Transient Expression of Antibodies in Suspension Plant Cell Suspension Cultures is Enhanced When Co-transformed with the Tomato Bushy Stunt Virus p19 Viral Suppressor of Gene Silencing

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Two distinct transient expression approaches were compared with assess the impact of the viral suppressor p19 on a recombinant protein production performed in Nicotiana benthamiana *suspension culture. A parental* N. benthamiana *cell line was transiently transformed with either an* Agrobacterium *containing a gene construct for a murine IgG1 (R514) or concurrently with two* Agrobacteria *containing R514 or p19. In addition, a stably transformed* N. benthamiana *cell line that constitutively expresses p19 was transformed with R514-containing* Agrobacterium. *The parental* N. benthamiana *cell line that had been co-cultivated with both p19 and R514 achieved the highest yield of IgG1 (1.06 mg IgG1/kg FW; 0.024% TSP) compared with that obtained without p19 (0.61 mg IgG1/kg FW; 0.014% TSP). The* N. benthamiana *cell line that had been stably transformed with p19 only reached 0.25 mg IgG1/kg FW (0.009% TSP) when co-cultured with R514-containing* Agrobacterium. *Dual agroinfiltration of* N. benthamiana *leaves with p19 and R514 was also performed to assess for* Agrobacteria *efficiencies and 147.7 mg IgG1/kg FW were obtained. Therefore, our results demonstrate that transient co-transformation of plant cell suspension culture with two transformation vectors is feasible and that the use of the viral suppressor of silencing p19 significantly raises the production of the protein of interest.* © 2010 American Institute of Chemical Engineers *Biotechnol. Prog.,* 26: 1534–1543, 2010
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Introduction

Microbial[1] and animal cell[2] platforms are broadly used for the production of biopharmaceuticals, which now account for over 20% of all new pharmaceuticals on the market.[3] However, the plant system is gaining in interest among emerging platforms because of its various advantages compared with established platforms.[4] Molecular farming platforms using stable genetically transformed plants or transient transformation (agroinfiltration) by modified *Agrobacteria* strains have been shown to be efficient for the production of recombinant proteins.[5] The most studied plant species for recombinant protein production are *Nicotiana* species (*Nicotiana tabacum* and *Nicotiana benthamiana*),[6] rice (*Oryza sativa*),[7] soybean (*Glycine max*),[8] and to a lesser extent tomato (*Solanum lycopersicum*).[9] *In vitro* culture, either of plant cell suspensions[10] or hairy roots,[11] has also been shown to be a valuable platform for producing bioactive human proteins.[5] *In vitro* plant cell culture has important advantages over other platforms such as the use of low cost and completely defined culture media[12] under aseptic culture conditions that do not promote infectious agents such as viruses and prions[13] nor the presence of insects and microbes.

As for the other *in vitro* production platforms, stable recombinant plant cell lines can be easily obtained and

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master cell banks generated.[14] Recent studies also demonstrated the potential of transient transformation of plant cells by co-cultivation with an *Agrobacterium* strain containing an expression vector coding for a protein of interest.[15,16] Although the proportion of transformed (i.e., productive) cells may be less than when stable recombinant cell lines are used, a transient expression approach can speed up and facilitate transformation vector screening as well as overall bioprocess management. A high reproducibility level for the bioprocess can also be achieved, avoiding possible loss of cell productive capacity by mutations or genetic rearrangement after multiple cell divisions. It may thus represent a lower risk level, in terms of cell line stability and production reproducibility, to culture a parental cell line all along the culture cascade and only start recombinant protein production at the last bioprocess step. Moreover, a transient strategy may also enable rapid response to punctual market needs, from gene identification to protein production; the development of stable recombinant eukaryote cell lines is generally time-consuming (several months) while the development of transformed *Agrobacteria* can be achieved within 2 weeks.[17,18]

Various metabolic engineering strategies were developed to increase the bioactivity and yield of a recombinant protein. Vézina et al.[19] reported the production of antibodies with human-like *N*-glycans by transiently co-expressing in tobacco leaves a chimaeric human beta1,4-galactosyltransferas and Kim et al.[20] showed an enhanced production of recombinant human granulocyte-macrophage colony stimulating factor in stable rice cell suspensions co-expressing a synthetic serine proteinase inhibitor II. Higher bioproduct levels have even been observed from transient expression due to a "burst" of gene expression[21] but final recombinant protein yields are usually low, partly because of post-transcriptional gene silencing (PTGS). PTGS acts as an adaptive immune system targeted against viruses,[22] but it can also affects transgene expression, since, like viruses, transgenes that are expressed under the control of a strong promoter, produce high level of identical RNAs. Interestingly, recent studies showed that PTGS limitation can be overcome using virus-encoded suppressor proteins.[23] Many plant viruses include proteins which role is to suppress RNA silencing in the host cell.[24] Various viral suppressors, such as p19 that had been identified in the tomato bushy stunt virus (TBSV),[23] were shown to interfere with plant cell natural defense mechanisms. Viral suppressors were thus used in an attempt to improve the yield in recombinant protein production and the most promising method relies on the co-expression of viral suppressors such p19 which prevent silencing of the transgene.[23] Therefore, an "optimized" production platform may require the simultaneous expression of many transgenes. This can either be achieved by developing a modified cell line (having all the transgenes but that for the recombinant protein of interest) which can then be transiently transformed for a recombinant protein of interest, by using a single vector allowing for the transfer of multiple genes, or by a co-transformation where multiple *Agrobacteria* strains are used simultaneously to transfer all the required transgenes. The successive transformations approach is tedious and time-consuming since multiple plant cell line regeneration and selection steps are required for each transgene addition. The successful co-transformation of plant leaves with many *Agrobacterium* strains containing different vectors has been reported with two,[23,25] four,[26] and even six different strains[27] used simultaneously.

Therefore, various technological approaches are available to develop optimal transient production of a recombinant protein. In the present work, we have applied for the first time, to the best of our knowledge, the dual agroinfiltration concept to a *N. benthamiana* plant cell suspension cultured in shake flask. Plant cells were co-cultured for 12 days with an *Agrobacterium* strain that contained the expression vector for a mouse IgG1 antibody or with two *Agrobacteria* cell lines; one containing the p19 viral suppressor and one containing the IgG1 antibody-coding genes (heavy and light chains). The performance of the dual agroinfection process was compared with that of transient agroinfiltration of the parental *N. benthamiana* plant leaves and co-culturing an IgG1 antibody-coding *Agrobacterium* of a stable recombinant *N. benthamiana* cell line that stably expressed p19.

Material and Methods

Plant cell line development

N. benthamiana leaves were sterilized by a 5 min immersion in 70% ethanol followed by a 5 min immersion in a 0.5% hypochlorite solution. Leaves were washed in sterile water three times and cut in small disks with a scalpel blade. The disks were placed in the dark on solid MS NAA KIN medium, a variant of the MS[28] medium containing 30 g/L glucose, 4 g/L Phytagel, 2 mg/L α-naphthalene acetic acid, and 0.05 mg/L kinetin. Few weeks later, ~2 g of calluses were transferred into 10 mL of liquid MS NAA KIN medium in 50 mL Erlenmeyer flasks that were agitated on a rotary shaker at 120 rpm in the dark. Liquid medium was regularly added and the cells transferred to a bigger Erlenmeyer until a final 150 mL of cell suspension was obtained in 500 mL Erlenmeyer flasks.

