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NICOTIANA WILD TOBACCO SPECIES – NEW PRODUCENTS FOR RECOMBINANT PROTEINS EXPRESSION

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Summary. The development of heterologous expression methods in plants is a promising way to produce recombinant valuable proteins. The aim of this work was to analyse different species of the genus *Nicotiana* for new producers of recombinant proteins and optimisation of heterologous expression conditions for further study of the regulation of reporter gene expression during transient genetic transformation. All Australian tobacco species of the genus Nicotiana (*N. benthamiana, N. excelsior, N. debneyi, N. exigua, N. maritima, N. simulans, N. amplexicaulis, N. excelsior, N. excelsiana and N. rustika*) can be used for the expression of various recombinant proteins. The profile of GFP accumulation and fluorescence activity of many species is comparable to the profile of GFP fluorescence in *Nicotiana benthamiana* plants.

Keywords: genes heterologous expression, recombinant proteins, *Nicotiana* species (*N. benthamiana, N. excelsior, N. debneyi, N. exigua, N. maritima, N. simulans, N. amplexicaulis, N. excelsior, N. excelsiana* and *N. rustika*), transient genetic transformation, GFP.

Introduction

A large number of protein-based pharmaceutical products are used in modern medicine. The biotechnological production of therapeutically valuable recombinant human proteins (enzymes, hormones, antibodies, etc.) is of particular interest because they have a limited natural source. Currently, microorganisms are mostly used as producers of recombinant proteins. Despite the examples of successful use of bacterial production systems, prokaryotic biosynthetic systems have a number of disadvantages: lack of glycosylation, protein folding (packaging), accumulation of bacterial toxins in the culture solution, etc.

The development of heterologous expression methods in plants is a promising way to produce recombinant valuable proteins. Plant systems have a number of advantages over bacterial systems, including: cost-effectiveness, availability of plant material, the possibility of consuming plant food crops, stability of eukaryotic proteins in plant cells, the possibility of glycosylation, and the absence of bacterial pathogens and toxins. However, a significant disadvantage of using transgenic plant systems for stable protein synthesis is the low levels of recombinant product accumulation (about 1%). In this case, transient expression of the transferred genes, without their stable integration into the genome, is another alternative approach [1, 2, 3]. Transient expression is carried out by *Agrobacterium*-mediated introduction of plasmid vectors into plant cells without their integration into the genome. Over a certain period of time, the introduced transgene is expressed, after which the foreign DNA is eliminated.

Although in recent years the possibility of successful transient expression of heterologous proteins in many species of cultivated plants has been shown, the wild Australian tobacco species *Nicotiana benthamiana* Domin remains a model species for studying transient gene expression in

plants [4]. Therefore, we focused on the search for wild Australian representatives of the genus *Nicotiana*, which are endemic species of Australia, belonging to the genus *Nicotiana*, family *Solanaceae*. Due to their morphological and physiological properties, species of the *Nicotiana* genus are often characterised by resistance to many plant pathogens, including viral pathogens and fungi. For example, *Nicotiana debneyi* has been shown to be a donor of resistance genes to black root rot (*Chalara elegans*) and downy mildew (*Peronospora tabacina* Adam) found in the *N. tabacum* genome [5]. The transfer of resistance between closely related species to pathogens is studied on the example of the genus *Nicotiana* [6].

The aim of this work was to analyse and test the prospects of using representatives of different species of the genus *Nicotiana* for the selection of highly productive plant species in terms of biomass, search for new producers of recombinant proteins and optimisation of heterologous expression conditions for further study of the regulation of reporter gene expression during transient genetic transformation.

Materials and methods

Seeds of Australian tobacco plants of the genus *Nicotiana* (*N. benthamiana, N. excelsior, N. debneyi, N. exigua, N. maritima, N. simulans, N. amplexicaulis, N. excelsior, N. excelsiana* and *N. rustika)* were provided by the National Collection of Plant Tissue Cultures *In Vitro* of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine by Dr. Belokurova V. B. Plants of all species were grown in a greenhouse for 2 months. The reporter gene gfp, encoding green fluorescent protein (GFP), was used to develop effective working methods for transient expression of foreign genes in tobacco plants [7, 8]. In the experiments, both transcriptional vectors and viral vectors (carrying elements of the genomes of plant viruses TMV (Tobacco mosaic virus) and PVX (Potato virus X) were used. The 35S promoter of CaMV (*Cauliflower mosaic virus*) was used in transcription vectors. Infiltration of plants was carried out according to the standard method. The overnight culture of bacterial cells was precipitated and resuspended in an infiltration buffer of 10 mM $MgSO_4$. Infiltration was carried out into the leaf blade using a syringe [2].

