rDNA Methylation in *Hypericum perforatum* Diploids and Tetraploids

Jana Halušková[a,*], Benye Liu[b], and Ludger Beerhues[b]

[a] Institute of Biology and Ecology, Faculty of Science, P. J. Šafárik University, Mánesova 23, 04167 Košice, Slovakia.
Fax: +42 15 56 33 73 53. E-mail: janaha@kosice.upjs.sk
[b] Institute for Pharmaceutical Biology, TU Braunschweig, Mendelssohnstrasse 1, 38106 Braunschweig, Germany

* Author for correspondence and reprint requests

Z. Naturforsch. **58 c,** 895–897 (2003); received August 25, 2003

The effect of the gene dosage on the expression of rRNAs was studied in *Hypericum perforatum*. The methylation levels of rDNA were analysed using the isoschizomers *MspI* and *HpaII* and eleven additional methylation-sensitive enzymes. No differences in rDNA methylation were observed between diploids and tetraploids at an early ontogenetic stage.

Key words: Hypericum perforatum, rDNA Methylation, Gene Dosage Effect

Introduction

The fate of redundand genes resulting from genome duplication is poorly understood. In *Arabidopsis*, some homologous genes in naturally occurring and newly generated polyploids were subjected to silencing (Lee and Chen, 2001; Comai *et al.,* 2000). The silenced genes were hypermethylated and could be reactivated by blocking DNA methylation, suggesting that epigenetic regulation is involved in the expression of orthologous genes in polyploid genomes. The methylation of plant DNA was originally reported to occur predominantly at cytosines of symmetrical sequences such as CG and CNG. More recently, however, genome sequencing has revealed frequent methylation of cytosines in non-symmetrical sites. The primary sequence of DNA can be modified by methylation of either cytosine or adenine (Finnegan *et al.,* 1998).

Hypericum perforatum (HP) is an important medicinal plant containing various pharmacologically active secondary metabolites (Whiskey *et al.,* 2001). Recently, HP somaclones were found to exhibit fairly extensive cytogenetic variation (Brutovská *et al.,* 1998). RFLP analysis using *EcoRV* and heterologous rDNA probes revealed natural rDNA variation (Halušková and Čellárová, 1997). The dependence of some phenotypic traits on the ploidy of HP somaclones was reported (Čellárová *et al.,* 1997). This raised the question whether the gene dosage might affect the expression of the genes encoding the traits.

Here we studied HP diploids derived from tissue culture and tetraploids for differences in the expression of rRNA genes manifested by rDNA methylation changes. Gene silencing due to an increased gene dosage might lead to transcriptional inactivation by methylation of some rDNA copies in tetraploids, resulting in different restriction patterns generated by isoschizomers and other methylation-sensitive enzymes.

Experimental

Plant material and cytogenetic analysis

Tetraploid plants were derived from seeds of *Hypericum perforatum* cv. Topas; diploid plants represented the R_3 progeny of two different diploid somaclones (Čellárová *et al.,* 1994). Plants were grown on basal RM medium (Linsmaier and Skoog, 1965) without plant growth regulators at 22 °C, 1100 lux irradiation (6.8 μm m^{-2} s^{-1}), 40% relative humidity and 16/8 h photoperiod. The chromosome number was determined in root tips of plants with 5–6 pairs of foliage leaves employing classical cytogenetic procedures (Brutovská *et al.,* 1998).

DNA isolation and methylation analyses

Leaf DNA was isolated at the above ontogenetic stage using the method of Haberer *et al.* (1996). Each analysis used 400–500 ng DNA. Digestion and Southern blotting were performed as described by Maniatis *et al.* (1989). The isoschizomers *MspI* and *HpaII* digest CCGG but not mCmCGG and mCCGG. Sequences with internal cytosine methylation, CmCGG, are digested by *MspI* but not by *HpaII*. The other enzymes possessing sensitivity to cytosine methylation have target sequences that either contain CG or CNG (*SmaI*, *XhoI*) or could overlap with CG (*SacI*, *Sau3A*, *BamHI*, *EcoRI*). In one case, cytosine in

the restriction site is located in the non-symmetrical sequence CXX, with X representing any base except G (*XbaI*). Enzymes with sensitivity solely to adenine methylation were also employed (*EcoRV*, *NdeI*, *SpeI*). The enzyme *DpnI* digests the target sequences GmATC and GmATmC but not GATC and GATmC.

As the nucleotide sequence of the HP rDNA unit, except the IGS (intergenic spacer) region, has been established (AF455674, AF206934, AF479122), the number of restriction sites within the rDNA unit could be determined for all the enzymes employed. These enzymes have at least one or more restriction sites in the coding sequences of the 18SrDNA, 5,8SrDNA, 25SrDNA or in the ITS1 or ITS2 spacer regions. The enzymes recognizing a tetranucleotide target sequence find even 20–30 restriction sites.

