

Comparison of Several *Nicotiana* Species as Hosts for High-Scale *Agrobacterium*-Mediated Transient Expression

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Abstract: *Agrobacterium*-mediated transient expression may be regarded as a promising method for inexpensive large-scale production of recombinant proteins. We optimized the protocol of transient expression in *Nicotiana benthamiana* and compared six Australian species of *Nicotiana* as hosts for transient expression. The transient expression of GFP under 35S CaMV promoter was observed in all species tested, although the GFP content in leaves of *N. benthamiana*, *N. exigua*, and *N. excelsior* was significantly higher (3.8, 3.7, and 2.0% TSP, respectively). Usage of viral-based expression system resulted in considerable increase of GFP accumulation in *N. excelsior* and *N. benthamiana* (63.5 and 16.2% TSP, respectively). We displayed that *N. excelsior* has the best characteristics in regard to biomass yield as well as GFP accumulation level for both types of the expression cassettes tested.

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Keywords: *Nicotiana benthamiana*; *Nicotiana excelsior*; *Agrobacterium*; transient expression; GFP

INTRODUCTION

Plants as source of recombinant proteins have important advantages over microbial or animal cell systems. Plant cells, unlike bacteria, are able to produce proteins with post-translational modifications, as well as correctly folded and assembled multimeric proteins, for example, antibodies (Stoger et al., 2002). In contrast to animal cells, plants are free from human pathogens like viruses and prions, so the recombinant proteins of plant origin are considered to be safer. In some cases, the therapeutic proteins produced in plants can be used directly without any purification as edible vaccines (Larrick and Thomas, 2001).

The main obstacle on the way of using transgenic plants for high-scale production of recombinant proteins is the low level of foreign gene expression in case of stable integration

into plant nuclear genome (resulting in the foreign protein level usually lesser than 1% of total soluble protein (TSP)) (Daniell et al., 2001), often due to the transcriptional or post-transcriptional gene silencing of the transgenes (Fagard and Vaucheret, 2000; Yu and Kumar, 2003). Plastid transformation allows achieving considerably higher level of recombinant protein expression (Maliga, 2003). However, it usually takes many months or even years to obtain the plants with transgenic plastids. This may be an important drawback for production of some recombinant proteins, for example, patient-specific cancer vaccines, which should be generated during several weeks.

Transient gene expression in plants allows accumulation of large amount of recombinant proteins within a very short (days) time (Gleba et al., 2005; Marillonnet et al., 2004, 2005). This method was successfully applied for formation of tumor-specific recombinant antibodies in tobacco leaves (Galeffi et al., 2005; Vaquero et al., 1999). We can outline two major strategies to enhance production of transiently expressed recombinant proteins in plants: more expensive and time-consuming approach includes modification of the expression vector system including construction of viral-based vectors. This system consists of three elements delivered in plant by simultaneous infiltration of *Agrobacterium* carrying different plasmids. The viral genes and regulatory elements are combined with the gene of interest inside the plant cell with the help of the *Streptomyces* phage PhiC31 site-specific recombinase. The resulting DNA molecule contains the viral genes of the RNA-dependent RNA polymerase and moving protein. The reporter gene GFP is driven by the subgenomic promoter of a viral coat protein. Therefore, the resulting vector cannot move systemically because it lacks for the coat protein, but is able to move cell-to-cell due to the moving protein (Marillonnet et al., 2004). The recent publications demonstrated perspectives of this approach: optimized construction of viral-based vectors delivered in plant by *Agrobacterium* infiltration allowed to

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achieve the level of green fluorescent protein (GFP) more than 50% TSP (Marillonnet et al., 2004, 2005) and the level of human growth hormone up to 10% TSP (Gils et al., 2005).