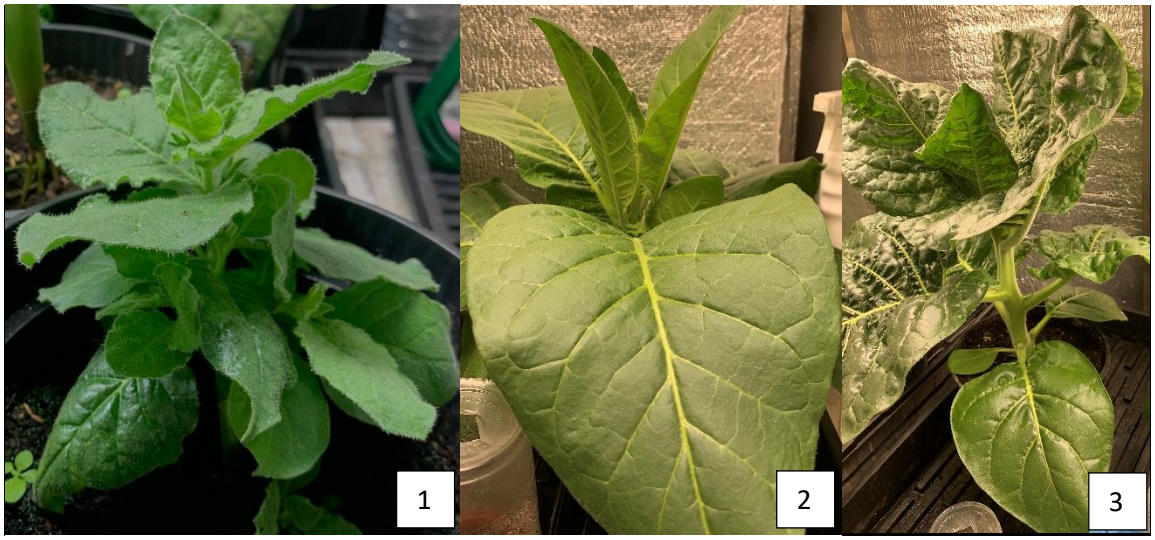


Figure 1. Plants used for transient genetic transformation by agrobacterial infiltration with a needleless syringe: 1 - *Nicotiana benthamiana*; 2 – *N. tabacum*; 3 – *N. rustica*

Results and discussions

Plants of the genus *Nicotiana* L. are considered to be the best targets for agrobacterial transient transformation, although other valuable crops such as rice, wheat, alfalfa, and potatoes are

also used [9-14]. The most commonly used tobacco species for transformation is *N. benthamiana*, which is a model for transient gene expression. Using *N. benthamiana* plants, various recombinant proteins have been synthesised, some of which are vaccines based on immunoglobulin (IgG) antibodies for the treatment of lymphoma patients. By using transient expression methods, a rapid synthesis time and high yield of recombinant proteins was achieved, which ranged from 0.5 to 4.8 g/kg of fresh material [12, 13].

In addition, other types of tobacco, such as *N excelsior*, *N. excelsiana*, etc. were used in experiments, especially when using the method of vacuum infiltration of plants [15, 16]. Tobacco (*N. tabacum*) is often used in scientific research [16]. One of the factors limiting the commercial success of recombinant protein production technology in tobacco may be low gene expression. To solve this particular problem, certain high-yielding agronomic lines are selected from a wide range of available tobacco germplasm that will significantly increase the expression of heterologous genes [17].

