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# Tissue-culture-responsive and autotetraploidy-responsive changes in metabolic profiles of cucumber (*Cucumis sativus* L.)

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**Abstract.** Somaclonal variation commonly occurs during *in vitro* plant regeneration and may introduce unintended changes in numerous plant characters. In order to assess the range of tissue-culture-responsive changes on the biochemical level, the metabolic profiles of diploid and tetraploid cucumber $R_1$ plants regenerated from leaf-derived callus were determined. Gas chromatography and mass spectrometry were used for monitoring of 48 metabolites and many significant changes were found in metabolic profiles of these plants as compared to a seed-derived control. Most of the changes were common to diploids and tetraploids and were effects of tissue culture. However, tetraploids showed quantitative changes in 14 metabolites, as compared to regenerated diploids. These changes include increases in serine, glucose-6P, fructose-6P, oleic acid and shikimic acid levels. Basing on this study we conclude that the variation in metabolic profiles does not correlate directly with the range of genome changes in tetraploids.

**Key words:** autotetraploids, *Cucumis sativus*, metabolic profiling, somaclonal variation.

## Introduction

The procedure of *in vitro* regeneration is commonly used in research and in commercial propagation, including generation of transgenic plants. This procedure quite frequently induces somaclonal variation, which changes numerous plant characters, most often as an unintended effect (Karp 1991). Many factors have been described as potentially responsible for somaclonal variation since the publication of the fundamental article by Larkin and Scowcroft (1981), and generally most can be classified as stress factors. This mutagenic stress-response mechanism can be described as a programmed loss of cellular control, leading to ploidy changes, chromosome rearrangements, DNA methylation, and point mutations (Phillips et al. 1994). Additionally, plants regenerated in tissue culture can harbor changes induced in the cells during tissue differentiation *in planta* before explantation (Colijn-Hooymans et al. 1994). Increase in ploidy level and changes in methylation pattern are somaclonal changes that are easily observed. Some types of *in vitro* culture of cucumber tissues often induce these kinds of changes (Pląder et al. 1998; Ładyżyński et al. 2002).

Autotetraploid cucumbers show many altered characters, as compared to their diploid counterparts. The changes include: bigger cells, thicker leaves (with more distinct serrate margins), thicker and poorly branching shoots, bigger flowers, smaller and shorter fruit, broader and thicker seeds, lower viability of pollen and seeds, and longer vegetative growth (Kubicki 1962). The effect of autotetraploidy is cultivar-dependent, and tetraploids

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may differ in the level of sex expression, fruit shape and time of flowering. Tetraploid cucumber genotypes also have a higher soluble solids content of fruits (30% greater) and higher marketable yield, as compared to their diploid counterparts (Mackiewicz and Malepszy 1996).

Regeneration *in vitro* seems to be an attractive model for comparing the effects of autotetraploidy and somaclonal variation in diploids because both types of changes can be generated in the same regeneration procedure. In order to assess the range of biochemical changes in such diploid and tetraploid plants, we compared their metabolic profiles with those of plants produced from seeds.

## Material and methods

All plants were of the highly inbred line (I15) of *Cucumis sativus* cv. Borszczagowski. $R_1$ generation plants were obtained by self-pollination of regenerants from leaf-derived callus as previously described (Ładyżyński et al. 2002; diploids and tetraploids were obtained in the same regeneration experiment and the ploidy level was determined by flow cytometry as described ibidem). In this study, we used 25 plants that were the progeny of 5 tetraploids, and 40 plants being the progeny of 8 diploids (5 plants per independently regenerated diploid or tetraploid parent). The control plants were the diploids that were solely generatively propagated. The $R_1$ diploids did not exhibit visible phenotypic changes when compared to the control. The tetraploids showed a common tetraploid phenotype (Kubicki 1962) with no apparent variation among plants analyzed. Plants were grown in the soil in environmentally controlled greenhouse chambers in 16 h light/8 h dark photoperiod in spring (March–April) at a constant light intensity of 120 μmol $m^{-2}$ $s^{-1}$. For metabolic profiling, mixed samples of 25 mg (fresh weight) were concomitantly collected (after about 6 h light) from 3 young, fully expanded leaves from each plant (leaves at nodes 3, 4 and 5 of tissue-culture-derived and control plants) at the stage of 5 nodes. Extraction and fractionation were performed as described previously (Fiehn et al. 2000). Metabolic profiles were acquired by GC/time-of-flight (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 i.d., 0.25 μm film) with a 10-m integrated pre-column (Restek, Germany). Peak detection and quantification of selective ion traces were accomplished by using the manufacturer's MassLab FindTarget Software. The changes in metabolite levels were analyzed for statistical significance with Student's *t*-test ($P < 0.05$). The metabolites tested constituted 5 groups: amino acids, organic acids, sugars, alcohols and other metabolites (Table 1).