On the other hand, amending of transient expression conditions and plant host characteristics allows further improving of the effectiveness of a given genetic construct. This way would be useful for fast preparative production of recombinant proteins in a laboratory lacking in complex of molecular biology tools. Recently, transient assays in lettuce, tomato, and *Arabidopsis* were described (Joh et al., 2005; Wroblewski et al., 2005). Here, we report the quantitative data on optimization of the transient expression protocol in *N. benthamiana*, a model species for transient expression assays, using GFP as a reporter, and comparison of six species of *Nicotiana* genus as hosts for GFP transient expression with regard to their biomass yield and transient expression level.

MATERIALS AND METHODS

Plant Materials

Seeds of *N. benthamiana*, *N. debneyi*, *N. excelsior*, *N. exigua*, *N. maritima*, and *N. simulans* were obtained from the National Germplasm Bank of World Flora of the Institute of Cell Biology and Genetic Engineering (Kyiv, Ukraine). In greenhouse, plants were grown at 20–25°C and 14 h light period (3,000–4,000 lux).

Bacterial Strains and Genetic Constructs

The plasmid pIC5290 carried the reporter synthetic *GFP* gene (Chiu et al., 1996) driven by the CaMV 35S promoter. Constructs pIC10881, pIC10570, and pIC7410 represent a viral-based module system which is described in details in the recent publications (Gils et al., 2005; Marillonnet et al., 2004; see also Introduction for more details). The plasmid pIC6692 (Marillonnet et al., 2005) contained the gene of the p19 protein of Tomato bushy stunt virus, a suppressor of post-transcriptional gene silencing (Voinnet et al., 2003). All the mentioned plasmids were generously donated by Icon Genetics GmbH (Halle/Saale, Germany).

Agrobacterium tumefaciens strain GV3101 transformed with individual constructs was grown overnight in LB medium supplemented with 50 mg/L of rifampicin and 50 mg/L of carbenicillin or kanamycin, and 100 µM of acetosyringone.

Transient Expression Assay

Plant infiltration was performed as it was described by Schob et al. (1997) with several modifications (Marillonnet et al., 2004): *A. tumefaciens* cells of overnight culture were centrifuged and resuspended with the infiltration buffer (10 mM MES, pH 5.5; 10 mM MgSO₄; 100 µM acetosyringone). The *Agrobacterium* suspensions harboring different plasmid vectors were mixed in the equal volumes so that

the final optical density (OD 600) of each suspension in the infiltration buffer amounted 1.0 for 35S expression cassette (pIC5290 and pIC6692) or 0.5 for viral-based cassette (pIC10881, pIC10570, pIC7410, and pIC6692). For typical assay, the leaves of greenhouse grown *N. benthamiana* plants were infiltrated with *Agrobacterium* mixture (50 ml/leaf) by using a syringe without a needle. After infiltration, the plants were further grown under greenhouse conditions. All experiments were carried out in four to six replications.

Isolation and Analysis of GFP

Accumulation of GFP in the infiltrated leaves was monitored with a hand-held black ray lamp (UVP, Upland, CA) and the infiltrated areas were cut out. The harvested plant material was frozen for storage at –70°C or analyzed immediately. Grinded leaf tissue (usually 100–200 mg) was extracted with 1 ml of 50 mM phosphate buffer (pH 7.0) in the Eppendorf tubes during 10 min in vortex. The extracts were centrifuged and the supernatants were used for protein analysis. The content of GFP was calculated by measurements of fluorescence intensity in dilutions of leaf extracts using fluorescence spectrophotometer Hitachi 850 (Hitachi, Tokyo, Japan) (excitation at 395 nm, emission at 509 nm) on the basis of standard values (GFP standard was generously granted by Icon Genetics GmbH). The background fluorescence of control extracts (from leaves infiltrated with bacteria carrying pIC6692 only) was subtracted from values of GFP containing extracts. The identity of GFP in the extracts to the standard was proved by recording their fluorescence spectra. The concentration of TSP was determined by the method of Bradford (1976).