Plant cell suspension culture

Plant cell suspensions were subcultured when the sedimented cell volume reached 70–80% of the total volume in 5 min (i.e., every 7 days). Subcultures were performed using a 1/3 dilution ratio (150 mL total volume) in 500 mL Erlenmeyer flasks kept at 27°C on rotary shakers (120 rpm) in the dark. All Erlenmeyer flasks were closed with a two-layer aluminum foil.

Development of a stable p19 expressing N. benthamiana *cell line*

Five milliliter of 7-day old *N. benthamiana* cells were transferred to 10 mL of fresh MS NAA KIN medium in a 50 mL Erlenmeyer. The *Agrobacterium* strain C58C1 containing the vector coding for p19 gene (C58C1-p19) from TBSV was grown at 27°C overnight in LB medium supplemented with 50 mg/L kanamycin. p19 was under a CaMV 35s promoter and a Nos terminator[23] (kindly provided by Dr. Baulcombe). The bacteria were centrifuged and resuspended to an O.D. of 0.8 at 600 nm in MS NAA KIN. Five days after inoculation of the plant cells, 0.6 mL of fresh *Agrobacterium* suspension culture was added. Acetosyringone was also added to a final concentration of 100 μM. The cells were cultivated with the bacteria for two additional days. On day 7, the cells were collected and transferred to a 15 mL sterile tube. After centrifugation (1 min, 300 rpm), the supernatant was discarded and replaced by fresh MS NAA KIN medium with 50 mg/L kanamycin and 200 mg/L cefotaxim. Cells were mixed in the new medium for

5 min before being recentrifuged. Washing steps were repeated three times. The cells were finally plated on Petri dishes containing solid MS NAA KIN medium with 50 mg/L kanamycin and 200 mg/L cefotaxim. The Petri dishes were sealed with Parafilm™ and incubated in the dark at 27°C for 2–3 weeks. Emerging calluses were transferred on fresh Petri dishes with selective medium. Few weeks later, calluses were transferred in selective liquid medium agitated at 120 rpm. After five subculture rounds, the use of antibiotics was stopped. Validation of the presence of the p19 gene was performed using a PCR analysis with p19-F′ (5′-ATGGAACGAGCTA TACAAGGAAACG-3′) and p19-R′ (5′-TTACTCGCTTTC TTTTTCGAAGGTC-3′) primers. Verification of a residual contamination by *Agrobacterium* was performed using the Agro-F: 5′-ATGC CCGATCGAGCTCAAGT-3′and Agro-R: 5′-CCTGACCCAAACATCTCGGCTGCCCA-3′ universal primers designed by Haas and coworkers.[29] The strains AgL1, C58C51, and LBA4404 were used as positive controls.

PCR analyses

DNA was extracted from the suspension cells using the technique described by Edwards et al.[30] PCR reactions were performed in a total volume of 25 μL (1 X GenScript PCR reaction buffer, 0.5 μM of each primer, 200 μM of each nucleotide), containing 1 μL (approximately 5 ng) of DNA, and 0.5 U of Taq DNA polymerase (GenScript). The PCR reactions were performed as follows: 1 μL of DNA and 11.5 μL of sterile water were first incubated at 94°C for 5 min to achieve complete denaturation of genomic DNA. Subsequently, 12.5 μL of reaction cocktail containing the other PCR components were added. Typical amplification conditions were: 35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 1 min, followed by a 5 min extension at 72°C.

Agroinfiltration of plants

N. benthamiana plants were grown in soil at 25°C under a photoperiod of 16 h of light and 8 h darkness. One-and-a-half-month old plants were used for agroinfiltration. *Agrobacteria* were grown at 28°C overnight in LB media supplemented with 50 mg/L kanamycin. The bacteria were centrifuged and resuspended to an $O.D._{600}$ of 0.8 in a solution of 10 mM $MgCl_2$ (Sigma) and 100 μM acetosyringone. Five different experimental conditions were tested. *N. benthamiana* plants were either left untreated, infiltrated with a solution of 10 mM $MgCl_2$, infiltrated with an *Agrobacterium* strain that did not contain any transformation plasmid (i.e., no T-DNA plasmid) (C58C1), infiltrated with a strain (AgL1) that contained the R514 vector (kindly provided by Medicago), or infiltrated with both a strain that contained the R514 vector and a strain (C58C1) that contained the vector for p19. The R514 vector encoded murine IgG1 heavy and light chain genes, each under a CaMV 35s promoter and Nos terminator; a variant from the R610 and R612 constructs.[19] For the combined inoculation of R514 and p19, the two bacterial suspensions were diluted at an $O.D._{600}$ of 0.8 and were mixed at equal volume. A minimum of five plants per experimental group was transformed for this experiment. Plants were agroinfiltrated by plunging them upside down in a beaker containing 500 mL of the *Agrobacterium* suspension and placed in a bell vacuum jar. Vacuum was maintained for 30 s before being quickly released. The surfaces of the leaves were gently dried using paper tissues. The leaves that were not properly infiltrated were cut to reduce the impact of a lesser transformation. For the sampling, three leaves were taken per experimental group. The leaves were taken randomly from different plants. The leaves were crushed in liquid nitrogen and stored at −80°C in cryovials. The whole experiment was performed twice with two distinct plant groups.

Co-culture of plant cells and Agrobacterium

For transient expression, 30 mL of fresh MS NAA KIN medium were inoculated with 15 mL of 7-days-old plant cell cultures in 250 mL Erlenmeyer flasks. On day 5 postinoculation, 45 mL of 2X MS NAA KIN were added to each flask. *Agrobacteria* were added 1 day after medium addition.

For the *N. benthamiana* parental cell line, one group was inoculated with a strain of *Agrobacterium* (C58C1) that did not contain any transformation plasmid (i.e., no T-DNA plasmid), another one with a strain of *Agrobacterium* (AgL1) containing the R514 vector coding for the production of the IgG1, and one group was inoculated simultaneously with two strains of *Agrobacterium*: one containing the R514 vector and one (C58C1) containing the vector coding for p19. For the *N. benthamiana* expressing p19 cell line, one group was inoculated with a strain without plasmid (C58C1) and another one with a strain (AgL1) containing the R514 vector. For both plant cell lines, one group without any bacteria was also kept as control. All seven experimental groups were performed in triplicates.

Agrobacteria were grown at 28°C overnight in LB medium supplemented with 50 mg/L kanamycin. The bacteria were centrifuged and resuspended to an $O.D._{600}$ of 0.8 in MS NAA KIN. Co-cultivation was initiated in suspension cultures by inoculating 0.5 mL of the corresponding resuspended *Agrobacterium*. Acetosyringone was added to each cell flask (including controls) to a final concentration of 100 μM. Cells were co-cultured in a dark shaker as described above. Around 15 mL of the co-cultured cells were harvested on days 0, 3, 6, 9, and 12 of co-culture. The samples were filtered to separate cells from medium. The latter was kept at −80°C while plant cells were weighted for the fresh weight (FW) assessment, crushed in liquid nitrogen with a mortar and pestle, and stored in cryovials at −80°C. The whole experiment was performed twice.

Protein extraction for analysis

One hundred (100) milligram of frozen and crushed cells were weighted in a cold 1.5 mL minitube. 50 μL of extraction buffer (50 mM Tris-HCl pH 8.00, 300 mM NaCl, 0.1% (v/v) Triton X-100, 0.01% (v/v) proteases inhibitor cocktail (Sigma P8849)) was then added, and the mix was crushed on ice for 1 min using a small pestle for 1.5 mL minitubes. The samples were centrifuged at 10,000 rpm for 10 min. The supernatants were transferred to fresh minitubes and stored at −80°C for future analysis. Total protein concentrations were measured using a bicinchonic acid protein assay kit (Pierce, cat# 23225). Samples were diluted in water prior testing.

