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Changes in protein spectra of transgenic plants carrying different *Agrobacterium tumefaciens* C58 T-DNA genes

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Abstract

A series of binary vector plasmids derived from the T-DNA of the *Agrobacterium tumefaciens* strain C58, carrying the five plant morphoregulatory genes 1, 2, 4, 5 and 6b in different combinations, was used in the transformation of *Nicotiana tabacum* leaf discs. Protein patterns of the transgenic tobacco analysed through SDS-PAGE have shown changes in the polypeptides with M_r: ~ 120, 60, 55, 43 and 27 kDa (for tobacco with transgene 4); ~ 60, 55, 43, 26-25, 21, 18 kDa (for tobacco with transgenes 1, 2 and 5); ~ 70, 60, 26, 25, 18 kDa (for tobacco with transgene 5); ~ 60, 55, 48, 26, 18 kDa (for tobacco with transgenes 4, 5, 6b); ~ 60, 55, 22 and 18 kDa (for tobacco with transgene 6b); ~ 60, 55, 43, 26 and 18 kDa (for transgenes 5, 6b); ~ 60, 55, 22, 18 and 16 kDa (for transgenes 4 and 6b). All types of transgenic plants showed quantitative changes in protein content. Mendelian segregation ratio to kanamycin resistance in the progeny of transgenic tobacco clones in the R1 generation was 3:1 except in transgenic tobacco carrying transgenes 1, 2 and 5.

Introduction

The common or core part of the T-DNA of most Ti plasmids carries five main morphoregulatory genes. Gene 1 (iaaM) codes for enzyme tryptophan mono-oxygenase which conditions the conversion of tryptophan to indolylacetamide. Gene 2 (iaaH) is for enzyme indolylacetamide hydrolase which converts indolylacetamide to IAA. Gene 4 (ipt) codes for isopentenyltransferase which catalyses an early step in

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Abbreviations: HV - high voltage, LV - low voltage, M_r - relative molecular mass, PAGE - polyacrylamide gel electrophoresis, SDS - sodium dodecylsulphate, TEMED-N,N,N',N - tetramethyl ethylene-diamine, TRIS - Tris-hydroxymethylaminomethane.
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cytokinin synthesis. For references see Ondřej *et al.* (1991). Gene 5 (ila) codes for enzyme which catalyses conversion of tryptophan to auxin analogue, indolyl-3-lactate (Körber *et al.* 1991). Gene 6b modifies the action of cytokinins in transformed tissues (Spanier and Schell 1989). Genes 5 and 6b thus both affect the activity of auxins and cytokinins. All phytohormones affect selectively the transcription activity of some plant genes and therefore they should affect the protein spectra of transformed cells.

The aim of our experiments was to look for the changes in the protein spectra in the leaves of transgenic plants carrying *Agrobacterium tumefaciens* T-DNA genes for phytohormone synthesis and genes 5 and 6b.

Material and methods

Chemicals used: TRIS, SDS, amonium persulphate, kanamycin, low M_r protein kit, *Coomassie Brilliant Blue R-250* and *G-250* were purchased from *Serva* (Heidelberg); TEMED, acrylamide and bisacrylamide were from *LKB Pharmacia* (Uppsalla, Sweden).

Bacterial strains: Series of *Agrobacterium tumefaciens* LBA4404 derived strains (Hoekema *et al.* 1983), carrying one of the following vector plasmids each: pCB1334 (carrying T-DNA pTi C58 gene 4), pCB1349 (genes 1, 2, 5), pCB3013 (gene 5), pCB3016 (genes 4, 5 and 6b), pCB3026 (gene 6b), pCB3028 (genes 5 and 6b) and pCB3029 (genes 4 and 6b) as described in Vlasák and Ondřej (1992) were used. All the vector plasmids also transfered chimeric kanamycin resistance gene into the plant genome.

Transformation of tobacco: *Nicotiana tabacum* L. cv. Samsun leaf discs and the regeneration of transgenic plants carrying individual T-DNA genes and their combinations has already been described in Ondřej *et al.* (1991). The scoring of segregation of a kanamycin resistance marker in the progeny transgenic plants was as described in Ondřej *et al.* (1989). Each transformed type was represented by a single clone.

