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Changes of shikimate pathway in glyphosate tolerant alfalfa cell lines with reduced embryogenic ability

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Abstract

Glyphosate tolerant cell lines were selected from highly embryogenic cell suspension culture of *Medicago sativa* L. Resistant cell lines showed significant reduction of embryogenic ability and during long-term culture in the presence of glyphosate gradual loss of this ability was observed. After glyphosate treatment the increased activity of 5-enolpyruvylshikimate-3-phosphate synthase in tolerant cell lines overcame the block in aromatic amino acid synthesis which was observed in control cell lines. Glyphosate caused marked increase in the content of shikimic acid in both control and tolerant cell lines but the accumulation of shikimic acid was considerably lower in tolerant calli. Significant qualitative and quantitative differences were found in the content of individual phenolic acids. The considerable decrease in the amount of cinnamic acid derivates and broader spectrum of hydroxybenzoic acids suggest in tolerant cell lines the activation of alternative pathway not regulated by phenylalanine ammonia lyase. The possible role of altered pool of phenolic acids on the embryogenic ability is discussed.

Introduction

Glyphosate is a potential inhibitor of the shikimate pathway enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) (Steinrücken and Amrhein 1980). *In vitro*

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Abbreviations: C-calli - control calli; 2,4-D - 2,4-dichlorophenoxyacetic acid; EPSPS - 5-enolpyruvylshikimate-3-phosphate synthase; f.m. - fresh mass; HPLC - High Performance Liquid Chromatography; KIN - kinetin; T-calli - glyphosate tolerant calli.
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selection for glyphosate resistance in higher plants has only yielded tolerant cell lines with increased EPSPS activity. In cell cultures of petunia, carrot and tobacco over-production of EPSPS was the result of amplification of genes encoding this enzyme (Shah *et al.* 1986, Shyr and Widholm 1990, Goldsbourgh *et al.* 1990). Amplification of EPSPS was maintained during regeneration from glyphosate tolerant cell lines in tobacco (Wang *et al.* 1991). In *Corydalis sempervirens* transcriptional or post-transcriptional alternations have led to the increased level of EPSPS in the absence of changes in EPSPS gene copy number (Holländer-Czytko *et al.* 1988). Inhibition of EPSPS activity is the only known primary site of glyphosate action. Consequently it causes reduction of free aromatic amino acid pool accompanied by significant changes of protein metabolism and of metabolism of secondary aromatic compounds (Hoagland *et al.* 1979, Holländer and Amrhein 1980). Glyphosate promoted IAA level in *Cyperus esculentus* leaves is explained as a consequence of increased level of IAA protecting phenolic acids observed in plants after glyphosate treatment (Cañal *et al.* 1987) and thus metabolism of auxins can be indirectly affected.

To obtain stable glyphosate tolerant cell lines with amplified EPSPS genes long-term exposure to glyphosate or stepwise selection procedure are efficiently used. Regeneration of plants in the presence of glyphosate is usually unsuccessful and also regeneration ability of glyphosate tolerant cell lines cultivated in the absence of glyphosate is highly reduced (Dyer *et al.* 1988, Wang *et al.* 1991). Significant reduction of embryogenic potential was also observed in glyphosate tolerant alfalfa cell lines in our experiments. Regenerated plants showed slightly increased tolerance to glyphosate but many phenotypical and caryological abnormalities occurred and the loss of fertility prevented to test their progenies. The long-term culture of alfalfa cell lines on glyphosate caused the gradual loss of embryogenic potential. Since little is known about metabolic alterations in plant cells upon long-term exposure to glyphosate we studied changes in EPSPS activity, in protein and phenylpropanoid metabolism in relation to the loss of embryogenic ability.

Materials and methods

Plant material: Embryogenic cell suspension culture of *Medicago sativa* L. ($2n = 4x = 32$) was initiated and maintained as described earlier (Binarová and Doležel 1988).

