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The heritable changes in metabolic profiles of plants regenerated in different types of *in vitro* culture

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Abstract

In this work we show how three types of cucumber *in vitro* cultures – leaf callus culture, cytokinin dependent cell suspension and liquid culture of meristematic clumps – influence the metabolite profiles of plants in the first generative progeny. Based on this study we conclude that there exists a specific and inheritable metabolic fingerprint reflecting the history of previous generations, probably related to specific stress factors accompanying the passage through different types of culture. The leaf callus culture generated the highest heritable differences in metabolite content and was the most distinctly separated cluster in PCA analysis. The smallest number of variable metabolites characterizes the plants regenerated from cytokinin dependent cell suspension whereas the liquid culture of meristematic clumps induced slightly more changes. Changes induced by these two culture types were not as pronounced as in the case of leaf callus culture. However the plants after these types of culture were well separated from the control on PCA diagram. The highest changes were over 2-fold increases in cystin and galactose-6-P and over 2-fold decreases in aspartate, myo-inositol, hydroxylamine, phosphate and putrescine. These changes concerned the plants, which were one generation after the leaf callus culture. The possible nature of observed heritable changes is discussed.

Abbreviations: CDS – cytokinin dependent cell suspension; 2,4-D – 2,4-dichlorophenoxyacetic acid; GC/MS – gas chromatography/mass spectrometry; LC – leaf callus culture; LMC – liquid culture of meristematic clumps; PCA – principal component analysis

Introduction

The plants regenerated *in vitro* may show variability in many characters such as morphological traits, pathogen resistance, content of certain compounds and many others. Many of these changes are heritable and are known as somaclonal variation (Larkin and Scowcroft, 1981). There are many factors influencing the frequency and type of somaclonal changes but according to Karp (1991) the dependence of such variability of culture type is the least understood. Generally, it is assumed that meristem and shoot primordia culture are the regeneration systems that give plants genetically stable and free of somaclonal variation.

The cucumber (*Cucumis sativus* L.) is an example where the influence of type of culture on somaclonal variation was recently under investigation (Burza and Malepszy, 1995; Plader et al., 1998; Ładyżyński et al., 2002). It was demonstrated, that two types of cell suspension, liquid meristematic clumps culture, recurrent leaf callus

regeneration and direct protoplast regeneration differently induce variation with a frequency ranging from 3.4 to 90% of lines.

Progress recently observed in analysis of metabolites also allows the relatively easy assessment of metabolite profiles of plant regenerated in different types of tissue culture. However we could not find this type of information in the literature. In this work we present the influence of three types of culture on metabolic profiles in the first generative progeny.

Materials and methods

All plants derived from the highly inbred line (I15) of *Cucumis sativus* cv. Borszczagowski and were grown on standard soil in computer directed greenhouse chambers in 16 h light/8 h dark photoperiod during spring season (March–April). Light intensity was fixed to 120 μmol m^{-2} s^{-1}. The plants, which passed *in vitro* regeneration, were R1 generation (obtained after self pollination of regenerants), fenotypically wild type, diploid, produced from leaf callus culture (LC), liquid culture of meristematic clumps (LMC) and cytokinin dependent cell suspension culture (CDS) as previously described (Ładyżyński et al., 2002). In the present work we used plants, which were the progeny of seven independent regenerants from LC, 11 independent regenerants from LMC and 11 independent regenerants from CDS. The results were compared with control measurements made on eight seed derived plants (propagated generatively only). For each independent regenerant we collected samples from five progeny plants. The samples were collected from three young, fully expanded leaves on each plant analyzed. The assayed plants did not show visible phenotypic changes as compared to the control.

