SECTION 7 - MOLECULAR BIOLOGY

Beta-glucuronidase in transgenic plants *in vitro* and *in situ*

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The fluorescence technique for *in vitro* assay of beta-glucuronidase in transgenic plants was modified concerning its reliability, sensitivity and possibility of quantitative evaluation. Using this procedure the stability of the GUS gene expression was studied in particular clones of *Agrobacterium* transformed *Nicotiana tabacum*. Some clones were GUS negative though they were kanamycin resistant. There are profound differences in GUS activity when comparing particular GUS positive clones. No substantial differences were found in GUS activity of different parts of the same plant. The freezing/defreezing procedure reduced GUS activity to 20 to 40 % of the control The fixation with Ca formol acted similarly. Only a small further decrease in GUS activity was observed after freezing/defreezing of the fixed material. However, no GUS activity was revealed in sections of trangenic plants prepared in the same way as used for *in situ* localization of intrinsing glycosidases. The indigogenic procedure was used for *in situ* studies. The incubation *in toto* resulted in patterns of differential substrate penetration rather than in patterns of differential gene expression, even if vacuum infiltration was used.

Induction of hypomethylated state of *Nicotiana tabacum* L. nuclear genome

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Cytosine methylation is a rather common epigenetic modification of eukaryotic DNA. In plant nuclear genomes cytosines are mostly methylated in CpG and CpXpG sequences (Gruenbaum *et al.* 1981). Eukaryotic genomes contain both hypermethylated and hypomethylated domains. Hypermethylated domains include mostly repetitive DNA sequences. Recently, we have initiated studies about induction of hypomethylated state of heavy methylated genomic domains (Bezděk *et al.* 1991). In these experiments hypomethylated state of the *N. tabacum* genome was induced *via* cultivation of calli on Murashige and Skoog's medium containing subtoxic concentrations of 5-azacytidine or ethionine. The hypomethylating effect was evaluated on two repeated DNA sequences cloned recently in our laboratory, HRS60 (Koukalová *et al.* 1989) and R8.1 (Kuhrová *et al.* 1991). Restriction endonucleases MspI and HpaII, differing in the sensitivity to methylation of their common recognition CCGG site, were used: DNAs of calli were digested with either of the enzyme and Southern hybridization was carried out using ^{32}P-labeled HRS60 or R8.1 as DNA hybridization probes. The state of DNA methylation was evaluated on the basis of hybridization patterns obtained. It was found that both 5-azacytidine and ethionine induced hypomethylated state but their effect differed: 5-azacytidine acted on both CpG and CpCpG sequences, whereas ethionine acted specifically on CpCpG. Hypomethylated state was rather stable in calli upon the removal of drugs. In order to analyze further the stability of induced hypomethylated state, the calli were

subjected to protoplast isolation. After the appropriate subcultivation in liquid K3 medium followed with agarose--K3 medium (Nagy and Maliga 1976) and calli formation on MS media, the state of DNA methylation was estimated in individual cell lines. A persistence of the induced hypomethylated state was found in several cell lines. In other lines DNA remethylation of different extent was observed. Remethylated DNA was typical for cell lines derived from ethionine-treated calli.

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Methylation state of rDNA in some species of the family *Brassicaceae* studied with the method of RFLP