**Table 1**. Metabolites identified and monitored during analyses of regenerated plants. Compounds marked with asterisks are autotetraploidization-responsive. Compounds in bold face did not change in regenerated diploids. The changes in metabolite levels were analyzed for statistical significance with Student's *t*-test ($P < 0.05$).

| Amino acids | Organic acids | Sugars | Alcohols | Other metabolites |
|---|---|---|---|---|
| cystine | arachidic* | fructose | glycerol | butanoate triOH* |
| aspartate | ascorbic | fructose-6-P* | glycerol-2-P | citrulline |
| GABA* | benzoic | galactose-6-P | indol | ethanolamine* |
| glutamic acid | fumaric | glucose | lyxitol | hydroxylamine* |
| leucine | galactonic* | **glucose-6-P*** | xylitol | organic phosphate* |
| ornithine | gluconic | maltose | | putrescine |
| phenylalanine | glycolic | mannose-6-P* | | spermidine |
| pipecolate | maleic | **raffinose*** | | urea |
| serine* | malonic | sucrose | | |
| threonine | oleic* | | | |
| valine | palmitic | | | |
| | pyrOH | | | |
| | shikimic* | | | |
| | stearic | | | |
| | succinic | | | |

## Results and discussion

Under certain conditions, plant regeneration from tissue culture generates numerous changes in the progeny. The regeneration of cucumber plants from leaf callus causes often their tetraploidization (Ładyżyński et al. 2002). In the present work, we analyzed diploid and tetraploid cucumbers, obtained from the same genotype and grown under identical conditions. Our analysis of metabolites indicated the existence of two separate effects: (1) direct changes related to the *in vitro* regeneration procedure; and (2) changes resulting from autotetraploidization.

Nearly all metabolites identified (46 per 48 identified) were altered in the progeny of regenerated diploid plants, as compared to the control (seed-derived plants). The only exceptions were glucose-6-P and raffinose. The most pronounced changes (by a factor > 2) were increases in galactose 6P and cystine levels and decreases in hydroxylamine, putrescine and aspartate levels (Figure 1). The profile of metabolite changes in tetraploids was similar (Figure 1). In this case, we observed changes in levels of 42 compounds of the 48 identified. As in diploid plants, the regenerated tetraploids showed the highest changes in galactose 6P, cystine, hydroxylamine, putrescine and aspartate levels (Figure 1). Our results clearly show that the regeneration process created a similar metabolic profile in diploids and tetraploids. However, 14 of these metabolites reacted significantly stronger in autotetraploids (Figure 2). We propose to call them the autotetraploidization-responsive metabolites. The remaining 32 metabolites changed their level similarly in both types of regenerated plants, as compared to the control. These metabolites reacted most likely to tissue culture and their profile reflected the specific regeneration method used (Filipecki et al. 2005). They are designated here as tissue-culture-responsive metabolites (Table 1). The increases of cystine and gluconic acid are typical examples of the response to tissue culture, and both metabolites are also known to be affected by stress. Gluconic acid is involved in plant defense and could be an indicator of stress response (Yun and Loake 2002). Numerous stress factors accompany *in vitro* plant regeneration. For instance, Carman (1995) lists 6 physiological stresses in meristem culture: (1) wounding; (2) desiccation during explantation; (3) osmotic stresses experienced during culture; (4) insufficient chemical energy for synthesizing macromolecules and for maintaining the turgor pressure and ionic gradients; (5) nutrient supplies less complex than those experienced in situ and causing diversion of energy supplies from growth and development to precursor biosynthesis; and (6) slow and variable rates of nutrient absorption across plasmalemmas. Therefore, the most probable source of observed changes is related to stress. The question of how this effect is transmitted to the next generation is still open. We suppose that at least part of the variation observed in cucumber has an epigenetic nature, e.g. the differential DNA methylation often observed after tissue culture, as reported for maize

