Gene Expression of Soybean Calli Culture Affected by Auxinoids with Various Chemical Structure

Elena Hlinkov' and Claudia Ruzickov'
Elena Hlinkov' and Claudia Ruzickov'

Department of Genetics
Faculty of Natural Sciences Comenius University
Mlynsk' dolina B-1
842 15 Bratislava
Slovakia

Hlinkov' E., and C. Ruzickov' (2000) Gene Expression of Soybean Calli Culture Affected by Auxinoids with Various Chemical Structure. Soybean Genetics Newsletter 27 [Online journal]. URL http://www.soygenetics.org/articles/sgn2000-006.htm (posted 11 May 2000.)

Introduction

Haberlandt's hypothesis (Haberlandt 1902) about totipotency of living plant cells meant a shift from a level of the hypothesis into a reality in the beginning of 60's when cultivation media for somatic cells of tobacco and carrot were prepared (Skoog et Miller 1957). Callus induction can be induced practically on all plant explants. Differences in a rate of cell conversion into a dedifferentiation are conditioned with the chemical structure of used exogenous growth regulators an ontogenetic stage of explant and concentration gradient between exogenous and endogenous growth regulators. The dedifferentiation rate is a function of genetic and epigenetic factors as a function of cultivation conditions (Hlinkov' 1998). Cytogenetic and morphology studies of the calli cultures of the various plant species showed strong heterogeneity in a chromosomal stability and the cell polymorphism (Gamborg et al. 1968; Shamina 1981; Sekerka 1988; Hlinkov' 1991; 1993). Differences in these parameters were conditioned not only by a genetic background of used plant cells but also by the concentration gradient of the exogenous synthetic auxins and cytokinin in the cultivation medium. The cultivation time in vitro conditions played the serious role in the same genetic material too. Calli cultures including soybean are characterized by prolonged time of a mitotic cycle compared to the dividing cells of the intact plans (2.5-5time) with lower mitotic index and higher level of methylation DNA (Phillips et al. 1990; Muller et al. 1990). Ultrastructure of the calli cells is

changed mainly chloroplasts and mitochondria (Bob'k 1990; Kuang et al. 1992). Possibility to the organogenesis is limited and regulated strongly by genetic background of used (primary) calli concentration gradient of the exo and endogenous growth regulators and the time of cultivation in vitro conditions. The soybean callus induced with the somatic cells of mature cotyledons lost their possibility to the organogenesis very quickly after several subcultivation (Hlinkov' unpublished date) when compared to the callus induced from immature soybean embryos or cotyledons (Lazzeri et al. 1987; Hartweck et al. 1988; Stejskal et Griga 1995). Because callogenesis as well as organogenesis are directly depended at the genetic background of the callus/explants and the ratio of the exo and endogenous growth regulators we were interested in how to change the gene expression of the young soybean calli as the function of the genetic background of used genotypes of the chemical structure of the exogenous auxinoids and the time of cultivation in vitro conditions in the stationary phase of the growth.

Materials and Methods

Primary callus induced on the middle segments of soybean cultivars Maple arrow line H-12 (obtained from Dr. Volenberg Institute of Plant Breeding Ottawa Canada) and Aida (Plant Breading Station Horn' Mo'tenice Czech Rep.) was cultivated on the mER medium with a supplement of 5mM 2 4-D and 50nM of kinetine (Hlinkov' 1993) for 6 weeks. Calli were growing in a darkness in a thermostat with the temperature 23±10C . Callus was friable without chlorophyll content. Genotype differences were manifested in the calli color. Maple arrow line H-12 callus was slightly orange while Aida callus had the color of a ripen lemon. The value of the mitotic index drifted about MI=15 9±2.2' and 2n= 20-80. Experimental media lacked 2 4-D and contained 3-benzyloxycarbonylmethyl-2-benzothiasolinone (BB) in the physiological interesting concentration 1mM; 1mM and 1nM. Kinetine concentration did not change. During 3 weeks of cultivation on the experimental media we concentrated on the changes in the growth index (GI) characteristic of the growth processes. Cell polymorphism dry mass and gene expression were studied in the stationary phase of the growth after 21 day cultivation.

