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INFLUENCE OF AGROINFILTRATION CONDITIONS ON THE TRANSIENT GREEN FLUORESCENT PROTEIN EXPRESSION IN *Nicotiana rustica* L. PLANTS

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Abstract

Transient gene expression in plants allows accumulation of large amount of recombinant proteins within a very short time. We optimized the protocol of transient green fluorescent protein (GFP) expression system in Aztec tobacco (Nicotiana rustica L.) plants. For transient transformation, the optimal optical density of the agrobacteria suspension for plant tissues infiltration was determined. The pSPV2303 genetic construct, contained the gfp gene from A. victoria under double 35S CaMV promoter, used for this purpose. The agrobacteria suspension was grown to OD = 1.0 initial optical density at wavelength 600 nm and the plants were infiltrated with the following final concentrations: 0.2; 0.4; 0.8; 1.0; 1.5; 2.0 without and with the addition of 4'-hydroxy-3',5'-dimethoxyacetophenone (acetosyringone) at 150 μM concentration. Spectrofluorometric and protein analysis were used for green fluorescent protein (GFP) detection. The highest level of expression obtained was using the OD = 1.0 agrobacteria optical density and there was not a detrimental effect on plant tissues.

Key words: *acetosyringone, agrobacteria optical density, green fluorescent protein (GFP), Nicotiana rustica L., transient transformation.*

INTRODUCTION

The heterologous gene expression system, mediated by agrobacteria in intact plant leaves, is a fast and productive method for analyzing gene expression. It can produce many heterologous proteins without the need to create stable transformants, which is difficult for many plant species. Furthermore, transient gene expression is not biased by position effect, which often occurs in stable transformation (Lee & Yang, 2006).

The heterologous gene expression system offers several advantages over stable expression. One of them is simplicity of execution. The expression of transient genes can be analyzed directly post agroinfiltration after several days (Kapila et al., 1997), subsequently the expression level decreases rapidly. This can be explained by the initiation of local silencing of RNA that blocks transgene expression (Johansen & Carrington, 2001; Voinnet et al., 2003) and is one of the main mechanisms of cell protection against foreign nucleic acids (Dougherty & Parks, 1995; Lindbo et al., 1993; Baulcombe, 2004; Tenllado et al., 2004). Specific viral proteins capable of inhibiting the mechanism of RNA silencing have been found in many plant viruses (Roth et al., 2004).

Transient expression is a common method for assessing the capacity of plant cells to produce a particular protein and⁄or to test various expression cassette arrangements (Sainsbury et al., 2009).

MATERIALS AND METHODS

Genetic construct

We used a pSPV2303 construct containing the *gfp* gene isolated from *A. victoria* encoding a Green Fluorescent Protein (GFP) under the control of a dual CaMV 35S promoter, a 5' untranslated (5'UTR) region from the Tobacco Mosaic Virus (TMV) and the CaMV 35S

terminator together with the polyadenylation signal and the 3'- untranslated region (3'UTR). The overnight culture of the genetic constructs were grown in LB medium (Bertani, 1951) supplemented with 50 mg/l rifampicin, 25 mg/l gentamicin, and 100 mg/l kanamycin.

Plant agroinfiltration

Plants of Aztec tobacco (*Nicotiana rustica* L.) were grown under greenhouse conditions at 25/18ºC and 16/8 photoperiod (day/night, respectively). Leaves of the middle tier of 4-week plants were used for infiltration.

To study the effect of different bacterial concentrations on the expression of *gfp* gene Aztec tobacco plants (*N. rustica* L.) transformed transiently. *Agrobacterium* overnight culture was grown to optical density of suspension OD_{600} = 1.0 and resuspended in infiltration buffer (10 mM $MgSO_4$, pH 5.6-5.8) with a final optical density of OD_{600} = 0.2; 0.4; 0.8; 1.0; 1.5; 2.0 without and with the addition of acetosyringone at 150 µM concentration. Plant infiltration was performed with a syringe (Sambrook et al., 1989).

Spectrofluorimetric analysis

The expression of the *gfp* gene was detected in the 7th day after infiltration visually and by spectrofluorometric analysis on a fluorescence spectrofluorometer "Fluorate-02-Panorama" (excitation at a wavelength of 395 nm, emission at 509 nm).

