THE FREQUENCY OF GENETIC REARRANGEMENTS DURING CARROT (*Daucus carota*) SOMATIC EMBRYOGENESIS IS DEPENDENT ON 2,4-D LEVELS AND DIMINISHED IN ITS ABSENCE

Ottó TOLDI – Kitti TÓTH-LENCSÉS – László KOVÁCS – Zsófia TÓTH – Ákos MENDEL – Adrienn KEREKES – Gábor GYULAI

Institute of Genetics and Biotechnology, Faculty of Agricultural and Environmental Sciences, Szent István University, Gödöllő 2103, Hungary

e-mail: toldi.otto@mkk.szie.hu

Abstract

For quantification of genetic variations occurring in plant tissue cultures, DNA sequence alterations and replicon-size changes were monitored through subsequent phases of the model carrot tissue culture system, from the 2,4-D-induced proembryiogenic cell cultures to regenerated plantlets, by RAPD and flow cytometry techniques. Banding patterns of random amplified DNA fragments and ploidy-level distributions of cultured cells were significantly different in the presence and in the absence of 2,4-D. In addition, there were marked differences between the cells induced with lower (1.0 mg/l) and higher (2.5 mg/l) doses of 2,4-D. Among those samples that were cultured in the absence of 2,4-D, the epicotyl (C2), hypocotyl control (H1) and the morphologically normal regenerants (IV2) showed identical banding patterns overlapping with the true-to-type seedling controls (N1, N2). In contrast, treatment of starting explants (H2) with 1.0 mg/l 2,4-D resulted in a marked 82% increase in the number of amplified fragments associated with an appearance of cell lines possessing unusual haploid-like and aneuploid-like DNA contents. The induction with 2.5 mg/l 2,4-D resulted in even greater increase in the number of DNA fragments (~100%) amplified from proembryiogenic cell cultures, while it had no further effect on ploidiy-level distributions compared to the treatment with 1.0 mg/l 2,-D. After the withdrawal of the synthetic auxin, banding patterns and ploidy-level distributions were gradually shifted back to the levels of controls resulting in true-to-type regenerants. Conclusions are in short, the combined use of RAPD and flow cytometry can make quantification of genetic variations typical of dedifferentiated plant cells possible. Quantification opens the window for comprehensive evaluation of different methods, treatments and bioactive compounds.

Keywords: genetic alterations, replicon size, ploidy-level distribution, plant tissue culture, somatic embryogenesis, genetic identity

Abbreviations: 2,4-D – 2,4-dichlorophenoxi acetic acid; DAPI – 4',6-diamidino-2-phenylindole; R_o plants – regenerated plants originating directly from in vitro tissue culture; RAPD - random amplification of polymorphic DNA technique; N1, N2, H1, H2, P1, P2, E1, E2, IV1, IV2 – sample set of the culture level RAPD and flow cytometry detailed in Table 1; mbn – mean band number per random amplified DNA sample

Introduction

Although a wide scale of genetic alterations can be obtained in plant tissue cultures, from point mutations to replicon-level rearrangements (for review see Smulders and de Klerk 2010), the occurrence of genetically non-identical off-types (somaclones) is relatively rare (Jain 2001, Gyulai et al. 2003, Joshi and Dhawan 2007). Among the components of a typical plant tissue culture medium mainly synthetic growth regulators were suggested as inducers of genetic alterations (Kaeppler et al. 2000) together with different abiotic stressors manifested typically in tissue cultures (Phillips et al. 1994, Guo et al. 2006). Our aim was to develop a technically simple and fast method, which, in theory, covers all potential genetic variations from sequence to replicon-size changes in order to allow estimations of the frequency of genetic rearrangements. Quantification of the frequency of genetic rearrangements can make different treatments

and effects comparable. There are evidences to support that 2,4-D, previously was used as an ingredient of Agent Orange herbicide to defoliate plants, induces irregular cell divisions (Fujimura and Komamine 1982, Nuti Ronchi et al. 1992ab,) an elevated genetic translocation frequency (Kaeppler et al. 2000) and reversible changes in the number of repetitions of the repetitive DNA sequences in plant cell cultures (Arnholdt-Schmitt, 1995), without severe negative impacts on the genetic identity of regenerants. Therefore, the carrot (*Daucus carota cv*. 'Nantes Duke') tissue culture system was chosen as model, where proembryogenic cell cultures are induced by 2,4-D at different concentrations (1.0 mg/l and 2.5 mg/l), and the subsequent plant regeneration via somatic embryogenesis starts after the withdrawal of 2,4-D. If our hypothesis is true, differences in the frequencies of genetic changes can be estimated comprehensively between 2,4-D-induced and non-induced cells and tissues, even finer variations could be identified in cell and tissue samples treated with 2,4-D at different concentrations. To test this hypothesis RAPD (Random Amplification of Polymorphic DNA) and flow cytometry techniques were combined for parallel monitoring of sequence and ploidy-level alterations.

