2009

Beneficial application of SMV viral elements for the expression of human interleukin 10 in plants

Fengtao Dai

Beneficial application of SMV viral elements for the expression of human interleukin 10 in plants

(Spine title: Application of SMV elements for human IL-10 expression)

(Thesis format: Monograph)

By

Fengtao Dai

Graduate program in Biology

A thesis submitted in partial fulfillment of the requirements of the degree of

Master of Science

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Low protein yield remains a rate-limiting factor in the commercial exploitation of the transgenic plant system for biopharmaceutical production. Plant viruses have evolved to permit high accumulation of viral proteins, in some cases up to 10-20% of total soluble protein (TSP) (Culver *et al.* 1993). Viral elements from *Soybean mosaic virus* (SMV) that may facilitate high-level expression of pharmaceutical proteins in plants were investigated. The results in this study indicate HC-Pro, a known virus-encoded gene silencing suppressor, has a beneficial effect on human interleukin 10 (IL-10) accumulation. Coexpression with HC-Pro resulted in an approximate 10-fold increase of IL-10 protein yield in *Nicotiana benthamiana*. The expression levels of IL-10 were not significantly affected in the presence of other viral proteins including P3, CI, 6K, VPg and CP. The plant-derived IL-10 was found to be efficiently assembled into homodimers, a biologically active form of IL-10.

Keywords: *Soybean mosaic virus*, viral elements, HC-Pro, human interleukin 10, cytosol-targeting, homodimer

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BSA	bovine serum albumin
CaMV 35S	*Cauliflower mosaic virus 35S* promoter
°C	Degrees Celsius
CP	the coat protein
CPMV	*Cowpea mosaic virus*
CI	the cylindrical inclusion protein
C-terminus	Carboxyl-terminus
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate (s)
dpi	days post infection
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ELP	elastic-like polypeptide
EPO	Erythropoietin
ER	endoplasmic reticulum
GFP	green fluorescent protein
GUS	β-glucuronidase
HA	hemagglutination
HC-Pro	the protease helper component
hGH	human growth hormone
HRP	Horseradish Peroxidase
IL-10	interleukin 10
6K1	the first 6 kDa peptide
6K	the second 6 kDa peptide or 6K2
kb	Kilobase
kDa	kiloDalton
LB	Luria-bertani
μg	Microgram
μL	Microlitre

μM	Micromolar
mg	Milligram
mL	Millilitre
mM	Millimolar
mRNA	messenger RNA
NIa	the nuclear inclusion A
NIb	the nuclear inclusion B
NLS	nucleus localization signal
N-terminal	Amino terminal
OD	Optical density
ORF	open reading frame
P1	the first protein
P3	the third protein
PBS (T)	phosphate-buffered saline (Tween-20)
PCR	Polymerase chain reaction
PTGS	Post-translational gene silencing
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SMV	*Soybean mosaic virus*
TBSV	*Tomato bushy stunt virus*
TCV	*Trunip crinkle virus*
TMV	*Tobacco mosaic virus*
UTR	untranslational region
VPg	the viral protein-genome linked
YFP	yellow fluorescent protein

Acknowledgement

I would like to thank the following people from Agriculture and Agri-food Canada and the University of Western Ontario. My foremost thank goes to my supervisor, Dr. Aiming Wang, who gave me invaluable advice, support and time for my master study in the past two years. Special thanks go to my co-supervisor Dr. Mark Bernards and my advisory committee member Dr. Jim Karagiannis, for their advice and critical reading of my thesis. I also would like to express my sincere gratitude to my advisory committee member, Dr. Rima Menassa for her priceless expert advice on my thesis. I am grateful to Angelo Kaldis for expert advice concerning IL-10 work; Alex Molnar for editing figures for my thesis; Linda Huang for her helpful discussion and technical support; and other fellow labmates.

Last but not least, I would like to thank my family. I could always count on them for support and encouragement.

Chapter 1. Introduction

1.1. Biopharmaceutical production in plants

Drug production in the early and mid 20th century was dominated by chemical synthesis (Lienard *et al.* 2007). In the early 1970s, the development of recombinant DNA technologies led to biosynthesis of therapeutic molecules that were too complicated for synthetic chemistry. Conventional production systems such as transgenic bacteria, yeast and mammalian cells have been applied to manufacture the majority of therapeutic proteins in the pharmaceutical industry (Chu and Robinson 2001, Swartz 2001).

However, no such production system completely satisfies the rigorous standards of industry. Bioactivity, safety and cost-effectiveness are the three critical factors of therapeutic protein manufacture. Co- and post-translational modifications of proteins, such as proteolytic cleavage, protein folding and glycosylation, are required for the majority of therapeutic proteins to maintain their stability, bioactivity, solubility and pharmacokinetics (Gomord and Faye 2004). In microbial expression systems, complex recombinant proteins are often poorly folded and processed, resulting in low biological activity (Crabb and Bolin 1999). Although mammalian cell systems are able to synthesize functionally equivalent proteins, the high risk of animal pathogen contamination along with expensive investment impedes their development (Ma *et al.* 2003).

Plant-based systems have emerged as a viable alternative to traditional expression systems. They offer a number of distinct advantages over traditional systems, such as minimal capital investment, reduced risks from mammalian pathogen contamination and the capability to execute most of the post-translational modifications on heterologous proteins in fashions that are identical or similar to those of mammalian systems. The ability of plants to produce bioactive mammalian therapeutic proteins is illustrated by haemoglobin (Dieryck *et al.* 1997), cytokine Erythropoietin (EPO) (Conley *et al.* 2009b) and antibodies (Ma and Hein 1995). A wide variety of other transgenic plant-derived biopharmaceuticals have been reported (Table 1). Further, plants have great capacity to scale-up production through existing farm infrastructures including established cultivation, harvesting, storage and processing systems, making plants a potential inexpensive source of biopharmaceuticals. It was estimated that 10- to 50-fold lower cost can be achieved by producing therapeutic protein in plants, compared to *E. coli* (Kusnadi

Table 1. Examples of biopharmaceuticals produced in plants.

Class	Protein	Expression Level	Ref.
Antibody	an anti-Foot-and-mouth disease virus scFv[2]	0.4% TSP[1]	(Joensuu *et al.* 2009)
	Human anti-Rhesus D IgG1 antibody	0.6% TSP	(Bouquin *et al.* 2002)
Vaccine	Hepatitis B virus envelop protein	0.05% TSP	(Richter *et al.* 2000)
	Cholera toxin B subunit	0.04% TSP	(Jani *et al.* 2002)
Pharmaceutical protein	Human erythropoietin	<0.0015% TSP	(Conley *et al,* 2009a, Conley *et al.* 2009b)
	Human haemoglobin	0.05% TSP	(Dieryck *et al.* 1997)
	Human epidermal growth factor	0.3% TSP	(Bai *et al.* 2007)

[1]TSP, Total Soluble Portein

[2]scFv, a single chain variable antibody fragment

et al. 1997). These attributes make transgenic plants suitable factories for therapeutic protein manufacturing.

1.2. Low product yield is the bottleneck for molecular pharming development

Although the use of transgenic plants as biopharmaceutical factories is promising, there remain significant challenges limiting their commercial exploitation. Currently, insufficient protein accumulation is a critical limiting factor in the economic value of transgenic plants. Low expression levels of foreign proteins in transgenic plants - at level much lower than 1% of total soluble protein (TSP) in most cases (Table 1) - are commonly reported (Doran 2006).

Low product yield poses a significant challenge for plant expression systems to economically compete with other production systems. Consequently, sufficient protein yield must be achieved before plant-based systems can be exploited as suitable and feasible alternatives to current existing production systems for the large-scale production of biopharmaceuticals. A diverse number of factors influence heterologous protein accumulation in plant systems. Despite the fact that much progress has been made in coping with each of these factors, such as applying strong promoters to enhance the transcript level, using native 3' and 5'–UTRs (untranslated regions) to improve protein translation (Kawaguchi and Bailey-Serres 2005) and targeting recombinant proteins to subcellular compartments, e.g. ER (Menassa et al. 2001) and seed protein storage vacuole (Reggi *et al.* 2005), to prevent protein degradation , insufficient accumulation of recombinant proteins in many cases were still often reported. In many cases, these frustrations prompted molecular pharming scientists to develop novel strategies in the hope of substantially increasing therapeutic protein accumulation in plants.

1.3. Viral vectors for recombinant protein production in plants

Among the strategies adopted to improve recombinant protein production in plants is the application of plant positive-sense RNA viral vectors. Plant viruses have evolved an ability to produce and accumulate high levels of viral proteins, in some cases up to 10-20% of TSP (Turner and Foster 1995). Vectors based on full-length viral genomes have been engineered as plant expression vectors for recombinant proteins,

taking advantage of their innate capacity for high-level genome amplification and concomitant large copy numbers of transgenes in infected plants. However, limitations, such as limited insert size (Avesani *et al.* 2007) and vector instability (loss of transgene) (Varsani *et al.* 2006), still accompany this complete virus vector strategy. More importantly, problems related to low protein accumulation (usually reaching only 0.04-0.3% of TSP), remain unsolved.

Further improvements in viral vectors have been made in order to promote protein yield. Viruses were deconstructed and an integrated system was rebuilt. In these "deconstructed viruses", viral elements which allow for rapid and high-level expression, e.g., potent promoters and translation enhancers, are maintained while other viral functions can be excluded from this system or provided by non-viral components (Gleba *et al.* 2007, Gleba *et al.* 2004). The inefficient or undesirable viral functions such as infectivity, cell to cell movement and assembly of virion particles can be excluded. Other robust and valuable machines such as replication/amplification, gene silencing suppression and systemic spread can be supplemented by other approaches. For example, replication/amplification ability can be supported by the viral elements placed on separate vectors. Vacuum agro-infiltration offers an alternative to efficiently introducing the DNA copy of viral vector into plant cells. This "deconstructed virus" strategy reflects attempts to build up a rapid and high-throughput viral expression vectors for heterologous protein expression. Furthermore, in this "deconstructed virus" system, diverse modifications were also made on the core viral elements in order to greatly exploit their abilities to boost the expression of proteins of interest in plants

Much higher expression levels of plant-made proteins have been reported by using these second generation viral vectors. For example, bioactive human growth hormone (hGH) reached approximately 10% of TSP in *Nicotiana benthamiana* with a *Tobacco mosaic virus* (TMV)-based system, in which functional replicon was assembled from two separate non-functional provector modules delivered by *Agrobacterium* (Gils *et al.* 2005). However, assembly of functional replicons was not necessitated and large quantities of biomass were still obtained. For instance, high accumulation of a full size IgG (human anti-HIV antibody 2G12) and vaccine HBcAg were achieved by using a *Cowpea mosaic virus* (CPMV) RNA-2-derived vector in the absence of RNA-1-derived

replication functions. (Sainsbury and Lomonossoff 2008). In addition, massive increases resulting from the removal of the in-frame start codon at 115 of the 5' leader sequence of CPMV RNA-2 were reported and IgG and HBcAg expressed by this modified vector accumulated >10% and 20% of TSP respectively.

So far, the use of destructed plant positive-sense RNA viral vectors has been demonstrated as the most efficient approach to achieve the high yield of foreign proteins in plants.

1.4. Viral genetic elements for high expression of heterologous proteins

Although there are many advantages, the application of genetically engineered virus-based expression systems raises concerns about biosafety issues (Pogue *et al.* 2002, Scholthof *et al.* 2002) when they are developed for commercial-scale manufacture of desired proteins. The potent ability of plant viral vectors to produce high levels of their own viral proteins and even heterologous proteins prompted us to consider how plant viruses manage to express proteins at high yields and what viral elements are required for their efficient expression. Any such identified genetic element could potentially be exploited for boosting therapeutic protein production. Furthermore, this approach may minimize concerns about environmental risks

Viruses are known for their minimalistic genomes. Although small, the compact and highly efficient genomes allow effective replication and expression of viral genomes, successfully competing for resources with their host cells. Plant viral genomes contain genetic elements like strong transcription promoters (Hohn and Futterer 1992, Sanger *et al.* 1990), and translational enhancers (Turner and Foster 1995). These elements are used by viruses to elevate the expression of viral genes to required high levels (Mushegian and Shepherd 1995).

To date, several viral genetic elements necessary for efficient expression have been uncovered and exploited. *Cauliflower mosaic virus* (CaMV) *35S* promoter is an excellent example. This potent promoter has been engineered and widely used by plant-related research to direct efficient, near-constitutive expression of transgenes in plants. Extensive analysis of the CaMV *35S* promoter sequence delineated the elements contributing to the high gene expression. An enhancer element was found inside the

domain B of the *35S* promoter, which harbors a number of binding sites for cellular *trans*-acting factors (Hohn and Futterer 1992).

Translational enhancers are another important genetic element for virus competitiveness, particularly for positive-strand RNA viruses, genomic RNAs of which are translated early upon infection. A number of plant viral 5' UTRs were discovered to have substantial translational enhancement capacity and a number of properties contribute to their translational enhancement effects. Besides the fact that the 5' UTR of TMV lacks the secondary structure and thus facilitates ribosome scanning for translation initiation (Gallie, 1993), the interaction of the CAA element in the 5' UTR with a specific cellular protein was thought to be involved in the enhancement (Gallie and Walbot 1992). Other viral 5' UTRs and their abilities to enhance translation have been investigated and modified for further application on plant transgene engineering. A hypertranslatable (HT) leader sequence was constructed by deleting the in-frame AUG at the 5' leader sequence of CPMV RNA-2 (Sainsbury and Lomonossoff, 2008). As mentioned above, high product yields, 10% and 20% respectively, were observed for IgG and HBcAg by using this modified leader sequence. But the mechanism of enhancement is unknown. The enhancing capacity of 5' UTRs from different viruses depends on their structures and further studies are underway to investigate their mechanisms and potential application.

In addition to 5' UTRs, many 3' UTRs of plant viruses were also discovered to be involved in translation enhancement, although their activities were first thought to be limited to stabilizing RNA levels (Mitchell and Tollervey 2001). The 3' cap-independent translation element (CITE) located in the 3' UTR of *Barley yellow dwarf virus* RNA is able to enhance translational initiation by delivering translation initiation factor eIF4F to the 5' end (Treder *et al.* 2008). In the presence of the 5' UTR of *Turnip crinkle virus* (TCV), the TCV 3' UTR confers translational enhancement by attracting ribosomal subunits (Stupina *et al.* 2008). Recent reports reveal that the 3' UTRs of many plant viruses have translational enhancers, which recruit translational factors or ribosomes by different mechanisms (Miller *et al.* 2007).

These promoters and UTRs exemplify the utilities of plant viral elements for producing large quantities of recombinant proteins. To date, most of the viral elements employed derive from the regulatory regions of viral genomes, e.g., the promoter, 5'

UTR and 3' UTR. Although such regulatory elements are able to greatly increase the efficiency of transcription and translation, high protein yield or effective virus multiplication is not guaranteed.

