INSTITUTO TECNOLÓGICO Y DE ESTUDIOS SUPERIORES DE MONTERREY

CAMPUS MONTERREY
DIVISIÓN DE BIOTECNOLOGIA Y ALIMENTOS
PROGRAMA DE GRADUADOS EN BIOTECNOLOGIA

TECNOLÓGICO DE MONTERREY®

"Expression of the Recombinant Human Granulocyte-Colony Stimulating Factor (rhG-CSF) with Zera® in NT1 cells, and suppression of gene silencing by the p19 protein of Tombusvirus."

TESIS

PRESENTADA COMO REQUISITO PARCIAL PARA OBTENER EL GRADO ACADEMICO DE:

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POR:

ISRAEL ALFONSO RAMIREZ ALANIS

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Los miembros del comité de tesis recomendamos que el presente proyecto de tesis presentado por el Q.B.P. Israel Alfonso Ramírez Alanis, sea aceptado como requisito parcial para obtener el grado académico de:

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Abreviations

%	percentage
(IL)-2	human interleukine 2
(IL)-4	Human interleukine 4
°C	Celsius grade
µL	Microliter
A	adenine
bia	antibiotic bialaphos
Bp	Base pairs
BSA	Bovine serum albumine
C	citosine
CHO cells	Chinese Hamster Ovary Cells
$CuSO_4$	Cupper sulfate
CymRSV	*Cymbidum ringspot tombusvirus*
dH2O	Distilated wáter
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide tri-phosphate
dsDNA	Denaturing double stranded DNA
EDTA	Ethylendiamin tetra acetic acid
EtOH	ethanol
G	guanine
G-CFU	Granulocyte-Colony Forming Unity
gly G-CSF	Glicosilated human granulocyte colony stimulating factor
GM-CFU	Granulocyte/Monocyte-Colony Forming Unity
GM-CSF	Granulocyte/Macrophage-Colony Stimulating Factor
GS	Glutamine synthetase

hG-CSF	Human Granulocyte-Colony Stimulating Factor
HNE	human neutrophil derived elastase
hr.	Hour
Kb	kilobase
KH_2PO_4	Monopotassium phosphate
LB-Medium	Luria Bertani Medium
MCS	Multiple cloning site
MES	2-N-morpholinoethanesulfonic acid
mg/ml	Milligrams per mililiter
$MgSO_4$	Magnesium sulfate
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
mRNA	messenger ribonucleic acid
MS salts	Murishage and Skoog
NaCl	Sodium clorhidre
NaOH	Sodium hydroxide
NG- GSCF	Non Glicosilated human granulocyte colony stimulating factor
NT1	*Nicotiana tabacum*
NTK	NT-1 medium containing 50 µg/mL kanamycin
OD600	Optical density at 600 nm
ON	Over night
ONC	Over night cultura
ORFs	Open Reading frames
PAT	phosphinothricin acetyltransferase
PBS	Phosphate buffer saline
PCR	Polimerase chain reaction

PEG MW 7500	Polyetilenglicol molecular weight 7500
PTGS	post-transcriptional gene silencing
PVDF	Polyvinylidene fluoride
r.p.m	Revolution per minute
rhG-CSF	Recombinant Human Granulocyte-Colony Stimulating Factor
RISC	RNA-induced silencing complex
RT	Room temperature
s	segunde
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interferring RNA
ssDNA	two single stranded DNA
ssRNA	two single stranded RNA
T	thiamine
TAE	Tris-Acetat-EDTA
TBE	Tris-Borat-EDTA
TCA	Trichloroacetic acid
TCV	Turnip Crinkle Virus
uF	Microfaradays
UTR	Unstraslated región
UV	Ultre violet
Vol	volume
Vol.	Voltius
w/v	Weight/volume
YM	Yeast malt medium
ZEB	Zera Extraction Buffer
Ω	Ohms

1. INTRODUCTION

1.1 Human Granulocyte-Colony Stimulating Factor (hG-CSF)

The Granulocyte-Colony Stimulating Factor is the main cytokine that regulates granulopoiesis. The function of this cytokine is the production of granulocytes, having effect on the proliferation, differentiation and activation of the neutrophil stem cells, specifically on the GM-CFU (Granulocyte/Monocyte-Colony Forming Unity) and G-CFU (Granulocyte-Colony Forming Unity) (Fig. 3). In this way, it is possible to elevate the levels of mature and active neutrophils in the organism. Such neutrophils are white cells or leucocytes, which are in charge of protecting the human body by phagocytosis, killing bacteria that get to invade tissues, as well as other microorganisms such as fungi (Fig.1)

1).

The mature hG-CSF is a 19.6 kDa glycoprotein, made up 174 aminoacids, two intramolecular disulphide bonds, one free cystein at the residue 17, and a carbohydrate chain bound to the Thr amino acid at residue 133 (Fig. 2). Monocytes, macrophages, stromal cells, endotelial cells and fibroblasts are the cells responsible for the production of the hG-CSF. The expression of this cytokine is driven under the exposure to endotoxins and inflammation reactions (2, 3, 4, 5, 1, 6, 7).

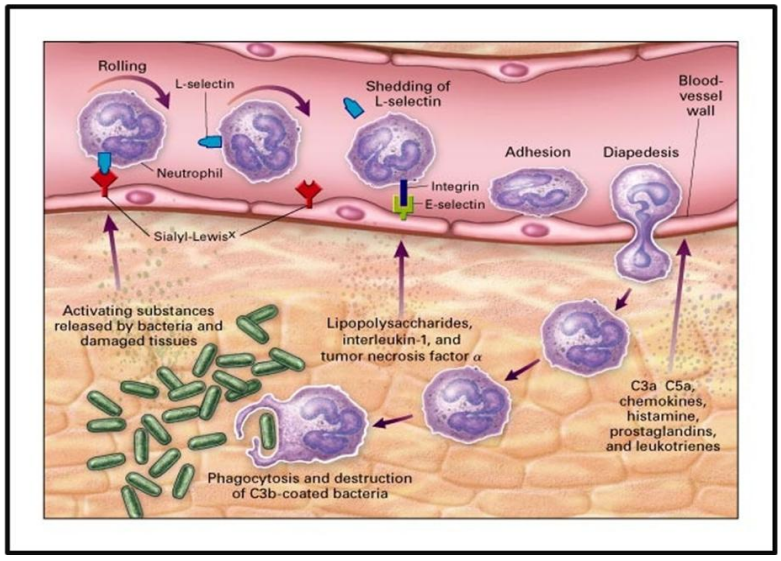


Figure 1. Phagocytic activity and chimiotaxis of mature neutrophils, stimulated by endotoxins and inflamation. Taken from Peter et al., 1984 [2].

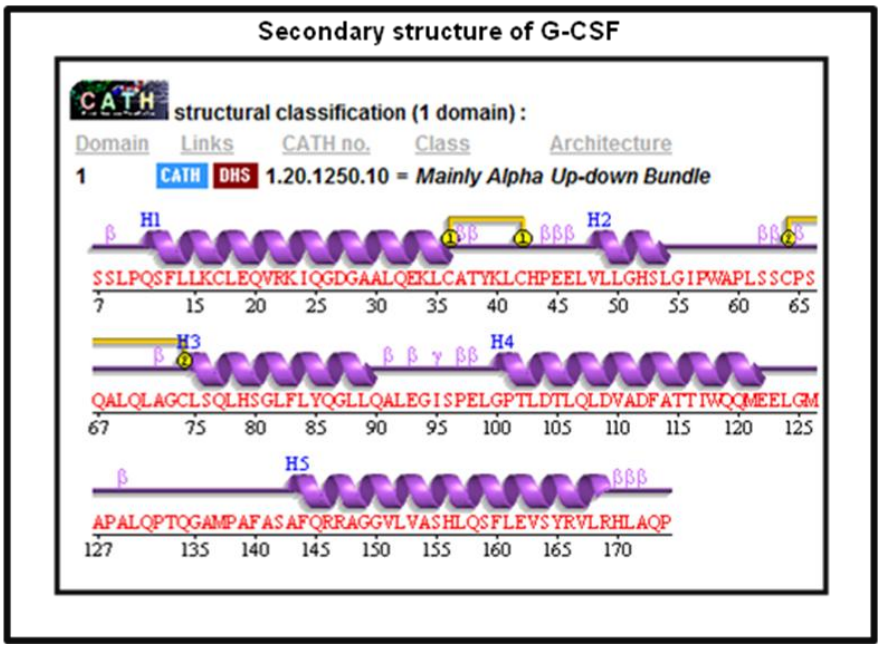


Figure 2. Secondary structure of the human Granulocyte-Colony Stimulating Factor (hG-CSF) (Taken from CATHDB Nr 1.20.1250.10 [7]).

1.2 Clinic Issues

Due to the short half-life of neutrophils, hG-CSF possesses an essential role maintaining a basal level of neutrophils in the body. In case of infection, hG-CSF also increases considerably the amount of neutrophils, regulating their production by activating pluripotent stem cells from the bone marrow (Fig. 3). hG-CSF is also capable of prolonging the half-life of neutrophils, increasing their functional capacity and stimulating neutrophil mobilization from the bone marrow to blood and tissues (8, 9, 10, 11).

Due to the activity of the hG-CSF on the neutripils and progenitor cells, this cytokine plays an important role protecting the human organism against bacterial, fungal and viral infections; by regulating the production of functional and mature neutrophils. Because of this, hG-CSF is used as a pharmaceutical therapeutic for the treatment of patients with neutropenia, thus reducing the risk of infections. Such treatment provides a tremendous benefit in the case of patients with cancer exposed to chemotherapy and radiotherapy.

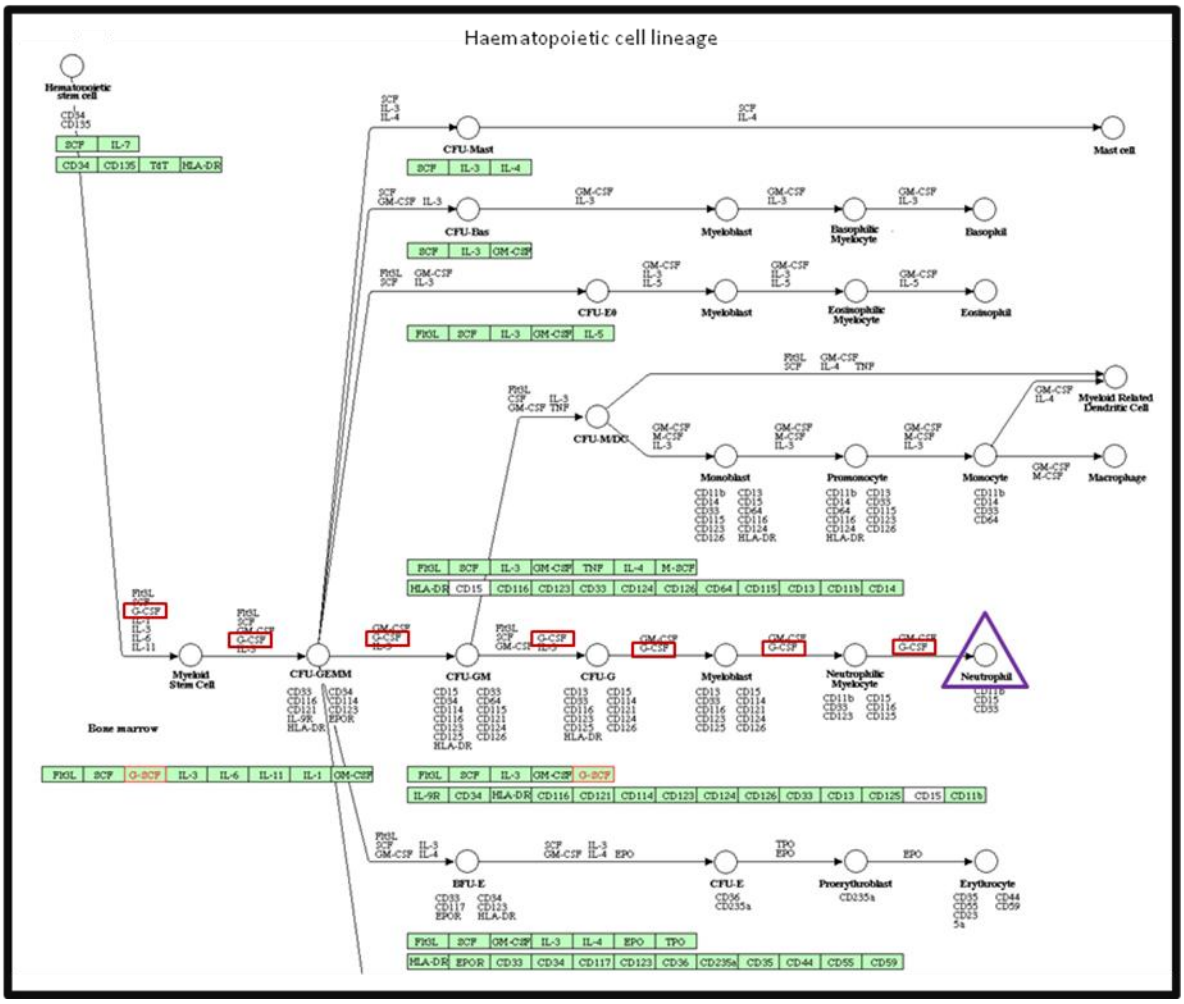


Figura 3. Human Granulocyte-Colony Stimulating Factor effect on Granulocyte/Macrophage-Colony Forming Cells (GM-CSC) and Granulocyte-Colony Forming Cells (G-CSC) (taken from KeggDB [11]).

Patients with neutropenia, caused by other pathologies, may also receive hG-CSF treatment in order to elevate white cells levels. This treatment could even be applied for the mobilization of haematopoietic stem cells in transfusions.

With the use of this haematopoietic growth factor, chemotherapy dose may be elevated, providing for a more efficient treatment of cancer patients with citotoxic chemicals. Without the usage of the hG-CSF, chemotherapy is usually limited due to the haematological toxicity, resulting in deficient cancer treatment and consequently, a possible suspension of such treatment in case of nosocomial infections (12).

1.3 Economic Impact

Due to the positive clinical impact that results form the use of recombinant hG-CSF, it is in high demand all over the world. In the year 2000, more than US$ 2 billion in sales of this drug were registered globally. Currently, rhG-CSF is one of the most widely sold pharmaceutical proteins, and it is the most common pharmaceutical therapeutic used in cancer treatment.

Treatment with rhG-CSF alone can reduce patient hospitalization (25.3 days *vs* 29.8 days) in case of nosocomial infection and, current with the use of antibiotics an even greater reduction is observed (14.5 days *vs* 18.6 days). Such reductions economically impacts hospitals and patients, lowering costs in antibiotics and hospitalization expenses (13, 14). Due to these beneficial factors, the economical value of rhG-CSF is very high, and thus its production for clinical use is very important.

1.4 Recombinant Human G-CSF Expression Systems

rhG-CSF has been produced using diverse hosts, such as *Escherichia coli*, yeasts and mammalian cells. Currently, rhG-CSF is commercially available as non-glycosylated protein (NG-GCSF), produced in *Escherichia coli* (Neupogen by Amgen Inc.). The bacterial presentation does not possess the same molecular structure compared with the natural one, since the bacterial translational mechanisms are not as developed as those in mammals. For this reason, Neupogen is non-glycosylated, the glycosylated version (gly G-CSF) is produced in Chinese Hamster Ovary Cells (CHO cells) by Aventis Pharma, and is commercially called Granocyte. The last pharmaceutical presentation is structurally more similar to the human one (15).

1.5 Importance of the Proteic Structure

It is important to determine the possible effects that structural differences in the recombinant protein might confer on patients. It is stipulated that glycosylation represents important consequences in terms of cytokine efficacy. Diverse studies *in vitro* and *in vivo* reinforce this hypothesis. Because of the structural differences present in the commercially available rhG-CSFs (Table 1), a study was performed, in which the biological activity of this cytokine was tested in

humans (127 patients with lymphoma or myeloma). In this study, it was found that in order to obtain 2 x 10^6 $CD34^+$ cells/Kg (constitutive level known as optimal for a good quality cytaphereris event), it was necessary to administer 1 vial of Granocyte per day (an average of 3.5 μg/kg/day), compared with 10 μg/kg/day of Neupogen for the same result. Therefore, at least 3 times more Neupogen were needed in the same period of time to obtain the same physiological effect (15, 16).

Table 1. Structural characteristics according to the source of the rhG-CSF[17].

Lenograstim (Granocyte)	Deglycosylated Lenograstim	Filgrastim (Neupogen)
Authentic glycosylated recombinant human granulocyte colony-stimulating factor (rhG-CSF)	Deglycosylated rhG-CSF	Non-glycosylated rG-CSF
Expressed in Chinese Hamster Ovary Cells (CHO)	Expressed in CHO cells	Expressed in *Escherichia coli*
Has the authentic primary amino acid sequence of human G-CSF (174 amino acids)	Has the authentic primary amino acid sequence of human G-CSF (174 amino acids)	Poypeptide sequence is not authentic to that of human G-CSF because of N-terminal methionine extension (175 amino acids)
Proportion of isoform structures (monosialo- and disialoforms) corresponds to that in G-CSF obtained from the human cell lines	Deglycosylation results in shift in isoelectric point (due to removal of sialic acid residues)	Isoelectric point similar to that of deglycosylated rhG-CSF
Greater resistance to inactivation (at neutral pH) by temperatura or physiochemical changes than deglycosylated rhG-GCSF	Shift in isoelectric point results in loss os stability and resistance to thermal/physiochemical inactivation	Thermal/physiochemical stability profile corresponds to that of deglycosylated rhG-CSF

When comparing the pharmaceutical effect, using the same level of dose (10 μg/kg/day, dose used in cytapheresis events), 27 % more $CD34^+$ cells can be obtained when using Granocyte, compared with Neupogen (18, 19). Such data are very important for patients who will receive cytapheresis. The Granocyte superiority over Neupogen is somehow expected, since Granocyte contains 27 % additional biological units in 1 μg, compared with 1 μg of Neupogen (18, 19). However, the previous results were obtained from studies achieved with healthy patients. In a study made with mammary cancer patients, similar results were obtained, using 8 μg/kg/day in the case of Neupogen, and 6.2 μg/kg/day for Granocyte (20).

The glycosylation effect on actin polymerization in neutrophils has been analyzed in another study. This is important since it is required for neutrophil mobilization (21, 22). It was discovered that a

negative effect is seen on the actin assembling, when using Filgrastim (Neupogen). This negative effect induced high levels of F-actin in circulating neutrophils, causing an altered morphology (indicating a spontaneous cellular polymerization) and a diminished motility response against chemotactic stimuli. These effects were not observed when using Lenograstim (Granocyte). Due to the latter data, it is assumed that the presence of a carbohydrate can modify the biological activity of the rhG-CSF, since a modified ligand-receptor interaction could impact the transmembranal signaling (23).

There are some other factors, inherent to the patient, which could contribute to the biological activity differences between the glycosylated and non-glycosylated rhG-CSF. It has been demonstrated that the non-glycosylated rhG-CSF is susceptible to degradation caused by the human neutrophil derived elastase (HNE) in the serum. Such degradation produces a reduction in the biological activity of the cytokine. The glycosylated cytokine also suffers degradation by the HNE, but this enzymatic destruction occurs at a slower rate. This data was corroborated by producing a non-glycosilated G-CSF in yeast, and a glycosylated G-CSF which was enzymatically treated in order to remove the carbohydrate. In this study, it was found that the glycosylic residue was critical for the cytokine protection against protease degradation (24).

1.6 Pharmaco-Economical Studies of the Commercially Available Recombinant G-CSF

In other studies, it has been determined that 1 vial of Granocyte 34 or 2 vials of Neupogen 30 are required, in order to obtain the same efficacy (25). In a study performed by Martin-Christin et al., 2001, it was possible to estimate the dialy costs when using the commercially available rhG-CSF (average prices in France). The hypothesis was based on considering an average patient of 65 kg with a corporal surface area of 1.7 m^2, using the prescription recommendations indicated by these products (Table 2).

Table 2. Daily costs of treatment (DCT) by Granocyte and Neupogen, according to indication, for an average patient of 65 kg and 1.7 m^2, with a price per vial of € 100 for both Granocyte 34 (263 µg/vial) and Neupogen 30 (300 µg/vial) [26].

Indications	Granocyte		Neupogen	
	Dosage	DCT (€)	Dosage	DCT (€)
Reduction in duration of chemotherapy-induced neutropenia	150 µg/m^2	96.9	5 µg/kg	108.4
Reduction in duration of neutropenia following auto- or allobone marrow transplantation	150 µg/m^2	96.9	5 o 10 µg/kg	108.3 or 214.5
Mobilization of hematopoetic stem cells in circulating blood associated with chemotherapy	150 µg/m^2	96.9	5 µg/kg	108.3
Mobilization of hematopoetic stem cells in circulating blood with G-CSF alone	150 µg/kg	247.1	10 µg/kg	214.5

1.7 rhG-CSF Expressed in Yeasts

In contrast to the intracellular production of recombinant proteins by *E. coli*, the production of recombinant proteins in yeasts offers as an advantage that yeast possesses a secretion system similar to that of higher order eukaryotes. Such secretion systems can be manipulated in order to increase the secretion of recombinant polypeptides. By using such secretion systems, the recovery/purification process can be simplified and, therefore the recovery levels can be significantly elevated.

rhG-CSF has been produced, using *Saccharomyces cerevisiae* as an expression system, in batch-fed cultures with galactose as an expression inducer for the recombinant protein. The recombinant protein concentration achieved after 40 hours was over 60 mg/L, in cultures of high cellular density (more than 70 g/L) and high plasmid stability (> 90 %). The production of this recombinant protein did not alter the cell growth of the yeast and the biological activity observed was similar to that obtained using *E. coli* as expression system (27, 28). Although, no consisted studies about glycosylation were accomplished, it was estimated that the rhG-CSF did not present any O-glycosylation.

However, the secreted rhG-CSF was usually found as large multimers in the medium, due to strong hydrophobic interactions. Such multimers diminish the specific union activity to the receptor of

the rhG-CSF, thus causing undesired problems in the process of recovery/purification. In order to avoid such problems, it is necessary to modify the medium by adding a non-ionic surfactant (Tween 80), and to raise the pH to its maximum level of 6.5 (29).

Other yeast systems have been used for the production of rhG-CSF, such as *Pichia pastoris*, in which an expression level of 10 mg/L has been obtained, using a biomass of 10 g/L in a shaking flask. However, this production platform presented a similar problem as with *S. cerevisiae*. Nevertheless, changes in the medium pH as well as adding Tween 80 to the medium, were not useful to diminish the problem. On the other hand, using a process to denature the proteins through the use of guanidine-HCl and subsequent separation and renaturation by filtration gel chromatography, it was possible to recover more than an 80 % of the rhG-CSF in its monomeric form (30).

1.8 Plant Platforms for the Production of the rhG-CSF

Plant expression systems offer several advantages for the production of pharmaceuticals over the already well established bacterial or mammalian systems. In contrast with other systems, plant expression systems possess the capability of producing proteins and other biological products at low cost, and plants are usually not vectors for pathogens that could put human health at risk (31, 32, 33).

Compared to bacterial systems, the plant platforms do possess the capability to perform post-translational modifications. Such modifications may be indispensable for the correct function of recombinant proteins, especially mammalian proteins. Additionally, the production costs of recombinant proteins, in plant platforms, can be 10 to 50 times lower compared with fermentation systems based on *E. coli.* Due to the advantages mentioned above, plant production platforms can offer a promising potential for the production of high cost pharmaceuticals of limited availability, in a more economic manner (34).

1.9 Plant Cell Platforms for the Production of rhG-CSF.

In contrast to the use of whole plants, as recombinant proteins production systems, the usage of cell cultures present the possible advantage of being maintained in containers and maintained

under laboratory conditions. Under such conditions, ethical issues concerning environmental contamination and gene transfer to other organisms are avoided.

1.9.1 NT1 cells

The usage of NT1 tobacco cells (*Nicotiana tabacum*) as a platform for the production of recombinant proteins, represents a system of fast growth cells. Furthermore, containing the cells in a controlled environment, such as on solid media or in bioreactors, allows the use of good manufacturing practices at the laboratory level. The latter represents an important advantage when it comes to avoiding the possible escape of transformed species to the environment, which is a possibility in the case of *in planta* methods, where field productions is required, or perhaps, even though less likely, in a greenhouse environment. Growth of NT1 liquid or solid medium cultures should eliminate any issue of recombinant species escaping to the environment or inadvertent horizontal gene transmission to other species (35). The NT1 cellular system has been used as expression platform for the production of erythropoietine (36), hepatitis B surface antigen (HBsAg) (37, 38), *Escherichia coli* intimine (39), arabic gum protein (40) and Norwalk virus capside (41).

In recent experiments, the production of the rhG-CSF has been achieved in tobacco suspension cultures. In the last system, a production of 105 µg/L of rhG-CSF has been obtained, after 9 days from the suspension culture initiation. However, after the ninth day, the production of the rhG-CSG was diminished, while the cellular growth of the transgenic line rose dramatically. The latter suggests that the contact of proteases in the medium with the cytokine, during a long period, could have caused such production effect (42).

Recently, several plant cell suspension platforms have been used for the production of a variety of exogenic proteins. Some examples are: recombinant antibodies (43, 44, 45), human interleukine (IL)-2 and IL-4 (46), ricin (47), Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF) (48), and human α-antitrypsine (49). Nonetheless, the data obtained in these studies, shows that the levels of recombinant protein recoveries are significantly lower (approximately 100 to 300 µg/L) compared with the levels obtained when using whole plant platforms or organ cultures, such as leaves, seeds or roots (42).

In studies achieved later on by Yang et al., 2006, it was possible to obtain a higher production of the rhG-CSF. After 13 days of culturing rice cells in suspension, a concentration of 2.5 mg/L (0.7% of the total secreted protein) was achieved, using the α-amilase 3D promoter, which is inducible in the absence of sucrose. In this way, the expression and secretion to the medium of the rhG-CSF was achieved due to the signal sequence of the leader peptide Ramy3D. It was also observed that protein degradation in the medium was very low (50). Furthermore, the biological activity of the secreted cytokine was similar to that observed by the rhG-CSF expressed in *E. coli* (51), however, no studies to determine the O-glycosylation were performed.

1.10 Strategies to Elevate the Expression Levels of Recombinant Proteins in Plant Systems

Recombinant protein plant production systems still present the disadvantage of low expression levels, when compared to bacterial, primitive eukaryotic, insect and animal cells. For this reason, many researchers have focused on improvement of the recombinant protein expression levels in plant cells. Once, the protein expression levels become competitive, plant systems could be considered as protein production platforms, like the animal cells platforms. Other aspects that must be considered for the development of a competitive plant system are: culture maintenance lower cost, easier protein purification and relatively low possibility of product contamination (51).
In order to improve the expression levels of recombinant proteins in plants, it is mandatory to study the diverse factors that drive the expression of the protein. Among those factors, strong promoters, enhancers, and preferential codons for the host cell, are important elements which can improve the expression of a foreign gene. However, other mechanisms are also responsible for the silencing of the genetic expression. These must be seriously considered in order to obtain high expression levels. Some silencing mechanisms are localized at the mRNA level or at the post-translational level (52, 53).

1.10.1 Suppression of RNA silencing

RNA silencing, or RNA interference (RNAi), is an evolutionary defense system developed by a wide range of eukaryotic organisms, which acts as a regulation system for the expression of genes. In plants, RNAi plays an important role as an immune system against the attack of viruses. Plants

have developed this mechanism, also called post-transcriptional gene silencing (PTGS), which is triggered by dsRNA intermediates. In the first step, the dsRNA is processed into siRNAs of 21-26 nucleatides by the RNase III-like enzyme DICER. siRNAs act later, together with a nuclease complex called RNA-induced silencing complex (RISC), in the recognition of mRNAs by sequence homology, causing thereafter the degradation of the targeted sequence (Fig.4) (54, 55).

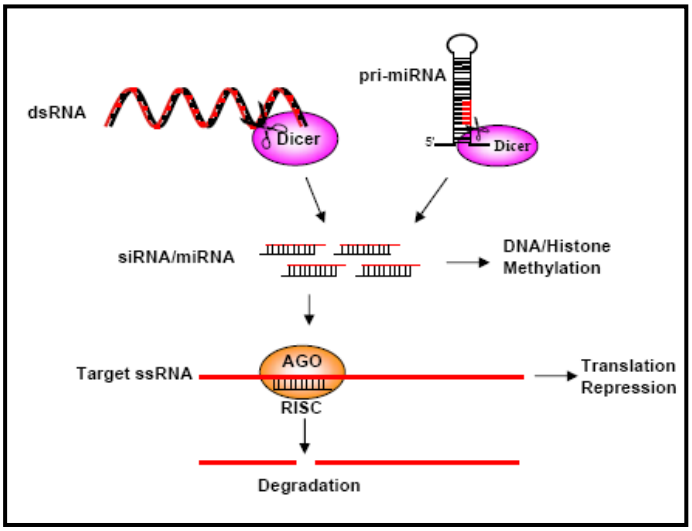


Figure 4. Basic pathway of RNA silencing. Taken from Pazhouhandeh et al., 2007 [55].