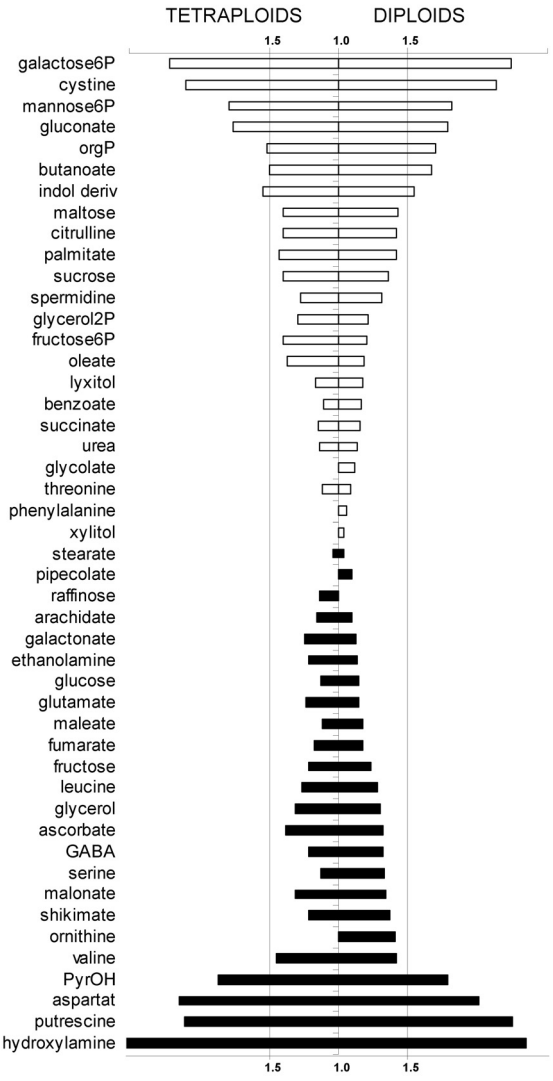


Figure 1. Metabolic profiles of diploid and tetraploid cucumber progeny derived from tissue culture, as compared to the seed-derived control. Empty bars represent the ratio of regenerated plants to control plants (compounds whose level increased after culture), filled bars represent the inverse ratio (compounds whose level decreased after culture). Based on mean values for 40 diploids and 25 tetraploids, statistically significant (Student's *t*-test, $P < 0.05$).

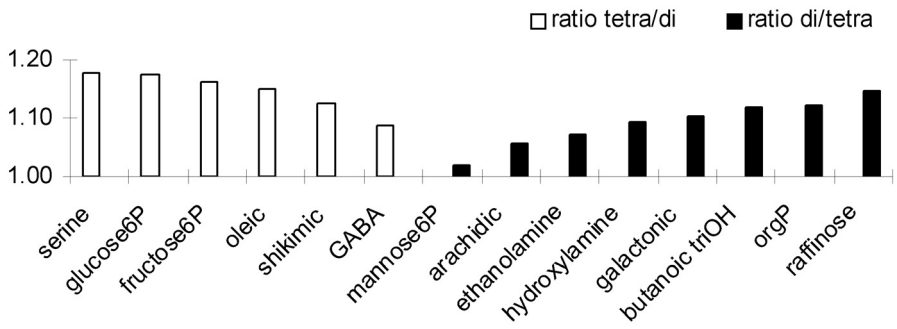


Figure 2. Metabolic profile of tetraploid cucumber plants derived from tissue culture, as compared to diploid plants after the same type of culture. Hollow bars represent the ratio of regenerated tetraploid plants to regenerated diploid plants (compounds whose level increased in tetraploids), solid bars represent the inverse ratio (compounds whose level decreased in tetraploids). Based on mean values for 40 diploids and 25 tetraploids, statistically significant (Student's *t*-test, $P < 0.05$).

(Kaeppler and Phillips 1993), tomato (Smulders et al. 1995), and many other species. These changes were quite frequent (up to 39% of independently regenerated plants) and stably inherited (Kaeppler and Phillips 1993).

Using the GC/MS we were able to identify quite a large number of metabolites changing upon regeneration *in vitro* or after tetraploidization induced therein. These changes were detectable in the $R_1$ progeny, thus showing their heritable nature. The effect of autotetraploidization involves 14 compounds (out of 48 inspected), including 6 with increased levels and the 8 with decreased levels (Figure 2). The remaining, autotetraploidization-responsive metabolites showed only slight changes. This was unexpected considering the previously observed numerous morphological and physiological differences between diploids and tetraploids of the same genotype (Mackiewicz and Malepszy 1996). Moreover, there is growing evidence about gene-specific effects of duplication on gene expression via several mechanisms: transcriptional gene silencing caused by hypermethylation, and posttranscriptional silencing by RNAi (Schubert et al. 2004; Adams and Wendel 2005). The other possible silencing mechanisms in polyploids include higher-order changes in chromatin structure, transposon activation, or general regulatory mismatch. These mechanisms do not exclude the possibility of additive consequences of DNA duplication. The increase in soluble solids content of tetraploid cucumbers (Mackiewicz and Malepszy 1996) is most probably associated with elevated concentrations of fructose-6P, glucose-6P, shikimic acid and oleic acid, which are known as metabolic intermediates. The shikimic acid, for example, is an intermediate in the biosynthesis of aromatic amino acids, phenolics and flavonoids, and consequently its concentration could influence many other substances, including plant hormones (Knaggs 2003). In fact, an increase in several compounds has been described as an effect of autotetraploidy. This applies, for example, to the concentration of sucrose in sugar beet (Negovsky 1974), proteins in ryegrass (Nelson and Rouquette 1981) and pea (Kacperek 1986). This shows that the autotetraploidization effect may comprise the changes in sugars or proteins reflected by differences in some metabolites.