Other unknown sources of variability, derived from both the protein of interest and the plant system present challenges to overall protein yield. Plant-based therapeutic proteins tend to show a substantial decrease of production when compared to reporter proteins (for instance, GFP and GUS), which are naturally more stable compared to pharmaceutical proteins. In other words, the efficacy of strategy also relies on the nature of the target protein (Gils *et al.* 2005). In addition, plants possess defense systems against transgene expression or virus propagation (Mittler *et al.* 1995, Roy-Barman *et al.* 2006). Plants have developed diverse strategies against viral infection and multiplication. For example, post-transcriptional gene silencing (PTGS) is a vital part of the plant adaptive immune system against virus infection via sequence-specific mRNA degradation (Lindbo *et al.* 1993, Metzlaff *et al.* 1997). Virus-induced small interfering RNAs (siRNAs) base-pair to their target mRNA and result in mRNA degradation, thus preventing it from producing a protein (Ahlquist 2002). On the other hand, viruses have evolved count-defensive strategies against the detrimental effects of plant defense as well. The versatile viral proteins residing in the coding region are thought to play some important roles in these mechanisms. For instance, the 19-kDa protein (P19) encoded by *Tomato bushy stunt virus* (TBSV) acts as a suppressor of virus-induced gene silencing. P19 homodimers sequester 21-nucleotide (nt) duplex siRNA, preventing the programming of the RNA-induced silencing complex (RISC) (Omarov *et al.* 2007). Suppressing gene silencing by P19 allows efficient TBSV infection in its hosts. Such powerful elements can be used to counteract the plant defense system to ensure efficient transgene expression. More investigations are needed to explore more such genetic elements for appropriately increasing product yield for proteins of interest.

1.5. Potyvirus and potyviral proteins

Among the 34 known plant virus families, Potyvirus is the largest and most agriculturally important group and causes significant losses in agricultural crops. Potyviruses, the major genus in *Potyviridae*, share a similar genomic structure and

expression strategy (Goldbach 1992). The potyviral genome consists of a single-stranded, positive-sense RNA, approximately 10,000 nucleotides in length, with a VPg (viral protein genome linked) covalently attached to its 5' end and a poly (A) tail at the 3' end. The RNA genome encodes an approximate 350 kDa polyprotein which is cleaved by its three proteases, P1, HC-Pro, and NIa-Pro, into ten mature proteins (Urcuqui-Inchima *et al.* 2001). These ten mature viral proteins starting from the N terminus are P1, HC-Pro, P3, 6K1, CI, 6K, VPg, NIa-Pro, NIb, and CP (Riechmann *et al.* 1992) (Figure 1). These ten viral proteins are involved in the viral infection process, during which these viral proteins work together against the defense system of the plant or recruit host factors to replicate the virus. In view of the extremely tiny viral genome, one protein may perform various functions to accomplish virus propagation, in other words, viral proteins are versatile. Understanding the functions and mechanisms of these potyviral proteins involved in virus life cycle sheds light on their utilities for heterologous protein production in plants. Unfortunately, the function(s) of each viral protein involved in the viral life cycle are far from fully understood, and more research is needed to clearly interpret their roles in the viral life cycle before they can be exploited for the production of recombinant proteins in plants.

1.6. Human interleukin 10 as a therapeutic agent

Human interleukin 10 (IL-10) is a cytokine discovered in 1989 (Fiorentino *et al.* 1989). In the last two decades, it has been implicated as a powerful cytokine of crucial importance in immunoregulation. This has led to numerous clinical trials and ultimately its use in practice (Asadullah *et al.* 2003).

Human IL-10 is normally produced in the secretory system by various immune cell populations. Monocytes (de Waal Malefyt *et al.* 1991a) and macrophages (Spits and de Waal Malefyt 1992) are the major sources of IL-10. Co- and post-translational modifications of human IL-10 involve the cleavage of a signal peptide and then the formation of two intra-chain disulfide bonds within monomers, resulting in the biologically active homodimer of IL-10 with a molecular mass of 37 kDa (Delve *et al.* 1998). IL-10 has one potential N-linked glycosylation site but is not glycosylated

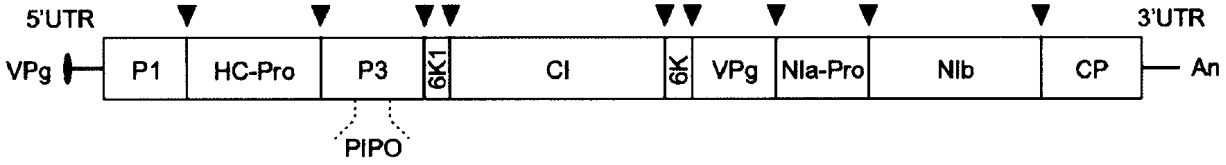

Figure 1. Potyvirus genome organization. The viral genome contains a VPg and a poly (A) at its 5' and 3' end, respectively. The single open reading frame of the RNA genome encodes a large 340 – 370 kDa polyprotein, which is cleaved by its three virus-encoded proteases P1, HC-Pro and NIa, into ten mature proteins designated by boxes. Indicated in or below boxes are the names of the 10 viral proteins, from the N terminus including the first protein (P1), the helper component/protease (HC-Pro), the third protein and pretty interesting *Potyviridae* ORF (P3-PIPO), the first six kDa protein (6K1), the cylindrical inclusion (CI), the second six kDa protein (6K), the viral protein-genome linked (VPg), the nuclear inclusion A (NIa-Pro), the nuclear inclusion B (NIb), the coat protein (CP). Arrows present the positions where three viral proteases cleave the polyprotein. P1 and HC-Pro are responsible for autoproteolytic cleavage at their respective C termini and NIa all the other proteolytic sites.

(Vieira *et al.* 1991). This is in contrast to murine IL-10 which is glycosylated at the N-terminus.

The major immunological function of IL-10 is to alleviate and terminate the inflammatory response through suppressing the expression of inflammatory proteins such as pro-inflammatory cytokines, chemokines and antigen-presenting molecules (de Waal Malefyt *et al.* 1991a, de Waal Malefyt *et al.* 1991b, Fiorentino *et al.* 1991a, Fiorentino *et al.* 1991b, Romagnani 1995). In addition to mediating cellular immunity, IL-10 regulates the differentiation and proliferation of several immune cells, e.g., lymphocytes, monocytes, natural killer cells, and dendritic cells (Allavena *et al.* 1998, Buelens *et al.* 1997, Cai *et al.* 1999).

The impacts of IL-10 on inflammatory factors and immune cells are further supported by experimental research in animals. IL-10 application was observed to be beneficial on IL-10 knockout mice (Kuhn *et al.* 1993, Rennick and Fort 2000), inflammatory animal models for colitis (Herfarth *et al.* 1998, Herfarth *et al.* 1996) and pancreatitis (Van Laethem *et al.* 1995) and autoimmune disease models such as diabetes mellitus (Pennline *et al.* 1994), encephalomyelitis (Rott *et al.* 1994) and Rheumatoid arthritis (Persson *et al.* 1996, Tanaka *et al.* 1996).

Furthermore, several clinical trials suggest that IL-10 has a favorable effect on autoimmune and inflammatory diseases and has been developed for the treatment of Crohn's disease (Deventer *et al.* 1999), rheumatoid arthritis (Bulpitt *et al.* 1999) and psoriasis (Asadullah *et al.* 1999, Asadullah *et al.* 2000).

Its promising clinical value has raised the interest of producing IL-10 in transgenic plants in order to meet the great demand. To date, however, IL-10 does not accumulate at a satisfying level in plants. IL-10 serves as a good representative of a biopharmaceutical protein that is in need of further improvements with respect to its production in plants.

1.7. Objectives of this research

The goal of this project was to identify the *Soybean mosaic virus* (SMV, a member of potyvirus) genetic elements that may be beneficial for the production of large quantities of IL-10 in plants. In order to evaluate the general utilities of SMV elements on

IL-10 accumulation, various genetic elements (e.g., six SMV cistrons, including *HC-Pro, P3, CI, 6K, VPg* and *CP*) were expressed as fusions or coexpressed with IL-10 in *Nicotiana benthanmiana* using transient expression, a rapid analysis approach for detecting gene expression without positional effects on transgenes. Yellow fluorescent protein (YFP) was tagged to fusion proteins to visualize their expression patterns. IL-10 concentrations were further quantitated by enzyme-linked immunosorbant assays (ELISA). Finally, the plant-derived IL-10 was determined if it was assembled to its biologically active form, homodimer in plants.

Chapter 2. Materials and methods

2.1 Plant materials

Nicotiana benthamiana were grown in a Conviron growth cabinet with 16 h light and 8 h dark at 22°C with 80% relative humidity. Five to six-week old *Nicotiana benthamiana* were used for agroinfiltration.

2.2. IL-10 plant expression vectors construction

Gateway cloning technology was used for the generation of IL-10 plant expression vectors (Figure 2). All plasmid vectors were based on two previously described pEarleygate vectors 100 and 101 (Earley *et al.* 2006), both of which contain of an enhanced CaMV *35S* promoter and the octopine synthase terminator (*ocs3'*). Expression plasmids were generated in two different configurations, as fusion or as coexpression (Figure 3). As fusions or as coexpression, the functions of SMV viral proteins were expected to facilitate the IL-10 protein accumulation in plants. HC-Pro, as a gene silencing suppressor (Anandalakshimi *et al.* 1998), may help overcome the post-transcription gene silencing, which has been shown to limit recombinant protein accumulation. P3 and 6K have membrane binding domains (Eiamtanasate *et al.* 2007, Schaad *et al.* 1997), which were expected to anchor the IL-10 protein to a favorable environment to prevent protein from degradation. CI forms conical structure (Rodriguez-Cerezo *et al.* 1997), such a structure may help protect IL-10 protein from degradation. VPg shuts off host protein synthesis (Leonard *et al.* 2000), which may be in IL-10's favor. The high expression of CP protein (Culver *et al.* 1993) may help increase IL-10 expression. To construct these fusion expression vectors, IL-10 fusions (*IL-10* was fused to the 3' end of each SMV gene to generate translational fusions) were inserted to pEarleygate 101. After the gateway LR recombination reaction, the inserts were fused to the *YFP* gene in pEarleygate 101, allowing expression of the IL-10 protein and its fusions with the C-terminal YFP tag. To construct coexpression vectors, *IL-10* alone (as a control) was inserted into pEarleygate 101 and *HC-Pro* alone to pEarleygate 100, which has no YFP tag or HA tag. The *HC-Pro*–containing plasmid was used for coexpression with the control plasmid.

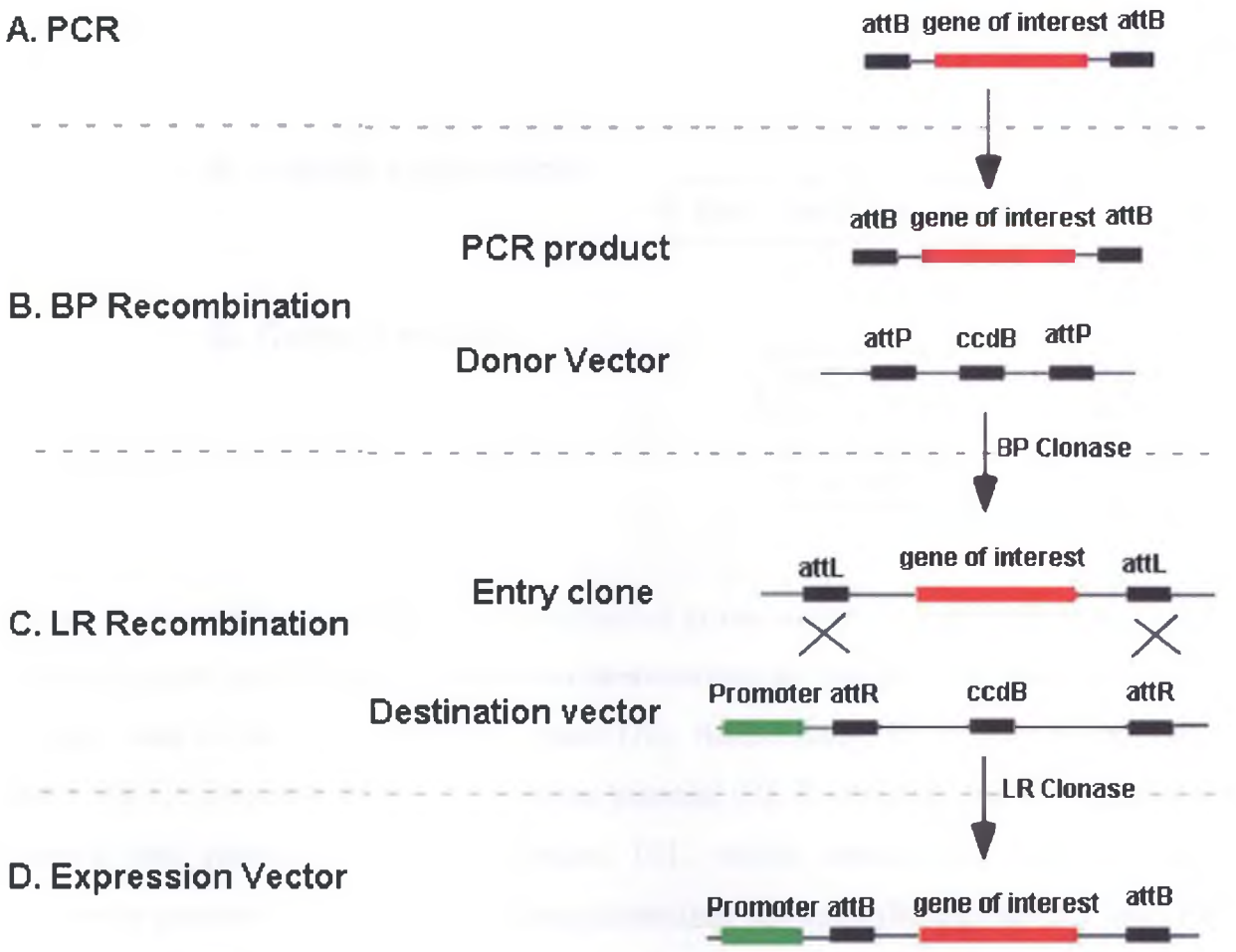

Figure 2. Gateway cloning. In the gateway cloning system, target sequences are transferred to gateway vectors with the aid of the lambda recombination system. (A) Genes of interest are amplified by PCR with 25 bp *att*B recognition sites on both 5' and 3' ends. (B) Recombination between an *att*B-PCR product and an *att*P substrate (donor vector) results in an *att*L-target gene-containing entry clone. Such a process is called BP recombination and is catalyzed by BP Clonase. (C) Recombination between an *att*L entry vector and an *att*R substrate (destination vector) results in an *att*B-target gene-containing expression clone (D). Such a process is called LR recombination and is catalyzed by LR Clonase.

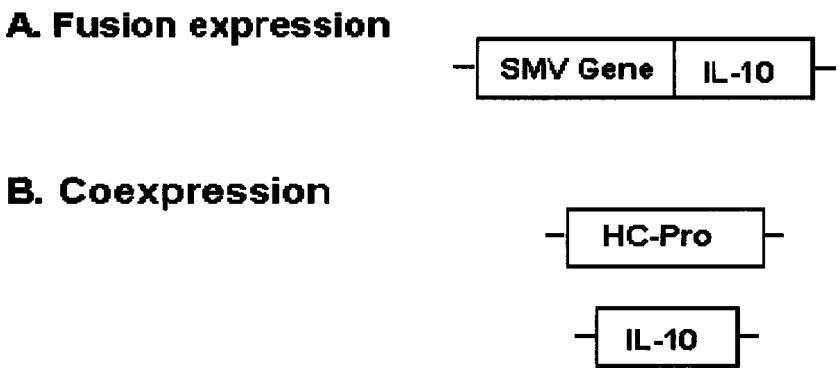

Figure 3. Expression plasmids were constructed in two different configurations. Plasmid constructs were introduced into *Nicotiana benthamiana* as translational fusions where IL-10 was fused to the 3' end of SMV genes (A); Additionally, IL-10 was co-introduced with SMV HC-Pro on a separate expression plasmid (B). *IL-10* alone and its fusions were inserted into gateway vector pEarleygate 101, which contains an in-frame *yellow fluorescent protein* (*YFP*) gene and hemagglutination sequence (ha-tag) at its 3' end. HC-Pro was inserted into pEarleygate 100, which has no *YFP* gene. Both vectors consist of an enhanced *Cauliflower mosaic virus 35S* (CaMV *35S*) promoter and the octopine synthase transcription termination signal (*ocs 3'*).