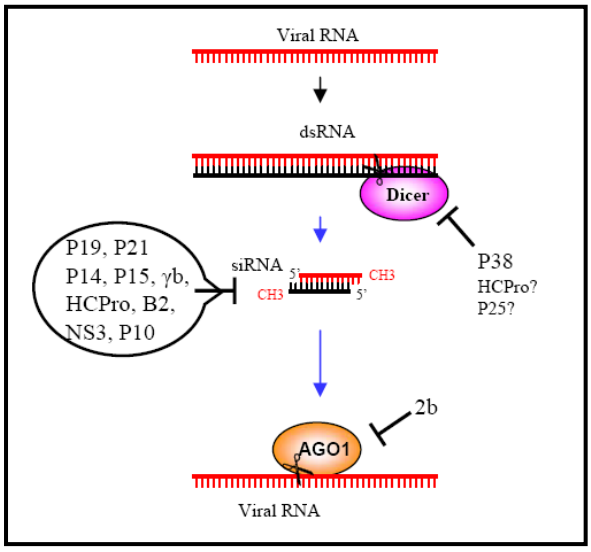


Figure 5. Different suppressors act at ifferent stages of the post-transcriptional gene silencing (PTGS).Taken from Pazhouhandeh et al., 2007 [55].

However, viruses can overcome this defense mechanism by developing proteins that suppress diverse steps (Fig. 5) of the PTGS mechanism. *Cymbidum ringspot tombusvirus* (CymRSV) is a phytovirus, which genome is formed by five open reading frames (ORFs) in a positivesense ssRNA. The 19 kDa protein (p19) is a peptide expressed by the ORF5 and has shown to present RNA-silencing suppression activity (Fig. 6)(56).

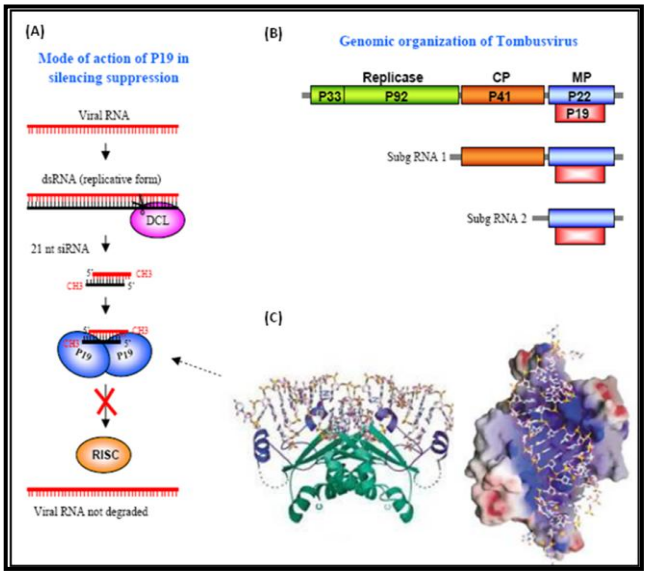


Figure 6. (a) The mechanism of action of P19 in suppression of silencing by binding and sequestering siRNA so as to prevent them from being loaded to RISC. (b) Genomic organization of Tombusvirus ~4.8 kb positive-sense ssRNA. P19 is expressed from subgenomic 2 by leaky scanning of the first AUG. (c) crystal structure of P19 dimer shows binding to 21 nt siRNA. Taken from Pazhouhandeh et al., 2007 [55].

PTGS is also a common plant response present in transgenic plants, in which high copy number transgene rearrangements can occurleading to dsRNA production or perhaps to the high expression of transgenes, which is recognized as a viral behavior. Thus, the expression of the transgene may be efficiently silenced, and the transgenic organism loses the capability to produce the recombinant product. However, it has been demonstrated that the coexpression of viral RNA-silencing suppressor proteins, such as the p19 protein, alleviates the host silencing response, allowing a dramatically enhancing transgene expression (53, 57).

1.10.2 Protein degradation

In order to avoid this kind of silencing, investigators also use signaling strategies, expressing fusion proteins which can be secreted to the media or directed to certain compartments within the plant cell. The Zera® (γ-Zein ER accumulating domain, 58) protein represents an efficient and universal strategy for the production of recombinant proteins. This protein accumulates the protein of

interest in organelles derived from the endoplasmic reticulum instead of secreting the protein to the extracellular medium (Fig. 7 and 8), even though Zera® does not possess a signal peptide for retention in ER (KDEL/HDEL). Using this kidnapping and storing system, the protein of interest can be stored in the cytoplasm, and it is possible to avoid the common protein degradation mechanisms, which are carried out as a response by the plant host cell, without representing any damage to the host (59).

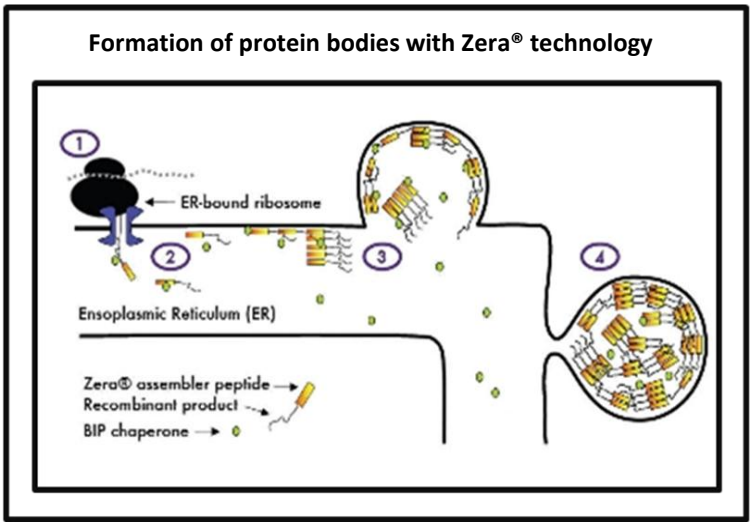


Figure 7. Protection mechanism confered by the fusión protein Zera. 1: mRNA translation (fusión of Zera peptide with the recombinant protein of interest). 2: Internalization into the endoplasmic reticulum through signal peptide. The Zera peptide interacts with the ER membrane, it folds and is later associated with chaperons, so that the formation *de novo* of storage organelles can be induced. 4: The fusion protein Zera and the protein of interest are kidnapped in the protein body like organelles, and isolated from the proteolitic degradation. Taken from EraBiotech [58].

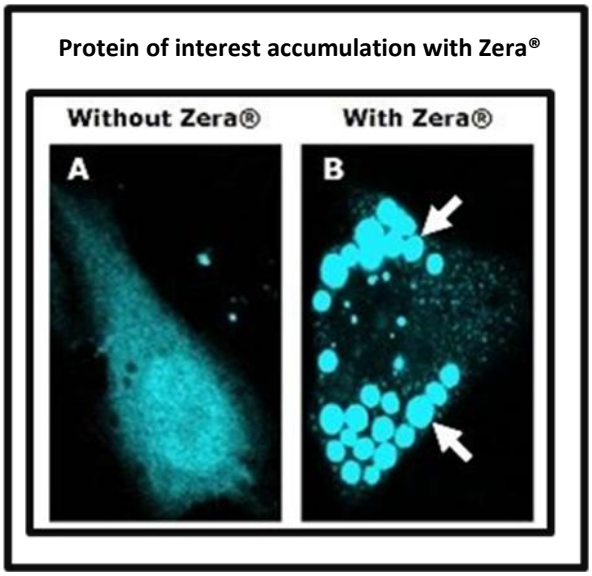


Figure 8. Accumulation of protein of interest in transfected mammalian cells. A: Recombinant proteins without Zera diffused along the cell. B: Fluorescent proteins fused to Zera localized in inclusion organelles, storage structures that contain high quantities of the protein of interest in a soluble and functional state. Taken from EraBiotech[58].

The structure of the protein bodies, formed by the fusion of Zera® with the protein of interest, possess many of the storage advantages present in the authentic protein bodies in cereals. Among these advantages, scientists have found the presence of eukaryotic chaperons to facilitate the recombinant protein folding; high densities, which allow a simple recovery/purification by capturing these protein bodies (Fig. 9 and 10); and isolation of the content of the protein bodies (recombinant proteins) from the proteolitical and enzymatic activities in the cytoplasm (59).

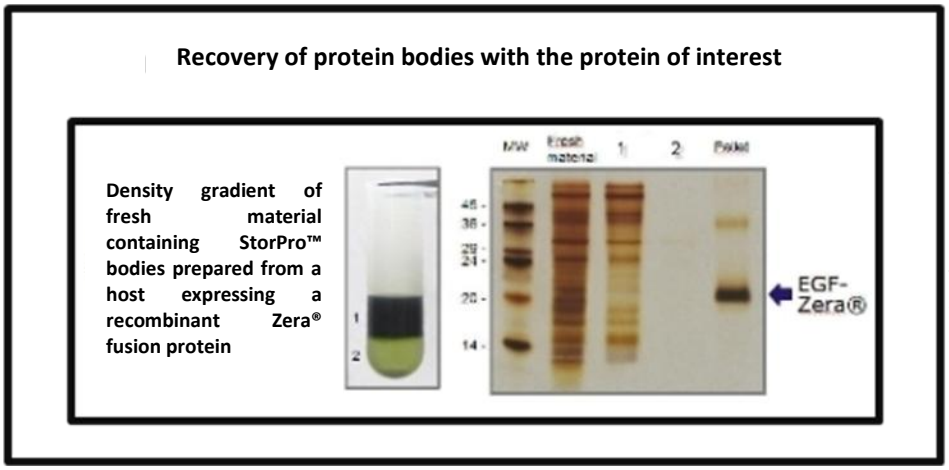


Figure 9. Recovery of the inclusión bodies containing the protein of interest. The high density of the protein of interest fused with Zera, allows an easy recovery. Taken from EraBiotech[58].

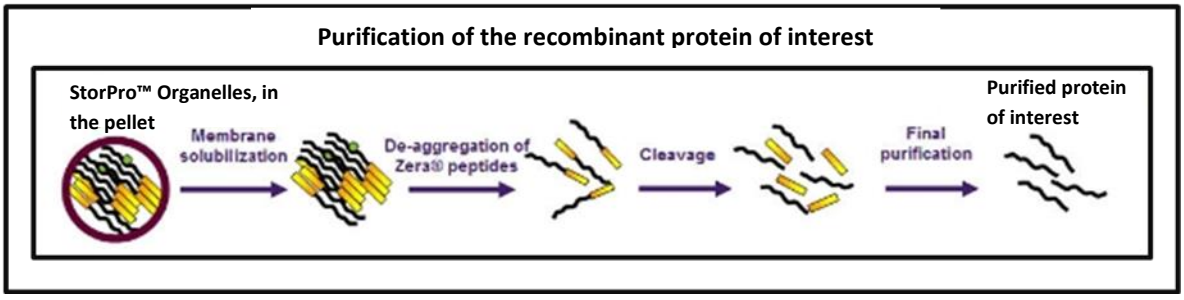


Figure 10. Purification of the protein of interest fused with Zera. Due to the high densities, a high recovery rate of the protein bodies, and protection against protein degradation, the purification of the protein of interest can be achieved by the cleavage of the fused recombinant protein, obtaining high levels of production. Taken from Erabiotech[58].

Recent studies have demonstrated that the Zera technology can be used in a wide variety of eukaryotic hosts, as well as for the expression of a wide range of recombinant proteins. By using fluorescent reporter proteins fused to Zera, correct expression has been observed in epidermal leaf cells in tobacco, and CHO cells; as well as in fungal hosts like *Trichoderma reesei*; and insect

cells as *Spodoptera* (Sf9), where the recombinant protein Zera-DrRED baculovirus has been expressed (59).

It has also been demonstrated that Zera offers stability to pharmaceutical proteins, due to encapsulation in protein bodies. Different recombinant pharmaceutical proteins fused to Zera, such as calcitocine, epidermal growth factor and human growth hormone, have been successfully expressed in different mammalian host cells, such as HEK2937 human cells, CHO cells, and Cos1 primate cells. The expression level impact of the production of recombinant proteins fused to Zera has also been explored using epidermal growth factor (accumulated 100 x higher vs. the production without Zera), and the human growth factor (accumulated 13 x higher vs. the production without Zera), expressed in transgenic tobacco cells (59). The fused Zera-proteins remained stable after 5 months of storage, in the plant (tobacco) leaves under freezing or dry (37°C) conditions (59). In other experiments, the *Yersinia pestis* F1-V antigen fused to Zera (ZeraF1-V) has been expressed in NT1 tobacco cells and Alfalfa (52).

Due to the difficulty and cost issues related to downstream processes of biopharmaceuticals, Torrent et al., 2009 determined that the expressed proteins fused to Zera, possessed high density. This physical characteristic can be taken in consideration in order to facilitate the protein purification by density gradients. The product can further be analyzed by gel electrophoresis and immunoblotting using antibodies anti-Zera.

1.10.3 NT1 cells transformation mediated by Agrobacterium tumefaciens

Agrobacterium tumefaciens is the causative agent of the neoplastic disease crown gall in plants. During the decade of 1980, extensive genetic analyses were conducted in order to identify the bacterial genes involved in virulence. During the last years, genomic technologies have revealed additional information involved with the role of virulence genes and new genes that more subtly influence transformation. The results of those genomic analyses have allowed scientists to better understand how *Agrobacterium* interacts with plant host cells. The development of an integrative knowledge of how *Agrobacterium* manipulates its hosts can increase the probability of successful transformation out-comes (60).

During the *Agrobacterium tumefaciens* infection, the T-DNA fragment from the Ti plasmid is translocated as a single-stranded DNA sequence (transferred strand or T strand) into the plant cell. Such process is similar to bacterial conjugation. Once the T-DNA is translocated to the plant cell

nucleus, it integrates into the plant genome (61, 62). The expression of the genes located in the T-DNA is responsible for the plant cell transformation into a tumor cell, which later results in a tumor formation (crawn gall). Due to this capability of transferring DNA from bacteria to plant cell, *Agrobacterium tumefaciens* is the most commonly DNA delivery tool for genetic engineering in plants (62).

The infection process of *Agrobacterium* is regulated by a set of chromosome-encoded genes (*chv*), which are responsible for the attachment of the bacterium to the plant cell; *vir* genes encoded by the Ti plasmid, which function in *trans*, involved in the generation, transfer, and integration of T strands into the plant genome; as well as other plant genes and molecules. In a general approach, the infection mechanism first starts with the release of plant molecules by wounded cells, which stimulates the expression of *vir* genes (63). VirA, an inner membrane protein, detects the signal molecules and autophosphorylates itself at the His-474 residue. Once activated, VirA phosphorylates in turn the cytoplasmic VirG protein at the Asp-52 residue. The activated VirG protein, a DNA binding protein, acts as a transcriptional factor of the *vir* genes by binding the *vir* boxes present upstream of all *vir* operons. The original T-DNA fragment of the Ti plasmid carries genes that specify the synthesis of tumor-specific compounds called opines. Ti plasmids are then classified according to the kind of opine that they encode, thus based on the opine utilized, Ti plasmids are divided as octopine, nopaline, agropine, and succinamopine types (61). However, the *vir* genes regions of different Ti plasmids are well conserved in sequence and are functionally cross-reactive (62).

The T-DNA is flanked by the known left- and right borders, which are recognized by the VirD1-VirD2 endonuclease complex. VirD2 remains covalently bound to the 5' end of the T-DNA through its Tyr-29 residue. Once the whole T-DNA is nicked, the T-DNA-VirD2 complex is translocated to the plant cell by a *vir* region-encoded type IV secretion apparatus, which consists of VirD4 and 11 different VirB proteins. The single-stranded DNA-binding protein VirE2 is transported from *Agrobacterium* to the plant cell by the same type IV secretion apparatus, and once the T-DNA-VirD2 complex enters the plant cell, VirE2 coats the T strand to protect it against nucleolytic degradation. Both VirD2 and VirE2 have nuclear localization signals that help the T strand import into the nucleus, where it is integrated to the plant chromosomal DNA randomly (Fig. 11)(62).

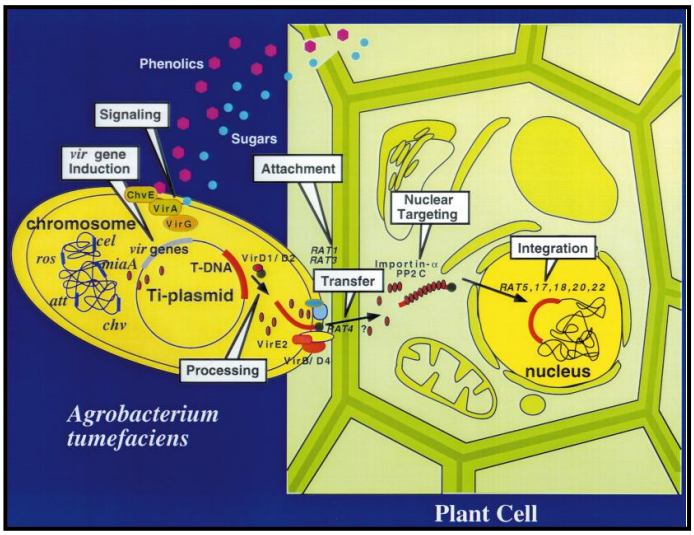


Figure 11. Schematic diagram of the Agrobacterium infection process. Critical steps that occurto or within the bacterium (chemical signaling, *vir* gene induction, and T-DNA processing) and within the plant cell (bacterial attachment, T-DNA transfer, nuclear targeting, and T-DNA integration) are highlighted, along with genes and/or proteins known to mediate these events. Taken from Gelvin et al., 2000 [63].

Increasing the transformation rate of NT1 cells mediated by *Agrobacterium* is a critical issue when using certain recombinant proteins, and such is the case when using the Zera fusion protein. As reported by Alvarez et al., 2009[52], the outcome of NT1 calli, transformed with a transgene that encodes a Zera fusion protein, can take until 15 weeks to obtain 100 outcomes, and the number of developed calli is relatively lower compared with those transformed with a transgene which encodes the same protein of interest but without Zera. Therefore, the time required for the outcome of transformed NT1 calli using the Zera technology represents a stress factor for the plant cells development. However, the time and number of calli outcome can be reduced and increased, respectively, by selecting supervirulent *Agrobacterium* strains.

Therefore, the present research project intends to create a plant cell production platform, using an NT1 cell culture for the expression of the rhG-CSF. In order to accomplish competitive expression levels, the Zera technology, together with the suppression of the mRNA silencing mechanism, driven by the viral protein p19, will be used. Due to the phenotypical impact that the Zera and p19 proteins might produce to the plant cell, the transformation protocol mediated by *Agrobacterium tumefaciens* will be optimized, and the most virulent *A. tumefaciens* will be chosen, in order to increase the outcomes of transgenic cell lines.

2. OBJETIVES

2.1 General Objective:

This research project has as purpose the expression of the recombinant human Granulocyte-Colony Stimulating Factor (rhG-CSF) in plant cells (NT1), fused with Zera for protein body-like structions formation and suppression of gene silencing in transformed NT1 cells through the expression of the p19 gene.

2.2 Specific Objectives:

Construction of the p19 expression cassette, using the pPS1:NPTII binary vector.

Pre-transformation of NT1 cells, mediated by *Agrobacterium*, with the pPS1p19 binary vector.

Optimization of NT1 cell transformation mediated by *Agrobacterium*, using the p19 as model transgene.

Determination of the most virulent *Agrobacterium* strain, using p19 as model transgene.

Design and sequence optimization (recodification) of the *zeraG-CSF* gene.

Construction of the ZeraGCSF^T expression cassette, using the pGPTV:BAR binary vector.

Transformation of the NT1 cells mediated by *Agrobacterium*, with the ZeraGCSF^T expression cassette.

Super transformation of the NT1:p19 lines mediated by *Agrobacterium*, with the ZeraGCSF^T expression cassette.

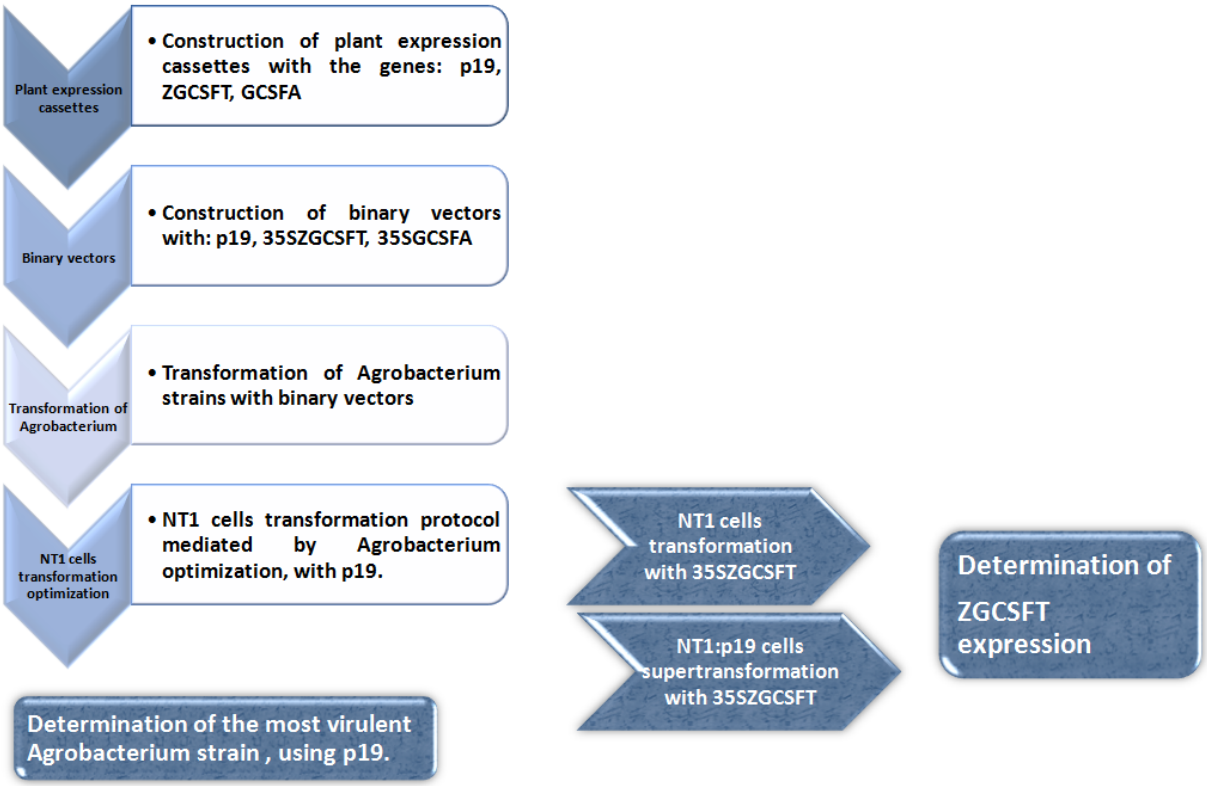
Detection of stable transgenic lines by determination of expression cassettes presence in plant genome DNA, using PCR.

Detection of ZeraG-CSF^T expression, by detecting mRNA using reverse transcriptase PCR.

Detection of ZeraG-CSF^T protein, using serological techniques.

3. MATERIALS AND METHODOLOGY

3.1 General Strategy



3.2 Water and Sterilization

Solutions preparations were accomplished using double distilled water (Laboratorios Monterrey, S.A. de C.V.). When needed, solutions and media were autoclaved using 15 p.s.i. for 20 min, at 120°C. In case autoclaving was not suitable for the solution or media, these were sterile filtrated.

3.3 Bacterial Manipulation

3.3.1 *Escherichia coli* strains

In order to create the different vectors used in this work, the following *Escherichia coli* strains were used to replicate and isolate plasmids (Table3).

Table 3. *E. coli* strains used in this work.

Strain	Genotype	Plasmid	Reference
DH5α	F- endAI hsdRJ7 (r-, mit) supE44 thi-J ArecAl gyrA96 relAI deoR A(lacZYA-argF)-U169 480dlacZAM15	pIBT210.1p19 pPS1 pPS1p19 pUC57EkGCSF^{T} pUC57MfGCSF^{A} pUC57Ls pUC35SZeraF1-V pUC35SF1-V p35SZeraF1-V bin p35SF1-V bin p35SZGCSF^{T} bin	Alvarez et al., 2009
Dam-/Dcm-	*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)* Tet^{S} *endA1 rspL136* (Str^{R}) *dam13::Tn9* (Cam^{R}) *xylA-5 mtl-1 thi-1 mcrB1 hsdR2*		NEB c#2925

3.3.2 *Escherichia coli* growth and maintenance

The *E. coli* strains were all grown at 37°C, using Luria-Bertani (LB, Sigma) broth medium (10g/L bacto tryptone, 10 g/L NaCl and 5g/L bacto yeast extract) and in agitation at 250 rpm. Agar plates were prepared using LB broth medium supplemented with 15 g/L of agar for bacterium. Respective antibiotic was added after agar solution cooling in water to approximately 50°C (Modified from Sambrook et al., 2001[64]).

3.3.3 Calcium competent *Escherichia coli*

An overnight *E. coli* culture would be diluted 1:100 with the Solution I and placed in shaker at 37ºC until it reaches the Log phase (OD_{600} 0.5-0.6 Abs). The cells must fill over a 50 mL tube, placed 10 min over ice and finally centrifuged (6000 rpm, 4°C, 10 min). The developed pellet would be re-

suspended with 0.5 ml Solution I, 2.5 ml Solution II added and kept frozen by 100 µl aliquots at -80ºC (Modified from Sambrook et al., 2001 [64]).

Solution I:	$MgSO_4$	10mM (sterile filtrated)
	Glucose	0,2 % (sterile filtrated)
		Up to 100 ml LB-Medium
Solution II:	$MgSO_4$	12 mM (sterile filtrated)
	Glycerin	36,0 % (sterile filtrated)
	PEG MW 7500	12,0 % (sterile filtrated)
		Up to 100 ml LB-Medium

3.3.4 *Escherichia coli* transformation by heat shock

The whole ligation reaction would be diluted into 100 µl competent *E. coli* Cells and the incubated for 30-60 min. over ice. The reaction is afterwards placed 90 s in a 42ºC Heat block and after that placed 2 min over ice. 800 µl LB-Medium are then added to the reaction and then the culture is placed in a shaker at 37ºC for incubation to allow expression of the antibiotic resistance development determinant, for example Ampiciline. And then usually incubated during one hour. For erythromycin, the culture must be incubated for 4 or 5 hours. 100 µl from the transformation reaction tube are then plated over preheated LB selection plates and the plates must be incubated over night at 37ºC. The grown clones are pricked into selective medium and by means of PCR tested (Modified from Sambrook et al., 2001[64]).

3.3.5 Electrocompetent *Escherichia coli*

Inoculate a single colony from an LB plate into 5 mL LB. Incubate the liquid culture over night (ON) in agitation, at 37°C, 250 r.p.m. The next day, transfer 1 mL from the ON culture into 1 L LB broth medium. Incubate the culture at 37°C, 250 r.p.m. Grow cells until the culture reaches an OD_{600} 0.5-0.8 (log phase growth). Transfer the cells to 50 mL conical tubes, pre-cooled on ice. Spin the cultures at 4000 r.p.m., 25 min, 4°C. Remove supernatant immediately, and resuspend the pellets gently with 160 mL ice-cold $_dH_2O$. Spin the cultures at 4000 r.p.m., 25 min, 4°C. Remove supernatant, and resuspend the pellets gently in 80 mL ice-cold $_dH_2O$. Spin the cultures at 4000

r.p.m., 25 min, 4°C. Remove supernatant, and resuspend the pellets in 80 mL ice-cold 10% glycerol. Spin the cultures at 4000 r.p.m., 10 min, 4°C. Remove supernatant, and resuspend the pellets gently in 5 mL ice-cold 10% glycerol. Aliquot the culture in pre-chilled 1.5 mL tubes. Store the cells at -80°C (Modified from Sambrook et al., 2001[64]).

3.3.6 *Escherichia coli* transformation by electroporation

Thaw cells on ice. Add 1 µL plasmid. Incube the mixure on ice for 1 min. Electroporate (Gene Pulser Xcell Electroporation System, Bio-Rad) using 24 kV, 200 Ω, and 2 mm cuvettes, previously cooled. Add immediately 1 mL LB. Pass the electroporated bacteria to a 1.7 mL tube and incubate in agitation at 250 r.p.m., 37°C for 1 hr. After the incubation time, the cells are plated out on agar plates containing the respective antibiotic, and incubated at 37°C (Modified from 65).

