateway cloning technology does not use restriction and ligation, but is based on site-specific recombination, carried out *in vitro* (Hartley et al., 2000). Cloning is directional and highly efficient, from a PCR fragment to entry vectors containing compatible recombination sites (*att*B and *att*P), and from this entry vector to a destination vector (involving *att*L and *att*R sites), thereby facilitating gene cloning. Indeed, many libraries and genes are being cloned in entry vectors, including e.g. the *Arabidopsis* library from the European consortium AGRIKOLA (*Arabidopsis* genomic RNAi knockout line analysis) project (Watson et al., 2005). Transient gene expression approaches, such as the viral vector or the *Agrobacterium tumefaciens* transient assay (ATTA) also represent important tools in this functional genomics endeavor. These approaches avoid the generation of multiple lines of transgenic plants, which is time and space consuming and laborious. Binary vectors containing an array of plant selectable markers, promoters, GFP fusions and hairpin generating constructs, all compatible with the Gateway cloning technology are available and have been successfully used (Curtis and Grossniklaus, 2003; Karimi et al., 2005; Karimi et al., 2002; Wesley et al., 2001). A TRV-based viral vector compatible with the Gateway technology has also been developed and successfully tested for gene silencing using a tomato EST library (Liu et al., 2002).

The TMV-GW and PVX-GW vectors described here could be inoculated using *Agrobacterium tumefaciens* (agroinoculation). Although agroinoculation is easier and more economical than inoculation with *in vitro* RNA transcripts; it implies an extra *Agrobacterium* transformation/selection step. Besides, *in vitro* transcripts inoculation may be conveniently exploited for high-throughput screening of a cDNA library by analyzing individual inoculation foci, as approached by Escobar *et al.* (2003). Therefore, the choice of inoculation method may depend on the experimental approach. For heterologous protein production, a fast, high level expression of the target gene would be ideal. This can be achieved by infiltrating bacterial suspensions in leaf tissues, with a syringe or by vacuum infiltration of the aerial parts of the plant (Marillonnet et al., 2005). These methods are particularly attractive since one can expect to collect material for protein extraction as early as a few days after inoculation. Systemically infected tissues are also suitable for high-level expression from viral vectors; however, problems associated with insert instability may be present, especially for larger inserts (over 1 kb) (Awram et al., 2002; Chapman et al., 1992).

N. benthamiana leaves infiltrated with PVX-GW-GFP showed intense GFP fluoresce apparent from every cell type within the infiltrated leaf areas. Expression followed TMV

infiltration, however was reduced to a few individually transformed cells, indicating that transcription of the viral cDNA from the T-DNA and the subsequent onset of viral infection, starting from translation of the viral replicase is not as efficient as for the PVX-based infectious DNA. Similar results have been described previously by Marillonnet et al. (2005), who showed that the delivery of TMV from *Agrobacterium* can be substantially further improved by altering the TMV sequence by removing putative intron recognition sites. Another approach for TMV vector improvement was achieved by DNA shuffling of the movement protein, resulting in virus that could spread faster both from cell-to-cell and systemically (Toth et al., 2002).

Viral vectors allow high level transient expression of foreign genes in differentiated tissues. Long, cumbersome tissue culture procedures are avoided and a large number of plants can be easily inoculated and analyzed after a few days following plant inoculation. These features make viral vectors very useful for studies of gene function either through virus induced gene silencing (VIGS) or by the subcellular localization of a target protein. Subcellular localization studies can provide additional information on the function of a target gene (Escobar et al., 2003). A common approach for such studies is to obtain a fusion with a reporter gene to facilitate the detection of the chimeric resulting protein. The GFP gene for its convenience as a non destructive, easily detectable reporter gene has been widely used either as a C or N terminal fusion (Curtis and Grossniklaus, 2003; Escobar et al., 2003). We have modified the PVX-GW vector to allow the easy cloning of a candidate gene to obtain a GFP at the N terminus of the fusion protein. To validate the use of the PVX-GFP-GW construct vector was recombined to the coat protein gene of the ToCMoV resulting in a CP-GFP fusion. LSM images showed that the fusion protein was targeted to the nucleus. The subcellular localization of this protein had not been studied before, however, a putative nuclear localization signal was indicated from its coding sequence (SG Ribeiro, unpublished).Nuclear targeting of the ToCMoV capsid protein is consistent with the CP targeting of other geminiviruses, such as African cassava mosaic virus (Unseld et al., 2001) and Tomato yellow leaf curl virus (Kunik et al., 1998). The PVX-GFP-GW vectors, therefore, were shown to be a useful and reliable tool for to study subcellular localization of proteins.

Inoculation of PVX-GFP resulted in intense fluorescence that could be detected after 4-5 days from a large number of cells, due to the high efficiency agroinoculation of the PXV vector. For the GFP gene fusion, however, the relatively large size can result in a rather unstable insert, and the resulting fusion protein may not be detected in systemically infected leaves (Chapman et al., 1992; Pogue et al., 2002). Larger gene fusions can also reduce the number of expressing cells in the inoculated leaf, as observed for a GFP:GUS fusion (data not shown). Still, as information on subcellular localization can be obtained from a small number of inoculated cells, and the viral replicon ensures high expression, this should not constitute a limitation to the system.

Viral vectors have been an important tool to help elucidating gene function in plants though VIGS (Burch-Smith et al., 2004). The most commonly used vector for VIGS is TRV-derived. This vector is particularly efficient, inducing a very effective and consistent silencing, possibly due to its silencing imprinting at the meristematic tissue (Liu et al.,

2002; Ratcliff et al., 2001). PVX and TMV-based vectors have also been used for VIGS and to study the mechanisms of RNA silencing in plants (Angell and Baulcombe, 1999; Faivre-Rampant et al., 2004; Kumagai et al., 1995). The PVX-GW vector containing the partial sequence of the *pds* gene was shown to trigger RNA silencing in *N. benthamiana*. Silenced areas are typically patchy and leaves still show large green areas (Figure 3), which seems to be characteristic of the PVX as a silencing inducer (Angell and Baulcombe, 1999; Faivre-Rampant et al., 2004). Plants inoculated with the TMV-GW vector containing the *pds* gene fragment showed mild chlorosis in contrast with the more extensive bleached areas [Figure 3 and Kumagai et al. (1995)]. Both PVX and TMV are less efficient initiators of VIGS than TRV, although they may be improved by cloning inverted repeated sequences (Lacomme et al., 2003). Moreover, both TMV and PVX can be co-infected with TRV, broadening the possibilities for RNA silencing and for plant-virus interaction studies.

In addition to the use of the PVX-GW and TMV-GW vectors for heterologous protein production and gene function analysis we described a fast transient method to assess gene constructs for their potential to induce virus resistance. By expressing a single chain antibody against the nucleoprotein N of TSWV in transgenic *N. benthamiana* plants, Prins et al. (2005) showed that high level resistance to TSWV infection could be obtained. Here the same scFv (N56) gene was tested via the PVX-GW to validate this expression system for testing resistance strategies. Transient expression of the N56 scFv led to a considerable delay of TSWV infection. Transient expression of the N gene upon inoculation of PVX-GW-N was even more effective in hampering TSWV infection (Figures 5b). After 30 days, the N protein could not be detected in plants systemically infected with PVX in contrast to the PVX CP, indicating that the heterologous expression of the N gene from the PVX-GW vector in the infiltrated leaf either blocked TSWV infection or virus titers were too low to be detected by Western blot. Expression of the N gene from the PVX viral vector may have induced the RNA silencing machinery leading to subsequent inhibition of TSWV infection by targeting N mRNA.

The potential use of viral expression systems to screen large numbers of gene constructs, e.g. different single-chain clones, or constructs containing pathogen-derived sequences against different virus isolates, is demonstrated. A similar approach was recently tested by (Donini et al., 2005) in plants inoculated with a PVX vector carrying a killer peptide (which) fused to the coat protein (CP). Plants were inoculated with the PVX-CP-killer peptide fusion and obtaining resistance to different challenged bacterial and fungi phytopathogens, thereby demonstrating the efficacy of the tested peptide and the efficiency of the PVX-based display method.

Screening of such constructs can lead to an increase in efficiency of time and effort, as a result of the cloning through Gateway and of the simplicity of this transient expression assay. Testing different single chain antibody clones, which may vary both on their stability and neutralizing efficacy (Prins et al., 2005) can help to quickly screen potentially most successful clones prior to high numbers of plant transformation. Likewise, a candidate gene or sequence designed to achieve resistance through RNAi can be easily tested using this system. Cumbersome, low efficiency cloning steps are avoided, as well as the generation of several lines of transgenic plants. Selection of the best candidate genes would provide

potential candidates for further developing and testing a transgenic approach for pathogen resistance.

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Chapter 3

Expression and localization of Chicken anemia virus (CAV) proteins in plant cells

Abstract

Chicken anemia virus (CAV) is an important pathogen of chicken worldwide, causing severe anemia and immunodeficiency. Its small single-stranded DNA genome (2.3 kb) encodes three proteins: VP1, the only structural protein, VP2 a protein phosphatase, and VP3, also known as apoptin, which induces apoptosis. In this study, CAV proteins were expressed in plants as an alternative for recombinant protein production in animal cells. Additionally, the effect of VP3 expression was tested to evaluate possible involvement in programmed cell death in plants. The CAV genes were cloned in binary vectors with the Green fluorescent protein (GFP) as N terminal fusion, and into a Potato virus X (PVX) and Tobacco Mosaic Virus (TMV)-based vectors. *Nicotiana benthamiana* plants were inoculated with *Agrobacterium tumefaciens* containing the binary vector constructs or the PVX and TMV constructs. Upon transient expression GFP:VP1 and GFP:VP2 were observed throughout the nucleoplasm, whereas VP3 formed compact aggregates within the nucleus, indicating functional nuclear localization signals in all three proteins. An intense fluorescence was observed for VP2 and VP3 fusions, whereas GFP:VP1 fluorescence remained faint and was only detected in a limited number of cells. Co-expression of GFP:VP1 and VP2 had a marked alteration on the distribution of GFP:VP1, forming large VP1 aggregates throughout the nucleus, indicating an interaction of the two CAV proteins. No visible alteration on GFP pattern was detected upon co-expression of GFP:VP1 and VP3, or with GFP:VP2 and VP3. Leaves infiltrated with binary vectors expressing CAV proteins showed no visible phenotype. However, plants infected with PVX or TMV-based vectors expressing VP3 displayed strong necrosis and wilting, suggesting an involvement of VP3 in cell death in plants. A direct association with programmed cell death in plants could, however, not be established. Overall, our results show that all CAV proteins can be expressed in plant cells, though expression of VP1 needs to be further optimized before testing its potential as (edible) subunit vaccine.

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Lacorte C, Lohuis D, Goldbach, R and Prins M. Expression and localization of Chicken anemia virus (CAV) proteins in plant cells

Introduction

Chicken anemia virus (CAV) is an important pathogen in chickens, causing severe immunodeficiency and anemia (Noteborn, 2004; Todd et al., 2001). The virus presents a small single-stranded DNA genome (2.3 kb) and has been classified in the Circoviridae family, being the only species assigned for the genus *Gyrovirus* (Todd et al., 2001). The negative sense genome presents three partially overlapping ORFs. VP1 is the only structural protein found in purified particles (Todd et al., 1990); VP2 is a protein phosphatase and has been shown to interact with VP1, possibly helping VP1 assembly (Noteborn et al., 1998; Peters et al., 2002); and VP3, also known as apoptin (Noteborn, 2004; Noteborn et al., 1994). This protein induces apoptosis in infected chicken cells. Additionally, it was shown to cause apoptosis in human tumor cells, but not in normal human cells (Danen-Van Oorschot et al., 1997; Noteborn, 2005).

CAV has a narrow host range infecting chickens reared for the meat and egg industry worldwide. Economical losses can be very high as a consequence of outbreaks that cause mortality and morbidity due to secondary infections, and reduction in broiler weight (Adair, 2000; Todd et al., 2001). Difficulties in obtaining attenuated strains and the very slow growth rate and low titers that can be achieved from infected cell cultures have hampered the development of effective vaccines (Cunningham et al., 2001; Todd et al., 2001). The expression of VP1, VP2 and VP3 was achieved in insect cells inoculated with recombinant baculoviruses (Noteborn et al., 1998). Co-expression of VP2 and VP1 was essential for the recombinant induction of neutralizing antibodies (Koch et al., 1995; Noteborn et al., 1998) leading to the suggestion that VP2 would help VP1 –the sole viral structural protein- to achieve a proper stable conformation, by acting as a scaffold protein (Noteborn et al., 1998). The induction of neutralizing antibodies that could confer protection shows the viability of using recombinant subunit vaccines against CAV (Koch et al., 1995; Noteborn et al., 1998). However, for an effective vaccination schedule, and considering the large number of animals to be vaccinated, large amounts of the recombinant antigen would have to be produced, at low cost. An oral recombinant vaccine, for example, delivered through the drinking water or feed would be very adequate (Todd et al., 2001).

The expression of heterologous proteins in plants has become an attractive alternative for the more current expression systems based on animal, yeast or bacteria cell culture (Ma et al., 2003). A number of proteins of pharmaceutical and industrial applications have been produced in plants, where the low cost of production, easy scale-up, and low risk of contamination with animal pathogens are the main advantages (Ma et al., 2003; Rigano and Walmsley, 2005). Plants expressing a recombinant antigen can be used directly as an oral vaccine, which presents low cost and high stability. Therefore, in this study, the potential use of plants for the production of CAV proteins, to be used as a recombinant vaccine, was explored. For this purpose, *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* strains containing binary vectors or, alternatively, inoculated with PVX and TMV-based viral vectors carrying the CAV genes. In addition, the

expression of VP3 and its potential role in inducing programmed cell death (PCD) in plants was included in the experiments.

Materials and Methods

Bacterial strains and culture conditions

Escherichia coli strain XL1Blue was used for DNA cloning. Cells were grown in LB medium, in a shaker at 37°C. *Agrobacterium tumefaciens* were cultured in LB3 medium (Peptone, yeast extract, NaCl, KCl, pH7.0), at 28°C. Competent cells were transformed by electroporation using a BioRad apparatus, following the manufacturer's instructions.

DNA amplification and cloning

CAV genes were amplified by PCR from cloned DNA of the Cux-1 strain, obtained from Dr. Harry Flore (Lohmann Animal Health, Cuxhaven, Germany). Restrictions sites were included as extension on the primers for VP2 and VP3. PCR fragments were cloned on pGem-T easy vector (Promega) and then digested with *Bam*HI and *Sst*I, for cloning into a pCambia 2300 binary vector, containing the CaMV35S promoter and nos terminator. The sGFP gene (Chiu et al., 1996) was PCR amplified excluding the termination codon, and cloned into the *Nco*I site of the pCambiaCaMV35S-VP2 and pCambiaCaMV35S-VP3 to obtain *in frame* GFP C terminal fusions. VP2 and VP3 fragments were also cloned in the pEntr11 vector (Invitrogen). VP1 was amplified with primers containing the Gateway attB1/attB2 (Invitrogen) extensions. PCR fragment was recombined with pDonr207 using the PB clonase mix (Invitrogen), as recommended by the manufacturer. Entry vectors (pDonor207 and Entr11) containing the VP1, VP2 or VP3 genes were recombined with destination vectors using the LR clonase mix (Invitrogen). Destination vectors used were the binary vectors pK2GW7 and pK7WGF2 (Karimi *et al.*, 2002) and a Gateway compatible PVX-based vector (PVX-GW and PVX-GFP-GW) (Chapter 3). Recombination reactions were transformed into *Agrobacterium tumefaciens* strain GV3101. The PVX-GW vectors were transformed in a GV3101 strain containing the pSoup plasmid (Hellens et al., 2000). DNA restriction and cloning was done essentially as described by Sambrook et al. (1989).

Plant material and inoculation conditions

Nicotiana benthamiana plants (4-5 week-old) were used for *Agrobacterium tumefaciens* transient assay (ATTA). Young, fully expanded leaves were infiltrated with *Agrobacterium* suspension using a 5 ml syringe without needle at the abaxial surface. Bacterial suspensions were obtained from an overnight culture (600 µl) centrifuged and resuspended in 3 ml induction medium [10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.0 g/l of $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.2% (w/v) glucose, 0.5% (v/v) glycerol, 50 µM acetosyringone, and 10 mM morpholineethanesulfonic acid (MES), pH 5.6]. After overnight incubation at 28°C,

cultures were centrifuged and resuspended in 5 ml MS medium (Murashige and Skoog, 1962) containing 10 mM MES and 150 µM acetosyringone and the OD_{600} adjusted to 0.5. Co-inoculation with a suppressor of gene silencing, HcPro, was tested by mixing 1 volume of strain LBA4404 pBin-HcPro (Bucher et al., 2003) with 2 volumes of strains carrying the binary vectors with the CAV genes. Strains carrying the sGFP gene, either in a binary vector or in a viral vector were included as controls. The avirulent *Agrobacterium* strain A136 carrying a pCambiaCaMV35S-sGFP plasmid was used a negative control. For each construct, 2-3 plants were inoculated and each experiment was repeated at least 3 times. After infiltration, plants were maintained in a growth chamber at 25°C and 12 h photoperiod.

Sample preparation and microscope imaging

Samples from infiltrated tissues were mounted with water on a glass slide. Images were obtained with a digital camera (CoolSNAP™) mounted on an epifluorescence microscope (Leica), with UV light and a blue filter set (465 nm). LSM images were obtained in a Zeiss LSM 510 confocal microscope and the software Zeiss LSM Data Server. GFP fluorescence was observed through excitation by a blue light laser light (488 nm) and emission through a 505-530 bandpass filter. For DAPI staining, samples were incubated in 0.1 M phosphate buffer with 10 mg/ml DAPI for 15-20 min at 60°C, to facilitate the penetration of the dye (Malerba et al., 2003). Samples were observed in an epifluorescence microscope, using a 380 nm filter set.

Western blot analysis

Four leaf discs (9 mm) were collected with a cork borer and macerated with a pestle in a tube with 200 µl of phosphate-saline buffer (PBS), pH 7.2. Samples (10 µl per well) were loaded on a 12% SDS-acrylamide gel on a electrophoresis apparatus (BioRad) and run at 150 V. The gels were blotted to an Immobilon membrane (Millipore), using semi-dry transfer (BioRad). Membranes were incubated for 1 h in 5% (w/v) non-fat milk, in PBS, and subsequently incubated with an anti-GFP polyclonal antibody (1:3000). An alkaline-phosphatase conjugate anti-rabbit antibody (Sigma) was used as a secondary antiserum (1:4000). The blot was developed by adding CSPD and incubating 5 min at room temperature, 10 min at 37°C, and exposed to X-ray film, for 30 min.