Leaves of 4 month old plants cultivated *in vitro* were homogenized in liquid nitrogen to a fine powder. Each sample was comprised of 500 mg of fresh leaf material. The powder was placed into cold test tubes (-10 °C) and overlayered with 2.5 cm^3 of cool 0.1M Na-phosphate buffer pH 7.0. After 1 h extraction in -1.2 °C, the mixture was centrifuged in a refrigerated centrifuge for 15 min at 5 700 *g*. 50 µl of supernatant was used for the determination of protein concentrations after Bradford (1976). The volume 2.5 cm^3 of supernatant was overlayered with a 5 fold higher volume of acetone and precipitated in -20 °C for 24 h. Sediment was centrifugated in a refrigerated centrifuge at 4 500 *g* for 10 min. Washing of the protein extract by acetone was repeated three times in 5 h intervals. Proteins were then dried in a cooled dessicator under vacuum. The mass of crude protein extracts was determined gravimetricaly and standardized for 1 g fresh leaf mass. For SDS-PAGE, proteins were separated by HV and LV electrophoresis. Samples were prepared according to Laemmli (1970). Gradient gels 10/20 % according to Smith

(1988) were used. HV electrophoresis was performed on *LKB Broma* apparatus and for the LV electrophoresis; the electrophoresis unit for gels with a dimension of 150 × 150 × 1.2 mm was used. M_r of proteins and their subunits was interpolated from the calibration curve for low M_r protein kit *Serva.* Protein spectra were scanned on *Beckman DU-65* spectrophotometer. Isolation was repeated three times.

Results

The transgenic regenerated plants showed slight phenotype differences when compared with untransformed control plants. When their shoot segments were put on MS medium, the callogenesis and regeneration potential differed according to the type of the T-DNA segments introduced.

The Mendelian segregation ratios to kanamycin resistance markers in the progeny of transgenic clones used for protein analysis are given in Table 1. There was found clear-cut monogenic inheritance in six out of 7 offsprings. In the progeny of the transformant carrying genes 1, 2, 5, clone 1349-1, a segregation ratio between monohybridism and dihybridism appeared. One possible explanation for this aberrant ratio would be the integration of T-DNA into two sites followed by a decrease of expression, as already shown by Matzke and Matzke (1990).

Table 1. Segregation ratios of kanamycin resistance in the offspring of transgenic plants.

Transformant	Segregation ratio green : white seedlings	Theoretical ratio	χ^2
1334-4	622 : 196	3 : 1	0.47
1349-1	1 598 : 234	between 3 : 1 and 15 : 1	
3026-2	809 : 261	3 : 1	0.21
3016-4	565 : 212	3 : 1	2.15
3028-2	260 : 92	3 : 1	0.24
3029-7	747 : 247	3 : 1	2.36
3013	453 : 135	3 : 1	1.31

Agreement with theoretical segregation ratio 3 : 1 was found in all cases except 1349-1.

The expresion of the newly introduced genes has shown quantitative and qualitative differences on the level of proteins in the leaves of the transgenic plants (Figs. 1, 2). In all cases the quantity of the proteins is higher in comparison with the untransformed control plants. For transgenic tobacco carrying genes 4; 5; 4, 5, 6b; 5, 6b and 4, 6b these differences are significant. Patterns of transgenic tobacco proteins are shown in HV and LV SDS-PAGE (Fig. 1, Table 2). Untransformed control plants (line 1) showed 4 deeply stained bands and few less prominent ones. The deeply stained bands correspond to proteins with the following M_r: I-50 kDa, II-43 kDa, III-36 kDa, IV-15,5 and 15 kDa. There are some less prominent bands in the zone of higher M_r which correspond approximately to 120 and 70 kDa. For transgenic tobacco with gene 4 (ipt), line 2, showed a decrease in quantity of proteins with