3.3.7 Dam-/Dcm- *Escherichia coli* (NEB C2925I) (For solutions, see below)

DNA methyltransferases (MTases) that transfer a methyl group from S-adenosylmethionine to either adenine or cytosine residues, are found in a wide variety of prokaryotes and eukaryotes. Methylation should be considered when digesting DNA with restriction endonucleases because cleavage can be blocked or impaired when a particular base in the recognition site is methylated. In prokaryotes, MTases have most often been identified as elements of restriction/modification systems that act to protect host DNA from cleavage by the corresponding restriction endonuclease. Most laboratory strains of *E. coli* contain three site-specific DNA methylases. Dam methylase is a methylation that occurs at the N^6 position of the adenine in the sequence GATC. Dcm methylase is a methylation that occurs at the C^5 position of cytosine in the sequences CCAGG and CCTGG.

Methyltransferase deficient chemically competent *E. coli* cells suitable for growth of plasmids free of Dam and Dcm methylation. Note that *dam*$^-$ strains are not recommended as a host for primary cloning/ligation. The *dam* mutation can result in an increased mutation rate in the cell and a

reduction in the transformation efficiency. DNA should be maintained in a *dam*$^{+}$ strain unless there is a specific need for DNA free of Dam or Dcm methylation.

Thaw a tube of dam-/dcm- Competent *E. coli* cells on ice until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice. Add 1-5 µl containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix. Place on ice for 5 minutes. Do not mix. Pipette 950 µl of room temperature SOC (Super Optimal Broth [SOB] with Catabolite repression) into the mixture. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate. Warm selection plates to 37°C. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC. Spread 50-100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or at 25°C for 48 hours (66).

SOB: 2% Vegetable peptone (or Tryptone)
0.5% Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$

SOC: SOB + 20 mM Glucose

3.3.8 *Agrobacterium* strains

Table 4. Agrobacterium strains used in this work (*67*).

Strain	Chromosomal Background	marker gene	Ti plasmid	marker gene	Opine	Bin plasmid
LBA4404 LpPS1p19 Lp35SZF^{T}	TiAch5	rif	pAL4404	spec / strep	Octopine	 pPS1p19 p35SZF^{T}
GV3850 GpPS1p19	C58	rif	pGV3850 (pTiC58onc.genes)	carb	Nopaline	 pPS1p19
EHA105 EpPS1p19 Ep35SZF^{T}	C58	rif	pEHA105(pTiBo542 DT-DNA)	-	Succinamopine	 pPS1p19 p35SZF^{T}
AGL-1 ApPS1p19 Ap35SZF^{T}	C58,RecA	rif, carb	pTiBo542DT-DNA	-	succinamopine	 pPS1p19 p35SZF^{T}

3.3.9 *Agrobacterium tumefaciens* growth and maintenance

The *A. tumefaciens* strains were all grown at 28-30°C, using LB broth medium (10g/L bacto tryptone, 10 g/L NaCl and 5g/L bacto yeast extract) or Yeast Malt (YM) broth medium (400 mg yeast extranct, Sigma; 10 g mannitol, Sigma; 100 mg NaCl, 200 mg $MgSO_4 \bullet 7H_20$, 500 mg KH_2PO_4) and in agitation at 150-250 rpm. Agar plates were prepared using LB broth medium supplemented with 15 g/L of agar. Appropriate antibiotic was added after agar solution cooling in water to approximately 50°C (Modified from Virgili-López et al., 2008[68]).

3.3.10 Electrocompetent *Agrobacterium tumefaciens*

To 1 mL of ON culture, add 300 mL of LB (add appropriate antibiotic). Incubate 28°C, 150 rpm, 24 hrs, until OD600 0.5. Transfer the culture on ice for 15 min. Centrifuge 4500 rpm, 4°C, 10 min. Discard supernatant. Resuspend in 50 mL ice-cold MilliQ water (two tubes). Centrifuge 4500 rpm, 4°C, 10 min. Discard supernatant. Wash with 10 % Glycerol (25mL) for 1 h. Centrifuge 4500 rpm, 4°C, 10 min. Discard supernatant. Resuspend in 3 mL 10 % Glycerol. Aliquot in 50 µL and store at -80ºC (Modified from Virgili-López et al., 2008[68]).

3.3.11 *Agrobacterium tumefaciens* electroporation

Add 2 µL of plasmid from a miniprep to the electrocompetent culture. Transfer to precooled cuvette. Electroporate (25 uF, 100 ohm, 1.8 KVolts). Immediately add 1 mL LB and resuspend. Pass the cells to a 15 mL tube, incubate for 4 h, 28°C, 100 rpm. Take two plates with LB and appropriate antibiotic and saw 200 µL on one of the plates. Centrifuge the culture for 15 min, 3000 rpm, 28 °C. Eliminate the supernatant, resuspend the cells with the medium that was left in the tube. Saw it on the plate. Incubate at 28°C, for 1 to 4 days (Modified from 69).

3.3.12 Glycerin cultures

Freeze Medium: 50% Glycerine
2.9% NaCl

Activate bacterial culture (5 – 20 mL) and incubate it in agitation ON with the respective conditions according to the strain. Stop the culture when it reaches a OD_{600} of 0.5-0.6. Spin the culture at 3000 r.p.m., 4°C, 10 min. Discard supernatant. Resuspend the pellet in 1 mL LB. Transfer the resuspended pellet into a sterilized 2 mL (safe cap) tube. Add 1 mL of sterilized freeze medium (1:1). Mix by inversion, and stock at -80°C (Modified from Ramírez-Alanis et al., 2007 [70]).

3.4 Cloning Methods

3.4.1 Primers

Table 5. Oligonucleotides used in this work.

Primer	Sequence	Tm	Homology with	Source
35S F1-V Fow	5'- GCGTGAAGCTTGCATGCCTGC -3'	62.5	5' end of 35S promoter	52
35S F1-V Rev	5'- TAAGATCAGTCATGACTATCGTTCGT -3'	55.3	3' end of F1-V gene	
Zera Fow	5'- AGGTCGACATCATGAGGGTGTTG -3'	58.9	5' end of Zera gene	
Zera Rev	5'- CCTCTAGAATCCATGGTCTGGC -3'	56.9	3' end of Zera gene	
VSP Fow	5'- TTCGAAAAAGAGTCTAGAAAGAGCTC -3'	54.6	5' end of VSP sequence	
VSP Rev	5'- TCTCGAATTCGCTTCAAGACG -3'	55.1	3' end of VSP sequence	
p19 Fow	5'- GGAGAGGAGCTCGAGAATTAATTC -3'	54.8	5' end of TEV sequence	

p19 Rev	5'- TGTTGAGAGAGCTCTTACTCGC -3'	56.3	3’ end of p19 gene	
Up19	5’ CTATACAAGGAAACGACGCTA 3’	51.8	Inner 5’ extreme of p19 gene	**Present work**
Lp19	5’ GGGCATCCTCTTGATACATTA 3’	51.8	Inner 3’ extreme of p19 gene	
UpGCSF	5'- CCATCACAAGCACTGCAACT -3'	56	Inner 5’ extreme of G-CSF gene	
LoGCSF	5'- ATGGGAAGCAACCAAGACAC -3'	55.6	Inner 3’ extreme of G-CSF gene	
UpF1V	5’- CTTACTGGACATGGCAGCAG -3’	55.9	Inner 5’ extreme of F1-V gene	
LoF1V	5’- GCTTCTGGCATCTCCATGAT -3’	55	Inner 3’ extreme of F1-V gene	
UpTEV	5'- GCATTCTACTTCTATTGCAGC -3'	51.5	Inner 5’ extreme of TEV sequence	
LoVSP	5'- TAGTGCATATCAGCATACCT -3'	50.4	Inner 3’ extreme of VSP sequence	

Primers were designed using the software Integrated DNA Technologies (71).

The following criteria apply for the oligonucleotide choice:

- GC-content approximately 50%
- Size range of 18-28 bp
- Avoid palindrome sequences
- Avoid homologies between the primers
- G or C at the 3´OH-End for stabilizing of the ignition of the PCR reaction; however avoid longer GC-succession at the 3´OH-ends.

The primer melting temperature (Tm) can be calculated according to the following formula:

$$Tm = (A+T) \times 2°C + (G+C) \times 4°C$$

This formula applies only for primers of a size between 18 and 24 nucleotides. The optimal annealing temperature lies approximately 2-5ºC under the lower of both calculated melting points. If unspecific PCR-Products are amplified, the annealing temperature must be raised. If the product is missing, the temperature must be reduced. If one begins with a very small template concentration or desires to obtain a larger amount of product, the number of cycles can be increased.

3.4.2 Plasmid vectors

3.4.2.1 pUC57

The G-CSF sequences were *de novo* synthesized by GenScript, using the pUC57 vector to deliver the synthesized sequences. This vector is a 2710 bp plasmid and confers resistance against Amp. The EcoRV restriction site, within the multiple cloning site (MCS), is commonly used for the insertion of the synthesized sequences, or the XbaI-BamHI for orientation purposes, in the case of this project (GenScript Inc.).

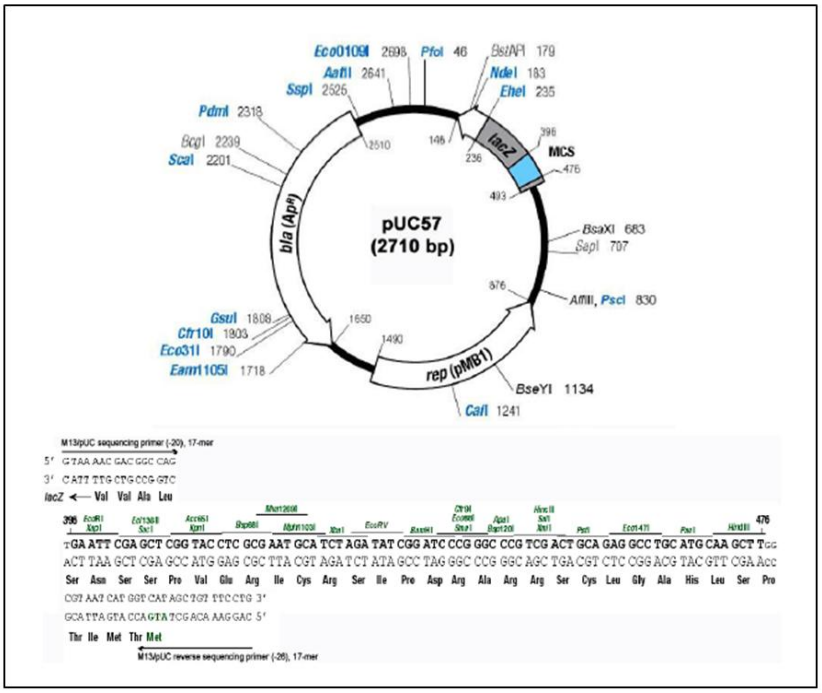


Figure 12. pUC57 plasmid vector.

3.4.2.2 pUC18

pUC18 is a plasmid vector suitable for cloning and for the dideoxy DNA sequencing method. It has multiple cloning sites in the lac Z' region. Since the multiple cloning site is inserted into the 5' end of the lac Z' gene, insertion of foreign DNA in this region will destroy the alpha-complementation activity of the lac Z' gene product, making the screening for white colonies on plates containing X-Gal and IPTG possible. Recombinant plasmids generate white colonies while non-recombinant

plasmids generate blue colonies. pUC18 is identical to pUC19 except that the polylinker is inserted in the opposite orientation. The pUC18 vector contains a Amp reporter marker and it has a size of 2686 bp.

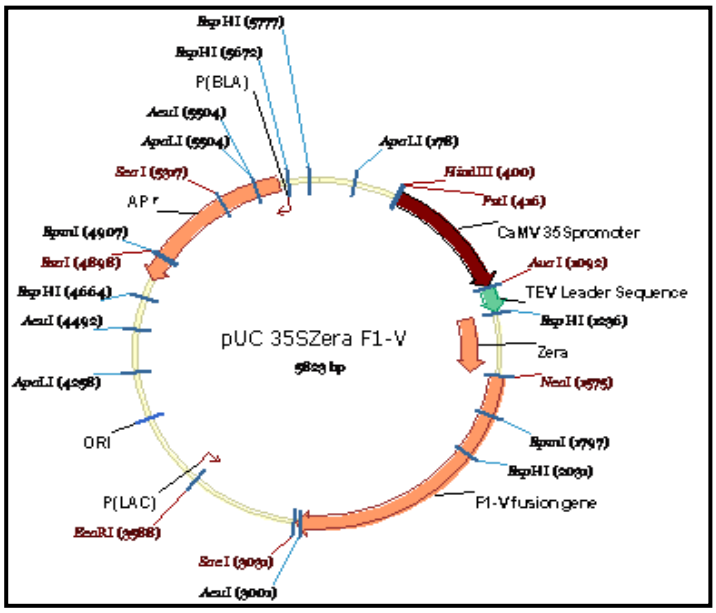


Figure 13. pUC35SZeraF1-V, pUC18 plasmid vector containing the expression cassette 35SZeraF1-V.

The construction of the cassette of expression containing the G-CSF sequence was carried out by using the pUC18-35SZeraF1-V plant expression vector for its future isolation and insertion into the binary vector. This vector is a derivative from the pUC18 plasmid vector, and it has a size of 5823 bp.The pUC18-35SZeraF1-V vector contains in its expression cassette, the Calliflower Mosaic Virus 35S Promoter, which triggers a strong and constitutive expression of the gene of interest, particularly in dicotyledonous plants but also in monocots. Furthermore, it has been observed that a 2x CaMV 35S promoter triggers an enhanced gene expression (for sequence, see Anexe data) (72). In order to provide mRNA stability and affinity to the ribosome for efficient translation, a 5' UTR region (5' untranslated region) was added, which is derived from the TEV 5'-UTR (Tobacco Etch Virus). Additionally, there is a 3' UTR comprised of sequence from the soybean vegetative storage (vspB) to provide a polyadenylation site. Finally, this vector contains the *Zera-F1-V* fusion gene sequence, which was used to substitute the F1-V part for the EkG-CSF part (Fig. 13) (52).

3.4.2.3 pIBT201.1

The pIBT210.1 plasmid is a plant expression vector. The expression cassette contains the strong constitutive CaMV 35S promoter, tobacco etch virus 5'-UTR and the soy bean *vspB* 3' region. This plasmid confers resistance against Amp and it has a size of 4051 bp. This plasmid was used for the isolation of the expression cassette containing the *p19* gene. The pIBT210.1p19 plasmid vector has a size of 4553 bp (Fig. 14) (52).

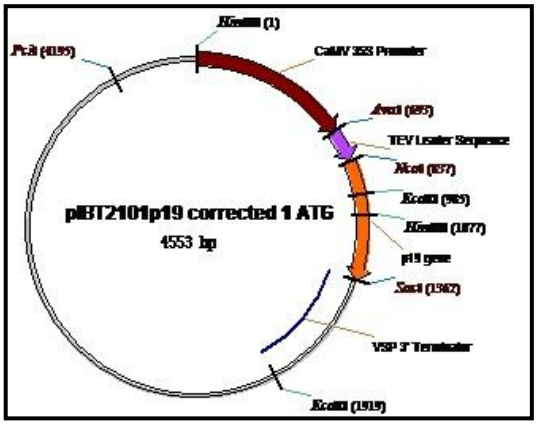


Figure 14. pIBT210.1p19 plasmid vector containing the expression cassette 35Sp19.

3.4.2.4 pGEM

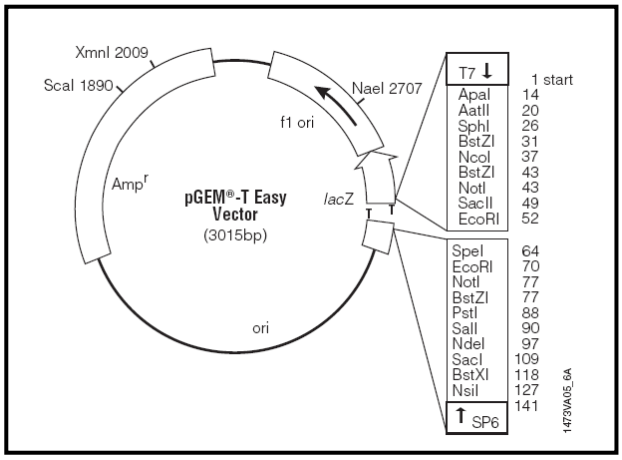


Figure 15. pGEM-T Easy plasmid vector used for cloning purposes and sequencing.

The pGEM®-T Easy plasmid is a linearized vector, which has a single 3'-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated with poly-A tails. The pGEM-T Easy vector is a high-number copy vector, containing T7 and SP6 RNA polymerase promoters, flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase (the *lacZ* gene) as in pUC18. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates. The pGEM-T Easy plasmid confers resistance against Amp and it has a size of 3015 bp. This plasmid was used for sequencing purposes (Fig. 15) (73).

3.4.3 Binary vectors

3.4.3.1 pGPTV:BAR

The binary vector pGPTV:BAR (Glucuronidase Plant Transformation Vector) is a 13400 bp vector used for plant transformation. This vector is compatible with the hosts *E. coli, Agrobacterium tumefaciens,* and vascular plants. This plasmid contains a selectable marker gene for plant transformation within the T-DNA and close to the left border of the T-DNA, providing selection for transfer of the complete T-DNA from the right border, through the gene of interest and the plant selectable marker, to the left border and thus increasing the probability of having the gene of interest transferred to the plant cells which are expressing the selection gene (74).

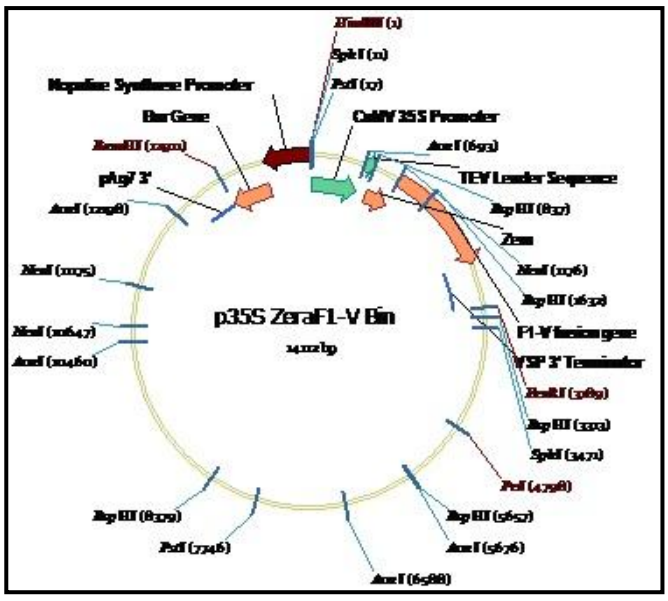


Figure 16. p35SZeraF1-V binary vector.

The marker genes used by this vector are *npt2,* conferring resistance against Kan in the bacterium and *bar* for plant cells, which confers resistance against phosphinothricin (PPT). This herbicide triggers plant cell death by accumulating toxic levels of ammonium, and thus acting as an inhibitor of the main enzyme from the metabolic pathway of nitrogen assimilation, glutamine synthetase (GS). Intracellular peptidases activate PPT by removing the Alanine residue, the glufosinate compound acts as inhibitor of the glutamine synthetase. The *bar* gene encodes the phosphinothricin acetyltransferase (PAT) enzyme, which is responsible for the glufosinate detoxification by the acetylation of the amino group, providing thus resistance to the plant cell against the herbicide.

In this work, the pGPTV:BAR binary vector was used to clone the *ZeraGCSF* transgene, using the p35SZeraF1-V bin previously used by the lab, and thus substituting the expression cassette containing the *ZeraF1-V* gene by the one containing the ZeraGCSF sequence. The p35SF1-V bin contains the Soybean Vegetative Storage Protein 3' Terminator (*vspB* 3')(Genbank: M37529.1)(Fig. 16)(See Anexes) (52).

3.4.3.2 pPS1:NPTII

The pPS1 binary vector is a 12599 bp vector used for plant transformation. This vector drives expression with a doubled CaMV 35S promoter and carries a tobacco etch virus translational enhancer to further increase transgene expression. The selection for both, bacterium and plant cells, is driven by the *nptII* gene, which confers resistance against kan. This binary vector was used to clone the p19 sequence (Fig. 17) (52).

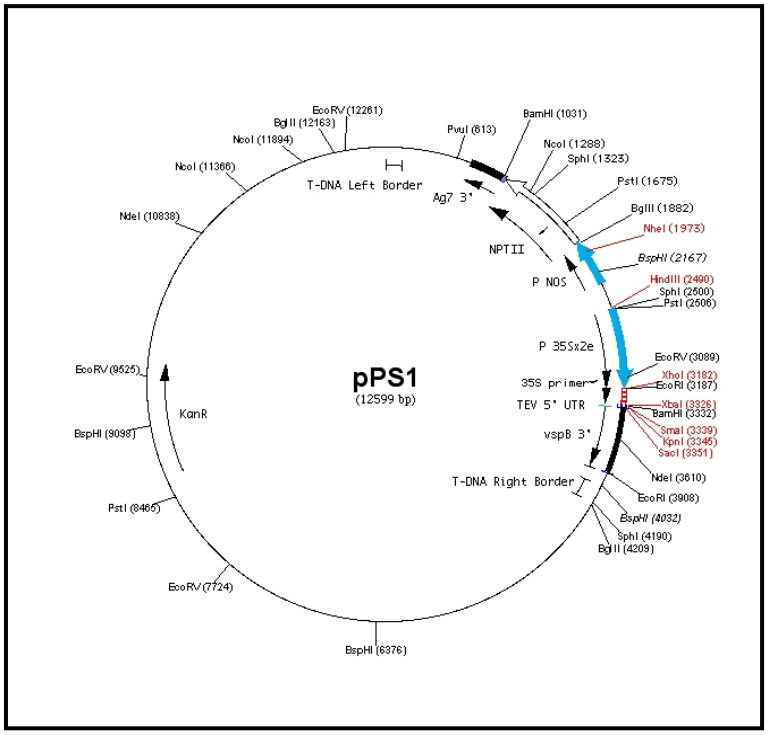


Figure 17. pPS1 binary vector.

3.4.4 Sequences

3.4.4.1 p19 sequence from Tombusvirus

The p19 sequence inserted into the pIBT210.1 vector (pIBT201.1p19) was described by Alvarez et al., 2008. The p19 sequence was previously amplified and cloned into the pIBT210.1 vector via PCR (Fig. 18).

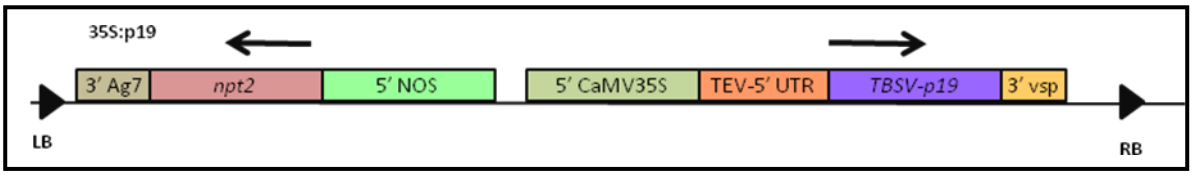


Figure 18. p19 expression cassette. *TBSV-p19* represents the original nucleotide sequence from the Tomusvirus *p19* gene. The expression is driven by the CaMV 35S promoter.

3.3.4.2 ZeraG-CSF^{T}

The G-CSF sequences were *de novo* synthesized, and provided by GenScript in the pUC57 vectors. The variant number II of the human G-CSF (75) was chosen as the gene variant for the expression of this cytokine. This variant is formed by 174 amino acids and is the one expressed in the CHO cells platform (NCBI: NM_172219, NP_757373.1). Since the variant II rhG-CSF would be fused to the Zera protein, a cleavage site for the enterokinase between both elements was designed for purification purposes (Fig. 19).

```
        10         20         30         40         50         60
MRVLLVALAL LALAASATST HTSGGCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL
        70         80         90        100        110        120
PPPVHLPPPV HVPPPVHLPP PPCHYPTQPP RPQPHPQPHP CPCQQPHPSP CQTDDDDKTP
       130        140        150        160        170        180
LGPASSLPQS FLLKCLEQVR KIQGDGAALQ EKLCATYKLC HPEELVLLGH SLGIPWAPLS
       190        200        210        220        230        240
SCPSQALQLA GCLSQLHSGL FLYQGLLQAL EGISPELGPT LDTLQLDVAD FATTIWQQME
       250        260        270        280        290
ELGMAPALQP TQGAMPAFAS AFQRRAGGVL VASHLQSFLE VSYRVLRHLA QP
```

Figure 19. Zera-rhG-CSFtransgene amino acid sequence. The Zera sequence is localized at the N-terminal extreme, and the C-terminal extreme contains the rhG-CSF. In the middle of both sequence, a enterokinase cleavage site was designed (gray) (Winstar software).

The human G-CSF contains an O-glycosylation site at the Thr 133 residue. Even though the plant cells, as eukaryotic cells, possess the mechanisms necessary to perform such post-translational modification, the fusion of the rhG-CSF to the Zera protein is likely to produce O-glycosylation, which occurs in the Golgi post Zera mediated protein body formation, which occurs in the ER. Nevertheless, the glycosylation site was left for future cloning purposes (Fig. 20).

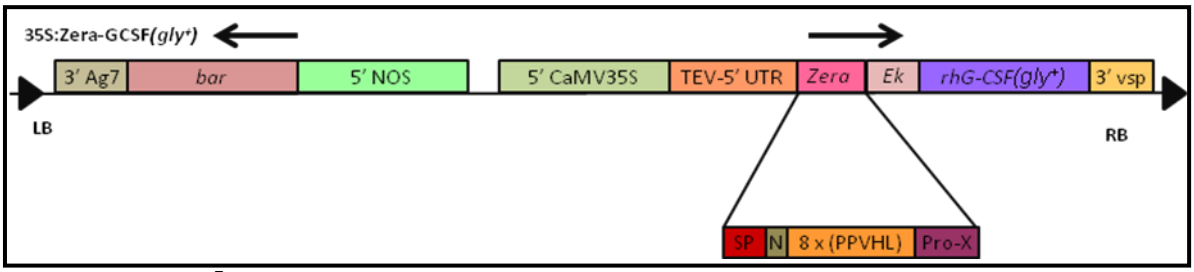


Figure 20. ZeraG-CSF^{T} expression cassette. rhG-CSF(gly^{+}) represents the recodified nucleotide sequence, which encodes the original amino acids sequence from the hG-CSF, the glycosylation site is maintained at the 133 residue (T). The Ek box represents the Enterokinase cleavage site, which follows the Zera sequence.

3.4.4.2 G-CSF^{A}

The rhG-CSF mature sequence without the N-terminal Zera fusion was also synthesized *de novo*, and provided by GenScript using the pUC57 vector. The same variant of the hG-CSF was used to design this sequence, however an additional amino acid was added at the 5' end (Met) to allow gene translation initiation, converting this sequence into a sequence with 175 amino acids. The mature rhG-CSF sequence was also synthesized without encoding the glycosylation site, by changing the Thr codon for an Ala codon (Fig. 21).

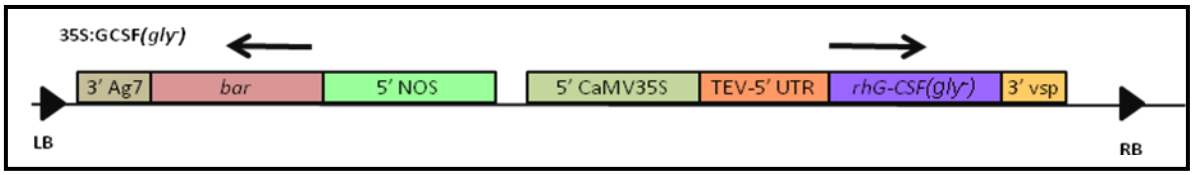
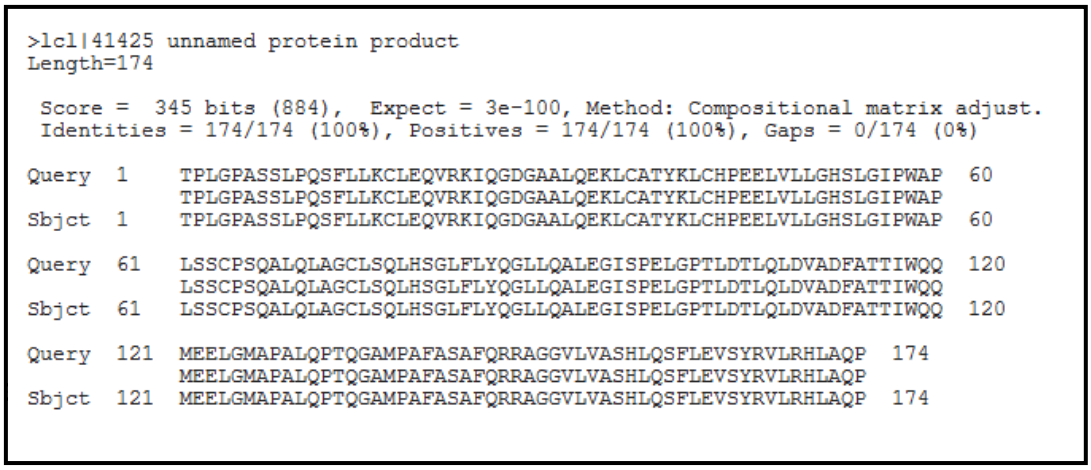


Figure 21. G-CSF^{A} Cassette of expression. rhG-CSF(gly^{-}) represents the codon optimized synthetic nucleotide sequence, which encodes the original amino acids sequence from the hG-CSF, however the glycosylation site has been removed by amino acid change at the 133 residue(T/A).