The SDS–PAGE analyses of GFP containing extracts were carried out according to Baulcombe et al. (1995). The crude protein extracts were mixed with 3× loading buffer and applied on 12% gel without prior heat denaturation. The GFP was visualized directly after electrophoretic separation with a hand-held black ray lamp. For Coomassie staining, protein extracts mixed with 3× loading buffer were boiled during 5 min prior to SDS–PAGE analysis (Blakesly and Boezi, 1977).

RESULTS AND DISCUSSION

Optimization of Transient Expression in *N. benthamiana* Using 35S Promoter Cassette

Transient expression is often carried out in *N. benthamiana* (Thomas et al., 2003; Voinnet et al., 2003). In spite of development of the effective infiltration protocol for greenhouse-grown plants (Marillonnet et al., 2004; Yang et al., 2000), no systemic studies on the biotechnological characteristics of the host were performed. In our initial experiments, greenhouse-grown plants of *N. benthamiana* were infiltrated with *Agrobacterium* harboring the plasmid with the reporter gene coding for GFP driven by the CaMV 35S promoter (pIC5290). The GFP was selected as a reporter

protein because of its stability and fast and easy detection in native plant. To avoid post-transcriptional gene silencing, *Agrobacterium* harboring vector with the gene encoding p19 protein of Tomato bushy stunt virus, a suppressor of gene silencing (pIC6692), was co-infiltrated with the reporter gene (Voinnet et al., 2003). In these conditions, GFP accumulation in *N. benthamiana* reached the maximal level on the 3rd day after infiltration and remained stable to the 8th day (data not shown), that is in agreement with the recent data (Voinnet et al., 2003; Wroblewski et al., 2005), so we measured GFP content in the leaves on the 4th day.

Since plant senescence is known to alter gene expression and endogenous phytohormone balance that, in its turn, may cause change of plant susceptibility to viral/bacterial infection and/or accumulation of foreign proteins (Buchanan-Wollaston et al., 2005; Kim et al., 2005; Schwechheimer and Schwager, 2004), our first task was to determine the plant developmental stage optimal for transient expression. We tested *N. benthamiana* plants of three ages: young plants with six to eight fully expanded leaves (4–6 weeks old), plants shortly before flowering (8–10 weeks old), and intensively flowering plants with several flowers (>10–12 weeks depending on the growth conditions). We found that the level of GFP accumulation depended on *N. benthamiana* developmental stage, increasing during vegetative growth (1.9% TSP in young plants compared with 3.8% TSP in plants before flowering) and diminishing at inflorescence (0.5% TSP) (Fig. 1a). Similar tendency was mentioned in the recent publication when the viral-based vector system was used (Marillonnet et al., 2005). Thus, *N. benthamiana* plants immediately before flowering are optimal for recombinant protein production by means of transient expression.

Another factor that can influence the concentration of recombinant protein is the age of infiltrated leaves. After infiltration of eight fully expanded upper (young) leaves, we observed the highest GFP levels in the 2nd–4th upper leaves. The 1st, 5th, and 6th leaves demonstrated moderate level of GFP production while the oldest leaves accumulated considerably lower levels of the reporter protein (Fig. 2).

Moreover, one should remark that the effective infiltration into the 1st leaf is sometimes precluded by too thin leaf lamina.

The obtained data suggest that in the optimal conditions, transient expression of the reporter gene under CaMV 35S promoter is maximal in the 1st–6th leaves of *N. benthamiana* plant shortly before flowering (Fig. 3). In these conditions, plants can produce about 0.4 mg of recombinant GFP per 1 g of raw biomass during 4 days (about 1 mg/plant).

Transient Expression in *N. benthamiana* Using Viral-Based Module Vector System

In the developed conditions, we also tested a viral-based module vector system (Gils et al., 2005; Marillonnet et al., 2004). As in the case with 35S promoter driven system, the suppressor of gene silencing was co-infiltrated with the reporter gene.