M_r = 120, 60, 56-55 and 43 kDa. The contribution of proteins with M_r 30-20 kDa was higher, more prominent being bands 22 and 18 kDa (Fig. 1). The protein spectrum of transgenic tobacco with incorporated T-DNA of the plasmid pCB 1349 with genes 1 (iaaM) and 2 (iaaH), as shown both electrophoresis (Fig. 1, line 4) differed in the proteins with M_r 60-49 kDa, 36, 21 and 18 kDa. Differences were both quantitative and qualitative in comparison with the control and with the previous variant. The tobacco clone which carried the genome T-DNA of pCB3013

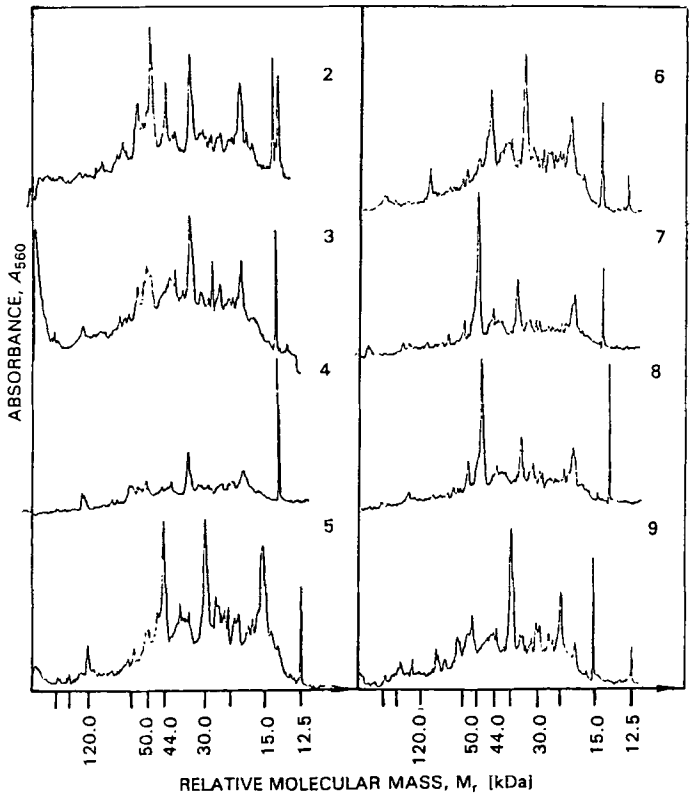

Fig. 1. Protein spectra of transgenic tobacco plants in high voltage electrophoresis. Abscissa - M_r as determined by extrapolation of calibration curve, ordinate - absorbance A scanned at λ = 560 nm, A_{max} = 0.6.

	c_p [g l^{-1}]
2 - *Nicotiana tabacum* L. cv.Samsun,	1.46
3 - *Nicotiana tabacum* L. cv Samsun, pCB1334	2.34
4 - *Nicotiana tabacum* L. cv Samsun, pCB1349	1.92
5 - *Nicotiana tabacum* L. cv Samsun, pCB3013	3.42
6 - *Nicotiana tabacum* L. cv Samsun, pCB3016	2.46
7 - *Nicotiana tabacum* L. cv Samsun, pCB3026	2.38
8 - *Nicotiana tabacum* L. cv Samsun, pCB3028	2.46
9 - *Nicotiana tabacum* L. cv Samsun, pCB3029	3.06

Table 2. Quantitative distribution of proteins in the spectra of transgenic plants.