In order to optimize the expression the gene of interest, it is advisable to perform a sequence modification (recodification), so that the preferential codons from the host plant cells can be used, and therefore increase the expression at the translational level (see Annexes). The recodification of the transgene sequences encodes the same amino acids sequence as the original sequence (76)(Fig. 22).



```
>lcl|41425 unnamed protein product
Length=174

 Score =  345 bits (884),  Expect = 3e-100, Method: Compositional matrix adjust.
 Identities = 174/174 (100%), Positives = 174/174 (100%), Gaps = 0/174 (0%)

Query  1    TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAP  60
            TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAP
Sbjct  1    TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAP  60

Query  61   LSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQ  120
            LSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQ
Sbjct  61   LSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQ  120

Query  121  MEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP  174
            MEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP
Sbjct  121  MEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP  174
```

Figure 22. rhG-CSF amino acids sequence, after codon optimization, compared to the amino acids sequence encoded by the original human nucleotide sequence (Modified using NCBI Blast [76]).

The Polymerase Chain Reaction (PCR) performs an automatic procedure for the duplication of any DNA section between two oligonucleotide primers, which border the complementary strands in both directions. Primers corresponding to approximately 20 bp of the 5' ends of the DNA sequence to be amplified are used. Heat stable polymerases, e.g. the Taq-Polymerase from *Thermus aquaticus*, the Tfl-Polymerase from *Thermus flavus* or the Tth-Polymerase from *Thermus thermophilus*, are used. DNA-polymerases need a small double stranded sequence with free 3´OH-ends comprised of a primer bound to a template, in order to synthesize the complementary DNA-Sequence. The prepared DNA synthesis products are denaturized at 94ºC and amplified after the oligonucleotides annealing (at a range of 48-60ºC). The cycles of denaturing, hybridization ("Annealing") and DNA-Synthesis ("Elongation") are repeated, whereby an exponential increase of the target sequence is possible.

<u>The steps are as follows:</u>

Denaturing: double stranded DNA (dsDNA) is placed at 94ºC and denatured into two single stranded DNA molecules (ssDNA).

Annealing: hybridizing of one and/or two (i.d.R. two) specific target sequence oligonucleotide-primers to complementing sequences; at this step, the chosen temperatures for such primers depend on the melting temperature and the oligonucleotide homology, which should range between 48-60ºC.

Elongation: Polymerization of the DNA-sequence which lies between the oligonucleotide primers, at the primers' 3´OH-End is performed by the thermostable Polymerase at 72ºC; the elongation time must take approximately 1 min. per 1 Kb DNA.

Standard PCR Reaction (GoTaq® Green Master Mix – Promega)

Component	Volume	Final Concentration
GoTaq® Green Master Mix (2x)	12.5 µl	1x
Upstream primer (10 µM)	0.25-2.5 µl	0.1-1.0 µM
Downstream primer (10 µM)	0.25-2.5 µl	0.1-1.0 µM
Template-DNA	1-5 µl	<250 ng
Nuclease-Free Water to	25 µl	

Depending upon the primers and template used, the given conditions entered into the Thermocycler vary. With standard conditions under which positive results are usually obtained, the following apply:

3.4.7 High Fidelity PCR amplification

DNA sequence amplification of high quality, e.g. for cloning purposes, must be performed using a high fidelity DNA polymerase enzyme. In this work, the Finnzymes' Phusion® High-Fidelity DNA Polymerase was used for such purposes. The Phusion DNA Polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity-enhancing domain. The error rate of this DNA polymerase is determined to be 4.4 x 10^7, which is approximately 50-fold lower than that of *Thermus aquaticus* DNA polymerase, and 6-fold lower than that of *Pyrococcus furious* DNA polymerase.

General High Fidelity PCR Reaction (Phusion® HF DNA Polymerase – Finnzymes)

Component	Volume	Final Concentration
Phusion® HF Buffer (5x)	4 µl	1x
dNTPs (10 mM)	0.4 µl	200 µM each
Upstream primer (10 µM)	1 µl	0.5 µM
Downstream primer (10 µM)	1 µl	0.5 µM
Template-DNA	1-5 µl	1 pg-10 ng
Phusion® DNA Polymerase	0.2 µl	
Nuclease-Free Water to	20 µl	

3.4.8 Fast PCR amplification

For routine and high throughput PCR applications, e.g. plant PCR screening, the Finnzymes' Phire®Hot Start II DNA Polymerase was used. This polymerase is faster, robust, and capable of amplifying long DNA fragments with high yields. Such features are achieved by incorporating a ds-DNA-binding domain, which allows short extension times (10-15 s/kb), improves yields and increases fidelity 2-fold compared to Taq DNA polymerase.

General Fast PCR Reaction (Phire® Hot Start II DNA Polymerase – Finnzymes)

Component	Volume	Final Concentration
Phire® Reaction Buffer (5x)	4 µl	1x
dNTPs (10 mM)	0.4 µl	200 µM each
Upstream primer (10 µM)	1 µl	0.5 µM
Downstream primer (10 µM)	1 µl	0.5 µM
Template-DNA	1-5 µl	1 pg-10 ng
Phire® Hot Start II DNA Polymerase	0.4 µl	
Nuclease-Free Water to	20 µl	

3.4.9 *Escherichia coli* colony PCR

A specific test of several clones by means of PCR is made possible by lysing samples of one or more colonies together (pooled reaction) to isolate template DNA. One and/or more clones (by collection PCR) are diluted into 50-100 µl $_{dd}H_2O$. The resuspended colonies are heated 10 min. at 100ºC. Afterwards, the cell debris is pelleted by centrifugation (15000 rpm; 5 min.; RT). The supernatant will contain DNA that can be used as template (2-5 µl Lysed for a volume reaction of 50 µl) (Modified from Ramírez-Alanis et al., 2007 [70]).

3.4.10 *Agrobacterium tumefaciens* colony PCR

For *Agrobacterium tumefaciens* colony quick screening PCR after electroporation, bacterial lysate may also be used for the PCR reaction. A single colony is dissolved in 50 µL nuclease-free H_2O. The dissolved colony is heated 10 min, at 100°C. The cell debris is centrifuged at 15000 r.p.m., 5 min, RT. The supernatant (5 µl) can be used as DNA template for PCR (20 µl reaction) (Modified from Ramírez-Alanis et al., 2007[70]).

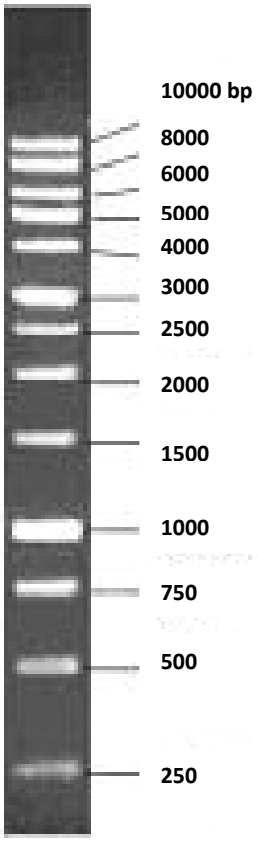
3.5 Recombinant DNA Techniques

3.5.1 Agarose gel

50 x TAE-Buffer:	Tris	242,0 g
	Glacial acetic acid	57,1 ml
	0.5 M EDTA pH 8,0	100 ml
		Up to 1000 ml H_2O_{deion}
10 x Sample's Buffer:	Blue Bromophenol	0,2%
	EDTA	0,2 M
	Glycerine	50,0%
Ethidium bromide Stock solution	10 mg/ml H_2O *Mutagen; kept at 4ºC and light protected	

1% Agarose gel production

Either 1 x TAE (Tris-Acetate-EDTA) or 1 x TBE (Tris-Borate-EDTA) can be used as running Buffers. 1.0 g of agarose is diluted in 100 ml of the running Buffer and heated briefly, until the agarose is totally in solution. After the temperature drops to approximately 50ºC, the gel can be poured on a gel forming bed and incubated at RT for some 30-45 min. in order to polymerize. The DNA samples must be mixed with 1/10 Vol. Sample Buffer and pipetted into the gel holes. The electrophoresis separation takes place at 160 V for approximately 40 min. The gel must then be placed into an ethidium bromide TAE bath (500 ml TAE + 50 µl ethidium bromide (10mg/ml) for about 15 min., and the separated DNA fragments can be finally observed under a UV-light and photographed. In order to determine the size of the badns observed in the gels run in this work, a 0.25-10 Kb DNA ladder was used as marker.



1.0 % Agarose

3.5.2 Gel extraction (Promega A9281)

The purification of DNA fragments produced by digestion reactions was achieved by using the Wizard® SV Gel and PCR Clean-up System. Load and run the agarose gel. Weigh a 1.5 mL microcentrifuge tube for each DNA to be isolated. Visualize and photograph the DNA using a long-wavelength UV lamp and an intercalating dye such as ethidium bromide. Excise the DNA fragment of interest in a minimal volume of agarose using a clean scalpel or razor blade. Transfer the gel slice to the weighed microcentrifuge tube and record the weight. Subtract the weight of the empty tube from the total weight to obtain the weight of the gel slice. Note: The gel slice may be stored at 4°C or at –20°C for up to one week in a tightly closed tube under nuclease-free conditions before purification. Add Membrane Binding Solution at a ratio of 10µl of solution per 10mg of agarose gel slice. Vortex the mixture and incubate at 50–65°C for 10 minutes or until the gel slice is completely dissolved. Vortex the tube every few minutes to increase the rate of agarose gel

melting. Centrifuge the tube briefly at room temperature to ensure the contents are at the bottom of the tube. Once the agarose gel is melted, the gel will not resolidify at room temperature. Place one SV Minicolumn in a Collection Tube for each dissolved gel slice.

Transfer the dissolved gel mixture or prepared PCR product to the SV Minicolumn assembly and incubate for 1 minute at room temperature. Centrifuge the SV Minicolumn assembly in a microcentrifuge at 16,000 × *g* (14,000rpm) for 1 minute. Remove the SV Minicolumn from the Spin Column assembly and discard the liquid in the Collection Tube. Return the SV Minicolumn to the Collection Tube. Wash the column by adding 700µl of Membrane Wash Solution, previously diluted with 95% ethanol, to the SV Minicolumn. Centrifuge the SV Minicolumn assembly for 1 minute at 16,000 × *g* (14,000rpm). Empty the Collection Tube as before and place the SV Minicolumn back in the Collection Tube. Repeat the wash with 500µl of Membrane Wash Solution and centrifuge the SV Minicolumn assembly for 5 minutes at 16,000 × *g*. Remove the SV Minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the flowthrough. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. Carefully transfer the SV Minicolumn to a clean 1.5ml microcentrifuge tube. Apply 50µl of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 16,000 × *g* (14,000rpm). Discard the SV Minicolumn and store the microcentrifuge tube containing the eluted DNA at 4°C or –20°C (77).

3.5.3 PCR clean-up (Promega A9281)

Amplify target of choice using standard amplification conditions. Add an equal volume of Membrane Binding Solution to the PCR Amplification. Continue protocol according to step 9 on, from the “Gel Extraction” protocol (77).

3.5.4 DNA digestion

Restriction enzymes used:

Restriction enzyme	Restriction site	Provider
AvaI	C'YCGRG	NEB
BamHI	G'GATCC	
BspHI	T'CATGA	
EcoRI	G'AATTC	
FauI	CCCGC$(N)_4$'	
HindIII	A'AGCTT	
NcoI	C'CATGG	
SacI	GAGCT'C	
SapI	GCTCTTC$(N)_1$'	
SmaI	CCC'GGG	
SmlI	C'TYRAG	
XhoI	C'TCGAG	
XbaI	T'CTAGA	
XmaI	C'CCGGG	

Restriction endonucleases are elementary components of the restriction- and modificationsystems that bacteria use to protect themselves from foreign DNA invation (bacteriophage) while, at the same time, permitting low frequency uptake of foreign DNA to promote genetic variability. The use of TypeII Restriction Endonucleases makes possible the dsDNA sequence-specific cutting. Depending on the restriction enzymes used, different buffers and temperature conditions (37ºC and 30ºC) are needed. One Restriction Enzyme Unit generally cuts 1µg of Phage DNA completely in one hour under optimal reaction conditions. There is a distinction between the partial digest, by which small enzyme quantities are used and not all sites are cut, and the complete digest, by which a surplus of the reaction enzymes are used and all sites are cut.

Plasmid-DNA	1 µg
10x-Puffer	4 µl
Reaction enzyme	1-2µl (10-20 Units)
	Up to 20 µl H_2O_{steril}

The digestion is usually incubated from 1 to 2 hours at 37ºC. Evaluation of efficiency of digestion is performed by separation and visualization of bands via agarose gel electrophoresis.

3.5.5 Ligation

In order to construct a DNA recombinant molecule, T4-DNA-Ligase is required. This enzyme forms phosphodiester bonds between the free 3'OH-end and the free 5'-phosphate end of dsDNA. Both sticky-ends and blunt-ends can be bound or ligated, although blunt ended ligation is less efficient. Recombinant DNA molecules are frequently obtained from the insertion of the specific DNA Fragment (Insert) into a cloning vector. PCR products can be ligated with vectors, when sticky-ends or blunt-ends are created after digesting the DNA with restriction enzymes.

Different vector:insert proportions must be tested, and the optimal proportion for a successful ligation must be selected. In this work, the T4 DNA Ligase from Invitrogen was used, following the protocol below.

	Cohesive Ends
5x Ligase Reaction Buffer	4 µL
Insert:Vector Molecular Ratio	3:1
Vector Ends	3-30 fmol
Insert Ends	9-90 fmol
Total DNA	0.01-0.1 µg
T4 DNA Ligase	0.1 unit
Autoclaved distilled water	To 20 µL
Temperature	23-26°C
Time	1 h

3.5.6 Small scale plasmid isolation (Miniprep) (Solutions described below)

Add 1.7 mL of overnight culture into a microfuge tube. Centrifuge max speed, 1 min. Repeat steps 1 and 2 (total of 3-5 mL). Add 0.2 mL ice-cold Solution I, resuspend with tip. Add RNase. Vortex. Incubate 5min at RT. Add 0.4 mL Solution II (Fresh). Invert 5 times, incubate 5 min RT. Add 0.3 mL

ice-cold Solution III. Invert 5 times, incubate on ice for 10 min. Centrifuge 5 min max speed. Prepare new eppendorf tubes, and pass supernatants (without removing any of the white pellet debris). Fill up with ice-cold isopropanol. Invert and incubate 2 min at RT. Centrifuge 5 min max speed. Pour off supernatant, dry on paper. Add 1 mL ice-cold 70% Ethanol. Invert, and centrifuge 1 min max speed. Pour off supernatant, dry on paper. Speed vac, 10 min, with heat. Resuspend with 20-50 uL nuclease free water or TE buffer. Meassure DNA concentration at OD_{260}.

Solution I	5 mM Glucose (MERCK) 25 mM Tris-HCl (Promega) 10 mM EDTA (Promega) pH 8.0 sterile filtered
Solution II	1% SDS (Fisher) 0.2 N NaOH (Fresh)
Solution III	3 M Potassium acetate (Fisher) 57.5 mL glacial acetic acid (Especialidades Químicas S.A. de C.V.)
TE Buffer	10 mM Tris-HCl 1 mM EDTA

3.5.7 Small scale large plasmid isolation (Miniprep for *Agrobacterium*)(Solutions described below)

Centrifuge 4.5 mL of the ON *Agrobacterium* culture to collect cells. Add 100 µL Solution I, RNase A, and vortex. Add 200 µL Solution II. Invert 5 times, and incubate for 5 min at RT. Add 150 µL Solution III, add 150 µL chloroform. Invert 5 times. Incubate 10 min on ice. Centrifuge 10 min. Pass supernatant to new tubes and add 200 µL Precipitation Solution. Invert 5 times, incubate 15 min on ice. Centrifuge 5 min. Drain and resuspend in 20 µL nuclease free water (Modified from Virgili-López et al., 2008[68]).

Solution I	5 mM Glucose
	25 mM Tris-HCl
	10 mM EDTA
	pH 8.0
	sterile filtered
Solution II	1% SDS
	0.2 N NaOH
	(Fresh)
Solution III	3 M Potassium acetate
	57.5 mL glacial acetic acid
Precipitation Solution	30% 8000MW PEG (Sigma)
	1.5 M NaCl

3.5.8 Genomic DNA extraction from plant cells

Solution:

2% CTAB Solution	
100 mM Tris-HCl pH 8	1.21 g Tris
2% CTAB (Sigma)	2 g CTAB
20nM EDTA pH8	0.74 g EDTA
1.4 M NaCl	8.182 g NaCl
1% PVP (sigma)	1 g PVP
	100 mL H2O

Procedure

Weigh 0.1 – 0.25 g NT1 callus and add 400 µL CTAB. Disrupt tissue and vortex. Incubate at 65°C, 20 min. Vortex and add 400 µL Chloroform. Vortex and Centrifuge 14000 rpm, 5 min. Pass supernatant to new tube. Add 300 µL Isopropanol (equal vol) and mix. Centrifuge 14000 rpm 5 min. Decant supernatant and add 300 µL 75% Ethanol and mix. Centrifuge 14000 rom, 5 min. Dry

the pelleted DNA by using a speed vac equipment. Resuspend in 40 µL H_2O. Add 1 µL RNase (10 mg/ml) and 37°C 1 min. Use DNA as template for PCR (Modified from Stewart et al., 1993[78]).

3.5.9 DNA ethanol precipitation

This protocol can be used to concentrate DNA, or to change the buffer in which the DNA is suspended. It can also be coupled with phenol chloroform extraction for purifying nucleic acids. This protocol also works for RNA precipitation (take care to use RNAse free materials in this case).

Materials

- 3M NaOAc pH 5.2
- 95% Ethanol
- Glycogen (optional)

Procedure

Add 0.1 volumes of 3M Sodium Acetate solution to 1 volume of DNA sample. Add 1ul Glycogen to the DNA sample. Add 2 volumes of 95% EtOH to the DNA Sample. Store the solution overnight at -20°C or for 30 minutes at -80°C. Centrifuge the solution at maximum speed for at least 15 minutes. Decant and discard the supernatant. (Optional) Add 1 ml of 70% EtOH to the pellet and let sit for 5 minutes. (Optional) Centrifuge the sample at maximum spped for 5 minutes. (Optional) Decant and Discard the supernatant. Air-dry the pellet for 10-15 minutes at room temperature until all liquid is gone. Resuspend in desired volume of water or buffer (Modified from Sambrook et al., 2001[64]).

3.5.10 Determination of DNA concentration

DNA concentration was determined by measuring the absorbance, using spectrometry at 260 nm wavelength. To determine the concentration of DNA, the Nanodrop Thermo Scientific instrument was used (Modified from Sambrook et al., 2001[64]).

3.6 Construction of the Expression Cassettes

3.6.1 Cloning p19 from pIBT210.1p19 into pPS1

The TEVp19 nucleotide sequence is flanked by the restriction sites XhoI and SacI, at its 5'- and 3'-ends respectively. In order to clone the TEVp19 sequence, into the pPS1:NPTII binary vector, the p19Fow and p19Rev primers are used to PCR the sequence out. After PCR product clean-up, the amplified TEVp19 sequence is digested with XhoI and SacI, so that the sticky-ends can be created. Simultaneously, the pPS1 binary vector is also digested with the same restriction enzymes for further ligation and creating the pPS1p19 binary vector (Fig. 23).

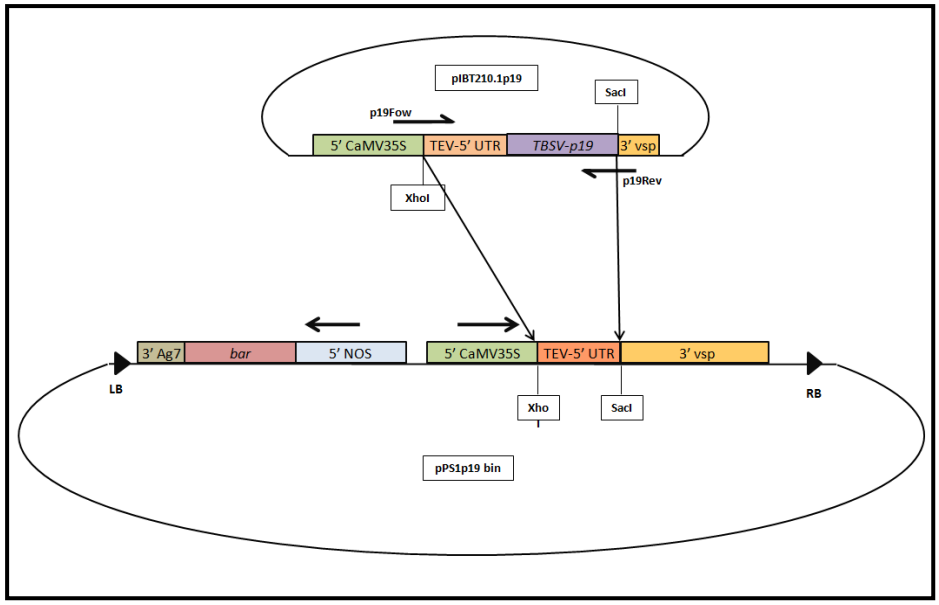


Figure 23. Construction of the 35Sp19 expression cassette.

3.6.2 Cloning p19 into pGEM

In order to insert a nucleotide sequence into the pGEM-T EASY vector, the insert sequence must be amplified with poly-A residues, so that the vector backbone and DNA insert can be ligated (Fig. 24).

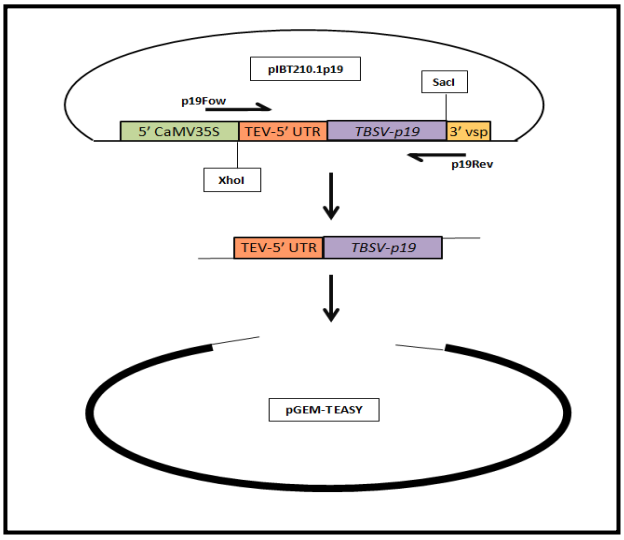


Figure 24. Construction of the pGEM plasmid vectors.

3.6.3 Fusing G-CSF^{T} with Zera in the expression cassette

The construction of the expression cassette containing the ZeraG-CSF^{T} is based on replacing the F1-V gene, present in the pUC35SZeraF1-V vector, leaving the G-CSF^{T} fused to Zera in the expression cassette driven by the CaMV35S Promoter. The F1-V gene is replaced by G-CSF^{T} using the restriction enzymes NcoI and SacI, which flank at the 5- and 3-ends, respectively, the EkG-CSF^{T} sequence in the pUC57Ek G-CSF^{T}, and F1-V in the pUC35SZeraF1-V (Fig. 25).

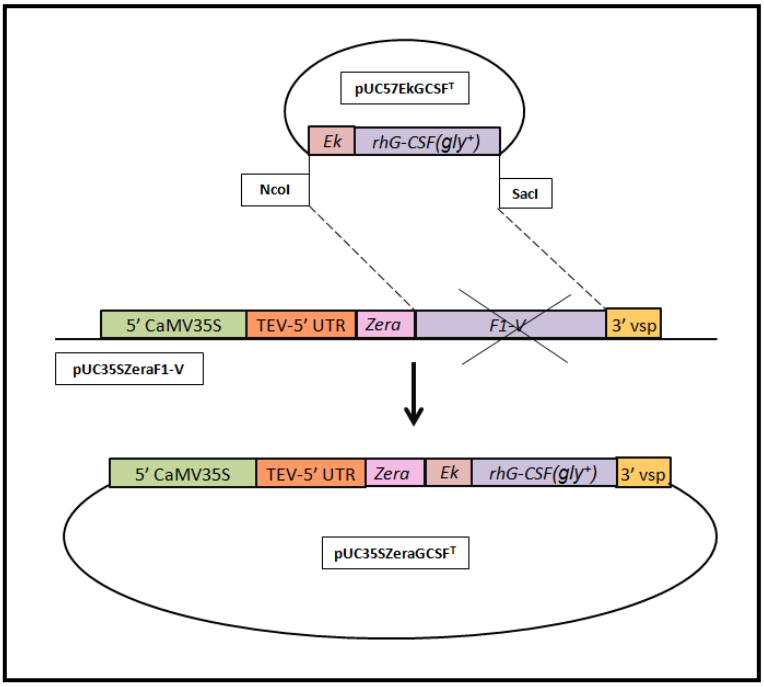


Figure 25. Construction of the 35SZGCSF^{T} expression cassette in pUC18.

3.6.4 Cloning 35SZeraGCSF^{T} expression cassette into pGPTV:BAR

The further insertion of the expression cassette containing the gene *Zera G-CSF*^{T} gene into the binary plasmid pGPTV:BAR is achieved by using the restriction enzymes HinddIII and EcoRI, which flank the both expression cassettes (35SZeraF1-V and 35SZera G-CSF^{T}) at their 5'- and 3'-ends respectively. Thus, the expression cassette 35SZeraF1-V present in the p35SZeraF1-V binary vector is substituted by the 35SZeraG-CSF^{T} from the pUC35SZeraG-CSF^{T} (Fig. 26).

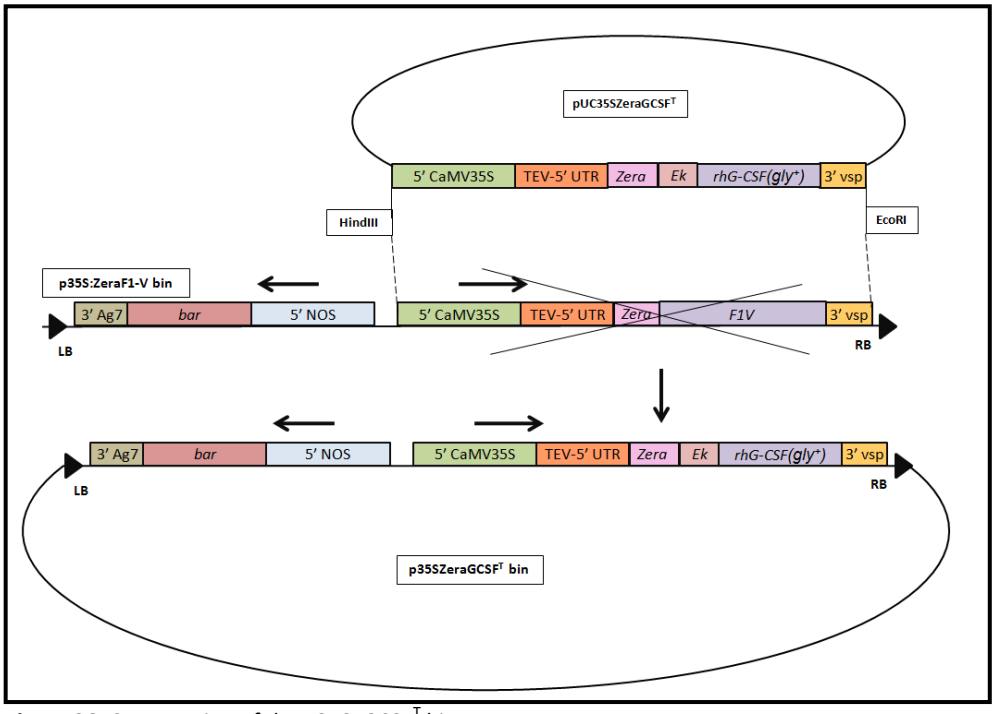


Figure 26. Construction of the p35SZGCSF^{T} binary vector.

3.6.5 Cloning G-CS F^{A} in the expression cassette

The construction of the G-CSF^{A} expression cassette is based on using the restriction sites flanking the G-CSF^{A} sequence, BspHI and SacI (5'- and 3'-end, respectively). The same restriction sites flank the F1-V gene present in the expression cassette 35SF1-V in the pUC35SF1-V. Thus, by replacing the F1-V gene, the pUC35SG-CSF^{A} vector would be created. Further substitution of the whole expression cassette 35SF1-V in the p35SF1-V binary vector, would also be carried out by using the same strategy as in 3.5.4 in order to create the p35SG-CSF^{A} binary vector (Fig.27).