Materials and methods

Plant material, culture conditions and sampling

Surface-sterilized carrot (*Daucus carota cv*. 'Nantes Duke') seeds were used as starting explants. After *in vitro* germination on growth regulator-free solid MS medium (Murashige and Skoog 1962), hypocotyl sections were excised and divided to 3 groups. The control group was placed into growth regulator-free liquid MS medium, the second group of excised hypocotyls was placed into liquid MS medium supplemented with 1.0 mg/l 2,4-D, while the third group was transferred into liquid MS medium supplemented with 2.5 mg/l 2.4-D. The list of samples was shown in Table 1. The control hypocotyl segments of the first group were labelled as H1, the second group incubated with1.0 mg/l or 2.5 mg/l 2,4-D were labelled as H2.The 2,4-D-induced hypocotyl segments were removed after sufficiently dense embryogenic suspension cultures were formed in liquid MS medium containing 2,4-D. Young proembryogenic suspensions (P1) were incubated for 15 days in either 1.0 mg/l or 2.5 mg/l 2,4-D. In the case of old proembryogenic suspensions (P2) the duration of the incubation was 30 days. Subculturings were performed weekly.

To induce embryogenesis and plant regeneration, embryogenic cell clusters (P1) were separated from 2,4-D-containing induction media and were transferred into growth regulator-free liquid MS media. Somatic embryos at the stage of heart-torpedo (E1) were obtained 15 days after the withdrawal of the 2,4-D, while elongated-torpedo stage embryos (E2) appeared after 30 days-incubation in growth regulator-free media. For plant regeneration, the mature somatic embryo cultures were placed to darkness without subculturing and shaking. Morphologically normal (IV2) and abnormal plantlets showing symptoms of undetermined polarity (IV1) were selected for further analysis as R_0 generation. Besides these morphological alterations, the most important difference between the IV1 and IV2 plantlets was that the IV1 plantlets were non-viable after the removal from tissue culture.

Flow cytometry

Cell nuclei were isolated mechanically as described by Dolezel et al. (1989) and Mitykó et al. (1995). Approximately 10 mg tissues were chopped with a scalpel in a glass petri dishes containing 2 ml lysis buffer LB01 (pH 7.5) supplemented with 2 µg/ml DAPI. Settled suspension cells were resuspended in lysis buffer LB02 and left at room temperature for 15 min. The cell nuclei were released from the cells by syringing twice through a 25-gauge needle. Cell fragments were filtered through a 21 µm nylon filter and kept on ice until the analysis. The flow cytometer was adjusted so that the diploid-like peak of the nuclei isolated from the control was set as channel 100. Flow cytometric

 DOI: 10.18380/SZIE.COLUM.2014.1.2.109

Table 1. Description of the sample set examined by RAPD and flow cytometry techniques. Liquid or agar solidified MS media were used for *in vitro* maintenance of tissue cultures and 2,4-D was used at 1.0 mg/l or 2.5 mg/l concentrations for inducing the growth of proembryogenic mass

Sample set	Treatment types (1.0 mg/l or 2.5 mg/l 2,4-D)
N1 - seedling for control	*in vivo*, greenhouse conditions
N2 - seedling for control	
C2 - epicotyls excised	*in vitro*, growth regulator-free liquid MS
H1 - hypocotyls (not-treated)	
H2 - hypocotyls (treated)	*in vitro*, liquid MS + 1.0 mg/l or 2.5 mg/l 2,4-D
P1 - young proembryogenic suspension (treated)	
P2 - old proembryogenic suspension (treated)	
E1 - young embryo suspension	*in vitro*, growth regulator-free liquid MS
E2 - old embryo suspension	
IV1 - somatic embryo derived abnormal plantlets	*in vitro*, growth regulator-free solidified MS
IV2 - somatic embryo derived true-to-type plantlets (S_1-S_{12})	

measurements were performed with a Partec CA-II computerized compact flow cytometer. The amounts of nuclear DNA were calculated on the basis of the areas under the plotted graphs by using SIS Soft Imaging Software.

DNA extraction

Total DNA was isolated by homogenizing 1 g FW of plant materials with phenol/chloroform/isoamyl alcohol (25/24/1; v/v/v) in liquid nitrogen containing pre-chilled mortars as described by Dellaporta et al. (1983). The extracted DNA was treated with RNAase A (10 mg/ml) for 1h at 37°C. The DNA was then extracted again with phenol/chloroform/isoamyl alcohol (25/24/1; v/v/v), following isopropanol precipitation and 70% ethanol wash. The pellet was dissolved in 50 µl sterile ddH_2. The final concentration of DNA (~20 ng μl^{-1}) was measured by a Hoefer TKO-100 fluorimeter.

RAPD conditions

Fifty 10-mer RAPD primers (kits A, B from Operon Technologies, Almeda, California) were used for PCR amplifications. RAPD reactions were performed in a volume of 50 µl containing 100 ng of extracted DNA, 1.0 µM of primer, 200 µM of dATP, dTTP, dGTP, dCTP (Pharmacia), 1/10 volume 10xPCR-buffer (Boehringer Mannheim). Taq DNA polymerase (Boehringer Mannheim) concentration was 3.0 units per sample. For the RAPD cycling conditions, samples were first heated to 94 °C for 3 min. before entering a 40-cycle PCR procedure of 94 °C for 1 min., 36 °C for 2 min. and 72 °C for 3 min., followed by 72 °C for 5 min. and closed at 4 °C. Amplifications were carried out using a PCT-100/60 thermal cycler (MJ Research Inc). Amplified DNA fragments were separated by gel electrophoresis at 100V for 7h in a 1.5-2% agarose gel with a TBE buffer. Gels were stained with ethidium bromide and fragment patterns were photographed under UV light for further analysis. RAPD patterns were analyzed by the *restml* program of the PHYLIP

3.5c software package. The mean band number (*mbn*) for each sample was calculated.

