BRIEF COMMUNICATION

Activities of ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase, and oxygen evolution in transgenic tobacco plants

J. KVĚTOŇ

Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, and Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic

Abstract

Three clones of tobacco transformed with the T-DNA of Agrobacterium rhizogenes Ri plasmid A4b cultivated in vitro on a solid agar medium neither showed pronounced morphological diversity nor significantly differed in chlorophyll (Chl) contents from control plants. However, the transformation caused a 27 to 83% decay in leaf oxygen evolution and in both ribulose-1,5-bisphosphate carboxylase (RuBPC) and phosphoenolpyruvate carboxylase (PEPC) activities. Therefore, the transformation brought about a reduction of active PEPC as well as activable RuBPC amounts in plant tissues. Individual clones did not mutually differ. In tobacco transformed with A. rhizogenes strain TR101 and grown in soil only the mean leaf area tended to reduce. Chl contents, Chl a/b ratio, oxygen evolution, and activities of both RuBPC and PEPC were insignificantly affected by the transformation.

Additional key words: Agrobacterium rhizogenes, Nicotiana tabacum, Ri plasmids, T-DNA, young, mature and old leaves.

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T-DNA of Agrobacterium rhizogenes Ri plasmids A4b bears genes for agropine synthesis and utilisation (Petit et al. 1983) and consists of two regions, TL and TR, that integrate independently into a plant genome. The TR has genes for auxin synthesis (Offringa et al. 1986). The TL contains rolA, rolB, rolC, and rolD genes the expression of which may not only affect the metabolism of phytohormones but also sensitise plant cells to phytohormone action (e.g., Estruch et al. 1991, Dehio et al. 1993, Nilsson et al. 1993, Schmülling et al. 1993). These genes in integration bring about the formation and growth of roots. The Ri plasmid TR101 is of a mannopine type and has a single T-DNA part homologous to TL of A4b (Filetici et al. 1987). Therefore the transformation of plants with Ri plasmids may cause complex physiological modifications by shifting the balance of phytohormone amounts and their activities. The introduction of T-DNA genes from the Ri plasmid A4b into the tobacco genome did not cause statistically significant differences in many structural and physiological traits of the plants (Siffel et al. 1988, Tichá et al. 1988). The transformation only broadened their variability range. In comparison with control plants, a higher transpiration rate but still a constant total net photosynthetic rate were found in tobacco clones of the T-phenotype transformed with T-DNA of the Ri plasmid TR101 and transplanted into ex vivo conditions (Václavík et al. 1988).

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Abbreviations: ATP - adenosine 5'-triphosphate; Chl - chlorophyll; NADH - β-nicotinamide adenine dinucleotide, reduced; PEPC - phosphoenolpyruvate carboxylase; Ri - root inducing; RuBPC - carboxylating operation of ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; Tricine - N-tris(hydroxymethyl)methylglycine.

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Fax: (+420) 38 5300356, e-mail: queton@umbr.cas.cz

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The objectives of this study were to find whether the introduction of Ri plasmid T-DNA genes into the tobacco genome induces some changes in the photosynthetic carboxylation mediated by RuBPCO, in the non-photo autotrophic carboxylation by PEPC, in oxygen evolution by leaf pieces, in Chl contents, and in leaf area. I studied both the original transformants in vitro (A4b) and groups of differently old leaves of the transformants transferred into soil (TR101).

Control and transgenic tobacco (Nicotiana tabacum L. cv. White Burley) regenerants transformed with A. rhizogenes Ri plasmid A4b or TR101 were kindly provided by Dr. M. Ondrej (IPMB, ASCR, České Budějovice, Czech Republic). Methods of transformation, selection of transgenic plants and their cultivation in vitro on the Murashige and Skoog medium without sucrose are described in Tichá et al. (1988) and Václavík et al. (1988). Plants transformed with the Ri plasmid TR101 were transferred into pots with soil and grown in a greenhouse (Václavík et al. 1988). All the plant types were taken for analysis about 40 d after their last transplantation.