Growth index assay:

Six week-old calli both soybean genotype were pretreated by a cold (70C) on

a synchronization in the mitotic cycle. After 48 hours partially synchronized calli were divided into small portions. The mass of inoculum (m0) was obtained gravimetrically in the sterile conditions. Inoculums with the mean weight 250x50mg were transferred on the experimental media with BB 5 pieces per Petri dish (x 10cm). Every 7th day we measured inoculum weight and calculated growth index (GI). GI was calculated from an equation GI= mi /m0 (i-th day of cultivation).

Determination of the cells polymorphisms :

In the stationary phase of the growth (21-st day of cultivation) one sample per experimental medium was resuspended in the sterile solution of 2.5% glucose. The length of the cells was measured with a micrometer on the microscope Biolar B (PZO Warszawa Poland) and recalculated from a calibration curve for 20x objective. Statistical distribution was made for the length of the 500 cells with 30mm intervals per sample.

Dry mass assay:

In the stationary growth phase the weight of the samples was measured for every concentration of used BB and dried at 950C in a vacuum drier within 24 hours. Obtained dry mass was recalculated on 1g of the fresh mass of callus per sample.

Isolation quantification and separation of the general soluble proteins:

Proteins were isolated from the homogenized calli samples (500mg) for every variant into nondeneturated 0.1M Na-phosphate buffer according to Hlinkov' et al. (1995). Quantitative content of the soluble proteins was determined by Bradford's spectrocolorimetrical methods (Bradford 1976). Samples for 1-SDS-PAGE were prepared according to Laemmli (1970). SDS-PAGE denatured proteins was carried out in 12.5% discontinuous slab gel system according to Smith (1988). A-PAGE of acidic proteins was carried out on the gels with the same reagents only b-mercaptoethanol and SDS were from the solutions omitted. Acidic peroxidases (E.C. 1.11.1.7) were detected according to Vallejos (1983). Silver staining of the gel slabs was carried out using standard nonspecific silver method according to Nesterenko et al. (1994). Molecular mass of denatured proteins and polypeptides was determined from calibration curve for the broad range

protein kit of Biolabs (New England U.S.A.). Haemoglobine (H Mr~ 68kDa; polypeptide: 52; 30; 14.5 and 14 0 kDa Serva Heidelberg Germany) bovine serum albumin (Al; Mr~ 66.7 and 66.2 kDa; Imuna 'ari'sk' Michalany Slovakia) and lysozyme (Ly; Mr~14.3; Sigma St. Luis U.S.A.) were used as molecular mass standards for A-PAGE. Electrophoresis was done in the Midi Protean gel apparatus of LKB Pharmacia (Uppsala Sweden). The experiments were repeated 3-times.

Results and Discussion

The physiological interesting concentration of tested BB showed that the both young soybean calli reacted differently on the BB during 3 weeks cultivation compared to 2 4-D in the control media (Fig.1a b). The first changes were detected in a duration of lag-phase by calli of both genotype at the highest BB concentration used. The minimal time interval necessary to gain double amount of the callus biomass was longer at 100% for 1mM BB in both genotypes compared to calli growing on the media with 2 4-D. This effect but not so evident was observed in the "old" calli cultures of Haploppapus gracilis and Crepis capillaris long-term cultivated in vitro conditions for more than 4 years (Hlinkov' 1990 1991). The concentration of 1mM BB in the medium showed not optimal for the soybean calli. It is contrary result which we received by the induction of the primary callus (Hlinkov' 1998) . The growth curve lost their S-shape and the calli biomass increase was lower compared to 2 4-D in the control medium and 1mm BB. Stationary phase of the growth was reached more quickly but with lower growth effect. Maple arrow line H-12 callus (MA) changed its color probably as the result of the accumulation of the secondary metabolities. 1mM concentration of BB in the cultivation medium did not change significantly basic characteristics of the growth curve compared to 2 4-D. It was useful for activation proliferation of the old calli cells and suspension cultures of the various plant species (Hlinkov' 1990 1991). The 1nM concentration of BB prolonged lag-and exponential phase of the growth for MA calli. Genotype differences between Aida' callus (A) and MA callus were evident in an input to the exponential phase of the growth. The increase of the MA calli biomass was smaller compared to both the control and A callus values. The growth curve of A calli for this concentration of BB in the exponential phase was lower too and the increment of calli biomass differs from the control values. The effect of the used concentrations of BB on the growth processes of the