The 25SrDNA probe was amplified by PCR using the primers JF09 (5′-GCG AGC GAA CCG GGA TAA GCC C-3′) and JF10 (5′-CGG AAT TTA CCG CCC GAT TGG GG-3′) (Yokota *et al.,* 1989). Digoxigenin-labeling of probes, hybridisation and fluorescence detection were performed using the DIG High Prime Labeling and Detection Starter Kit II according to the manufacturer's instructions (Roche, Germany).

Results and Discussion

Our analyses demonstrated that, with all enzymes used, no polymorphic bands occurred in the hybridisation profiles of tetraploid plants when compared to those of diploid plants. In both diploids and tetraploids, the isoschizomers *HpaII* and *MspI* led to different hybridisation patterns, indicating mostly internal cytosine methylation in the rDNA CCGG sequences. The profiles obtained with *HpaII* pointed to the presence of some non-methylated CCGG restriction sites.

The enzyme *SmaI* with the target sequence CCCGGG failed to completely digest the DNA from plants of both ploidies, suggesting internal 3′-cytosine methylation in most *SmaI* restriction sites. Similarly, incomplete digestion of diploid and tetraploid DNA was found with *DpnI*, demonstrating that no or only few GmATC sequences are present.

In conclusion, our results indicate that there are no differences in rDNA methylation between HP diploid and tetraploid plants at the early ontogenetic stage. Regardless of double gene dosage, all rDNA copies in tetraploids appear to be expressed. A similar observation was made by Polizzi *et al.* (1998) who used methylation-sensitive isoschizomers to analyse rRNA gene methylation during endosperm development in triploid and endoreduplicated nuclei of *Triticum durum*. Irrespective of the gene dosage, active transcription of all rDNA copies occurred.

Acknowledgements

J. H. is grateful to the Deutscher Akademischer Austauschdienst (DAAD) for a research scholarship. We thank Prof. E. Čellárová, Institute of Biology and Ecology, P. J. Šafárik University in Košice, for providing seeds of *H. perforatum* plants of different ploidy. The primers JF09 and JF10 were kindly provided by Dr. I. Schubert, IPK, Gatersleben, Germany.

Brutovská R., Čellárová E., and Doležel J. (1998), Cytogenetic variability of *in vitro* regenerated *Hypericum perforatum* L. plants and their seed progenies. Plant Sci. **133**, 221–229.

Čellárová E., Daxnerová Z., Kimáková K., and Halušková J. (1994), The variability of the hypericin content in the regenerants of *Hypericum perforatum* L. Acta Biotechnol. **14**, 267–274.

Čellárová E., Brutovská R., Daxnerová Z., Bruňáková K., and Weigel R. C. (1997), Correlation between hypericin content and the ploidy of somaclones of *Hypericum perforatum* L. Acta Biotechnol. **17**, 83–90.

Comai L., Tyagi A. P., Winter K., Holmes-Davis R., Reynolds S. H., Stevens Y., and Byers B. (2000), Phenotypic instability and rapid gene silencing in newly formed *Arabidopsis* allotetraploids. Plant Cell **12**, 1551–1567.

Finnegan E. J., Genger R. K., Peacock W. J., and Dennis E. S. (1998), DNA methylation in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**, 223–247.

Haberer G., Fischer T. C., and Torres-Ruiz R. A. (1996), Mapping of the nucleolus organizer region on chromosome 4 in *Arabidopsis thaliana*. Theor. Appl. Genet. **85**, 190–196.

Halušková J., and Čellárová E. (1997), RFLP analysis of *Hypericum perforatum* L. somaclones and their progenies. Euphytica **95**, 229–235.

Lee H.-S., and Chen Z. J. (2001), Protein-coding genes are epigenetically regulated in *Arabidopsis* polyploids. Proc. Natl. Acad. Sci. USA **98**, 6753–6758.

Linsmaier E. M., and Skoog F. (1965), Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. **18**, 100–127.

Maniatis T., Fritsch E. F., and Sambrook J. (1989), Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Polizzi E., Natali L., Muscio A. M., Giordani T., Cionini G., and Cavallini A. (1998), Analysis of chromatin and DNA during chromosome endoreduplication in the endosperm of *Triticum durum* Desf. Protoplasma **203**, 175–185.

Whiskey E., Werneke U., and Taylor D. (2001), A systematic review and meta-analysis of *Hypericum perforatum* in depression: a comprehensive clinical review. Int. Clin. Psychopharmacol. **16**, 239–252.

Yokota J., Krawata T., Iida J., Kato A., and Tanifuji S. (1989), Nucleotide sequence of the 5,8S rRNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. J. Mol. Evol. **26**, 294–301.