Measurement of IgG1 by ELISA

The ELISA measurement of IgG1 was adapted from Khoudi et al.[31] The microplate wells (Costar 3369) were coated overnight with a goat antimouse IgG1 heavy chain specific antibody (Sigma M-8770) diluted at 2.5 μg/mL in carbonate buffer (50 mM, pH 9.6). Blocking was achieved through a 1 h-

incubation in a 1.0% casein-containing phosphate buffer-saline (PBS) (PBS-casein) at 37°C. For each plate, a standard curve was prepared with 0, 1, 2, 3, 4, 5, 7, and 10 ng/mL of purified IgG1 murine standard (Sigma, M-9269). When performing the immunoassays, all dilutions (control and samples) were performed in PBS-casein solution and applied directly to the coated wells. Plates were incubated with 50 μL of diluted samples and protein standard for 1 h at 37°C. After three washes with 0.1% Tween-20 in PBS (PBS-T), the plates were incubated with a goat antimouse IgG-peroxydase conjugate (Bio-Rad Laboratories, CA, 170-5047) in PBS-casein for 1 h at 37°C. The washes with PBS-T were repeated and the plates were incubated with a 3,3′,5,5′-Tetramethylbenzidine liquid substrate for ELISA (Sigma, T0440). The reaction was stopped by the addition of 50 μL of 1 N HCl and the absorbance was measured at 450 nm with a Victor^{3}V microplate reader (PerkinElmer). Each sample and control was assayed in triplicate and the concentrations were intrapolated in the linear portion of the standard curve.

Glucose concentration determination

Glucose was measured using a YSI 2700 Select Biochemistry Analyser equipped with a YSI 2365 membrane. Calibration of the instrument was performed every six sample measurement with a commercial standard at 2.5 g/L of glucose (RSI 27250).

qPCR analysis

RNAs were extracted as follows: 1 mL of Tri-Reagent (Sigma T9424) was added to 200 mg of frozen and crushed cells in a 1.5 mL minitube and vortexed for 1 min. After 5 min incubation (room temperature), 0.2 mL of chloroform was added and mixed. After 15 min incubation (room temperature), the samples were centrifuged (4°C, 15 min, 12,000*g*). The supernatants were transferred to fresh minitubes and followed by 0.5 mL isopropanol addition. The samples were incubated for 10 min at room temperature before centrifugation (4°C, 10 min, 12,000*g*). The supernatants were discarded, the pellets washed with 1 mL EtOH 70% and centrifuged (4°C, 5 min, 7,500*g*). The supernatants were discarded, and the pellets were air-dried for 10 min at room temperature, resuspended in 50 μL DEPC-treated water and stored at −80°C until use. RNA quantification by reverse transcription and qPCR analysis was performed by the Genomics Core Facility at IRIC (Montréal, QC). Two (2) μg of total RNA was reverse transcribed in a final volume of 20 μL using High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems) as described by the manufacturer. The primers used for this analysis are as follows: IgG1 (UPL # 71) Forward 5′-TACTGTGGAGTGGCAGTGGA-3′, Reverse 5′-TGATGGGCTGAGTGTTCTTG-3′ p19 (UPL # 7) Forward 5′-GTTGGGATGGAGGATCAGG-3′ Reverse 5′-CTCGGACTTTCGTCAGGAAG-3′ Actin (UPL # 67) Forward 5′-TGGAACAGGAATGGTCAAGG-3′ Reverse 5′-AGGGAATACAGCTCGTGGAG-3′, Ubiquitin (UPL # 39) Forward 5′-TGACTGGGAAGACCATCACC-3′ Reverse 5′-CCTTGTCCTGGATCTTAGCTTTT-3′. PCR reactions were performed in 384 well plate formats using 2 μL of cDNA sample (25 ng), 5 μL of the Fast Universal qPCR MasterMix (Applied Biosystems), 2 μM of each primer, and 1 μM of a UPL probe in a total volume of 10 μL. The ABI PRISM®7900HT Sequence Detection System (SDS; Applied Biosystems) was used to detect the amplification level and was programmed as follows: an initial step of 3 min at 95°C was followed by 45 cycles of 5 s at 95°C and 30 s at 60°C. All reactions were run in triplicate and the average Cts values were used for quantification. The actin and ubiquitin genes were used as endogenous controls. The relative quantification of target genes was determined using the ΔΔCT method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control gene (Ubiquitin) ($\Delta CT = Ct_{target} - Ct_{Ubiquitin}$) and compared with a calibrator: $\Delta\Delta CT = \Delta Ct_{Sample} - \Delta Ct_{Calibrator}$. Relative expression (RQ) was calculated using the SDS 2.2.2 software (Applied Biosystems) based on the formula: $RQ = 2^{-\Delta\Delta CT}$.

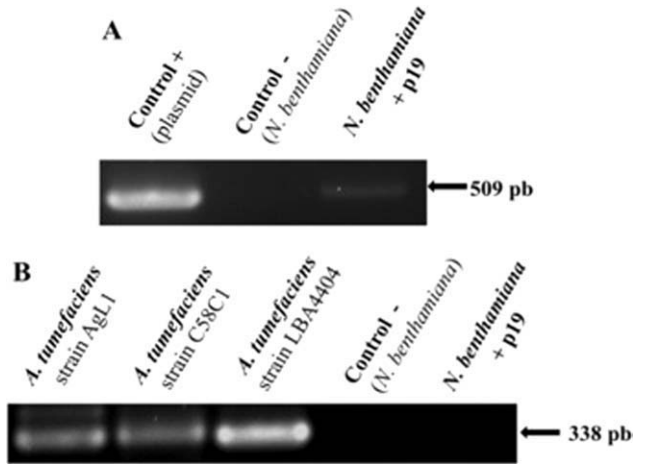

Figure 1. Validation of the established *N. benthamiana* cell line expressing the viral suppressor p19.

A: PCR assay to detect the presence of the p19 gene, B: PCR analysis to detect a possible residual contamination with the *Agrobacterium* used to modify the cells. *Agrobacterium* strains AgL1, C58C1, and LBA4404 were used as positive controls.

Results and Discussion

Development of a stable p19 expressing N. benthamiana *cell line and its analysis*

Presence of p19 transgene in the stable cell line was verified by PCR analysis (Figure 1A). To ensure that the PCR fragment was related to the presence of the gene in the plant genome and not by a residual *Agrobacterium* contamination, PCR tests were also conducted to detect the presence of *Agrobacterium* (Figure 1B). Kanamycin selection of transformants ensured that most of the cells contained the transgene. Also, due to the transformation method (coculture), the stable cell line for p19 contained a pool of cells with different levels of expression mainly caused by the variation in the insertion sites of the transgenes and/or the number of transgenes per cell. Furthermore, the selection process may have favored cells with low p19 expression levels as a high expression may hinder cellular mechanisms to the long term. This has been considered in the interpretation of the results.