Results

Expression and subcellular localization of CAV-GFP fusion proteins

N. benthamiana plants were infiltrated with *Agrobacterium* cultures containing binary vectors with GFP C terminal fusions to the VP1, VP2 and VP3 genes of CAV, hereafter referred to as GFP:VP(1, 2 or 3). Three days after infiltration the leaves were visualized under UV light using an epifluorescence microscope. GFP:VP1 fusion was observed as a

weak fluorescence signal localized in the nucleus. This fluorescence was detectable from a limited number of epidermal cells. Co-inoculation of GFP:VP1 with a vector containing HcPro, a strong suppressor of RNA silencing (Anandalakshmi et al., 1998; Voinnet et al., 2003), substantially increased the number of cells from which fluorescence could be visualized and also the intensity of the fluorescence. The GFP:VP1 fusion was primarily localized in the nucleoplasm and nucleolus, as shown in Figure 1a. Leaves inoculated with GFP:VP2 and GFP:VP3 examined under the epifluorescence microscope revealed that both protein fusion were exclusively localized to the nucleus. Nuclear localization was confirmed by staining the tissues with DAPI, as shown for GFP:VP3 (Figure 1cd). Western blot analysis confirmed the convincing expression of the GFP:VP2 and GFP:VP3 in infiltrated leaves, whereas the signal from GFP:VP1 was very low (Figure. 2). Detailed cytology using the LSM showed small compact aggregates of GFP:VP3 clustered in the nucleus, while GFP:VP2 had a nucleoplasmic distribution and was excluded from the nucleolus (Figure 1bc). Co-expression of GFP:VP2 or GFP:VP3 with HcPro showed no alteration in the pattern of the fluorescence, but a marked increase in fluorescence intensity, which persisted up to 10 days.

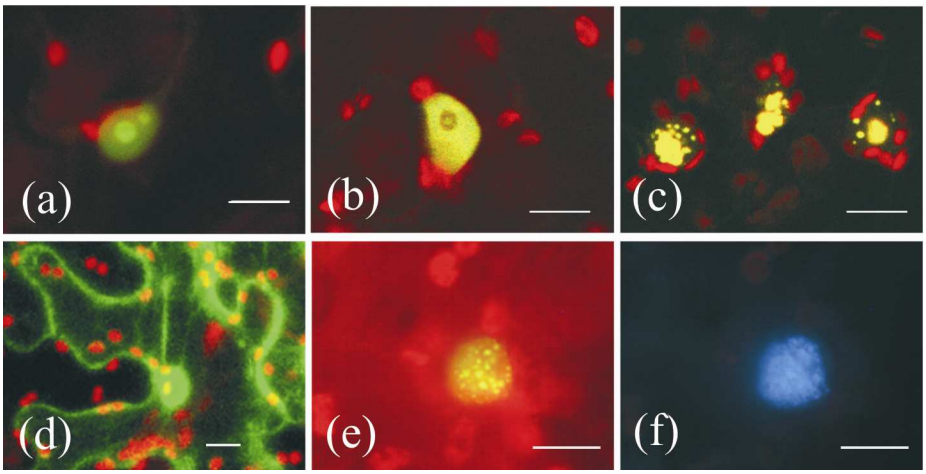

Figure 1: Expression of CAV:GFP fusion proteins in *N. benthamiana* leaf cells. LSM images showing GFP fluorescence in the nucleus of cells expressing (A) GFP:VP1; (B) GFP:VP2; (C) GFP:VP3 or (D) free GFP. Epifluorescence image showing the nucleus of a cell in a leaf expressing GFP:VP3 with a GFP filter (E) and a DAPI filter (F). Bar = 10 µm.

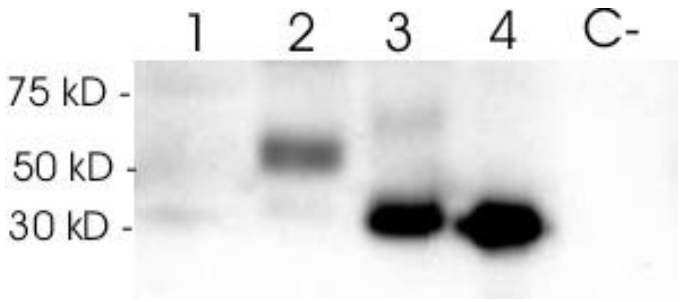

Figure 2: Western blot from *N. benthamiana* leaves infiltrated with *A tumefaciens* strains carrying various CAV:GFP fusion constructs. The blot was incubated with antiserum against GFP. Lane 1: GFP:VP1 (co-infiltrated with HcPro); lane 2: GFP:VP2; lane 3: GFP:VP3; lane 4: free GFP; C-: control: non infiltrated plant.

Co-infiltration of GFP:VP1 and VP2 / VP-3

To tested possible effects of co-expressing CAV proteins, GFP:VP1 was co-infiltrated with binary vectors carrying VP2 or VP3 (devoid of the GFP fusion). A suspension of the *Agrobacterium* strain carrying the HcPro gene was also included (a ratio of 2:1:1 of VP1:VP2/VP3:HcPro). In leaves co-infiltrated with GFP:VP1 and VP2, the fluorescence pattern of GFP:VP1 was clearly altered. From a faint fluorescence evenly distributed in the nucleoplasm and a more intense signal from the nucleolus (Figure 1a), co-expression of GFP:VP1 and VP2 lead to the formation of several aggregates inside the nucleus (Figure 3a), suggesting an interaction between VP1 and VP2. No alteration in the GFP:VP1 fluorescence pattern was seen when co-infiltrated with VP3 (Figure 3b). Triple co-infiltration of GFP:VP1 with VP2 and VP3 followed the pattern of aggregate formation as observed for co-expression of GFP:VP1 and VP2 (Figure 3c). The inverse combinations, VP1 and GFP:VP2 or GFP:VP3, were also tested but did not cause any noticeable alteration on the fluorescence pattern of VP2 or VP3 when compared to the pattern observed for these proteins when expressed alone (results not shown). Also co-infiltration of GFP:VP2 and VP3 or GFP:VP3 and VP2 also did not reveal any noticeable change of GFP fluorescence pattern.

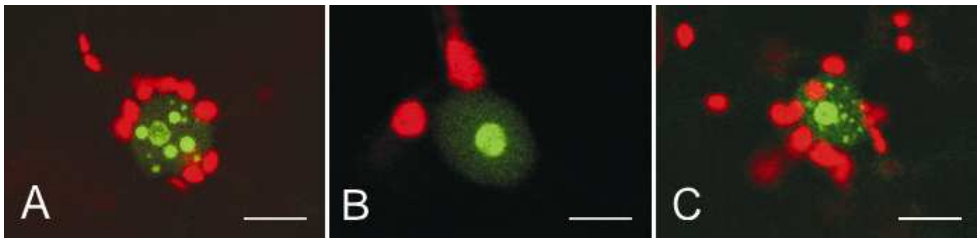

Figure 3: LSM images of *N. benthamiana* leaf cells showing GFP:VP1 fluorescence in the nucleus. Leaves were co-infiltrated with *Agrobacterium* lineages carrying GFP:V1 and VP2 (A), GFP:VP1 and VP3 (B) and GFP:VP1 plus VP2 and VP3 (C). Bar = 10µm.

Expression of CAV proteins from a PVX-based viral vector

CAV genes VP1, VP2 and VP3 were cloned into a PVX-based vector. This PVX replicon contains a GFP gene and the *attR* sites for recombination using the Gateway LR clonase (Chapter 2). *Agrobacterium* containing these PVX-based GFP fusions, hereafter referred to as PVX-GFP:VP(1,2 or 3), were agroinoculated in *N. benthamiana* and GFP fluorescence was monitored. PVX-GFP:VP2 showed identical fluorescence patterns to those observed in leaves infiltrated with the binary vector constructs. For PVX-GFP:VP1, GFP fluorescence was observed in a very limited number of cells, showing both nuclear and cytoplasmic localization (data not shown). Co-inoculation of PVX-GFP:VP1 and VP2 (with an *Agrobacterium* strain containing a binary vector) did not alter this VP1 localization. The differences in expression pattern and efficiency could be due to the large insert size of GFP:VP1 (~ 2 kb) leading to instability of the foreign sequence (Chapman et al., 1992). Expression of VP1 using this PVX-based expression vector was not further explored.

Plant cell death induced by VP3 expression

Plants inoculated with PVX-GFP:VP3 expressed the fusion proteins after 3 days. However, infiltrated areas severely wilted after 4 days and died after 7-10 days. Systemically infected leaves showed small necrotic spots (Figure 4). Similar results were obtained with PVX-VP3 (lacking the GFP fusion partner). Observation of PVX-GFP:VP3-infected tissues under the epifluorescence microscope showed an area of dead tissue surrounded by a ring of cells expressing GFP:VP3, suggesting cell death following VP3 expression as the virus moved from cell-to-cell (Figure 4). DNA extracted from infiltrated area after 3, 6 and 10 d.p.i. was analyzed in agarose gel electrophoreses, but no low molecular weight DNA laddering pattern typical for PCD was observed (data not shown). Expression of VP3 from a TMV-based vector was also tested and also in this case plants showed severe wilting and died after 10 days (Figure 4).

Since no evident sign of cell death was observed in leaves agroinfiltrated with GFP:VP3, or VP3, expressed from binary vectors, the relation between PVX or TMV infection and VP3 expression was further studied. For this purpose, leaves were co-inoculated with a strain containing the PVX vector without a foreign gene insert (pGR107) and a strain carrying the VP3 gene or the GFP:VP3 (binary vectors). In addition, leaves infiltrated with GFP:VP3 or VP3, were mechanically inoculated after 3 days with extracts from PVX or TMV infected plants. In all these inoculations no evident signs of cell death were observed. No difference in the pattern of GFP fluorescence from agroinoculated GFP:VP3 was noticed after co-infiltration with either PVX or TMV.

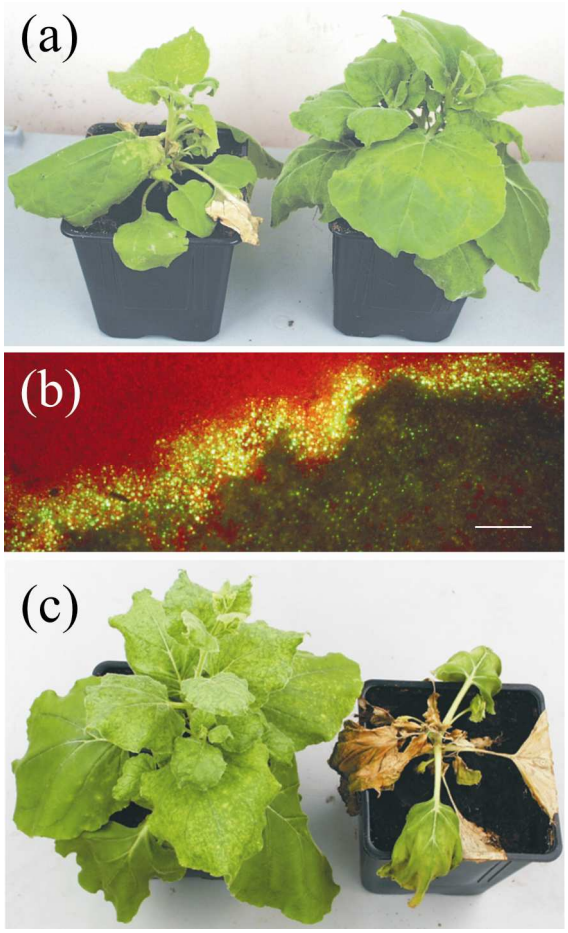

Figure 4: *N. benthamiana* plants systemically infected (10 d.p.i.) with PVX-GFP:VP3 (left) and PVX (right) (a). PVX-GFP:VP3 infected leaf tissue showing a row GFP fluorescing

cells and dead cells (b). *N. benthamiana* plants systemically infected (15 d.p.i.) with TMV:VP3 (left) and wt TMV (right) (c).

Discussion

The expression of CAV proteins in *N. benthamiana* leaves was evaluated. VP2 and VP3 GFP-fusions were readily observed at high levels, but the VP1 fusion was always detected at low levels and in only a few cells. VP1, VP2 and VP3 fused to GFP all showed nuclear localization, which indicates that the known nuclear localization signals of the three CAV proteins are functional in plants. CAV proteins all possess putative nuclear localization signals and are known to be directed to the nucleus in infected chicken cells where virus assembly takes place (Adair, 2000). However, this nuclear localization is not always observed, since VP3 e.g. does not localize in the nucleus of normal human cells (Noteborn, 2004).

VP1 is the only structural protein found in CAV purified particles and is the main choice as candidate antigen for a recombinant vaccine (Cunningham et al., 2001; Koch et al., 1995). Expression of VP1 from *E. coli* was attempted but recombinant proteins were unstable leading to truncated products (Pallister et al., 1994). Successful expression of VP1 was obtained in insect cells inoculated with a recombinant baculovirus. It was also shown that co-expression of VP1 and VP2 was necessary to induce neutralizing antibodies (Koch et al., 1995). The association of VP1 and VP2 was also demonstrated by co-immunoprecipitation assays, leading to the suggestion that VP2 could act as a chaperone, helping VP1 to achieve the correct conformation necessary for stability and effective epitope presentation that would lead to neutralizing antibodies (Noteborn et al., 1998). The need for a correct VP1 conformation or proper particle assembly is also suggested by the fact that monoclonal antibodies poorly recognized denatured VP1 in western blot experiments. VP1 expressed alone in insect cells was also poorly recognized in contrast to co-expressed VP1 and VP2 (Koch et al., 1995; Noteborn et al., 1998).

Besides its interaction with VP1, and its possible role as a chaperone or scaffold protein, VP2 was shown to be a dual protein phosphatase (Peters et al., 2002; 2005). However, as these studies were carried out *in vitro*, the role of VP2 in CAV infection is still not established (Noteborn, 2005). As shown in this chapter, in *N. benthamiana* cells, co-expression with VP2 clearly alters the (GFP:)VP1 distribution within the nucleus, indicating a possible interaction between these proteins. On the other hand, the reverse combination did not cause VP2 to assemble in a pattern similar to VP1, which might suggest that the association is only transient and leads to a different location of VP1 after refolding by VP2. No alteration was observed by co-expressing GFP:VP1 and VP3 or VP2 and VP3, when either one was expressed as GFP fusion. Further research will be needed to test whether co-expression of VP2 would also be required for proper VP1 conformation in plants, suitable to induce neutralizing antibodies.

Co-expression of CAV genes and HcPro had a marked effect on the expression level and no visible alteration on subcellular localization. Co-expression with suppressors of RNA

silencing has been shown to boost expression level of many different genes by protecting mRNA from degradation by RNA silencing pathways, which are induced as a plant response to T-DNA transformation process (Bucher et al., 2003; Voinnet et al., 2003). Despite the increase in expression level of VP1 by co-infiltration with HcPro, the current expression system is as yet insufficiently efficient for production of high amounts of VP1 in plants. Optimizing VP1 expression level and/or protein stability will be necessary to encourage further experiments to evaluate a recombinant plant-produced VP1.

Besides testing expression of VP1 as a potential candidate for a recombinant vaccine against CAV, the effect of VP3 expression was tested for a possible involvement in programmed cell death (PCD) in plants. Programmed cell death is a complex process involved in many developmental and physiological processes. In animal cells, PCD or apoptosis, has been extensively studied in recent years, and is essentially distinct from plant PCD (Hengartner, 2000; van Doorn and Woltering, 2005). Nevertheless, some morphological features related to apoptotic animal cells including chromatin condensation, cell shrinkage and degradation of DNA into nucleosomal fragment sizes resulting in a laddering pattern have also been observed in plants and are generally described as "apoptotic-like" (Huckelhoven, 2004; Ryerson and Heath, 1996; van Doorn and Woltering, 2005). Based on sequence similarity, most genes known to be involved in apoptosis in animals are not present in plants, (Higashi et al., 2005; Huckelhoven, 2004), but some studies have shown that expression of apoptosis related genes in plants, like the Bcl-2 family, causes PCD related effect. Expression of *bcl-2* and other apoptosis antagonists were shown to block HR in plants, thereby interfering with resistance to different fungi, bacterial and viral pathogens (Dickman et al., 2001; Greenberg and Yao, 2004). The expression of Bax, an apoptosis inducing gene of the Bcl-2 family, caused a typical HR reaction in *N. benthamiana* and like in animal apoptotic cells, localized in mitochondria (Huckelhoven, 2004; Lacomme and Santa Cruz, 1999).

The VP3 gene of CAV causes apoptosis in infected chicken cells and in transfected human tumor cells, but not in normal human cells (Danen-Van Oorschot et al., 1997). This fact has called the attention for the potential use of VP3 as a drug in cancer therapy (Noteborn, 2005). Induction of apoptosis by VP3, also called apoptin, is p53 and Bcl-2 independent and involves an activation of caspases (Rohn and Noteborn, 2004). Redirecting VP3 to the nucleus of human cells by a heterologous NLS is not sufficient for the induction of apoptosis, as this may require phosphorylation of VP3 by a tumor specific kinase (Rohn et al., 2002). In the nucleus, VP3 forms aggregates, interacting with the heterochromatin (Leliveld et al., 2004; Leliveld et al., 2003b). These aggregates seem to be a common feature of VP3, and are also observed *in vitro* and when heterologously expressed in *E. coli*. These aggregates were shown to be very stable and to retain the apoptosis induction activity (Leliveld et al., 2003a).

In *N. benthamiana* cells, VP3 is targeted to the nucleus and locally forms large aggregates. When expressed from a binary vector no evidence of cell death was observed. However, when expressed from a viral vector, either TMV or PVX, areas expressing VP3 showed extensive necrosis, eventually leading to plant death. No DNA laddering was observed from these infected tissues. However, this does not exclude PCD induction as a result of

VP3 expression, since DNA laddering is often not observed in plant PCD related processes (Hengartner, 2000; Lacomme and Santa Cruz, 1999; van Doorn and Woltering, 2005).

The different responses obtained from VP3 when expressed from a binary vector and a viral vector could result from the higher expression level using the viral replicon. In that case, PCD could be triggered after passing a threshold level of VP3 accumulation. It is also possible that VP3 interacts with viral components or is processed during PVX or TMV infection and thus turning the molecule active for inducing PCD. In that case, however, this "activation" of VP3 or any related response involving host-pathogen interaction (e.g. HR) would not be achieved by expression of VP3 *in trans*, since no cell death was observed in leaves co-inoculated with strains carrying the PVX (no insert) and a binary vector carrying the VP3, or by expression of VP3 from a binary vector in PVX infected tissue.

Cell death observed in viral vector infected tissue expressing VP3 could result from a necrotic reaction not related to PCD. Necrotic reactions are frequently observed upon viral vectors-based expression of different genes unrelated to PCD (Gleba et al., 2004; Pogue et al., 2002). Furthermore, PCD in plants may not be easy to demonstrate or to distinguish from a necrotic reaction and further biochemical analysis would be necessary (Greenberg and Yao, 2004; van Doorn and Woltering, 2005). Therefore, we consider it premature to assign any involvement of VP3 in PCD in plants as yet. Nevertheless, the relatively high level of expression of VP3 in plant cells, particularly from binary vectors, offers an attractive alternative source for apoptin production. Considering its importance as a potential in anti-cancer therapy, purification of VP3 from plant extracts and biological activity tests are promising enough to warrant further studies.