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The role of 5-methylcytosine in the regulation of gene activity in eukaryotic cells has become recently apparent (Cedar 1988). Results obtained with animal cells show that DNA methylation patterns of genes are related to their expression, the unmethylated genes being typical for tissues of expression. Plant genomes have about 30 % of cytosine residues methylated in sequences CpG and CpXpG. Nevertheless information about the tissue-specificity of DNA methylation pattern in plants is rather rare. In our experiments, the DNA methylation pattern of rDNA, coding ribosomal RNA, was compared in two different plant tissues: young seedlings and differentiated leaves. Four species of the family *Brassicaceae* were studied: *Brassica oleracea, B. campestris, B. napus* and *Raphanus sativus*. The DNAs of these plants were digested using the combinations of endonucleases EcoRI + MspI or EcoRI + HpaII. EcoRI cuts DNA into fragments having on average 4096 bp. Within them, the tetranucleotides CCGG were digested with methylation of cytosine residues in CCGG recognition site. Southern blot hybridization was then carried out with ^{32}P labeled 25S-rDNA probe (Kiss *et al.* 1989). According to the hybridization patterns obtained, the methylation state of DNAs was evaluated. Resulting hybridization patterns were species-specific and demonstrated that a large fraction of rDNA genes was heavily methylated. However, no differences in the DNA methylation pattern were found when comparing DNAs of young seedlings and differentiated leaves.

References:
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Insertion of *Agrobacterium* plasmid genes 5, rolB and rolC affects regeneration capacity of potato stem explants

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Three *Agrobacterium* plasmid born genes (Ti plasmid gene 5, Ri plasmid genes rolC and rolB) were transmitted into the genome of two potato cultivar stem explants which strictly differ in their natural regeneration ability *in vitro*, under the effect of exogenously applied phytohormones (Opatrná *et al.* 1990). Under the same conditions of stem internode section culture (SIS) on the medium MS containing 6-BAP, adenine and IAA the process bud/shoot formation (initiation time, number of regenerating explants, number of buds per one explant) in control and *Agrobacterium* treated segments was studied. Transformed plants were derived from shoots, cloned *in vitro* and "secondary" regeneration capacity of their SIS was analyzed. No effect was observed in well regenerating cv. Lada using genes under their own promotor, only some variations in the total number of buds per explant were induced by the same genes driven by CaMV promotor. Pronounced variability was found in the regeneration capacity of the SIS taken from some randomly selected transformants. Naturally low regeneration ability of the cv. Karin explants was pronouncedly increased by the insertion of genes rolB, rolC and, in particular, gene 5. In all cases, transformed plants were obtained and cloned *in vitro*. However, secondary regeneration capacity of the SIS taken from them was in most cases again low. The analysis of the hormonal (auxin, cytokinin) content of the transformants is being done to understand the mechanism of the action of the products of inserted genes.

Reference:

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Leaf morphology, anatomy and ultrastructure in transgenic tobacco (*Nicotiana tabacum* L. cv. Samsun) plantlets

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With the aim of elucidating the effect of gene 4 transfer (and thus an enhanced internal cytokinin content in the transgenic plants) on leaf architecture, leaves of tobacco seedlings (C) and transgenic plants (T) carrying in their genome gene 4 for cytokinin synthesis from T-DNA of pTiC58 and the gene for kanamycin resistance, were compared. Both the C and T plantlets were cultivated for two months under *in vitro* conditions on Linsmaier-Skoog solid medium with 3 % saccharose in an air-conditioned chamber (16 h photoperiod, photon fluence rate 30 μmol m^{-2} s^{-1}, temperature 20 °C). C and T plantlets reached in average the height of 63 mm with 10 to 11 leaves per stalk, T plantlets did not form roots. Significant quantitative differences between individual C plants, and much more between individual T plants were found. C plants had longer and broader leaves which resulted in a larger leaf area in comparison to T plants. But leaf form expressed as the leaf length-to-width ratio, and also leaf thickness did not differ significantly: only a tendency to thicker leaves in T appeared. In leaf cross sections in C and T plantlets, a relatively thick epidemis (lower epidermis was

somewhat thinner with more stomata than the upper one) and a mesophyll consisting of one layer of poorly differentiated palisade parenchyma cells and two to three spongy parenchyma layers were found. Large intercellular spaces were present in the mesophyll. chloroplasts of T plantlets exerted a tendency to more developed system of thylakoids, smaller plastoglobules and larger starch inclusions than C plantlet chloroplasts. The insertion gradients, *i..e.* the differences between upper, middle and lower leaves, were more pronounced in C plantlets.