2.2.1. Gene amplification

All amplifications were conducted in a Techne TC-412 thermocycler (Techne Inc.). Primers used in this study are listed in Table 2. Fragments of *IL-10, HC-Pro, P3, CI, 6K, VPg* and *CP* were amplified using specific primer pairs (*IL-10*, primers 1 and 2; *HC-Pro*, primer 3 and 4 or 5; *P3*, primer 6 and 7, *CI*, primers 8 and 9; *6K*, primers 10 and 11; *VPg*, primers 12 and 13; *CP*, primers 14 and 15) respectively. Additionally, *IL-10* was amplified with 18 base pairs (bp) of *HC-Pro, P3, CI, 6K, VPg* or *CP* for fusion genes construction (primers 2 with 16, 17, 18, 19, 20 or 21). Reactions (50 µL) were set up with 50 ng of plasmid pCaMter X_IL-10_GFP (a generous gift from Dr. Rima Menassa, Agriculture and Agri-food Canada) or plasmid SMV-L, 2 units of Phusion polymerase (Finnzymes), 1× Phusion buffer, 200 µM of each dNTP and 2 µM of each primer. PCR was conducted with a 55°C melting temperature for 30 cycles. The PCR products were analyzed by agarose gel electrophoresis and amplified DNA fragments with the correct size were gel extracted using gel extraction kit (QIAGEN).

2.2.2. Fusion genes construction

The same melting temperature and cycle numbers were used in bridge-overlap-extension PCR for constructing in frame fusion genes including *HC-Pro-IL-10, P3-IL-10, CI-IL-10, 6K-IL-10, VPg-IL-10* and *CP-IL-10* using primers 22 and 23. *IL-10* was fused to the 3' end of each of SMV genes selected in this study. Fusion fragments corresponding to the predicted size were gel extracted. After amplification, *IL-10, HC-Pro* and fusion fragments were subjected to cloning using Gateway vectors (Invitrogen).

2.2.3. Cloning IL-10 and fusion genes into Gateway binary vectors for plant transformation

Gateway BP recombination reactions were performed to transfer PCR products to the donor vector pDONR™221 and entry clones according to manufacturer's instruction. The entry clones were transformed into *E. coli* host DH5α. *E. coli* transformants were cultured in liquid LB medium containing 100 µg/mL kanamycin. Plasmids were isolated with Miniprep Kit (Qiagen) and correct inserts were confirmed by DNA sequencing. For sequencing short fragments, M13 forward and reverse primers (primers 24 and 25) were

used. For long fragments, additional primers (for *HC-Pro* and *HC-Pro-IL-10*, primer 26; for *P3-IL-10*, primer 27; for *CI-IL-10*, primers 28 and 29) were used.

Because both entry clones and destination vectors have neomycin phosphotransferase II (*npt* II) gene (Kanamycin resistance gene), the *npt* II must be cut out before LR reaction. Therefore, identified entry clones with correct sequences were subjected to restriction digestion with *Mlu*I. The larger fragments (containing genes of interest) were gel extracted and then transferred to binary destination vector pEarleygate 100 or 101 via LR recombination reactions following standard condition and procedures recommended by the supplier (Invitrogen). To generate plant expression plasmids, genes were introduced between the *CaMV 35S* promoter and the octopine synthase terminator (*ocs* 3') within the T-DNA borders of these two gateway vectors.

2.3. Introduction of plant expression vectors into *Agrobacterium*

Purified plant expression vectors were transformed into *Agrobacterium* EHA105 via electroporation.

2.4. *Agrobacterium*-mediated plant transformation and leaf tissue sampling

Agrobacterium transformant culture was grown in LB medium with 100 μg/mL kanamycin and 25 μg/mL rifampicin to log phase (OD_{600}=0.8-1.0) at 28°C. Cells were collected by centrifuging at 3,500 rpm for 15 minutes, washed twice with vacuum infiltration medium (10 mM MES, 10 mM $MgCl_2$) and resuspended with infiltration medium to a final OD_{600} of 0.8. *Nicotiana benthamiana* were vacuum infiltrated with various constructs. Particularly, six *Nicotiana benthamiana* were coinfiltrated with two constructs: pIL-10 and pHC-Pro. Leaf tissues (0.25 g) were collected and pooled together from six plants infiltrated with the same construct (s) on each of six consecutive days post infiltration (dpi). These samples were subjected to screening for potential enhancers for IL-10 accumulation using Western blot and ELISA analysis. Based on a preliminary screening, putative enhancers were chosen for further characterization of IL-10 expression. Four 7 mm leaf discs (about 25 mg fresh weight) from each plant (six plants in total) infiltrated with each plant expression vector or the control were collected for six

days. Samples were frozen immediately with liquid nitrogen and kept in a -80°C freezer for subsequence analyses.

2.5. Confocal Imaging

Plant tissue was imaged under a Leica TCS SP2 inverted confocal microscope with a 63× water immersion objective. Fluorescence was detected by a krypton/argon laser. YFP, excited at 514 nm, was captured at 525-650 nm.

2.6. Transient expression analysis of IL-10 expression

2.6.1. Protein extraction

Frozen leaf tissues were homogenized with a tissue mill (Retsch Inc., MM301). The resulting powder was dissolved in 12 volumes (v/w) of protein extraction buffer (phosphate-buffered saline (PBS) pH 7.4, 0.05% v/v Tween 20, 2% w/v polyvinylpolypyrrolidone (PVPP), 4 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/mL leupeptin) (Menassa *et al.* 2001). DTT was excluded from samples subjected to non-reducing SDS-PAGE separation. Samples were vortexed thoroughly before subjecting to centrifugation at 14,000 ×rpm for 10 min at 4°C. The supernatant fraction containing total soluble proteins was collected. Because 6K has a membrane binding domain, the fusion protein 6K-IL-10 is a membrane protein. Therefore, the supernatant obtained from the construct p6K-IL-10 was further centrifuged at 14,000 rpm for 30 min at 4°C, resulting in a crude membrane fraction. Total protein samples subjected to Western blot and ELISA analysis were quantitated by the Bradford method.

2.6.2. Total protein determination by Bradford Assay

Undiluted Bradford reagent Coomassie G-250 Dye (Sigma) was added to a 96 well plate (200 μl/well) and undiluted samples were added to plate of dye (3 μl/sample). The plate was read at 595 nm using a microplate reader (Bio-Rad, 550) following 5 minutes' room-temperature incubation. The sample protein concentrations were determined based on a standard curve obtained by plotting the measured absorbance of the protein-dye conjugates versus the known concentrations of bovine serum albumin (BSA).

2.6.3. Western blot for characterizing IL-10 protein and profiling expression pattern

Protein samples for non-reducing SDS-PAGE separation were not heated. All other samples were heated to 95°C for 5 minutes. Proteins were separated by 10% SDS-PAGE gel at room temperature and then electrotransferred onto PVDF membrane (Bio-Rad) on ice by wet electroblotting. The membrane was blocked with blocking buffer (5% w/v fat-free milk in PBST (0.05% v/v Tween-20 in PBS) at room temperature for 1 hour. The membrane was probed with polyclonal anti-human IL-10 antibodies (R&D systems, AF-217-NA) at 1:5000 dilution in the blocking buffer at room temperature for 1 hour. Horseradish Peroxidase (HRP) conjugated anti-goat IgG (Bio-Rad, 172-1034) was used as the secondary antibody with a 1:5000 dilution. The signal was detected by ECL (Amersham Pharmacia). Between incubations, the membranes were washed three times with PBST.

2.6.4. Determination of IL-10 protein concentration

The concentration of IL-10 expressed in *N. benthamiana* was quantitated by a sandwich ELISA. ELISA plates (Nunc^{TM}) were incubated with purified rat anti-human IL-10 (BD Pharmingen^{TM}, 554705) at the concentration of 2 μg/mL (0.1 M Na_2HPO_4 buffer, PH 9.0) overnight at 4°C. The plates were blocked with blocking buffer (1% w/v BSA in PBST) at room temperature for 1 hour. Recombinant human IL-10 (BD Pharmingen^{TM}, 554611), which was used to calibrate the assay as a positive control, and plant protein extracts with various dilutions with blocking buffer were added into the plates and incubated overnight at 4°C. The plates were incubated at room temperature with 0.5 μg/mL biotinylated anti-human and viral IL-10 antibody (BD Pharmingen^{TM}, 554499) for 1.5 hour and then 1:2500 dilution of avidin-horseradish peroxidase conjugate (BD Pharmingen^{TM}, 554058) for 1 hour. After each incubation with different reagents, the plates were washed with PBST four times. The plates were developed with ABTS substrate solution (0.1 M anhydrous citric acid, 0.03% w/v 2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), pH 4.35, 0.03% v/v H_2O_2 (added immediately before use)). The absorbance values were determined by a microplate reader (Bio-Rad, 550) at 405 nm.

Table 2. Primers used in this study. All oligonucleotides were synthesized by Operon (Eurofins MWG Operon, AL, USA).

Number	Primer sequence 5' to 3'
1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAGCCCAG GCCAGGGCACCCAGT
2	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTTCGTATCTT CATTGTCATG
3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCCCAAAA TCCTGAAGCTCAGT
4	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAACCAACT CTGTAGAATTTCATC
5	GGTGCCCTGGCCTGGGCTACCAACTCTGTAGAATTTCATC
6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTGAAGT GCAACAGAGAATGA
7	GGTGCCCTGGCCTGGGCTCTGTGCGGAGACATCCTCTGAT
8	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAGTCTTGA TGAGATTCAGAACA
9	GGTGCCCTGGCCTGGGCTCTGTAATTGGACTGCATTCAAA
10	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAGCAAAC ATGAGGTGAGTAAGT
11	GGTGCCCTGGCCTGGGCTTTGAGTTGATACTGGCTCGCGT
12	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGAAAG AAGAGGCAGATACAAA
13	GGTGCCCTGGCCTGGGCTTTCCATTTCAACTCTTTC
14	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAGGC AAGGAGAAGGAAGGAG
15	GGTGCCCTGGCCTGGGCTCTGCTGTGGGCCCATGCC
16	AAATTCTACAGAGTTGGTAGCCCAGGCCAGGGCACCCAGT
17	GAGGATGTCTCCGCACAGAGCCCAGGCCAGGGCACCCAGT
18	AATGCAGTCCAATTACAG AGCCCAGGCCAGGGCACCCAGT

19	GAGCCAGTATCAACTCAAAGCCCAGGCCAGGGCACCCAGT
20	GAAAGAGTTGAAATGGAAAGCCCAGGCCAGGGCACCCAGT
21	GGCATGGGCCCACAGCAG AGCCCAGGCCAGGGCACCCAGT
22	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC
23	GGGGACCACTTTGTACAAGAAAGCTGGGTC
24	GTAAAACGACGGCCAG
25	CAGGAAACAGCTATGAC
26	GATCAGAAAGAATCCAAACGGA
27	CGGTGCGAGCCTTTGTGAGGTT
28	GCTGACATGATCCAACATGGAA
29	GTGAAAGTACCTTTTTATGTGC

Chapter 3. Results

3.1. Construction of IL-10 plant expression vectors

3.1.1. Gene amplification and chimeric gene construction

Genes, including *IL-10*, *HC-Pro*, *P3*, *CI*, *6K*, *VPg* and *CP*, were first cloned by PCR individually (Figure 4A). Fusion genes, including *HC-Pro-IL-10, P3-IL-10, CI-IL-10, 6K-IL-10, VPg-IL-10* and *CP-IL-10*, were obtained by bridge-overlap-extension PCR (Figure 4B). PCR products were confirmed by agarose gel electrophoretic analysis. The amplified DNA fragments consistent with the predicted size were gel purified and used for subsequent cloning.

3.1.2. Plant expression vectors construction using Gateway technology

Gel-purified PCR products of *IL-10*, *HC-Pro*, *HC-Pro-IL-10, P3-IL-10, CI-IL-10, 6K-IL-10, VPg-IL-10* and *CP-IL-10* were first inserted into gateway donor vector, pDONR™221, creating entry clones, in which the inserted genes were verified by DNA sequencing. Entry clones were digested with *Mlu*I and the digested products were separated by agarose gel (Figure 5). The resulting larger fragments containing the genes of interest were purified and further recombined into gateway destination vectors, creating IL-10 plant expression plasmids (Figure 6). All transgenes were placed under the control of the enhanced CaMV *35S* promoter and the octopine synthase transcription termination signal (*ocs 3'*). The *YFP* gene in pEarleygate 101 allowed expression of IL-10 protein and its fusions with the C-terminal YFP tag.

3.2. The effects of SMV elements on transient expression of IL-10 in *Nicotiana benthamiana*

3.2.1. Imaging IL-10 and its fusions expression by confocal microscopy

To better understand the effects of SMV genetic elements on IL-10 expression in plants, YFP was tagged to IL-10 and its fusion proteins as described above. This would allow for the visualization of the subcellular localization and the expression of IL-10 and its fusions in *Nicotiana benthamiana* leaf epidermal cells agro-infiltrated with these YFP-containing expression vectors using confocal microscope.