Selection *in vitro*: Cell suspensions in the exponential growth phase with a density around $2\text{-}10\times10^{-4}$ living cells cm^{-3} were plated on BL medium (Blaydes 1966) containing 5 μM 2,4-D and 1 μM KIN. Growth inhibiting concentration of glyphosate evaluated in preliminary experiments was 1.5 - 2 mM. Plated cells were cultivated on control and glyphosate media in the dark at 26 °C.

Long-term culture of calli after selection: After three weeks culture both control (C) and glyphosate tolerant (T) cell lines were isolated and maintained on Bl medium supplemented with 5 μM 2,4 D and 1 μM KIN for 3 months in the absence of glyphosate. To study the stability of tolerance to glyphosate T-calli were exposed for one week to glyphosate and then harvested for enzyme assays, amino and phenolic

acid analyses (I). Part of T-calli was further cultured on glyphosate medium for one year. Long-term cultured T-calli were then cultured on the medium with or without glyphosate for 3 months and, in the exponential phase of growth, harvested for biochemical analysis (II).

C-calli were in parallel cultured on control medium, exposed to glyphosate and harvested for analyses in the same time schedule as T-calli.

5-enolpyruvylshikimate-3-phosphate synthase assay: Extraction and enzyme purification were done according Steinrücken *et al.* (1986). EPSPS activity was measured by the determination of inorganic phosphate using malachite green dye assay method according Lanzeta *et al.* (1979). Protein concentration was determined according Bradford (1976).

Amino acid analysis: Free amino acids were extracted with 80 % ethanol at 27 °C for 24 h. The samples were analysed by the means of automatic amino acid analyser AAA 339 (*Microtechna*, Praha, Czech Republic).

Determination of phenolic acids: Phenolic acids were extracted as described earlier (Cvikrová 1988). Briefly, free (F1), ester-bound (F2-released after alkaline hydrolysis) and glycoside-bound phenolic acids (F4-released after acid hydrolysis) were obtained from a methanol extract of tissue ground in liquid nitrogen. The fraction of cell wall-bound phenolic acids (F3) was obtained after alkaline hydrolysis of the residual material after methanol extraction. Phenolic acids were analysed by the means of HPLC using a *Pye Unicam PU 4002-Video Liquid Chromatograph* with a *Spherisorb 5 ODS* column. Elution conditions were described by Cvikrová *et al.* (1991). The column eluate was monitored at 260 and 300 nm using a *Multichannel detector PU 4021*. Authentic compounds (*Serva, Germany*) were used as references for quantitative analyses.

Determination of shikimic acid: Shikimic acid was extracted by modified method of Lydon and Duke (1988). A *Spherisorb 5 ODS* column and HPLC solvents identical as in phenolic acid analysis were used in the shikimic acid assay. The column eluate was monitored at 230 nm and RT for shikimic acid was 7.00 min (Fig. 2). Authentic compound (*Serva*) was used for quantitation.

Presentation of results: Absolute values of EPSPS activity, the levels of phenolic acids and of shikimic acid depended largely on the physiological state of subcultivated calli. Therefore the results of one representative experiment are presented. All experiments were repeated at least three times with 3 parallel determinations with deviations not exceeding 10 to 15 %.

Results and discussion

Characterization of glyphosate tolerant cell lines: T-cell lines retained unchanged tolerance to glyphosate even after 3 months growth in its absence. Regeneration ability of T-cell lines was reduced (as compared with C-lines) but during the first three months after selection it was still possible to regenerate plants. Slight decrease

in the sensitivity to glyphosate varied among individual regenerated plants. Many morphological abnormalities often accompanied by caryological instabilities were observed in alfalfa plants regenerated from T-cells (data not shown), similarly as described in the experiments of Dyer *et al.* (1988), Singer and McDaniel (1985) and Wang *et al.* (1991). T-cell lines lost gradually their embryogenic potential during one year culture on glyphosate (II); calli became soft, white and their growth rate decreased.