Dual agroinfiltration of N. benthamiana *plants with both p19- and IgG1-containing* A. tumefaciens *bacterial strains significantly enhances IgG1 production in leaves*

To evaluate the transformation efficiency of the two *A. tumefaciens* cell lines (IgG1 and p19), both were tested on wild-type *N. benthamiana* plants by agroinfiltration

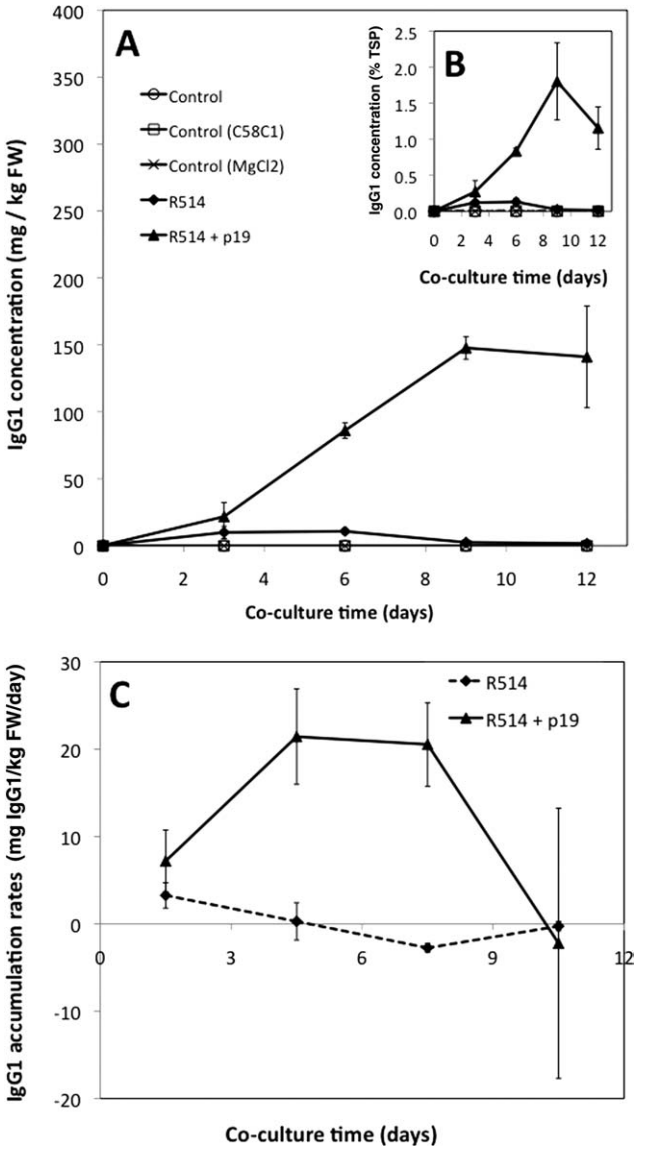

Figure 2. Concentration and accumulation rates of cellular IgG1 in agroinfiltrated *N. benthamiana* leaves.

A: Concentration (mg IgG1/kg FW), B: Concentration (% TSP) and C: Accumulation rates [mg IgG1/(kg FW/day)]. Control: plant leaves that had not been infiltrated; Control (C58C1): plant leaves infiltrated with the *Agrobacterium* C58C1 strain that did not contain a Ti-plasmid; Control ($MgCl_2$): plant leaves infiltrated with a 10 mM $MgCl_2$ solution only. Control R514: plant leaves infiltrated with the *Agrobacterium* AgL1 strain containing the R514 vector for IgG1 production; R514 + p19: plant leaves infiltrated with *Agrobacterium* AgL1 strain containing the R514 vector for IgG1 production and *Agrobacterium* C58C1 strain containing the vector for the p19 viral suppressor.

technique.[32] Maximum yields in IgG1 were obtained for the simultaneous infiltration of R514- and p19-containing *A. tumefaciens* (Figures 2A,B). On day 9, IgG1 concentration in the leaves reached a maximum value of 147.7 mg IgG1/kg FW. This corresponded to 1.80% TSP. This yield in IgG1 was almost 14 times higher than the maximum yield obtained on day 6 without p19 (10.8 mg IgG1/kg FW; 0.13% TSP). IgG1 accumulation rates were calculated between each sampling times in order to compare the productivity levels (Figure 2C). The antibody accumulated during the first 3 days at a rate of 3.26 ± 1.51 (SEM) mg IgG1/kg FW/day for R514 only transformation ($r^2 = 0.70$; $n = 4$ data points), and its concentration stabilized from day 3 to 6. From day 6, the IgG1 concentration decreased to reach undetectable level at day 9. In the case of the dual agroinfiltration procedure (i.e., R514- and p19-containing *Agrobacteria*), the antibody accumulated at a constant rate of 21.00 ± 1.74 mg IgG1/kg FW/day (r^2 =0.97; $n = 6$) between day 3 and day 9 and decreased thereafter (Figure 2C). These results unambiguously demonstrated that the R514 and the p19 *A. tumefaciens* bacterial strains were effective and that the addition of p19 both increased and prolonged recombinant protein production, in accordance with previous reported results.[23]

Effects on plant cell growth of co-cultivating N. benthamiana *suspension cells with* A. tumefaciens

Two common *Agrobacteria* strains (AgL1 for R514 and C58C1 for p19) were used to assess the impact of co-culture with *N. benthamiana* suspension cells. During the first week of co-cultivation (R514 or the combined R514 and p19 *A. tumefaciens* strains), plant cells growth was not significantly affected by the presence of the *Agrobacteria* strain(s) (Figures 3A,B), with average specific growth rates of 0.089 ± 0.0061/day ($r^2 = 0.82$, $n = 49$) and 0.085 ± 0.0057/day ($r^2 = 0.87$, $n = 34$) for the parental and the stable p19 *N. benthamiana* plant cell lines, respectively (the contribution of the presence of the *Agrobacteria* to the measured biomass was considered negligible). Thus, during the first week of culture, no significant differences could be observed between a wild-type cell line and a cell line that had been transformed to constitutively express p19. However, on day 12, cultures corresponding to *N. benthamiana* parental cell line (Figure 3A) exposed to R514 and to the dual R514/p19 showed a lower final biomass when compared to control. The co-culture of the parental *N. benthamiana* with a T-DNA plasmid-free *Agrobacterium* (C58C1) (control-C58C1 culture) resulted in an even more pronounced decrease in biomass concentration at day 12. The co-culture of the stable p19 *N. benthamiana* cell line with T-DNA-free plasmid *Agrobacterium* (C58C1) showed a similar reduction of biomass on day 12 (Figure 3B) but, surprisingly, co-cultivation with *Agrobacterium* R514 did not seem to affect plant cells growth for the 12 day culture duration (Figure 3B). The high cell death observed in all flasks containing the T-DNA plasmid-free *Agrobacterium* (C58C1) could possibly be attributed to a more rapid bacterial growth due to the absence of metabolic burden caused by the T-DNA plasmid or be related to a potentially higher virulence of the C58C1 strain, when compared with the AgL1 strain. Nonetheless, these results indicated that, in most cases, long-term co-cultivation of plant cells with *Agrobacteria* can be detrimental to the cells. In fact, many of the flasks containing *Agrobacteria* turned brown or black by day 12 as opposed to a light yellow color for control cultures (no bacteria), hence suggesting a large-scale hypersensitive cell death response as observed during an incompatible plant-pathogen interaction.[33] However, the presence of *Agrobacteria* did not significantly affect glucose consumption (a decrease similar to control culture was observed). Interestingly, glucose concentration in the culture media was not limiting in any cultures. That is, residual concentration above 5 g/L glucose were measured for all cultures (data not shown), conditions that are thought to be nonlimiting.