Protein analysis

Water-soluble proteins were isolated in extraction buffer (PBS), consisting of the following elements: 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl, pH 7-7.5 (Sambrook et al., 1989). Plant tissues of *N. rustica* L., in which *gfp* gene expression was detected, were triturated with extraction buffer in a pre-chilled mortar (+4ºC) at a 3:1 (v/w) ratio (300 µl of buffer per 100 mg of tissue), after which the suspension was precipitated (+4°C, 14000 rpm, 30 minutes). The supernatant was used to determine protein concentration by Bradford protein assay (Bradford, 1976) and for separation of proteins in polyacrylamide gel under native and denaturing conditions.

All statistical analyzes were performed in Microsoft Office Excel, determining the mean and standard deviation for each experiment. For the statistical processing of the spectrofluorometric analysis, the average value was determined through the logarithmic transformation of the spectrofluorometer parameters.

RESULTS AND DISCUSSIONS

This study was conducted to develop an effective system of transient transformation for heterologous protein synthesis in Aztec tobacco (*N. rustica* L.) tissue.

Genetic construct and plant agroinfiltration

We tested six different concentrations of bacterial suspension with pSPV2303 vector (Figure 1) and the effect of acetosyringone on heterologous protein expression.

The fluorescence of the GFP protein (Figure 2) was detected 7 days after infiltration. Tissues of plants infiltrated with *Agrobacterium tumefaciens* GV3101 strain and tissues that were not infiltrated were used as a negative control.

Figure 1. Schematic representation of the pSPV2303 vector T-DNA

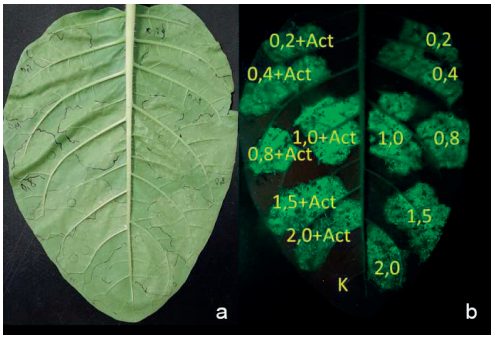

Figure 2. Transient expression of the *gfp* gene: a) leaf 7 days after infiltration (day light); b) leaf 7 days after infiltration (ultraviolet light)

Spectrofluorometric analysis

Scholz et al. (2000) showed a positive correlation between the level of fluorescence and the accumulation of recombinant GFP protein, as level of the *gfp* gene expression and explained the non-linear nature of this process, which is confirmed by our experiments between the spectrofluorometric analysis of GFP fluorescence and the determination of water-soluble protein concentration level.

Leuzinger et al. (2013) showed that low concentration of OD_{600} = 0.12, demonstrated

Agrobacterium strain balances between maximum delivery of gene construct without causing tissue necrosis and cell death. We showed that the *gfp* gene expression at low bacterial concentration, OD_{600} = 0.2, was the poorest (Table 1) and equaled 0.48 ± 0.03. Shamloul et al. (2014) used a bacterial suspension of 0.5 optical density at 600 nm wavelength.

Table 1. Fluorescent level of GFP protein in *N. rustica* L. tissue (relative units)

	Mean	-Cl	+Cl
OD_{600} = 0.2	0.48	0.03	0.03
OD_{600} = 0.2+Act150 µM	0.48	0.02	0.02
OD_{600} = 0.4	0.55	0.01	0.02
OD_{600} = 0.4+Act150 µM	0.55	0.02	0.02
OD_{600} = 0.8	0.99	0.04	0.04
OD_{600} = 0.8+Act150 µM	1.00	0.03	0.03
Kgv3101	0.02	0.00	0.01
K leaf tissue	0.01	0.00	0.01
OD_{600} = 1.0	1.02	0.04	0.04
OD_{600} = 1.0+Act150 µM	1.02	0.02	0.02
OD_{600} = 1.5	0.99	0.03	0.03
OD_{600} = 1.5+Act150 µM	0.99	0.04	0.04
OD_{600} = 2.0	0.61	0.03	0.04
OD_{600} = 2.0+Act150 µM	0.61	0.01	0.01

In our experiments we showed that the *gfp* gene expression increased depending on the increasing of suspension optical density. When we used OD_{600} = 0.4 fluorescence level was 0.55 (+0.02; -0.01), and was higher than at OD_{600} = 0.2. Spectrofluorometric analysis showed (Figure 3) that the highest level of the *gfp* gene was showed when the optical density of suspension OD_{600} = 0.8; 1.0; 1.5 were used and equaled 1.02 ± 0.04; 0.99 ± 0.04; 0.99 ± 0.03, respectively. But at the maximum bacterial suspension concentration, what we used in our experiments (OD_{600} = 2.0) for agroinfiltration, the protein GFP fluorescence level in the tissues was 0.61 (+0.04; -0.03), which is less than when was used the bacterial suspension with OD_{600} = 1.5; 1.0; 0.8. Shamloul et al. (2014) showed that none of the tested concentration of acetosyringone induced a significant increase in GFP fluorescence or protein production compared with control, where induction media contained no acetosyringone for *Nicotiana benthamiana* L. This was also confirmed by our experiments on *N. rustica* L.