5-enolpyruvylshikimate-3-phosphate synthase activity: T-cell lines yielded higher EPSPS activity than C-lines even after 3 months growth in the absence of glyphosate (Fig. 1). Exposure to glyphosate led in the C-cell lines to the reduction of EPSPS activity to more than 50 %, while in the T-cell lines, 20 % increase was observed. The elevated level of EPSPS activity in tolerant cells is known to overcome glyophosate inhibition of the enzyme (Smart *et al.* 1985). It was found out that the overproduction of EPSPS is stable during the culture without glyphosate (Singer and McDaniel 1985, Dyer *et al.* 1988). The amplification rate for EPSPS genes correlated with glyphosate concentration and the length of exposure and varied among different plant cell cultures (Shyr and Widholm 1990, Goldsbrouhg *et al.* 1990). Surprisingly no further increase in EPSPS activity was observed in the T-cell lines of alfalfa after one year culture on medium with glyphosate (compared with the activity in the T-cell lines isolated from glyphosate after 3 week selection in I, Fig. 1). The ratio between EPSPS activity of C and T-cell lines was similar regardless of the length of culture in the presence of glyphosate (Fig. 1).

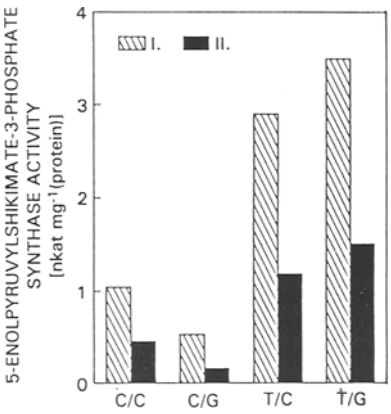

Fig. 1. The effect of glyphosate on EPSPS activity in control (C) and tolerant (T) alfalfa calli cultivated on control (C) and glyphosate (G) media.
I.- four months after selection;
II.- long-term culture.
Each value represents the mean of 3 replicates. C/C - control calli on control medium;
C/G - control calli on medium with glyphosate; T/C - tolerant calli on control medium;
T/G - tolerant calli on medium with glyphosate.

Amino acids: Control cell lines showed after exposure to glyphosate about four-fold reduction in free phenylalanine and tyrosine contents. No significant reduction of aromatic amino acid levels was observed in T-cell lines (I) after exposure to glyphosate (Table 1).

Long-term culture of both C and T-cell lines (II) caused significant decrease in the pool of free amino acids (compared with the results of I). After exposure to glyphosate the synthesis of aromatic amino acids in the C-calli was strongly (ten-fold) inhibited while in the T-calli long-term cultured with glyphosate the levels of

phenylalanine and tyrosine were increased (Table 1). Similar increase of aromatic amino acids after glyphosate treatment was described without deeper explanation in tobacco cells long-term selected on glyphosate (Dyer *et al.* 1988).

Table 1. Content of amino acids in control and tolerant alfalfa calli [μmol g^{-1} (f.m.)].

Amino acid	Control calli		Tolerant calli	
	control medium	medium with 2.5 mM glyphosate	control medium	medium with 2.5 mM glyphosate
I. four months after selection				
Asp	305 ± 44	78 ± 6	181 ± 14	332 ± 34
Pro	641 ± 80	711 ± 55	821 ± 87	945 ± 65
Ala	655 ± 105	232 ± 78	1295 ± 250	644 ± 69
Tyr	1251 ± 190	328 ± 83	771 ± 89	663 ± 69
Phe	2396 ± 400	579 ± 89	1547 ± 280	1356 ± 160
II. long-term culture				
Asp	131 ± 18	142 ± 20	521 ± 80	221 ± 30
Pro	672 ± 61	624 ± 89	120 ± 18	564 ± 80
Ala	648 ± 89	399 ± 89	190 ± 38	648 ± 45
Tyr	170 ± 10	35 ± 15	96 ± 14	278 ± 59
Phe	1021 ± 210	148 ± 21	311 ± 24	895 ± 79