Overall, our results show that all CAV proteins can be expressed in plant cells. Current expression levels of VP1 may be too low to be exploited as an oral vaccine, but optimizing expression level in plant cells to obtain recombinant VP1 protein capable of inducing neutralizing antibodies, most likely by co-expression with VP2, represents an attractive low cost strategy towards novel, affordable vaccines against CAV.

Acknowledgments

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Chapter 4

High expression of recombinant human erythropoietin in plants using TMV and PVX-based viral vectors

Abstract

EPO is a hormone acting as a growth factor for erythrocytes and has an important therapeutic application for anemia resulting from chemotherapy, AIDS or chronic renal failure. Here we show that a synthetic *epo* gene, with a codon usage optimized for plant expression, can be efficiently produced in plants using Tobacco mosaic virus (TMV) or Potato virus X (PVX) as expression vehicles. The expressed EPO was shown to be primarily present in the apoplast confirming the functionality of its signal sequence in plants. Furthermore, the apparent molecular mass of the EPO protein in western blot analysis suggested the protein to be extensively glycosylated. Expression of recombinant EPO from the TMV – but not the PVX - vector induced a strong necrotic reaction in both the inoculated leaves and systemically infected tissues. This necrotic reaction was also not observed when EPO was retained in the endoplasmatic reticulum due to the addition of a C-terminal KDEL. Overall, the results obtained demonstrate that high levels of glycosylated EPO can be obtained in plants using viral vectors, thus representing a novel alternative for large scale production.

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Introduction

Over the past two decades the production of recombinant proteins in plants has been recognized as an attractive alternative for bacterial, yeast or animal cell-based heterologous expression systems (Hood et al., 2002; Stoger et al., 2005). Low cost and easy scale-up production has attracted the interest of the pharmaceutical industry. An additional bonus is that plant-derived products have a lower risk of being contaminated with animal pathogens, which meets the demand of the growing market for animal-free reagents (Ma et al., 2003).

A number of recombinant proteins have been successfully produced in plants for different purposes. These include proteins derived from pathogens, to be used as antigens in subunit vaccines; an array of antibodies for therapeutic and diagnostic application, either as full length or single chain variable fragments (scFv); proteins of industrial use (e.g. enzymes); and proteins for therapeutic use (Ma et al., 2003; Stoger et al., 2005). In most cases plant-produced recombinant proteins are identical or similar to the original or currently used product. In plant cells post-translational modifications, particularly in glycosylation, may differ compared to animal cells,. This may represent a limitation, although many plant-produced (glyco)proteins have been shown to retain equivalent activity (Brooks, 2004; Gomord et al., 2005; Ma et al., 2003).

Erythropoietin (EPO) is a hormone controlling red blood cell production (erythropoiesis). EPO is produced by the kidney, up-regulated by hypoxia, and acts on the erythroid progenitor cells in the hemopoietic organs (Fisher, 2003; Jelkmann, 1992). The human EPO gene is composed of five exons and four introns, encoding a precursor protein of 193 amino acids (Lai et al., 1986). The N-terminal signal peptide consisting of 27 residues directs EPO to the endoplasmatic reticulum (ER) and through the Golgi secretion pathway during which it is glycosylated. Currently, recombinant human EPO (rhEPO) is produced in mammalian cells and has important therapeutic applications in patients suffering of anemia resulting from chemotherapy, AIDS or chronic renal failure (Fisher, 2003; Jelkmann, 2000). However, due to the limited production capability and high cost production from animal cells, EPO treatment is expensive and its routine use to reduce the frequency of red blood cells transfusions is not cost-effective (Hood et al., 2002; MacLaren and Sullivan, 2005).

In the past, plant-based EPO expression has been achieved in tobacco cell suspension culture and transgenic plants of tobacco and *Arabidopsis* (Cheon et al., 2004; Matsumoto et al., 1995). However, the reported expression levels in cultured tobacco cells were very low (up to 0.0026% total soluble protein). Yet, EPO was show to be correctly processed and targeted to the apoplast. The mature protein proved to be glycosylated and was shown to be active *in vitro* but not *in vivo*, possibly due to reduced half-life of the protein (Matsumoto et al., 1995). Interestingly, the expression of EPO in transgenic tobacco and *Arabidopsis* plants caused male sterility, suggesting a possible side effect of the EPO protein in plants. Transgenic tobacco plants expressing EPO also showed retarded vegetative growth and plants were dwarfed (Cheon et al., 2004).

Plant expression vectors based on replicating viruses have been successfully used for the expression of a number of foreign genes, leading to high levels of protein production

(Awram et al., 2002; Pogue et al., 2002). Another advantage of these replicative expression systems is that the recombinant protein can be harvested a few days after plant inoculation, thereby avoiding the cumbersome and time consuming generation of transgenic plants. However, the size of the foreign gene sequence may represent a limitation, and larger inserts may render the vector unstable (Donson et al., 1991; Pogue et al., 2002). EPO is a relatively small protein and, therefore, constitutes a good candidate for evaluating expression from such viral vectors. This communication reports the expression of EPO in *Nicotiana benthamiana* plants using TMV and PVX-based vectors.

Materials, results and discussion

Synthesis of the Erythropoietin (EPO) coding sequencing and cloning into TMV and PVX-based viral vectors

The coding sequence of human EPO was optimized for plant codon usage and synthesized by overlap extension PCR using a set of 12 overlapping 70-mer primers and verified by sequence analysis. The resulting EPO sequence retained the sequence for the 27 amino acid signal peptide. Restriction sites for *Pac*I and *Sun*I flanking the *epo* sequence were used for cloning into an adapted pBinTMV-30B vector (Shivprasad et al., 1999). This binary vector, which was inoculated to plants using *Agrobacterium tumefaciens* consists of a cDNA of a hybrid TMV with the coat protein of TMGMV, under transcriptional control of a CaMV 35S promoter and a nos terminator. A hammerhead-type ribozyme was included to assure proper cleavage of the TMV transcript (Shivprasad et al., 1999) (Figure 1a). The resulting construct, referred to as TMV-EPO, was transformed into *Agrobacterium tumefaciens* strain GV3101. The EPO gene was also cloned into *Nco*I and *Not*I sites of pEntr11 vector (Invitrogen) and the resulting construct, pEntr-EPO, recombined with the Gateway-compatible expression vector PVX-GW (Chapter 3) (Figure 1c) using the Gateway LR clonase mix (Invitrogen) essentially as recommended by the manufacturer, except that the final reaction volume was scaled-down to 5 µl. The PVX-EPO expression vector was transformed into *Agrobacterium* strain GV3101 strain containing the pSoup helper plasmid (Hellens et al., 2000). DNA restriction and cloning was done essentially as described (Sambrook et al., 1989). The pEntr-EPO construct was also recombined into the bacterial expression vector pDest-17 (Invitrogen), thus providing the expressed EPO with an N-terminal hexa-histidine tag, allowing purification using Talon columns (BD Biosciences, USA).

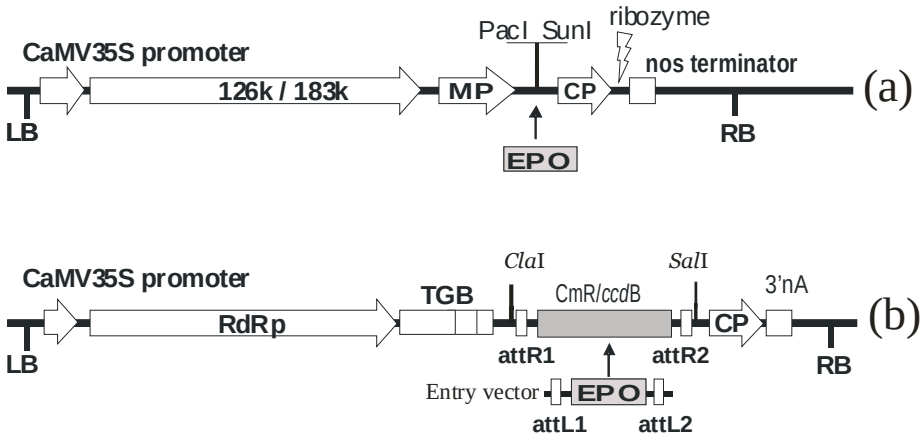

Figure 1: Schematic diagram of the TMV and PVX-based vector in binary vectors for agroinoculation. (a) TMV-based vector indicating the open reading frames (126/183; MP-movement protein; CP – coat protein), the EPO gene was clone into PacI and SunI sites. (b) PVX vector indicating the open reading frames (RdRp - RNA dependent RNA polymerase; TGB – triple gene box; CP – coat protein). The attR1 and attR2, flanking the chloramphenicol resistance gene (CmR) and *ccd*B genes, are Gateway sites for recombination with the attL1 and attL2 flanking the EPO gene on an entry vector.

Agroinoculation of N. benthamiana *plants*

Nicotiana benthamiana plants (4 leaf-stage, 4-5 week-old) were used for providing the viral vectors through agroinoculation. Young, fully expanded leaves were infiltrated with *Agrobacterium* suspension at the abaxial surface using a 5 ml syringe without needle. Bacterial suspensions were obtained from an overnight culture (600 µl) centrifuged and resuspended in 3 ml induction medium [10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.0 g/l of $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.2% (w/v) glucose, 0.5% (v/v) glycerol, 50 µM acetosyringone, and 10 mM morpholineethanesulfonic acid (MES), pH 5.6]. After overnight incubation at 28°C, cultures were centrifuged and resuspended in 5 ml MS medium (Murashige and Skoog, 1962) containing 10 mM MES and 150 µM acetosyringone and the OD_{600} adjusted to 0.5. For each construct tested, 3-4 plants were inoculated and each experiment was repeated at least 3 times. After infiltration, plants were maintained in a growth chamber at 25°C and 12 h photoperiod.

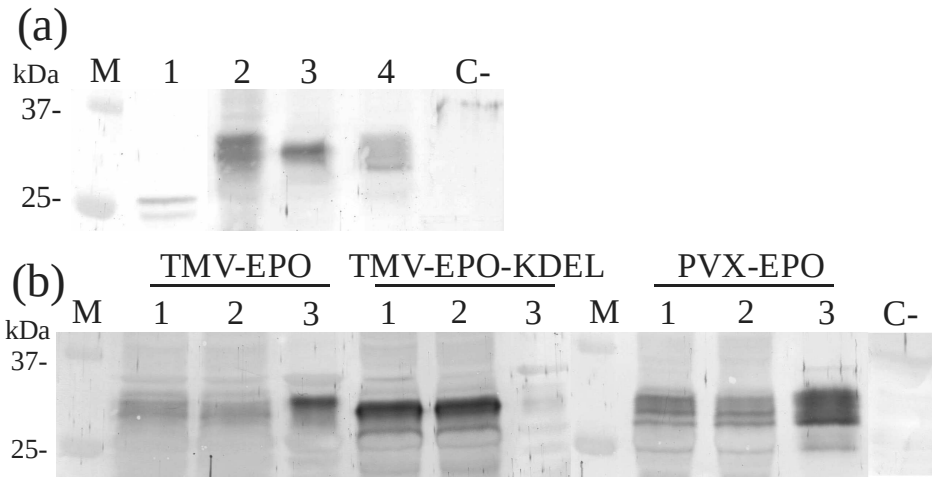

Figure 2: Western blot analysis of leaf extracts of *N. benthamiana* plants inoculated with different EPO-expressing viral constructs. Panel (a): (1) *E. coli* expressed EPO; (2 - 4) extracts from plants infected with TMV-EPO (2); TMV-EPO-KDEL (3) or PVX-EPO (4). Panel (b): extracts from plants inoculate with TMV-EPO, TMV-EPO-KDEL and PVX-EPO. Panels 1, 2 and 3 correspond to total leaf extracts (1), extracts from the symplast (2) and apoplast (3), respectively. M: molecular size markers; C-: non-inoculated plant.

Western blot analysis of TMV-EPO and PVX-EPO infected leaves

EPO expression in inoculated leaves was analyzed by Western blot 6 days post infection (d.p.i.) and in systemically infected leaves 10-15 d.p.i. Two leaf discs (approximately 50 mg fresh weight) were macerated with a plastic pestle in a tube with 130 µl of phosphate-saline buffer (PBS). Protein concentration was determined using the Biorad kit (Biorad, USA), based on the Bradford assay. Samples (15 µl per well) were separated by electrophoresis in a 12% polyacrylamide gel by electrophoresis at 150 V. The gels were blotted to an Immobilon membrane (Millipore), using semi-dry transfer (Biorad). Membranes were incubated for 1 h in 5% (w/v) non-fat milk in PBS, and subsequently incubated with an anti-EPO polyclonal antibody (1:500; Sigma). An alkaline-phosphatase conjugated anti-rabbit antibody (Sigma) was used as a secondary antiserum (1:2000). The blot was developed by adding BCIB/NBT and incubated for 20-30 min at room temperature.

Western blot analysis revealed the presence of EPO in samples from plants infected with TMV-EPO and PVX-EPO (Figure 2). In both cases the size of the expressed EPO was

approximately 32 kDa. EPO purified from *E. coli* showed a size of approximately 24 kDa, indicating that plant produced EPO is being glycosylated (Figure 2a). The size of EPO from cultured tobacco cells or tobacco and *Arabidopsis* transgenic plants was also approximately 32 kDa (Cheon et al., 2004; Matsumoto et al., 1995). EPO isolated from human urine and recombinant human (rH)-EPO expressed in mammalian cells, show an apparent size of approximately 34 kDa, accounted for the carbohydrate portion of the protein, which represents 40 % of the total mass of the mature protein (Lai et al., 1986).

Based on the intensity of the bands on Western blot the amount of EPO present in plants infected with TMV-EPO was estimated to be around 0.5% of total soluble protein, using a dilutions series of bacterial-produced EPO as standard (data not shown). Hence, demonstrating a 200 times improved expression compared to what was previously reported for cultured transgenic tobacco plants and protoplast expression studies (Matsumoto et al., 1995). This increase in expression is likely due to two alterations, the adaptation of the amino acid codon usage to plant codons and the use of a viral based expression system.

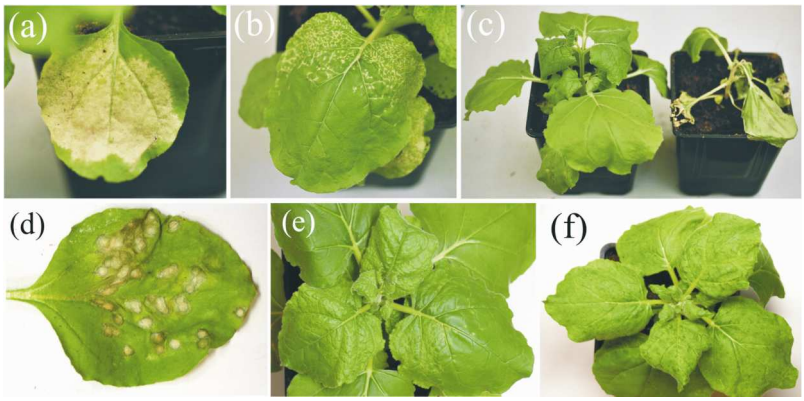

Figure 3: Phenotypic effect of EPO expression on infected *N. benthamiana* plants using TMV or PVX as viral expression vector. (a) Leaf inoculated with TMV-EPO showing necrosis of the infiltrated areas, 6 d.p.i.; (b) Leaf systemically infected by TMV-EPO, 10 d.p.i. and showing necrosis of the veins; (c) Plants infected with TMV wildtype (left) and TMV-EPO (right), 20 d.p.i.; (d) Leaf mechanically inoculated with extracts from TMV-EPO infected plant, 6 d.p.i.; (e) Plant systemic infected with TMV-EPO-KDEL; (f) Plant systemic infected with PVX-EPO.

EPO expression in N. benthamiana *plants induces necrosis*

Leaves agroinoculated with TMV-EPO showed necrotic areas 4-5 d.p.i. (Figure 3a). In the systemically infected leaves, necrosis was observed mainly at the veins and plants wilted and died 15-20 d.p.i. (Figure 3bc). Extracts from leaves showing necrotic areas were used for a next passage onto plants, to confirm the necrosis to be caused by the viral construct. Again necrotic lesions were evident in inoculated leaves (Figure 3d) while these were not observed in plants inoculated with TMV-GFP (data not shown). To further investigate the necrotic effect of EPO expression, a set of 5 plants was inoculated with TMV-EPO and maintained at 18°C and 12h photoperiod. After 10-15 days plants showed mild TMV symptoms but no necrosis was observed. Western blot analysis of these plants revealed a lower amount of EPO again suggesting a correlation between expression level and the necrotic effect caused by EPO in *N. benthamiana* plants (Figure 2b). Consistent with this result, the lower expression levels obtained with PVX-EPO (Figure 2a) also never coincided with necrosis (Figure 3f).

The underlying mechanism by which EPO causes necrosis in *Nicotiana benthamiana* plants infected with TMV-EPO is unclear. In both transgenic tobacco and *Arabidopsis* plants *epo* expression resulted in deformations of the flowers and male sterility (Cheon et al., 2004). Transgenic tobacco plants were stunted, with reduced internodes resulting in a dwarfed phenotype (Cheon et al., 2004). These pleiotropic effects of *epo* expression were suggested to be associated with alterations in sugar signaling but no experimental data was presented to support this notion (Cheon et al., 2004).

EPO accumulates in the apoplast of infected N. benthamiana *plants*

The *epo* gene construct utilized contained the coding sequence for the 27 amino acids N-terminal signal peptide for ER localization and upon translational expression this sequence has been shown to be correctly cleaved in tobacco cell suspension cultures (Matsumoto et al., 1995). To investigate whether the produced EPO is excreted to the apoplast, leaf samples of plants systemically infected with TMV-EPO or PVX-EPO were immersed in distillated water and vacuum-infiltrated (~500 mBar) for 5 minutes. Infiltrated leaf tissue was subsequently tissue-blotted, placed in a 10 ml syringe and centrifuged in a centrifuge tube (15 min, 3500 rpm), yielding 200-300 µl of protein sample from the apoplast. The remaining leaf tissue ("symplast sample") was macerated in PBS and strained through Miracloth. Western blot analysis of symplast and apoplast samples from leaves inoculated with TMV-EPO and PVX-EPO showed that EPO is present mainly in the intercellular fluid and in only smaller amounts in cellular extracts (Figure 2b). The apoplast localization of EPO is in accordance with results obtained from tobacco protoplast cultures expressing EPO (Matsumoto et al., 1995). In cultured cells, however, EPO was not secreted to the medium, suggesting that in these experiments the protein was retained at the cell wall (Matsumoto et al., 1995)

Effect of the KDEL retention signal on EPO accumulation

Subcellular localization may greatly influence the accumulation levels of recombinant proteins in plant cells (Conrad and Fiedler, 1998). The addition of a C-terminal KDEL sequence for ER retention has been shown to potentially (further) increase expressed protein levels, as a result of the oxidizing environment of the ER (Conrad and Fiedler, 1998; Schouten et al., 1997). To evaluate the effect of subcellular localization on EPO accumulation, the *epo* gene was PCR amplified to add the coding sequence of a C-terminal KDEL sequence. The resulting construct, TMV-EPO-KDEL, was agroinoculated in *N. benthamiana* and its expression evaluated after 6 days.