calli culture of the both soybean genotypes in the stationary phase represented Fig.2. The increase of the A calli biomass by the concentration of 1mM BB in the medium was statistically significant. Inhibition effect of the concentration 1mM BB on the growth reflected genotype differences and was statistically significant.

Effect of all used BB concentrations was also presented on the cell level. The cell length polymorphisms of both calli cultures was BB changed (Fig.3a b). The maximum in the cell length for the 1mM BB was shifted to the lower values and reached lmax '(60-90mm) when compared to the cells of the calli cultures growing on the control media with the 2 4-D. Cells were smaller and the mean length was 65mm. The highest and smallest concentration of BB (1mM and 1nM) influenced content of the cells with size larger than 100mm. This result indicated that values of the GI could be changed as a consequence of the cell enlargement and the accumulation of the secondary metabolites too. Cell populations with the small size in both soybean calli (~ 70-75%) could be indicate on their higher mitotic and metabolic activity.

The genetic background of both calli the concentrations of the used auxinoids and their chemical structure affected the amount of the dry mass (Tab.1). The water capacity of the cells was higher for callus A and vice versa the quantity of the insoluble compounds was higher for callus MA. Genotype differences were not registered in the general content of the soluble proteins isolated from the calli growing on the media with 2 4-D (Tab. 1) as it was in the primary calli (Hlinkov' 1998). Qualitative and quantitative differences of the proteins and polypeptides were identified only by separation A-PAGE and SDS-PAGE (Tab. 2a b). The protein patterns of calli in the stationary phase of the growth represented actual gene expression as the complicated function of genetic and epigenetic factors chemical structure of used auxinoids and their concentration as well as time cultivation in vitro conditions. 9-week old callus of both soybean genotypes cultivated on the media with 2 4-D differed from the primary callus in many bands. Differences in the nonspecific proteins and polypeptides common for both genotypes had Mr~66; 60; 52-50; 42-40; 34; 26-24; 21; 20.2-20.04; 17; 15; and 14 kDa (Tab. 2a b). Bands for the some soluble subunits of lectine and a b and g conglycinine were missing. Rubisco gene products and proteins connected with the light harvesting complex I and II were strongly depressed (Nap et al. 1993 ; Ikeuchi et al. 1991; Chen et al.1990 ; Ku-Chuan Hsiao et al. 1988).