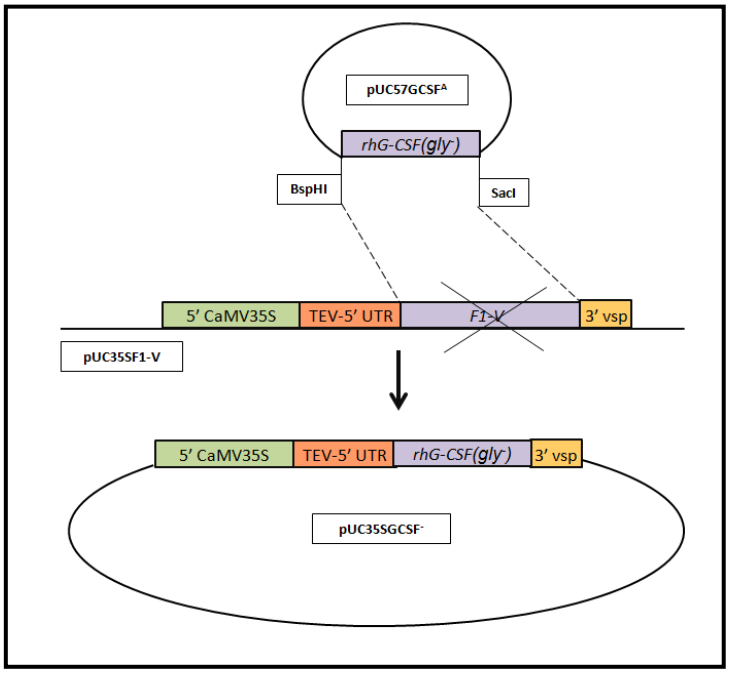


Figure 27. Construction of the 35SGCSF^{A} expression cassette, using pUC18.

3.7 Transformation of Plant Cells

3.7.1 Growth and maintenance of NT1 cells (For medium see below)

Maintain callus (**52**) on plates of NT1 medium in the dark at 23 – 24C. Transfer small clumps to fresh medium every 3 weeks by taking an amount the size of a pea, and placing it on the medium. Five pieces this size can be cultured per plate. It is strongly recommended that a few plates of callus are maintained as a back-up for material to initiate new suspensions in the event suspension cultures become contaminated, which can occur easily.

When callus has grown up to approximately the size of a quarter, add 1.6 to 1.8 grams to a 250 ml flask containing 48 ml of KCMS liquid medium. This should be done under sterile conditions. Place flask on a shaker at 100 RPM, at 23 – 24C in the dark. If a shaker is not available in the dark, wrap flask in aluminum foil. Subculture to fresh medium every 7-10 days by transferring 2 ml (use a 10-ml wide-bore pipet) of the 7- or 10-day-old culture to a 250 ml flask containing 48 ml of KCMS liquid medium.

Component	Quantity for 1 L
MS Salts (PhytoTechnology Laboratories)	4.3 g
Myoinositol (MERCK)	100 mg
Thiamine HCl (1 mg/ml)(PhytoTechnology Laboratories)	1.3 mL
2,4-D (1mg/ml) (Sigma)	200 µL
KH_2PO_4	200 mg
Kinetin (1mg/ml) (Sigma)	100 µL
Sucrose	30 g
pH	5.5 ± 0.3

3.7.2 *Agrobacterium* mediated NT1 cells transformation

Preparation of Plant Cell Material

Three to four days prior to transformation, subculture a 1-week-old NT-1 suspension culture (2 ml of NT-1 culture into 40 ml NT-1 medium). Culture in the dark at 23 to 25C on a gyratory shaker at 100 rpm.

Preparation of Agrobacterium

Streak Agrobacterium from glycerol stock onto a LB plate with spectinomycin (50 mg/l) or appropriate selection for plasmid of interest. Incubate at 30C for 24 to 48 hours.

Two days before transformation pick some colonies from the plate and culture in 3 ml YM medium containing 50 mg/l spectinomycin or appropriate selection. Incubate slanted, in a 30C shaking incubator at 250rpm.

Transformation

Measure OD_{600} of *Agrobacterium* culture. Optimum OD_{600} equals 0.5 - 0.6. Add acetosyringone (PhytoTechnology Laboratories) to the 3-(or 4)-day-old NT-1 cell culture: 1 µl of a 20 mM stock solution made in 70% ethanol is added for each ml of cell culture. Acetosyringone stock solution needs to be made fresh prior to use. Abrade the NT-1 cells by repeatedly pipetting (20 times) with a 10 ml-wide bore, sterile disposable plastic pipet. This wounds the cells and can improve transformation efficiency. Add 100 µl of *Agrobacterium* to each of the petri dishes (60 x 15 mm) containing 4 ml of the abraded NT-1 cell culture. However, do not add Agro to one plate, so that it

can serve as a non-transformed control. Wrap plate in parafilm and incubate in the dark on a shaker at 100 rpm at 23 - 25C (19°C optimum if possible) for 4 days.

Selection of Transformants

Transfer cells to a 50 ml conical centrifuge tube. Bring final volume of suspension to 9 mL using NTT medium (NT-1 medium containing 300 µg/mL timentin). Mix and centrifuge at 1000 rpm for 10 minutes in a centrifuge equipped with a swinging bucket rotar. Pipet off supernatant. Resuspend in NTC and repeat wash twice more for a total of 3 washes. After final wash and centrifuging, resuspend pelleted cells in 16 mL NTK (NT-1 medium containing 50 µg/mL kanamycin).

Plate 2 mL of cells onto each of NTTK medium plates (150 x 15 mm-disposable petri plates) containing NT-1 agar medium supplemented with 8 g/L Agar/Agar, 50 µg/L kanamycin (add a filter sterilized solution after autoclaving the medium and then allowing it to cool until it is cool enough to pour). Wrap plates with parafilm and culture at 23 - 25C in dark. Plates may be left open in the hood to allow excess liquid to evaporate.

After 4 - 6 weeks transformed calli may appear and can then be selected and subcultured to fresh NTK (plates containing NT-1 agar medium supplemented with µg/mL kanamycin). Wrap plates with parafilm and culture at 23-25C in dark.

	Per liter
MS salts	4.3 g
MES stock (20X)	50 ml
B1 inositol stock (100X)	10 ml
Miller's I stock	3 ml
2,4-D (1 mg/ml)	2.21 ml
Sucrose	30 g
pH to 5.7 $\pm$ 0.03	

B1 Inositol Stock (100x)(1 liter)

Thiamine HCl (Vit B1) - 0.1 g

Myoinositol - 10 g

MES (20x) (1 liter)

MES (2-N-morpholinoethanesulfonic acid) – 10 g

Miller's I (1 liter)

KH_2PO_4 – 60 g

Alternatively, YM in powder form can be purchased (Gibco BRL; catalog #10090-011). To make liquid culture medium, add 11.1 g to 1 liter water.

3.8 RNA Manipulation

3.8.1 Total RNA extraction (FastRNA Pro Green Kit – FastPrep) (MPBio 6020-600)

For each 100-300 mg NT1 sample to be processed, add 1 ml RNApro Solution to a green-cap tube containing Lysing Matrix D. Add 100-300 mg plant sample to the tube containing RNApro Solution and Lysing Matrix D. Securely close the cap to prevent leakage in the next step. NOTE: The volume of the sample with 1 ml of RNApro Solution must provide approximately ¼ inch (5mm) airspace in the matrix tube to allow for effective homogenization and to prevent sample leakage and/or tube failure. DO NOT overfill the matrix tube. If the sample is too large for processing in a single tube, divide the sample and process using multiple tubes.

Process the sample tube in the FastPrep instrument for 40 seconds at a setting of 6.0. If the user experimentally determinates that additional processing time is required, the sample should be incubated on ice in the Lysing Matrix tube for at least 2 minutes between successive FastPrep Instrument homogenizations to prevent sample heating and possible RNA degradation.

Remove the sample tube and centrifuge at a minimum of 12000 x g for 5 minutes at 4°C or room temperature. Transfer the upper phase to a new microcentrifuge tube. Avoid transferring the debris pellet and lysing matrix. Incubate the transferred sample 5 minutes at room temperature to increase RNA yield. Add 300 µl of chloroform (NO isoamyl alcohol). Vortex 10 seconds. Incubate 5

minutes at room temperature to permit nucleoprotein dissociation and increase RNA purity. Centrifuge the tubes at a minimum of 12000 x g for 5 minutes at 4°C. Transfer the upper phase to a new microcentrifuge tube without disturbing the interphase. If a portion of the interphase is transferred, repeat the centrifugation with the upper phase, and transfer the new upper phase to a clean microcentrifuge tube. NOTE: Samples containing large amounts of cellular mucopolysaccharides can be re-extracted with chloroform (isoamyl alcohol may be included with the chloroform [CHCl3:IAA, 24:1, v:v]) to increase RNA purity. Alternatively, a lithium chloride precipitation may be used. Add 500 µl of cold absolute ethanol to the sample; invert 5x to mix and store at -2°C for at least 30 minutes. Centrifuge at a minimum of 12000 x g for 15 minutes at 4°C and remove the supernatant. The RNA will appear as a white pellet in the tube. If the pellet is floating the sample may be recentrifuged to place the pellet at the tube bottom. Wash the pellet with 500 µl of cold 75% ethanol (made with DEPC-H_2O). Remove the ethanol, air dry 5 minutes at room temperature (DO NOT completely dry the RNA) and resuspend the RNA in 100 µl of DEPC-H_2O for a short-term storage.

RNA resuspended in DEPC- H_2O is generally stable for up to a year at -80°C. For longer term storage RNA samples may be stored at -20°C as ethanol precipitates. Ethanol precipitates must be pelleted and the RNA resuspended in aqueous solution prior to use. NOTE: RNA does not evely distribute in ethanol and can lead to inconsistent RNA amounts between samples when equal volumes are pipetted. In situations where precise amounts of RNA are required, resuspend the RNA in DEPC- H_2O and measure the concentration by OD_{260} before proceeding. Incubate 5 minutes at room temperature to facilitate RNA resuspension. Determine the RNA concentration:

a. Dilute 5 µl of the purified RNA into 495 µl of DEPC- H_2O.
b. Read the OD_{260} using DEPC- H_2O as a blank.

Aliquote and store the RNA solution at -70°C. The RNA integrity and an estimation of the yield can be determined by analyzing a portion of the RNA sample using gel electrophoresis. Add 1 µg RNA in 9 µl DEPC- H_2O, heat to 65°C for 5 minutes, add gel loading buffer and load the sample on a 1.2% agarose gel containing 2.2 M formaldehyde in MOPS buffer. The sample is run at ~ 80 volts for 30 minutes. Ethidium bromide may be added to the denatured RNA sample at 10 µg per milliliter prior to gel loading or the gel may be ethidium bromidestained and destained following electrophoresis and visualized under UV light. The quality of the RNA is determined by the appearance of ribosomal RNAs as sharp, distinct bands. Heterogenous-sized messenger RNA may

appear as diffuse ethidium staining between and below the ribosomal bands. Small RNA species such as tRNA and 5S RNA may be present in varying amounts at the dye front (79).

3.8.2 Determination of RNA concentration

RNA concentration is best determined by measuring the absorbance of an aqueous solution at a wavelength of 260 nm. A 1 ml solution with an A260 of 1 absorbance unit contains 40 µg of RNA. RNA should have a A260/A280 minimum ratio of 1.7 or higher. It is very important to avoid exogenous ribonuclease contamination during the preparation, storage and handling of the RNA samples before cDNA synthesis (79).

3.8.3 Determination of RNA quality

Total RNA concentration and quality can be estimated on a native agarose gel by loading a small sample in 1X RNA Loading Buffer next to varying amounts of the included control RNA. The most visible bands are the ribosomal subunits running approximately at 1.8 and 0.9 kb on a 1.2% agarose TBE gel. (Figure 28).

Protocol

Mix equal volumes of RNA and 2X RNA Loading Buffer, heat at 70°C for 5 minutes, put briefly on ice and run on a 1.5% agarose, 1X TBE gel. Stain and photograph the gel on a UV transilluminator. 2X RNA Loading Buffer: 90% deionized formamide, 0.05% bromophenol blue, 1X TBE: 89 mM Tris Base 89, mM Boric Acid, 2 mM EDTA (pH 8.0) (79).

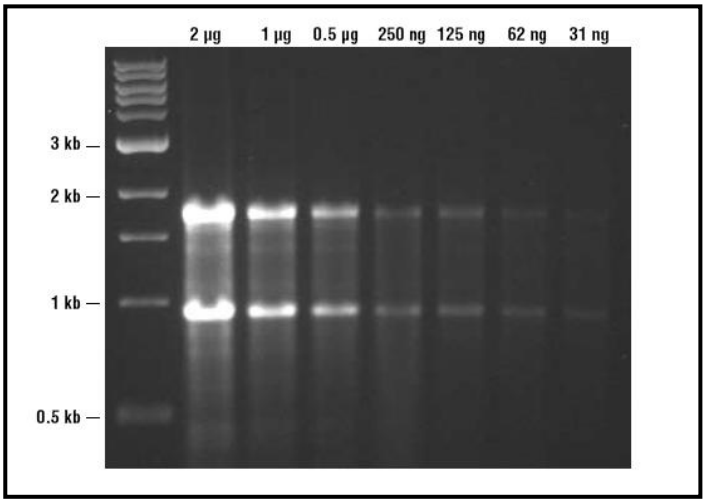


Figure 28. Determination of RNA quality (79).

3.8.4 Reverse transcriptase

First Strand Synthesis Protocol:

In order to avoid ribonuclease contamination use proper precautions, including the use of autoclaved tubes, baked glassware, ultra-pure solutions, sterile pipette tips and latex gloves during manipulations.

Thaw system components and put on ice. The 10X RT Buffer can be warmed briefly at 42°C and vortexed to dissolve any precipitate. In a sterile microfuge tube add:

Total RNA 1–10 µl (1 ng–2 µg)

Primer dT23VN 2 µl

dNTP Mix 4 µl

Nuclease-free H20 to a total volume of 16 µl

Heat for 5 minutes at 70°C. Spin briefly and put promptly on ice.

RNA/primer/dNTP mix from step 1 16 µl

10X RT Buffer 2 µl

RNase Inhibitor 1 µl

M-MuLV Reverse Transcriptase 1 µl

Final volume 20 µl

Incubate at 42°C for one hour. Inactivate the enzyme at 95°C for 5 minutes. Add 1 µl (2 units) of RNase H and incubate at 37°C for 20 minutes to degrade the RNA. Heat at 95°C for 5 minutes to inactivate the enzyme. Dilute reaction to 50 µl with water and use 2–5 µl for each PCR reaction (79).

Notes:

Using poly(A)+ RNA

Use 1 to 100 ng of poly(A)+ RNA. The amount required depends on the abundance of transcripts in the sample (79).

3.9 Protein Manipulation

3.9.1 Total soluble protein extraction

Weigh 0.250 g of fresh callus. Put in a FastPrep tube with 1 ceramic bead. Add Zera Extraction Buffer (ZEB: 100mM TrisHCl pH 8.0, 5% SDS, 5%β-mercaptoethanol, 200mM NaCl, 0.2% protease inhibition cocktail, Sigma), 5 ml ZEB per 1 g of fresh callus (1.25 ml per 0.25 g Callus). Disrupt the sample at 4 m/s for 15 s. Incubate the sample in shaking at RT for 1 h. Boil the sample for 20 min. Centrifuge at max speed (approx. 14000 rpm) for 20 min. Use supernatant to a new tube and/or pellet for protein analysis (52).

3.9.2 Quantification of total soluble proteins (Lowry Method)

Solution	Components
A Sodium carbonate solution	2% Na_2CO_3 0.1 N NaOH
B Cupper sulfate solution	1.56% $CuSO_4 \cdot 5H_2O$
C Sodium and phosphate tartrate Solution	2.37% NaK tartrate
D Solution	100 mL Solution A 100 mL Solution B 100 mL Solution C
1 N Folin-Ciocalteu's Phenol	1 Vol 2 N Reactant: 1 Vol H_2O (FRESH!)

Prepare standard curve. Take 0.2 mL sample + 2 mL D Soltuion (Mix well). Incubate 10 min RT. Add 0.2 mL 1N Folin-Ciocalteu's phenol reactant. Incubate 30 min RT. Measure OD_{660nm}.

3.9.3 RC DC Lowry method (Reducing agent and detergent compatible)

Blank tube: 1 mL H2O, Sample tube: 200 µL sample + 800 µL H_2O (0.1M NaCl). Add 0.1 mL DOC Solution (0.15% Na Deoxycholate). Mix well, incubate 10 min RT. Add 0.1 mL TCA Soltuon (72% w/v Trichloroacetic acid). Mix well. Centrifuge 5-10 min maximum speed and decant. Add 1 mL D Solution (from Lowry Method and continue the protocol as indicated).

3.9.4 Protein concentration

To 1 Vol of total soluble protein solution, add 0.1 Vol of 2% DOC (Na deoxycholate, detergent). Vortex and let sit for 30 min at 4°C. Add 0.1 Vol of Trichloroacetic acid (100% TCA: 454 µL H_2O + 1 g TCA). Vortex and let sit O.N. at 4°C. Centrifuge 15 min at 4ºC, 15000 g. Discard supernatant, and dry on paper. Wash with cold 90% acetone. Discard supernatant, and dry on paper. Repeat step VII 2 times. Resuspend in 1x PBS.

3.9.5 SDS-PAGE

Solutions	Components
10% APS (1ml)	1ml $_dH_2O$ 100mg ammonium persulphate
1.5M Tris, pH 8.8 (100ml)	18.121g Tris Base add 75ml $_dH_2O$ adjust pH with HCl, final volume of 100ml.
1M Tris, pH 6.8 (100ml)	12.114gr Tris Base add 75ml $_dH_2O$ adjust pH with HCl final volume of 100ml.
SDS 10% (100ml) **sonicate to dissolve**	10gr SDS in 100ml
5X Tris-Glycine Buffer (1000ml)	15.1gr Tris Base 94gr Glycine 5gr SDS

1X Tris-Glycine Buffer (1000ml)	200ml 5X Tris-Glycine Buffer 800ml $_dH_2O$
Loading buffer	125mM Tris pH 7.4 20% Glycerol 4% SDS 0.04% bromophenol blue

10% Polyacrylamide Gel (40% Acrylamide)

Solution	**Stacking gel (mL)**		**Resolution gel (mL)**	
	2.5	**1.25**	**10**	**5**
H2O	1.81	.905	4.8	2.4
Acrylamide	.31	.155	2.5	1.25
1M Tris	.31	.155	-	-
1.5 Tris	-	-	2.5	1.25
10% SDS	.025	.0125	.1	.05
10% APS	.025	.0125	.1	.05
Temed	.005	.0025	.1	.05

Resuspend the soluble protein sample in the loading buffer (10 µL sample: 10 µL loading buffer). Vortex the sample. Incubate the simple at 95ºC for 5-10 min. Incubate the sample in agitation at RT for 30 min. Load 15 µL sample per gel well.

3.9.6 Western-Blot

Once the total soluble protein extraction has been accomplished, run a SDS-PAGE under the established conditions and initiate the transfer procedure.

Transfer

Transfer buffer (keep at 4C)

200 ml methanol

200 ml Tris- Glycine 5X

H_2O until 1 liter

Tris- Glycine buffer 5X: 15.1 g Tris Base, 94 g glycine (electrophoresis grade) and 50 ml 10X SDS, check the pH about 8.3. Add H_2O until 1 liter.

PBST: 1X PBS + 0.1% Tween 20

Cut a corner of the PVDF membrane and leave it 30 seconds in methanol (in fume hood), rinse in water and leave it in transfer buffer for 10 – 15 minutes (together with 4 pads, 2 filter papers special for Western and the gel after electrophoresis running). After this, open the cassette for the transfer with the black part down. Add the pads, eliminate bubbles, filter paper, eliminate bubbles, gel, eliminate bubbles, membrane, eliminate bubbles, filter paper, eliminate bubbles, pad, eliminate bubbles, close cassette. Put the cassette into the apparatus and put some transfer buffer. Add a blue cooling package that is always at -20C. Fill completely with transfer buffer. Put the electrophoresis cell on a glass try with ice (especially if the transfer will be overnight), add a stir bar and leave on a magnetic stirrer for continuous agitation. Run the transfer 2 hs at 50V (the transfer buffer has to be always cold during the 2 hs of transfer) or overnight at 17V with a lot of ice around the cell.

After the transfer, stain the gel use the commercial PageBlue Protein Staining Solution (Fermentas cat# R0571). Simply soak the gel in the solution during at least 2 hours (you can leave it overnight if it's too late) and observe the stained bands. No destaining required.

Leave the membrane O.N. in 1% dry milk (DM) in PBST or 2 hs at 2.5% DM in PBST at room temperature.

Detection

If you did not incubate the membrane in blocking buffer (dry milk in PBST) the day before, do it now. Leave the membrane 2 hs at 2.5% DM in PBST at room temperature.

Wash membrane 3x with $PBST^{0.1\%}$, 10 min each. Add primary antibody anti-GCSF (1:100) diluted in PBS. Incubate in agitation for 2 h at RT. Wash the membrane 3x with $PBST^{0.1\%}$, 10 min each. Add secondary antibody anti-IgG (1:10000). Incubate in agitation for 1 h at RT. Wash the membrane 3x with $PBST^{0.1\%}$, 10 min each. Dissolve 15 mg diaminobezidine in 30 mL PBS (prechilled 30 sec in a microwave). Filter the diaminobendizine solution. Add 60 µL H_2O_2. Pour the diaminobenzidine over the membrane, agitate gently, and observe bands (52).

3.9.7 Dot-Blot

Place from 2-20 µL total soluble protein extraction sample on the nitrocellulose membrane. Let sample dry at air for approximately 20 min. Incubate the membrane with SuperBlock T20 (PBS) Blocking Buffer (ThermoScientific) in agitation for 2 h at RT. Wash 3x with $PBST^{0.1\%}$, 5 min each. Add primary antibody anti-GCSF (1:100) diluted in PBS. Incubate in agitation for 1 h at RT. Wash 3x with $PBST^{0.1\%}$, 5 min each. Add secondary antibody anti-IgG (1:10000). Incubate in agitation for 1 h at RT. Wash 3x with $PBST^{0.1\%}$, 5 min each. Dissolve 15 mg diaminobezidine in 30 mL PBS (prechilled 30 sec in a microwave). Filter the diaminobendizine solution. Add 60 µL H_2O_2. Pour the diaminobenzidine solution over the membrane, agitate gently and observe presence of a spot.

3.9.8 ELISA

Carefully pour 100 µL of protein sample or standard into the well. Incubtate ON at 4º. Decant the liquid in the wells and dry on paper. Add 300 µL SuperBlock T20 (PBS) Blocking Buffer (ThermoScientific) per well. Incubate 2 h at RT. Wash 2x with 300 µL $PBST^{0.05\%}$, under agitation conditions. Wash 2x with 300 µL PBST, under agitation conditions. Add 50 µL primary antibody anti-GCSF (1:100) diluted in PBS. Incubate 1 h at RT. Repeat washing steps. Add secondary antibody anti-IgG (1:10000) diluted in $PBST^{0.05\%}$. Incubate 1 h at RT. Repeat washing steps. Add 50-100 µL 1-Step Ultra TMB-ELISA solution (ThermoScientific). Incubate in dark for until 30 min. Stop reaction with 50 µL HSO_4 or HCl. Read signal at OD_{450nm} (52).

4. RESULTS AND DISCUSSION

The objective of the present research work was to produce the rhG-CSF in NT1 cells by using strategies to elevate its expression, such as protein body like formation and suppression of the RNA silencing. Since the above mentioned strategies affect the appearance of successful plant cell transformation events, optimization of the transformation mediated by *Agrobacterium* as well as the selection of the most virulent *Agrobacterium* strain was accomplished. As the first attempt, the construction of the binary vector containing the *p19* gene was produced, and used to transform different *Agrobacterium* strains to determine the most virulent one. The same gene was also used, as model gene, to optimize the transformation protocol. Thereafter, the construction of the expression cassettes containing the *rhG-CSF* genes was initiated in order to create the binary vectors for the NT1 cells transformation mediated by *Agrobacterium*. Some of the healthy-appearance calli carrying the *p19* gene in the genome DNA determined by PCR, as well as untransformed NT1 cells, were transformed with the expression cassette carrying the *ZeraGCSF*^{T} gene. Once the calli started to appear, they were subcultivated on new agar plates, using the respective antibiotic, and the presence of the rhG-CSF gene was determined by PCR. Finally, the expression of the gene of interest was assessed by determining the presence of mRNA, since protein detection could not be achieved.

4.1 Design of the Expression Cassettes

In order to achieve the NT1 cells transformation and production of the rhG-CSF using the strategies mentioned in Materials and Methods 3.5, different expressions cassettes and thus plasmid and binary vectors must have been constructed.

4.1.1 p19 expression cassette

During the first step of the project, we wanted to produce the expression cassette containing the *p19* gene in the binary plasmid pPS1. In order to achieve this, the TEVp19 sequence was amplified from the pIBT210.1p19 plasmid, using a high fidelity DNA polymerase and the p19Fow (5'-GGAGAGGAGCTCGAGAATTAATTC -3') and p19Rev (5'- TGTTGAGAGAGCTCTTACTCGC -3') primers,

which flank the TEVp19 region. Once the fragment was amplified, it was purified and digested with XhoI and SacI restriction enzymes, leaving the TEVp19 fragment with sticky-ends compatible with the pPS1 binary vector, after digestion with the same restriction enzymes. Once the binary vector was open, releasing the TEV sequence from the expression cassettes, it was ligated with the digested TEVp19 sequence. After ligation, the whole ligation reaction was used to transform Ca competent *E. coli*. The detection of the clone bearing the correct construct was achieved by pool colony screening PCR, using the p19Fow and p19Rev primers. The clones used for the pool colony PCR reaction in which the TEVp19 fragment was amplified, were later used separately to prepare the same reaction and select the positive clones. Other PCR reactions using different primers (p35SF1VFow: 5'- GCGTGAAGCTTGCATGCCTGC -3' and p19Rev) and restriction enzyme digestions (XhoI and SacI) analysis were used to assure the correct insertion of the TEVp19 sequence into the pPS1 binary vector. A clone positive, for the previous criteria, was selected in order to amplify and purify the pPS1p19 binary plasmid, and totransform it into the *Agrobacterium* strains (Fig. 29).

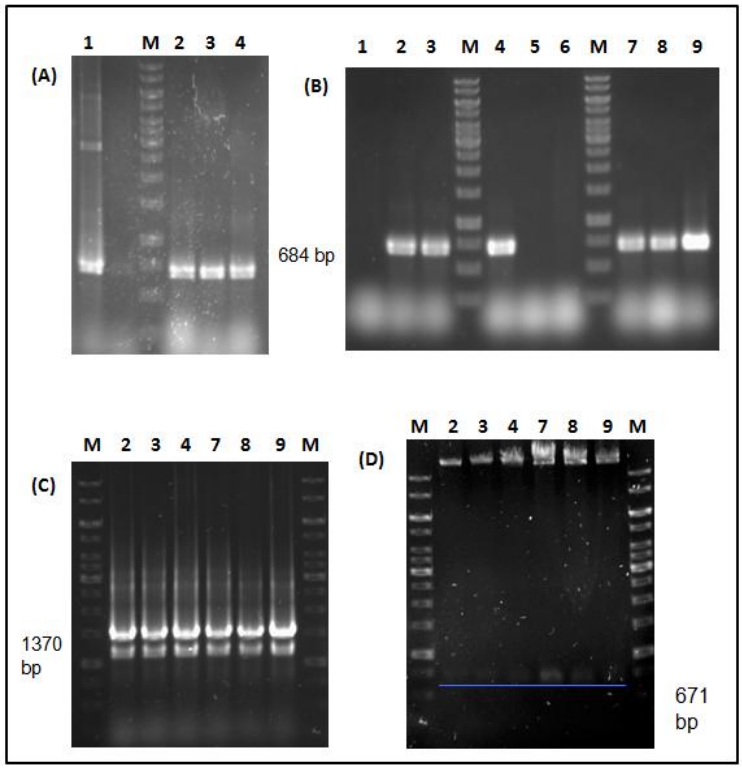


Figure 29: Detection and molecular characterization of pPS1p19 binary construct. A) Colony pool PCR using the p19Fow and p19Rev primers, the amplified TEVp19 fragment corresponds to a 684 bp band. Lane 1 corresponds to the (+) Control pIBT210.1p19 isolated plasmid. Lanes2-4 correspond to the pools containing the colonies 1 to 3, 4to 6, and 7 to 9, respectively. B) Individual colony PCR using the p19Fow and p19Rev primers, the amplified TEVp19 fragment corresponds to a 684 bp band. Lanes 1 to 9 represent each individual colony per lane, from colony to colony 9. C) Colony PCR using the p35SF1VFow and p19Rev primers, the amplified fragment conrresponds to a 1370 bp band. Lanes 1 to 6 represent the positive clones amplified by p19Fow and p19Rev, 2 to 4 and 7 to 9, respectively. D) Isolated pPS1p19 plasmids from positive PCR colonies digested with XhoI and SacI., the digested expected fragment presents a size of 671 bp. Lanes 1 to 6 correspond to the clones 2 to 4 and 7 to 9, respectively. 1 Kb DNA ladder; Marker (M).