Results

Genotype identity of somatic embryo originated carrot plants

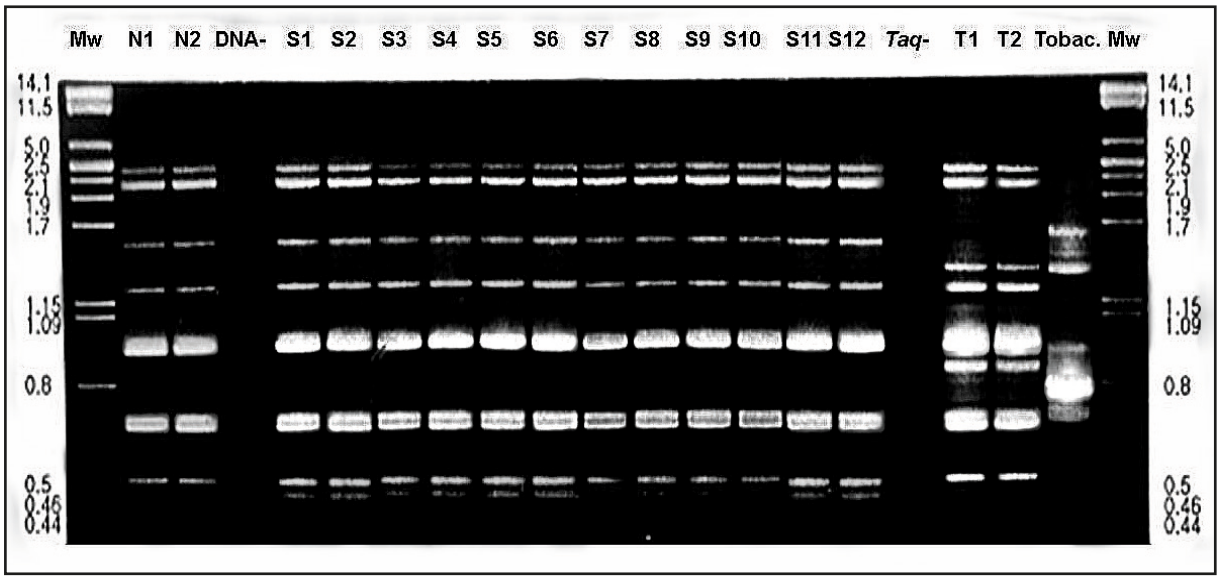
Genotype identity of 35 randomly selected R_O carrot plants regenerated from somatic embryos was tested by RAPD technique. Of the 35 regenerants, results of 12 are presented in Figure 1 (lanes S1-S12). The negative control group was formed by 2 greenhouse grown carrot plantlets (lanes N1, N2), while the positive control group consisted of T-DNA-inserted carrots (lanes T1, T2) and a tobacco sample (lane C3) as a taxonomically non-related species. Amplifications were obtained with all the primers, between 2-11 bands per primer, showing a homogenous genotype identity both in the control and in tissue culture-originated carrot plants, while tobacco always showed a different banding pattern. In *Agrobacterial* T-DNA carrying carrots only one primer, the OPB-11 (5'-GTAGACCCGT-3'), amplified a different banding pattern compared to control and tissue culture-originated plants.

The potential anomalies in the ploidy-level distributions of tissue culture-originated carrots were tested by flow cytometry using the same R_O plant material as in RAPD analysis. No differences were found between tissue culture-originated R_O and greenhouse grown control carrot plants (Figure 4AB sample group no. I., Table 2).

Quantification of the frequency of genetic alterations in 2,4-D-induced and control carrot tissue cultures

The whole *in vitro* plant tissue culture process was screened by RAPD and flow cytometry from the establishment of the embryogenic cell culture to the growth of the Ro regenerants. Twenty-five primers (OPA-01, OPA-08, OPA-12, OPA-16, OPA-17, OPA-19, OPA-20, OPB-01, OPB-02, OPB-03, OPB-05, OPB-11, OPB-12, OPB-16, OPB-18), which were randomly chosen from the 50 primers confirming homogenous uniformity of the R_0 plants, were used for fingerprinting. Amplifications were obtained with all the 25 primers between 11-26 bands per primer (Figure 2). Both the epicotyl (C2) and the hypocotyl controls (H1) incubated in the

Figure 1. Testing genotype identity of tissue culture-originated carrot plantlets by RAPD technique using 50 ten-mer OPB primers of which banding pattern of OPB-11 is presented. Lanes Mw - size marker in Kbp (lambda phage DNA digested with PstI); lanes N_1 and N_2 – greenhouse grown carrot plantlets; lane DNA^- - no-template DNA; lanes S_1 to S_{12} - carrot plantlets regenerated via somatic embryo genesis; lane Taq^- - no-Taq DNA polymerase; lanes T_1 and T_2 - T-DNA-carrying carrot controls; lane Tobac - tobacco (Nicotiana tabacum) control plants



 DOI: 10.18380/SZIE.COLUM.2014.1.2.109

Table 2. Ploidy-level (replicon size) distributions (% of analyzed nuclei) and sequence-level genetic alterations, quantified by changes in *mbn*, during carrot plant regeneration via somatic embryogenesis. The standard deviations were less than 5% of the mean value in each case.