Analysis of symplast and apoplast samples from these plants confirmed that EPO-KDEL was not secreted and thus, as the functional signal sequence remained, most likely retained in the ER (Figure 2b). Interestingly, in contrast to plants expressing the secreted EPO, plants inoculated with TMV-EPO-KDEL did not show any necrosis in the infiltrated or systemically infected leaves, (Figure 3e). As shown by Western blot analysis, EPO with the c-terminal KDEL addition accumulated to a lower level than secreted EPO (Figure 2a), which might add to the suggestion of a threshold expression level for EPO to induce necrosis. Interestingly, EPO-KDEL had a smaller size than the secreted EPO (Figure 2b), though still appeared larger than the bacterial EPO, indicating, as might be expected, a partial glycosylation. EPO has three potential sites for *N*-linked glycosylation and a single site for an *O*-linked glycan (Lai et al., 1986). *N*-linked glycosylation occurs in the ER, by the addition of mannose residues, in the Golgi apparatus where these residues are further processed. *O*-linked glycans are added only in the Golgi apparatus (Brooks, 2004; Gomord and Faye, 2004). Plants can synthesize complex glycans structures, but these display some different features when compare to human glycans (Brooks, 2004; Ma et al., 2003). These differences include addition of β(1,2)xylose and α(1,3)fucose residues in plants, while terminal sialic acid and galactose are lacking (Brooks, 2004; Chen et al., 2005a; Gomord and Faye, 2004; Ma et al., 2005a). For many recombinant proteins produced in plants these differences did not represent a limitation, as their biological activity appears to be equivalent to the animal-produced molecules (Ma et al., 2003). However, for some therapeutic proteins, including EPO, sialylated N-glycans are important determinants for protein stability and *in vivo* activity, as the non-sialylated protein is cleared from the blood stream faster (Ma et al., 2003; Takeuchi and Kobata, 1991). It has also been demonstrated that hyperglycosylated EPO has an increased serum half-live and *in vivo* biological activity (Egrie and Browne, 2001). Also recombinant EPO production in mammalian cells is affected by variations in posttranslational processing, and biologically active EPO accounts for only 20-25 % of the total produced protein (Brooks, 2004; Grabenhorst et al., 1999). This limited output and elevated cost of production from animal cells are main challenges for the industry and has created a demand for alternatives sources of production (Hood et al., 2002; Joshi and Lopez, 2005). Like other non-mammalian cells, differences in glycosylation profiles may impose limits for plant-produced protein. Due to different glycosylation, it may be expected that also in this recombinant expression system, *in vivo* activity will be lower than that of purified human EPO. Further improvement of the

biological activity of plant-based production of human recombinant EPO may be obtained by expressing these constructs in plants with altered '*humanized*' glycosylation pathways or *in vitro* application of post-translational modifications by enzymatic addition of sialic acid residues or synthetic polymers (Bakker et al., 2001; Gomord et al., 2005).

Beside its well characterized erythropoietic activity, EPO has recently been implicated in a range of neural protective effects (Erbayraktar et al., 2003; Leist et al., 2004). Indeed, chemically desialylated erythropoietin (asialoEPO) was shown be tissue-protective without showing potential complications associated with thrombosis as a consequence of high levels rhEPO administration. Thus, EPO-related molecules with alternative glycosylation, such as produced in plants, could be exploited to confer neural tissue-protection and be used in this alternative fashion.

We have shown here that EPO can be produced in plants accumulating at levels 200 times higher than previous results by optimizing amino acid codon usage and viral vectors, thereby making large scale production of this protein feasible. Future experiments involving *in vivo* testing of plant-produced EPO activity will reveal its potential in erythropoiesis or neural tissue protection.

Acknowledgments

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Chapter 5

Genetic stability and spatial distribution of Tobacco mosaic virus-based vectors in *Nicotiana benthamiana* plants

Abstract

Plant virus-based expression systems have great potential for heterologous protein production. In many cases, however, expression is hampered by genetic instability of the inserted sequence. To identify putative deletion mutants of a Tobacco mosaic virus (TMV)-based vector and to study their spatial distribution in infected *Nicotiana benthamiana* plants we used a TMVmGFP construct allowing a simple monitoring assay using GFP fluorescence and lugol staining. In systemically infected leaves GFP-deficient mutants were observed only in leaves at a later stage of infection, showing a mottled pattern of GFP fluorescence. Extracts from these leaves revealed to contain a high proportion of non-(GFP) expressor mutants. Functional (GFP-expressing) TMVmGFP virus and derivative non-expressor mutants were shown to be strictly spatially separated, which was confirmed by passage inoculation and RT-PCR. This spatial distribution was also observed by co-inoculation of TMV-constructs carrying distinct GFP genes with different fluorescence properties. Deletion mutant were shown to be capable of systemically infecting plants significantly faster than the original TMVmGFP virus, thereby rapidly out-competing the latter in co-inoculation experiments. Implications of the observed insert stability and the mutually spatial exclusion of functional TMV-GFP and its dysfunctional mutants for the use of viral vectors in plants are discussed.

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Lacorte C, Lohuis D, Goldbach, R and Prins M. Genetic stability and spatial distribution of Tobacco mosaic virus-based vectors in *Nicotiana benthamiana* plants

Introduction

Plant viral vectors represent a promising strategy for foreign gene expression in plants. Compared to transgenic expression of foreign proteins these transient expression systems take advantage of the high replication rate of plant viruses allowing high production rates of heterologous protein in relatively short time intervals, usually days or weeks (reviewed by Pogue et al., 2002; Scholthof et al., 2002). A number of proteins of pharmaceutical and industrial application have thus been successfully produced (Pogue et al., 2002). Viral vectors have also been used to study gene function, either through loss of function, by virus induced gene silencing (VIGS), or by overexpression of the target gene (Burch-Smith et al., 2004; Voinnet, 2005).

Some commonly used viral vectors, such as Tobacco mosaic virus (TMV) (Donson et al., 1991) and Potato virus X (PVX) (Chapman et al., 1992), were modified from cloned cDNA sequences by adding an extra subgenomic promoter as well as restriction sites at which a foreign gene sequence can be cloned (Scholthof et al., 2002). Early versions of the TMV vector containing a duplication of the subgenomic promoter of its coat protein (CP) cistron were shown to be unstable (Donson et al., 1991), but a chimeric virus containing the duplicated subgenomic promoter and coat protein gene from a heterologous tobamoviruses, e.g. Odontoglossum ring spot virus (ORSV) or Tomato mild green mosaic virus (TMGMV) led to TMV-based vectors that could infect the plant systemically, producing high amounts of foreign protein (Donson et al., 1991; Shivprasad et al., 1999) (Chapter 2, Chapter 4). Further improvements of TMV-based vectors were approached by *in vitro* selection of DNA shuffling of their movement protein gene or by optimizing vector inoculation by removing cryptic intron border sequences or by modifying the host to supply some viral function *in trans* (Mallory et al., 2002; Marillonnet et al., 2004; Toth et al., 2002).

Despite progress on vector development, genetic instability of the inserted sequence can be a limitation for routine use of plant viral vectors. In a large population of viruses deletion mutants may arise as a result of non-homologous recombination and may constitute a subpopulation within the infected plant. Analysis of putative mutant isolates from passage-inoculated plants showed that, in general, these mutants had large deletions in the foreign gene insert sequences (Rabindran and Dawson, 2001). This genetic instability is directly related to the size of the insert, although other factors such as the sequence of the insert and aspects of viral host interaction are also involved and so far poorly understood (Pogue et al., 2002; Schneider and Roossinck, 2001; Toth et al., 2002; Zhong et al., 2005). Besides the molecular aspects leading to the generation of deletion mutants, viral vectors are also subject to complex population dynamics in infected plants. Studies on aspects such as genetic structure and spatial distribution of viral populations in infected plants are scant (Garcia-Arenal et al., 2001), particularly on the context of a viral vector designed for foreign protein production.

In this study, a TMV-based vector carrying the green fluorescent protein (GFP) gene as a visual marker was used to evaluate the genesis and next spatial distribution of GFP mutants in infected *Nicotiana benthamiana* plants.

Materials and methods

TMV vectors and GFP cloning

The mGFP5 and GFP S65T (Haseloff et al., 1997; Prasher, 1995) genes were amplified by PCR using specific primers containing a *Pac*I and a *Sun*I sites as 5' extensions of the forward and reverse primers, respectively. GFP amplified fragments were cloned into the pGEM-T Easy vector (Promega). *Pac*I/*Sun*I digested fragments were purified from agarose gel and ligated to pRB2, containing the TMV-based vector, 30B (Figure 1) (Shivprasad et al., 1999). Ligation reactions were transformed in *Escherichia coli* DH5α cells, which were plated on LB agar medium containing ampicillin (50 mg/l). Selected colonies were grown in LB medium for plasmid DNA isolation. The presence of the GFP insert was confirmed by restriction analysis and sequencing. These clones are referred to as TMVmGFP and $TMVGFP_{S65T}$.

In vitro *transcripts and virus inoculation*

Clones TMVmGFP and $TMVGFP_{S65T}$ were linearized by digestion with *Ehe*I and *in vitro* transcripts were obtained using the T7 mMESSAGE mMACHINE^{TM} kit (Ambion, Austin, USA) following the manufacturer's instructions. RNA concentration and quality were measured in a spectrophotometer and checked by agarose gel electrophoresis.
Leaves from young *Nicotiana benthamiana* plants (4-6 leaves stage, 4-5-week old) were mechanically inoculated with 10-15 µg of viral RNA transcripts resulting in GFP fluorescing foci (2-3 mm) after 3-4 days. Three individual foci were excised under UV light and used as TMVmGFP founder inoculum. Plant extracts were prepared by grinding leaf tissues in a mortar in 9 volumes (w/v) of 0.02 M sodium phosphate buffer, pH 7.0. Extracts were strained through miracloth and stored at -20°C. Plants were mechanically inoculated with diluted extracts (10^{-4}, unless otherwise stated) by dusting with carborundum and gently rubbing the leaves with a glass spatula. After inoculation, plants were rinsed with tap water and incubated in a growth chamber at 25±2°C and a 12 h photoperiod. A total of 6 to 9 plants were used for inoculation, in three independent experiments.

RT-PCR conditions

Total RNA was prepared using Trizol® (Invitrogen). Reverse transcription was done as described by Hall *et al.* (2001). A primer specific to the TMV movement protein (5'-GCTACTGTCGCCGAATCGG-3') was used to initiate cDNA synthesis. This primer was also used for PCR, together with a coat protein specific one (5'-GCGATCCAAGACACAACCC-3'). PCR products were analyzed on agarose gel electrophoresis, cloned into the pGEM-T easy vector (Promega), and sequenced.

Figure 1: Schematic representation (not to scale) of TMV hybrid vector 30B carrying the GFP gene (TMVmGFP). The position of the primers used for RT-PCR are indicated. MP-movement protein; CP - TMGMV coat protein; grey box - TMV 3'UTR.

Detection of GFP mutants in TMVmGFP viral populations

The frequency of non expressing GFP mutants (generated by either point mutations or deletion) in a sample from a TMVmGFP infected plant was evaluated by mechanical inoculation of test plants (*N. benthamiana*). Inoculated leaves from these plants were harvested 4 days post inoculation (d.p.i.) and photographed under UV light using a geldoc apparatus (BioRad). Subsequently, leaves were bleached in 96% ethanol for 30 min at 80°C, transferred to a Petri dish, rinsed with lactic acid and incubated in a lugol solution (10% potassium iodine in lactic acid) for 10 minutes (Lindner et al., 1959). After rinsing in tap water, leaves were transferred to a plastic bag and the water carefully drained. Images of the stained leaves were obtained using a flatbed scanner (HP PSC1219). The frequency of GFP mutants in a given sample, therefore, was estimated by comparing the number of GFP fluorescing foci and the total number of foci stained by lugol, on inoculated leaves of test plants.

GFP detection and photography

GFP fluorescence was monitored using a hand-held long wave UV lamp (365 nm). Photographs of GFP fluorescence from infected plants were produced under UV light (Philips HPW, 125W) with a Nikon camera using a Kodak 400 ISO film and an orange filter (040; BW Filters). Close-up and microscope digital images were obtained with a CoolSNAP™ camera adapted to a stereomicroscope (M3Z; Leica) with a GFP-plus filter set (excitation 480 nm, barrier filter 510 nm) or to a microscope (Laborlux S, Leica) using either a GFP (excitation 490 nm, barrier filter 515 nm) or a DAPI filter set (excitation 385 nm, barrier filter 430 nm).

Results

Identification of TMVmGFP mutants in inoculated and systemically infected leaves

The genetic structure of a TMV-based viral vector population in infected *N. benthamiana* plants was evaluated using GFP as inserted marker. In this viral vector the TMV coat protein was replaced by the TMGMV coat protein resulting in a hybrid vector (30B) which has enhanced genetic stability and increased expression of the foreign insert gene (Figure 1) (Shivprasad et al., 1999).

A simple method based on GFP phenotype was used to identify GFP mutants. Leaf extracts to be evaluated were mechanically inoculated on *N. benthamiana* test plants. Inoculated leaves of these plants showed detectable fluorescent foci after 3-4 days and were collected, photographed under UV light and subsequently stained with lugol. The lugol staining method for identification of inoculation foci is based on alterations of starch location in virus infected cells (Lindner et al., 1959). By comparing the patterns of GFP expressing foci and that of lugol stained foci it is possible to determine the ratio of foci expressing GFP and those corresponding to GFP mutants (i.e. non fluorescing foci) (Figure 2).

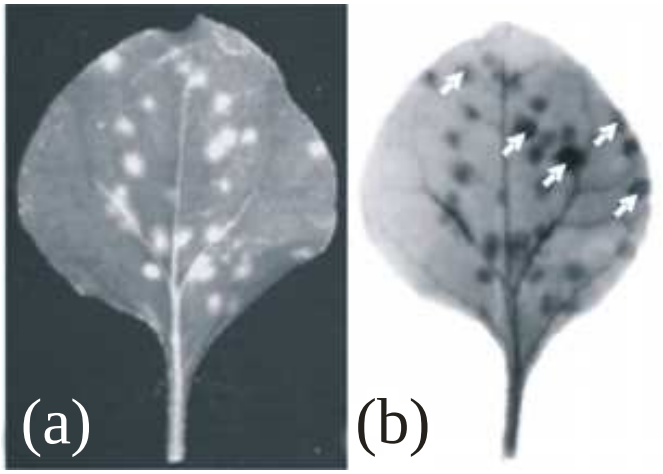

Figure 2: Identification of putative GFP deletion mutants in test plants passage-inoculated with a systemic leaf sample from a transcript inoculated plant (20 d.p.i.). (a) Passage-inoculated leaf 4 d.p.i. under long UV light, showing GFP expressing foci. (b) Same leaf after lugol staining. TMV infection foci that do not show GFP fluorescence are indicated by arrows.

In vitro transcripts were inoculated and after 4 days GFP fluorescing foci were excised, macerated and used as founder inoculum. Leaves inoculated with this founder inoculum were collected at 5 and 20 d.p.i. and tested for the presence of non-expressor mutants, by inoculating test plants. The frequency of non-expressor mutants increased from zero to 20%, for leaves collected after 5 and 20 days, respectively (Figure 4a). These inoculated test plants were also evaluated for systemic GFP fluorescence, after 10-15 days. All plants inoculated with extracts obtained from leaves at 5 d.p.i. (a total of 8 plants) showed systemic GFP fluorescence, whereas test plants inoculated with extracts from leaves 20 d.p.i. (a total of 8 plants) showed no GFP fluorescence in the systemic leaves, despite typical TMV symptoms.

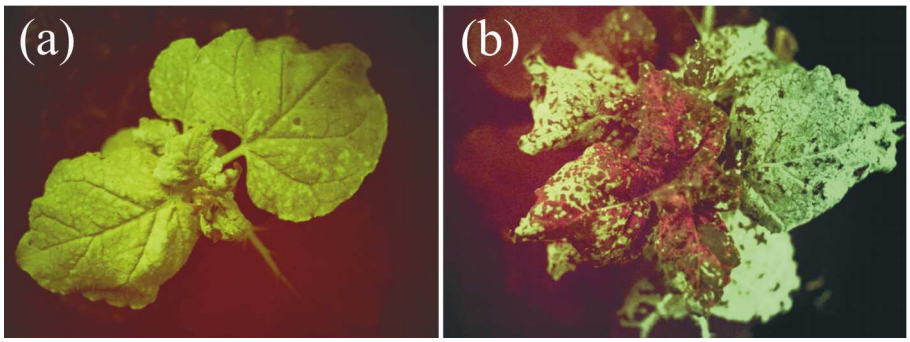

Figure 3: GFP fluorescence from *N. benthamiana* plants systemically infected with TMVmGFP. (a) Plants showing GFP fluorescence in primarily infected leaves early in infection. (b) The same plant in a later stage of infection showing a mosaic of GFP fluorescing and non-fluorescing tissues.

Plants inoculated with a 5 d.p.i. extract, and revealing systemic infection fully coinciding with GFP fluorescence, were also used to evaluate for the presence of non-expressor mutants. Samples of GFP expressing areas (10 mm leaf discs) were collected from infected emerging leaves (i.e. 5^{th} and 6^{th} leaves, Figure 3a) at 10 day intervals (10, 20 and 30 d.p.i.). Surprisingly, no GFP mutants were found in these extracts, after inoculating test plants and assaying leaves for GFP and lugol stained foci (using three test plants for each time point, in three independent experiments). All test plants became systemically infected and showed intense GFP fluorescence at 10 d.p.i. Samples collected from the top leaves (i.e. 8^{th} to 12^{th} leaves) of older plants (20-30 d.p.i.), however, showed a large proportion of GFP mutants. Typically, these leaves showed a mosaic or mottled pattern of GFP fluorescence (Figure 3b) and testing extracts of these leaves resulted in a large proportion (40-90 %) of non-expressor foci (Figure 4a). Further testing of such leaves was done by carefully collecting samples (~2 mm diameter) under UV light from GFP expressing and non-expressing areas,

by puncturing the leaves with a cut pipette tip. Extracts from these samples were inoculated on test plants and examined after 3-4 days. Plants inoculated with samples from GFP fluorescing areas showed no defective mutants when tested with the GFP/lugol assay, whereas non GFP expressing foci were seen in test plants inoculated with samples of non-fluorescent areas, though these plants developed strong symptoms, confirming that the non-fluorescent GFP areas did contain virus. To further characterize these putative non-expressor mutants, samples were collected from systemically infected plants and RNA was extracted for RT-PCR. Agarose gel electrophoresis of PCR amplified products from separate samples from 6 different leaves revealed one single band of a smaller size relative to the control sample from GFP expressing plant (Figure 5).