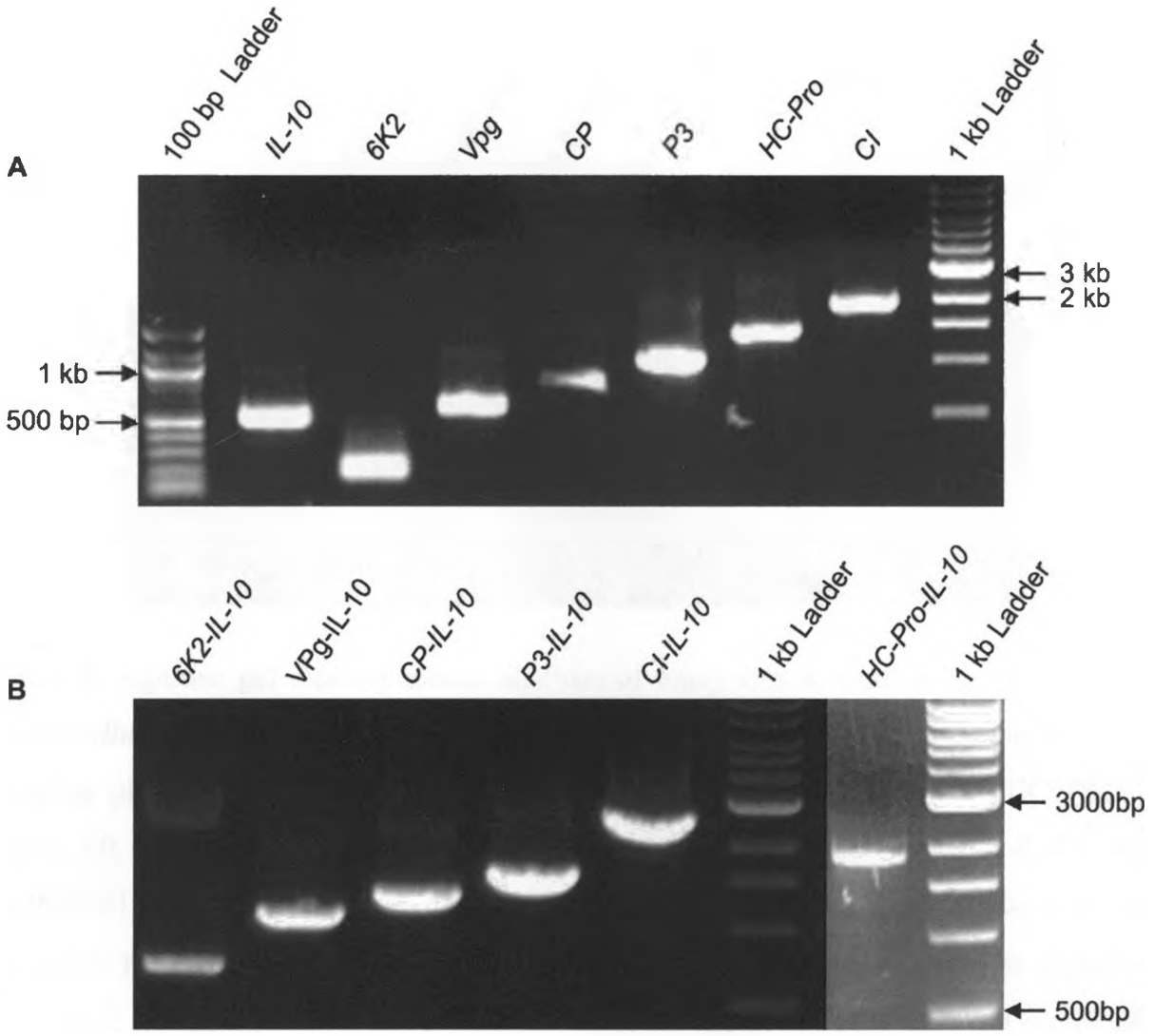

Figure 4. Agarose gel electrophoretic analysis of PCR amplification products of *IL-10*, SMV genes and fusion genes. (A) PCR products of *IL-10* and SMV genes. The first and last lanes are the 100 bp ladder and the 1kb ladder respectively, and the rest are *IL-10* gene and six SMV genes, including *6K*, *VPg*, *CP*, *P3*, *HC-Pro* and *CI*. Fragment sizes were as expected, 544, 223, 644, 869, 1115, 1445, 1976 bp respectively. (B) Bridge-overlap-extension PCR products of fusion genes. Sizes of chimeric fragments were as expected, 703, 1114, 1339, 1585, 2446, 1915 bp respectively.

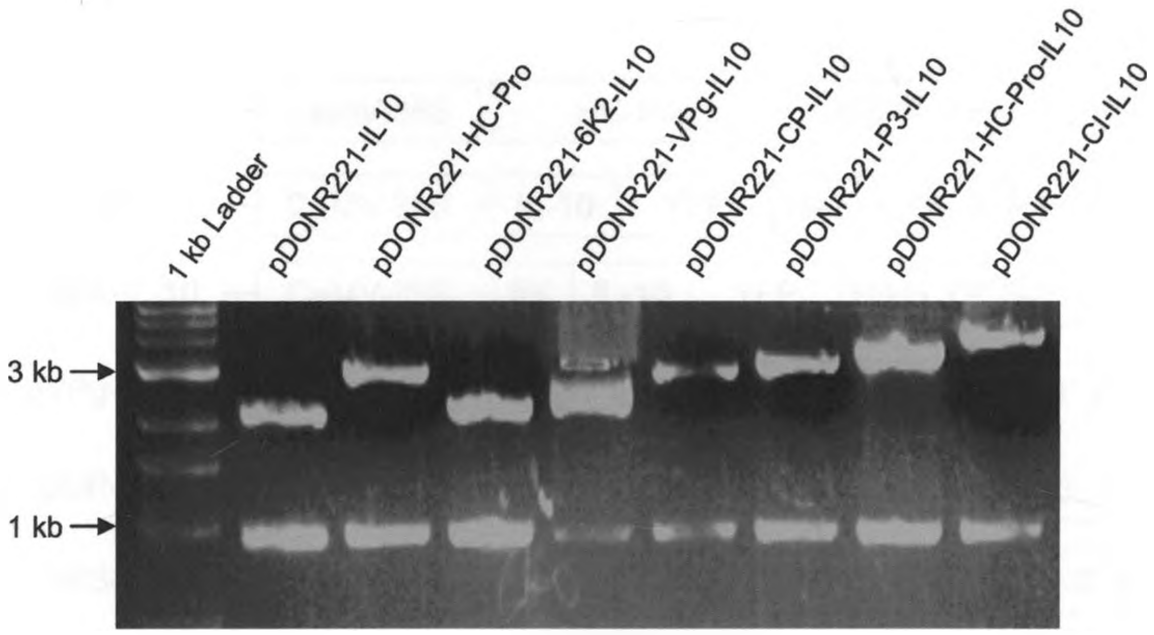

Figure 5. Agarose gel electrophoretic analysis of entry clones digested with restriction enzyme *Mlu*I. The first lane is 1 kb DNA ladder and the rest are the digested entry clones, including pDONR221-IL-10, pDONR221-HC-Pro, pDONR221-6K-IL-10, pDONR221-VPg-IL-10, pDONR221-P3-IL-10, pDONR221-CP-IL-10, pDONR221-CI-IL-10 and pDONR221-HC-Pro-IL-10. The digestion of entry clones with *Mlu*I resulted in two fragments: the large ones contained the genes of interest and the sizes were as expected, 2160, 3050, 2319, 2730, 3155, 3201, 3531, 4062 bp respectively; the small ones, 932 bp, contained the neomycin phosphotransferase II (*NPT* II) gene.

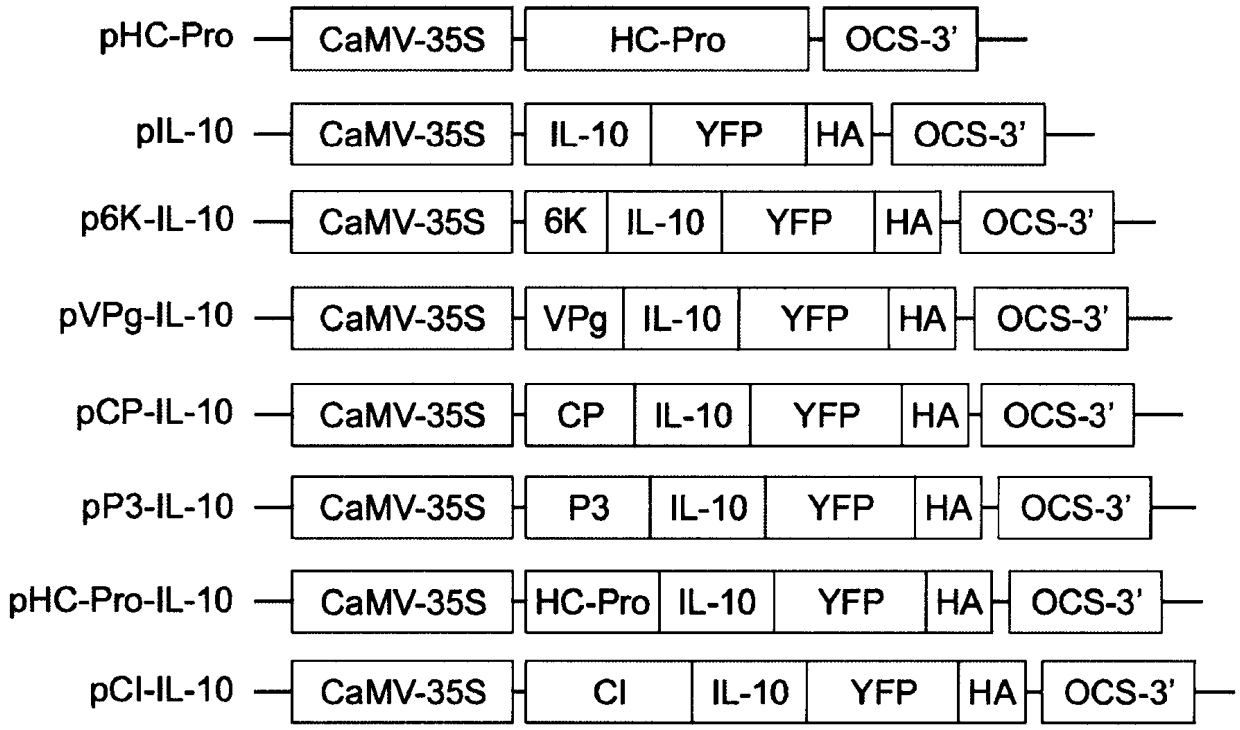

Figure 6. Schematic representation of the plant expression vectors for human interleukin 10 (IL-10) transient expression in *Nicotiana benthamiana*. All transgenes were placed under the control of the enhanced *Cauliflower mosaic virus* 35S (CaMV 35S) promoter and the octopine synthase transcription termination signal (*ocs 3'*). For fusions, *IL-10* was fused to the 3' end of SMV genes (Please refer to figure 1 for SMV genes' abbreviations). Names of constructs are designated by the abbreviations "p" for plasmid and the gene which the construct contains. YFP, yellow fluorescent protein; HA, hemagglutinin epitope.

Fluorescence visualized by confocal microscope revealed the subcellular localization of the fluorescently labeled protein in leaf cells. In the absence of signal peptide and viral proteins, IL-10-YFP fusion expressed by the control construct pIL-10 was detected in cytosol and nuclei (due to diffusion) (Figure 7A). When co-expressed with HC-Pro, IL-10 presented the same distribution pattern in the cell as the control (Figure 7B). However, the localization pattern of IL-10 changed and was dependent on its N-terminus fusion partners when it was expressed as fusions containing different viral elements. Potyviral HC-Pro was distributed throughout the cytoplasm of infected tobacco leaves or protoplasts (Mlotshwa *et al.* 2002). P3, CI and CP were also found in cytoplasm (Rodrı´guez-Cerezo *et al.* 1993, Rojas *et al.* 1997, Riedel *et al.* 1998). In the case of P3, the localization of this protein is virus strain-dependent. The TVMV P3 localizes to cytoplasm (Rodrı´guez-Cerezo *et al.* 1993) while the TEV P3 was found to localize in the nucleus (Langenberg and Zhang 1997). The SMV P3 is on the ER network (Cui and Wang, unpublished data). CI forms aggregates in cytoplasm. CP diffuses across the infected cells. 6K was recently demonstrated to traffic to chloroplasts and attach to the surface of chloroplasts (Wei and Wang, unpublished data). A nucleus localization signal located between residues 40 and 49 in the VPg domain (Schaad *et al.* 1996) was able to translocate the protein VPg to the nuclei and VPg forms inclusion bodies in the nuclei of potyvirus infected cells (Riedel *et al.* 1998). The observed localizations of IL-10 fusion proteins were in good agreement with the known localizations of its fusion partners. Fusions HC-Pro-IL-10-YFP, P3-IL-10-YFP and CI-IL-10-YFP CP-IL-10-YFP mainly localized to the cytoplasm (Figure 8). VPg-IL-10-YFP was evident exclusively in the inclusion bodies located in the nuclei (Figure 9). Colocalization of 6K-IL-10-YFP with chloroplasts was observed (Figure 10).

3.2.2. Screening for SMV elements that enhance IL-10 accumulation in *Nicotiana benthamiana*

3.2.2.1. Analysis of IL-10 protein and its fusions using immunoblotting

A time-course analysis was performed to examine the expression profile of IL-10 and its fusions expressed in *Nicotiana benthamiana*. Plant protein extracts were first separated by reducing SDS-PAGE and further analyzed by immunoblotting. The

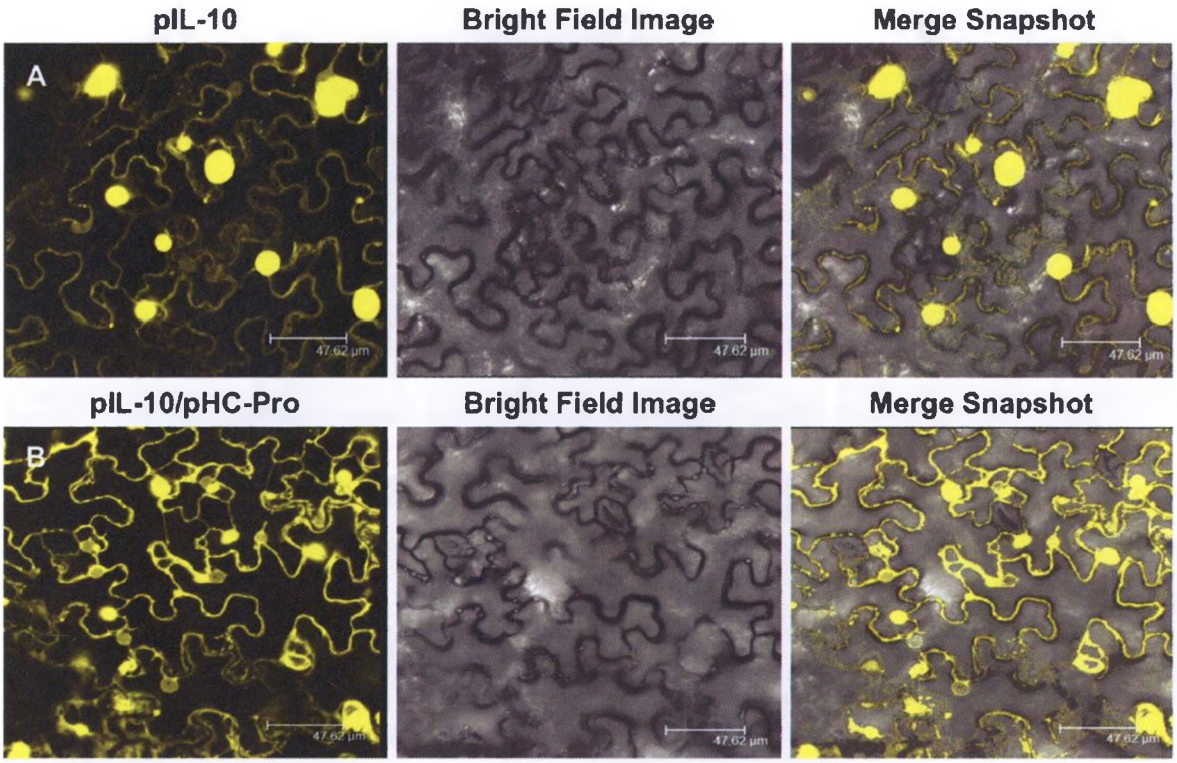

Figure 7. Subcellular localization of human interleukin 10 (IL-10) in *Nicotiana benthamiana* leaf epidermal cells in the absence or presence of HC-Pro. *Nicotiana benthamiana* leaves were agro-infiltrated with construct pIL-10 alone (A) or in combination with construct pHC-Pro (B) and visualized with confocal microscopy. In these constructs, yellow fluorescent protein (YFP) was fused to the C terminus of IL-10. HC-Pro: helper component/protease.