IgG1 accumulates in plant cells in cultures

Cellular IgG1 was monitored in all cell cultures and was undetectable until day 6 (Figure 4). The antibody was not detected at day 3 but the trend, with time, for the IgG1

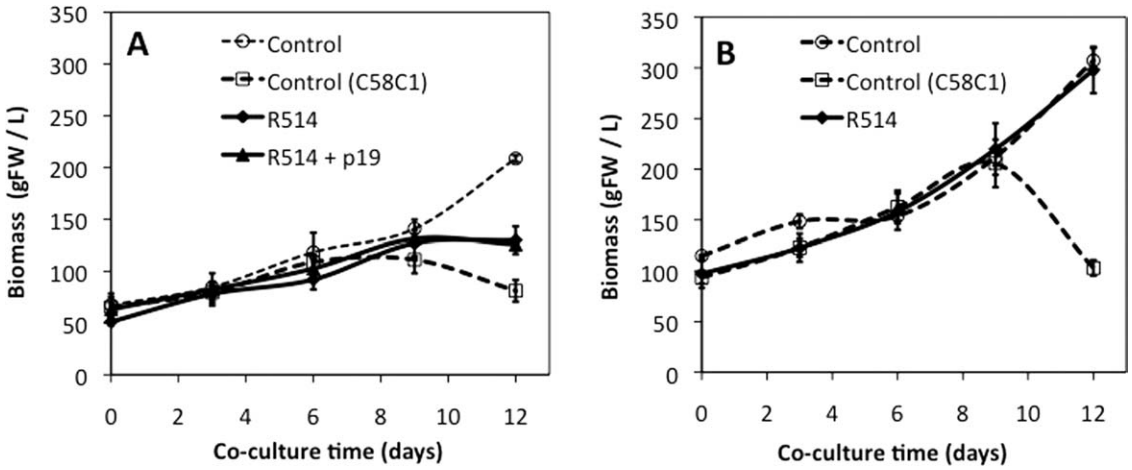

Figure 3. Growth of *N. benthamiana* suspension cells co-cultured with *Agrobacterium tumefaciens*.

A: *N. benthamiana* parental cell line, B: *N. benthamiana* cell line transformed to constitutively express the p19 viral suppressor. Control: plant cells grown without bacteria; Control (C58C1): plant cells grown with *Agrobacterium* C58C1 that did not contain T-plasmid; R514: plant cells grown with *Agrobacterium* AgL1 that contained the R514 vector for IgG1 production; R514 + p19: plant cells grown with *Agrobacterium* AgL1 that contained the R514 vector for IgG1 production and *Agrobacterium* C58C1 that contained the vector for the p19 viral suppressor.

cellular concentration suggests that the antibody production in all co-cultures was only above the detection limit of our ELISA protocol after around 3 days of co-culture. The co-cultures of the parental *N. benthamiana* cell line with R514 yielded 0.610 ± 0.028 mg IgG1/kg FW (0.014% TSP) on day 9 (Figures 4A,C). In that culture, the cell specific productivity in IgG1 reached a plateau between day 3 and day 9, with 0.100 ± 0.005 mg IgG1/kg FW/day ($r^2 = 0.98$, $n = 9$), and decreased thereafter (Figure 4E). Co-cultivating *N. benthamiana* parental cell line with the two *Agrobacteria* strains corresponding to R514 and p19 had a strong impact on IgG1 production with a maximum cell content of 1.064 ± 0.045 mg IgG1/kg FW (0.024% TSP; Figure 4A), the equivalent of a 75% increase, which was also reached on day 9. In that R514/p19 co-culture with the parental *N. benthamiana* cell line, the specific productivity in IgG1 increased linearly from day 3 and reached a maximum between day 6 and day 9 with 0.213 ± 0.036 mg IgG1/kg FW/day (Figure 4E). This corresponded to a 2 fold increase, when compared with that related to the co-culture with R514 only (Figure 4E). In the R514 co-culture with the p19 *N. benthamiana* cell line, antibody accumulation continuously increased and reached 0.25 ± 0.075 mg IgG1/kg FW on day 12 (Figure 4B). The specific productivity of IgG1 in the p19 cell line oscillated from 0.022 ± 0.013 to 0.035 ± 0.07 mg IgG1/kg FW/day between day 3 and day 12 (Figure 4E), a range of values which were 6 to 9 fold lower than those for the dual co-culture with R514 and p19. Interestingly, the antibody cell content increased from day 3 to day 6 at faster specific rates than for plant cell growth with 1.28 ± 0.079/day (r^2 =0.99, $n = 6$), 1.58 ± 0.097/day (r^2 =0.99, $n = 6$) and 1.16 ± 0.087/day (r^2 =0.98, $n = 6$) for the co-culture with R514, dual R514/p19 and with R514 and the p19 *N. benthamiana* cell line, respectively. Therefore, the specific rate of increase of the IgG1 cell content was higher when simultaneously co-cultivating with R514 and p19 (compare with the single use of R514). Then, from day 9, the cellular concentration of IgG1 decreased in the culture with the parental *N. benthamiana* cell line, concurrently with plant cell growth arrest (Figure 3A).

IgG1 secretion into the extracellular medium was clearly not favored and low concentrations of 1.76 ± 0.21, 1.42 ± 0.68, and 1.36 ± 1.23 μg/L were measured at day 9, respectively for the R514, the dual R514/p19 and the R514 with the p19 expressing cell line (Figures 5A,B). These low concentrations disabled sample dilution for ELISA analysis and resulted in increased measurement errors. The R514 genetic construction was designed to direct the antibody toward the secretory pathway. However, due to its large size (~150 kD), the IgG1 may have been trapped by the cell wall. Nevertheless, the observed trends are similar to those of cellular concentrations. Also, the specific rate of increase was similar for the three cultures involving IgG1 production with 0.19 ± 0.014/day ($r^2 = 0.96$, $n = 9$). This specific rate was 2 fold higher than that corresponding to cell specific growth rate, which may in turn suggest that the presence of the antibody in the medium was not only related to a cell division process where IgG1 is released from entrapment in the cell wall or cell lysis from death. On day 12, extracellular IgG1 levels decreased, more likely due to protease release in the medium from dying cells.

The p19 silencing suppressor is known to interfere with the plant mechanism responsible for the destruction of viral RNA and for the production of small interfering RNAs (siRNAs) involved in plant developmental pathways[34] and other mechanisms such as stress response or environmental changes.[35] In our case, p19 had no significant effect on plant cell growth rate, when either expressed in a transient or stable fashion. However, a significantly lower cell content in total soluble proteins (1.38 fold) was observed for the stable p19 transgenic *N. benthamiana* cell line, when compared with the parental cell line. The significant differences observed between the IgG1 specific yield and productivity for the parental and the p19 expressing *N. benthamiana* cell lines may be explained from differences in the availability of the p19 proteins within the cells. Indeed, p19 crystal structure showed that a homodimer of this viral suppressor can easily enclose and capture a 21-nt siRNA, thus preventing their interaction with the viral or transgene RNA and thus, their silencing.[36] Due to its mechanism of action, p19 must be expressed at high levels within the cells in order to interfere with siRNAs.[37,38] It is now acknowledged that recombinant protein levels are usually higher for transient expression than for nonselected stable cell lines due to a "burst" of expression occurring few days after infection in the transient mode.[39] A common hypothesis for this