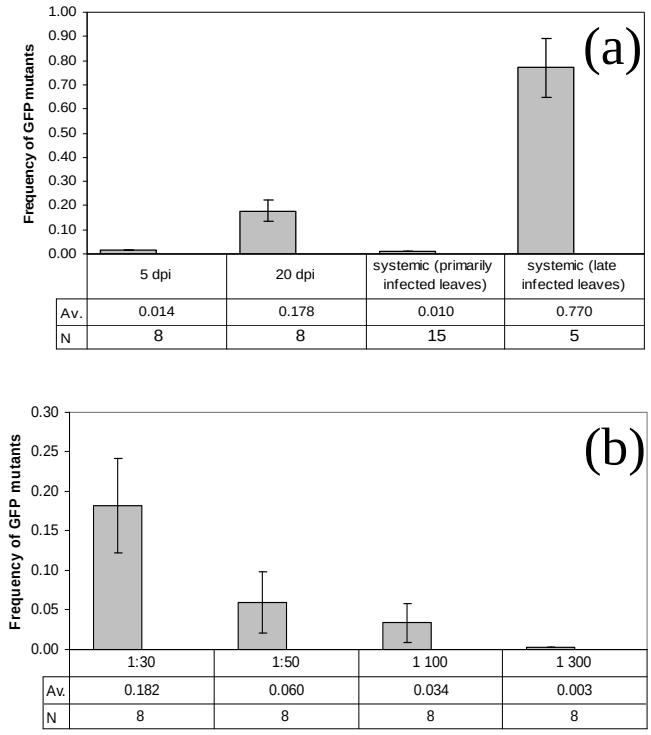

Figure 4: Frequency of GFP non-expressor mutants in infected *N. benthamiana* plants, estimated by passage inoculation of test plants. (a) Frequency of non-expressor mutants in extracts from inoculated leaves (5 and 20 d.p.i.) and from systemic infected leaves; primarily infected leaves and leaves from later stages of systemic infection, showing a mottled pattern of GFP fluorescence. (b) Co-inoculation of TMVΔgfp and TMVmGFP. The ratios (1:30 to 1:300) correspond to dilutions of extracts containing TMVΔGFP (10^{-5} to 10^{-6}) while keeping the same dilution for TMVmGFP ($3x10^{-4}$).

Relative fitness of TMVmGFP and a derivative deletion mutant

A GFP deficient mutant was randomly selected from a passage-inoculated plant that lacked GFP fluorescence. Sequence analysis of this mutant revealed a large deletion (~70%) of the C terminal region of the GFP gene. This mutant, referred to as TMVΔgfp, was used to obtain information about the competitiveness of such mutants compared to the original lineage (TMVmGFP).

Table 1: Frequency of TMV infection in plants after periodically removing the inoculated leaf.

Lineage[1]	Removal of the inoculated leaf (hours after inoculation)					
	46	49	52	57	64	68
TMVmGFP	0% (0/6)	0% (0/6)	0 % (0/6)	50% (3/6)	50% (3/6)	100%
TMVΔgfp	0% (0/6)	50 % (3/6)	50% (3/6)	100% (6/6)	100% (6/6)	n.t.[2]

[1] TMV lineages were inoculated in one leaf per plant, in two independent experiments

[2] n.t. not tested

To determine the time required for establishing a systemic infection, TMVΔgfp and TMVmGFP were separately inoculated on a set of plants and the inoculated leaf was detached after 46 to 68 hours, in 3-4 hours intervals (Table 1) showed that TMVΔgfp is capable of systemically infecting plants within 49-52 hours post infection, whereas TMVmGFP established a systemic infection not earlier than 57-64 hours post infection. In addition, deletion mutants appear to move faster from cell-cell, as suggested by the larger size of the corresponding lugol stained foci in passage inoculated test plants (Figure 2). Plants infected with TMVmGFP also showed milder symptoms as compared to TMVΔgfp infected plants.

Next, to evaluate their direct competition, plants were co-inoculated with TMVΔgfp and TMVmGFP, using inocula mixed in different ratios ranging from 1:1 to 1:3000 (TMVΔgfp:TMVmGFP), from initial concentrations of 10 µg virus per ml for each of the plant extracts. Plants inoculated with TMVΔgfp:TMVmGFP ratios of up to 1:30 did not show any systemic GFP fluorescence, despite clear TMV symptoms. Only when inoculum ratios of TMVΔgfp:TMVmGFP between 1:50 to 1:3000 were applied, all inoculated plants showed systemic GFP fluorescence (Figure 4b).

Co-inoculated TMV based vectors show a distinct spatial distribution

To further evaluate the observed spatial distribution of TMV strains in mixed infections, two TMV vector constructs containing different versions of GFP, mGFP and GFP_{S65T}, were co-inoculated on *N. benthamiana* leaves. TMVmGFP and $TMVGFP_{S65T}$ differ only in a single nucleotide, resulting in a substitution of amino acid Serine 65 with a Threonine (S65T). This replacement in the chromophore eliminates the long UV excitation peak (395 nm) and increases the extinction coefficient to the 475-490 nm region (Prasher, 1995). As a result, the GFP_{S65T} is excitable with blue light, but not with long UV light, whereas mGFP is excited both by long UV and blue light. This difference allows a simple screening of mixed infections. Co-inoculation of TMV vector containing different GFP versions resulted in systemically infected plants primarily showing leaves containing only one kind of GFP and, less frequently, leaves showing a patchy distribution of both GFP types (Figure 6abc).

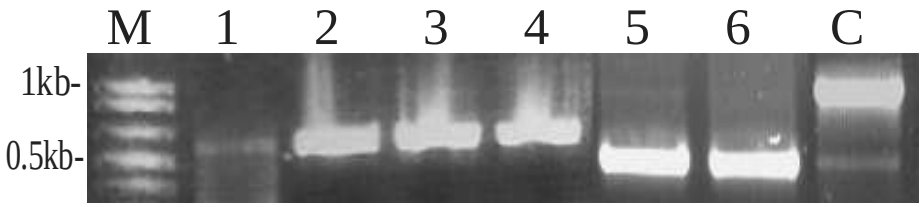

Figure 5: Reverse transcription PCR using TMV specific primers flanking the inserted foreign GFP gene (see Figure 1 for their genomic positions). (1-6) Amplification reaction of RNA extracted from different infected plants not presenting any GFP fluorescence. C: reaction from RNA extracted from a GFP fluorescing leaf.

Analysis of samples from such patchy areas using epifluorescence microscopy revealed distinct GFP fluorescence from cells from different areas that may be adjacent but do not overlap (Figure 6c, d, and e). To confirm this spatial separation of the two types of TMV-GFP viruses, samples from selected areas were carefully collected under the binocular and extracts from these tissues inoculated on a new set of plants. After 3-5 days, inoculated leaves were checked for their type of GFP fluorescence, under UV or blue light. For each individual sample tested, only one type of GFP was observed, confirming that in systemic infected tissues TMVmGFP and $TMVGFP_{S65T}$ were present in separate areas and mutually excluded each other.

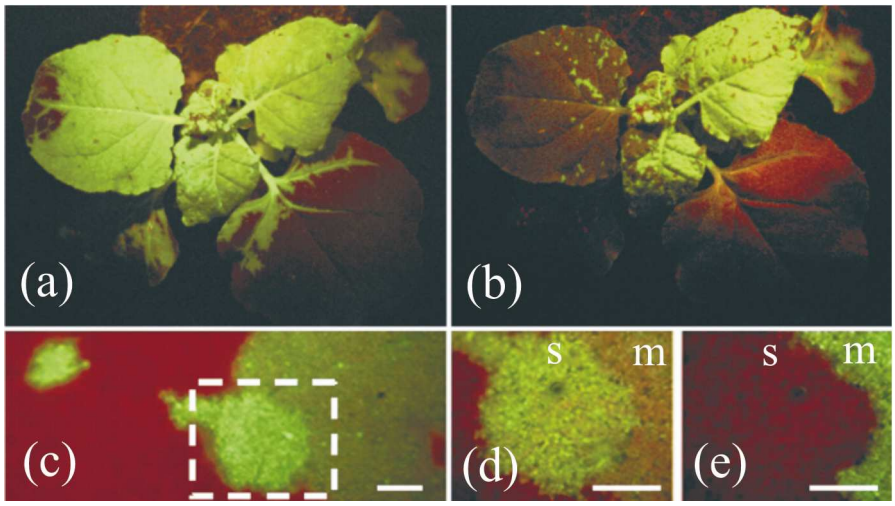

Figure 6: GFP fluorescence in N. *benthamiana* plants co-inoculated with TMVmGFP and TMVGFP$_{S65T}$, showing the spatial separation of the two constructs. (a) Systemically infected plant under blue light that excites both mGFP and GFP$_{S65T}$. (b) Same plant under long UV light, revealing areas expressing mGFP. (c to e) Co-infected leaf showing patchy areas of fluorescence of mGFP and GFP$_{S65T}$ (c). The boxed area indicates the region analyzed under epifluorescence microscope using (d) a 490 nm GFP filter that distinguishes both mGFP (m) and GFP$_{S65T}$ (s) and (e) a 385 nm filter that discerns only mGFP. Bars are 1 mm.

Discussion

A simple method based on GFP fluorescence and identification of primary infection foci was used to follow the occurrence and spatial distribution of deletion mutants from an inoculated TMV-based viral vector. The GFP gene was chosen because of its convenience as an easy detectable, non destructive reporter gene and for its relatively small size (720 nt). By inoculating test plants and comparing the total number of GFP expressing foci on inoculated leaves with the total number of primary infection foci stained by lugol it was possible to estimate the frequency of GFP-deficient mutants in a given sample.

Genomes of RNA viruses are known for being highly prone to mutation as a result of the high error rate of RNA-dependent RNA polymerases (due to the lack of proof-reading) and their short generation time (Garcia-Arenal et al., 2001; Roossinck, 2005; Schneider and Roossinck, 2001). Nevertheless, Kerney *et al*. (1993) sequence-analyzed several lineages of a TMV-derived viral vector and found relatively low rates of point mutation ($<10^{-4}$) in regions corresponding to the insert,. These mutation rates were no different from the

mutation rates observed in functional regions within the TMV genome. The inserted sequences, however, are not essential to the virus and may, moreover, interfere with viral replication and efficiency of movement. Deletion mutants arising from non-homologous recombination will tend to replicate faster and move more efficiently than the original insert-carrying genomes, thus forming a subpopulation within the infected plants which may have a significant selective advantage over the insert-carrying genomes (Donson et al., 1991; Kearney et al., 1993; Pogue et al., 2002; Rabindran and Dawson, 2001). The screening for GFP mutants in inoculated leaves and systemic infected plants revealed different patterns and a high frequency of mutants was found particularly in systemic infected leaves showing a mottled pattern of GFP.

The original TMVmGFP and a spontaneous deletion mutant (TMVΔgfp) were compared in fitness in parallel and by competition experiments. Systemic infection by this mutant occurred significantly (on average 10 hours) faster that TMVmGFP (Table 1). Moreover, on inoculated leaves, the GFP mutant formed larger infection foci as visualized by lugol staining (Figure 2), suggesting a more rapid cell-to-cell spread by the mutant from cell-to-cell (Figure 2). The TMVmGFP also induces milder and delayed symptoms in relation to the GFP deletion mutant or to the "empty" TMV-based vector. Co-inoculation experiments also confirmed the competitive disadvantage of TMVmGFP relative to TMVΔgfp. Testing different inoculum ratios showed that at least a 50 fold excess of TMVmGFP over TMVΔgfp was necessary to obtain plants showing systemic GFP fluorescence.

The relative stability of an inserted sequence within a viral vector sequence, in general, depends on a number of factors, including the size and sequence of the insert and host-virus interactions (Pogue et al., 2002; Schneider and Roossinck, 2001; Scholthof et al., 2002; Toth et al., 2002). Larger inserts (>1.0 kb) tend to be lost shortly after inoculation usually being restricted to a few cells at the inoculation site (Dawson et al., 1989; Donson et al., 1991). Smaller inserts can be stably maintained for several passages, nevertheless, the addition of an insert as short as 200 nt was shown to cause some effect on movement (Pogue et al., 2002). The nucleotide sequence of the foreign insert may also influence insert stability, possibly due to the formation of secondary structures favoring non-homologous recombination during replication (Nagy and Simon, 1997; Toth et al., 2002). These dsRNA secondary structures may also be recognized by the host gene silencing machinery (Lacomme et al., 2003; Molnar et al., 2005; Zhong et al., 2005).

Insert instability is a drawback for the use of viral vectors as foreign gene expression vehicle. At the same time, this instability may be considered a positive feature for their biosafety, assuring limited spread of inserted genes into the environment (Pogue et al., 2002). Rabbindan and Dawson (2001) compared the competitiveness of wildtype TMV with a TMV-derived vector containing a duplicated subgenomic promoter but no insert, and demonstrated the latter to be less competitive and virulent. Our results extend these observations showing that an insert-containing TMV vector is less virulent than a derived non-expressor deletion mutant.

Besides selection, genetic drift can also strongly influence viral populations, as shown for TMV, Cucumber mosaic virus (CMV), and Wheat streak mosaic virus (WSMV) (Hall et

al., 2001; Li and Roossinck, 2004; Sacristan et al., 2003). Because only small numbers of virions are transported through the phloem, the effective population (N_e) in an infected leaf is much smaller than the census number, creating a genetic bottleneck (French and Stenger, 2005; Garcia-Arenal et al., 2001; Hall et al., 2001; Li and Roossinck, 2004; Sacristan et al., 2003). Selection and genetic drift both seem to be operating in a leaf inoculated with mixed populations of TMV vectors. The higher fitness of TMV molecules lacking a full insert (TMVΔgfp), as specified by higher replication capacity and faster movement, increases their likelihood of becoming the founder genotype when their frequency in the effective population reaches a threshold (Hall et al., 2001; Li and Roossinck, 2004). As a consequence, systemically infected plants will not express the desired heterologous protein. The effect of genetic bottleneck on systemic infection was also shown in plants co-inoculated with TMVmGFP and TMVGFP$_{S65T}$. These two lineages carry GFP genes differing only in a single amino acid substitution and, therefore, most likely have similar fitness. Although leaves were co-inoculated with the same virus titers, systemic distribution of each strain was clearly stochastic and individual leaves became infected predominantly by either one of the lineages. These data are consistent with results reported by Sacristan *et al.* (2003) which quantitatively estimated the genetic bottleneck by co-inoculating natural populations of TMV.

Although GFP-expressing virus populations were observed to be relatively stable in the first systemically infected leaves, leaves from later stages of systemic infection revealed large proportions of GFP mutants. These leaves showed a typical mosaic or mottled pattern of GFP fluorescence and further analysis of these areas showed that the populations were spatially restricted forming demes. Such separation was confirmed by passage inoculation of samples from GFP and non-GFP expressing areas and RT-PCR analysis. This spatial separation, probably resulting from selection and genetic drift imposed by the dynamics of phloem transport and loading-unloading processes, will determine the typical mottled pattern of GFP-fluorescent and non-fluorescent areas in late infected leaves. Spatial distribution was also observed in plants co-inoculated with TMVmGFP and TMVGFP$_{S65T}$. Co-inoculation of these lineages did not result in a mixed systemic infection, as discussed above, but leaves showing some patches of distinct GFP fluorescence were observed. The areas were strictly spatially separated as confirmed by differences in GFP fluorescence and by passage inoculating samples isolated from each individual area. Although not observed in the tissues examined, it is possible that some localized co-infection occurs in single occurred in cells at the border regions, as was demonstrated for potyviruses labeled with green and red fluorescent proteins (Dietrich and Maiss, 2003).

Spatial distribution of viral lineages in a mix-inoculated plant has been recognized since the early days of experimental plant virology (McKinney, 1929). More recently, using molecular analysis or fluorescence approaches this spatial distribution was further studied for a whole range of viruses including *Alfalfa mosaic virus* (AMV) (Hull and Plaskitt, 1970), *Potato virus X* (PVX) (Dietrich and Maiss, 2003; Diveki et al., 2002), WSMV (Hall et al., 2001), CMV (Li and Roossinck, 2004; Takeshita et al., 2004), and different potyviruses (Dietrich and Maiss, 2003). For all these viruses, despite their different taxonomic positions and host ranges, it has been demonstrated that co-inoculation of similar

virions resulted in spatial exclusion, each lineage occupying separate demes within the systemically infected plant. This spatial exclusion has been associated with cross-protection, a general phenomenon that has been known and exploited for many years as a successful strategy to manage virus resistance in several cultures. Its biological basis is not well understood, and more recently it has been suggested to involve RNA silencing and would operate in a manner analogous to pathogen derived resistance strategies (Dietrich and Maiss, 2003).

In this study, the TMV-based vector was used as a model to study the spatial distribution of deletion mutants in viral populations in infected plants. Because of the strong selection advantage and the genetic drift imposed by phloem transport, deletion mutants are likely to be present during late systemic infection, resulting in a typical mottled pattern of GFP fluorescence. Upon serial passage, the frequency of such deletion mutants in the inoculum is an important determinant to assure the maintenance of an effective viral population containing the desired gene for heterologous protein expression. Above a threshold frequency these mutants are more likely to constitute the founder population, resulting in systemically infected plants that do no longer express the desired heterologous protein. Furthermore, the observed spatial exclusion of viral genomes carrying distinct inserts may impose restrictions for using viral vectors for co-expression of proteins within the same cell, e.g. heavy and light chains of full length antibodies, fluorescence complementation analyses and protein complementation or subunit assembly. Therefore, besides the molecular aspects of viral infection and the development of efficient constructs, understanding of host-viral vector interaction is needed to lead to better management and use of these vectors for combinations of heterologous protein production in plants.

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Chapter 6

The nucleoprotein of *Tomato spotted wilt virus* as protein tag for easy purification and enhanced production of recombinant proteins in plants

Abstract

Upon infection, Tomato spotted wilt virus (TSWV) forms ribonucleoprotein particles (RNPs) that consist of nucleoprotein (N) and viral RNA. These aggregates result from the homopolymerization of the N protein, and are highly stable in plant cells. These properties feature the N protein as a potentially useful protein fusion partner. To evaluate this potential, the N gene was fused to GFP (green fluorescent protein), either at the amino or carboxy terminus, in binary vectors. *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* and evaluated after 4 days, revealing an intense GFP fluorescence under UV light. Microscopy analysis revealed that upon expression of the GFP:N fusion a small number of large aggregates were formed, whereas N:GFP expression lead to a large number of smaller aggregates scattered throughout the cytoplasm. A simple purification method was tested, based on centrifugation and filtration, yielding a gross extract that contained large amounts of N:GFP aggregates, as confirmed by GFP fluorescence and Western blot analysis. These results show that the homopolymerization properties of the N protein can be used as a fast and simple way to purify large amount of proteins from plants.