Ten to twenty leaf discs (5 - 10 cm²) cut from a leaf were extracted by a Potter & Elvejem all glass homogenizer (Kavalier, Šázař, Czech Republic) in 5 cm³ of an ice-cold medium containing 50 mM Tricine, 10 mM MgCl₂, 0.15 mM 2-mercaptoethanol, 10 mg cm⁻³ polyvinylpyrrolidone, 0.5 mM ethylenediaminetetraacetic acid, at pH 8.0 (KOH). The crude homogenate was filtered through paper filters 595 s and 602 s (Schleicher & Schuell, Feldbach, Switzerland). The filtrate was kept in crushed ice until assayed. Enzyme activities were determined at 30 °C in a final volume of 1 cm³ by continuous monitoring co-factor (NADH) oxidation at 340 nm using a double beam spectrophotometer (PU 8800, Philips - Pye Unicam, Cambridge, UK). The RuBPC activity assay was based on the method of Lilley and Walker (1974). The reaction mixture contained: 50 mM Tricine and 20 mM MgCl₂ at pH 8.0 (KOH), 5 mM ATP, 5 mM phosphocreatine, 0.2 mM NADH, 42 nkat cm⁻³ glyceraldehyde phosphate dehydrogenase, 67 nkat cm⁻³ phosphoglycerate phospho-kinase, 17 nkat cm⁻³ creatine phosphokinase, 0.5 mM ribose-5-phosphate, 10 mM NaHCO₃, and 50 mm² of the enzyme extract. The PEPC activity was measured using the conventional coupled enzyme assay (Lane et al. 1969). The medium contained: 50 mM Tricine and 5 mM MgCl₂ at pH 8.0 (KOH), 0.2 mM NADH, 37 nkat cm⁻³ malate dehydrogenase, 1 mM phosphoenolpyruvate, 5 mM NaHCO₃, and 50 mm² of the extract. For measurement of oxygen evolution, leaf discs (2.5 - 3.5 cm²) were cut by a keen blade into 1 - 2 mm³ pieces and infiltrated with distilled water for 1 min using a vacuum pump. When exposed to continuous light in a chamber with the reaction mixture (50 mM Hepes, 330 mM mannitol, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM ethylenediaminetetraacetic acid disodium salt, and 20 mM NaHCO₃), the leaf pieces produced oxygen that was measured by the Clark-type probe: Chl amounts were estimated spectrophotometrically in 80 % (v/v) acetone extracts by the double-wave method (Arnon 1949). Statistical significance of differences between transformic and control plants was analysed using Mann-Whitney’s non-parametric test (SOLO software, release 3.1).

Three independent lines denoted A4b-1, A4b-2, and A4b-17 of the transgenic tobacco regenerants transformed with Ri plasmid A4b T-DNA and cultivated in vitro on a solid agar medium were randomly selected for experiments. In the variants A4b-1 and A4b-2, Tichá et al. (1988) found insignificant changes in morphological, anatomical, and growth parameters. Similarly in this study, the transformants of all the three clones differed neither in morphology, Chl contents nor Chl a/b ratio from the control plants (Fig. 1). On the other hand, the oxygen evolution rate and the activities of both RuBPC and PEPC (normalized per unit of Chl and/or