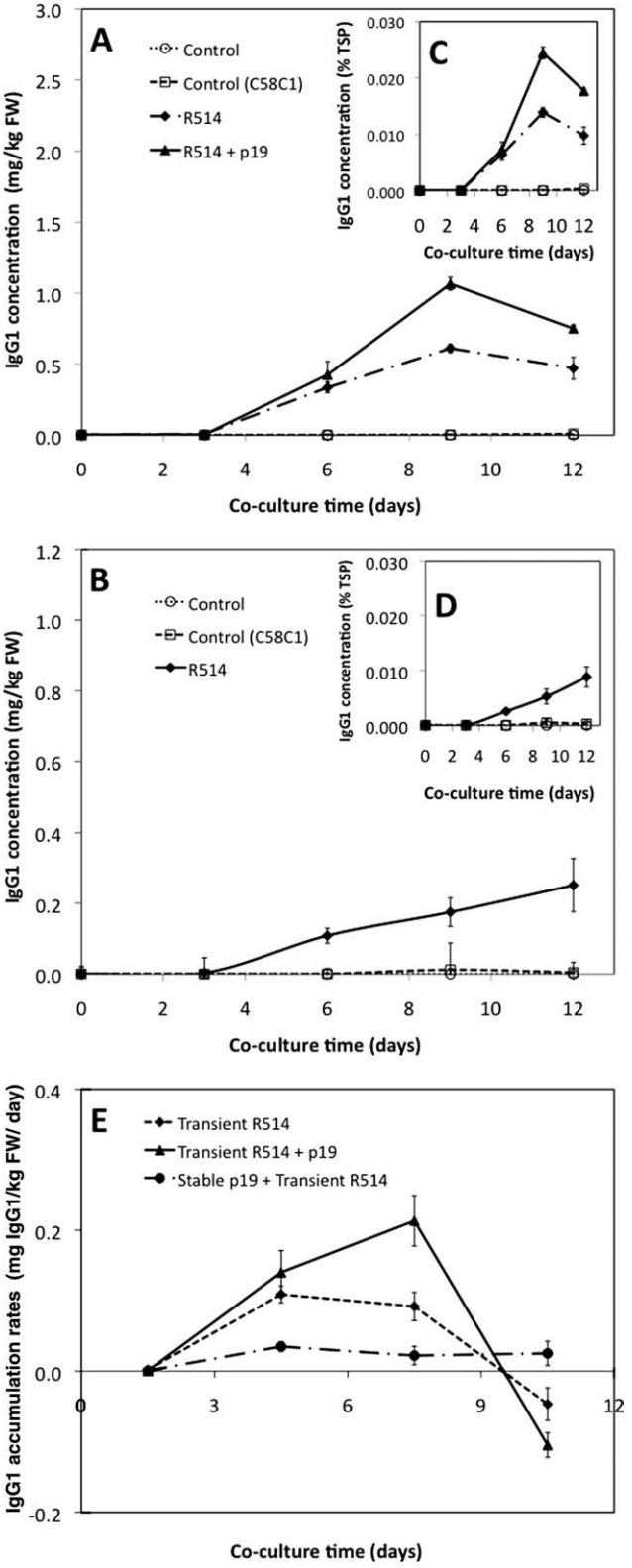

Figure 4. Concentration and accumulation rates of cellular IgG1 for *N. benthamiana* suspension cells co-cultured with *A. tumefaciens*.

A: Concentration (mg IgG1/kg FW) in *N. benthamiana* parental cell line, B: Concentration (mg IgG1/kg FW) in *N. benthamiana* cell line transformed to express the viral suppressor p19 constitutively, C: Concentration (% TSP) in *N. benthamiana* parental cell line, D: Concentration (% TSP) in *N. benthamiana* cell line transformed to express the viral suppressor p19 constitutively, and E: Accumulation rates for both plant cell lines (mg IgG1/kg FW/day). Same conditions as Figure 3 applied.

phenomenon is that, during the first days of infection, many nonintegrated copies of the T-DNA reach the nucleus and are transcribed.[40] To validate this hypothesis, a qPCR analysis of the p19 RNA level was performed on the *N. benthamiana* cell line that had been transiently transformed with p19 and on the p19 expressing *N. benthamiana* stable cell line (Figure 6A). Gene expression level was determined using assays designed with the Universal Probe Library from Roche (www.universalprobelibrary.com). This technology utilizes short hydrolysis probes of eight or nine bases. Using Locked Nucleic Acid (LNA) nucleotide chemistry, these short probes retained high melting temperature characteristic of normally observed for longer probes. Because probes are only eight or nine bases long, each probe can statistically hybridize to over 7,000 transcripts; thus, a set of 100 probes only can enable the quantification of virtually any transcript in a transcriptome. As expected, the level of expression of p19 in the stable cell line remained almost constant over the 12 days of co-culture. This result for p19 stable cell line was then used as a reference to compare with the transient p19 transformation. In the dual transformation co-culture, higher levels of p19-RNA were indeed observed, reaching almost 25 times the level of the stable p19 *N. benthamiana* cell line on day 12. This can be explained by the fact that over time, more and more plant cells are transformed. Note that all our samples were standardized using ubiquitin as an expression reference. This means that levels obtained for the dual transformation are in fact much higher that what is shown in Figure 6A. For example, on day 3, if only 1% of the cells are transformed, the p19 RNAs they produce are diluted among 99% of nontransformed cells. Because it is impossible, under our experimental conditions, to know the exact percentage of transformed cells due to the absence of a specific marker (i.e., GUS, GFP, etc.), it is only possible to conclude that the expression level of p19 was significantly higher for the transiently transformed cells than for the stable cell line after 9 days and longer.

The co-infection approach thus seems more effective while it interferes with the silencing mechanism and results in higher yields than when using a stable p19 expressing cell line. To complete this study, IgG1 accumulation was followed in the *N. benthamiana* parental cell line transiently transformed with the R514 construction only or with both R514 and p19, and in the p19 expressing *N. benthamiana* cell line transformed with R514 (Figure 6B). When compared with the IgG1 protein levels shown in Figure 4, it appears that the RNA levels evolved similarly for the parental cell line from day 0 to 9. On day 3, a detectable RNA level was observed in all experimental groups, but IgG1 levels were still close to the detection limit of the ELISA assay. On day 6, IgG1-RNAs levels for the two groups with the parental cell line were similar, which is also reflected by similar IgG1 levels (Figure 4A). The presence of the viral suppressor p19 on day 9 has almost doubled the RNA level in dual transformation group and the IgG1 quantity observed in the cells with p19 has also exhibited a ~2-fold increase. The RNA protection from PTGS effect of p19 has thus resulted in higher RNA levels. The p19 *N. benthamiana* expressing cell line that had been transformed with R514 showed a similar trend in its IgG1-RNA levels as the R514 transformed parental cell line showing that the presence of low levels of p19 had few effects on IgG1-RNAs. However, on day 12, the parental cell line with R514 (only) showed an increase of RNA level but a reduction of the cells IgG1

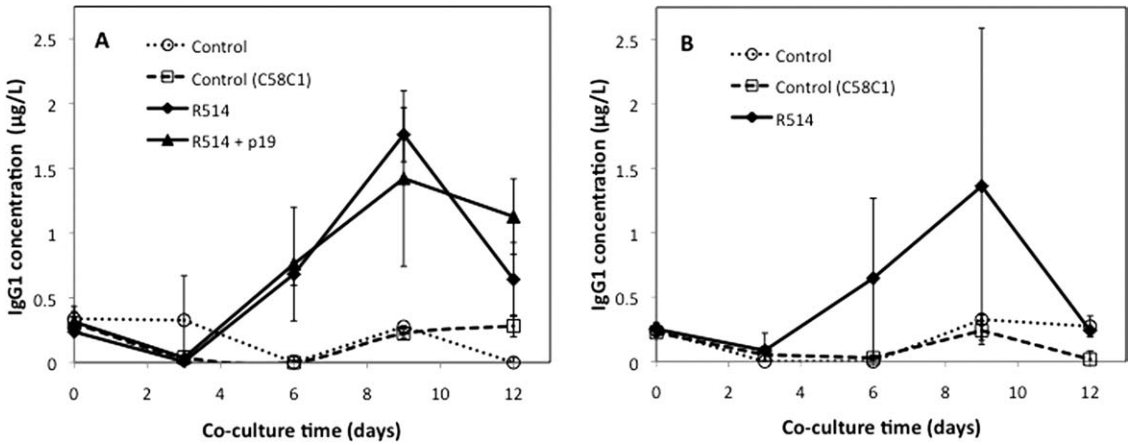

Figure 5. Concentration of extracellular IgG1 for *N. benthamiana* suspension cells co-cultured with *A. tumefaciens*.