Tissue culture and individual samples	DNA content per sample (% of the analyzed samples)				Mean band number (*mbn*)	
	1C haploid-like	1C-2C aneuploid-like	2C diploid-like	tetraploid-like cells	1.0 mg/l 2,4-D	2.5 mg/l 2,4-D
N_1 (---)	-	-	100.0	-	11.33	11.33
N_2 (---)	-	-	100.0	-	11.33	11.33
C2 (*grf*)	-	-	100.0	-	11.33	11.33
H1 (*grf*)	-	-	100.0	-	11.33	11.33
H2 (+2,4-D)	64.9	24.9	10.2	-	20.00	24.00
P1 (+2,4-D)	58.3	30.9	10.8	-	20.00	25..00
P2 (+2,4-D)	58.3	30.9	10.8	-	18.66	25.66
E1 (*grf*)	28.1	45.4	26.5	-	17.00	19.00
E2 (*grf*)	-	-	88.8	11.2	15.66	16.66
IV1 (*grf*)	-	-	88.8	11.2	13.66	13.66
IV2 (*grf*)	-	-	100.0	-	11.33	11.33

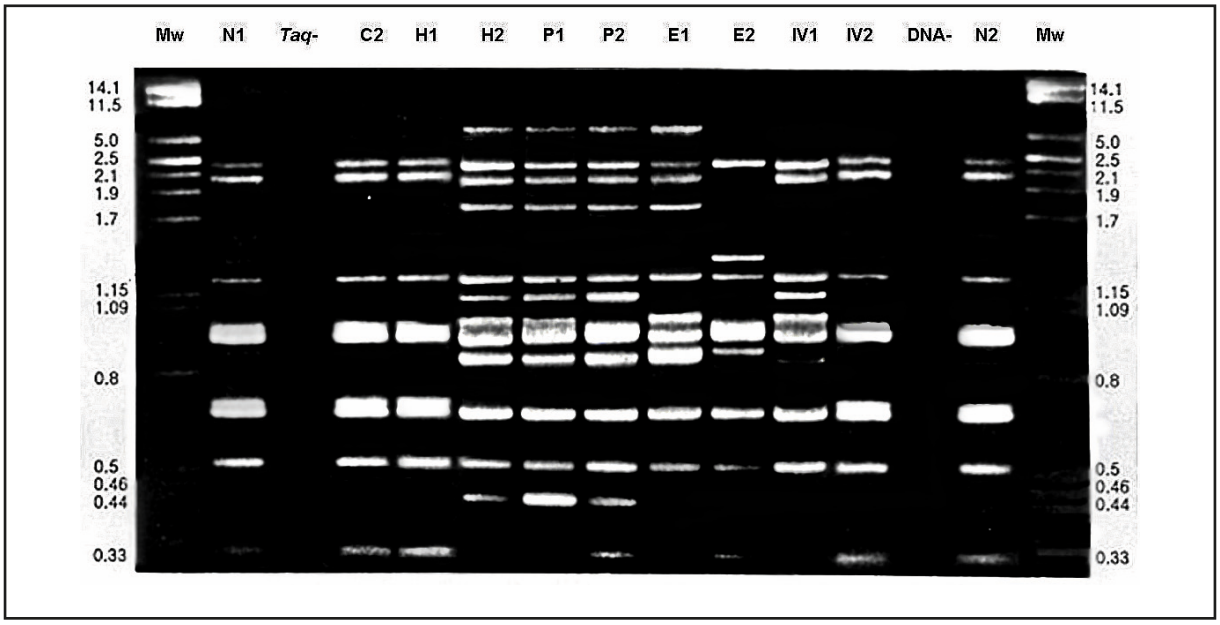
(---): greenhouse grown seedlings;

(*grf*): tissue culture incubated on growth regulator-free media;

(+2,4-D): tissue culture incubated on 1.0 mg/l or 2.5 mg/l 2,4-D containing media;

absence of 2,4-D, as well as the morphologically normal regenerants (IV2), showed identical banding patterns corresponding to each other and to the greenhouse grown, individual controls (N1, N2). For statistical analysis, the banding pattern values of the sample group consisting of sample N1, C2, H1, IV2 and N2, incubated in the absence of 2,4-D, was anchored as the root of the similarity tree generated by the *restml* program of PHYLIP 3.5c software (Figure 3AB). In this sense, the cultures incubated with 1.0 mg/l 2,4-D (H2, P1, P2) represented the top of the similarity tree. Between these two sample groups, which were the least similar to each other in terms of the number of amplified DNA fragments, torpedo-stage embryo cultures (E1, E2) and the morphologically deviant regenerants (IV1) formed a statistically intermediate sample group that settled down on the stem of the similarity tree. Based on the comprehensive banding pattern analysis by the '*restml*' program of PHYLIP 3.5c, *the in vitro* carrot plant regeneration process could be divided into proembryogenic or 2,4-D induction phase (H2, P1, P2), somatic embryo differentiation phase (E1, E2) on growth regulator-free tissue culture medium, and finally plant regeneration phase again in the absence of 2,4-D (Figure 3A). We have stated that the mean band number (*mbn*) of the control sample group (N1, C2, H1, IV2, N2) was identical within the sample group and the lowest compared to the other two groups (Table 2). The *mnb* of proembryogenic cultures induced with either 1.0 mg/l or 2.5 mg/l 2,4-D showed a dramatic increase 82% (1.0 mg/l 2,4-D) and 100% (2.5 mg/l 2,4-D) respectively. In the intermediate sample group, the *mbn* gradually decreased (E1=17.0 bands; E2=15.66 bands; IV1=13.66 bands) until the number of amplified fragments returned to the starting value in regenerated plantlets (IV2) which corresponds to the parameters of the individual controls (N1, N2). In each case the increased *mbn* characteristic of the 2,4-D-induced (H2, P1, P2) and the intermediate (E1, E2, IV1) sample groups appeared in a way that the amplified fragment sets of these samples included all the bands of the N1, C2, H1, IV2, N2 control sample group and some extra amplificates were found besides them. Even within the 2,4-D-treated cultures the 2.5 mg/l 2.4-D-induced ones showed significantly