Apr. M_r [kDa]	m_{p1} [%]	[μg]	m_{p2} [%]	[μg]	m_{p3} [%]	[μg]	m_{p4} [%]	[μg]	m_{p5} [%]	[μg]	m_{p6} [%]	[μg]	m_{p7} [%]	[μg]	m_{p8} [%]	[μg]
150							1.05	0.343								
120			1.4	0.328			2.14	0.732	1.47	0.362	1.36	0.324	1.2	0.295	1.57	0.480
100															1.51	0.462
90	1.36	0.198	1.56	0.365	1.58	0.303	2.23	0.763								
70	5.57	0.813	2.1	0.491			4.54	1.553	2.01	0.494	1.8	0.428	1.6	0.394	5.74	1.756
67					3.11	0.597										
66	2.32	0,338s														
65											3.37	0.802			4.34	1.328
60			3.1	0.725	3.79	0.727	3.59	1.228	4.86	1.196			4.07	1.001		
50	5.24	0.765	3.6	0.842	4.18	0.803	6.83	2.336	10.67	2.625	12.94	3.080	12.65	3.112	3.99	1.221
43	5.59	0.816,					5.06	1.731								
41			3.3	0.772							4.1	0.976				
40			3.8	0.889			3.66	1.252								
38													4.33	1.065	11.49	3.165
36	8.55	1.248	9.96	2.331	8.71	1.672	9.97	3.410	10.38	2.553	8.04	1.91	7.69	1.892	3.28	1.004
28			3.47	0.812	3.53	0.677										
26			3.69	0.864	7.54	1.486	3.02	1.033	3.01	0.741	4.74	1.128	3.458	0.851		
23							3.12	1.067					3.27	0.804		
22																
21	10.98	1.603	7.49	1.752	11.49	2.206	4.70	1.607	3.46	0.851	6.35	1.511	2.57	0.632	6.89	2.108
20																
18	3.54	0.517	4.56	1.067	4.9	0.941			3.46	0.851	3.52	0.838			2.78	0.851
17													2.63	0.647		
16													6.23	1.533		
15.5	3.76	0.549	4.08	0.955			10.90	3.728	9.22	2.268	3.52	0.838				
15	3.91	0.571			3.45	0.662	5.3	1.813			4.14	0.985	3.23	0.794	4.53	1.386
12.5															2.27	0.695
12			2.06	0.482			5.7	1.949	3.79	0.932	3.13	0.745	1.11	0.270	1.84	0.551

Abbreviations: m_{pi} - mass of protein bands in HV electrophoresis; m_{p1} - 14.6 μg (control), m_{p2} - 23.4 μg (*N. tabacum* + pCB1334), m_{p3} - 19.2 μg (*N. tabacum* + pCB1349), m_{p4} - 34.2 μg (*N. tabacum* + pCB3013), m_{p5} - 24.6 μg (*N. tabacum* + pCB3016), m_{p6} 23.8 μg (*N. tabacum* + pCB3026), m_{p7} - 24.6 μg (*N. tabacum* + pCB3028), m_{p8} -30.6 μg (*N. tabacum* + pCB3029). The deviation of M_r determinations: up to 90 kDa ± between 90 kDa - 15 ± 1.5 kDa, less than 15 kDa ± 0.5 kDa.

(gene 5) line 5, showed several quantitative differences in the protein spectrum regarding to untransformed control. Pattern has showed changes in 120, 70, 60, 55, 38-35, 26-23 and 15.5 kDa proteins. The portion of 60-55 kDa proteins was decreased, meanwhile the portion of proteins with M_r 26 kDa was increased.

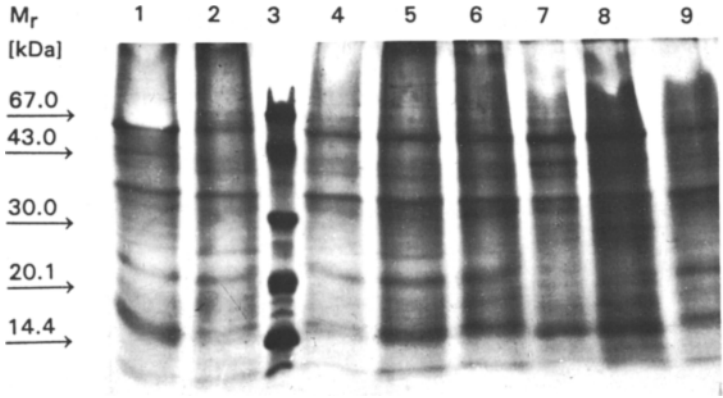

Fig. 2. Low voltage electrophoresis of proteins of transgenic tobacco plants.