Abbreviations: Ala - alanin; Asp - asparagin; Pro - proline; Phe - phenylalanine; Tyr - tyrosine

Shikimic acid and phenolic acids: Glyphosate caused significant increase in the content of shikimic acid in both C and T-calli (Table 2, Fig. 2). The T-calli (I) contained only 80 % of the amount of shikimic acid found in the C-calli after glyphosate treatment. The decline in shikimic acid content was more pronounced in

Table 2. The effect of glyphosate on shikimic acid content in control (C) and tolerant (T) alfalfa callus cultures cultivated on control (C) and glyphosate (G) media.

	Shikimic acid [nmol g^{-1}(f.m.)]			
	C/C	C/G	T/C	T/G
I. four months after selection	0	113.56 ± 8.02	0	97.13 ± 5.26
II. long-term culture	0	102.14 ± 6.90	trs	62.18 ± 3.84

C/C - control calli on control medium; C/G - control calli on medium with glyphosate; T/C - tolerant calli on control medium; T/G - tolerant calli on medium with glyphosate; trs - traces of shikimic acid

the T-calli long-term cultured on glyphosate (60 % of the content determined in the C-calli in II). No shikimic acid accumulation was detected in C and T-calli without glyphosate treatment.

The shikimic acid pathway is responsible for the synthesis of most phenolic compounds found in higher plants. The effect of glyphosate on shikimate and

hydroxybenzoate accumulation in treated plants was described (Lydon and Duke 1988) while data describing the alteration of phenolic metabolism pattern in glyphosate tolerant plants or cell lines are still missing. In addition to *p*-hydroxybenzoic, vanillic, anisic and salicylic acids (found also in C-calli), in T-calli in all phenolic fractions also gallic, 2,3-dihydroxybenzoic and gentisic acids were detected (Table 3). The broader spectrum of benzoic acids that we found in the T-calli is in agreement with the results obtained in glyphosate treated yellow nutsedge (Cañal *et al.* 1987a) or pigweed and velvetleaf (Lydon and Duke 1988).

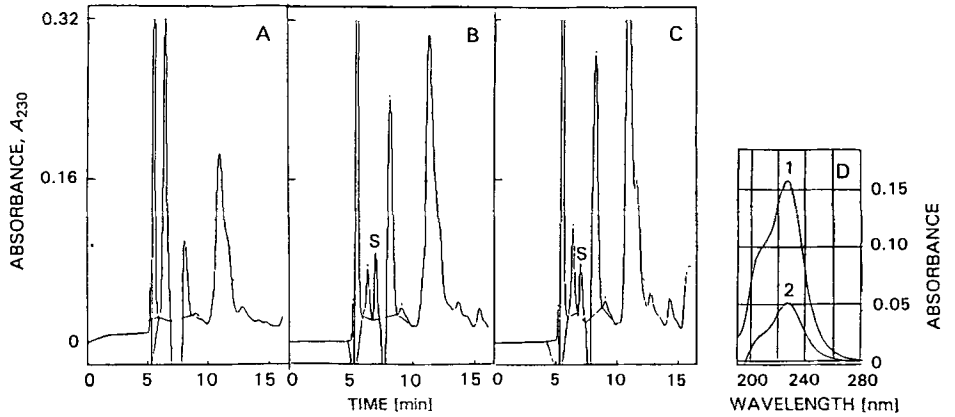

Fig. 2. HPLC analysis of shikimic acid in acid-hydrolysed extract of control and tolerant alfalfa calli cultured on control and glyphosate supplemented media four months after selection. Each profile represents an equivalent amount of extract, normalized on a volume of extract per mg of tissue basis. A - control calli on control medium (no detectable amounts of shikimic acid was found); B - control calli on medium with glyphosate; C - tolerant calli on medium with glyphosate; D - UV spectra of standard (1) and shikimic acid isolated from tolerant calli (2);S - shikimic acid.