Figure 2. Monitoring the whole tissue culture process by RAPD technique from the establishment of proembryogenic cell cultures with 1.0 mg/l or 2.5 mg/l 2,4-D to regeneration of carrot plantlets via somatic embryogenesis on growth regulator-free tissue culture media. Banding patterns amplified with OPB-11 primer were presented as in Figure 1. The loading order of samples followed the characteristic stages of tissue culturing given in Table 1. *Lanes Mw* - size marker in Kbp (lambda phage DNA digested with *Pst*I); *lanes* N_1 *and* N_2 – greenhouse grown carrot plantlets; *lane DNA*$^-$ - no-template DNA; *lanes C2* to *IV2* – cell culture-level samples described in Table 1; *lane Taq*$^-$ - no-Taq DNA polymerase.



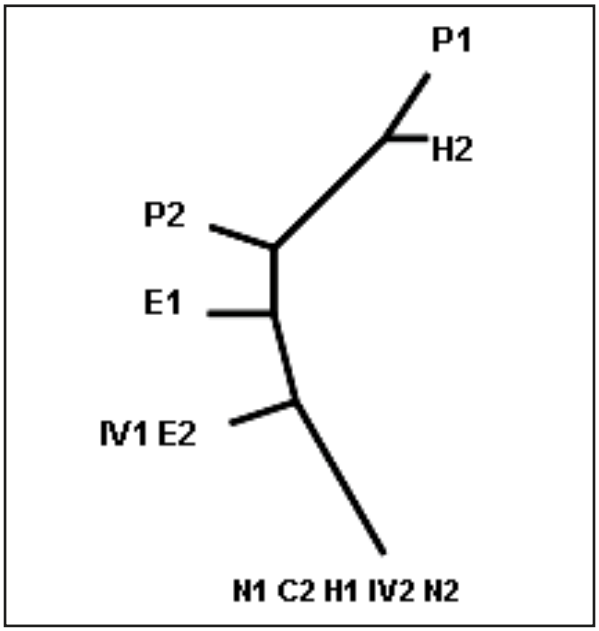
higher *mnb* value matching over a 20% increase compared to 1.0 mg/l 2.4-D induced ones (Table 2, Figure 3B).

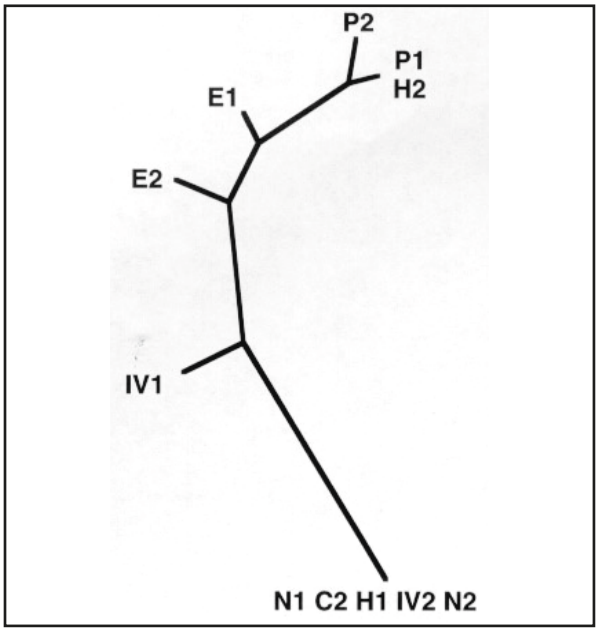
Alterations in ploidy-level distributions correlate not only with the absence and presence of 2,4-D, but also with its concentrations