Infiltration of *N. benthamiana* with the constructs described above allowed obtaining higher level of the reporter protein (16.2% TSP) in comparison with 35S promoter expression system, although the maximal level of GFP in leaves was detected considerably later (at 14–18 days post infiltration) due to slow spread of moving protein from cell to cell. We found that the optimal for transient expression of viral-based vector system plant development stage and leaf position were similar with those tested for 35S promoter cassette (data not shown). These data are in agreement with the data published by Marillonnet for similar constructions (Marillonnet et al., 2004). Moreover, the faster and more effective viral-based expression system was recently described (Marillonnet et al., 2005).

Searching for the Best Host for Transient Expression

Australian species *N. benthamiana* is known to be a model host for transient expression (for both *Agrobacterium* or viral-based systems) (McCormick et al., 1999; Voinnet et al.,

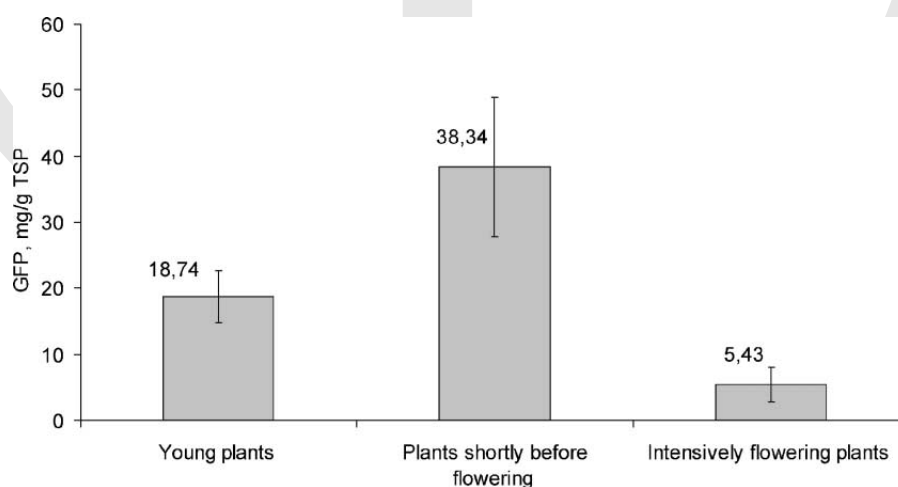


Figure 1. [XXX^{Q2}](#).

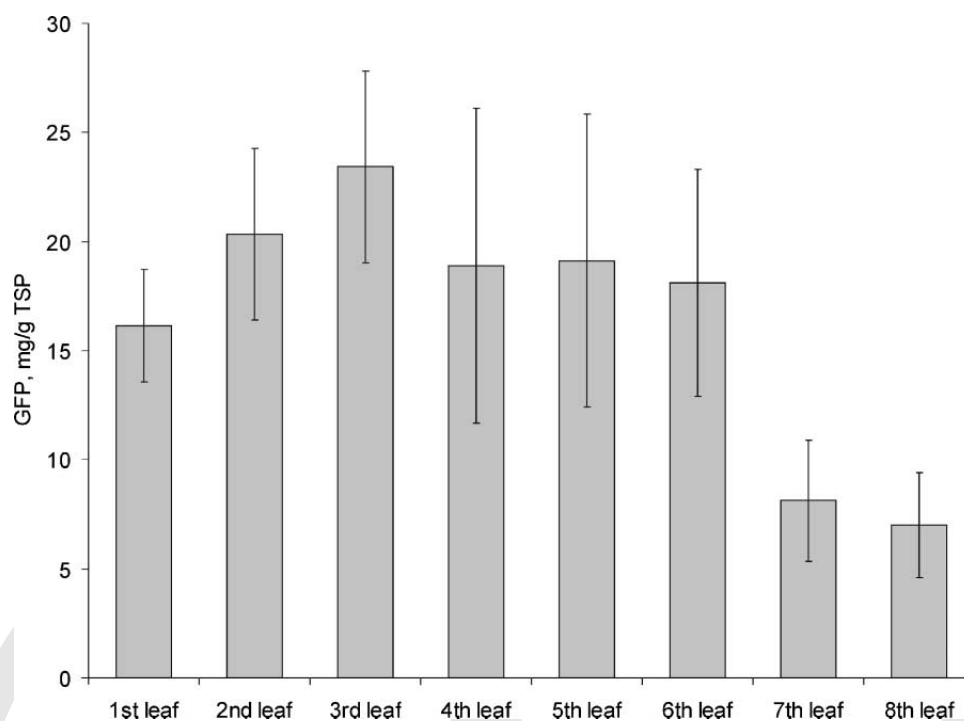


Figure 2. XXX.