The gene expression of calli reacted differently on the used auxinoids independently of the model soybean genotype (Tab.2a b). Fast reaction on the used auxinoid in a proteosynthesis noted Jones (1994). mRNA induced with auxinoid 2 4-D appeared after some hours of their application and the auxin-binding (AB) proteins were localized in connections with many cell compartments (Tian et al. 1995; Guilfoyle et al. 1993; Jones 1994). From the group of auxin-binding proteins there were identified proteins with molecular mass Mr~ 60; 42/40; 28/27; 25; 24 ; 22 and 11kDa. Ainley et al. (1988) identified genes aux22 and aux28 in the soybean genome. Guilfoyle et al. (1993) found their homology in Arabidopsis thaliana. Protein p60 (biochemical function-hydrolase) was identified in the connection with the nucleus. Its content depended on the used auxinoid and the time cultivation in vitro conditions independently of the genotype . AB proteins p42/40 as well as p50 probably play the role in the influx of the exogenous auxins into the cells and with a high probability participated on the structure of the receptor channels because their quantity strongly changed with the concentration and the structure of the used synthetic auxinoid. Their amount in the patterns independently of plant species is connected with the cell wall. In soybean protoplasts this bands are fully missing (Hlinkovï¿½ unpublished date). The soluble auxin-biding protein p24 connected with the raf endoplasmatic reticulum identified Tian et al. (1995). Quantitative content of this protein changed the used concentration of BB independently of genotype. Its synthesis was BB arrested and instead of them there were synthesized proteins p22 and p25. BB affected biosynthesis of the protein p28 too. The amount of this protein was higher when compared to the p28 protein content from the protein extract isolated from the calli growing on the medium with 2 4-D. Biochemical function of the soluble AB protein p25 was determined immunofluorescently for soybean as glutation-S-transferase (Jones 1994).

Interesting situation was observed in the calli for the tyrpsin inhibitor (ti subunits : 20.04 and 20.2 kDa). 2 4-D was depressed ti gene expression in the calli culture of both soybean genotypes. Expression of this gene reflected genotype difference as well as used chemical structure of exogenous auxinoids and time interval of cultivation in vitro conditions. Synthesis of the trypsin inhibitor was inhibited in A calli while in the MA calli were proteins with this Mr synthesized for 1mM and 1mM BB. The content of both tyrpsin subunits in MA calli was connected with the higher amount of proteins with Mr~27; 21; 17 and 16 kDa. This fact as well as the changes in color and dry mass of MA calli for the concentration of 1mM BB in culture medium

indicated that metabolic pathway for ti-gene and accumulation of secondary metabolites are connected with some mitotic inhibitors. Connection of BB with some inhibitors in calli culture as well as the general level of gene expression in the contrary to the primary calli represented the decrease of among of peroxidases and their qualitative content (Tab. 1). Guilfoyle et al. (1993) indicated that mRNA content connected with exogenous auxinoids changed in the dependence of ontogenetic development of tissue and used auxinoid. We received near results for the soybean calli (influence of time and count of subcultivation in vitro condition) on the base of physiological and the protein analyses. We slightly complemented these knowledge about possible genetic differences the role of the chemical structure of used auxinoid' and time of cultivation in vitro conditions . It is possible that the differences in the quality and quantity of mRNA in the soybean calli as well as the level of the soluble vegetable proteins induced difficulties connected with the induction of the organogenesis. Products of many biosynthetic pathways in the callus culture are missing or their concentration compared to the primary callus induced on the slices of the mature as well as the immature cotyledons are very low. It is very probable that silence genes in calli cultures can changed direction of many biosynthesis pathways .

Conclusions

On the basis of the obtained results we can note that:

gene expression of the soybean calli changed in a consequence of the long-term in vitro cultivation independently of used genotype and chemical structure of used auxinoid;
chemical structure of exogenous auxinoids induced gene expression of the different auxin-binding proteins but it was not genotype independent;
optimal concentration of 3-benzyloxycarbonylmetyl-2-benzothiasolinone for the callus cultivation was 1mM in the difference of 1mM concentration which was unoptimal ;
BB affected quantitative and qualitative content of proteins with peroxidase activity independently of genotype;
content of secondary metabolities and insoluble proteins induced in the calli by BB reflected genotype differences;
in the genotype dependence of the calli culture 1mM and 1mM BB affected

content of tyrpsin inhibitors.
amount of the vegetable soluble proteins in the calli decreased with the long-term cultivation independently of the used auxinoid;
proteins connected with the expression of the chloroplast genome were arrested in the calli as the function of a long-term cultivation independently of soybean genotype and used auxinoid.
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