For the flow cytometry, the same sample set was used as for the RAPD. Similarly to the controls (N1, N2), epicotyl- (C2) and non-induced hypocotyl cultures (H1) incubated in the absence of 2,4-D showed a diploid 2C DNA content (Figure 4AB sample group no.I., Table 2). A common characteristic of the cultures incubated with 2,4-D (H2, P1, P2) was the coexistence of the continuously present 2C DNA containing (G0/G1 phase cells), 2C-4C DNA containing (S phase cells), and 4C DNA containing cell lines (G2+M phase cells) with the aberrant 1C DNA containing (58.3-64.9% of the cells), and 1C-2C DNA containing (24.9-30.9% of the cells) ones (Figure 4AB sample groups no. II., III., Table 2). In those cell cultures in which the higher concentration of 2,4-D (2.5 mg/l) was used for inducing the growth of proembryogenic mass, the ploidy-level distributions showed even more extreme rearrangements (Figure 4B sample groups no. II., III, Table 2). However, after the withdrawal of 2,4-D, the 1C DNA containing cell line gradually disappeared (E1=28.1%, E2=0%, IV1=0%, IV2=0%) together with the 1C-2C DNA containing aneuploid-like cell line (E1=45.4%, E2=0%, IV1=0%, IV2=0%) (Figure 4AB sample groups no. IV., V., Table 2). At the same time, the diploid 2C DNA containing cell line gradually became dominant (E1=26.5%, E2=88.8%, IV1=88.8%) after the withdrawal of 2,4-D, during differentiation of somatic embryo-originated plantlets. As a result of the above mentioned process, the regenerated plants (IV2) showed the same 2C DNA contents as the individual control plants independently of the applied 2,4-D concentrations (Figure 4AB sample group no. I., Table 2).

 DOI: 10.18380/SZIE.COLUM.2014.1.2.109

Figure 3. Statistical distances between carrot tissue cultures (see Table 1) incubated with 1.0 mg/l 2,4-D **(A)** or 2.5 mg/l 2,4-D **(B)** calculated from the mean band numbers generated by RAPD analyses and processed with the *restml* program of PHYLIP 3.5c software package. Roots of dendrograms were fixed to samples showing genetic alterations at the lowest frequency (N1, C2, H1, IV2).





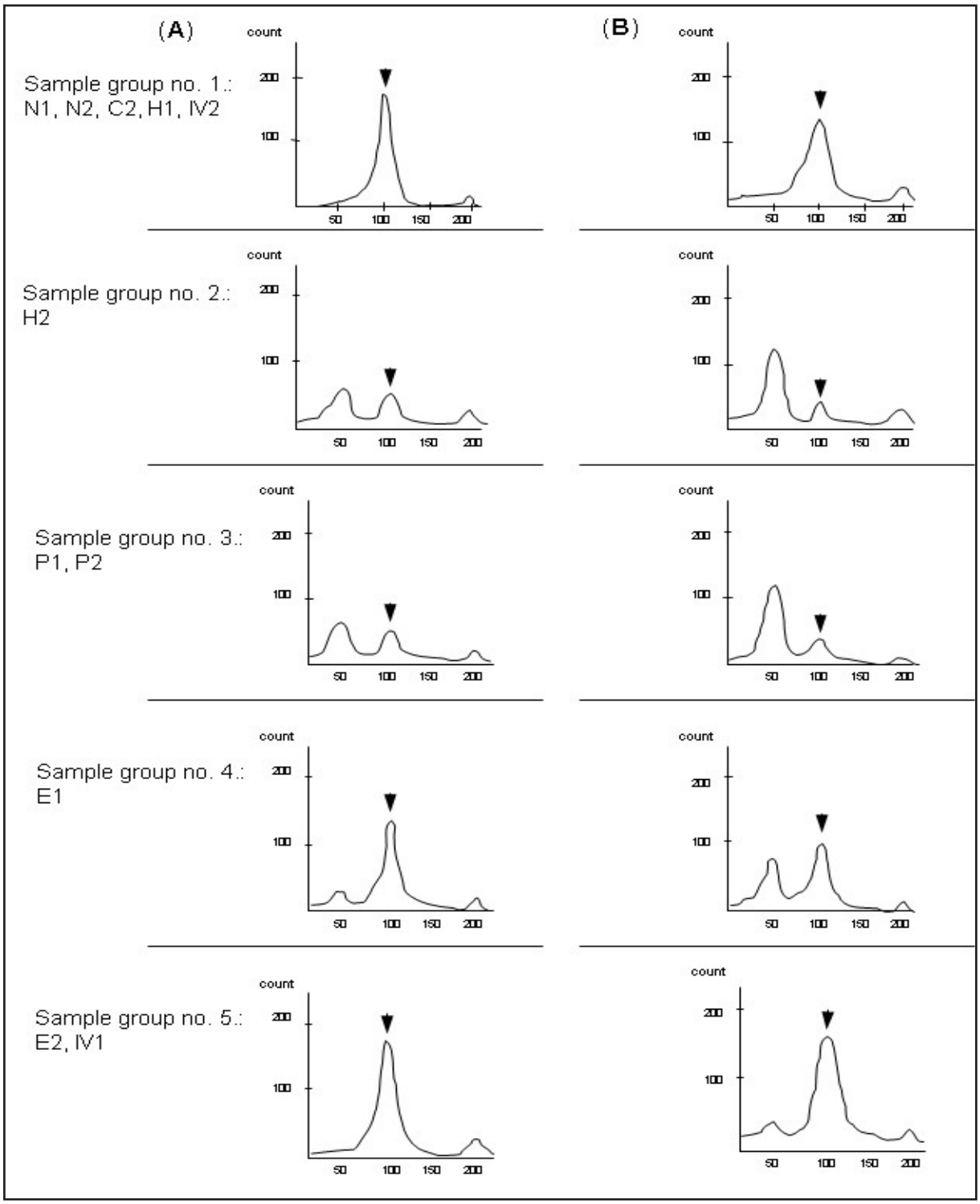
Discussion

Comparative analysis of tissue culture-originated plants based on RAPD technique is a widely applied method for the evaluation of genotype identity (Martins et al. 2004, Saker et al. 2006, Guo et al. 2007). Our examinations were different from the foregoings in one aspect; it was not a stationary relation comparing banding patterns of two treatments or sample groups, but a dynamic approach analysing two complete plant regeneration systems. The theory behind this approach came from the realisation that if the RAPD fragment patterns of individual mother plants were identical, the fragment patterns of bulk samples of tissue cultures originating from them should be also identical. If an *in vitro* culture became different from its individual mother-plant, it means that the *in vitro* tissue culturing resulted in such genetic changes that are detectable by culture-level application of RAPD technique.