2003), but has a rather small biomass yield which hinders its application for large-scale production of recombinant proteins. An optimal host should combine high level of transient expression with possibility to obtain quickly

necessary amounts of raw material. Additionally, the plant should be easily grown in field/greenhouse and be adapted for *Agrobacterium* infiltration procedures (thick pulpy leaves with thin epidermis are preferable).

To detect such host, we tested six different species of *Nicotiana* genus (*N. benthamiana*, *N. debneyi*, *N. excelsior*, *N. exigua*, *N. maritima*, and *N. simulans*, all Australian species) using simple expression system containing GFP under CaMV 35S promoter as well as the viral-based module constructs. In all experiments, p19 suppressor of silencing was co-expressed with the *GFP* gene to increase the reporter protein accumulation (Voinnet et al., 2003). The experiment was performed with the equal expression conditions: 2nd–4th youngest leaves of plants shortly before flowering were infiltrated with *Agrobacterium* and GFP level was monitored at the 4th and 18th dpi for 35S promoter driven or viral-based constructions, respectively.

The accumulation of reporter protein was observed in all investigated species if it was expressed under control of the CaMV 35S promoter, although GFP content in leaves of *N. benthamiana*, *N. exigua*, and *N. excelsior* was significantly higher (3.8 ± 1.0 , 3.7 ± 1.4 , and $2.0 \pm 0.5\%$ TSP, respectively) than in other species tested (Fig. 4a). Although *N. benthamiana* shows the best level of the reporter gene expression among the tested species, the difference is not significant and it turned out that it is possible to obtain considerably more recombinant protein from one plant of *N. exigua* or *N. excelsior* (approximately 3–4 mg) than from *N. benthamiana* (approximately 1 mg) due to better biomass yield of the former species (Fig. 4c). Although plant biomass can depend on growth conditions, the above

COLOR



Figure 3. GFP expression in *N. benthamiana* at the 5th day after infiltration with *A. tumefaciens* harboring pIC5290 and pIC6692 (under UV-light). Leaves (2nd–6th) were maximally filled with *Agrobacterium* suspension.