4.1.2 p38 expression cassette

The *p38* gene, from the Turnip Crinkle Virus (TCV), is another RNA silencing suppressor protein (55). We tried to use this gene as another alternative in order to determine whether the phenotypic impact on the NT1 cells, might be favorable or not, as compared to p19. Since the storage conditions of the stab containing the *Agrobacterium* strain GV3101 with the p35Sp38 binary vector were not optimal (temperature), we had to confirm the presence and integrity of the strain with its binary vector.

As the first attempt, we performed standard PCR using the p35SF1VFow (5'-GCGTGAAGCTTGCATGCCTGC -3') and VSPRev (5'- TCTCGAATTCGCTTCAAGACG -3') primers (with homology to the 5'-end of the CaMV35S promoter and 3'-end to the VSP terminator, respectively). The expected amplified fragment must have had a size of 2460 bp, if the p38 gene were present in the sequence; however the amplified band was approximately 1600 bp size. The amplified fragment was purified and submitted to different digestion reactions. Xba and SmaI were used as negative control restriction enzymes that should not cut the amplified sequence. HindIII and FauI should give a band pattern of 1010 bp, 955 bp, 495 bp. Instead we obtained a band pattern of approximately 800 pb. By using the AvaI restriction enzyme, we would expect to obtain a digestion pattern of 732, 693, 673 and 363 bp. Instead, we observed a digestion pattern of approx. 1500, 1300 and 400bp (Fig. 30).

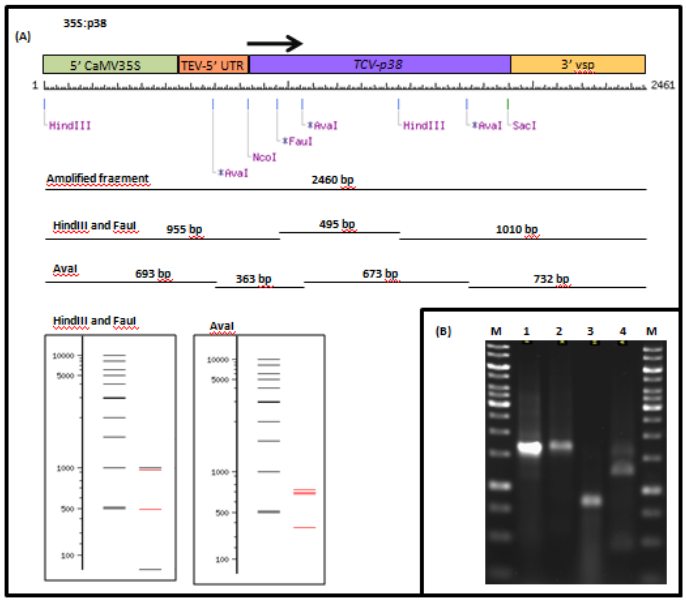


Figure 30: Analysis of the p35Sp38 binary construct. A) Virtual characterization of p35Sp38 using HindIII, FauI, and AvaI. B) PCR amplification using p35SF1VFow and VSPRev primers and enzymatic digestions. Lane 1: PCR amplification. Lane 2: (-) Control digestion, using XbaI and SmaI. Lane 3: HindIII and FauI digestion. Lane 4: AvaI digestion. 1 Kb DNA ladder; Marker (M).

The digestion patterns with the restriction enzymes selected were based on a putative cassette of expression conformed by the CaMV35S Promoter, 5'-UTR TEV, p38, 3'-VSP terminator. However, the restriction sites chosen for the digestion pattern, are primarily localized in p38. Nevertheless, we needed to make sure that the amplified fragment was derived from the putative p35Sp18 binary vector and not from other DNA structures, such as chromosomal DNA or Ti-plasmid. For this reason, we had to implement a miniprep for *Agrobacterium*, in order to isolate and visualize the binary vector and large plasmids (Ti-plasmid). The modified miniprep for *Agrobacterium* protocol is explained in detail in the chapter of Materials and Methods 3.7.2. By using the modified miniprep for large plasmids, we determined the presence of a DNA fragment, which size is similar to that showed by the binary vectors (13-14 kb) and also the presence of the Ti-plasmid. According to Hellens et al 2000, the *A. tumefaciens* strain GV3101 must be resistant only to rifampicin, and to kanamycin due to the binary plasmid. However we observed that our putative *A. tumefaciens*GV3101 p35Sp38 was resistant against ampicilin and rifampicin. We never succeeded in growing it with kanamicin in the medium, suggesting that the bacterium did not contain the p35Sp38 binary vector.

Due to the nature of the putative *Agrobacterium* GV3101 p35Sp38 strain, we needed to determine whether the rest of the *A. tumefaciens* strains (LBA4404, GV3101, EHA105) were actually free of binary vector, but did contain the Ti-plasmid und thus virulent genes, so that we could be able to use them to clone the binary vectors containing the p19 and the G-CSF genes. By using the modified miniprep protocol for large plasmids, we observed the presence of binary vectors size-like bands and presence of Ti-plasmids.

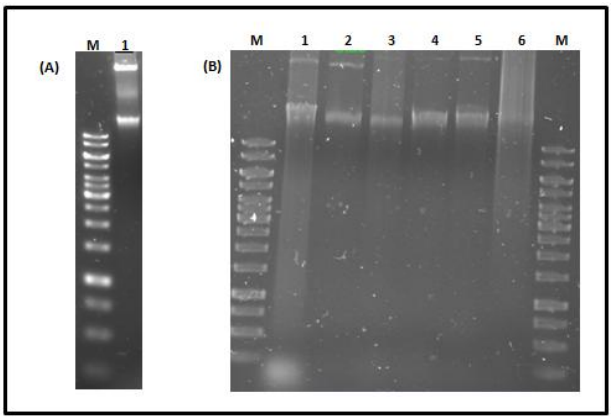


Figure 31: *Agrobacterium* miniprep (for large plasmids). Bands above 10 Kb represent the presence of binary vectors (approx. 13-14 Kb). Higher bands represent the isolated Ti-Plasmids. A) Lane 1: Miniprep of *A. tumefaciens* GV301 p35Sp38. B) Miniprep of supposed *A. tumefaciens* strains wihtout binary vectors. Lane 1,2: LBA4404. Lane 3,4: GV301. Lane 5,6: EHA105. 10 Kb DNA ladder; Marker (M).

The last suggests that the strains recovered also from the stabs maintained in unfavorable conditions might have been contaminated by other *Agrobacterium* strains or mislabeled. For this

reason, new *Agrobacterium* strains (LBA4404, AGL1, EHA105, and GV3850) needed to be obtained and continuously monitored (absence of binary vector and presence of Ti-plasmid) (Fig. 31).

4.1.3 ZeraG-CSF^T expression cassette

The construction of the 35SZeraGCSF^T expression cassette was achieved by cutting and isolating the EkGCSF^T (enterokinase-GCSF^T) sequence from the pUC57EkGCSF^T plasmid vector, by using the restriction sites NcoI and SacI, which flank the EkGCSF^T at the 5'- and 3'-ends respectively. The pUC35SZeraF1-V (having the pUC18 as backbone) was also linearized by using the same restriction sites (NcoI and SacI), and thus taking the F1-V sequence out of the vector. The pUC35SZera backbone, which contains the expression cassette and the Zera sequence, was later ligated with the EkGCSF^T fragment. The ligation reaction was used to transform Ca competent DH5α *E. coli*. The pUC35SZGCSF^T clones were screened by PCR, using the p35SF1VFow (5'- GCGTGAAGCTTGCATGCCTGC -3') and pVSPRev (5'- TCTCGAATTCGCTTCAAGACG -3') primers (amplifying an approx. 2203 bp band corresponding to the expression cassette 35S-TEV-Zera-EkGCSF^T-VSP), and the p35SF1VFow and p35SF1VRev (5'- TAAGATCAGTCATGACTATCGTTCGT -3') primers were used to amplify the CaMV35S promoter as positive control (Fig. 32).

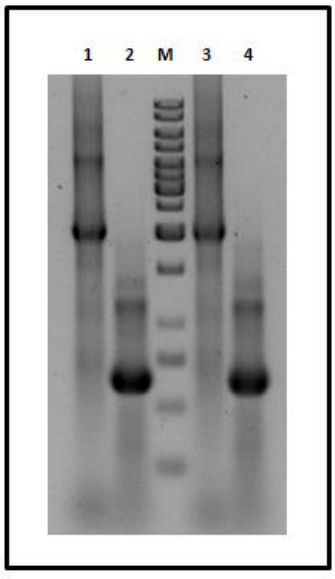


Figure 32: pUC35SZGCSF^T plasmid vector characterization. PCR amplification of pUC35SZGCSF^T. The amplification of the whole expression cassette containing EkGCSF^T is represented as a 2203 bp band. CaMV35S promoter amplification as a (+) control shows a band of approx. 600 bp. Lane 1 and 2: PCR from clone 1, amplification of the expression cassette and 35S promoter, respectively. Lane 3 and 4: PCR from clone 2, amplification of the expression cassette and 35S promoter, respectively. 10 Kb DNA ladder; Marker (M).

After the selection of the *E. coli* pUC35SZGCSF^T strain, the whole expression cassette 35SZGCSF^T was isolated by using the restriction sites HindIII and EcoRI, which flank the expression cassette at the 5'-end of the 35S promoter and 3'-end of the VSP terminator, respectively. Simultaneously, the p35SZeraF1-V binary vector was linearized by the same restriction sites, thus taking the whole expression cassette containing the F1-V gene out from the pGPTV:BAR backbone. The linearized pGPTV:BAR backbone was ethanol precipitated, and later ligated with the expression cassette 35SZGCSF^T, in order to create the p35SZGCSF^T binary vector. 2 µL from the ligation reaction were used to transform electrocompetent DH5α *E. coli*. The *E. coli* p35SZGCSF^T bin clones were analyzed by screening PCR using different primer sets (UpTEV/LoVSP and UpGCSF/LoVSP). The isolated binary vector was also digested with the restriction enzymes HindIII and EcoRI for the whole expression cassette, HindIII and SacI for the 35S-TEV-ZGCSF^T region, and NcoI with SacI for the EkGCSF^T sequence (Fig. 33).

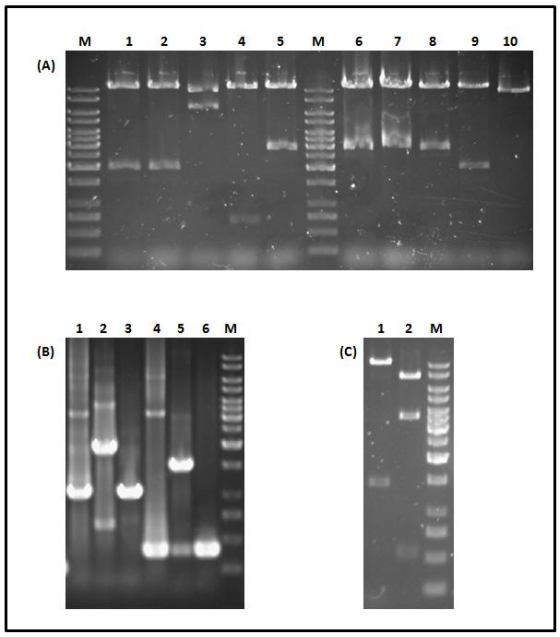


Figure 33: p35SZGCSF^T binary vector characterization. A) Binary vector isolation and digested with HindIII and EcoRI, producing a 2203 pb fragment.. Lanes 1 to 10 represent different clones. B) PCR amplification from the binary vector isolated from clone 1. Lanes 1 to 3 correspond to PCR reaction using the primers UpTEV and LoVSP. In lane 1 the pUC35SZGCSF^T was used as (+) control (approx. 1000 bp). In lane 2 the p35SZeraF1-V bin was used as (-) control (approx. 2000 bp). In lane 3 the isolated plasmid from clone 1 p35SZGCSF^T was used. Lanes 4 to 6 correspond to PCR reaction using the primers UpGCSF and LoVSP. In lane 3, the pUC35SZGCSF^T was used as (+) control (approx. 400 bp). In lane 5 the p35SZeraF1-V bin was used as (-) control (approx. 1500 bp). In lane 6 the isolated plasmid from clone 1 p35SZGCSF^T was used. C) Binary vector isolation from clone 1 p35SZGCSF^T bin and digested with: Lane 1, HindIII and SacI to cut the 35S-TEV-ZGCSF^T fragment (approx. 1500 bp). Lane2, NcoI and SacI to cut the EkGCSF^T fragment (approx. 580 bp). 10 Kb DNA ladder; Marker (M).

4.1.4 G-CSF^{A} expression cassette

The attempt of creating the expression cassette 35SG-CSF^{A}, consisted in isolating the G-CSF^{A} sequence and following a similar strategy as the one used for 35SZGCSF^{T}. G-CSF^{A} stands for the mature nucleotide sequence of the protein with no gycolysation site encoded. The G-CSF^{A} fragment would be isolated from pUC57GCSF^{A}, by using the restriction sites BspHI and SacI, which flank the G-CSF^{A} sequence. However, using both enzymes at the same time would create two sequences of similar size (536 and 453 bp), being only the first one of interest, and the second one a byproduct of the enzymatic digestion. In order to avoid the possible insertion of the false fragment (not G-CSF^{A}), into the expression cassette in the pUC18 vector, we first digested the pUC57GCSF^{A} with SacI, creating a single fragment of 561 bp, which contains the G-CSF^{A} sequence, and a 2683 bp corresponding to the pUC57 backbone. After G-CSF^{A} fragment isolation, it was digested with BspHI and ethanol precipitated, in order to prepare the fragment for its insertion into the expression cassette. The pUC35SF1-V was linearized using the same restriction enzymes, the pUC35S backbone was isolated, leaving the F1-V sequence out of the expression cassette, and prepared for the insertion of G-CSF^{A}, to create the pUC35SGCSF^{A} plasmid vector. 2 µL from the ligation reaction was used to transform electrocompetent DH5α *E. coli*, and the clones were analyzed with screening PCR using the UpTEV and LoVSP primers to confirm the insertion of the G-CSF^{A} fragment into the expression cassette (Fig. 34).

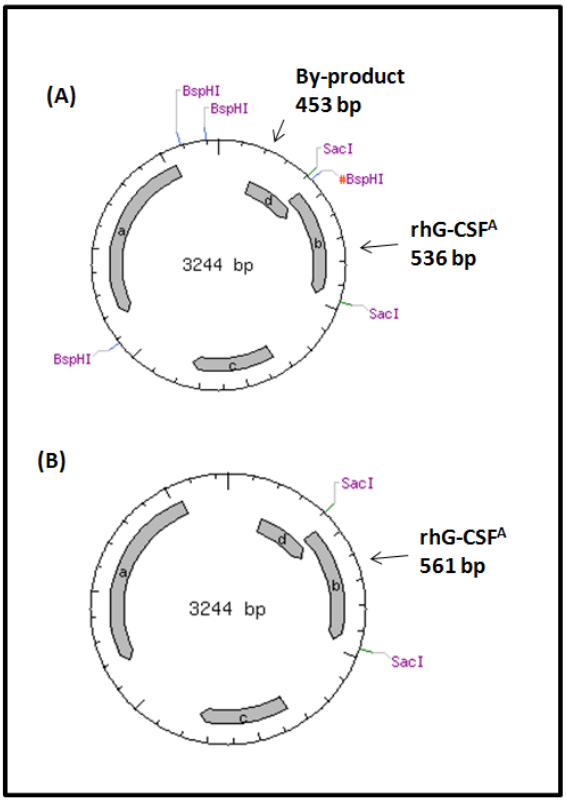


Figure 34: pUC57GCSF^{A} digestions strategies. A) Production of a by-product when using the flanking restriction sites BspHI and SacI. B) Production of a single and size isolated fragment using only SacI.

Despite all the efforts, no clone resistant to ampicilin contained the correct construct. After sequence analysis, it was determined that the 5'-end BspHI restriction site of the G-CSF^{A} sequence was dam methylated, and thus site recognition by the BspHI restriction enzyme could not be

achieved. In order to avoid bacterial dam methylation at this site, and allow the digestion of the fragment, a dam$^-$/dcm$^-$ *E. coli* strain was needed to clone and amplify the pUC57GCSF^A plasmid without methylation. So far, all attempts to transform the Ca competent dam$^-$/dcm$^-$ *E. coli* strain have failed, but efficient transformation of this strain will be needed in order to allow the construction of the 35SGCSF^A expression cassette (Fig. 35, 36).

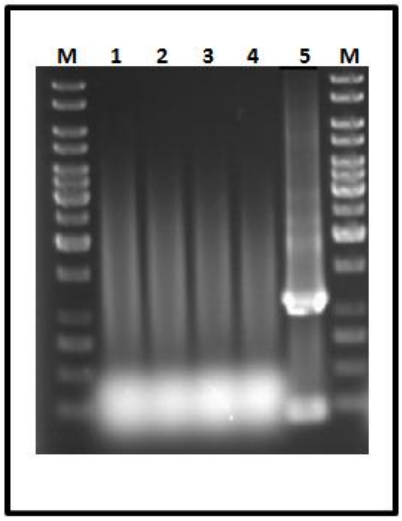


Figure 35 pUC35SGCSF^A construct. A) Pool PCR with UpTEV and LoVSP primers. Lanes 1: clones 1-10. Lane 2: clones 11 to 20. Lane 3: clones 21 to 30. Lane 4: clones 31 to 40. Lane 5: (+) Control: pUC35SZGCSF^T, corresponds to a approx. 1600 bp. 10 Kb DNA ladder. Marker (M).

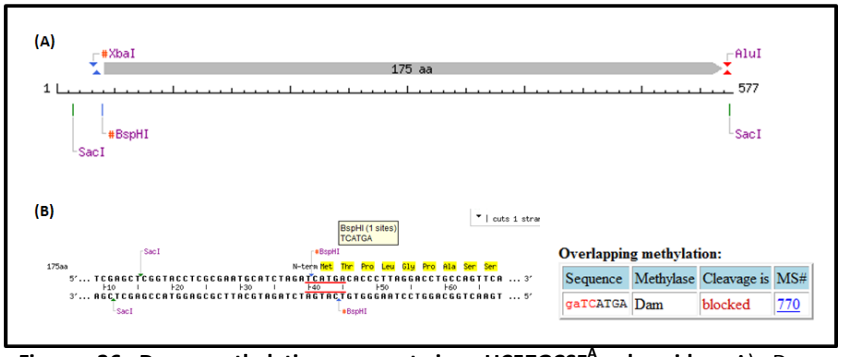


Figure 36 Dam methylation present in pUC57GCSF^A plasmid. A) Dam methylation detected at BspHI restriction site, 5'-end of G-CSF^A sequence. B) Dam methylated sequence, next to the Met codon sequence of GCSF^A.

4.1.5 *Agrobacterium* electroporation with pPS1p19 binary vector

The constructed pPS1p19 binary vector was amplified and isolated in *E. coli* for its electrotransformation in *Agrobacterium tumefaciens*. Since we wanted to determine the most virulent strain, we transformed four different strains, in order to determine which one could raise the number of transformation events and shorten the time for calli out-comes. For this approach,

we used the *Agrobacterium* strains LBA4404, AGL1, EHA105, and GV3850. After colony recovery under the respective antibiotic selection, the clones were analyzed by screening PCR using the p19Fow and VSPRev primers (Fig. 37).

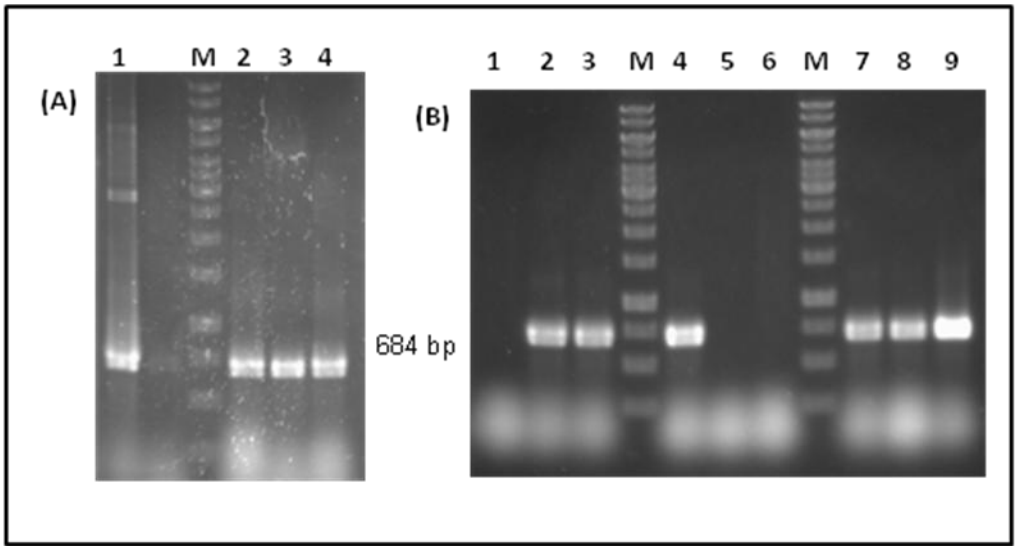


Figure 37. Selection of Agrobacterium strains with pPS1p19 binary vector. PCR amplification using p19Fow and p19Rev primers, the amplified band corresponds to 684 bp. *A) Lanes 1 to 5: Agrobacterium tumefaciens LBA4404 clones 1 to 5. B) Lanes 1 to 4: Agrobacterium tumefaciens AGL1 clones 1 to 4. Lanes 5 to 8: Agrobacterium tumefaciens EHA105 clones 1 to 4. Lanes 9 to 12: Agrobacterium tumefaciens GV3850 clones 1 to 4. 10 Kb DNA ladder. Marker (M).*

4.1.6 *Agrobacterium* electroporation with p35SZGCSF^T binary vector

The p35SZGCSF^T binary vector, after amplification and isolation in *E. coli*, was used to transform electrocompetent *A. tumefaciens* LBA4404 and AGL1. AGL1 and LBA4404 were chosen for transformation with p35SZGCSF^T because they proved to be the most effective when transforming in tobacco NT1 cells with the p19 gene. The LBA4404 and AGL1 p35SZGCSF^T bin clones were characterized by screening PCR using the UpTEV and LoVSP (5'- GCATTCTACTTCTATTGCAGC -3' and 5'- TAGTGCATATCAGCATACCT -3', respectively), UpTEV and LoGCSF (5'- ATGGGAAGCAACCAAGACAC -3'), UpGCSF (5'- CCATCACAAGCACTGCAACT -3') and LoVSP primer sets, after growth under the respective antibiotic selection (Fig. 38).

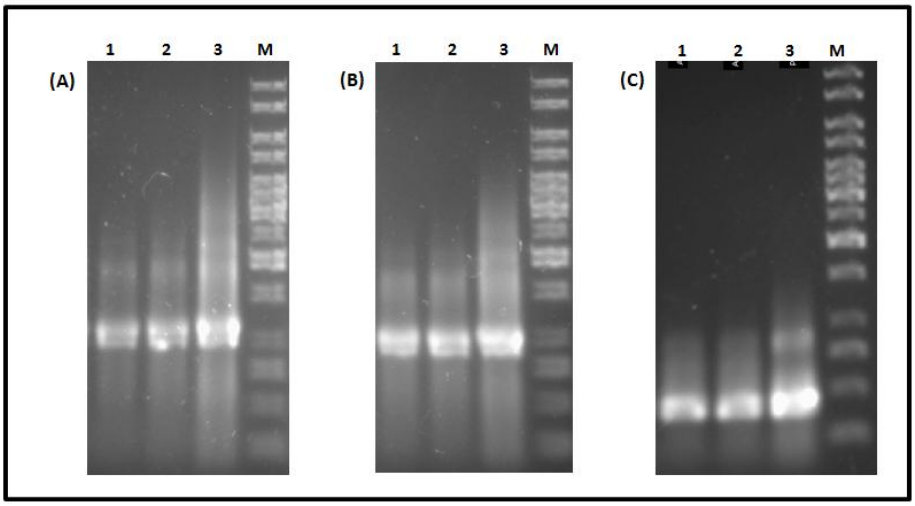


Figure 38. Selection of *Agrobacterium* AGL1 with p35SZGCSF^T binary vector. PCR amplification using: A) UpTEV and LoVSP primers (1038 bp): Lane 1, clone 1. Lane 2, clone 2. Lane 3, (+) control p35SZGCSF^T. B) UpTEV and LoGCSF primers (911 bp): Lane 1, clone 1. Lane 2, clone 2. Lane 3, (+) control p35SZGCSF^T. C) UpGCSF and LoVSP primers (403 bp): Lane 1, clone 1. Lane 2, clone 2. Lane 3, (+) control p35SZGCSF^T. 10 Kb DNA ladder. Marker (M).

4.2 Optimization of the Transformation Protocol Mediated by *Agrobacterium*

The time required for NT1 calli out-come, after transformation mediated by *Agrobacterium,* is usually 6 weeks (46). However, it was observed by Alvarez et al., 2009 that in the case of expressing recombinant proteins fused to the Zera peptide, the time for NT1 calli out-come can take even 15 weeks, in order to reach a considerable amount of calli in comparison to those obtained, where the Zera peptide was not used. It was also observed that time, as well as the number of outcomes, were strain dependant. For this reason, we tried to optimize the transformation protocol and to determine the most virulent *A. tumefaciens* strain, among the new strains recently obtained in the lab and hence not used before by our group (AGL1 and GV3850) (Table 6).

Table 6. Disarmed *Agrobacterium tumefaciens* strains defined by the *Agrobacterium* chromosomal background and the Ti plasmid they harbour (67).

Strain	Chromosomal Background	marker gene	Ti plasmid	marker gene	Opine
LBA4404	TiAch5	rif	pAL4404	spec / strep	Octopine
GV3850	C58	rif	pGV3850 (pTiC58onc.genes)	carb	Nopaline
EHA105	C58	rif	pEHA105(pTiBo542DT -DNA)	-	Succinamopine
AGL-1	C58,RecA	rif, carb	pTiBo542DT-DNA	-	succinamopine

4.2.1 Selection marker for NT1 cells transformed with p35SZGCSF^T

As the first attempt, we needed to determine the minimum inhibitory concentration (MIC) for the bar selection marker. In the previous studies achieved by Alvarez et al., 2009, the herbicide/antibiotic bialaphos (bia) was used for the selection of the transformed calli carrying the *bar* selection marker gene in the expression cassette. However, there are alternative selective agents available that represent a more economic option and can be used for *bar* selection, such as phosphinothricin (PPT). For this reason, we needed to determine the MIC for PPT, as selection marker for the NT1 cells transformed with the expression cassette 35SZGCSF^T (carrying the *bar* selection marker gene).

The MIC determination for PPT (Table 7) was based on the bialaphos concentration used for the selection step by Alvarez et al., 2009, 10 µg/mL bialaphos in the selection agar plates, and 5µg/mL bialaphos in agar plates for subcultures once the presence of the transgene was determined. In this case, PPT presented a similar MIC pattern as bialaphos, when using NT1 cells and the transgenic lines resistant to bialaphos F1V-4, EHZ-1 and LBZ-13 (these lines express the F1-V or ZeraF1-V recombinant protein (52)).

Table 7. Phosphinothricin minimum inhibitory concentration (MIC) determination.

	Line							
	NT1		**F1V-4**		**EHZ-1**		**LBZ-13**	
Antibiotic Concentration	Bia	PPT	Bia	PPT	Bia	PPT	Bia	PPT
0 µg/mL	+	+	+	+	+	+	+	+
1 µg/mL	+	+	+	+	+	+	+	+
5 µg/mL	-	-	+	+	+	+	+	+
10 µg/mL	-	-	+	+	+	+	+	+

4.2.2 Optimization of the transformation protocol.

The optimized transformation protocol for NT1 cells mediated by *Agrobacterium* used in this work, was developed with the p19 gene as model transgene and *nptII* as selection marker. Based on the results obtained from this experiment, we identified the most virulent *Agrobacterium* strain. The data obtained, was used for the transformation of NT1 cells and supertransformation of some selected NT1:p19 lines. As a general approach, we used the protocols established by Mayo et al.,

2006[35] and Alvarez et al., 2009[52], and based on those protocols; we made certain modifications shown in the table below.