The increased frequency of point mutations and tranlocation events, the irregular cell divisions caused by 2,4-D (Fujimura and Komamine 1982, Nuti Ronchi et al. 1992ab), as well as the decreased replicon size due to a 2,4-D-induced reversible reduction in repetitive DNA sequences (Arnholdt-Schmitt, 1995) have already been proven (Kaeppler et al. 2000, Smulders and de Klerk 2010). In our case, it is also probable that the different RAPD patterns in the proembryogenic (H2, P1, P2) and somatic embryo cultures (E1, E2) were stimulated by the 2,4-D treatment, because of two reasons. First, other factors of the *in vitro* culture had no effect on these parameters, becasue genetic alterations could not be detected in cell cultures maintained in the absence of 2,4-D. Second, the quantification of the frequency of genetic alterations showed a dose effect of 2,4-D, namely, incubation of cell cultures with higher concentration of 2,4-D resulted in an increase in the number of random amplified DNA fragments compared to incubation at lower 2,4-D concentrations (Table 2, Figure 3AB).

Based on the quantified results of RAPD analyses, a hypothesis was devised to describe the most likely mechanism associated with

Figure 4. Flow cytometry plots of carrot tissue cultures (see Table 1) incubated with 1.0 mg/l 2,4-D **(A)** or with 2.5 mg/l 2,4-D **(B)**. The peak of diploid (2n) DNA volume was adjusted to the channel 100. Arrow heads indicate diploid (2n) genome.



reversible changes in DNA sequences and ploidy-level distributions detected in our carrot tissue culture system. The results of culture-level RAPD and flow cytometry showed that the diploid starting genotype became a mixture of different ploidy-level deviant cell lines consisting of a 1C haploid-like, 1C-2C aneuploid-like and 2C diploid cell lines (Figure 4AB sample

 DOI: 10.18380/SZIE.COLUM.2014.1.2.109

groups no. II., III.). Meanwhile, the number of random amplified DNA fragments of these cell lines, possissing unusual ploidy-level distributions, contained the amplified DNA fragments characteristic to the starting 'genotype' and different numbers of additional fragments depending on the presence and concentration of 2,4-D (Figure 2, 3AB). The appearance of 1C haploid-like and 1C-2C aneuploid-like cell lines in 2,4-D-induced proembryogenic cultures might be a consequence of the reversible loss of a marked proportion of repetitive DNA sequences descibed by Arnholdt-Schmitt (1995). Although the chromosome number of these cell lines is diploid, their DNA contents (replicon size) are varied and can be reduced up to half (1C DNA content). In spite that the molecular machinery of the above process still unknown, these data are concordant with and may explain our flow cytometric results. Omitting 2,4-D from culture media led to the disappearance of aberrant cell lines, which resulted in a disarrangement of the original ploidy-level distribution and RAPD pattern (Figure 2, Figure 4AB sample groups no. IV., V.).

We suppose that 2,4-D plays a central role in triggering these genetic alterations in two ways. First, as a direct inducer of irregular cell divisions (Fujimura and Komamine 1982, Nuti Ronchi et al. 1992ab) and reduction of the number of repetiitions within repetitive DNA sequences (Arnholdt-Schmitt 1995). Second, 2,4-D acts also as auxinic herbicide triggering oxidative strees, abnormal growth, senescence, and plant death (Song 2014). Physiological stress associtated with *in vitro* tissue culturing, thought to be the second most important among the potential inducers of genetic alterations, exacerbating with the fact that 2,4-D has no catabolism in plants, making it capable for prolonged actions (Wright et al. 2010). However, genetic alterations can be stimulated not only by induction with exogenous effectors, but also by disturbing endogenous repaire mechanisms through a defficiency in suppression of repeat-induced point mutations (Phillips et al. 1994) and activation of plant retrotransposons (Grandbastien 1998).

The sensitvity and the resolution of our approach, the paralel use of RAPD (Random Amplification of Polymorphic DNA) and flow cytometry techniques supported by PHYLIP 3.5c and SIS Soft Imaging softwares, allowed a complete cover of all genetic alterations that, in theory, can be perceived in tissue cultures. This combined approach made quantification of genetic variations typical of dedifferentiated plant cells possible. opening a window for comprehensive evaluation of different methods, treatments and bioactive compounds.