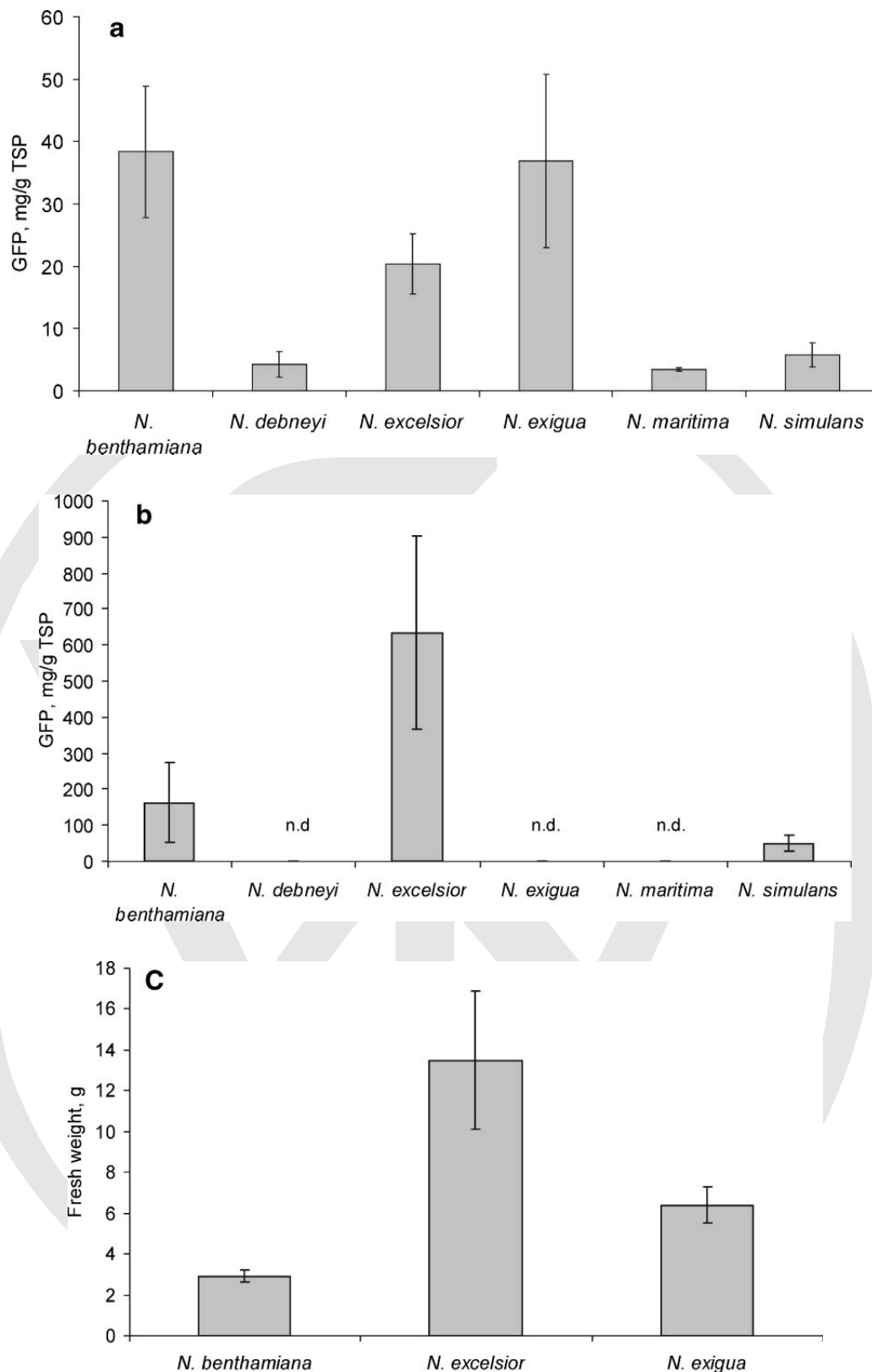


Figure 4. XXX.

mentioned ratio between biomass/recombinant protein yield in the tested species should remain constant. Therefore, *N. exigua* and *N. excelsior* may be regarded as more effective hosts for large scale transient expression of

foreign genes driven by the CaMV 35S promoter in comparison with *N. benthamiana*.

Usage of the viral-based module vector system resulted in much higher GFP levels in *N. benthamiana* and *N. excelsior*