Extraction and fractionation was performed as described previously (Fiehn et al., 2000). Metabolic profiles were acquired by GC/TOF MS analysis using an Agilent 6900 gas chromatograph coupled to a LECO time-of-flight mass spectrometer (Leco, St. Joseph, USA) on a 30 m RTX-5 column (250 μm ID, 0.25 μm film) with 10 m integrated pre-column, Restek (Germany). Peak finding and quantification of selective ion traces was accomplished using the instrument's MassLab FindTarget Software. The differences between metabolites were determined to be statistically significant by the performance of *t*-test ($p < 0.01$). The metabolites monitored constituted five groups: amino acids, organic acids, sugars, alcohols, sugaralcohols and other metabolites (Table 1). For principal component analyses (PCA) the Statgraphics software (Manugistics, Rockville, USA) was used. The results of these analyses were then presented as a three- or two-dimensional graphical display of the data in which a single sample or metabolite is represented by a point in three- or two-dimensional space, respectively.

Results

Regeneration of plants in the tissue culture generates or conserves numerous morphological, physiological and genetical changes, which can be observed in the progeny. In our experiments we monitored the biochemical changes in morphologically unchanged progeny of plants, which were regenerated via three different methods. For this purpose we used the metabolic profiling technique using the GC/MS analyzes with a quite simple and rapid sample preparation. This allowed us to evaluate the regeneration systems in respect of the ability to cause somaclonal, biochemical changes, which could be a result of induced genetic or heritable epigenetic variability. We identified and compared 78 metabolites in the progeny of regenerated plants. Each tissue culture system had its characteristic set of changed compounds: 55 in LC, 35 in CDS, 41 in LMC (Table 1). Changes in CDS and LMC R1 progeny were not only less numerous but usually not so pronounced like in progeny of LC derived plants. The weakest changes were observed in CDS plants.

In the R1 progeny of regenerated in LC culture plants we could find many well distinguishable markers which were specifically changed in these plants or changes in CDS and LMC plants were of much lower intensity.

In the group of amino acids the characteristic marker for LC regeneration system was elevated content of cystin by a factor of 2.13. Cystin is known to be a compound readily reacting on stress conditions. The other markers of callus-derived plants are aspartate, and tryptophan, which were decreased by 50 and 39%, respectively. CDS and

Table 1. The mean values of metabolite change ratios in the progeny of plants regenerated in different culture conditions as compared to seed derived control

Metabolite	LC	$\pm\sigma_n$	CDS	$\pm\sigma_n$	LMC	$\pm\sigma_n$	Pattern
Amino acids							
Aspartate	0.50	0.01	ns	–	0.95	0.01	I
Beta-alanine	ns	–	ns	–	0.94	0.01	VI
Cystine	2.13	0.01	ns	–	ns	–	IV
GABA	0.76	0.01	0.96	0.01	0.96	0.01	I
Glutamate	0.88	0.03	ns	–	0.96	0.00	I
Leucine	0.78	0.01	0.83	0.02	0.84	0.02	II
Ornithine	0.71	0.03	1.10	0.01	1.08	0.01	III
Phenylalanine	1.06	0.01	0.94	0.01	ns	–	VII
Serine	0.75	0.02	0.95	0.01	0.94	0.00	I
Threonine	1.09	0.02	ns	–	ns	–	IV
Tryptophane	0.61	0.02	0.85	0.01	0.90	0.01	II
Tyrosine	0.77	0.05	ns	–	ns	–	I
Valine	0.71	0.00	0.92	0.01	0.95	0.00	II
Organic acids							
Arachidic	0.91	0.01	ns	–	0.95	0.01	VII
Ascorbic	0.77	0.01	0.79	0.02	0.63	0.03	VII
Benzoic	1.17	0.02	ns	–	1.16	0.03	VII
Citramalic	ns	–	1.03	0.01	0.98	0.00	VII
Citric	0.65	0.03	0.89	0.01	0.91	0.01	II
Fumaric	0.85	0.01	1.12	0.01	1.14	0.01	III
Galactonic	0.89	0.01	1.04	0.01	ns	–	VII
Gluconic	1.79	0.01	ns	–	ns	–	IV
Lactic	0.68	0.03	1.12	0.02	ns	–	VII
Maleic	0.86	0.01	1.06	0.01	1.15	0.01	III
Malic	0.74	0.01	1.07	0.01	1.05	0.01	III
Malonic	0.75	0.01	ns	–	0.91	0.01	VII
Palmitic	1.41	0.02	1.04	0.01	ns	–	IV
Shikimic	0.74	0.02	1.04	0.01	ns	–	III
Stearic	0.96	0.00	1.04	0.01	ns	–	III
Succinic	1.17	0.02	ns	–	1.13	0.01	VII
Sugars							
Fructose	0.82	0.01	ns	–	1.21	0.01	III
Fructose-6-P	1.21	0.01	ns	–	0.94	0.01	IV
Fucose	0.75	0.03	ns	–	0.97	0.01	I
Galactose-6-P	2.25	0.01	ns	–	0.89	0.01	IV
Glucose-6-P	ns	–	1.04	0.01	0.95	0.01	VII
Glucose	0.88	0.01	ns	–	1.22	0.01	III
Maltose	1.42	0.02	1.39	0.02	1.20	0.01	V
Mannose-6-P	1.82	0.00	1.16	0.01	ns	–	V
Sucrose	1.36	0.02	1.11	0.01	1.07	0.00	V
Trehalose	0.59	0.03	1.07	0.01	1.16	0.02	III
Alcohols							
Glycerol	0.78	0.01	0.90	0.01	ns	–	II
Glycerol-2-P	1.22	0.02	ns	–	ns	–	IV
Indol derivatives	1.56	0.02	1.06	0.01	0.92	0.02	IV
Inositol-2-P	1.32	0.01	0.92	0.01	ns	–	IV