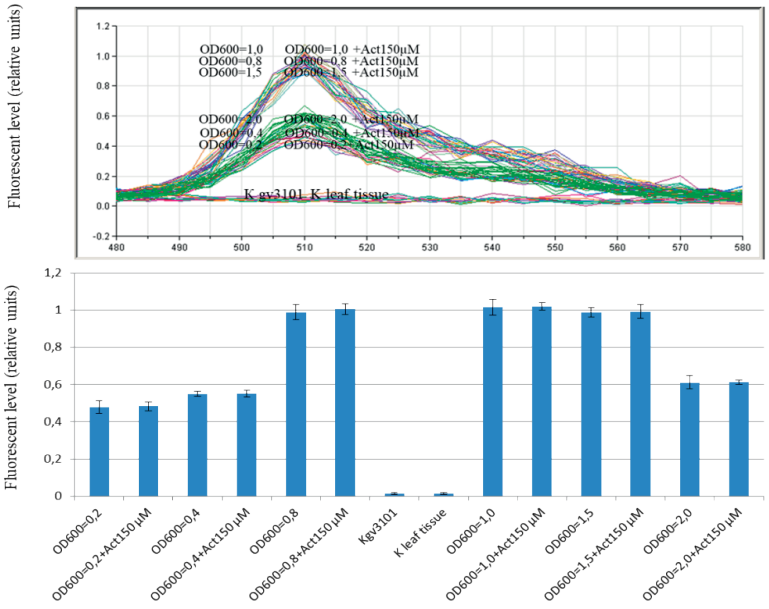

Figure 3. Spectrofluorometric analysis of GFP expression

Protein analysis.

Concentration of total water-soluble proteins (µg/ml) in *N. rustica* L. tissue (Figure 4), showed that the protein concentration (Table 2) during transient expression of the *gfp* gene was different depending on the optical density of bacterial suspension what was used for agroinfiltration.

Indicators of protein analysis are completely identical to spectrofluorometric, where the GFP protein had the highest expression level when the OD_{600} = 0.8, 1.0 or 1.5 were used, and the lowest when the bacterial concentration of OD_{600} = 0.2 was used.

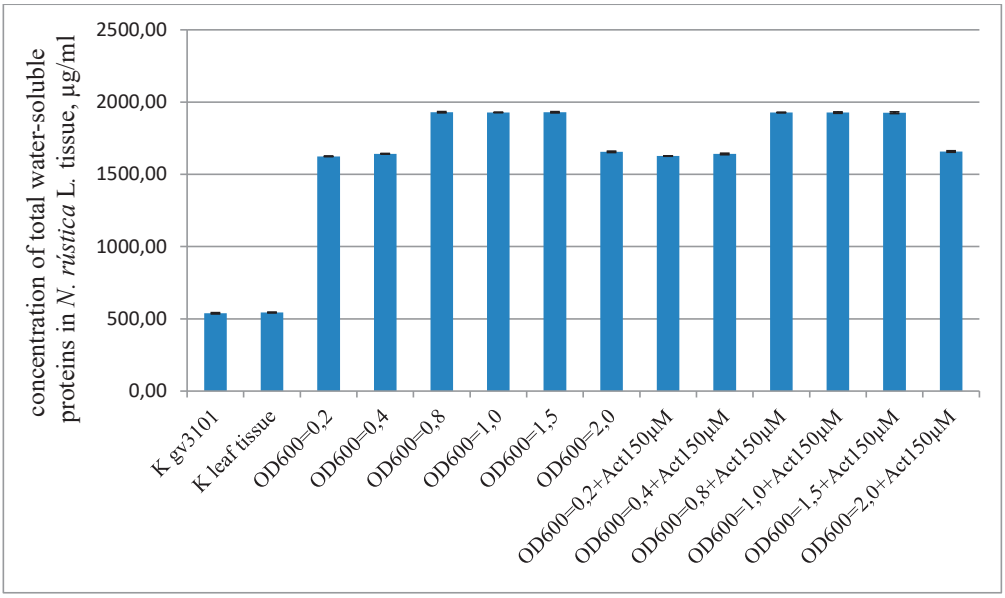

Figure 4. Concentration of total water-soluble proteins in *N. rustica* L. tissue, µg/ml