The results of infiltration were recorded starting from day 2 after infiltration (dpi, days post-infiltration) and continued at intervals of 2 days until day 14 (for transcriptional vectors) and day 28 (for viral vectors). For the transcriptional vectors, the onset of gfp gene expression was recorded starting from day 2 for *N. benthamiana* and from day 3 for the other species, which corresponded to the onset of GFP fluorescence in leaf tissues. The maximum level of fluorescence was observed on day 8 for *N. benthamiana* and day 9-10 for the other species, followed by a plateau and subsequent silencing of gfp gene expression. When using viral vectors, the onset of GFP fluorescence was observed somewhat later: on day 5 after infection for *N. benthamiana* and on days 7-8 for other species. The maximum level of GFP fluorescence in the tissues was observed on day 9 after infection for *N. benthamiana* and on days 10-14 for other species.

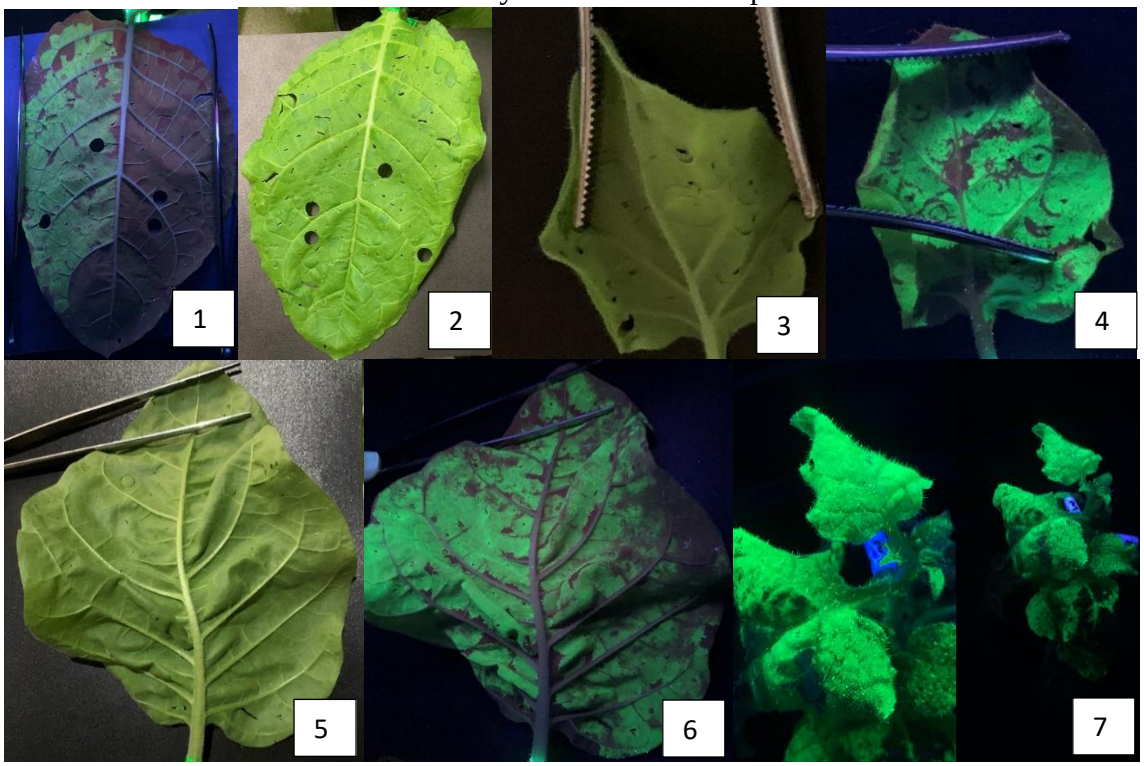


Figure 2. Visual assessment of the temporal expression of the gfp gene in tissues of different tobacco species after 4 days of infiltration: 1-2 - *Nicotiana tabacum* leaf under UV (1) and visible (2) illumination; 3-4 - *Nicotiana benthamiana* leaf under visible (3) and UV (4) illumination; 5-6 - *Nicotiana rustica* leaf under visible (5) and UV (6) illumination; 7 - Viral infection on day 7-8 of infection in *Nicotiana benthamiana* plant

At the same time, 2 weeks after infection, the development of systemic infection was observed in plants for most species, which spread up the shoots through the vascular system. In plants that have shortened internodes and form a rosette, systemic infection was observed visually by GFP fluorescence in leaves located above the infiltration site (*N. debneyi, N. exigua*). GFP fluorescence was also observed in flowers (corolla and stamen filaments). The presence of GFP protein was confirmed by spectrofluorimetry and non-denaturing acrylamide gel electrophoresis. One month later, the plants still showed systemic distribution of GFP in young tissues.