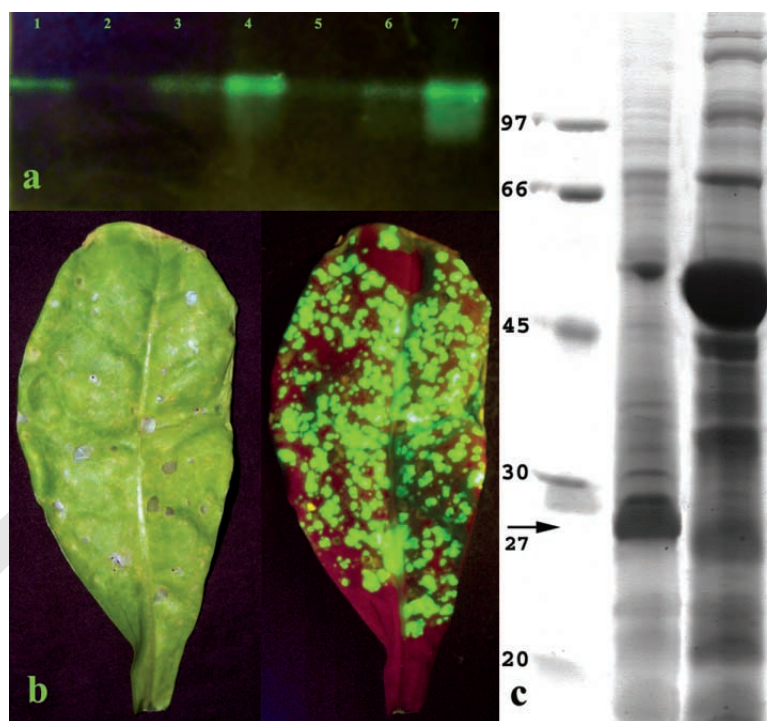


Figure 5. GFP accumulation in leaves and GFP SDS-PAGE analyses: (a) GFP fluorescence in crude protein extracts without prior heat denaturation (under UV-light); 1—standard GFP, 2—control plant of *N. benthamiana* (infiltrated with pIC6692 only), 3—*N. benthamiana* infiltrated with pIC5290 and pIC6692 (35S cassette), 4—*N. benthamiana* infiltrated with pIC10881, pIC10570, pIC7410, and pIC6692 (VIR cassette), 5—control plant of *N. excelsior* (infiltrated with pIC6692 only), 6—*N. excelsior* infiltrated with 35S cassette, 7—*N. excelsior* infiltrated with VIR cassette; (b) GFP accumulation in *N. excelsior* leaf infiltrated with VIR cassette (18 dpi) (left—under visible light, right—under UV light); (c) Coomassie staining of proteins (with prior heat denaturation); 1—molecular weight marker proteins, 2—*N. excelsior* infiltrated with VIR cassette; 3—control (not infiltrated) plant of *N. excelsior*; GFP is indicated with arrow.

(16.2 ± 11.2 and $63.5 \pm 26.8\%$ TSP, respectively), while no GFP accumulation was detected in the infiltrated leaves of *N. debneyi*, *N. exigua*, and *N. maritima* (Fig. 4b). The reasons why such viral vectors may have difficulties starting the replication cycle were discussed recently (Marillonnet et al., 2005).

Protein extracts from the infiltrated areas were analyzed by SDS/PAGE with or without prior heat denaturation. The last method allows direct detection of GFP fluorescence in the gel (Fig. 5a). The best GFP accumulation levels obtained with *N. excelsior* were as high as about 70% TSP (Fig. 5b), as it was determined by fluorescence measurements. In this case, recombinant GFP replaced normally most abundant plant protein, the large subunit of Rubisco (Fig. 5c). Taking into account that the useful biomass yield of this species almost fivefold surpasses the corresponding mean of *N. benthamiana*, *N. excelsior* may be regarded as the best host for transient expression of viral-based vectors among the species tested.

In conclusion, the reported results show that the effectiveness of a given genetic construct may depend significantly on the choice and state of the host plant. For *N. benthamiana*, a model host for transient expression for both *Agrobacterium* or viral-based expression systems, the obtained data suggest that the transient expression of the reporter gene is maximal in the 1st–6th leaves of *N. benthamiana* plant of age shortly before flowering. In these conditions, plants can accumulate the reporter protein at the level of 3.8 and 16.2% of TSP, if

CaMV 35S promoter or viral-based cassette was used, respectively. We tested several *Nicotiana* species as hosts for transient expression. Among them, *N. excelsior* displayed the best characteristics in regard to biomass yield as well as GFP accumulation level for both types of the expression cassettes tested.

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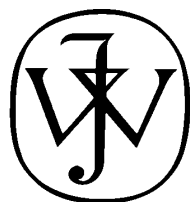
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