Table 1. (Continued)

Metabolite	LC	$\pm\sigma_n$	CDS	$\pm\sigma_n$	LMC	$\pm\sigma_n$	Pattern
Lyxitol	1.18	0.01	ns	–	1.04	0.01	IV
Xylitol	1.04	0.01	ns	–	ns	–	IV
Sugaralcohols							
Ribitol	1.49	0.04	1.03	0.00	1.04	0.01	IV
Meso-inositol	ns	–	ns	–	1.06	0.01	VI
Myo-inositol	0.37	0.00	ns	–	1.02	0.01	I
Sorbitol	ns	–	0.96	0.01	0.92	0.01	VI
Other metabolites							
Butanoate tri-OH lct	1.67	0.03	1.08	0.01	1.09	0.01	V
Butanol-2-amino-4-OH	ns	–	ns	–	0.94	0.01	VI
Citrulline	1.42	0.02	1.26	0.03	1.16	0.02	V
Ethanolamine	0.89	0.01	ns	–	ns	–	I
Hydroxylamine	0.43	0.00	ns	–	0.96	0.00	I
Org-P	1.70	0.03	1.17	0.02	ns	–	V
Phosphate	0.41	0.02	1.04	0.00	1.02	0.00	I
Pipecolate	0.91	0.01	0.85	0.01	0.90	0.00	VII
Putrescine	0.45	0.01	ns	–	1.08	0.01	III
Spermidine	1.31	0.01	0.96	0.01	ns	–	IV
Urea	1.14	0.01	1.46	0.02	ns	–	VII

Only significant (t -test, $p < 0.01$) differences are given (ns – not significant; $\pm\sigma_n$ – standard error, the 0.00 value of σ_n means that it is below 0.005). The common patterns of changes are numbered: I – strong decrease in LC, in CDS and LMC changes ns or very small; II – decreases in all culture types LC > CDS > LMC, in LMC sometimes ns; III – decrease in LC, increase elsewhere, in CDS often intermediate value or ns; IV – strong increase in LC, in CDS and LMC changes ns or very small; V – increases in all culture types LC > CDS > LMC, in LMC sometimes ns or = CDS; VI – LMC increases or decreases, other ns or smaller than LMC; VII – non classified; often LC and LMC reacts similarly whereas CDS ns. The following metabolites did not show significant changes in either of regeneration systems and they are not listed in the table – amino acids: alanine, asparagine, glutamic acid, glycine, isoleucine, lysine, proline; organic acids: caffeic, cinnamic, glyceric, isocitric, oleic; sugars: raffinose, xylose, xylulose; alcohols: galactitol, glycerol-3-P; other: homoserine.