References

Arnholdt-Schmitt B, Physiological aspects of genome variability in tissue culture. II. Growth phase-dependent quantitative variability of repetitive *BstN*I fragments of primary cultures of *Daucus carota* L., Theor Appl Genet 91, 1995, 816–823. DOI: http://dx.doi.org/10.1007/bf00220965

Dellaporta SL, Wood J and Hick JB, A plant DNA minipreparation: Version II, Plant Mol Biol Rep 1, 1983, 19-21. DOI: http://dx.doi.org/10.1007/bf02712670

Dolezel J, P Binarova, S Lucretti, Analysis of nuclear DNA content in plant cells by flow cytometry, Biologia Plantarum 31/2, 1989, 113–120. DOI: http://dx.doi.org/10.1007/bf02907241

Fujimura T, A Komamine: Molecular aspects of somatic embryogenesis in a synchronous system. In: Plant tissue culture 1982: Proceedings, 5th International Congress of Plant Tissue and Cell Culture held at Tokyo and Lake Yamanake, Japan, July 11-16, 1982, edited by Akio Fujiwara, p. 105–106.

Grandbastien M-A, Activation of plant retrotransposons under stress conditions. Trends in Plant Science 3(5), 1998, 181–187. DOI: http://dx.doi.org/10.1016/s1360-1385(98)01232-1

Guo WL, Gong L, Ding ZF, Li YD, Li FX, Zhao SP and Liu B, Genomic instability in phenotypically normal regenerants of medicinal plant *Codonopsis lanceolata* Benth. et Hook. f., as revealed by ISSR and RAPD markers, Plant Cell Reports 25(9), 2006, 896-906. DOI: http://dx.doi.org/10.1007/s00299-006-0131-8

Guo WL, Wu R, Zhang YF Liu XM, Wang HY, Gong L, Zhang ZH and Liu B, Tissue culture-induced locus-specific alteration in DNA methylation and its correlation with genetic variation in *Codonopsis lanceolata* Benth. et Hook.f, Plant Cell Reports 26, 2007, 1297-1307. DOI: http://dx.doi.org/10.1007/s00299-007-0320-0

Gyulai G, Z Mester, J Kiss, Szemán L, Heszky L, A Idnurm, Somaclone breeding of reed canarygrass (*Phalaris arundinacea* L), Grass and Forage Sci 58, 2003, 210–215. DOI: http://dx.doi.org/10.1046/j.1365-2494.2003.00372.x

Jain SM, Tissue culture-derived variation in crop improvement, Euphytica 118, 2001, 153-166.

Joshi P and Dhawan V, Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay, Biologia Plantarum 51(1), 2007, 22-26. DOI: http://dx.doi.org/10.1007/s10535-007-0005-0

Kaeppler SM, Kaeppler HF and Rhee, Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43, 2000, 179-188. DOI: http://dx.doi.org/10.1007/978-94-011-4183-3_4

Larkin PJ, WR Scowcroft, Somaclonal variation - a novel source of variability from cell cultures for plant improvement, Theor Appl Genet 60, 1981, 197–214. DOI: http://dx.doi.org/10.1007/bf02342540

Martins M, Sarmento D and Oliveira MM, Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers, Plant Cell Reports 23, 2004, 492-496. DOI: http://dx.doi.org/10.1007/s00299-004-0870-3

Mitykó J, Andrásfalvy A, Csilléry G, Fári M, Anther- culture response in different genotypes and F1 hybrids of pepper (*Capsicum annuum* L.). Plant Breeding, 1995, 114:78–80. DOI: http://dx.doi.org/10.1111/j.1439-0523.1995.tb00764.x

Murashige T, F Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol Plant 15, 1962, 473–497. DOI: http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nuti Ronchi VN, L Giorgetti, M Tonelli, G Martini, Ploidy reduction and genome segregation in cultured carrot cell lines, I. Prophase chromosome reduction, Plant Cell Tiss Org Cult 30, 1992a, 107–114. DOI: http://dx.doi.org/10.1007/bf00034303

Nuti Ronchi VN, L Giorgetti, M Tonelli, G Martini G, Ploidy reduction and genome segregation in cultured carrot cell lines. II. Somatic meiosis, Plant Cell Tiss Org Cult 30, 1992b, 115–119. DOI : http://dx.doi.org/10.1007/bf00034304

Phillips RL, Kaepplert SM and Olhof P, Genetic instability of plant tissue cultures: Breakdown of normal controls. Proc. Nati. Acad. Sci. USA 91, 1994, 5222-5226.

Saker MM, Adawy SS, Mohamed AA and El-Itriby HA, Monitoring of cultivar identity in tissue culture-derived date palms using RAPD and AFLP analysis. Biologia Plantarum 50(2), 2006,198-204. DOI: http://dx.doi.org/10.1007/s10535-006-0007-3

Smulders MJM and de Klerk GJ, Epigenetics in plant tissue culture. Plant Growth Regulation 63(2), 2011, 137-146. DOI: http://dx.doi.org/10.1007/s10725-010-9531-4

Song Y, Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide, Journal of Integrative Plant Biology 56(2), 2014, 106–113. DOI: http://dx.doi.org/10.1111/jipb.12131

Wright TR, Shan G, Walsh TA, Lira JM, Cui C, Song P, Zhuang M, Arnold NL, Lin G, Yau K, Russell SM, Cicchillo RM and Peterson MA, Simpson DM, Zhou N, Ponsamuel J, Zhang Z, Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes, Proc Natl Acad Sci USA 107, 2010, 20240–20245. DOI: http://dx.doi.org/10.1073/pnas.1013154107

 DOI: 10.18380/SZIE.COLUM.2014.1.2.109