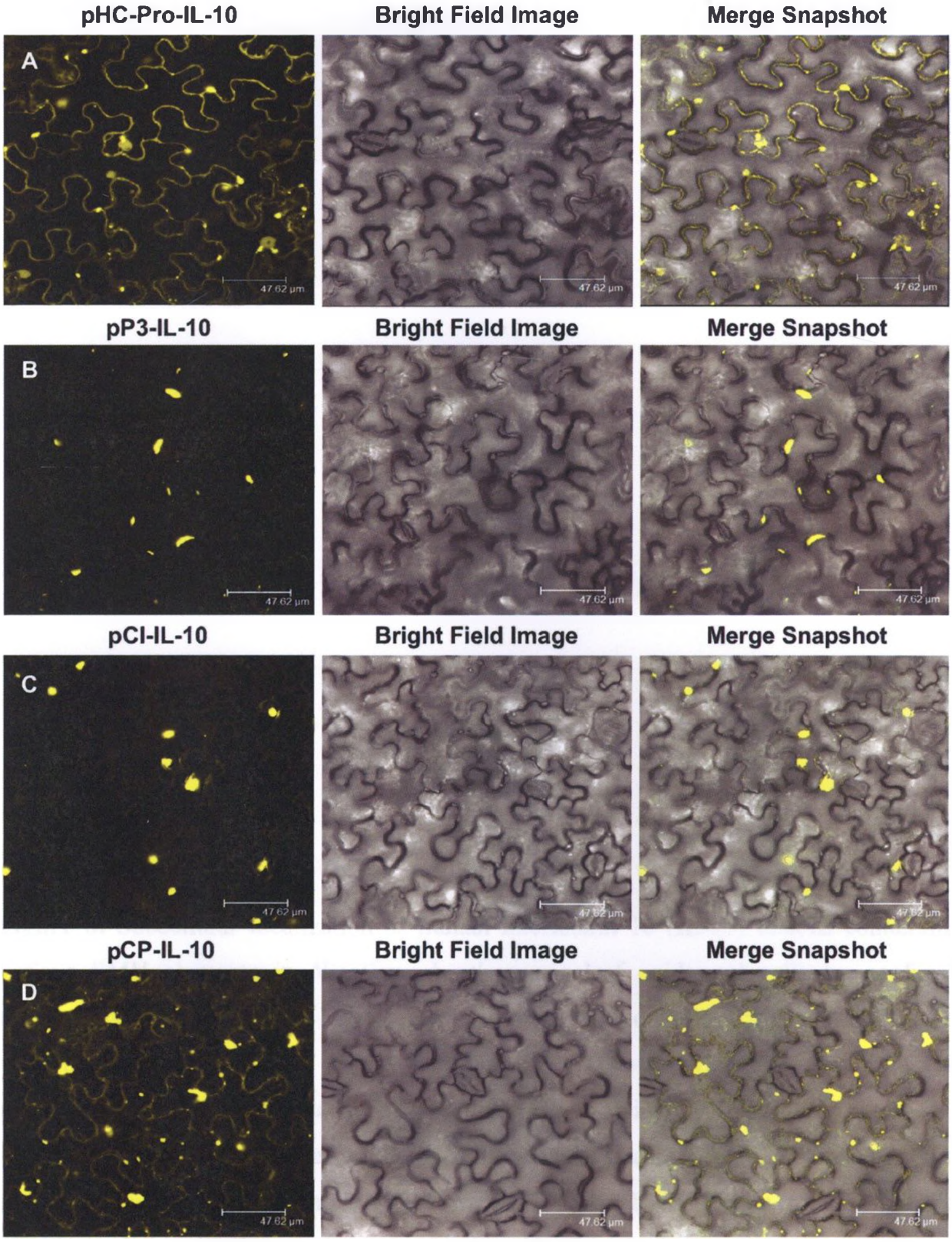
pHC-Pro-IL-10
Bright Field Image
Merge Snapshot
A
47.62 µm
pP3-IL-10
Bright Field Image
Merge Snapshot
B
47.62 µm
pCI-IL-10
Bright Field Image
Merge Snapshot
C
47.62 µm
pCP-IL-10
Bright Field Image
Merge Snapshot
D
47.62 µm

Figure 8. Subcellular localization of human interleukin 10 (IL-10) fusions containing various SMV elements in *Nicotiana benthamiana* leaf epidermal cells by confocal microscopy. *N. benthamiana* leaves agro-infiltrated with constructs pHC-Pro-IL-10 (A), pCP-IL-10 (B), pP3-IL-10 (C) and pCI-IL-10 (D) were visualized by confocal microscopy. In these constructs, yellow fluorescent protein (YFP) was fused to the C terminus of IL-10. From the left to the right: fluorescent image, bright-field image, merge snapshot. The arrows indicate the localization of the IL-10 fusion proteins in the cytoplasm. Bar = 47.62 μm. 63× magnification. HC-Pro, the helper component/protease; P3, the third protein; CI, the cylindrical inclusion; CP, the coat protein.

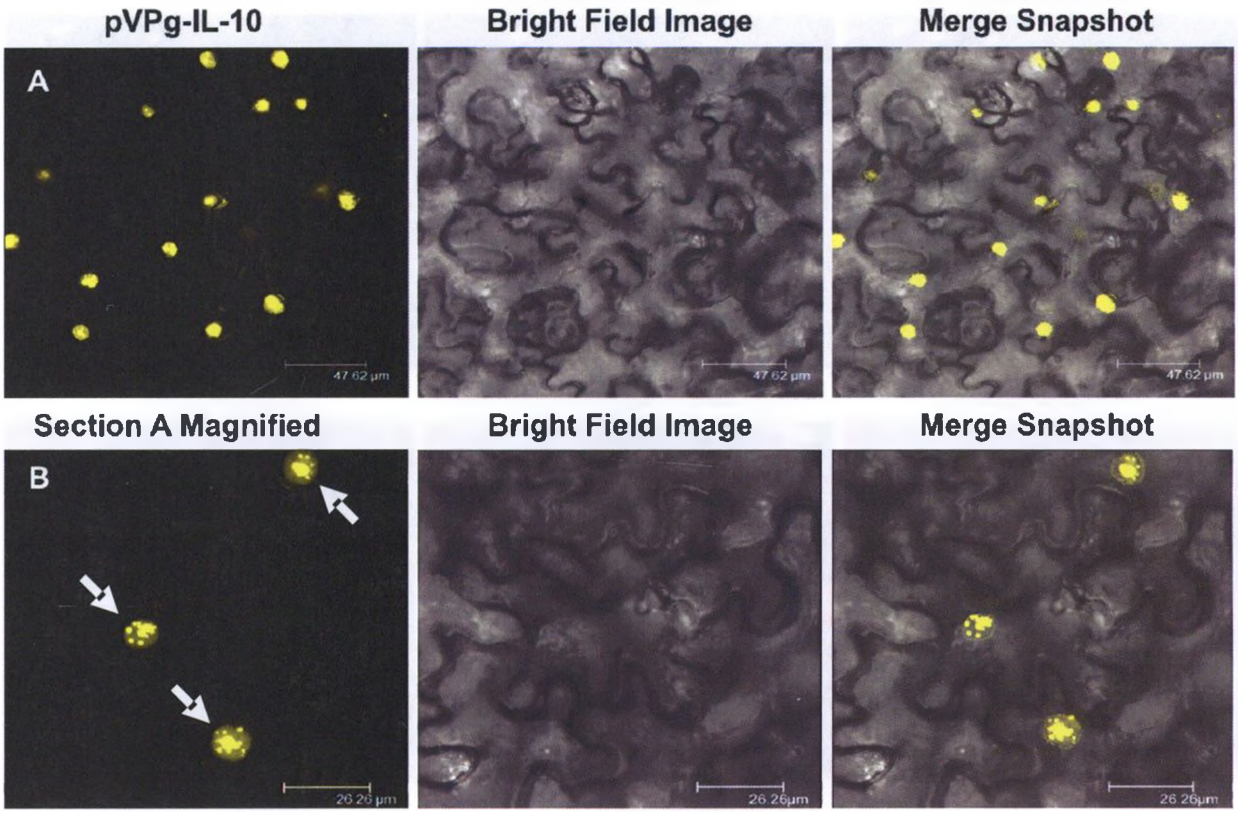

Figure 9. Subcellular localization of human interleukin 10 (IL-10) N-terminus fused with SMV viral protein-genome linked (VPg) in *Nicotiana benthamiana* leaf epidermal cells by confocal microscopy. *N. benthamiana* leaves agroinfiltrated with construct pVPg-IL-10 were visualized by confocal microscopy. In this construct, yellow fluorescent protein (YFP) was fused to the C terminus of IL-10. (A) From left: fluorescent image, bright field image, merged snapshot. (B) Magnified image. The arrows indicate the location of the fusion protein VPg-IL-10-YFP in the nucleus. 63× magnification.

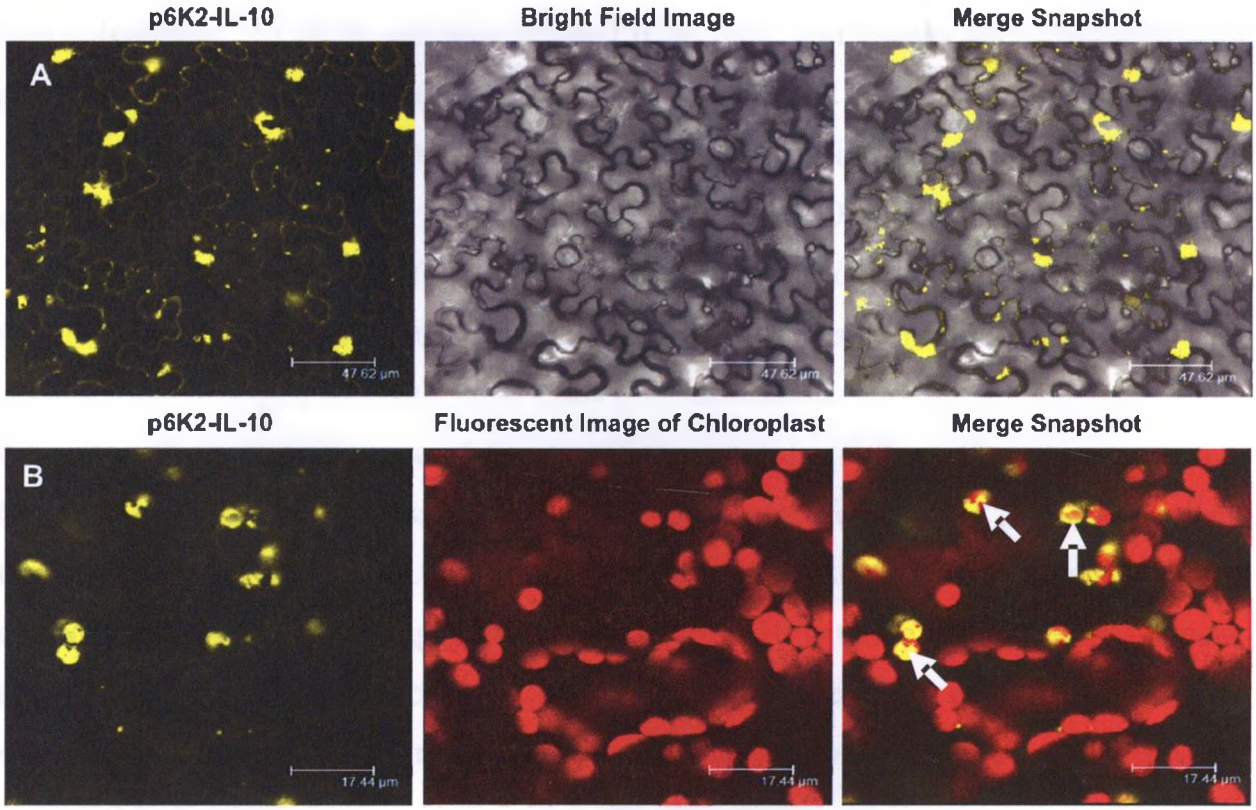

Figure 10. Subcellular localization of human interleukin 10 (IL-10) N terminus fused with the second 6 kDa protein (6K) of SMV in *Nicotiana benthamiana* leaf epidermal cells by confocal microscopy. *N. benthamiana* leaves agroinfiltrated with construct p6K-IL-10 were visualized by confocal microscopy. In this construct, yellow fluorescent protein (YFP) was fused to the C terminus of IL-10. (A) From left: yellow fluorescent image, bright field image and merged image. (B) From left: yellow fluorescent image, red fluorescent image of Chloroplast, merged yellow and red signals. The arrows indicate the colocalization of fusion protein 6K-IL-10-YFP with chloroplast. 63× magnification.

polyclonal IL-10 antibody detected the positive control IL-10-ELP_{30} (a generous gift from Jussi Joensuu), a 31 kDa fusion protein containing IL-10 and 30 repeats of elastic-like polypeptide (ELP). In samples isolated from plant leaf tissues agro-infiltrated with plasmids pIL-10, p6K-IL-10 and pCP-IL-10 or coinfiltrated with two plasmids, i.e., pIL-10 and pHC-Pro, proteins recognized by the IL-10 antibodies corresponded to the predicted molecular mass for the monomeric form of IL-10-YFP (46.5 kDa), 6K-IL-10-YFP (52.5 kDa), CP-IL-10-YFP (76.4 kDa) and IL-10-YFP (46.5 kDa), respectively (Figure 11). Moreover, a typical transient transgene expression profile was observed for plasmid pIL-10. IL-10 was detectable 1 day post infiltration (dpi), remarkably increased 2 dpi and reached its peak accumulation level at 2 and 3 dpi, followed by a steady decline (Figure 11). A similar expression profile was also found for plasmid pCP-IL-10, albeit the expression level of CP-IL-10-YFP was remarkably reduced in comparison with the control (Figure 11). Interestingly, coexpression of IL-10 with HC-Pro extended the peak time of the IL-10 accumulation and delayed the occurrence of the decline. In *Nicotiana benthamiana* leaf tissues agro-infiltrated with plasmid p6K-IL-10, IL-10 expression appeared to increase steadily from 1 to 4 dpi. But overall the IL-10 level seemed to be lower that the control. Due to the appearance of obvious leaf necrosis after 4dpi, no leaf tissues were sampled and analyzed further. No IL-10 expression was detectable from leaves agro-infiltrated with constructs pP3-IL-10, pCI-IL-10 or pVPg-IL-10 (Figure 12).

3.2.2.2. Screening for SMV elements that enhance IL-10 protein yield

Based on the preliminary observation of IL-10 expression profile from various IL-10 expression constructs, IL-10 reached a relatively high expression level at 3 dpi. Accordingly, IL-10 expression in *Nicotiana benthamiana* leaves agro-infiltrated with different IL-10 expression constructs (at 3 dpi) was further quantitated by ELISA. The resulting ELISA data is summarized in Figure 13. As shown in Figure 13, coexpression of SMV HC-Pro greatly increased IL-10 protein yield in *Nicotiana benthamiana*. However, IL-10 fusions containing six SMV elements, namely, HC-Pro, P3, CI, 6K, VPg and CP, resulted in a decline in IL-10 accumulation. The opposite results of HC-Pro on IL-10 accumulation suggest that coexpression of HC-Pro *in trans* has a beneficial effect on the accumulation of recombinant proteins in plants.