As a result of the work, the stage of plant development at which the highest level of GFP reporter protein accumulation is observed was determined for all species. Also, the dynamics of transient expression of the GFP reporter gene was determined using both transcriptional and viral vectors. Since *N. benthamiana* has a small biomass compared to some other tobacco species, we searched for another host plant that would combine a significant biomass with a high level of transient expression.

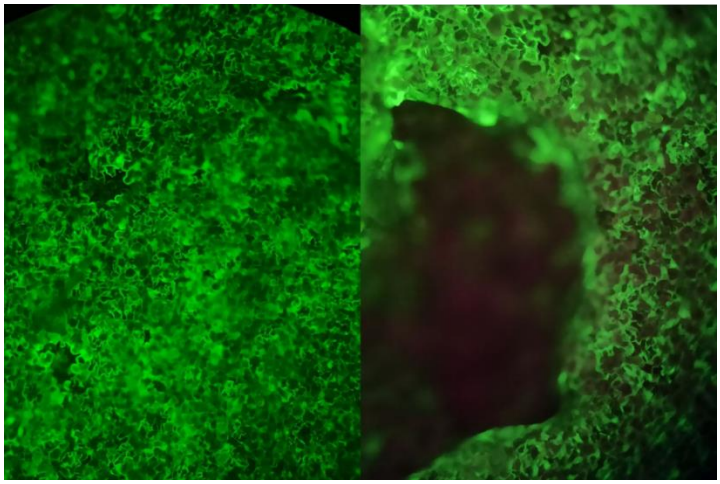


Figure 3. GFP of tobacco leaves on day 12 of agroinfiltration

A total of 10 species of this genus (*N. benthamiana, N. excelsior, N. debneyi, N. exigua, N. maritima, N. simulans, N. amplexicaulis, N. excelsior, N. excelsiana and N. rustika*) were tested and the species that showed the highest level of GFP reporter protein accumulation due to transient expression of the corresponding gene under the control of both transcriptional and viral vectors were identified. It should be noted that the use of viral vectors made it possible to significantly increase the GFP content due to transient expression of the corresponding gene in all plants of these species. Since the biomass of leaves of some species is comparable or exceeds that of *N. benthamiana,* and the expression of the GFP gene is almost comparable to that of *N. benthamiana,* these species can be used as alternative targets for the production of recombinant proteins by transient expression (*N. excelsior, N. debneyi, N. exigua, N. simulans and N. rustika*).

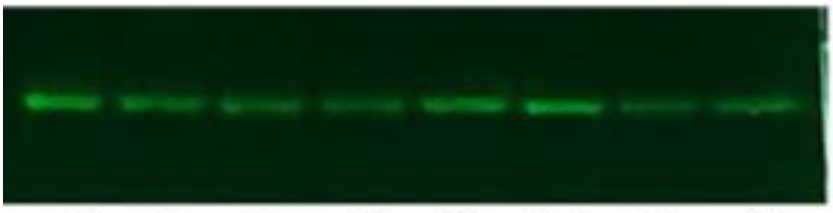


Figure 4. Electrophoretic separation of proteins in a polyacrylamide gel (different *Nicotiana* species). Results of native electrophoresis (detection of GFP protein in ultraviolet light)

Conclusions

Thus, all Australian tobacco species of the genus Nicotiana (*N. benthamiana, N. excelsior, N. debneyi, N. exigua, N. maritima, N. simulans, N. amplexicaulis, N. excelsior, N. excelsiana and N. rustika*) can be used for the expression of various recombinant proteins. The profile of GFP accumulation and fluorescence activity of many species is comparable to the profile of GFP fluorescence in *Nicotiana benthamiana* plants.

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