	c_p [g l^{-1}]
line 1 - *Nicotiana tabacum* cv. Samsun	1.46
line 2 - *Nicotiana tabacum* cv. Samsun + pCB1334 T-DNA	2.34
line 3 - M_r kit *Serva*: albumine (67 kDa), ovalbumine (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), alpha-lactoalbumine (14.4 kDa)	
line 4 - *Nicotiana tabacum* cv. Samsun + pCB1349 T-DNA	1.92
line 5 - *Nicotiana tabacum* cv. Samsun + pCB3013 T-DNA	3.42
line 6 - *Nicotiana tabacum* cv. Samsun + pCB3016 T-DNA	2.46
line 7 - *Nicotiana tabacum* cv. Samsun + pCB3026 T-DNA	2.38
line 8 - *Nicotiana tabacum* cv. Samsun + pCB3028 T-DNA	2.46
line 9 - *Nicotiana tabacum* cv. Samsun + pCB3029 T-DNA	3.06

The volume of the sample V_s 20 µl, U = 3 V cm^{-1}, I = 15 mA, concentration gradient 10/20 %, duration of electrophoresis 4.5 h.

T-DNA of plasmid pCB3016 (genes 4, 5 and 6b) changed tobacco protein spectrum in peptids with M_r: 60, 55, 48, 26, 21 and 18 kDa (Fig. 1, line 6). Transgenic tobacco obtained by transformation with T-DNA of the vector plasmid pCB3026 (gene 6b), line 7, showed some changes in the range of 70-43 kDa. The proteins with M_r 70 and 60 kDa were decreased meanwhile proteins with M_r 55, 43, 22 and 18 kDa were increased. T-DNA of the plasmid pCB3028 (genes 5 and 6b), line 8, differed from the control in 60, 55, 26, 23-21 and 18-17 kDa proteins. In comparison with the protein spectrum of the transgenic plant modified by plasmid pCB3016, there were differences in 70, 60, 55 kDa proteins and low M_r proteins from the range 30-20 kDa. The overall content of purified proteins differed only slightly and the relative ratio was equal in relation to the crude protein.

Transgenic tobacco carrying T-DNA of the vector plasmid pCB3029 differed from the control mostly in the proportion of high M_r components (Fig. 1, line 9, Fig. 2). Its spectrum showed clear-cut differences in proteins with M_r 150, 120, 70 and 60, 55 and 43 kDa, which were strongly depressed. Quantity of proteins with M_r 22-21,18 and 16 kDa was increased. In comparison with the action of the gene 4 carried into the plant genome by the T-DNA of the vector pCB1334, there were differences in protein with M_r 60, 55 and 27 kDa.

Discussion

The variation in the protein spectra observed are likely to be due to regulatory differences caused by the expression of T-DNA phytohormone synthesis genes 1,2 and 4 and/or interacting genes 5 and 6b. The effects might be on the level of transcription, translation and/or stabilities of mRNA or proteins. If plants and not tumors differentiate after transformation by T-DNA genes, it does not necessarily mean that the T-DNA genes were fully inactivated. The morphogenetic action of T-DNA genes could also be suppressed by plant regulation mechanisms. Amount of phytohormones in the transgenic plants is under control genes 1, 2 and 4 (Ondřej *et al.* 1989, Matzke and Matzke 1990, Beinsberger *et al.* 1991) and the plant response to phytohormones is modified by genes 5 and 6b (Spanier and Schell 1989, Körber *et al.* 1991). The morphoregulatory action of increased level of endogenous phytohormones could be suppressed by plant regulation mechanisms.

The possibility that T-DNA genes products contribute to observed protein spectra changes cannot be excluded. Barker *et al.* (1983) have studied nucleotide sequence of T-DNA of the octopine pTi 15 955 and identified the following proteins expressed from T-DNA genes: 74 kDa (gene 1), 49 kDa (gene 2), 27 kDa (gene 4) and proteins with M_r 30, 16, 15 and 5 kDa. The gene 4 in our experiments increase the proportion of 27, 21 and 17 kDa, which would indicate that some of them could be subunits of isopentenyltransferase. The indolyl lactate synthase subunits has M_r 26 kDa (Körber *et al.* 1991). An increase in the proportion of this band in transformants that carying gene 5 was also observed. Several other possible instances of the direct contribution of primary products of T-DNA genes could be inferred from our experiments, but they can be proven by immunobloting studies only.

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