Table 2. Concentration of total water-soluble proteins in *N. rustica* L. tissue, µg/ml

	Mean	± Deviation
K gv3101	538.33	4.73
K leaf tissue	543.67	3.21
OD_{600} = 0.2	1624.33	2.08
OD_{600} = 0.4	1642.33	2.31
OD_{600} = 0.8	1930.00	4.36
OD_{600} = 1.0	1928.33	1.53
OD_{600} = 1.5	1929.67	4.51
OD_{600} = 2.0	1655.67	4.93
OD_{600} = 0.2+Act150 µM	1626.67	1.53
OD_{600} = 0.4+Act150 µM	1641.00	5.29
OD_{600} = 0.8+Act150 µM	1927.67	2.52
OD_{600} = 1.0+Act150 µM	1927.33	4.93
OD_{600} = 1.5+Act150 µM	1926.67	6.11
OD_{600} = 2.0+Act150 µM	1658.00	5.29

To identify the GFP reporter protein, we further performed polyacrylamide gel electrophoresis to separate water-soluble proteins in the presence of sodium dodecyl sulfate under denaturing (with mercaptoethanol) and non-denaturing separation conditions (Figure 5).

Protein extracts were mixed with the sample buffer and applied to the wells with and without boiling denaturation.

The presence of the GFP reporter protein was observed immediately after electrophoresis by illuminating the gel with ultraviolet light using a hand lamp.

Detection in ultraviolet light (Figure 5a) showed the functional activity of the GFP reporter protein. Other proteins were visualized (Figure 5b) by staining the gel with a Coomassie reagent (Blakesly et al., 1977).

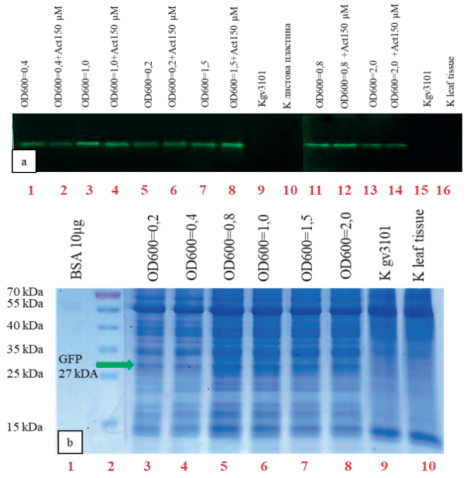

Figure 5. Electrophoretic separation of proteins in polyacrylamide gel: a) non-denaturation conditions (detection of GFP protein in ultraviolet light); b) denaturation conditions: 1-8; 11-14 - protein extracts of *N. rustica* L. tissue in which the *gfp* gene is transiently expressed under the control of different bacterial concentrations; 9,10; 15,16 - extracts of *N. rustica* L. tissue control plants infiltrated with *A. tumefaciens* GV3101 strain and uninfiltrated tissue; b) denaturing conditions: 1 - standard BSA (concentration 10 µg); 2 - a molecular weight marker; 3-8 - protein extracts of *N. rustica* L. tissue in which the *gfp* gene is transiently expressed under the control of different bacterial concentrations (the GFP protein is indicated by an arrow); 9, 10 - extracts of *N. rustica* L. tissue control plants infiltrated with *A. tumefaciens* GV3101 strain and uninfiltrated tissue

Aztec tobacco infiltrated by created construct with different optical density of suspension and recombinant GFP protein resulting from transient expression has the same molecular weight as standard GFP (Figure 5b).

Indicators of protein electrophoretic analysis are completely in line with spectrofluorometric and protein analysis, where the highest accumulation of recombinant GFP protein was when used bacterial suspension of 0.8, 1.0, 1.5 optical density at 600 nm wavelength

CONCLUSIONS

In our experiments we determined that the level of heterologous genes expression depends on the concentration of bacterial suspension, and has a positive character due to its increase from 0.2 to 0.4, then goes to the plateau, using 0.8; 1.0; 1.5 and declines at 2.0. An increase in the level of fluorescence coincides with an increase in the concentration of total water-soluble proteins in the Aztec tobacco tissues, which is confirmed by the study of other authors. The use of bacterial suspension concentrations of 0.8-1.0-1.5 leads to the highest expression of *gfp* gene in healthy plant tissues.

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