A: *N. benthamiana* parental cell line, B: *N. benthamiana* cell line transformed to express constitutively the viral suppressor p19. Same conditions as Figure 3 applied.

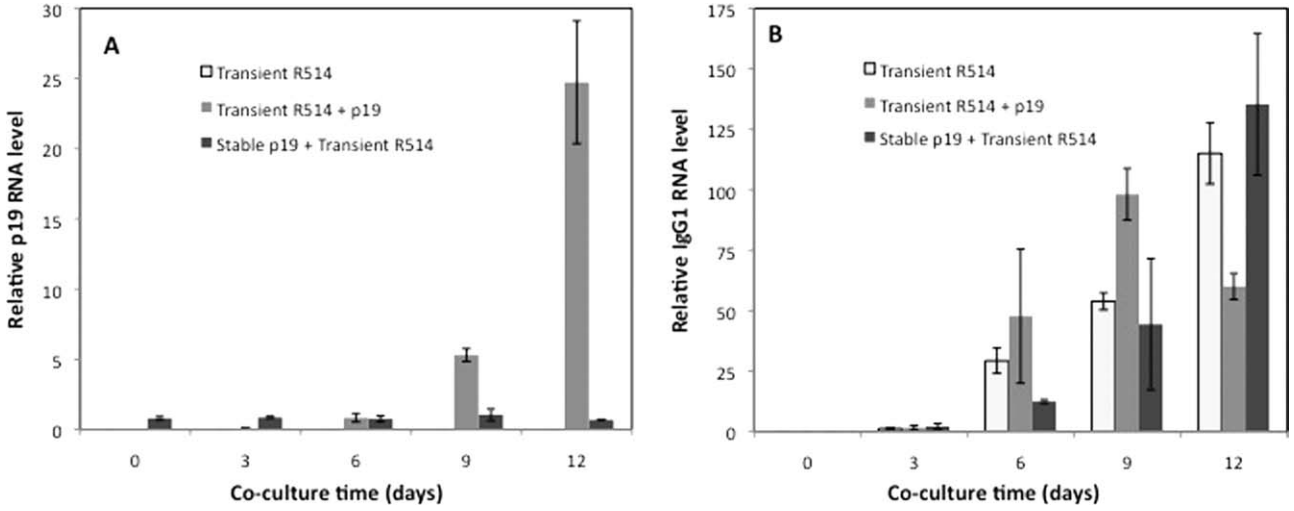

Figure 6. *N. benthamiana* suspension cells co-cultured with *A. tumefaciens*.

A: Relative p19 RNA level, B: Relative IgG1 RNA level.

level. Furthermore, for the group with both p19 and R514, even with the highest level of p19, a significant reduction of RNAs was observed. These results may be explained by plant cell death that has been observed in all co-cultures from day 12.

Approaches to improve co-culture productivity in recombinant protein

It is clear from this study that the *in vitro* co-culture approach needs to be improved to reach the production level observed for whole plant agroinfiltration. Nonetheless, our results showed that a transient dual transformation of suspension plant cells is feasible and is more efficient than transiently re-transforming an established modified cell line. Besides being fast and flexible, an entirely transient expression system has thus led to higher yields in antibody. To raise the yield in a recombinant protein, transformation vectors (the cassette, promoters, etc.) can be improved to be better adapted to the suspension plant cell culture conditions. One could think that encoding the viral suppressor p19 on the same T-DNA than the IgG1 heavy and light genes may result in higher yields because of the presence of the suppressor in all the producing cells. The use of such efficient multigene vectors has been recently reported in agroinfiltration of *N. benthamiana* leaves.[41] To maintain a vector size under 10 kb, which is preferable to improve yields during cloning procedure and facilitate construction development, Sainsbury et al.[41] successfully removed more than half of the plasmid backbone and some of the T-DNA region without compromising transient expression levels. This study utilizing the viral suppressor p19 encoded within the same T-DNA as the gene of interest had expression levels at least as high as those obtained with the use of multiple *Agrobacteria* lines.[41] Furthermore, for the multigene vectors, similar yields could be achieved with *Agrobacteria* concentration half the ones used in coculture.

The advantage of a co-culture suspension vs. an agroinfiltration process is the significant reduction of the required inocula size due to the fact that *Agrobacteria* cells will grow and thus continuously contribute to transform growing plant cells, which is not possible in plant leaves due to the limited nutrients in the intercellular space. However, our capacity to control the growth of *Agrobacterium* without affecting the continuous transformation of the plant cells is of prime importance to prolong the production phase with viable, growing and producing plant cells. Andrews and Curtis[16]

proposed the use of an auxotrophic *Agrobacterium* strain which growth was successfully controlled by the presence (growth) or absence (nongrowth) of cystein in culture medium. They were thus able to control the development of the bacteria for 3 to 5 days, a time period, however, for which we have not noticed any significant negative effect on plant cells in our experiments.[16] In addition to the use of an auxotrophic *Agrobacterium*, or as an alternative, the use of a bioreactor allowing for continuous fresh medium perfusion[42–44] enabling continuous bacterial cells washout may be highly efficient to control *Agrobacteria* populations.

Conclusion

In this study, we showed that a dual transient *Agrobacterium*-mediated transformation process of suspension plant cells, using a viral suppressor such as p19 in combination to an expression vector increases recombinant protein production by 2 fold when compared with that for the use of the expression vector alone. The continuous presence of *Agrobacteria* had no significant negative effects in terms of cell growth and extracellular protein production for the first 6 days of co-culture. However, plant cells in contact with the bacteria started to die thereafter and caused a decrease of cellular IgG1 concentration. We showed that, in our specific case, we were able to reach higher yields by co-cultivating plant cells with two *Agrobacteria* strains bearing the gene of interest and the p19 silencing suppressor, respectively. qPCR analyses showed that p19 expression level in this group were much higher than for cell line that was stably transformed with p19. This may, in part, explain the higher level of IgG1 produced due to the interference of p19 with PTGS. The p19 expressing *N. benthamiana* cell line showed lower yields more likely due to a negative effect of p19 on the plant cell mechanisms or to a expression level of p19 being lower than in the transient system. Extracellular levels of IgG1 remained low for all groups as it may be trapped by the cell wall. However, this result may indicate a low cell death level during the experiments.