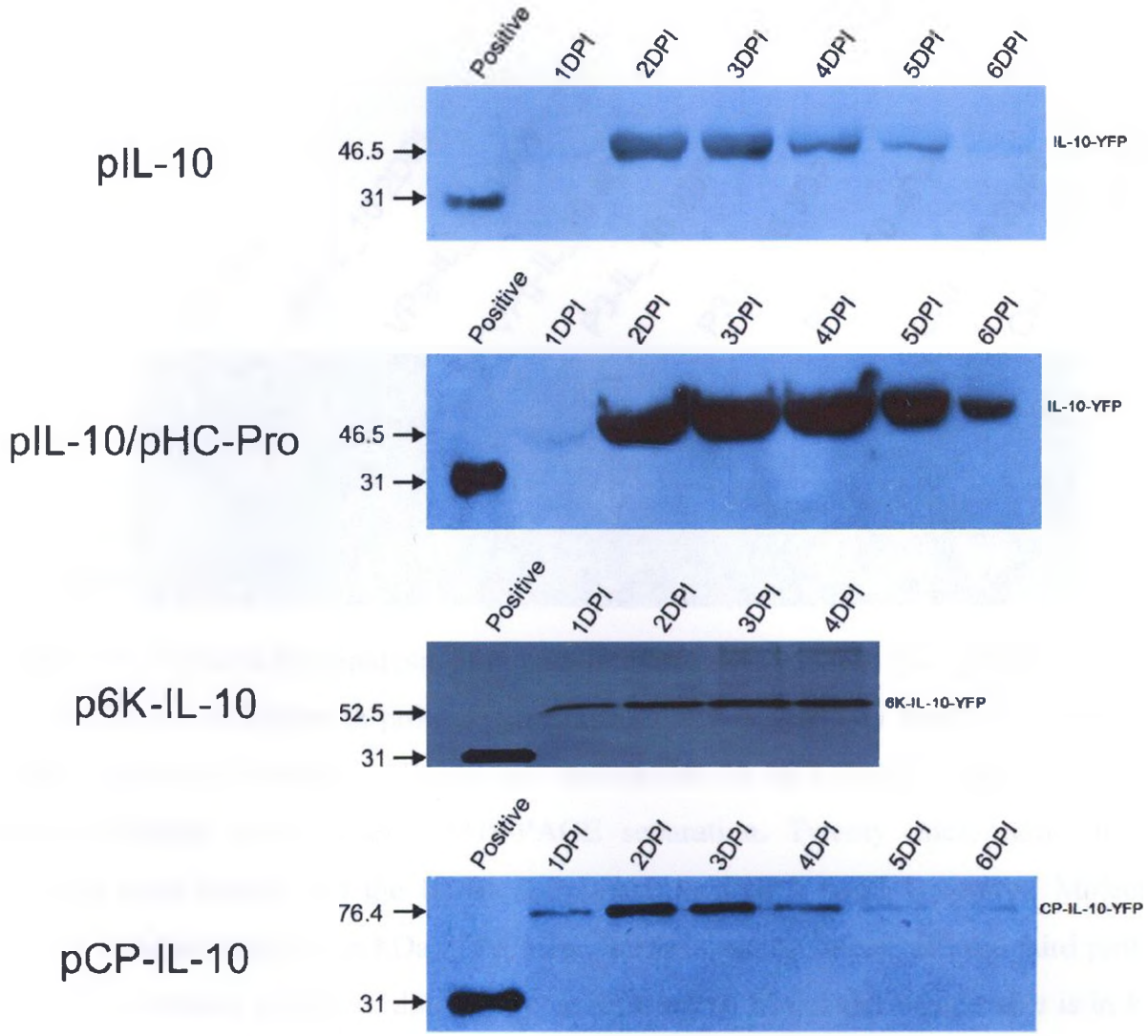

Figure 11. Western blot analysis of human interleukin 10 (IL-10) protein and its fusions in *Nicotiana benthamiana* and their expression profile in 6 days post infiltration (dpi). IL-10 from *N. benthamiana* leaves agroinfiltrated with construct pIL-10, pIL-10 and pHC-Pro, p6K-IL-10 or pCP-IL-10 was detected by immunoblotting after reducing SDS-PAGE separation. Twenty micrograms of total protein were loaded and the IL-10-ELP_{30} with a molecular weight about 31 kDa was used as a positive control. Molecular weight standard sizes are in kDa. ELP_{30}, a tag with 30 repeats of elastic-like polypeptide.

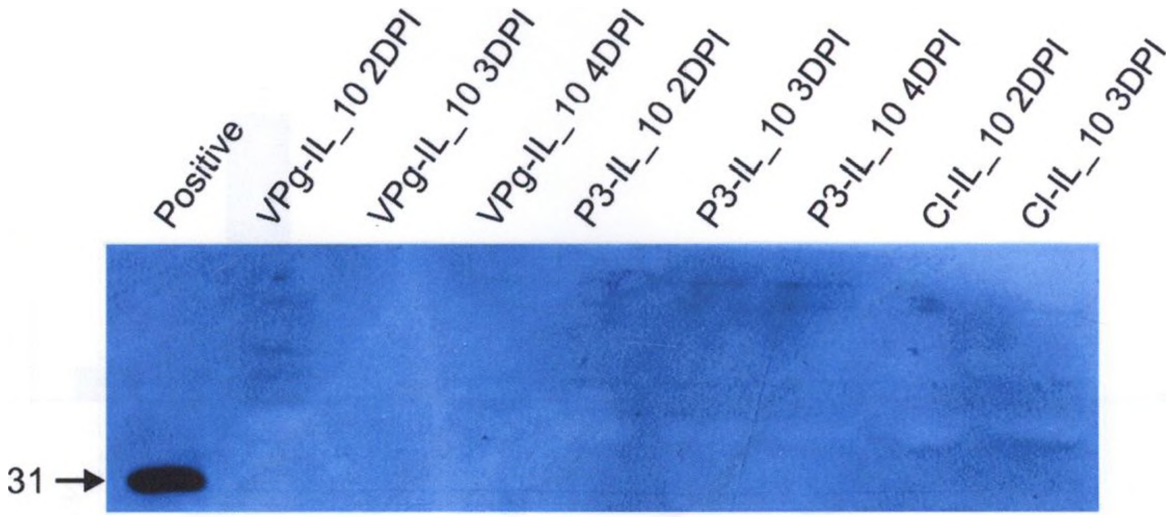

Figure 12. Western blot analysis of human interleukin 10 (IL-10) fused with SMV VPg, P3 and CI. IL-10 fusions in protein extracts from *N. benthamiana* leaves agro-infiltrated with expression vectors p-VPg-IL-10, pP3-IL-10 or pCI-IL-10 were subjected to immunoblotting after reducing SDS-PAGE separation. Twenty micrograms of total proteins were loaded and the IL-10-ELP_{30} was used as a positive control. Molecular weight standard sizes are in kDa. VPg, viral protein-genome linked; P3, the third protein; CI, the cylindrical inclusion; dpi, days post infiltration. Molecular weight size is in kDa. ELP_{30}, a tag with 30 repeats of elastic-like polypeptide.

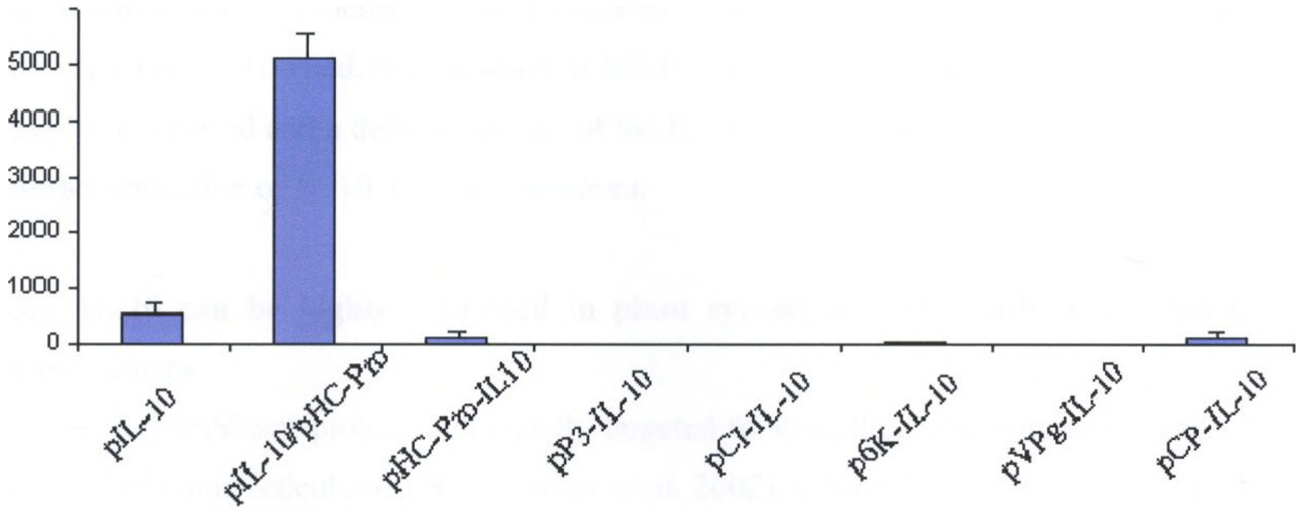

Figure 13. Quantitative analysis of transient human interleukin 10 (IL-10) expression from *Nicotiana benthamiana* leaves agro-infiltrated by various IL-10 expression constructs. Leaf tissues were harvested from six *Nicotiana benthamiana* plants three days post-infiltration (dpi) and the concentration of IL-10 was quantitated by a sandwich enzyme-linked immunosorbant assay (ELISA). Each column represents the average concentration of IL-10 with an error bar showing the standard deviation (n=6). TP, total protein.

3.3. HC-Pro allows high-level expression of IL-10 in *Nicotiana benthamiana*

IL-10 protein yield and its expression profile in *Nicotiana benthamiana* in thepresence or absence of HC-Pro were further determined by ELISA. When coexpressing with HC-Pro, the cytosol-derived IL-10 protein approached an average concentration of 5300 ng/mg TSP (i.e. 0.54% TSP) at 4 dpi (Figure 14), giving an approximate ten-fold increase of IL-10 protein yield compared with the control. Besides its impact on IL-10 yield, coexpression of HC-Pro resulted in a prolonged high-level peak expression period and a delayed decline of the IL-10 level. Thus, SMV HC-Pro enhances the accumulation of IL-10 in *N. benthamiana.*

3.4. IL-10 can be highly expressed in plant cytosol and efficiently assembled to homodimers

Recombinant proteins are usually targeted to subcellular compartments, such as the endoplasmic reticulum (ER) (Ramirez *et al.* 2002), chloroplasts (Rigano *et al.* 2009) and protein storage vacuoles (Mainieri 2004), which are considered favorable environments for protein accumulation and post-translational modification. On the other hand, few biopharmaceuticals, particularly therapeutic proteins, were reported to be highly or functionally expressed in the cytosol (Biocca and Cattaneo 1995, Biocca *et al.* 1994, Schillberg *et al.* 1999).

The gene *IL-10* in the construct pIL-10 contained no signal peptide for any organelle transport and the IL-10 protein was observed to localize to the cytosol of infiltrated leaf cells. In addition, the IL-10-YFP fusion can diffuse to nuclei as well (Figure 7). The above ELISA result indicated that IL-10 from the control accumulates 573 ng/mg, as high as that targeted to the ER (the ER-retained IL-10 neither with ELP or GFP nor in the presence of P19 in Conley, 2009). Furthermore, it reached about 5300 ng/mg when coexpressing with SMV HC-Pro, suggesting that the cytosol-targeted IL-10 is able to accumulate at high levels in plants. Except for the assembly of homodimers via formation of two disulfide bonds within monomers, IL-10 requires no other post-translational modifications such as glycosylation, hydroxylation or proteolytic cleavage (Vieira *et al.* 1991, Windsor *et al.* 1993) (cleavage of the signal peptide is not a concern for the IL-10 protein expressed by the construct pIL-10). To determine whether

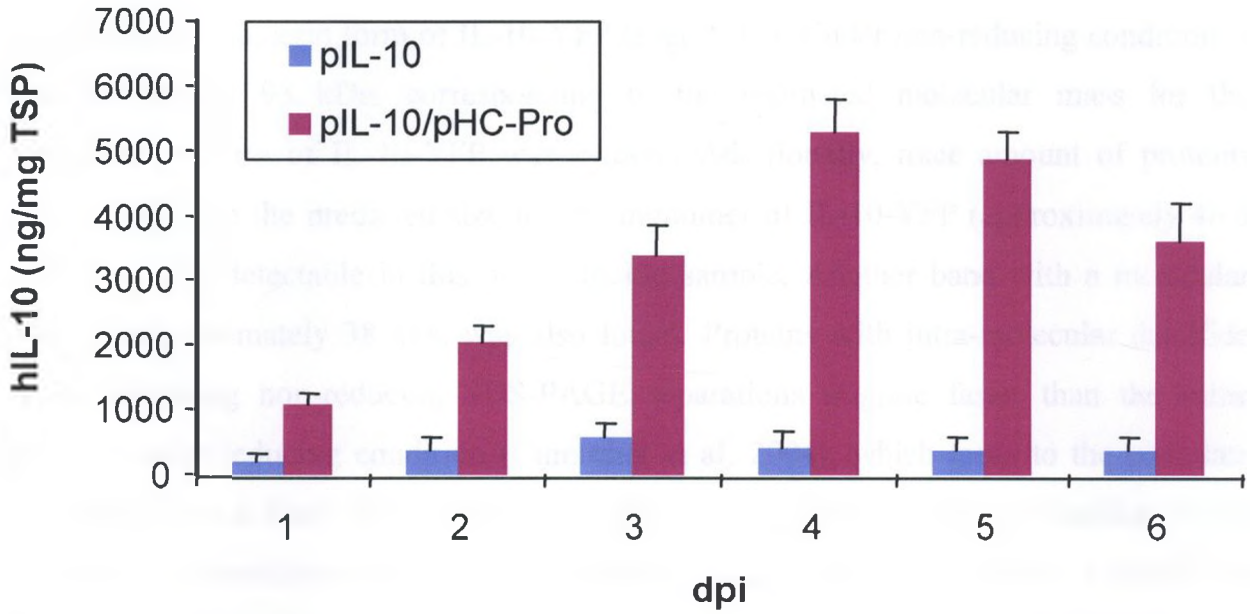

Figure 14. Time-course analysis of accumulation of human interleukin 10 (IL-10) in the absence or presence of HC-Pro following transient expression by agro-infiltration. Leaf tissues were harvested from 6 *Nicotiana benthamiana* plants agro-filtrated by construct pIL-10 alone or coinfiltrated with pIL-10 and pHC-Pro for 6 consecutive days post-infiltration (dpi) and the concentration of IL-10 was quantitated by a sandwich enzyme-linked immunosorbant assay (ELISA). Each column represents the average concentration of IL-10 and the error bar the standard deviation (n=6). HC-Pro, the helper component/protease; TSP, total soluble protein.

the plant-derived IL-10 was assembled into homodimer (the biological active form of IL-10), the plant protein extracts were analyzed by immunoblotting after both reducing and non-reducing SDS-PAGE separations. In the reducing protein sample, a major protein species was detected. This protein was consistent with the predicted molecular mass, 46.5 kDa for the monomeric form of IL-10-YFP (Figure 15). Under non-reducing condition, a band at around 93 kDa, corresponding to the estimated molecular mass for the homodimeric form of IL-10-YFP was evident. Additionally, trace amount of proteins corresponding to the predicted size for the monomer of IL-10-YFP (approximately 46.5 kDa) was also detectable in this non-reducing sample. Another band with a molecular mass of approximately 38 kDa was also found. Proteins with intra-molecular disulfide bonds following non-reducing SDS-PAGE separations migrate faster than the same proteins under reducing condition (Cumming et al. 2004), which leads to the postulate that this protein is likely the monomer of IL-10-YFP with intra-molecular disulfide bonds. Different concentration of DTT will be applied to verify this protein species. Overall, the high ratio of homodimers over monomers indicates this plant-derived IL-10 was efficiently assembled to homodimers.

Based on the above analyses, IL-10 was able to accumulate at high levels in the form of homodimers in plants, suggesting that this plant-based IL-10 is likely biologically active. Altogether, these results suggest that SMV HC-Pro is a viral element that may be used to enhance the accumulation of the biologically active IL-10 in *Nicotiana benthamiana*.