The critical points in the Mayo et al., 2006 protocol were (1) the amount of *Agrobacterium* inoculum of only 20 µL, compared with 100 µL used by Alvarez et al., 2009; (2) the wash steps (3x), for resuspending and centrifugating the cells were performed with 45 mL NTT medium according to Mayo (NT1 medium supplemented with 50 µg/mL timentin, see Materials and Methods 3.7.2), while in Alvarez, only 9 mL were used, avoiding a significant los of cells during the washes; and finally (3), Mayo used 4 mL NTT medium as the final resuspension volume and poured 2 mL on the NTTK (NT1 medium supplemented with 50 µg/mL timentin and kanamicin) agar plates, however, the number of cells lost during the washes was significant, leaving only two plates for calli selection with a high cell density on the surface. With this protocol, we obtained a total of 6 calli, which started to appear after 8 weeks. According to Alvarez, the loss of cells during the wash steps was diminished by using 9 mL NTT instead of 45 mL, and the high cell density present in the final volume, before pouring on plates, was solved by resuspending the cells in a final volume of 40 mL NTT instead of 4 mL. Finally, 5 mL of resuspended cells were poured on each selection plates, providing 8 selection plates, with cells evenly distributed and disaggregated. Nevertheless, when we used this protocol, we observed that the high medium volume of 5 mL poured on the selection plates was affecting the viability of the cells, and we were not able to recover any callus with any *Agrobacterium* strain (pPSp19 binary vector). On the other hand, we still had loss of cells during the wash steps, even when the washing volume was lowered from 45 mL to 9 mL NTT medium. The lost of cells during this step was mainly due to the poor pellet formation during centrifugation, thus causing the cells from the upper side of the pellet, to wash away while decanting the supernatant.

Based on the observations and results obtained in the two reported protocols, we decided that we should keep the same cell density poured on the selection plates, as specified by Alvarez et al., 2009, but diminish the final resuspension volume of NTT medium (from 40 mL to 16 mL), and thus the amount of liquid present on the selection plates (from 5 mL to 2 mL). Furthermore, the problem related with the weak formation of cellular pellet during the wash steps, and hence loss of cells, was solved by changing the centrifugal tube of 50 mL to one of 15 mL. The latter allowed the formation of a more compact pellet and diminished the loss of cells considerably. Finally, the

period of infection time was raised from 3 days to 4 days, increasing the potential of *Agrobacterium* to transform the plant cells (Table 8). We used 7 replicates for the transformation experiment, obtaining 8 selection plates from each one. This first version of the modified protocol to transform NT1 cells mediated by *Agrobacterium* resulted in the appearance of a total of 63 NT1:p19 calli (average of 7 replicates), with the AGL1 pPS1p19 strain demonstrating the best efficiency with 37 calli, followed by LBA4404 pPS1p19 with 16 calli and GV3850 pPS1p19 with 10. The total amount of outcomes was determined at the 8th week after infection, demonstrating that this protocol could increase the number of transformation events, compared with the previously reported protocols. Unexpectedly, the EHA105 pPS1p19 did not generate any callus (Fig. 39).

Table 8. Plant transformation mediated by *Agrobacterium* optimization (version 1).

Protocol	**Mayo et al., 2006[35]**	**Alvarez et al., 2009[52]**	**Version 1**
Factor			
		Infection step	
***Agrobacterium* Vol**	20 µL	100 µL	100 µL
Incubation Temp	23-25 ºC	23-25 ºC	23-25 ºC
Incubation time	3 d	3 d	4 d
		Selection step	
Washing containers	50 mL	50 mL	15 mL
Washing resuspension Vol	45 mL	9 mL	9 mL
Final resuspension Vol	4 mL	40 mL	16 mL
Plated Vol	2 mL	5 mL	2 mL
Strain used			
		Average of outcomes	
LBA4404 pPS1p19	1	0	16
AGL1 pPS1p19	0	0	37
EHA105 pPS1p19	2	0	0
GV3850 pPS1p19	1	0	10
Total	4	0	63
Time for first out-comes	8 weeks	12 weeks	8 weeks

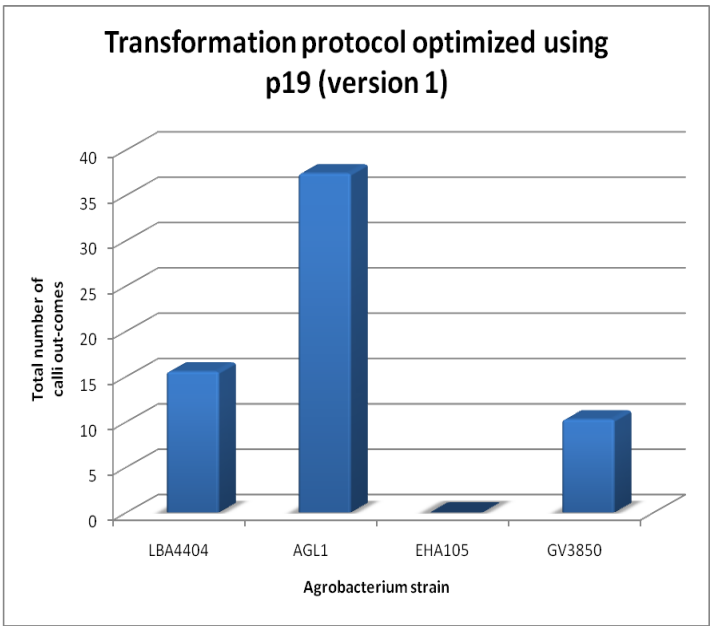


Figure 39. Transformation protocol optimization, using the p19 transgene (protocol optimization version 1).

4.2.3 Determination of *Agrobacterium* strains virulence

Once we had improved the transformation protocol, and obtained some insights about the virulence behavior of the *Agrobacterium* strains, we wanted to determine the virulence pattern of AGL1 pPS1p19, compared to the other strains, and to analyze the virulence capability of EHA105. In order to achieve this, and to try to increase the number of outcomes, we adjusted the incubation temperature during the infection step, by incubating at 19°C (Transformation protocol version 2, Table 9), instead of 23-25°C.

We performed the transformation experiments twice, and each experiment had three replicates. Using the transformation protocol version 2, we increased the number of transformant colonies (10-18 fold using AGL1 pPS1p19, and 6-13 fold using GV3850 pPS1p19) compared with the results obtained when using transformation protocol version 1. The effect of the change in the temperature might be correlated with the increase of *Agrobacterium* virulence. A similar effect has been observed by Dillen et al., 1997[80], since they determined a decreased transient expression (in *Nicothiana tabacum* leaves), as infection temperature increases from 22 to 25°C, low at 27°C and undetectable at 29°C. Dillen et al., 1997 detected the maximum expression levels when the infection was performed at 19 to 22°C. We also detected a shorter incubation time for calli

production, using transformation protocol version 2, which was from 4 to 5 weeks in general (Table 10). AGL1 pPS1p19 demonstrated to be the most virulent strain, followed by LBA4404- and GV3850 pPS1p19 (Table 11).

Table 9. Improvement of NT1 cells transformation mediated by *Agrobacterium*, by temperature incubation.

Protocol	Version 1	Version 2	
Factor			
		Infection step	
***Agrobacterium* Vol**	100 µL	100 µL	
Incubation Temp	23-25 ºC	19 ºC	
Incubation time	4 d	4 d	
		Selection step	
Washing containers	15 mL	15 mL	
Washing resuspension Vol	9 mL	9 mL	
Final resuspension Vol	16 mL	16 mL	
Plated Vol	2 mL	2 mL	
Strain used			
		Average of outcomes per plate	
LBA4404 pPS1p19	16	2	345
AGL1 pPS1p19	37	372	667
EHA105 pPS1p19	0	12	1
GV3850 pPS1p19	10	64	129
	63	450	1142
Time for first out-comes	8 weeks	4 weeks	5 weeks

Table 10. Time required for appearance of first NT1:p19 calli out-comes.

Protocol	Transformation Experiment	Replicate	LBA4404	AGL1	EHA105	GV3850
			Time for out-comes			
2	**1**	1	9	4	4	4
		2	NA	4	9	4
		3	NA	4	4	4
	2	1	5	5	9	5
		2	5	5	5	5
		3	5	5	5	5

We observed very few calli outcomes for LBA4404 in transformation experiment 1, using the protocol version 2, however this could be caused by mechanical stress suffered by the plant cells during cell abrasion. Concerning the virulence pattern of EHA105 pPS1p19, we observed a

considerable instability of this strain (Fig. 41), which is commonly considered as a supervirulent strain. EHA105 pPSp19 produced no or few calli in most of the replicates, using version 1 or 2 transformation protocol (Fig. 40). Nevertheless, there were two replicates in which this strain produced an elevated amount of calli outcomes, competitive to AGL1 (Table 11). Typically, incubation factors might affect the virulence and hence infection of *Agrobacterium.* In previous studies, it has been reported that the concentration of acetosyringone, used to stimulate and activate the *vir* genes, together with acidic conditions in the medium, might inhibit *Agrobacterium* growth during infection. In these studies, Fortin et al., 1992[81] observed *Agrobacterium* nopaline-type strains growth inhibition and presence of avirulent cells, when exposed to acetosyringone (60 µM) and acidic conditions (pH 5.6 to 6). Other strains, such as the octopine-type strains, were not affected for the same conditions. In our case, the final concentration of acetocyringone was 20 µM, and the pH of the NT1 medium is typically adjusted to pH 5.5 ±0.3. These might be factors that could impact the virulence of *Agrobacterium* strains, and thus the recovery of transgenic calli.

Diverse studies have focused on the genetic aspect of the *vir* genes, especially on *virA* and *virG*, observing a hypervirulence effect on strains with certain mutations or overexpressing the *virG* genes. However, a specified genetic characterization, determing what mutations in which genes could improve the virulence in *Agrobacterium* strains, seems to be more complex. Nevertheless, it has been demonstrated that Ti-plasmids may have evolved to optimize specific combinations of *virA, virG* and *vir* boxes, as the Ti-plasmid pTiBo542 in the C58 chromosomal background, which is hypervirulent on certain legume species, possibly because of the associated *virG* gene. On the other hand, despite all the genomic studies focused on the *vir* genes and Ti-plasmid backgrounds, it has been observed that *vir* gene induction and T-strand production by and transformation efficiency of particular *Agrobacterium* strains may not correlate well, and may not necessarily be reliable predictors of transformation efficiency (82).

Table 11. Determination of most virulent *Agrobacterium* strain. *Unusual or uncommon calli outcomes events obtained with EHA105.

Protocol	Transformation Experiment	Replicate	LBA4404	AGL1	EHA105	GV3850	
Version 1	1	1	16	49	0	20	
		2	20	22	0	5	
		3	18	55	0	15	
		4	15	49	0	13	
		5	2	48	0	9	
		6	28	20	0	5	
		7	10	19	0	5	
			109	262	0	72	Total
			16	37	0	10	Average
Version 2	1	1	6	391	5	73	
		2	0	370	19	31	
		3	0	354	*516	88	
			6	1115	24 (*540)	192	Total
			2	372	12	64	Average
	2	1	511	695	1	16	
		2	270	872	*464	280	
		3	254	433	1	92	
			1035	2000	2 (*466)	388	Total
			345	667	1	129	Average

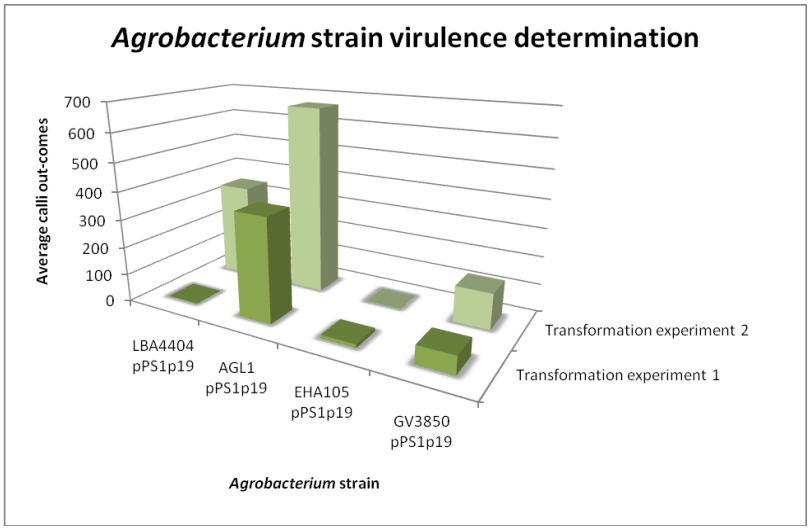


Figure 40. Determination of most virulent *Agrobacterium* strain.

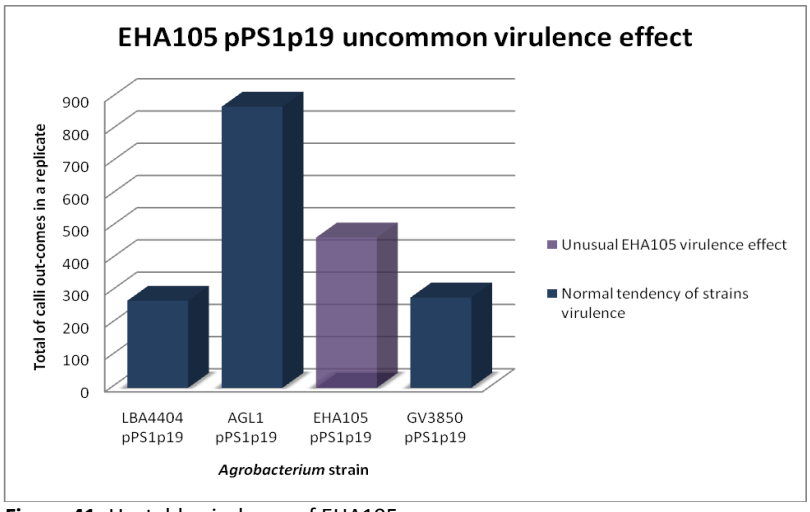


Figure 41. Unstable virulence of EHA105.

4.3 Stable Transformation of NT1 and NT1:p19 Cells with rhG-CSF

4.3.1 PCR screening for p19 transgene in NT1:p19 transgenic lines

The correct transformation of the recovered NT1 calli outcomes was confirmed by subculturing them under the respective selectable marker (Kan 50 gμ/mL) and by screening for insertion of the transgene. The screening PCR was achieved by isolation of the chromosomal plant DNA and using the fast DNA polymerase (Phire®Hot Start II DNA Polymerase) with the primers p35SF1VFow and pVSPRev, which amplify the expression cassette containing the p19 sequence. The transgenic lines obtained did not present any unfavorable phenotype, color and consistency was similar to that presented by NT1 cells, in both agar plates and suspension culture (Fig. 42).

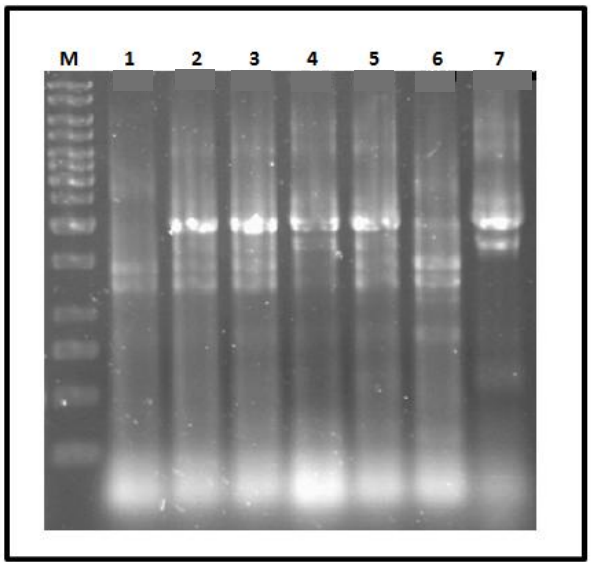


Figure 42. PCR screening for p19 transgene in NT1:p19 transgenic lines. PCR amplification using the primers p35SF1VFow and pVSPRev: (1928 bp). Lanes 1 to 5 correspond to transgenic lines 25 to 29, respectively. Lane 6 corresponds to (-) control NT1 chromosomal DNA. Lane 7 corresponds to (+) control pPS1p19 bin. 10 Kb DNA ladder. Marker (M).

4.3.2 NT1 transformation with ZeraGCSF

After the optimization of the transformation protocol mediated by *Agrobacterium* (Protocol optimization version 2), and the selection of the most virulent *Agrobacterium* strain (AGL1), we were able to successfully transform NT1 cells with the 35SZGCSF^{T} expression vector, which would allow the expression of the rhG-CSF^{T} fused to the Zera peptide. As observed previously with the calli transformed with p19 using the transformation protocol version 2, we obtained the first outcomes after 4-5 weeks of cell infection with AGL1 p35SZGCSF^{T} bin. This is a considerably shorter time as compared with the time reported by transforming NT1 cells with the ZeraF1-V transgene using the EHA105 strain, where it took 8 weeks for the first outcomes and up to 15 weeks to reach a number of 100 calli (52).

Nevertheless, we were not able to obtain a similar amount of calli outcomes as we did using the p19 gene. Instead we only obtained a maximum of 248 calli using AGL1 p35SZGCSF^{T}, at the end of 12 weeks. The insertion of the ZeraG-CSF^{T} transgene into the NT1 chromosomal DNA was determined by calli outcomes on selection plates using phosphinothricin (NTTP timentin 50 µg/mL

and PPT 10 µg/mL) and subculturing them under the same selection conditions (PPT 5 µg/mL). Thereafter, the calli were screened by fast PCR using the Phire®Hot Start II DNA Polymerase, and the primers UpGCSF and LoVSP, which amplify a fragment of 263 bp (Fig. 43).

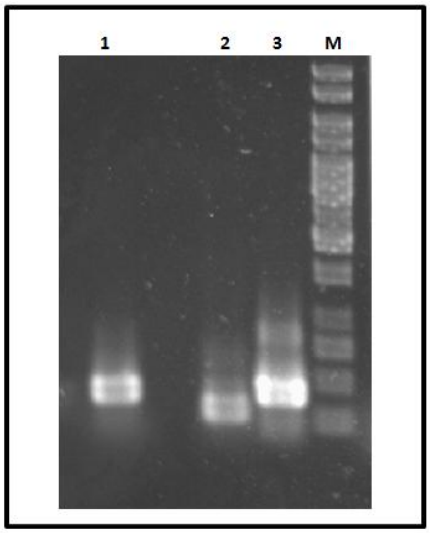


Figure 43. PCR screening for ZeraGCSF^T transgene in NT1:ZGCSF^T transgenic lines. PCR amplification using the primers UpGCSF and LoVSP: (263 bp). Lane 1 corresponds to NT1:ZGCSF^T transgenic line 1. Lane 2 corresponds to (-) control NT1 chromosomal DNA. Lane 3 corresponds to (+) control p35SZGCSF ^{T} bin. 10 Kb DNA ladder. Marker (M).

4.3.3 NT:p19 lines supertransformation with ZeraGCSF^T

Since post-transcriptional gene silencing (PTGS) has previously been observed in transgenic plants, we used the p19 gene to address this problem and assure the expression of the rhG-CSF. It has been observed by Alvarez et al., 2009 that the expression of recombinant proteins can be achieved after PTGS activation by expressing the p19 gene. However, we wanted to create NT1 lines that were already expressing this viral suppressor protein and were phenotypically viable, to be later supertransformed with any transgene, and avoid in this way the RNA interference or gene silencing. We selected 4 different NT1:p19 transgenic lines (NT1:p19 lines: 26, 27, 28, 29), which were selected for a healthy condition (color, consistency and growth)(fig. 44) and had p19 integrated in the genomic DNA.

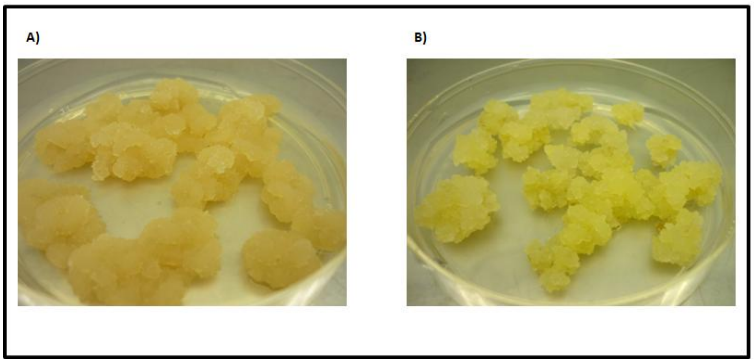


Figure 44. Phenotype of transgenic lines. A) NT1 calli. B) NT1:p19-ZGCSF^{T} 47 calli.

The NT1:p19 transgenic lines were supertransformed with AGL1 p35SZGCSF^{T}, but the selection conditions were changed, using 5 µg/mL instead of 10 µg/mL, since we considered that the expression of the p19 suppressor protein and the rhG-CSF^{T} fused to Zera recombinant protein might already represent too much stress for the plant cells. Finally, we compared the amount of calli obtained by transforming NT1 with p19 and supertransforming the NT1:p19 lines with ZeraGCSF^{T} (Fig. 45).

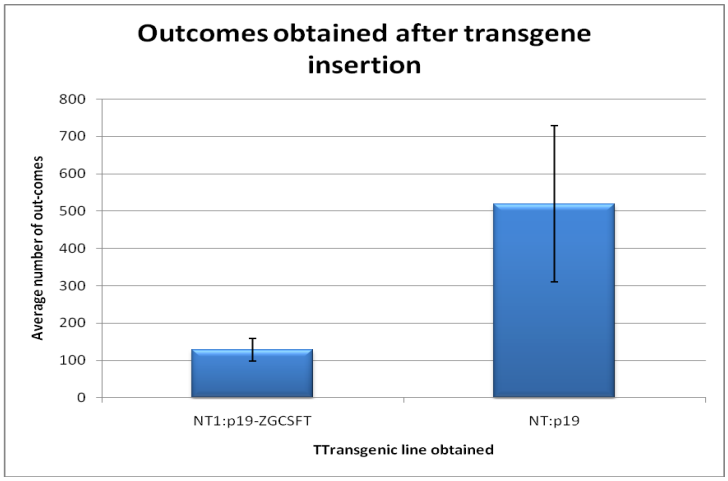


Figure 45. Calli outcomes after transformation with different transgenes.

We started to recover supertransformed NT1:p19-ZGCSF^{T} calli after 4 weeks of infection (Table 12). The amount (average of three replicates) of calli obtained at the end of 12 weeks of incubation were as few as 50, using NT:p19 29, and from 121 to 177 calli, using the other NT1:p19

lines (26, 27, 28). The amount of calli obtained using NT1:p19 29 might be affected due to a strong microbial contamination carried since the activation of the transgenic NT1:p19 line for the transformation experiment, which appeared in a considerable number of selection plates. The transgenic NT1:p19-ZGCSF^{T} were analized by screening PCR using the Phire®Hot Start II DNA Polymerase and the primers UpTEV and LoVSP, showing presence of both, p19 and ZeraGCSF^{T} genes. The supertransformed lines phenotype was similar to NT1 and to the transgenic lines p19 and ZGCSF^{T} (Fig. 46).

Table 12. Time required for appearance of first NT1:p19-ZGCSF^{T} calli outcomes

	Transgenic NT1:p19 lines			
	NT1:p19 26	NT1:p19 27	NT1:p19 28	NT1:p19 29
Average of NT1:p19-ZGCSF^{T} out-comes	121	177	128	50
Weeks for first out-comes appearance	4	4	4	4

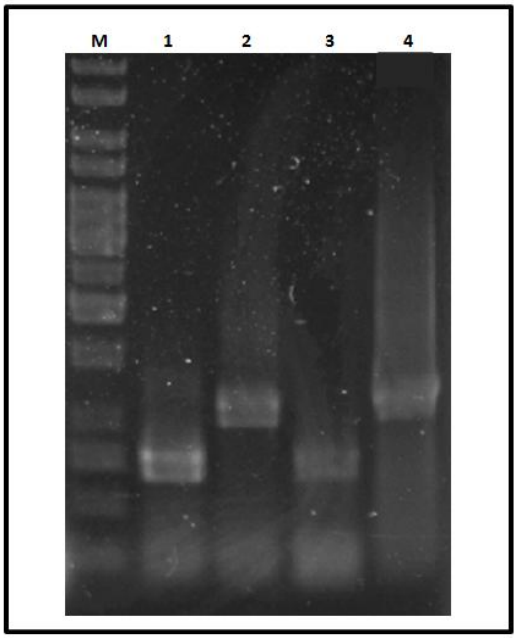


Figure 46. PCR screening for ZeraGCSF^{T} transgene in NT1:p19-ZGCSF^{T} transgenic lines. PCR amplification using the primers UpTEV and LoVSP: (911 bp). Lane 1 corresponds to NT1:p19 transgenic line 27. Lane 2 corresponds to NT1:p19-ZGCSF^{T} transgenic line 1. Lane 3 corresponds to (-) control NT1 chromosomal DNA. Lane 4 corresponds to (+) control p35SZGCSF ^{T} bin. 10 Kb DNA ladder. Marker (M).

4.4 ZeraGCSF^T Expression

Finally, we tried to detect the protein expression from NT1:ZGCSF^T and NT1:p19- ZGCSF^T. Total soluble protein was extracted using a FastPrep machine and the Zera Extraction Buffer (ZEB, see Materials and Methods 3.9.1). Detection of the extracted recombinant protein was assessed using the polyclonal anti-GCSF antibody produced in goat (Sigma G5046), and the ImmonoPure®-Mouse anti-Goat (minimal cross-reactivity to mouse, human and rabbit proteins) conjugated horseradish peroxidase (ThermoScientific 31400).

We first tried to detect ZeraGCSF^T by Western Blot (described in Materials and Methods 3.9.6), but despite all the adjustments we made to the technique, we were not able to detect the protein. For this reason, we tried to use transgenic lines expressing the recombinant antigen ZeraF1-V previously produced and analyzed by Alvarez et al., 2009, in which levels of protein expression were determined by using Western Blot. Following the same specifications to determine ZeraF1-V, and using the monoclonal anti-V and anti-F antibodies produced in mouse (USBiological Y2052-40 and Y2052-09 respectively), and its respective horseradish peroxidate conjugate (USBiological I1904-65N), we tried to adjust the Western Blot technique using ZeraF1-V transgenic lines to later detect ZeraGCSF^T. Nevertheless, we were not able to detect the antigen ZeraF1-V either.

Due to the unsuccessful ZeraF1-V detection, we considered that our inability to identify protein may be related to protein manipulation techniques (protein extraction or Western Blot), or due to a failure with the immune-detection systems we were using (antibodies/conjugates systems). However, protein degradation was not the cause for the absence of protein detection, since SDS-PAGE gels showed defined bands. For this reason, we tried to use Dot Blot and ELISA techniques, the first one being faster and simpler to perform and the second one being more sensitive. Using the Dot Blot technique, we could determine that the immune-detection systems (antibodies/conjugates) were effectively working, using the rhG-CSF produced in *E. coli* (Sigma G0407), and we determined the antibodies concentrations for immuno-detection in nitrocellulose membranes (see Materials and Methods 3.9.7) detecting as low as 5 ng rhG-CSF. However, we were not able to detect protein expression in the transgenic lines (ZeraGCSF^T and ZeraF1-V).

Using the ELISA assay, we could also determine that we could use the immune-detection systems, having as detection limit 1.56 ng rhG-CSF. However, we were not able to detect neither ZeraGCSF^T nor ZeraF1-V (using the respective antibodies with concentrations indicated by the manufacturer).

Once we established the detection limits for the standard protein, we tried to detect the recombinant protein using different conditions, in order to determine whether the extraction buffer or post-extraction handling might affect the detection system. We assume that the detection of the standard rhG-CSF might be affected by residues of strong reducing and detergent agents of the ZEB, despite of the protein concentration and washes with acetone to eliminate it, since the OD_{450} in total soluble protein preparations (with or without concentration, inoculated with the rhG-CSF) was considerably lower than that obtained with the standards dissolved in PBS (Fig. 47, 48).

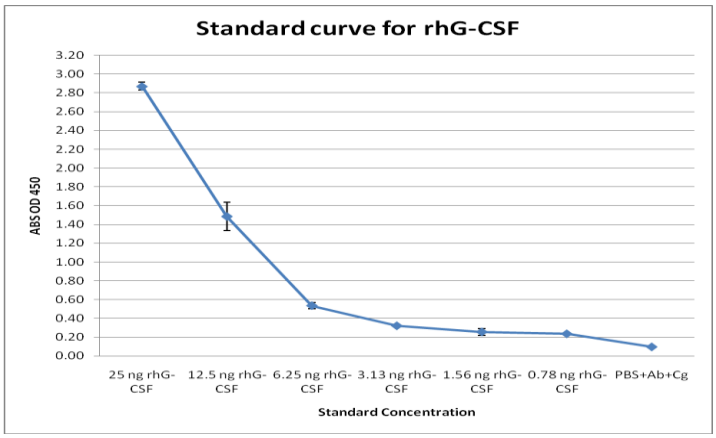


Figure 47. Determination of limit of detection, ELISA.

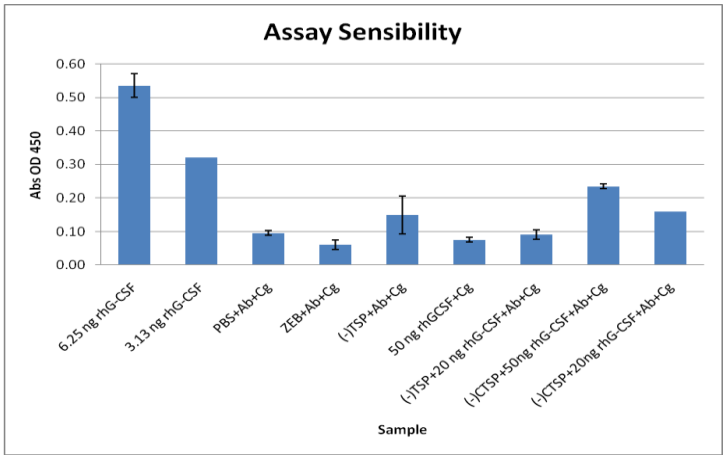


Figure 48. ELISA sensitivity.