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Literature Cited

1. Jana S, Deb JK. Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Appl Microbiol Biotechnol*. 2005;67:289–298.
2. Walsh G. Biopharmaceuticals: recent approvals and likely directions. *Trends Biotechnol*. 2005;23:553–558.
3. Jungbauer A. Editorial: biopharmaceutical technologies. *Biotechnol J*. 2007;2:647.
4. Shekhawat UKS, Ganapathi TR, Srinivas L, Bapat VA, Rathore TS. *Agrobacterium*-mediated genetic transformation of embryogenic cell suspension cultures of *Santalum album* L. *Plant Cell Tissue Organ Cult*. 2008;92:261–271.
5. Ma J, Drake P, Christou P. The production of recombinant pharmaceutical proteins in plants. *Nat Rev Genet*. 2003;4:794–805.
6. Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, Bacci C, Desiderio A, Benvenuto E, Donini M. Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. *Plant Biotechnol J*. 2008;7:59–72.
7. Sardana R, Dudani AK, Tackaberry E, Alli Z, Porter S, Rowlandson K, Ganz P, Altosaar I. Biologically active human GM-CSF produced in the seeds of transgenic rice plants. *Transgenic Res*. 2007;16:713–721.
8. Ganapathi TR, Sunil Kumar GB, Srinivas L, Revathi CJ, Bapat VA. Analysis of the limitations of hepatitis B surface antigen expression in soybean cell suspension cultures. *Plant Cell Rep*. 2007;26:1575–1584.
9. Agarwal S, Singh R, Sanyal I, Amla DV. Expression of modified gene encoding functional human α-1-antitrypsin protein in transgenic tomato plants. *Transgenic Res*. 2008;17:881–896.
10. Magnuson NS, Linzmaier PM, Reeves R, An G, Hayglass K, Lee JM. Secretion of biologically active human interleukin-2 and interleukin-4 from genetically modified tobacco cells in suspension culture. *Protein Expr Purif*. 1998;13:45–52.
11. Liu C, Towler MJ, Medrano G, Cramer CL, Weathers PJ. Production of mouse interleukin-12 is greater in tobacco hairy roots grown in a mist reactor than in an airlift reactor. *Biotechnol Bioeng*. 2009;102:1074–1086.
12. Hellwig S, Drossard J, Twyman RM, Fischer R. Plant cell cultures for the production of recombinant proteins. *Nat Biotechnol*. 2004;22:1415–1422.
13. De Wilde C, Van Houdt H, De Buck S, Angenon G, De Jarger G, Depicker A. Plants as bioreactors for protein production: avoiding the problem of the transgene siliencing. *Plant Mol Biol*. 2000;43:347–359.
14. Schmale K, Rademacher T, Fischer R, Hellwig S. Towards industrial usefulness - cryo-cell-banking of transgenic BY-2 cell cultures. *J Biotechnol*. 2006;124:302–311.
15. Fuentes A, Ramos PL, Meilyn Rodriguez CA, Ramirez N, Pujol M. Development of a highly efficient system for assessing recombinant gene expression in plant cell suspensions via *Agrobacterium tumefasciens* transformation. *Biotechnol Appl Biochem*. 2004;39:355–361.
16. Andrews LB, Curtis WR. Comparison of transient protein expression in tobacco leaves and plant suspension culture. *Biotechnol Prog*. 2005;21:946–952.
17. Kapila J, De Rycke R, Van Montagu M, Angenon G. An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci*. 1997;122:101–108.
18. Joh LD, VanderGheynst JS. Agroinfiltration of plant tissues for production of high-value recombinant proteins: an alternative to production in transgenic crops. *J Sci Food Agric*. 2006;86:2002–2004.
19. Vézina L-P, Faye L, Lerouge P, D'Aoust M-A, Marquet-Blouin E, Burel C, Lavoie P-O, Bardor M, Gomord V. Transient co-expression for fast and high-yield production of antibodies with human-like *N-glycans* in plants. *Plant Biotechnol J*. 2009;7:442–455.
20. Kim T-G, Lee H-J, Jang Y-S, Shin T-H, Yang M-S. Co-expression of proteinase inhibitor enhances recombinant human granulocyte-macrophage colony stimulating factor production in transgenic rice cell suspension culture. *Protein Expr Purif*. 2008;61:117–121.
21. Gleba Y, Klimyuk V, Marillonnet S. Viral vectors for the expression of proteins in plants. *Curr Opin Biotechnol*. 2007;18:134–141.
22. Ratcliff F, MacFarlane S, Baulcombe D. Gene silencing without DNA:RNA-mediated cross protection between viruses. *Plant Cell*. 1999;14:689–702.
23. Voinnet O, Rivas S, Mestre P, Baulcombe D. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J*. 2003;33:949–956.
24. Roth B, Pruss G, Vance V. Plant viral suppressors of RNA silencing. *Virus Res*. 2004;102:97–108.
25. Plesha MA, Huang T-K, Dandekar AM, Falk BW, McDonald KA. High-level transient production of a heterologous protein in

plants by optimizing induction of a chemically inducible viral amplicon expression system. *Biotechnol Prog*. 2007;23:1277–1285.
26. Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL. Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proc Natl Acad Sci USA*. 2003;100:4939–4944.
27. Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, Klimyuk V, Gleba Y. Rapid high-yield expression of full-size IgG antibodies in plants coinfected with non competing viral vectors. *Proc Natl Acad Sci USA*. 2006;103:14701–14706.
28. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant*. 1962;15:473–497.
29. Haas JH, Moore LW, Ream W, Manulis S. Universal PCR primers for detection of phytopathogenic *Agrobacterium* strains. *Appl Environ Microbiol*. 1995;61:2879–2884.
30. Edwards K, Johnstone C, Thompson C. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res*. 1991;19:1349.
31. Khoudi H, Laberge S, Ferullo J-M, Bazin R, Darveau A, Castonguay Y, Allard G, Lemieux R, Vézina L-P. Production of a diagnostic monoclonal antibody in perennial alfalfa plants. *Biotechnol Bioeng*. 1999;64:135–143.
32. Simmons CW, VanderGheynst JS, Upadhyaya S. A model of *Agrobacterium tumefaciens* vacuum infiltration into harvested leaf tissue and subsequent *in planta* transgene transient expression. *Biotechnol Bioeng*. 2009;102:965–970.
33. Atkinson MM, Huang J-S, Knopp JA. Hypersensitivity of suspension-cultured tobacco cells to pathogenic bacteria. *Phytopathology*. 1985;75:1270–1274.
34. Chuck G, Candela H, Hake S. Big impact by small RNAs in plant development. *Curr Opin Plant Biol*. 2009;12:81–86.
35. Bonnet E, Van de Peer Y, Rouzé P. The small RNA world of plants. *New Phytol*. 2006;171:451–468.
36. Baulcombe DC, Molnar A. Crystal Structure of p19—a universal suppressor of RNA silencing. *Trends Biochem Sci*. 2004;29:279–281.
37. Qiu W, Park J-W, Scholthof HB. Tombusvirus p19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage feature that influence pathogenicity. *Mol Plant Microbe Interact*. 2002;15:269–280.
38. Canto T, Uhrig JF, Swanson M, Wright KM, MacFarlane SA. Translocation of Tomato bushy stunt virus p19 protein into the nucleus by ALY proteins compromises its suppressor activity. *J Virol*. 2006;80:9064–9072.
39. Collens JI, Mason HS, Curtis WR. *Agrobacterium*-mediated viral vector-amplified transient gene expression in *Nicotiana glutinosa* plant tissue culture. *Biotechnol Prog*. 2007;23:570–576.
40. Narasimhulu SB, Deng X-B, Sarria R, Gelvin SB. Early transcription of *Agrobacterium* T-DNA Genes in tobacco and maize. *Plant Cell*. 1996;8:873–886.
41. Sainsbury F, Thuenemann EC, Lomonossoff GP. pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol J*. 2009;7:682–693.
42. De Dobbeleer C, Cloutier M, Fouilland M, Legros R, Jolicoeur M. A high-rate perfusion bioreactor for plant cells. *Biotechnol Bioeng*. 2006;95:1126–1137.
43. Su WW, Arias R. Continuous plant cell perfusion culture: bioreactor characterization and secreted enzyme production. *J Biosci Bioeng*. 2003;95:13–20.
44. Su WW, He BJ, Liang H, Sun S. A perfusion air-lift bioreactor for high density plant cell cultivation and secreted protein production. *J Biotechnol*. 1996;50:225–233.

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