LMC derived plants did not show such drastic changes in the amino acid content.

While monitoring organic acids fluctuations we observed a strong increase by 79% in gluconic acid and 35% decrease in citric acid content in LC derived plants. Interesting changes were observed in the case of ascorbic acid which decreased, in all regeneration systems tested but the strongest decrease was not as usually in LC plants (23%) but in CDS progeny (37%). Decreases in ascorbic acid were the highest among metabolites tested in plants after LMC and CDS culture (Table 1).

The progeny of plants regenerated from LC manifests the highest variation also in case of sugars, alcohols, sugaralcohols and other metabolites. Quite striking is high content of galactose-6-P increased by 125% which is the highest shift observed in our screen, and mannose which increased by 82%. The same plants showed a decrease in trehalose by 41%. Two other sugars, fructose and glucose, which showed a decrease in LC plants react in opposite way in plants after LMC culture, i.e. they increased by 21 and 22% and this were the highest positive reactions in plants after LMC culture.

In sugars and sugaralcohols the maximal changes were again observed in LC derived plants progeny whereas in CDS and LMC plants the differences were rather small. The indol derivatives showed here increase by 56% and myo-inositol decrease by 63% (the highest decrease among all compounds tested).

In the group of other metabolites we observed diverse changes. The most significant decreases were observed for hydroxylamine, phosphate and putrescine (by 57, 59 and 55%, respectively) and these changes could be the markers of LC culture

similarly to the biggest increase in that group in case of butanoate (by 69%). Here we could distinguish the only clear marker of the CDS regenerants progeny – the highest urea content (increased by 46%). The other metabolites content in CDS culture behaved usually as an intermediate value between LC culture and the LMC culture.

Several metabolites changed in similar fashion in three regeneration systems tested. Therefore the cluster analysis was done identifying seven patterns of changes (Table 1). First three patterns showed decreased level of a given metabolite in LC derived plants whereas in CDS and LMC they were not significantly changed (pattern I), in CDS and LMC the metabolite was also decreased but to much lower extent (pattern II) or in CDS and LMC there was an opposite reaction – an increase in a given metabolite level (pattern III). The following two patterns are characterized by an increase of metabolite level in LC derived plants whereas in CDS and LMC there were no changes (pattern IV) or there was also an increase but weaker than in LC (pattern V). Pattern VI characterizes weak although specific changes in LMC derived plants. In the last group there were different changes which were difficult to classify as patterns I–VI, but they often represented similar metabolite level in LC and LMC, distinct from CDS (Table 1).

Analyzing the patterns of metabolites in plants of a different tissue culture origin within the metabolite groups we could find different distribution of these patterns. For instance patterns I and II were dominating among amino acids. Among organic acids however we observed the opposite reaction: in the case of seven compounds (per 16 in the group) there was a slight shift in CDS and LMC whereas in LC plants most of these substances decreased (dominating was pattern III). Among sugars the dominating patterns were IV and V showing the increasing tendency for compounds. In sugaralcohols and other metabolites it was difficult to find any common pattern of changes – there are increases, decreases or no reaction (mainly in CDS). Alcohols grow strongly only in LC plants (pattern IV). Nonrandom distribution of the metabolite change patterns in some metabolite groups may suggest the somaclonal interference with defined metabolic pathways. To demonstrate this expression analyzes of enzymes involved in various pathways are needed.

Our results indicate the existence of a specific metabolic fingerprint reflecting the history of previous generations. We tested it applying the PCA tool to our combined data set (Figure 1). Four distinct clusters are clearly visible. Each cluster specifically corresponds to the regeneration system used to obtain parents of the plants analyzed. Two groups are separated along first component axis: LC and LMC + CDS + control. The control plants separate from CDS and LMC regenerated plant progeny along the second axis, whereas the CDS and LMC plants are separated along the third component axis. When variables (metabolites) were plotted on the two-dimensional PCA diagram, some patterns arbitrary selected in Table 1, could be distinguished, which confirmed that criteria used for pattern selection were adequate (Figure 2). Metabolites on the right side of the diagram were down-regulated in LC derived plants (patterns I, II and III) whereas those on the left side were up-regulated (patterns IV and V). The best separated metabolites on the PCA diagram showed the highest changes in LC derived plants which demonstrated again that changes observed after LC culture were dominating. Urea (the clearest marker of CDS derived plants) and ascorbate (the biggest change in LMC derived plants) were well separated along component three axis of the metabolite PCA diagram (data not shown). This component accounted for 2.6% of variance only, whereas first two could explain as much as 93.2% of variance.