Since different assays to try to detect the recombinant protein failed, we determined the expression of the transgene by detecting mRNA, using reverse transcriptase PCR. As the first attempt, we isolated total RNA, and determined the quality of the isolated RNA by detecting the typical rRNA bands in gel electrophoresis. 2000 ng of RNA were loaded to the gel, in order to have good rRNA bands resolution (Fig. 49). After RNA quality determination, 1000 ng of total RNA were used to produce cDNA using the M-MuLVRT Polymerase and 5 µL from cDNA were used for DNA amplification using the respective primers (UpGCSF and LoGCSF for ZeraGCSF^{T}, UpF1V and LoF1V for ZeraF1-V) and the Phire®Hot Start II DNA Polymerase (Fig. 50, 51)(see Materials and Methods 3.8).

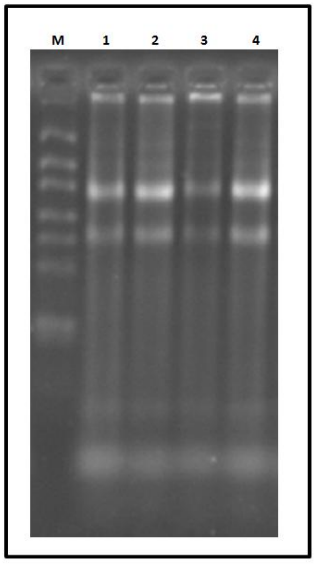


Figure 49. RNA quality determination. 2000 ng total RNA per well. Ribosomal subnits bands of 1.8 and 0.9 Kb. Lane1 1 corresponds to NT1. Lane 2 corresponds to LBZ11, expressing ZeraF1-V. Lane 3 corresponds to NT1:ZGCSF^{T} 79. Lane 4 corresponds to NT1:p19-ZGCSF^{T} 4. Marker (M).

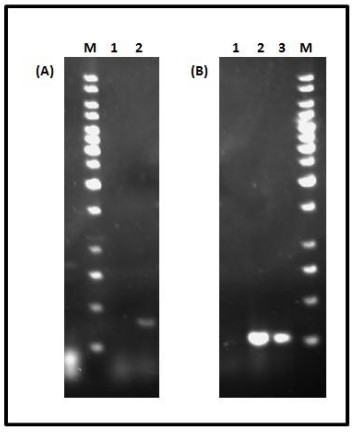


Figure 50. Reverse Transcriptase-PCR for detection of ZeraF1-V and ZeraGCSF^{T} (supertransformed NT1:p19 line). 1000 ng total RNA was used to create the cDNA, and 5 µL were used for the PCR reaction. A) PCR with UpF1V and LoF1V (384 bp) for detection of ZeraF1-V. Lane 1 corresponds to NT1. Lane 2 corresponds to LBZ11, expressing ZeraF1-V. B) PCR with UpGCSF and LoGCSF (276 bp) for detection of ZeraGCSF^{T}. Lane 1 corresponds to NT1. Lanes 2 and 3 correspond to NT1:p19-ZGCSF^{T} 38 (NT1:p19 29 supertransformed line). 10 Kb DNA ladder. Marker (M).

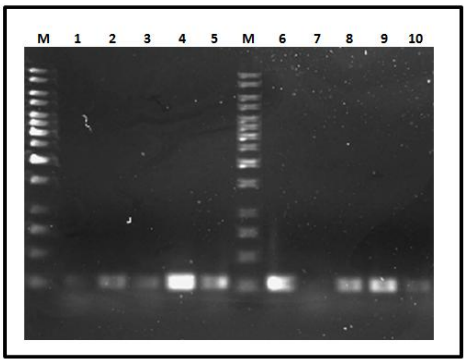


Figure 51. Reverse Transcriptase-PCR for detection of ZeraGCSF^T (NT1: ZGCSF^T lines and NT1:p19- ZGCSF^T). 1000 ng total RNA was used to create the cDNA, and 5 µL cDNA were used for the PCR reaction. PCR with UpGCSF and LoGCSF (276 bp) for detection of ZeraGCSF^T. Lanes 1 and 7 correspond to (-) control NT1. Lanes 2 to 5 correspond to NT1:ZGCSF^T lines 79, 98, 105, 110. Lanes 6, 8 to 10 correspond to NT1:p19-ZGCSF^T lines 4, 12, 13, 15 (NT1:p19 27 supertransformed lines). 10 Kb DNA ladder. Marker (M).

Using the previously described methodology, we were able to detect ZeraF1-V as well as ZeraGCSF^T transcripts. We selected some of the transgenic lines that we previously used for protein detection: LBZ11 (expressing ZeraF1-V), NT1:ZGCSF^T 79, 98, 105, 110 (expressing ZeraGCSF^T), NT1:p19-ZGCSF^T 4, 12, 13, 15 (NT1:p19 27 line supertransformed with ZeraGCSF^T), and NT1:p19-ZGCSF^T 38 (NT1:p19 29 line supertransformed with ZeraGCSF^T). All of them showed correct band amplification for the respective cDNA sequence.

The obtained results indicate the correct expression of the ZeraGCSF^T transgene at transcriptional level, but no detection of the recombinant protein using different protein detection techniques was achieved. Although this might be the case present in transgenic plant cells by activation of the PTGS mechanism, as previously reported by Alvarez et al., 2009, we were not able to detect protein expression in transgenic lines previously successfully analyzed and characterized by expressing ZeraF1-V, nor in transgenic lines supertransformed with p19 and ZeraGCSF^T, in which gene silencing should be suppressed. Hence, we presume that the fused Zera recombinant protein multimers are not efficiently separating along the extraction protocol or the protein levels are below the detection limits, and optimization of protein extraction, protein detection techniques, and ZeraGCSF ^{T} multimers separation should be addressed.

5. CONCLUSIONS

The present research project had as its purpose the design of the recombinant human Granulocyte-Colony Stimulating Factor (rhG-CSF) gene for its expression in plant cells (NT1), using different strategies to optimize obtaining transgenic lines and recombinant protein production. In order to achieve the purpose of this work, we implemented a series of molecular and plant cell culture techniques.

In the first part of this effort, we constructed the different expression cassettes for the plant cells transformation. The p19 expression cassette, which encodes the suppressor protein from the Tombusvirus, was inserted into the pPS1 binary vector, and cloned into the *Agrobacterium* strains LBA4404, AGL1, EHA105, and GV3850. The p19 suppressor was used as a model protein for the experiments related to the optimization of the transformation protocol mediated by *Agrobacterium*, as well as to determine the most virulent *Agrobacterium* strain.

The *Agrobacterium* strain AGL1 showed the most virulent pattern, providing the highest number of outcomes along the transformation experiments. The number of outcomes obtained after NT1 cell infection was also improved by implementing changes in the transformation protocol. We observed an increase in the appearance of outcomes, when infecting cells at 19°C and a reduction in loss of plant cells during the wash steps, after infection, by improving the pellet formation. Usually, it took 4 weeks to obtain NT1:p19 calli. The changes implemented in the transformation protocol allowed an increase in the number of outcomes 10-18 fold, when using the AGL1 pPS1p19 strain, at the end of 12 weeks, compared to the protocol we initially implemented based on the Mayo et al 2006 and Alvarez et al 2009 protocols.

The optimization of the transformation protocol and the selection of the most virulent *Agrobacterium* strain allowed the successful transformation and recovery of calli containing the ZeraGCSF^{T} transgene (using AGL1 p35SZGCSF^{T}). We also observed a considerable reduction in time of incubation of the infected plant cells, when transfected with the ZeraGCSF^{T} transgene, obtaining the first outcomes at the 4th week after transformation. The maximum amount of calli outcomes of NT1:ZGCSF^{T} was 248 calli; and for the supertransformed NT1:p19-ZGCSF^{T} lines, we obtained a maximum of 128 calli, at the end of the 12th week.

Unfortunately, we were not able to achieve the construction of the 35SGCSF^{A} expression cassette due to the unanticipated presence of Dam methylation at the restriction site (BspHI) located at the first codon of the nucleotide sequence, a result of nucleotides immediately preceding the synthetic gene sequence and found on the vector pUC57. Since this restriction site is used for the insertion of the transgene into the 35S expression cassette, we could not achieve the correct isolation and ligation of the G-CSF^{A} nucleotide sequence. Future attempts will be based on transforming the pUC57GCSF^{A} into a dam-/dcm- *E. coli* strain, which does not produce dam methylation, and will allow the restriction digestion of the G-CSF^{A} fragment.

The selection of all transgenic lines was based on the detection of the transgene in the chromosomal plant DNA, amplified by PCR, as the first step. The positive PCR lines (for the ZeraGCSF^{T}) were subcultured on agar plates using the respective antibiotic (PPT), for further protein and mRNA analysis. We were not able to detect the presence of the recombinant ZeraGCSF^{T} protein using different immunological techniques (Western Blot, Dot Blot, or ELISA). However, we were also not able to detect ZeraF1-V, a recombinant antigen expressed by NT1 cells previously produced and positively analyzed. Nevertheless, we were able to detect ZeraF1-V mRNA, by reverse transcriptase-PCR, in the calli expressing the antigen ZeraF1-V; and ZeraGCSF^{T} mRNA, in several NT1:ZGCSF^{T} and NT1:p19-ZGCSF^{T} transgenic lines. Hence, we assume that the protein extraction methodology might not be successfully degrading the protein body-like structures formed by the Zera fused recombinant proteins. Therefore, future efforts must be focused on protein extraction in order to successfully identify the protein of interest. Once we are able to detect the presence of the protein of interest, we will need to analyse the protein levels of the transgenic lines obtained, in order to select the transgenic lines with the highest protein expression levels. After performing the selection of the best lines, efforts will be addressed on purifying the Zera-GCSF^{T} protein, and releasing the G-CSF^{T} from the Zera part, trying to renaturate the cytokine for future biological activity *in vitro* assays.

6. LITERATURE

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7. ANNEXES

7.1 p19 nucleotide sequence (525 nt)

```
1     catggaacgagctatacaaggaaacgacgctagggaacaagctaacagtg
51    aacgttgggatggaggatcaggaggtaccacttctcccttcaaacttcct
101   gacgaaagtccgagttggactgagtggcggctacataacgatgagacgaa
151   ttcgaatcaagataatcccccttggtttcaaggaaagctggggtttcggga
201   aagttgtatttaagagatatctcagatacgacaggacggaagcttcactg
251   cacagagtccttggatcttggacgggagattcggttaactatgcagcatc
301   tcgatttttcggtttcgaccagatcggatgtacctatagtattcggtttc
351   gaggagttagtatcaccgtttctggagggtcgcgaactcttcagcatctc
401   tgtgagatggcaattcggtctaagcaagaactgctacagcttgccccaat
451   cgaagtggaaagtaatgtatcaagaggatgccctgaaggtactgaaacct
501   tcgaaaaagaaggcgagtaagagct
```

7.2 p35Sp38 expression cassette (2461 nt)

35S (696 bp): 1 to 696

TEV (144 bp): 697 to 838

p38 (1075 bp): 839 to 1896

VSP (557 bp): 1897 to 2461

```
1     aagcttgcatgcctgcaggtcaacatggtggagcacgacactctcgtcta
51    ctccaagaatatcaaagatacagtctcagaagaccagagggctattgaga
101   cttttcaacaaagggtaatatcgggaaacctcctcggattccattgccca
151   gctatctgtcacttcatcgaaaggacagtagaaaaggaagatggcttcta
201   caaatgccatcattgcgataaaggaaaggctatcgttcaagaatgcctct
251   accgacagtggtcccaaagatggacccccacccacgaggaacatcgtgga
301   aaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgata
351   acttttcaacaaagggtaatatcgggaaacctcctcggattccattgccc
401   agctatctgtcacttcatcgaaaggacagtagaaaaggaagatggcttct
451   acaaatgccatcattgcgataaaggaaaggctatcgttcaagaatgcctc
501   taccgacagtggtcccaaagatggacccccacccacgaggaacatcgtgg
551   aaaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgat
601   atctccactgacgtaagggatgacgcacaatcccactatccttcgcaaga
651   cccttcctctatataaggaagttcatttcatttggagaggacctcgagaa
701   ttaattctcaacacaacatatacaaaacaaacgaatctcaagcaatcaag
751   cattctacttctattgcagcaatttaaatcatttcttttaaagcaaaagc
801   aattttctgaaaattttcaccatttacgaacgatagccatggaaaatgat
851   cctagagtccggaagttcgcatctgatggcgcccaatgggcgataaagtg
901   gcagaagaagggctggtcaaccctaaccagcagacagaaacagaccgccc
951   gcgcagcgatggggatcaagctctctcctgtggcgcaacctgtgcagaaa
1001  gtgactcgactgagtgctccggtggcccttgcctaccgcgaggttaccac
1051  ccagcctcgggtctctactgccagggacggcataaccagaagcggttctg
1101  aactgatcacaaccttgaagaagaacactgacactgaacctaagtacacc
```

```
1151  acagctgtgcttaacccaagcgaacccggaacattcaaccagctcattaa
1201  ggaggcggcccagtatgaaaaataccgattcacgtcactcagatttaggt
1251  actcccccatgagcccttcaaccaccggaggcaaggtggctctggcattc
1301  gatcgagatgccgccaaacctccgcccaacgacctcgcttccctctacaa
1351  catagagggttgtgtatctagcgtgccctggacagggtttattttgaccg
1401  tcccaacagattctactgaccgctttgtggcggatggtatcagcgatcca
1451  aagcttgtcgatttcggcaagctcatcatggccacctacggccaaggagc
1501  caatgatgccgcccaactcggtgaagtgcgagtcgagtacaccgtgcagc
1551  tcaagaacagaactggctcaaccagcgacgcccagattggggacttcgca
1601  ggtgttaaggacggacccaggctggtttcatggtccaagaccaaggggac
1651  agctgggtgggagcacgattgtcattttctcggaaccggaaacttctcgt
1701  tgacattgttctacgagaaggcgccggtctcggggctagaaaacgcagac
1751  gcctctgacttctcggtcctgggagaagccgcagcaggtagtgtccaatg
1801  ggcaggagtgaaggtagcagaaaggggacaaggcgtgaaaatggtcacaa
1851  ctgaggagcagccaaagggtaaatggcaagcactcagaatttaggagctc
1901  tctcaacaatctagctagagtttgctcctatctatatgtaataaggtatg
1951  ctgatatgcactattcaaataggagcattagctatgtttgttaatgtcac
2001  tttatgttatgtgggtaagtcacctaagacactccacgtacctacgttgt
2051  tgtctcttaccggctttaataaatcttctgcccttgttccatatttacta
2101  attatccctttcttcactaaaagaaaattgttatcattaagtattagtct
2151  ttagaacatatgaggtctttaattgggtaggttttacaaattaactaata
2201  taaaatgtcataaaatccacgtggttaaacaaatgcagaaaatcgacgtc
2251  gtctattggaccgacagttgctattaatataatgggccaccatagtagac
2301  tgacaaataaattacctgacaacatcgtttcactaaataacaaacacaaa
2351  aagggagtgcattttccagggcatttttgtaataaaaaacagttaaaagg
2401  gagtgcaatagaaatataggggtgtggaaatagtgatttgagcacgtctt
2451  gaagcgaattc
```

7.3 hG-CSF (variant 2) mature protein encoding nucleotide sequece (522 nt) and amino acid sequence (174 aa)

```
1     acccccctgggccctgccagctccctgccccagagcttcctgctcaagtg
51    cttagagcaagtgaggaagatccagggcgatggcgcagcgctccaggaga
101   agctgtgtgccacctacaagctgtgccaccccgaggagctggtgctgctc
151   ggacactctctgggcatcccctgggctcccctgagcagctgccccagcca
201   ggccctgcagctggcaggctgcttgagccaactccatagcggccttttcc
251   tctaccaggggctcctgcaggccctggaagggatctcccccgagttgggt
301   cccaccttggacacactgcagctggacgtcgccgactttgccaccaccat
351   ctggcagcagatggaagaactgggaatggcccctgccctgcagcccaccc
401   agggtgccatgccggccttcgcctctgctttccagcgccgggcaggaggg
451   gtcctggttgcctcccatctgcagagcttcctggaggtgtcgtaccgcgt
501   tctacgccaccttgcccagccc
```

```
TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPE
ELVLLGHSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQ
ALEGISPELGPTLDTLQLDVADFATTIWQQMEELGMAPALQPTQG
AMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP
```

7.4 Zera (γ-zein) nucleotide sequece (339 nt) and amino acid sequence (113 aa)

```
1     atgagggtgttgctcgttgccctcgctctcctggctctcgctgcgagcgc
51    cacctccacgcatacaagcggcggctgcggctgccagccaccgccgccgg
101   ttcatctaccgccgccggtgcatctgccacctccggttcacctgccacct
151   ccggtgcatctcccaccgccggtccacctgccgccgccggtccacctgcc
201   accgccggtccatgtgccgccgccggttcatctgccgccgccaccatgcc
251   actaccctactcaaccgccccggcctcagcctcatccccagccacaccca
301   tgcccgtgccaacagccgcatccaagcccgtgccagacc
```

```
MRVLLVALALLALAASATSTHTSGGCGCQPPPPVHLPPPVHLPPPVHLPPPVHLPPPVHLPPPVHL
PPPVHVPPPVHLPPPPCHYPTQPPRPQPHPQPHPCPCQQPHPSPCQT
```

7.5 bar gene nucleotide sequece

GenBank: DQ211346.1

7.6 Preferential codon analysis

Table 14. Preferential codon analysis.

		cDNA hG-CSF	Codones preferenciales para Tabaco			
AA	**codon**	**wild type**	**version 1**	**wt %**	**v1 %**	**N tabacum**
						%
Ala (A)	gca	3	6	16	32	31
	gcc	13	3	68	16	17
	gcg	1	1	5	5	8
	gct	2	9	11	47	44
%=	5	**19**	**19**	**100**	**100**	**100**
Arg (R)	aga	0	2	0	40	32
	agg	1	2	20	40	26
	cga	0	0	0	0	11
	cgc	3	0	60	0	8
	cgg	1	0	20	0	8
	cgt	0	1	0	20	16
%=	20	**5**	**5**	**100**	**100**	**101**
Asn (N)	aac	0	0	**0**	**0**	40
	aat	0	0	**0**	**0**	60
%=	0	**0**	**0**	**0**	**0**	**100**
Asp (D)	gac	3	2	75	50	32
	gat	1	2	25	50	68
%=	25	**4**	**4**	**100**	**100**	**100**
Cys (C)	tgc	4	2	80	40	43

	tgt	1	3	20	60	57
%=	20	**5**	**5**	**100**	**100**	**100**
Gln (Q)	caa	2	9	12	53	58
	cag	15	8	88	47	42
%=	6	**17**	**17**	**100**	**100**	**100**
Glu (E)	gaa	3	5	33	56	54
	gag	6	4	67	44	46
%=	11	**9**	**9**	**100**	**100**	**100**
Gly (G)	gga	3	5	21	36	34
	ggc	6	2	43	14	17
	ggg	3	2	21	14	15
	ggt	2	5	14	36	34
%=	7	**14**	**14**	**100**	**100**	**100**
His (H)	cac	3	2	60	40	39
	cat	2	3	40	60	61
%=	20	**5**	**5**	**100**	**100**	**100**
Ile (I)	ata	0	0	0	0	25
	atc	4	2	100	50	25
	att	0	2	0	50	50
%=	25	**4**	**4**	**100**	**100**	**100**
Leu (L)	cta	1	3	3	9	10
	ctc	6	4	18	12	14
	ctg	20	5	61	15	12
	ctt	2	11	6	33	26
	tta	1	3	3	9	14
	ttg	3	7	9	21	24
%=	3	**33**	**33**	**100**	**100**	**100**
Lys (K)	aaa	0	1	0	25	49
	aag	4	3	100	75	51
%=	25	**4**	**4**	**100**	**100**	**100**
Met (M)	atg	3	3	100	100	100
%=	33	**3**	**3**	**100**	**100**	**100**
Phe (F)	ttc	5	3	83	50	42
	ttt	1	3	17	50	58
%=	17	**6**	**6**	**100**	**100**	**100**
Pro (P)	cca	0	5	0	38	40
	ccc	10	2	77	15	13
	ccg	1	1	8	8	10
	cct	2	5	15	38	37
%=	8	**13**	**13**	**100**	**100**	**100**
Ser (S)	agc	8	1	57	7	13
	agt	0	3	0	21	17
	tca	0	4	0	29	23
	tcc	3	2	21	14	14
	tcg	1	0	7	0	7
	tct	2	4	14	29	26
%=	7	**14**	**14**	**100**	**100**	**100**
Ter (.)	taa	0	0	0	0	41
	tag	0	0	0	0	19
	tga	0	0	0	0	39
%=	0	**0**	**0**	**0**	**0**	**99**

Thr (T)	aca	1	3	14	43	33
	acc	6	2	86	29	19
	acg	0	0	0	0	9
	act	0	2	0	29	39
%=	14	**7**	**7**	**100**	**100**	**100**
Trp (W)	tgg	2	2	100	100	100
%=	50	**2**	**2**	**100**	**100**	**100**
Tyr (Y)	tac	3	2	100	67	43
	tat	0	1	0	33	57
%=	33	**3**	**3**	**100**	**100**	**100**
Val (V)	gta	0	1	0	14	17
	gtc	2	1	29	14	17
	gtg	3	1	43	14	25
	gtt	2	4	29	57	41
%=	14	**7**	**7**	**100**	**100**	**100**
Total		**174**	**174**			**1159 cds**

7.7 Synthesized EkG-CSFT

```
1     gatgatgatgataagacacccttaggacctgccagttcattgccacagag
51    tttcttgctcaagtgcttagagcaagttaggaagattcagggtgatggcg
101   cagcgcttcaagagaaactttgtgctacctacaagttgtgtcatccagag
151   gaactggtgttactcggacactctctgggcattccttgggctccactgtc
201   tagttgtccatcacaagcactgcaactggcaggttgcttgtcacaacttc
251   atagcggacttttcctctatcaggggctccttcaggcactagaagggatc
301   tcccctgagttgggtccaactttggacacacttcaacttgatgttgctga
351   ctttgctactaccatctggcaacagatggaagaactaggaatggcacctg
401   ctcttcagccgacacaaggtgctatgcctgcctttgcttctgcctttcag
451   aggagagcaggaggtgtcttggttgcttcccatcttcagtcattccttga
501   agtatcttacagagttctacgtcaccttgctcaaccctga
```

DDDDKTPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLL
GHSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDT
LQLDVADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSF
LEVSYRVLRHLAQP.

7.8 Synthesized rhG-CSFA

```
1     tcatgacacccttaggacctgccagttcattgccacagagtttcttgctc
51    aagtgcttagagcaagttaggaagattcagggtgatggcgcagcgcttca
101   agagaaactttgtgctacctacaagttgtgtcatccagaggaactggtgt
151   tactcggacactctctgggcattccttgggctccactgtctagttgtcca
201   tcacaagcactgcaactggcaggttgcttgtcacaacttcatagcggact
251   tttcctctatcaggggctccttcaggcactagaagggatctcccctgagt
301   tgggtccaactttggacacacttcaacttgatgttgctgactttgctact
351   accatctggcaacagatggaagaactaggaatggcacctgctcttcagcc
401   ggcacaaggtgctatgcctgcctttgcttctgcctttcagaggagagcag
451   gaggtgtcttggttgcttcccatcttcagtcattccttgaagtatcttac
501   agagttctacgtcaccttgctcaaccctgatagagctc
```

MTPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSL
GIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLD
VADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVS
YRVLRHLAQP.

7.9 35SZGCSFT expression cassette (2285 nt) and amino acid codified sequence (292)

Zera: 1 to 694

5'-UTR TEV: 695 to 838

ZeraGCSF^{T}: 834 to 1720

3'-vsp: 1720 to 2285

```
1     aagcttgcatgcctgcaggtcaacatggtggagcacgacactctcgtcta
51    ctccaagaatatcaaagatacagtctcagaagaccagagggctattgaga
101   cttttcaacaaagggtaatatcgggaaacctcctcggattccattgccca
151   gctatctgtcacttcatcgaaaggacagtagaaaaggaagatggcttcta
201   caaatgccatcattgcgataaaggaaaggctatcgttcaagaatgcctct
251   accgacagtggtcccaaagatggacccccacccacgaggaacatcgtgga
301   aaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgata
351   acttttcaacaaagggtaatatcgggaaacctcctcggattccattgccc
401   agctatctgtcacttcatcgaaaggacagtagaaaaggaagatggcttct
451   acaaatgccatcattgcgataaaggaaaggctatcgttcaagaatgcctc
501   taccgacagtggtcccaaagatggacccccacccacgaggaacatcgtgg
551   aaaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgat
601   atctccactgacgtaagggatgacgcacaatcccactatccttcgcaaga
651   cccttcctctatataaggaagttcatttcatttggagaggacctcgagaa
701   ttaattctcaacacaacatatacaaaacaaacgaatctcaagcaatcaag
751   cattctacttctattgcagcaatttaaatcatttcttttaaagcaaaagc
801   aattttctgaaaattttcaccatttacgaacgatagtcatgagggtgttg
851   ctcgttgccctcgctctcctggctctcgctgcgagcgccacctccacgca
901   tacaagcggcggctgcggctgccagccaccgccgccggttcatctaccgc
951   cgccggtgcatctgccacctccggttcacctgccacctccggtgcatctc
1001  ccaccgccggtccacctgccgccgccggtccacctgccaccgccggtcca
1051  tgtgccgccgccggttcatctgccgccgccaccatgccactaccctactc
1101  aaccgccccggcctcagcctcatccccagccacacccatgcccgtgccaa
1151  cagccgcatccaagcccgtgccagaccgatgatgatgataagacaccctt
1201  aggacctgccagttcattgccacagagtttcttgctcaagtgcttagagc
1251  aagttaggaagattcagggtgatggcgcagcgcttcaagagaaactttgt
1301  gctacctacaagttgtgtcatccagaggaactggtgttactcggacactc
1351  tctgggcattccttgggctccactgtctagttgtccatcacaagcactgc
1401  aactggcaggttgcttgtcacaacttcatagcggacttttcctctatcag
1451  gggctccttcaggcactagaagggatctcccctgagttgggtccaacttt
1501  ggacacacttcaacttgatgttgctgactttgctactaccatctggcaac
1551  agatggaagaactaggaatggcacctgctcttcagccgacacaaggtgct
1601  atgcctgcctttgcttctgcctttcagaggagagcaggaggtgtcttggt
1651  tgcttcccatcttcagtcattccttgaagtatcttacagagttctacgtc
1701  accttgctcaaccctgacgagctctctcaacaatctagctagagtttgct
1751  cctatctatatgtaataaggtatgctgatatgcactattcaaataggagc
```

```
1801  attagctatgtttgttaatgtcactttatgttatgtgggtaagtcaccta
1851  agacactccacgtacctacgttgttgtctcttaccggctttaataaatct
1901  tctgcccttgttccatatttactaattatccctttcttcactaaaagaaa
1951  attgttatcattaagtattagtctttagaacatatgaggtctttaattgg
2001  gtaggttttacaaattaactaatataaaatgtcataaaatccacgtggtt
2051  aaacaaatgcagaaaatcgacgtcgtctattggaccgacagttgctatta
2101  atataatgggccaccatagtagactgacaaataaattacctgacaacatc
2151  gtttcactaaataacaaacacaaaaagggagtgcattttccagggcattt
2201  ttgtaataaaaaacagttaaaagggagtgcaatagaaatatagggggtgtg
2251  gaaatagtgatttgagcacgtcttgaagcgaattc
```

7.10 35SZGCSFT amino acid sequence (292 aa)

```
MRVLLVALALLALAASATSTHTSGGCGCQPPPPVHLPPPVHLPPPVHLPPPVHLP
PPVHLPPPVHLPPPVHVPPPVHLPPPPCHYPTQPPRPQPHPQPHPCPCQQPHPSP
CQTDDDDKTPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEEL
VLLGHSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPT
LDTLQLDVADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHL
QSFLEVSYRVLRHLAQP
```

7.11 pUC57 (2719 bp)

```
GenScript: SD1176
```

7.12 pUC18 (2686 bp)

GenScript: SD1162

7.13 pIBT210.1 (4051 bp)

```
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ttgagacttttcaacaaagggtaatatcgggaaacctcctcggattccat
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7.14 pPS1 (12599 bp)

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7.15 Transformation binary vector pBAR-35S, T-DNA region

GenBank: AJ251014.1

7.16 pGEM-T Easy (3000 bp)

Promega: A1360