Almost all the metabolites monitored here showed variation, which can be a result of somaclonal variation. Moreover, we reported the existence of a autotetraploidy-specific metabolic fingerprint, which is different from that of the regeneration procedure. The variation observed can originate from differential expression of the genome and not from alterations of the genome itself. The major challenge would be to find out the source of observed changes, for example to correlate them with the variation in transcript or protein product level of certain genes. At present this kind of analyses can be done using high-throughput methods, similar to the GC/MS method presented here, used for metabolic profiling.

## Conclusions

Metabolic profiling is a very sensitive tool showing a wide range of metabolic changes that were induced during plant regeneration *in vitro* and transmitted to the next generation ($R_1$). These changes are detectable even in plants showing no visible phenotype alterations.

Tissue culture shows a much stronger effect on metabolic profiles of regenerated cucumber plants than autotetraploidization induced therein.

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REFERENCES

Adams KL, Wendel JF, 2005. Novel patterns of gene expression in polyploid plants. Trends Genet 21: 539–543.

Carman JG, 1995. Nutrient absorption and the development and genetic stability of cultured meristems. In: Terzi M, Cella R, Falavigna A, eds. Current issues in plant molecular and cellular biology: Proceedings of the VIIIth International Congress on Plant Tissue and Cell Culture. Dordrecht, Kluwer Academic Publ: 393–403.

Colijn-Hooymans CM, Hakkert JC, Jansen J, Custers JBM, 1994. Competence for regeneration of cucumber cotyledons is restricted to specific developmental stages. Plant Cell Tiss Org Cult 39: 211–217.

Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L, 2000. Metabolite profiling for plant functional genomics. Nat Biotech 18: 1157–1161.

Filipecki M, Wiśniewska A, Yin Z, Malepszy S, 2005. The heritable changes in metabolic profiles of plants regenerated in different types of *in vitro* culture. Plant Cell Tiss Org Cult 82: 349–356.

Kaeppler SM, Phillips RL, 1993. Tissue culture-induced DNA methylation variation in maize. Proc Natl Acad Sci USA 90: 8773–8776.

Karp A, 1991. On the current understanding of somaclonal variation. Oxford Surv Plant Mol Cell Biol 7: 1–58.

Kasperek G, 1986. Artificial polyploids of the pea. Pisum Newslet 18: 32–33.

Knaggs AR, 2003. The biosynthesis of shikimate metabolites. Natural Prod Rep 20: 111–118.

Kubicki B, 1962. Polyploidy in muskmelons (*Cucumis melo* L.) and cucumbers (*Cucumis sativus* L.). Genet Pol 3: 161–179.

Larkin PJ, Scowcroft WR, 1981. Somaclonal variation – a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60: 197–214.

Ładyżyński M, Burza W, Malepszy S, 2002. Relationship between somaclonal variation and type of culture in cucumber. Euphytica 125: 349–356.

Mackiewicz HO, Malepszy S, 1996. Obtaining and characterization of tetraploid forms in cucumber – *Cucumis sativus* L. var. *sativus* and var. *hardwickii* Alef. Fol Hort 8: 3–10.

Negowsky NA, 1974. Some practical and theoretical aspects of sugar-beet polyploidy in the USSR. In: Polyploidy and induced mutants in plant breeding. Vienna, IAEA: 241–245.

Nelson LR, Rouquette FM, 1981. Neutral detergent, fiber and protein levels in diploid and tetraploid ryegrass forage. In: Proceedings of the XIX International Grassland Congress. Lexington, Kentucky, June 15–24: 234–237.

Phillips RL, Kaeppler SM, Olhoft P, 1994. Genetic instability of plant tissue cultures: Breakdown of normal controls. Proc Natl Acad Sci USA 91: 5222–5226.

Pląder W, Malepszy S, Burza W, Rusinowski Z, 1998. The relationship between the regeneration system and genetic variability in the cucumber (*Cucumis sativus* L.). Euphytica 103: 9–15.

Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R, 2004. Silencing in *Arabidopsis* T-DNA transformants: The predominant role of a gene-specific RNA-sensing mechanism versus position effects. Plant Cell 16: 2561–2572.

Smulders MJM., Rus-Kortekaas W, Vosman B, 1995. Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. Theor Appl Genet 91: 1257–1264.

Yun B-W, Loake GJ, 2002. Plant defense responses: current status and future exploitation. J. Plant Biotechnology 4: 1–6.