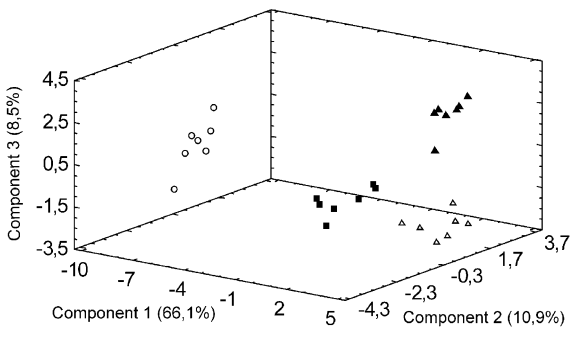

Figure 1. PCA of the metabolic profiles of leaves from progeny of plants regenerated in different culture conditions. Three vectors presented here account for 85.5% of the variability in the original data. Different colors represent plants one generation after regeneration procedure: open circles – LC, open triangles – LMC, black triangles – CDS, black squares – control.

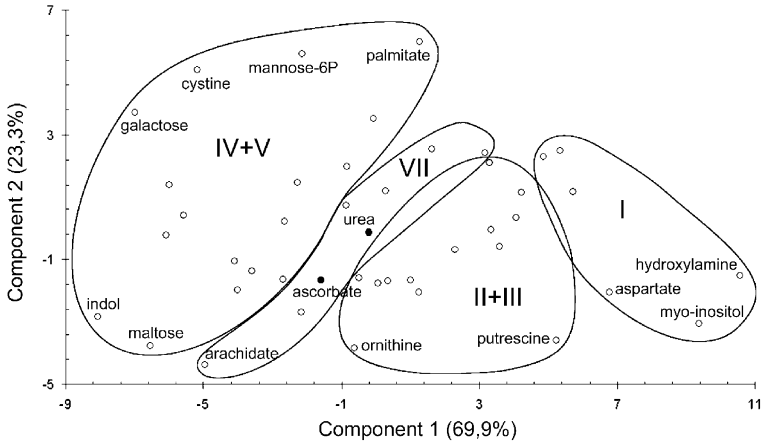

Figure 2. PCA of metabolites (open circles) used for discrimination of R1 progeny after different types of culture plotted on two-dimensional diagram. The most important metabolites with respect to the separation of differently regenerated plants (in R1 progeny) are labeled. Compounds representative for patterns defined in Table 1 (or groups of patterns) are separated mainly along first two component axes explaining 93.2% of variance. Black circles represent urea and ascorbate which could be well separated along component 3 axis (2.6% of variance, data not shown).

Discussion

All 78 monitored metabolites showed variation in plants obtained from parents regenerated via one of regeneration systems. Each tissue culture system had its characteristic set of changed compounds. Although many interesting hypotheses and conclusions could be drown from point-by-point analysis by looking at changes of specific metabolite after different types of culture, the clearest insight was gained after PCA. This revealed two important conclusions: first that plants regenerated by different methods can be distinguished on the basis of their metabolic complement even in the next generation; and second that leaf callus regeneration contributes the greatest metabolic changes among regeneration systems tested.

These changes most probably are the result of somaclonal variation, however we do not expect they all derive from mutations or other changes in the genome. Autotetraploidization and new phenotypes are quite often observed in plants regenerated *in vitro*. In our previous research there was shown that LC regeneration system quite efficiently induces tetraploids but not heritable morphological traits (Ładyżyński et al., 2002). Moreover the ploidy changes after LC culture are accompanied by characteristic changes in metabolic profiles (M. Filipecki et al., in preparation). Although there exists a group of autotetraploidization responsive metabolites, these changes are rather small and only quantitative, what was rather unexpected considering observed numerous morphological and physiological differences between di- and tetraploids of the same genotype (Mackiewicz and Malepszy, 1996; M. Filipecki et al., in preparation). In order to avoid the tetraploidy background, these plants were excluded from the presented analyses.