The level of phenolic acids in T-calli decreased with prolonged culture on glyphosate but there were not found any further qualitative changes (data not shown). The increase in the level of hydroxybenzoic acids, especially the appearance of gentisic acid, *p*-dihydroxyphenolic acid with possible auxin protection activity (Cañal *et al.* 1987b), can affect the balance of phytohormones and subsequently to influence the embryogenic ability of T-cells. The extremely low level of ferrulic acid in the cell wall-bound fraction of phenolics could explain the changed morphology of cells of T-calli, which were highly vacuolized and elongated and might be also connected with the loss of embryogenic potential. The oxidative coupling of phenolic side-groups between extensin (Fry 1986), feruoyl substituents of pectins and between structural polymers and other cell wall glycoproteins is probably prerequisite for successful morphogenesis (Fry 1990). The restriction of cell elongation and reduction of the cell wall plasticity might be also essential for somatic embryogenesis (Goldberg *et al.* 1986). We supposed, considering the high content of phenylalanine (which indicates the improved carbon flow through the shikimate pathway) that the

Table 3. Content of individual phenolic acids in control (C) and tolerant (T) alfalfa callus cultures four months after selection (I). F_1 - free phenolic acids; F_2 - ester-bound methanol soluble phenolic acids, F_3 - ester-bound cell wall phenolic acids, F_4 - glycoside-bound phenolic acids. Presented values are the means of 3 determinations with deviations not exceeding 15%.

		Phenolic acids [nmol g^{-1} (f.m.)]										
	Fractions of phenolic acids	Benzoic acid derivatives							Cinnamic acid derivatives			
		pHBA	VA	AA	SaA	2,3dHBA	GA	GaA	FA	pCA	SiA	CA
C-calli on control medium	F_1	1.23	0.83	0.18	1.33	-	-	-	0.72	0.63	0.40	0.23
	F_2	2.61	1.79	0.32	-	-	-	-	18.90	3.50	5.65	0.11
	F_3	1.04	0.49	0.11	-	-	-	-	1.10	0.43	-	-
	F_4	42.18	11.49	1.59	-	-	-	-	0.76	1.43	-	-
T-calli on 2.5 mM glyphosate	F_1	0.45	0.88	0.01	0.11	0.79	0.36	0.21	0.02	0.05	-	-
	F_2	2.25	1.27	0.03	-	1.46	4.44	2.54	0.53	0.32	0.86	-
	F_3	0.89	0.44	0.04	-	0.59	0.56	0.37	0.04	0.07	-	-
	F_4	7.69	11.23	-	-	2.87	4.04	4.20	-	-	-	-

Abbreviations: AA - anisic acid; CA - cinamic acid; pCA - p-coumaric acid; FA - ferulic acid; f.m. - fresh mass; GA - gentisic acid; GaA - gallic acid; 2,3dHBA - 2,3-dihydroxybenzoic acid; pHBA - p-hydroxybenzoic acid; SaA - salicylic acid; SiA - sinapic acid; VA - vanillic acid; benzoic acid derivatives - pHBA, VA, AA, SaA, 2,3dHBA, GA, GaA; cinnamic acid derivatives - FA, pCA, SiA, CA.

biosynthesis of cinnamic acid derivatives would be modified and increased. But the extremely low level of cinnamic acid as well as the increase in the number of individual benzoic acids suggest that the alternative biosynthesis of phenolic substances activated by glyphosate persisted in T-cell lines in spite of their stable tolerance to glyphosate based on increased EPSPS activity.

In conclusion, we can summarize that T-cell lines overcame the block in the synthesis of aromatic amino acids by the increased EPSPS activity which is stable during culture in the absence of glyphosate. High levels of benzoic acids and markedly decreased content of cinnamic acids in T-cells suggest the activation of alternative biosynthesis of phenolics, that persisted in long-term cultured T-calli in spite of considerably increased content of phenylalanine. The altered biosynthesis of phenolic acids might be one of the causes of the loss of embryogenic ability of T-cells.

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