Cristiano Lacorte, Simone G. Ribeiro, Dick Lohuis, Rob Goldbach and Marcel Prins

Introduction

Molecular farming is arising as an important expression system the production of high-valued recombinant proteins in plants. Over the last 10-15 years a large number of heterologous proteins have been expressed in plants, including antibodies, antigens, and other proteins of therapeutic and industrial application (Giddings et al., 2000; Ma et al., 2003; Stoger et al., 2005). The low costs, easy scale-up production and safety are some main advantages of this expression system as compared to others production system based on microorganism or mammalian cell culture (Hood et al., 2002; Ma et al., 2005b). A cost analysis for producing β-glucuronidase (GUS) in corn seeds estimated that the cost per gram is approximately 10 times lower than if production was carried out in *E. coli* (Hood et al., 2002). It was also estimated that the purification cost accounts for 94% of the total cost, being directly dependent on the expression level of the target protein (Evangelista et al., 1998). Considering that protein expression from plants is usually low, ranging from <0.1 % to 1% of the total soluble protein, purification cost can determine the commercial viability of a plant based platform for recombinant protein production (Menkhaus et al., 2004).

Much of the research on molecular farming has focused on protein expression level and post-translational modification of glycoproteins, which are major technical barriers (Daniell et al., 2002; Gomord and Faye, 2004; Stoger et al., 2005). Increasing recombinant protein expression levels has been tackled by different approaches, including the choice of efficient promoter sequences and enhancers, by using viral vectors or by optimizing codon usage and RNA stability (Giddings et al., 2000; Ma et al., 2003) (Chapters 1). At the protein level, the subcellular targeting of the recombinant protein can have a major effect on protein accumulation (Conrad and Fiedler, 1998; Hood et al., 2002). The use of gene fusions has also been shown to increase protein stability and yield. In plants, several proteins have been tested as a fusion tags including GUS, ubiquitin, heat-labile enterotoxin (LT) and green fluorescent protein (GFP) and oleosin (Hondred et al., 1999; Parmenter et al., 1995; Seon et al., 2002; Yasuda et al., 2005). Proteins to be used as tags typically show high stability and must comfort N or C-terminal fusions (Terpe, 2003). In the case of GFP and GUS, these tags also function as a reporter gene, which also facilitates the detection and purification of the fusion product (Terpe, 2003).

The nucleocapsid from Tomato spotted wilt virus (TSWV) is composed of the virally encoded nucleoprotein (N) and viral RNA, forming ribonucleoprotein particles (RNPs) (Prins and Goldbach, 1998). Nucleocapsids result from homopolymerization of N protein, are highly stable in plant cells, and can be easily purified from TSWV infected plants by ultracentrifugation (de Avila et al., 1990). These properties feature the N protein as a potentially useful protein fusion for plant-based expression. The aim of this study was to test the potential application of the N gene as a fusion tag, by evaluating gene fusion constructs with the GFP as a reporter gene.

Material and Methods

Agrobacterium *culture conditions and plant inoculation*

Agrobacterium tumefaciens strain GV3101 was grown in LB medium. Binary vectors were transformed into electrocompetent cells using a Biorad electroporator according to the manufacture's instructions. For plant inoculation, 2 ml of an overnight culture was centrifuged and resuspended in 5 ml of MS medium (pH 5.5) containing 150 µM of acetosyringone. The final volume was further adjusted to an A_{600}=0.5. To valuate the effect of RNA silencing suppressors on expression, *Agrobacterium* strains carrying either the NSs gene, from TSWV (Bucher et al., 2003) or the HcPro gene, from Cowpea aphid-born mosaic virus (CABMV) (Mlotshwa et al., 2002) were co-inoculated with the N fusions by mixing the bacteria suspensions (2:1 ration). As a control, the avirulent strain A136 was used. *Nicotiana benthamiana* plants (4-5 week-old) were infiltrated with *Agrobacterium* suspension using a needle-less syringe. Plants were kept in a growth chamber at 25 with a 12 hours photoperiod.

DNA constructs

The N gene from TSWV and the GFP gene (Chiu et al., 1996) ere amplified by PCR with a proof reading DNA polymerase (*Pfu*I, Promega). Forward and reverse primers for the N gene contained as extension the restriction sites *Nco*I/*Bam*HI and *Nco*I/*Not*I, respectively, whereas primers for sGFP primers contained *Nco*I and *Spe*I sites. The amplified fragments were purified, ligated to pGemT-easy (Promega) and electroporated into *Escherichia coli* strain XL1-Blue. Selected clones containing the N gene were digested with *Bam*HI and *Not*I and the purified fragment cloned into the into the Gateway™ entry vector, pENTR11 (Invitrogen). The GFP gene was cloned into the *Nco*I and *Xba*I sites of pEntr11. Another N gene fragment obtained by digestion with *Nco*I was cloned into the *Nco*I site of pEntr-GFP, generating a in frame N:GFP fusion. Vectors pEntr GFP and pEntr-N:GFP were recombined with the binary vector pK2GW7 (Karimi et al., 2002)using the LR recombinase (Invitrogen). Entry clone pEntr-N was also recombined with the binary vector pK7WGF2 (Karimi et al., 2002), generating a GFP:N fusion.

Protein extraction and Western blot

To purify the N-GFP fusions, 0.3 g of leaves were macerated with a pestle in a mortar, with 10 ml of cold 0.1 M potassium phosphate buffer (pH 7.0). The macerate was strained and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 5 ml of potassium phosphate buffer. The extract was transferred to a beaker and stirred for 1 hour at 4°C, in the presence of Nonidet P-40 (1%). Next, the extracts were strained through 11 µm nylon filter (Millipore), and collected in a 15 ml tube. Using a Pasteur pipette, a cushion of sucrose 20% was carefully added to the bottom of the tube,

and centrifuged for 10 minutes at 1,000 rpm. The pellet and the sucrose cushion fraction were collected, transferred to 1.5 ml centrifuge tubes, and centrifuged at 14,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml of potassium phosphate buffer. Samples from these extracts were maintained at 4°C and used for SDS-PAGE and Western blot analysis and for microscopic observation. Total protein extraction was done by macerating two leaf discs (30 mg) in a tube with 100 µl of PBS, using a plastic pestle. Protein loading buffer was added to these extracts and incubated for 3 minutes at 95°C. After protein separation by SDS-PAGE, proteins were transferred to a PVDF membrane (Millipore) using a semi-dry transfer apparatus (Biorad). The membranes were blocked in 10% BSA/PBS for 1 hour, and incubated with anti-GFP polyclonal antibody (1:2000 in 5% BSA/PBS), for 1 hour. After washing 3 times in PBS-Tween (0.1%) (PBS-T), the blots were incubated for 1 hour with an anti-rabbit secondary antibody conjugated to alkaline-phosphatase, and developed with BCIP/NBT. Protein concentration was estimated using a Fujifilm FLA 3000 scanner with a green laser (532 nm) and an O580 filter. A standard curve with purified GFP was used to estimate the amount of N:GFP and GFP:N present in the purified samples by using the Multigauge software. GFP was purified from *E. coli* culture using the TALON® CellThru Resin (Clontech), according to the manufacturer's instructions.

Detection of GFP fluorescence and imaging

Close-up UV images were obtained with a digital camera (CoolSnap) mounted on a stereo microscope (M3Z, Leica), with UV light and a blue filter set (465 nm). To facilitate microscopic imaging, leaf protoplasts from infiltrated leaves were prepared, essentially as describe by Kikkert et al. (1997). Leaf samples were mounted with water on a glass slide. Samples were analyzed under a Zeiss LSM510 laser scanning microscope (LSM), using a blue laser light at 488 nm and emission through a 505-530 bandpass filter.

Results and Discussion

A gene fusion approach based on the N protein from TSWV was explored in view of its potential to increase the stability of foreign proteins produced in plants. The rational behind this choice was the known high stability of this viral protein in plant cells, and on the fact that it can form homopolymers, thereby offering a possible purification alternative for a target protein as a gene fusion. The GFP gene was used as a model, for its convenience as an easy and efficient fluorescent reporter gene and its relatively small size.

The N gene was fused either to the N-terminus or to the C-terminus of GFP, generating the binary vectors pK7GW-N:GFP and pK2WG-GFP:N (Figure 1bc). The expression of these gene fusions was evaluated by transient gene expression after *Agrobacterium tumefaciens* infiltration in *N. benthamiana* leaves (ATTA). Four days after *Agrobacterium* inoculation, leaves were analyzed under UV light using a stereo microscope. Intense GFP fluorescence was observed in leaves infiltrated by both N-GFP fusions (Figure 2). Co-inoculation with the RNA silencing suppressors NSs or HcPro had a marked effect on fluorescence, when

compared to leaves co-inoculated with the avirulent *Agrobacterium* strain A136 (data not shown). The GFP fluorescence was monitored over time revealing that the fluorescence from GFP:N fusion was still visible as late as 30 days post infiltration, indicating a greatly increased stability of this protein *in planta* upon fusion to N.

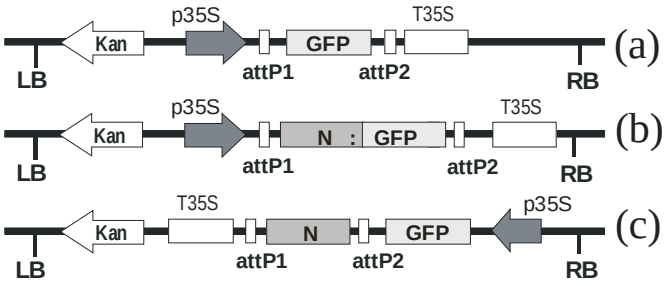

Figure 1: Schematic representation of the utilized binary vectors. Constructs (a) and (b), carrying the GFP gene and the N:GFP fusion are based on the destination vector pK2GW7. The fusion gene GFP:N was obtained upon recombination with the destination vector pK7WGF2.0 (c).

Leaves expressing the N fusions were analyzed under a laser scanning microscope, revealing marked differences in localisation between the N:GFP and GFP:N fusions. Large aggregates of intense fluorescence were seen in leaves expressing GFP:N, whereas smaller aggregates, scattered through the cytoplasm were present in leaf cells expressing the N:GFP fusion (Figure 2). Plants inoculated with the GFP gene showed the typical intense fluorescence from the cytoplasm and nucleus (data not shown).

The formation of N polymers has been studied by two-hybrid system and *in vitro* experiments leading to the mapping of N- and C-terminal regions that are essential for the interaction between N monomers (Kainz et al., 2004; Uhrig et al., 1999). Based on these studies, a “tail-to-tail” and “head-to head” organization of the N monomers has been proposed (Snippe et al., 2005b). The homotypic interaction of N and its interactions with other TSWV viral proteins have been studied through the expression of GFP fusions of the N gene in mammalian cells. Interestingly, in this cell system (BHK cells) no difference was observed between N- or C-terminal N:GFP fusions (Snippe et al., 2005a).

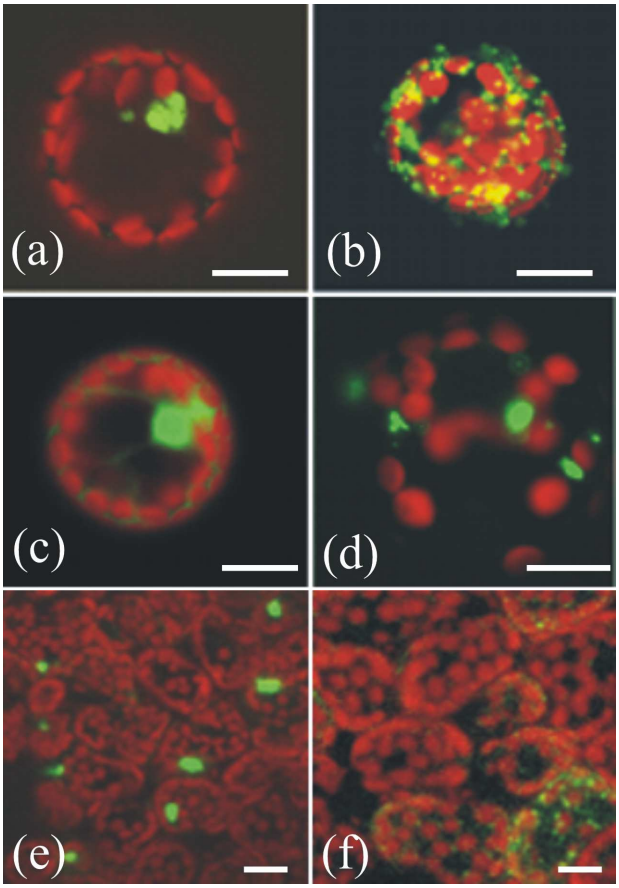

Figure 2: Laser scanning microscope (LSM) imaging of protoplasts and leaves of *N. benthamiana* expressing GFP:N (a), N:GFP (b) and free GFP (c). Immunolocalization of N using alexaR 504 fluorochrome anti-rabbit conjugated (d). Leaf cells expressing N:GFP (e) and N:GFP (f). Bar = 5 µm.

In plant cells, the N protein fused to the GFP C-terminus showed increased stability, besides providing a simple method for extraction. This extraction was based on the formation of the large aggregates of GFP:N and N:GFP fusions, and took advantage of the GFP fluorescence. The presence on the aggregates after each centrifugation or filtration steps was easily checked by fluorescence. In that way, it was verified that most aggregates were present in the pellet fraction, upon centrifugation of the leaf extracts. The resulting

extract contained starch grains, and some cell debris. In an attempt to separate the aggregates from starch grain, a (20%) sucrose cushion was added. Western blot analysis of these purified extracts confirmed the presence of the fusion proteins, but not the free GFP (Figure 3). Quantification of the purified N fusions evaluated by scanning of the immunodetected signal showed that the yield was approximately 7.0 µg/g of fresh weigh (FW). Total leaf extract of infiltrated leaves expressing GFP or GFP:N (Figure 3, lanes 3 and 6) contained approximately the same amount (6.5 µg/g FW), indicating that the fusion to the N gene does not lead to significant increase in GFP accumulation, but shows that purification is very efficient. Although this simple method for extraction of the large GFP:N and N:GFP aggregates still yielded a rather impure preparation, it can greatly facilitate further purification processing. A large volume of plant extract can be easily reduced to a concentrated precipitate, allowing the separation of this protein fusion complex from most cellular proteins, particularly soluble proteins, alkaloids, phenolic compounds and other undesirable substances (Gomord and Faye, 2004; Menkhaus et al., 2004).

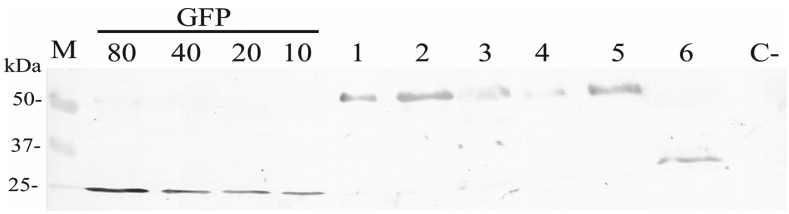

Figure 3: Western blot analysis of N gene fusions with GFP. Lanes 1 and 2: purified GFP:N fusion from infiltrated leaves; 3) Non purified leaf extract; 4 and 5) Purified N:GFP fusion; 6) Extract of leaves infiltrate with GFP; C-) Non-infiltrated leaves. GFP standards purified from *E. coli*; 80-10 ng per well are indicated on the left.

Protein purification methods for large scale production have been developed for some species, mainly corn and canola seeds (Kusnadi et al., 1998; Menkhaus et al., 2004). Large scale purification of apoplast proteins from tobacco leaves has also been used. Other methods for purification involve the rhizosecretion of the recombinant protein to the culture medium or the secretion of recombinant proteins from the leaf gutation fluid (Borisjuk et al., 1999; Gaume et al., 2003; Komarnytsky et al., 2000). To further increase yield and facilitate extraction, strategies based on gene fusion were developed. These include the fusion to oleosin, allowing the fusion protein to be recovered from oil bodies, and further treated with an endoprotease to liberate the recombinant target protein (Parmenter et al., 1995; Seon et al., 2002). Another approach is based on a fusion construct that contain an integral membrane-spanning domain leading the fusion protein to accumulate at the plasma membrane, thereby facilitating the extraction (Schillberg et al., 2000).

We have shown that the N protein as a fusion to GFP presents several advantages and can be further explored. Testing the N fusion tag with different proteins is essential as every protein is different and highly complex molecules. As a consequence, fusion proteins often do not function equally well with all partner proteins, and separating the fusion protein from the passenger may also be a challenge as the cleavage site may not be accessible (Esposito and Chatterjee, 2006). Nevertheless, the GFP:N (or N:GFP) fusion can also be useful for the production of small peptides, which are often unstable and do not accumulate within the cell (Cheng and Patel, 2004; Faber et al., 1996). A fusion with GFP and N and the target peptide could result in stable fusion aggregates that are easily extracted. This extract, free of most proteases and phenolic compounds, would subsequently be subjected to endoprotease cleavage, yielding the target peptide to be further purified by chromatography or affinity columns (Menkhaus et al., 2004)., The aggregated nature of the N protein fusion, however, may hamper the cleavage of the passenger peptide and further solublising may be required (Esposito and Chatterjee, 2006). Another potential application of this fusion system is to induce immune response, considering the large size and the stability of the aggregates, functioning as an epitope presentation system, may not be subjected to the same size and steric constrains of this system as from a virus-particle fusions (Canizares et al., 2005; Chatterji et al., 2002). In all, this investigation has demonstrated that the N protein as a fusion tag has a large potential for recombinant protein production and purification from plants.

Chapter 7

General discussion

The use of plants for recombinant protein production is an attractive alternative for the most commonly used production systems based on bacteria, yeast or animal cells. Over the past decades a large number of foreign proteins have been expressed in plants, including antibodies, antigens, and proteins of medical, veterinary and industrial applications (reviewed by e.g. Daniell et al., 2001; Ma et al., 2003; Stoger et al., 2005; Teli and Timko, 2004; Twyman et al., 2003).

There are some unique advantages for using plants as an expression system, but what makes plants an attractive option as a protein production system is largely the economical prospect (Hood et al., 2002). Growing plants for producing high-value recombinant proteins is very cost-effective, in contrast to conventional microbial fermentation and animal cells. The technology and infrastructure for cultivation are available and production can be easily scaled up without the need of large investments. Specially trained personnel is also not required (Hood et al., 2002). The economical prospect is not the only advantage. Plant derived products have reduced risk of contamination with pathogens that may infect animals or humans, and also with prions and toxins. This is an important issue in the production of biopharmaceuticals and meets the trends of the industry towards product lines that are animal-free (Hood et al., 2002). Despite these advantages, using plants for producing high-value pharmaceutical proteins still presents some problems and limitations that must be solved for this technology to become widely used and to reach its full potential. These limitations involve the expression level and post translational modifications of the foreign protein and regulatory issues.