Nevertheless the LC culture generates the highest differences in metabolite content and is the best separated cluster in PCA analysis. In presented data the smallest number of variable metabolites characterizes the CDS but surprisingly it was the most potent regeneration system in generating the new phenotypes (Ładyżyński et al., 2002). This culture was the closest cluster to the control after PCA (Figure 1). The LMC culture is characterized in turn by the intermediate number of changes, which are usually relatively small, not exceeding 22% (except ascorbic acid). However they were well separated from the control on PCA diagram (Figure 1).

Most likely part of observed changes derives from epigenetic variation or natural biological variation. This can originate from differential expression of the genome and not from alterations of genome itself. Unexpectedly these alterations are heritable without any visible morphological effect. It is difficult to define their source without

additional analyses. The phenomenon we observed is likely to be a result of the changes in DNA methylation. Regulation of gene expression via changes in DNA methylation is very potent regulatory mechanism often found in nature, e.g. in plant development (Reyes et al., 2002). The developmental processes are in turn the main targets of manipulations in tissue culture techniques. Reassuming, deregulation of natural methylation machinery influences expression of many genes. The expression changes can be manifested by the existence of a specific metabolic fingerprint. Indeed, the tissue culture induced DNA methylation polymorphism is often observed (Anderson et al., 1990; Kaeppler and Phillips, 1993; Smulders et al., 1995). These changes were quite frequent and stably inherited (Kaeppler and Phillips, 1993). The key factor responsible for mentioned deregulation of methylation machinery is stress (Phillips et al., 1994). Stress is also the most often pointed factor inducing the somaclonal variation after tissue culture. It includes wounding, desiccation, osmotic stress, insufficient nutrient supply and uptake (Carman, 1995). This however does not fully explain the major difference between the LC and other regeneration systems observed in our experiments. One possible cause of stronger effect in LC derived progeny could be the presence of a growth regulator 2,4-D in the medium, which in carrot cultures caused a dramatic elevation of cytosine methylation (LoSchiavo et al., 1989).

It was not surprising that some metabolites changed in a similar fashion (the characteristic metabolite expression patterns I–VII), which suggests the existence of common regulatory mechanisms. This is clear in the case of most amino acids and amines monitored, which mainly decreased and belonged to patterns I and II indicating the changes in expression or activity of enzyme (enzymes) engaged in nitrogen reduction or amino acid synthesis. The changes leading to cystine and urea shift in LC and CDS plants respectively or increases in most of sugars and alcohols content (patterns IV and V) were probably independent. The most likely is that observed changes were resultant of nonrandom changes in only a few loci but in order to see the segregation of the given trait this should be tested on a higher number of individuals within a line. There are two additional obstacles in deeper interpretation of our data: the relatively low number of identified metabolites and the lack of standardized compound concentrations, which should be considered in future experiments.

Various factors can also cause the temporal fluctuations of some monitored compounds here as a direct effect. Do these fluctuations and how could they be subsequently fixed to the heritable trait remains an open question. Observations on further inheritance of metabolite profiles as well as detailed expression analyses of genes involved in compound biosynthesis should shed more light on the nature of described differences.

These speculations evoke the habituation hypothesis (Meins, 1989) supported recently by results on maize seedlings exposed to cold stress (Steward et al., 2002) or pine trees exposed to radiation after Chernobyl nuclear accident (Kovalchuk et al., 2003). Such adaptive response to the stimulus leading to chromatin variations via e.g. methylation could be later fixed by genetic mutations as hypothesized by Jablonka and Lamb (1989). It is possible that base changes are initiated by methylation as reviewed by Phillips et al., (1994). However it is unclear to which extent (if any) this mechanism is significant in plants regenerated in tissue culture.

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