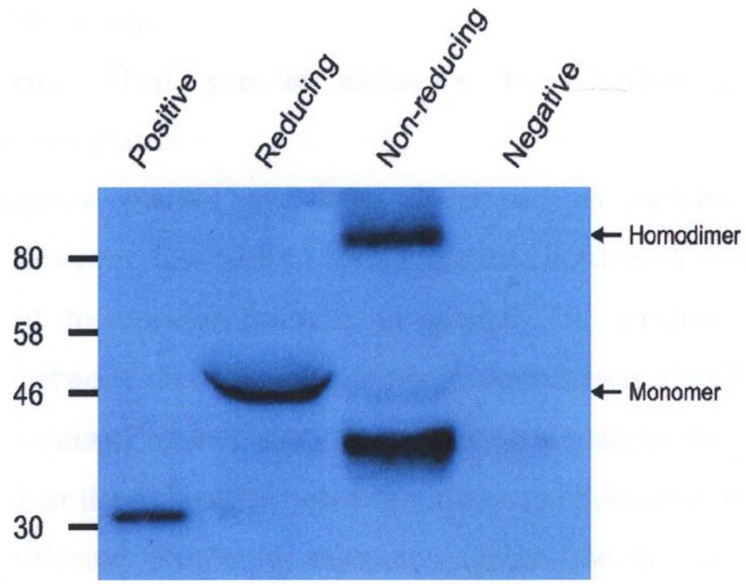

Figure 15. Analysis of plant-derived human interleukin 10 (IL-10) protein. Total soluble protein (TSP) from *Nicotiana benthamiana* leaves coinfiltrated with the construct pIL-10 and pHC-Pro was extracted under reducing condition (DTT was included in the protein extraction buffer) or non-reducing condition (no DTT in extraction buffer) and then subjected to Western blot analysis. TSP extracted under reducing condition from non-transgenic *Nicotiana benthamiana* was used as a negative control. IL-10-ELP_{30} was loaded as a positive control. Total protein extracts (10µg/lane) was loaded. Molecular weight marker is in kDa. The arrows mark the positions of the IL-10-YFP monomeric and dimeric proteins. Moreover, another band in the non-reducing sample with a molecular mass of approximately 38 kDa was postulated to be the monomers, which with intra molecular disulfide bonds migrate faster under non-reducing compared to reducing condition. DTT, dithiothreitol; ELP_{30}, a tag with 30 number of a pentapeptide repeat of elastic-like polypeptide.

Chapter 4. Discussion

4.1. Exploring viral genetic elements for improving recombinant protein accumulation in plants

Transgenic plants have been developed as factories for biopharmaceutical production. However, low protein yield remains a bottleneck problem to be solved for the production of therapeutic proteins in plants. The overall protein yield reflects a competition between the biosynthesis and accumulation of cellular proteins and that of the foreign protein of interest. Such competitions are similar to those between viruses and their hosts. Over the old battles between viruses and host cells, plant viruses have evolved highly sophisticated countering measures against the defense systems of plants. And surprisingly, viruses, with only a few genes, hijack host cellular machineries to manufacture progeny viruses and viral proteins which in some cases accumulate to 10-20% TSP. During infection, viruses could limit or shut off the host cellular protein synthesis through inactivating eukaryotic translation initiation factor eIF4E, the binding of which to mRNA is the rate-limiting step of cap-dependent initiation. This is a way viruses commandeer the host machinery to perform the viral cap independent translation initiation. Thus, lessons can be learnt from the ways viruses act against their hosts and more importantly, useful genetic elements can be exploited as good tools for genetic engineering to benefit recombinant protein production in plants. For instance, *CaMV 35S* promoter has been widely used to direct high-level transcription of foreign genes in plants and 5' UTRs from several viruses are used to promote translation efficiency. The applications of such viral elements have largely improved transgene expression. Most of the viral genetic elements employed in transgene expression reside in the regulatory sequences of viral genomes and the major roles they play are promoting transcription and translation.

However, high transcription and translation do not guarantee a high-level of protein accumulation, suggesting that there are other poorly understood factors affecting foreign protein accumulation in plant systems. There is not a positive correlation between high transcription and protein yield and post-transcriptional gene silencing (PTGS) has been revealed to contribute to this incorrelation. Foreign protein degradation and instability in plants have also been discovered to be responsible for low protein yield.

Therefore, besides increasing transcription and translation, more other factors should be taken into consideration.

In addition to regulatory elements, the coding regions of viruses hold versatile genetic elements, which counter diverse effects of the host defense system that prevents heterologous protein from accumulation in plants. Based on the data presented in this study, SMV HC-Pro is able to accumulate high-level IL-10 protein in plants. One possibility is that HC-Pro helps overcome host defenses such as PTGS, which has been revealed to play a role in transgene expression decline (Johansen and Carrington 2001, Kasschau and Carrington 1998). PTGS is a defense mechanism used by plants against invading viruses and involves sequence-specific RNA degradation. This phenomenon was first described in the early 1990s by plant scientists. Additional copies of a gene encoding chalcone synthase, a key enzyme for pigment production were introduced to petunia, with an expectation that over-expression of the transgene could lead to deeply colored flowers. However, fully or partially white flowers were observed, indicating that both the endogenous gene and the transgene were down-regulated or silenced (Napoli et al. 1990). Later investigations on transgene-induced PTGS revealed that high amounts of transgene transcript (mRNA) serve as efficient templates for plant-encoded RNA dependent RNA polymerases (RdRP) to synthesize double stranded RNA (dsRNA) (Dalmay et al, 2000, Chica and Macino, 2001), which has been found to trigger silencing phenomena in plants, fungi and animals.

During virus infection, high expression of viral genes also triggers PTGS, a process in which 21-25 nt small interfering RNA (siRNA) is cleaved from viral RNA by plant-made Dicer-like enzymes and along with additional proteins, down-regulates target genes. Viruses have evolved proteins that counter the plant silencing defense system. SMV HC-Pro is one of the gene silencing suppressors that have been identified. HC-Pro suppresses gene silencing via binding to 21-25 nt siRNA duplexes and miRNA (microRNA/microRNA*) intermediates (Lakatos *et al.* 2006).

In this project, transient expression of IL-10 in plants was achieved through agro-infiltration of an IL-10 expression plasmid in which the IL-10 gene is directed by a strong, constitutive promoter, the *CaMV 35S* promoter. High expression of foreign gene may trigger the silencing defense system of plants and this may contribute to a rapid decline of

gene expression a few days post agro-infiltration. Since SMV HC-Pro acts as a gene silencing suppressor during virus infection, coexpresison of HC-Pro likely accounts for elevated levels of IL-10 and a prolonged expression peak in *N. benthamiana* epidermal cells.

The observation of the effect of SMV HC-Pro on IL-10 protein accumulation in plants in this study demonstrates that viral genetic elements residing in the coding region of viral genomes can be potentially developed as powerful tools for transgene expression.

4.2. 6K-induced membranous vesicles protect IL-10 from degradation, but inhibit protein extraction efficiency

There is now increasing recognition of the contribution of protein degradation to low recombinant protein yield in plants (Doran 2006, Outchkourov *et al.* 2003). Proteolysis and protein turn-over play an important role in plant metabolism whereas it represents an obstacle to efficient recombinant protein production. Despite the fact that proteins retained in ER are kept away from the severely unfavourable environment of proteolysis in the cytoplasm, ER-derived proteins are reported to be constitutively transported to vacuoles for degradation (Tamura *et al.* 2004). Protein degradation remains a bottleneck problem to be solved for effective plant-derived protein production.

The SMV 6K peptide has a membrane binding domain and was shown to sediment with crude membranes fractions (Schaad *et al.* 1997). The 6K-induced membrane vesicles derived from the ER were observed as a result of interaction between 6K and the ER membrane via the central hydrophobic domain of 6K. Recently, 6K vesicles have been reported to form at ER exit sites (ERES) (Wei and Wang 2008) and further traffic to chloroplasts (Wei and Wang, unpublished data). Therefore, fusing IL-10 with 6K was expected to retain IL-10 in 6K vesicles and thus prevent exposure of IL-10 to the detrimental proteolytic environment of the cytoplasm, thereby stabilizing the protein. Besides, 6K itself forms homodimers via a disulfide bridge (Zamyatnin *et al.* 2006), which would facilitate the formation of IL-10 homodimer.

Despite all these promising advantages, 6K as a membrane protein challenges the efficiency of protein extraction. Usually, 500 μL of the crude membrane fraction was recovered from 10 mL plant tissue lysate (from 1 g plant tissue) but lower expression of

IL-10 from this concentrated plant extract was still observed. The considerably low recovery efficiency of membrane protein extraction impedes the development of 6K as a feasible strategy for enhancing IL-10 yield in plants. However, membranous vesicles are shelters for labile therapeutic proteins and thus it is still potentially a good tool for recombinant protein production.

To make good use of 6K-induced vesicles for recombinant protein production, the development of appropriate approaches, which rely on fundamental knowledge of viral replication processes, is required. The viral replication complex, including virus proteins and some host factors, such as eukaryotic initiation factor (iso) 4E, poly(A)-binding protein and eukaryotic elongation factor 1A, is anchored to 6K-induced membranous vesicles (Thivierge *et al.* 2008). Understanding the mechanisms of how these proteins are associated with 6K-induced vesicles may shed light on the utility of 6K for recombinant protein expression. In other words, if it was possible, recombinant protein without fusing to 6K, could be recruited inside the 6K-induced vesicles, which protect protein from the hazardous environment and on the other hand, protein extraction could be easy.

6K represents the SMV elements in this study which apparently are not beneficial for heterologous protein expression in plants. However, with increasing understanding of their functions and mechanisms, the utilities of these elements may be possible.

4.3. Plant-derived IL-10 is able to accumulate at high levels and efficiently assemble to homodimer

In this study, it is shown that recombinant IL-10 can accumulate at high levels in plants. The control expression construct used in this study (i.e., pIL-10) accumulated IL-10 protein at the level of 573 ng/mg, and when coexpressed with HC-Pro, IL-10 reached the level as high as 5300 ng/mg. This is very encouraging as IL-10 contains no specific signal peptides. It was further demonstrated that the plant-derived IL-10 forms homodimers, a biologically active form of IL-10 (Figure 15).

One of the strategies aimed at enhancing recombinant protein accumulation is the targeting of proteins to organelles, such as the ER, chloroplast and protein storage vacuoles. It has been demonstrated that higher levels of IL-10 protein accumulate in the ER lumen using transient expression systems. Heterologous proteins, if not targeted to a

specific subcellular organelle, often accumulate to low levels. However, organelle targeting is frequently associated with inappropriate post-translational modifications, which may negatively affect therapeutic protein functions. For instance, chloroplasts allow for high expression of IL-10. But, the chloroplast-derived IL-10 only shows the monomeric form, which severely impairs its biological activity (Menassa *et al.* 2004). Surprisingly, the IL-10 localized to cytosol in this study was able to accumulate high levels in plants in the presence of HC-Pro and to form homodimers. A YFP tag and a HA tag were added at the C-terminus of the IL-10 protein and whether these tags contribute to the enhancement of IL-10 protein yield in cytosol and the dimerization of IL-10 protein is not clear. Although some tags were originally developed for the purification of recombinant proteins, they were found to have abilities to increase foreign protein accumulation in plants, such as the 6× His tag (Menassa *et al.* 2004). Further investigations on the roles YFP and HA tag play in promoting IL-10 protein accumulation in plants are underway.

4.4. Conclusion

The goal of this thesis was the expression of high-level IL-10 protein in plants by utilizing SMV genetic elements. Among the six SMV genetic elements investigated in this study, SMV HC-Pro, a gene silencing suppressor, has been demonstrated to enable the high-level accumulation of IL-10 in *Nicotiana benthamiana,* when it was co-expressed with IL-10. In addition, the plant-derived IL-10 protein was efficiently assembled to homodimers, a form of IL-10 with biological activity.

Plant viruses have evolved mechanisms to overcome host resistant responses and successfully turn plants into their factories for manufacturing the progeny virus. Versatile viral proteins play important roles in these processes. Further studies are required to investigate more viral elements which could benefit biopharmaceutical production in plants.

References

Ahlquist, P. (2002) RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science*, **296**, 1270-1273.

Allavena, P., Piemonti, L., Longoni, D., Bernasconi, S., Stoppacciaro, A., Ruco, L. and Mantovani, A. (1998) IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. *Eur J Immunol*, **28**, 359-369.

Anandalakshimi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., Vance, V.B. (1998) A viral supressor of gene silencing in plants. *Proc. Natl. Acad. Sci. USA*, 95: 13079–13084.

Asadullah, K., Docke, W.D., Ebeling, M., Friedrich, M., Belbe, G., Audring, H., Volk, H.D. and Sterry, W. (1999) Interleukin 10 treatment of psoriasis: clinical results of a phase 2 trial. *Arch Dermatol*, **135**, 187-192.

Asadullah, K., Docke, W.D., Sabat, R.V., Volk, H.D. and Sterry, W. (2000) The treatment of psoriasis with IL-10: rationale and review of the first clinical trials. *Expert Opin Investig Drugs*, **9**, 95-102.

Asadullah, K., Sterry, W. and Volk, H.D. (2003) Interleukin-10 therapy--review of a new approach. *Pharmacol Rev*, **55**, 241-269.

Avesani, L., Marconi, G., Morandini, F., Albertini, E., Bruschetta, M., Bortesi, L., Pezzotti, M. and Porceddu, A. (2007) Stability of Potato virus X expression vectors is related to insert size: implications for replication models and risk assessment. *Transgenic Res*, **16**, 587-597.

Biocca, S. and Cattaneo, A. (1995) Intracellular immunization: antibody targeting to subcellular compartments. *Trends Cell Biol*, **5**, 248-252.

Biocca, S., Pierandrei-Amaldi, P., Campioni, N. and Cattaneo, A. (1994) Intracellular immunization with cytosolic recombinant antibodies. *Biotechnology (N Y)*, **12**, 396-399.

Bouquin, T., Thomsen, M., Nielsen, L.K., Green, T.H., Mundy, J. and Dziegiel, M.H. (2002) Human anti-rhesus D IgG1 antibody produced in transgenic plants. *Transgenic Res*, **11**, 115-122.

Buelens, C., Verhasselt, V., De Groote, D., Thielemans, K., Goldman, M. and Willems, F. (1997) Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor. *Eur J Immunol*, **27**, 756-762.

Cai, G., Kastelein, R.A. and Hunter, C.A. (1999) IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur J Immunol*, **29**, 2658-2665.

Chicas, A., Macino, G. (2001) Characteristics of post-transcriptional gene silencing. *EMBO reports*, **2**, 992-996.

Chu, L. and Robinson, D.K. (2001) Industrial choices for protein production by large-scale cell culture. *Current opinion in biotechnology*, **12**, 180-187.

Conley, A.J., Joensuu, J.J., Jevnikar, A.M., Menassa, R. and Brandle, J.E. (2009a) Optimization of elastin-like polypeptide fusions for expression and purification of recombinant proteins in plants. *Biotechnol Bioeng*, **103**, 562-573.

Conley, A.J., Mohib, K., Jevnikar, A.M. and Brandle, J.E. (2009b) Plant recombinant erythropoietin attenuates inflammatory kidney cell injury. *Plant Biotechnol J*, **7**, 183-199.

Crabb, W.D. and Bolin, J. (1999) Protein technologies and commercial enzymes. *current opinion in biotechnology*, **10**, 321-323.