![Fig. 1. The effect of transformation with T-DNA of A. rhizogenes Ri plasmids A4b on some physiological traits of tobacco. Values in transformants are expressed as percentages of respective controls. C - chlorophyll a+b contents, ab - chlorophyll a/b ratio, R - RuBPC activity, P - PEPC activity, O₂ - oxygen evolution. Vertical bars represent SE from at least 3 independent measurements. Asterisks indicate statistically significant differences between transformants and controls at 5 % probability level.](image-url)
leaf area) were 27 to 83% lower in transgenic plants than in controls (Fig. 1). However, the individual clones of transformants did not mutually differ. The constant Chl contents and the unchanged Chl \(a/b\) ratio indicated no effect of the transformation on pigment composition. But the transformation caused a depression of the quantum yield of oxygen evolution and also a reduction of the activable RuBPCO. As CO\(_2\) exchange rate and the growth did not significantly differ between the transgenic clones and the controls (Tichá et al. 1988), the decrease in active RuBPCO due to the transformation could imply a higher activation state of this enzyme in vivo under the conditions of plantlet cultivation at low irradiance (~40 μmol m\(^{-2}\) s\(^{-1}\)). RuBPC/PEPC activity ratio that increased 1.9 to 3.2 times in transgenic plants (Table 1) proves a larger relative drop of the non-autotrophic CO\(_2\) fixation (through PEPC) in comparison with the autotrophic one. Plantlets were cultivated in vessels covered with Al-foils which allowed merely a low gas exchange. This situation deprives plantlets of external CO\(_2\) and its level drops close to the compensation concentration shortly after the beginning of photoperiod (Pospišilová et al. 1989, Šantůček et al. 1991). Such conditions of growth affect carbon metabolism in plant tissues. Sallanon et al. (1995) found a more than 100% increase of RuBPC activity in walnut plantlets grown mixotrophically at low irradiance in vessels flushed with air containing atmospheric CO\(_2\) concentration (360 cm\(^3\) m\(^{-3}\)) compared to those in quasi confined vessels (lower CO\(_2\) concentration); PEPC activity remained unchanged and the RuBPC/PEPC activity ratio was then enhanced. RuBPC activity was higher for in vitro autotrophic plantlets of Rosa hybrida aerated with ambient CO\(_2\) concentration and/or CO\(_2\)-enriched air (2 500 cm\(^3\) m\(^{-3}\)) than for control in closed vessels. PEPC activity was stimulated only in the latter case (Genoud-Gourichon et al. 1996). Comparison of my results with the findings of these authors suggest that both the transgenic and control tobacco plantlets closed in cultivation flasks and suffering from the shortage of CO\(_2\) during the light period might be endowed with altered photosynthetic (RuBPC) as well as non-photosynthetic (PEPC) CO\(_2\) assimilation.

Table 1. Ratio of RuBPC/PEPC activities in transgenic tobacco transformed with Ri plasmids A4b or TR101. Means ± SE from minimum 5 replications.

<table>
<thead>
<tr>
<th>Transformant A4b</th>
<th>RuBPC/PEPC</th>
<th>Transformant TR101</th>
<th>RuBPC/PEPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.9 ± 0.3</td>
<td>young leaves</td>
<td>control</td>
</tr>
<tr>
<td>clone A4b-1</td>
<td>8.0 ± 1.1</td>
<td>TR101</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>clone A4b-2</td>
<td>9.2 ± 0.9</td>
<td>mature leaves</td>
<td>control</td>
</tr>
<tr>
<td>clone A4b-17</td>
<td>5.4 ± 0.8</td>
<td>TR101</td>
<td>3.2 ± 0.5</td>
</tr>
</tbody>
</table>

Fig. 2. The effect of transformation with T-DNA of A. rhizogenes Ri plasmids TR101 on some physiological traits of tobacco. Values in transformants are expressed as percentage of respective controls. C - chlorophyll \(a+b\) contents, LA - mean leaf area, R - RuBPC activity, P - PEPC activity, \(O_2\) - oxygen evolution. Statistical treatment is identical to that given in Fig. 1.

The tobacco transformants TR101 did not significantly differ from the controls in the amounts of Chl \(a\) and \(b\) and their ratio, in the oxygen evolution by leaf pieces, and in the RuBPC and PEPC activities (on a Chl and/or leaf area basis) in any leaf age class examined (Fig. 2). The RuBPC/PEPC activity ratio was insignificantly different between the transgenic and control plants and tended to a standard dependence on the
leaf insertion level in both plantlet types (Table 1). The mean leaf area was lower in all age categories but the differences were significant only for mature leaves (Fig. 2).

Physiological changes brought about by some transformation depend to a certain extent on the conditions of plant cultivation (Ooms et al. 1986). Some regulation mechanisms could lead to the normalization of plant gene expressions in the ex vitro regenerants grown in soil under standard environment. Moreover, the expression of transgenes decreases with time (Binns 1983). Hence during the relatively long period, which elapsed from the time of transformation to the realization of experiments, the activity of introduced genes could be gradually suppressed. Mannopine synthesis was used by Václavík et al. (1988) as a measure of expression of TR101 T-DNA genes in transgenic tobacco clones of the same origin. The authors indeed demonstrated a slowly decreasing synthesis of mannopine during the development of plants and also showed that the growth parameters tended to normalize as the plants grow.

References