In this thesis, different aspects related to the expression of recombinant protein production in plants were investigated. Transient expression assays based on *Agrobacterium* and viral vectors were used to express proteins in *N. benthamiana* leaves (Chapter 2). Several proteins of potential pharmaceutical value were expressed, i.e. proteins of Chicken anemia virus CAV) aiming to obtain a vaccine against this pathogen (Chapter 3), and human erythropoietin (Chapter 4). Despite the distinct features of the proteins and their ultimate purposes, the results obtained indicate the large potential of plants which need to be explored further. Therefore, in this chapter, the relevance of the expression level and proper glycosylation of target proteins for the viability of a plant-based production system is discussed further, in view of the results described in the previous chapters. Some aspects related to the transient expression system based on viral vectors and current strategies for improving the available vectors and increasing expression level are also discussed. Finally the implications on the use of viral vectors are considered in terms of biosafety.

The quest for increased protein yield and "humanized" post-translational modifications

The economic prospects and further advantages of producing recombinant proteins in plants do not mean that plant-based production represents a definitive solution for the production of every protein needed. Many proteins currently produced in bacteria, yeast or animal cells may never be produced in plants, for reasons that are both technical and economical (Ma et al., 2005b).Transgenic plant technology is relatively young, even though it has had large implications in the production chain (Castle et al., 2006; James, 2005). Crop plants currently being commercialized are considered as the first and second generation of transgenic plants, involving phenotypic modifications to improve agronomic traits. Some typical examples would be the expression of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), for glyphosate tolerance; the *Bacillus thuringiensis* Cry delta-endotoxins, for insect resistance; or RNA silencing to reduce the polygalacturonase enzyme activity in tomato fruits (Castle et al., 2006; Stewart and Mclean, 2005). The expression level for these genes may not be necessarily high to assure the desired phenotype.

For the third generation of transgenic plants, aiming at the production of proteins of pharmaceutical and industrial use, the challenge is to achieve high expression level of proteins, assuring that the final product is functional and safe, on a consistent basis. It has been suggested that an expression level of at least 1% of the total soluble protein in necessary for an economically commercial production (Kusnadi et al., 1998). Naturally that estimative depends on the product's market value and on the relative efficiency of down stream processing (i.e. purification efficiency and purity grade) (Evangelista et al., 1998).

The question that is raised then is: how to further increase recombinant protein production in plants? There is no direct answer to that, and it may be more a matter of decision making based on the current scientific knowledge and experience from different areas. The choice for an expression system does not follow a simple evaluation of the advantages and disadvantages of each system. Some basic guidelines are well established as, for example, the several protocols for plant transformation or for transient assays (Sharma et al., 2005). However, other factors regarding the gene construct and, particularly, the subcellular location of the target protein may have great influence of expression level, and may not be easily anticipated (Chikwamba et al., 2003; Doran, 2006; Drakakaki et al., 2006). Likewise, the purification process for the target protein must be considered (Menkhaus et al., 2004). Optimizing these factors do not assure that expression will be increased or the product will be efficiently purified and results are often unpredictable and inconsistent. In many cases, however, considerable improvements have been obtained, perhaps from the combination of several of these parameters. Choosing the proper promoter sequence, enhancers, leader sequences and polyadenylation signals, optimizing codon usage, removing cryptic introns and other mRNA destabilizing sequences and proper targeting of the protein are some parameters that were shown to be important. The stability of the target protein, the expression system and the plant system should also be considered, particularly in view of down stream processes. The use of fusion proteins can greatly contribute to increase protein stability. As demonstrated in Chapter 6, the fusion of GFP, as a model protein, with the nucleoprotein N from TSWV increased the protein stability *in planta*. It also provides a

simple method for purification of the recombinant proteins. This crude purification can substantially facilitate further downstream purification reducing productions costs and increasing market viability.

Having settled the parameters for increasing expression levels, evaluation should be carried out *in planta*. For that, transient expression system based on *Agrobacterium* infiltration (ATTA) or viral vectors are particularly convenient for a proof of concept or for small to medium scale production (Fischer et al., 1999; Voinnet et al., 2003). These assays are simple and the expression of the foreign protein can be assessed after a few days or a few weeks. This approach where different genes can be tested in a relatively short period of time is also greatly facilitated by the use of the Gateway cloning system (Chapter 2).

The use of viral vectors, as also shown in this thesis, have been demonstrated to be useful to assure high levels of expression. The high level of RNA that is produced as a result of the viral infection assures that proteins are accumulated in higher amounts. As demonstrated in Chapter 4, erythropoietin with plant-optimized codon usage was expressed from a viral vector at substantially increased level in relation to previously reported expression levels in transgenic plants. Also here correct targeting could be demonstrated using the original human signal sequence. By adding a C-terminal KDEL-encoding sequence the protein could be retained in the endoplasmic reticulum rather than excreted to the apoplastic fluid.

It should be noted that viral vectors do have their drawbacks. Viral vectors are often unstable and the inserted sequence may be readily deleted by non homologous recombination (Pogue et al., 2002; Shivprasad et al., 1999). Loss of the inserted sequence is usually associated with systemic infection (Chapter 5). In inoculated leaves, the foreign gene is stably maintained, although longer sequences (i.e. >1 kb) may show reduced efficiency. When *in vitro* RNA transcripts are used, the number of infected cells may be rather low, but agro-infection of a PVX vector, for example, assures high level of expression throughout the infiltrated leaf (Chapter 2). The high efficiency of agroinfection and high expression level expression that is maintained over a longer period of time (up to two weeks) make this simple transient expression system a useful approach to test candidate genes for their expression levels, as mentioned above, but also as bioassay for testing genes for pathogen resistance, for example, as demonstrated in Chapter 2.

The leaf transient assay was also used for co-expressing CAV VP1 and VP2 in plants (Chapter 3). Although the CAV capsid protein VP1 was shown to be expressed at very low levels, the correct targeting of both proteins to the nucleus and their interaction *in vivo*, demonstrate the versatility of this expression system. In contrast to VP1, the other CAV proteins, VP2 (a protein phosphatase and putative scaffold protein) and VP3 (a apoptosis inducing protein) were shown to be expressed much more efficiently, indicating that the intrinsic characteristics of VP1 might result in an unstable and therefore lowly expressed product.

The overexpression of a foreign protein can lead to the induction of cell death and necrosis of the infected tissue. This effect, sometimes unexpected, is more often observed when the protein is expressed from a viral vector. This effect was observed upon erythropoietin expression from the TMV vector (Chapter 4). The mechanisms related to the necrotizing

effect observed upon EPO expression in plants have remained unknown. The expression of EPO from the PVX vector or by maintaining the TMV-EPO infected plants at 18°C showed no necrotic response. Likewise the expression of EPO with the KDEL ER retention sequence, also showed a reduced necrotic response, thereby suggesting that the necrotic effect observed upon EPO expression from TMV-EPO is associated with the expression level and with the subcellular location of the protein.

A cell death-associated response was also observed upon TMV and PVX vector-mediated expression of the Chicken anemia virus VP3 protein (Chapter 3). The VP3 protein, also known as apoptin, is well known to induce apoptosis in animal cells. When expressed in plant cells, VP3 localizes to the nucleus, forming aggregates similar to those observed in animal cells. Considering also that expression of VP3 in plants results in cell death, it is tempting to implicate VP3 in programmed cells death. DNA analysis from leaves expressing VP3 however, did not show the typical laddering effect resulting from chromatin degradation of cells undergoing programmed cell death. This effect is not always observed in programmed cell death, or it may not be easily detectable as cells undergoing such process might constitute a rather small group within the tissue analyzed. Therefore, a possible involvement of VP3 in inducing programmed cell death in plants will need further studies, which might be rewarding by the possibility of further connecting animals and plants in relation to this important and complex process of programmed cell death.

RNA silencing inhibition

Between different lines of transgenic plants often show large differences in transgene expression levels. This variation is associated with the relative position and copy number of the integrated transgene and was later recognized was as being related to both transcriptional and post-transcriptional gene silencing mechanisms. Transient expression of foreign genes can also trigger gene silencing, most likely as a result of the high copy number of DNA transferred by *Agrobacterium*. The effect of gene silencing on ATTA experiments can be demonstrated by co-expression of viral suppressors of gene silencing, leading up to a 10 fold increase in gene expression level (Voinnet et al., 2003).

To further substantiate the advantages of transient expression systems based on agroinoculation of viral vectors additional experiments have been performed. Co-expression by agroinfiltration of a viral vector carrying the GFP reporter gene and a strong RNA silencing suppressor (HcPro) (Mlotshwa et al., 2002), leads to a greatly increased GFP fluorescence (Figure 1), similar to what has been found in ATTA experiments. Interesting was the effect of the presence of a viral RNA silencing suppressor on TMV infection, resulting in an increased number of cells that showed GFP expression, which could reflect either an elevated number of infected cells or an increased expression level in cells in which GFP expression would normally remain below the detection limit. A similar effect has been observed upon mechanical inoculation of RNA transcripts of Cowpea mosaic virus (CPMV) where the number of initially infected cells significantly increased when inoculation was done in leaves expressing HcPro, either from transient expression

(ATTA) or from transgenic plants (Silva, 2004). Compared to PVX-based vectors, agroinoculation of TMV vectors is less efficient and a relatively low number of infection foci are observed after 5-6 dpi (Chapter 2). The low efficiency observed for TMV vectors may be a consequence of the low transcription efficiency of TMV upon transfer of the T-DNA into the nucleus, due to destabilizing sequences and cryptic introns. Indeed, Removal of such sequences from the TMV genome greatly increased the inoculation efficiency upon agroinfection (Marillonnet et al., 2005).The increased area of GFP expression indicates that the efficiency of TMV infection by agroinfiltration can be further improved by the simple ectopic co-expression of a strong RNA silencing suppressor. It also confirms the importance and broad involvement of RNA silencing mechanisms on foreign gene expression in plants, also in the presence of viral vectors such as TMV and PVX, at least in the initiation of infection.

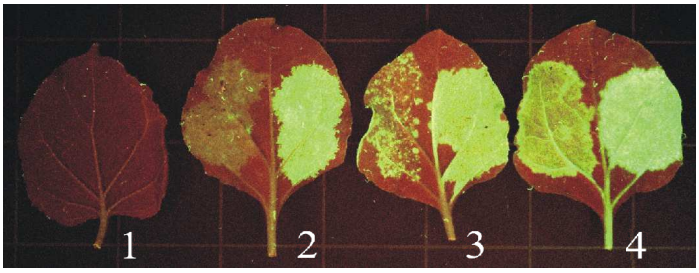

Figure 1: GFP fluorescence from (co-)agroinoculated *N. benthamiana* leaves with (right) or without (left) RNA silencing suppressor HcPro. 1) non inoculated control; 2) empty binary vector; 3) TMV-GFP; 4) PVX-GFP.

Optimizing the expression level of a target protein has been largely based on testing the effect of different expression systems and subcellular targeting. Nevertheless, no single individual factor may be sufficient to obtain an optimal protein level production, and this challenge must be approached in different ways (Doran, 2006). A good example of how the cumulative effect of technology can contribute to improved recombinant protein yield is provided by the transgenic corn seeds for avidin production. The avidin yield in these seeds, carrying both expression and subcellular targeting information, was further increased 150-fold in eight generations by conventional selection and backcross programs (Hood et al., 2002; Howard, 2005). In that way, the use of silencing suppressors and the alteration on the glycosylation pathways open novel possibilities for increasing protein accumulation with the proper glycosylation pattern. Further exploring the knowledge accumulated by functional genomics will create news options such as the manipulation of protein degradation pathways allow a further increased accumulation and yield of a recombinant protein in plants (Doran, 2006; Rivard et al., 2006)

Perspectives for the post translational modifications of plant made pharmaceuticals

Plant glycoproteins show slight differences from those produced in animal cells. They contain β(1,3)xylose and β(1,3)fucose, instead of β(1,6)fucose and lack the terminal galactose and sialic acid residues (Brooks, 2004; Gomord and Faye, 2004). For many glycoproteins produced in plants, particularly antibodies, these differences appeared to have minimal or no effect on their function, although concerns for possible immunogenic reactions were raised that still need to be further evaluated (Gomord et al., 2005; Hood et al., 2002; Joshi and Lopez, 2005; Ma et al., 2003).

The lack of sialic acid residues, however, may have a major effect for some proteins if produced in plants, including erythropoietin (EPO) (Chapter 4). The terminal sialic acid is an important determinant for the *in vivo* activity and serum half-life of EPO, as non-sialylated molecules are readily cleared from the blood stream by asialoglycoprotein receptors in the liver (Brooks, 2004; Fukuda et al., 1989).

To obtain proteins that are more "human-like", transgenic plants and cells expressing β(1-4)galactosyltransferase were successfully tested (Bakker et al., 2001; Palacpac et al., 1999). *Arabidopsis* mutants lacking key enzymes for the addition of fucose residues indicated that the absence of this residue did not disturb the biosynthesis of N-linked oligosaccharides. The α(1,3)fucosyltransferase and β(1,2)xylosyltransferase were knocked out in the moss *Physcomitrela patens*, allowing the production of proteins without fucose and xylose residues. Later, transgenic *Arabidopsis thaliana* knocked out plants were also obtained, which proved able to produce N-glycans lacking xylose and fucose (Strasser et al., 2004). For the addition of sialic acid residues, however, a transgenic based approach would be require, providing transgenic plants expressing the concerted action of at least five heterologous enzymes (Gomord et al., 2005; Joshi and Lopez, 2005). Nevertheless, the analysis of the *Arabidopsis* data base revealed genes that are potentially involved in sialylation and protein analysis detected some sialylated proteins, albeit in small proportion (2%) (Shah et al., 2003). These findings called the attention to a possible route for sialic acid production in plants, which can be further explored (Joshi and Lopez, 2005; Seveno et al., 2004).

Like other non-mammalian expression systems, expression of recombinant proteins from plants presents some potential complications due to differences in the glycosylation profile of the produced proteins in relation to human proteins (Grabenhorst et al., 1999). Reducing these differences is a challenge and a complex task as glycosylation depends on a series of enzymes and varies according to the physiological state of cell within subcellular compartments (Drakakaki et al., 2006; Gomord et al., 2005). Glycoproteins for which sialic acid residues are important determinants, as may be the case for EPO, chemical or chemo-enzymatic addition of sialic acid residues to a peptide are alternatives for post-translational engineering (Blixt et al., 2002; Chen et al., 2005b). For proteins destined for industrial application, the glycosylation differences may not have major implications, as long as the stability and function of the protein are maintained (Howard, 2005; Scheller and Conrad,

2005). Likewise, for the production of antigens, the glycosylation pattern may not be as important.

Biosafety and public acceptance of plant made pharmaceuticals

The use of genetic modified organisms (GMOs) has been subject of intense debate in society, leading to policies that vary from one county to another. Over the past 10 to 15 years the number of transgenic plants entering the food chain increased steadily, but that fact was not necessarily followed by general public perception towards acceptance (Castle et al., 2006; Davies, 2001; Stewart and Mclean, 2005). Although the use of transgenic plants for vaccine production was proposed as early as 1990, only in recent years regulatory agencies and the public were faced with the eminent use of this next generation of transgenic plants, renewing the debate concerning the biosafety of these plants (Kirk et al., 2005). Since 1991, over three hundred field trials with transgenic plants for the production of pharmaceuticals have been approved, mainly in the US, where a specific guidance for the pharmaceutical related products derived form genetically modified plants use was issued in 2002 (Elbehri, 2005; FDA, 2002).

The main concern from the regulatory agencies and the public in general is the potential risk that these products end up in the food chain, besides the possible environmental effects of gene flow and implications for incidental contact with other species (Elbehri, 2005; Kirk et al., 2005). Therefore, the selection of a host plant must be considered not only for the technical aspect, or it may be required additional measures to ensure an incidental mixing with other crops (Kirk et al., 2005; Mascia and Flavell, 2004). In that respect, the use of plants that are not in the food or feed chain, such as tobacco, is a great advantage (Mascia and Flavell, 2004). Most of the approved field tests for transgenic plants for the production of biopharmaceuticals, from 1991 to 2004, involved corn (70%), soybean (9%) and tobacco (5%) (Elbehri, 2005). The importance of choosing the host plant is evidenced by two incidents involving transgenic plants in the USA. The genetically modified corn Starlink, not approved for human consumption, was found throughout the food supply as well as in food export, leading to serious effect on the market. In another incident, an experimental field for transgenic plants producing biopharmaceuticals was not properly handled and nearly entered the food chain. These events call attention on flaws within the regulation for GMOs, and were a set back, likely influencing the approval policy concerning plant made pharmaceuticals (Stewart and Mclean, 2005). Alternatives to assure higher containment is to grow plants in greenhouses or carry out the production in bioreactors with culture plant cells (Hellwig et al., 2004).

For the use of viral vectors, there is no specific regulation and the approval for trials involving these vectors would follow the same GMOs regulations, varying among countries. The choice for the host plant is certainly an essential point, and it is likely that higher containment measures will be required. It is also expected that the public perception for such virus-based production could be more critical. Nevertheless, a company in the USA has got approval since 2002 for producing antibodies and other biopharmaceuticals in

an open area (160 hectares). The production is based on tobacco plants inoculated with a TMV-derived vector (Pogue et al., 2002).

Despite the arguable potential risk of gene flow through spreading of the virus to the environment, plant RNA viruses do not integrate into the host genome some viruses, such as TMV and PVX, do not have a known biological transmission vector (Pogue et al., 2002). Besides, as it has been shown for TMV, the virus containing the inserted sequence is substantially less virulent than the mutants that arise due to the genetic instability of the insert-containing virus (Chapter 5). These mutants, in turn, are less fit than the wilt type TMV virus, and are therefore likely to be rapidly out competed and eliminated from the viral population (Rabindran and Dawson, 2001). Therefore, the genetic instability of viral vectors, though challenging for foreign protein production, represents an intrinsic biosafety feature of the virus (Pogue et al., 2002; Rabindran and Dawson, 2001).

Approaches to further increase this biosafety include the development of expression systems based on transgenic plants containing an essential viral function that can be provided *in trans*. In that way, these plants are used as host for virus deficient in, for example, their viral RNA-dependent RNA polymerase (Bol et al. 2002) or cell-to-cell movement (Man and Epel, 2006). This complementary function may be under control of an inducible promoter and can also help to assure that virus infection is only accomplished under controlled circumstances. The use of vector viruses that are deficient in transmission has also been described (Marillonnet et al., 2005). As most of these viruses would not be infectious by mechanical inoculation, the use of *Agrobacterium*-based inoculation (agroinfection) can provide a safe inoculation/expression system. Leaves or even the whole aerial part of the plant can be infiltrated by vacuum, providing a large area of infected tissue that can be used for extraction, providing a safe and efficient small to medium scale production platform with further improved yields (Fischer et al., 1999; Marillonnet et al., 2005).

Setting up plants as platforms for biopharmaceuticals production

Producing proteins with the proper post-translational modifications and at enhanced expression levels involves technical achievements that may determine the biological activity and economical prospect of the product (Joshi and Lopez, 2005). Other barriers concern regulatory issues involved in handling genetic modified organisms in the context of producing biopharmaceuticals as well as all the regulations for producing, evaluating efficacy and safety, and conducting trials (Ma et al., 2005b). That long developmental route takes years and large investments until final approval.