Culver, J.N., Lehto, K., Close, S.M., Hilf, M.E. and Dawson, W.O. (1993) Genomic position affects the expression of tobacco mosaic virus movement and coat protein genes. *Proc Natl Acad Sci U S A*, **90**, 2055-2059.

Cumming R.C., Andon N.L., Haynes P.A., Park M., Fischer W.H., Schubert D. (2004) Protein disulfide bond formation in the cytoplasm during oxidative stress.*J Biol Chem.* **279**, 21749-21758.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., Baulcombe, D.C. (2000) An RNA-dependent RNA polymerase gene in Arabidopsis is required for Posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell, **101**:543-553

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G. and de Vries, J.E. (1991a) Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med*, **174**, 1209-1220.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M.G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. and de Vries, J.E. (1991b) Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med*, **174**, 915-924.

Delves, P.J. (1998) The role of glycosylation in autoimmune disease. *Autoimmunity*, **27**, 239-253.

Delves P, Roitt I. (1998) Encyclopedia of Immunology. 2nd Ed. San Diego: Academic Press.

Dieryck, W., Pagnier, J., Poyart, C., Marden, M.C., Gruber, V., Bournat, P., Baudino, S. and Merot, B. (1997) Human haemoglobin from transgenic tobacco. *Nature*, **386**, 29-30.

Doran, P.M. (2006) Foreign protein degradation and instability in plants and plant tissue cultures. *Trends Biotechnol*, **24**, 426-432.

Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J*, **45**, 616-629.

Eiamtanasate, S., Juricek, M., Yap, Y. (2007) C-terminal hydrophobic region leads PRSV P3 protein to endoplasmic reticulum. ***Virus Genes.*** 35:611-617.

Fiorentino, D.F., Bond, M.W. and Mosmann, T.R. (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*, **170**, 2081-2095.

Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M. and O'Garra, A. (1991a) IL-10 inhibits cytokine production by activated macrophages. *J Immunol*, **147**, 3815-3822.

Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W. and O'Garra, A. (1991b) IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol*, **146**, 3444-3451.

Gallie, D.R. (1993) Posttranscriptional regualtion of gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **44**, 28.

Gallie, D.R. and Walbot, V. (1992) Identification of the motifs within the tobacco mosaic virus 5'-leader responsible for enhancing translation. *Nucleic Acids Res*, **20**, 4631-4638.

Gils, M., Kandzia, R., Marillonnet, S., Klimyuk, V. and Gleba, Y. (2005) High-yield production of authentic human growth hormone using a plant virus-based expression system. *Plant Biotechnol J*, **3**, 613-620.

Goldbach, R. (1992) The recombinative nature of potyviruses: implications for setting up true phylogenetic taxonomy. *Arch Virol Suppl*, **5**, 299-304.

Gomord, V. and Faye, L. (2004) Posttranslational modification of therapeutic proteins in plants. *Curr Opin Plant Biol*, **7**, 171-181.

Herfarth, H.H., Bocker, U., Janardhanam, R. and Sartor, R.B. (1998) Subtherapeutic corticosteroids potentiate the ability of interleukin 10 to prevent chronic inflammation in rats. *Gastroenterology*, **115**, 856-865.

Herfarth, H.H., Mohanty, S.P., Rath, H.C., Tonkonogy, S. and Sartor, R.B. (1996) Interleukin 10 suppresses experimental chronic, granulomatous inflammation induced by bacterial cell wall polymers. *Gut*, **39**, 836-845.

Hohn, T. and Futterer, J. (1992) Transcriptional and translational control of gene expression in cauliflower mosaic virus. *Curr Opin Genet Dev*, **2**, 90-96.

Jani, D., Meena, L.S., Rizwan-ul-Haq, Q.M., Singh, Y., Sharma, A.K. and Tyagi, A.K. (2002) Expression of cholera toxin B subunit in transgenic tomato plants. *Transgenic Res*, **11**, 447-454.

Joensuu, J.J., Brown, K.D., Conley, A.J., Clavijo, A., Menassa, R. and Brandle, J.E. (2009) Expression and purification of an anti-Foot-and-mouth disease virus single chain variable antibody fragment in tobacco plants. *Transgenic Res*. (In press)

Johansen, L.K. and Carrington, J.C. (2001) Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant physiology*, **126**, 930-938.

Kasschau, K.D. and Carrington, J.C. (1998) A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell*, **95**, 461-470.

Kawaguchi, R. and Bailey-Serres, J. (2005) mRNA sequence features that contribute to translational regulation in Arabidopsis. *Nucleic Acids Res*, **33**, 955-965.

Kusnadi, A.R., Nikolov, Z.L. and Howard, J.A. (1997) Production of recombinant proteins in transgenic plants: Practical considerations. *Biotechnol Bioeng*, **56**, 473-484.

Langenberg, W.G., Zhang, L. (1997) Immunocytology shows the presence of tobacco etch virus P3 protein in nuclear inclusions. *J. Struct. Biol.* **118**: 243–247.

Le´onard, S., Plante, D., Wittmann, S., Daigneault, N., Fortin, M.G., Laliberte´, J.-F. (2000) Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. *J. Virol.* 74: 7730–7737.

Lienard, D., Sourrouille, C., Gomord, V. and Faye, L. (2007) Pharming and transgenic plants. *Biotechnol Annu Rev*, **13**, 115-147.

Lindbo, J.A., Silva-Rosales, L., Proebsting, W.M. and Dougherty, W.G. (1993) Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. *Plant Cell*, **5**, 1749-1759.

Ma, J.K., Hein, M.B. (1995) Immunotherapeutic potential of antibodies produced in plants. *Trends Biotechnology,* **13,** 522-527.

Ma, J.K., Hein, M.B. (2001) Immunotherapeutic potential of antibodies produced in plants. *Trends Biotechnology,* **13,** 522-527.

Ma, J.K., Drake, P.M. and Christou, P. (2003) The production of recombinant pharmaceutical proteins in plants. *Nat Rev Genet*, **4**, 794-805.

Menassa, R., Nguyen, V., Jevnikar, A., Brandle, J. (2001) A self-contained system for the field production of plant. *Molecular Breeding*, **8**, 177-185.

Menassa, R., Kennette, W., Nguyen, V., Rymerson ,R., Jevnikar, A., Brandle, J. (2004) Subcellular targeting of human interleukin-10 in plants. *J Biotechnol,* **108**:179-183

Metzlaff, M., O'Dell, M., Cluster, P.D. and Flavell, R.B. (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. *Cell*, **88**, 845-854.

Miller, W.A., Wang, Z. and Treder, K. (2007) The amazing diversity of cap-independent translation elements in the 3'-untranslated regions of plant viral RNAs. *Biochem Soc Trans*, **35**, 1629-1633.

Mitchell, P. and Tollervey, D. (2001) mRNA turnover. *Curr Opin Cell Biol*, **13**, 320-325.

Mittler, R., Shulaev, V. and Lam, E. (1995) Coordinated Activation of Programmed Cell Death and Defense Mechanisms in Transgenic Tobacco Plants Expressing a Bacterial Proton Pump. *Plant Cell*, **7**, 29-42.

Mlotshwa, S., Verver, J., Sithole-Niang, I., Gopinath, K., Carette, J., Van Kammen, A, Wellink, J. (2002) Subcellular location of the helper component-proteinase of Cowpea aphid-borne mosaic virus.*Virus Genes,* **25:** 207-216.

Mushegian, A.R. and Shepherd, R.J. (1995) Genetic elements of plant viruses as tools for genetic engineering. *Microbiol Rev*, **59**, 548-578.

Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell*, **2**, 279-289.

Omarov, R.T., Ciomperlik, J.J. and Scholthof, H.B. (2007) RNAi-associated ssRNA-specific ribonucleases in Tombusvirus P19 mutant-infected plants and evidence for a discrete siRNA-containing effector complex. *Proc Natl Acad Sci U S A*, **104**, 1714-1719.

Outchkourov, N.S., Rogelj, B., Strukelj, B. and Jongsma, M.A. (2003) Expression of sea anemone equistatin in potato. Effects of plant proteases on heterologous protein production. *Plant Physiology*, **133**, 379-390.

Pennline, K.J., Roque-Gaffney, E. and Monahan, M. (1994) Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clin Immunol Immunopathol*, **71**, 169-175.

Pogue, G.P., Lindbo, J.A., Garger, S.J. and Fitzmaurice, W.P. (2002) Making an ally from an enemy: plant virology and the new agriculture. *Annu Rev Phytopathol*, **40**, 45-74.

Ramirez, N., Ayala, M., Lorenzo, D., Palenzuela, D., Herrera, L., Doreste, V., Perez, M., Gavilond, J.V. and Oramas, P. (2002) Expression of a single-chain Fv antibody fragment specific for the hepatitis B surface antigen in transgenic tobacco plants. *Transgenic Res*, **11**, 61-64.

Reggi, S., Marchetti, S., Patti, T., De Amicis, F., Cariati, R., Bembi, B. and Fogher, C. (2005) Recombinant human acid beta-glucosidase stored in tobacco seed is stable, active and taken up by human fibroblasts. *Plant Mol Biol*, **57**, 101-113.

Richter, L.J., Thanavala, Y., Arntzen, C.J. and Mason, H.S. (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nature biotechnology*, **18**, 1167-1171.

Riechmann, J.L., Lain, S. and Garcia, J.A. (1992) Highlights and prospects of potyvirus molecular biology. *J Gen Virol*, **73** :1-16.

Riedel, D., Lesemann, D., Mai, E. (1998) Ultrastructural localization of nonstructural and coat proteins of 19 potyviruses using antisera to bacterially expressed proteins of plum pox potyvirus. *Archives of Virology,* **142:** 2133-2158.

Rigano, M.M., Manna, C., Giulini, A., Pedrazzini, E., Capobianchi, M., Castilletti, C., Di Caro, A., Ippolito, G., Beggio, P., De Giuli Morghen, C., Monti, L., Vitale, A. and Cardi, T. (2009) Transgenic chloroplasts are efficient sites for high-yield production of the vaccinia virus envelope protein A27L in plant cellsdagger. *Plant Biotechnol J,* 7:577-591.

Rodrı´guez-Cerezo, E., Ammar, E.D., Pirone, T.P., Shaw, J.G. (1993) Association of the non-structural P3 viral protein with cylindrical inclusions in potyvirus-infected cells. *J. Gen. Virol.* 74, 1945–1949.

Rojas, M.R., Murillo Zerbini, F.M., Allison, R.F., Gilbertson, R.L., Lucas, W.J. (1997) Capsid protein and helper component-proteinase function as potyvirus cell-to-cell movement proteins. *Virology* 237, 283–295.

Romagnani, S. (1995) Biology of human TH1 and TH2 cells. *J Clin Immunol*, **15**, 121-129.

Rott, O., Fleischer, B. and Cash, E. (1994) Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur J Immunol*, **24**, 1434-1440.

Roy-Barman, S., Sautter, C. and Chattoo, B.B. (2006) Expression of the lipid transfer protein Ace-AMP1 in transgenic wheat enhances antifungal activity and defense responses. *Transgenic Res*, **15**, 435-446.

Sainsbury, F. and Lomonossoff, G.P. (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiology*, **148**, 1212-1218.

Sanger, M., Daubert, S. and Goodman, R.M. (1990) Characteristics of a strong promoter from figwort mosaic virus: comparison with the analogous 35S promoter from cauliflower mosaic virus and the regulated mannopine synthase promoter. *Plant Mol Biol*, **14**, 433-443.

Schaad, M.C., Haldeman-Cahill, R., Cronin, S., Carrington, J.C. (1996) Analysis of the VPg-proteinase (NIa) encoded by tobacco etch potyvirus: effects of mutations on subcellular transport, proteolytic processing, and genome amplification. *J. Virol,* 70: 7039–7048.

Schaad, M.C., Jensen, P.E. and Carrington, J.C. (1997) Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. *Embo J*, **16**, 4049-4059.

Schillberg, S., Zimmermann, S., Voss, A. and Fischer, R. (1999) Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in Nicotiana tabacum. *Transgenic Res*, **8**, 255-263.

Scholthof, K.B., Mirkov, T.E. and Scholthof, H.B. (2002) Plant virus gene vectors: biotechnology applications in agriculture and medicine. *Genet Eng (N Y)*, **24**, 67-85.

Spits, H. and de Waal Malefyt, R. (1992) Functional characterization of human IL-10. *Int Arch Allergy Immunol*, **99**, 8-15.

Stupina, V.A., Meskauskas, A., McCormack, J.C., Yingling, Y.G., Shapiro, B.A., Dinman, J.D. and Simon, A.E. (2008) The 3' proximal translational enhancer of Turnip crinkle virus binds to 60S ribosomal subunits. *RNA*, **14**, 2379-2393.

Swartz, J.R. (2001) Advances in Escherichia coli production of therapeutic proteins. *current opinion in biotechnology*, **12**, 195-201.

Tamura, K., Yamada, K., Shimada, T. and Hara-Nishimura, I. (2004) Endoplasmic reticulum-resident proteins are constitutively transported to vacuoles for degradation. *Plant J*, **39**, 393-402.

Thivierge, K., Cotton, S., Dufresne, P.J., Mathieu, I., Beauchemin, C., Ide, C., Fortin, M.G. and Laliberte, J.F. (2008) Eukaryotic elongation factor 1A interacts with Turnip mosaic virus RNA-dependent RNA polymerase and VPg-Pro in virus-induced vesicles. *Virology*, **377**, 216-225.

Treder, K., Kneller, E.L., Allen, E.M., Wang, Z., Browning, K.S. and Miller, W.A. (2008) The 3' cap-independent translation element of Barley yellow dwarf virus binds eIF4F via the eIF4G subunit to initiate translation. *RNA,* **14**, 134-147.

Turner, R. and Foster, G.D. (1995) The potential exploitation of plant viral translational enhancers in biotechnology for increased gene expression. *Mol Biotechnol*, **3**, 225-236.

Urcuqui-Inchima, S., Haenni, A.L. and Bernardi, F. (2001) Potyvirus proteins: a wealth of functions. *Virus Res*, **74**, 157-175.

Van Laethem, J.L., Marchant, A., Delvaux, A., Goldman, M., Robberecht, P., Velu, T. and Deviere, J. (1995) Interleukin 10 prevents necrosis in murine experimental acute pancreatitis. *Gastroentero,*. **108**, 1917-1922.

Vieira, P., de Waal-Malefyt, R., Dang, M.N., Johnson, K.E., Kastelein, R., Fiorentino, D.F., deVries, J.E., Roncarolo, M.G., Mosmann, T.R. and Moore, K.W. (1991) Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proc Natl Acad Sci U S A*, **88**, 1172-1176.

Wei, T. and Wang, A. (2008) Biogenesis of cytoplasmic membranous vesicles for plant potyvirus replication occurs at the endoplasmic reticulum exit sites in a COPI- and COPII-dependent manner. *J Virol.* 82:12252-12264

Windsor, W.T., Syto, R., Tsarbopoulos, A., Zhang, R., Durkin, J., Baldwin, S., Paliwal, S., Mui, P.W., Pramanik, B., Trotta, P.P. and et al. (1993) Disulfide bond assignments and secondary structure analysis of human and murine interleukin 10. *Biochemistry*, **32**, 8807-8815.

Zamyatnin, A.A., Jr., Solovyev, A.G., Bozhkov, P.V., Valkonen, J.P., Morozov, S.Y. and Savenkov, E.I. (2006) Assessment of the integral membrane protein topology in living cells. *Plant J*, **46**, 145-154.