The production of biopharmaceuticals in plants also implies having to adapt and operate under current Good Manufacturing Practices (GMP) - a set of regulations, codes, and guidelines for the manufacture of medicinal products, medical devices, diagnostic products, foods products and active pharmaceutical ingredients. That also applies to plant-made vaccines for oral delivery (Kirk et al., 2005).This may not be an easy task, considering that

plants in the field are subjected to environmental variations, which can lead to inconsistencies of a product obtained, which implies that bioequivalence may not be achieved (Elbers et al., 2001; Stevens et al., 2000). One option is the production from contained greenhouses, hydroponics or plant cell cultures, assuring the production is carried out under controlled conditions and also in compliance with biosafety measures. However, this would imply an estimated increase of 10-20% (for green house facilities only, much more for cell culture) in the production costs and the plant-based system would loose an important benefit compared to other production platforms (Kirk et al., 2005).

These factors partially lead the industry to be cautious in their further investment in plants as an alternative source of plant-made pharmaceuticals. Nevertheless, the large number of new biopharmaceutical products that are entering the market and the growing demand for the available products has created a problem for the industry to couple with the low production capacity and the large investments needed for expanding the currently installed production facilities. That situation has been pointed as a motivation for the industry to search alternative production systems (Hood et al., 2002; Walsh, 2005).

The fact that some plant produced products, such as avidin, trypsin and β-glucuronidase, have already reached the market generates a positive signal to encourage the development of novel products. Also, the release for a draft guide line for plant-made pharmaceuticals in the USA (FDA) indicates that this production system is an eminent possibility of becoming adopted in the coming years (FDA, 2002). Indeed, recently, the first plant-made vaccine was approved (Mihaliak et al., 2004). This vaccine for poultry, against Newcastle disease virus, represents a landmark showing that the plant-made vaccines can get the approval from the regulatory agencies and shall pave the way for new products.

It is likely that plants will be increasingly important in the near future as a sustainable source of a variety of substances, including combustible fuel oil. The possibility of using plants also for the production of biopharmaceuticals and other industrial proteins can certainly benefit society. These benefits, however, may only become true by further developing the technology, which will require the initiative from scientists and the industry. Exploring new alternatives on scientific and innovative basis will certainly help further developing plant-based technology for recombinant protein production.

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Summary

Over the past 10 years the production of recombinant proteins in plants has become a promising and realistic alternative for the more established technologies which are often based on microbial bioreactors or animal cell cultures. Selected recombinant proteins are often high-valued biopharmaceuticals, antigens for vaccination purposes and therapeutic proteins such as hormones and cytokines. The growing demand for these products and the limiting production capacities have motivated the development of alternative production systems. Using plants for the production of heterologous proteins, either by transgenesis or by infecting them with protein-expressing viral vector constructs, could be very cost–effective. The technology for efficient cultivation is available and production can be easily scaled-up. Producing biopharmaceuticals from plants is also safer, as there is limited risk of contamination with human or animal pathogens.

As plant-based protein production systems still represent a developing area in biotechnology, some (potential) limitations still need to be overcome and new opportunities need to be explored. Expression levels of heterologous proteins produced in plants are frequently low, while their post-translational modification, in particularly their glycosylation, may differ from those produced in animal systems (reviewed in Chapter 1).

The research described in this thesis focused on the use of transient expression systems for heterologous protein production in plants. The choice for exploring and optimizing transient expression systems was based on both the convenience of a fast response and anticipated high expression levels by using viral vectors. To further exploit the advantage of a flexible convenient transient assay, viral vectors based on PVX and TMV were adapted through the Gateway™ (GW) recombination system to facilitate the insertion of target genes and tested by expressing marker genes (Chapter 2). The resulting vector (denoted PXV-GW) was successfully validated as expression vector and as VIGS (virus induced gene silencing) tool for plant gene knock-out studies. The PVX vector was also modified to contain the green fluorescent protein (GFP) gene upstream of the Gateway recombination sites, thereby allowing easy marker protein fusions.

In Chapter 3 the VP1, VP2 and VP3 proteins of Chicken anemia virus (CAV) were expressed in *N. benthamiana* plants aiming at a potential (oral) subunit vaccine against this virus. Expression was achieved using both viral vectors and the *Agrobacterium* transient assay (ATTA) and the subcellular localization of these proteins studied as GFP fusions. All three CAV proteins localized in the nucleus, demonstrating functional and autonomous nuclear localization signals. Co-expression of (non-fused) VP2 resulted in a markedly different distribution of GFP:VP1, indicating that these proteins interact within plant cells, similarly as observed in animal and insect cells. Leaves expressing CAV VP3 showed extensive necrosis, which might reflect its proposed function in (virus-induced) apoptosis in animal cells.

Chapter 4 describes the successful expression in plants of erythropoietin (EPO), a costly therapeutic product used among others in cancer and AIDS therapy, using both the TMV- and PVX-derived vectors. The generation of a synthetic EPO gene with optimized amino acid codons for expression in plants and the transient expression using viral vectors were important factors to obtain an expression level approximately 200 fold higher than previously reported. The secretion to the apoplast and extensive glycosylation of EPO in plants infected with TMV and PVX vectors was demonstrated. An EPO version provided with a C-terminal KDEL peptide sequence was shown to be retained in the endoplasmic reticulum, resulting in similar high expression levels and alternative glycosylation. Further research will demonstrate whether these products are biologically active *in vivo*.

Taking advantage of the high replication capacity of viruses, plant viral vectors represent a very efficient expression system. However, the use of these vectors is often hampered by the genetic instability of the inserted sequence. Deletion of (parts of) the inserted sequence may lead to systemically infected plants that no longer express the desired protein. The study described in Chapter 5 evaluated the vector instability in infected plants using a GFP-expressing TMV construct for easy monitoring. Furthermore a comparison was made between the fitness of the original GFP-expressing vector and a non-expressor deletion mutant derived from the same vector. In co-inoculation experiments it was also shown that the original vector and derived mutant occupy separate areas within the infected plant.

In Chapter 6 the nucleoprotein (N) of *Tomato spotted wilt virus* has been evaluated as potential fusion partner to increase protein stability and facilitate protein purification. The fusion of N to the GFP reporter protein was shown to be highly expressed and form large aggregates that accumulated for extended periods *in planta*, when compared to the non-fused GFP. Purification was achieved by simple centrifugation and filtration steps, thus confirming the anticipated application of the N protein as a fusion tag for increasing recombinant protein production in plants.

In Chapter 7 the major findings of the investigations reported in this thesis are discussed in view of the current limitations and challenges for further development of plant-based recombinant protein expression systems. Also, aspects related to biosafety regulations in the production of plant-based biopharmaceuticals and public acceptance of this technology are discussed.

Samenvatting

Gedurende de afgelopen tien jaar is het gebruik van planten en plantencellen voor de productie van recombinante eiwitten een veelbelovend en realistisch alternatief geworden voor gevestigde productietechnieken gebruikmakend van micro-organismen of dierlijke celsystemen. De aldus via heterologe expressiesystemen aangemaakte eiwitten zijn vaak hoogwaardige farmaceutische eiwitten, antigenen ten behoeve van vaccinproductie en therapeutische eiwitten zoals hormonen en cytokines. De groeiende vraag naar deze producten en de beperkte productiecapaciteit hebben de ontwikkeling van nieuwe productiemethoden gestimuleerd. Het gebruik van planten voor de productie van heterologe eiwitten, hetzij door transgenese of door ze te infecteren met virale genvectoren kan erg lucratief zijn. De technologie voor efficiënte productie is beschikbaar en de productie kan gemakkelijk opgeschaald worden. Het produceren van farmaceutica in planten is ook veiliger omdat er maar een beperkt risico op contaminatie met dierlijke of humane pathogenen bestaat.

Omdat biotechnologische eiwitexpressie in planten nog steeds in ontwikkeling is, moet een aantal (potentiële) problemen worden opgelost en nieuwe mogelijkheden geëxploreerd. Zo zijn de tot op heden behaalde expressieniveaus in transgene planten over het algemeen laag, terwijl de post-translationele modificaties van eiwitten, met name de glycosylering, kan verschillen van die in dierlijke systemen (Hoofdstuk 1).

Het in dit proefschrift beschreven onderzoek concentreerde zich op de transiënte eiwitexpressiesystemen in planten. De keuze om juist transiënte systemen te onderzoeken en te verbeteren was gebaseerd op zowel het gemak van snelle productie als ook de verwachte hogere expressie bij het gebruik van virale vectoren vergeleken bij transgene expressie. Aldus werden de bestaande gebruikersvriendelijke transiënte virale vectoren gebaseerd op het Tobaksmozaiekvirus (TMV) en het Aardappelvirus X (PVX) aangepast voor het gebruik van de Gateway^{TM} kloneringtechniek. Deze techniek maakte het mogelijk genen in te brengen met behulp van recombinatie en werd getest door de expressie van merkergenen te volgen (Hoofdstuk 2). De uit PVX ontwikkelde vector (PVX-GW) bleek niet alleen te voldoen als expressievector voor eiwitten maar tevens als werktuig voor VIGS (virus-geïnduceerde gen silencing) om gericht plantengenen mee uit te schakelen. De PVX vector werd ook aangepast om eiwitten gefuseerd aan het *green fluorescent protein* (GFP) te kunnen produceren.

In Hoofdstuk 3 werden de drie structurele eiwitten VP1, VP2 en VP3 van het Chicken anemia virus (CAV) in *N. benthamiana* tot expressie gebracht met als doel een mogelijk (oraal) vaccin tegen dit virus te ontwikkelen. De expressie werd bewerkstelligd met zowel virale vectoren als met de “transiënte *Agrobacterium* infiltratietoets” (ATTA) waarbij de subcellulaire lokalisatie zichtbaar werd gemaakt met GFP fusie-eiwitten. Alle drie virale eiwitten bleken in de celkern te accumuleren, waarmee de aanwezigheid van functionele

autonome kernlokalisatiesignalen in deze eiwitten kon worden aangetoond. Co-expressie van (niet gefuseerd) VP2 veroorzaakte een opvallende verandering in de intracellulaire lokalisatie van (GFP:)VP1, waaruit afgeleid mag worden dat deze eiwitten, net als in dierlijke en insectencellen, ook in de plantencel een onderlinge interactie aangaan. Bladeren waarin het CAV VP3 eiwit tot expressie werd gebracht gaven op uitgebreide schaal necrose te zien, dit zou met de veronderstelde functie van dit eiwit in (virusgeïnduceerde) apoptose in diercellen te maken kunnen hebben.

In Hoofdstuk 4 is de toepasbaarheid van zowel TMV- als PVX-afgeleide vectoren voor heterologe expressiedoeleinden verder getoetst aan de hand van het humane erythropoëtine (EPO), een kostbaar therapeutisch eiwit dat onder andere gebruikt wordt in kanker- en AIDS therapie. Uitgaande van een synthetisch EPO-gen met een voor planten geoptimaliseerd aminozuurcodongebruik werden expressieniveaus bereikt die ongeveer 200 maal hoger lagen dan eerder gerapporteerde resultaten in planten. Hierbij kon excretie naar de apoplast en glycosylering van het EPO-eiwit worden aangetoond. Een EPO-variant waaraan een carboxyterminaal KDEL tetrapeptide was gekoppeld bleek op te hopen in het endoplasmatisch reticulum. Dit ging gepaard met een vergelijkbaar hoog expressieniveau en een veranderd glycosyleringspatroon.

Zoals ook blijkt uit de resultaten van de Hoofdstukken 2 – 4 kunnen virale genvectoren vanwege hun hoge amplificatiesnelheid potentieel als zeer efficiënte expressiesystemen worden gebruikt. Echter, de intrinsieke genetische instabiliteit van de ingebrachte, niet-essentiële sequentie is vaak een beperkende factor. Deletie van (een deel van) de extra genetische informatie kan leiden tot systemisch geïnfecteerde planten die het gewenste eiwit niet meer tot expressie brengen. De in Hoofdstuk 5 beschreven proeven hebben deze complicatie nader onderzocht gebruikmakend van een TMV construct dat het eenvoudig te volgen fluorescerende GFP eiwit tot expressie brengt. Ook werd een vergelijking gemaakt tussen de fitness van de originele GFP producerende TMV vector en een spontane deletiemutant waarin GFP niet meer tot expressie kwam. Door middel van co-inoculatieproeven kon worden aangetoond dat de originele TMV-GFP vector en deze afgeleide mutant verschillende delen van de geïnfecteerde plant infecteerden en elkaar binnen de plant kennelijk uitsloten.

In Hoofdstuk 6 werd het nucleocapside-eiwit (N) van het Tomatenbronsvlekkenvirus (TSWV) onderzocht als mogelijke fusiepartner voor de verhoging van de stabiliteit van recombinante eiwitten en tevens hun opzuivering te vergemakkelijken. Fusie van N aan GFP bleek inderdaad te leiden tot verhoogde expressie en ophoping van grote, stabiele eiwitclusters *in planta*. Opzuivering kon worden bereikt door eenvoudige centrifugatie- en filtratietechnieken waarmee de verwachte toepassing van het virale N eiwit als fusiepartner voor verhoogde expressie van recombinante eiwitten kon worden aangetoond.

In Hoofdstuk 7 worden de belangrijkste bevindingen van dit proefschrift bediscussieerd in het licht van de huidige beperkingen en uitdagingen voor de expressie van recombinante eiwitten in planten. Ook komen aspecten zoals biologische veiligheid van in de plant geproduceerde biofarmaceutica en publieke acceptatie in dit hoofdstuk aan de orde.

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About the author

Cristiano Lacorte was born in Rio de Janeiro, Brazil, in 27-07-1964. He graduated in Biological Sciences at Universidade Santa Úrsula, where he was first interested in marine biology and started doing research on marine algi. From algi studies he moved to cormophytes and started doing tissue culture and plant transformation at the Universidade Federal do Rio de Janeiro. He did his MSc thesis on *Arachis hypogaea* tissue culture and transformation under supervision of Prof. Antonio Rodrigues Cordeiro, at the Department of Genetics.

In 1995 he went to Brasília for a training period at Cenargen-Embrapa and in 1995 got a position as a researcher at EMBRAPA-Hortaliças. In 1999 he returened to Cenargen, where he continued research on plant transformation and gene expression analysis. In 2001 he got a scholarship from CAPES for a PhD at Wageningen University where he developed the work presented in this thesis.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Cristiano Castro Lacorte
Date: 1 December 2006
Group: Virology, Wageningen University

	date	cp
1) Start-up phase		
► **First presentation of your project**		
Production of heterologous proteins in plant using viral vectors	Jan 2002	1.5
► **Writing or rewriting a project proposal**		
Expression of foreign proteins in plants using viral vectors	Mar 2002	6.0
► **Writing a review or book chapter**		
► **MSc courses**		
► **Laboratory use of isotopes**		
2) Scientific Exposure	*date*	*cp*
► **EPS PhD student days**		
EPS PhD Student day, Wageningen University	Jan 24, 2002	0.3
EPS PhD Student day, Utrecht University	Mar 27, 2003	0.3
EPS PhD Student day, Vrije Universiteit Amsterdam	Jun 3, 2004	0.3
EPS PhD Student day, Radboud University	Nov 5, 2005	0.3
► **EPS theme symposia**		
Theme 2 'Interactions between Plants and Biotic Agents', Leiden University	Dec 17, 2001	0.3
Theme 2 'Interactions between Plants and Biotic Agents', University of Amsterdam	Jan 10, 2003	0.3
	Dec 12, 2003	0.3
Theme 4 'Genome Plasticity', Wageningen University	Dec 9, 2004	0.3
Theme 4 'Genome Plasticity', Wageningen University	Dec 9, 2005	0.3
► **NWO Lunteren days and other National Platforms**		
Studiegroep nucleinezuren NWO-CW Lunteren	Dec 2001	0.6
Studiegroep nucleinezuren NWO-CW Lunteren	Dec 2002	0.6
Studiegroep nucleinezuren NWO-CW Lunteren	Dec 2003	0.3
Dutch annual virology symposium	Mar 2004	0.3
Nederlandse Kring voor Plantevirologie and the DPG Arbeitskreis Viruskrankheiten der Pflanzen (poster)	Mar 2005	0.3
► **Seminars (series), workshops and symposia**		
Symposium Current Themes in Ecology: Genomics for Nature	Nov 2001	0.3
Symposium Current Themes in Ecology: Global Ecology	Apr 2003	0.3
Seminar Frontiers in Plant science: Cell cycle control and plant development	Feb 2003	0.1
Symposium Current Themes in Ecology: Experimental Evolution, fundamental and applied	Apr 2004	0.3
Symposium Molecular advances in vaccinology	Oct 2005	0.3
Workshop: qPCR course for starters	Apr 2005	0.3
Symposium Bionanotechnology : Assembly and Nanostructures	May 2005	0.3
Seminar: Real-time PCR and gene expression analysis	Jun 2005	0.1
Flying seminar: A systems biology approach to understanding root development	Oct 2005	0.1
Symposium: Influenza and Pandemics	Apr 2006	0.3
► **Seminar plus**		
► **International symposia and congresses**		
International conference on ssDNA Viruses of Plants, Birds, Pigs and Primates. Saint Malo (France)	Sep 24- 25, 2001	0.6
International Congress of Virology - IUMS, Paris (France)	Jul 27-Aug 1, 2002	1.8
Introgression of Genetically Modified Plants into Wild Relatives and Its Consequences, Amsterdam (NL)	Jan 21-24, 2003	1.2
7th International Congress of Plant Molecular Biology, Barcelona (Spain)	Jun 23-28, 2003	1.8
Plant-derived vaccines and antibodies: potential and limitations, Veyrier du Lac (France)	Mar 21-24, 2004	1.2
XXXIX Annual meeting of the Brazilian Phytopathological Society	Aug 2006	1.5
► **Presentations**		
Nederlandse Kring voor Plantevirologie and the DPG Arbeitskreis Viruskrankheiten der Pflanzen (poster)	Mar 2005	0.7
International Congress of Virology - IUMS	Jul 2005	0.7
Invited presentation - EMBRAPA	Sep 2005	0.7
XXXIX Annual meeting of the Brazilian Phytopathological Society	Aug 2006	0.7
► **IAB interview**		
► **Excursions**		
Flora Holland	October 1, 2004	0.3
3) In-Depth Studies	*date*	*cp*
► **EPS courses or other PhD courses**		
Summer School Environmental signaling: *Arabidopsis* as a model	Aug 27-29, 2001	0.9
Disease Resistance in Plants	Oct 17-19, 2002	0.9
GFP and LUC: applications of 'light' reporters in biology	Apr 11-12, 2005	0.6
► **Journal club**		
Member of literature discussion group at Virology	2001-2004	3.0
► **Individual research training**		
4) Personal development	*date*	*cp*
► **Skill training courses**		
Dutch language course		3.0
► **Organisation of PhD students day, course or conference**		
► **Membership of Board, Committee or PhD council**		
Subtotal Personal Development		3.0
TOTAL NUMBER OF CREDIT POINTS*		**34.0**

** A credit represents a normative study load of 28 hours of study*

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