



SZENT ISTVÁN UNIVERSITY

**KARYOTYPING OF ENERGY WILLOW CHROMOSOMES, AND
CYTOLOGICAL AND MORPHOLOGICAL CHARACTERISATION
OF AUTOTETRAPLOID ENERGY WILLOW PLANTS**

Thesis of PhD dissertation

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1. RESEARCH BACKGROUNDS, AIMS OF THE RESEARCH

The short-rotation woody crops are one of the most important renewable bioenergy resources worldwide. The biomass of plantations planted for energy purposes can serve as a source of energy for various industries, either as green or as wood materials. Energy plants can be successfully grown in areas with extreme conditions because they have a good adaptability, rapid growth rate and tolerance towards wide soil variants. Woody energy plantations can be used for the recultivation and phytoremediation of soils and for the treatment of sewage sludge and sewage. The utilization of green energy can play a role in replacing non-renewable energy sources. The non-renewable energy sources are gradually depleted, however the green energy is economic, easily produced, and they have a minimal environmental impact.

Among the renewable energy sources to be utilized in Hungary, biomass has the greatest importance, which accounts for 65-80%. The use of biomass produced in woody energy plantations serve mainly for the production of heat and electricity, but it is also suitable for the production of bioethanol and biogas (Csipkés 2011). Today, Hungary has nearly 5.3 million hectares of agricultural area, of which is in approximately 4.3 million hectares the arable crop production. The proportion of disadvantaged areas reaches hundreds of thousands of hectares, but the energy crop plantations can be planted in these areas (Gyuricza et al. 2012). The total biomass stock of our country is 350-360 million tons. Most of the biomass is wood-based biomass, which includes not only woody energy plantations but also solid biomass from forestry (Vágvölgyi 2013).

In Hungary, poplar, black locust and willow plantations are primarily used for energy production purposes. The increasing demand for the renewable green energy focuses special attention on woody plants grown either in conventional forestry or in energy plantations established on marginal lands.

The main aims of the dissertation:

1. Production and propagation of autotetraploid energy willow plants (*Salix viminalis* L. $2n=76$) ENERGO genotype.
Cytological and physiological characterisation of autotetraploid willow plants.

The benefits of polyploidization can open new avenues for utilizing populations with high biomass yields and improved agronomic and energetic properties in energy crops. Furthermore, provides valuable information for determining and realizing further breeding aims.

2. Molecular cytogenetic characterisation of (*Salix viminalis* L. $2n=38$) ENERGO genotype using fluorescence *in situ* hybridisation (FISH)

In breeding for high biomass productivity, limited knowledge is available on the molecular cytogenetics of energy willow, which could be combined with genetic linkage mapping. In contrast to the progress in the molecular genetic mapping of the willow genome, the chromosomal karyotyping of this species has not been studied extensively. The combination of the genetic and physical mapping technologies is gaining increasing attention in research on the genomics of tree species and in related breeding programmes. The aim was to adapt the protocol of FISH-based chromosome karyotyping to willow plants. FISH method is based on labelled heterologous DNA probes allowing the identification of chromosomes or chromosome pairs as a first step in the prediction of the chromosome karyotype. The application of these DNA probes as molecular cytogenetic markers will provide new information to study the FISH patterns of the *Salix* chromosomes. The detailed cytogenetic analysis of individual chromosomes will lead to progress in the genome studies on the willow genome.

2. MATERIALS AND METHODS

2.1. Plant growth and materials

Our experiments were carried out with the energy willow variety ENERGO genotype (*Salix viminalis* L., $2n=2x=38$) which was bred and propagated by Kreátor 2005. Ltd. The propagation materials used in our experiment were 20 cm cuttings, which were clones with the same genetic background. The cuttings were stored in a cold room at 4°C from field harvest for experimental use. Before planting, cuttings were soaked in water for 48 hours at room temperature. The planting was done according to the cultivation technology used under the field conditions. The level of illumination in the greenhouse was approximately $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Plants were grown in the greenhouse at 16 hours light and 8 hours dark period. During this period, the temperature was between 18 to 22°C with optimal water supply. For our experiments, fresh buds were isolated. These buds were surface sterilization, and we produced the sterile cultures under laboratory conditions.

2.2. Production of autotetraploid willow plants by *in vitro* colchicine treatment of axillary buds

The surface sterilization of 2-4 cm isolated shoot pieces from the greenhouse was started in a laminar box after 10 minutes in sterile water. In a sterile Erlenmeyer flask, the explants were slowly shaken for 10 minutes in 0.2% HgCl_2 solution, and then the explants were soaked in slow shaking for 10 minutes in 70% ethanol solution. After soaking in ethanol, we washed with sterile water for 3×10 minutes, then placed the shoot pieces on sterile filter paper for drying.

From the sterile shoot pieces *in vitro* sterile plantlets were grown on a one-half strength concentration of hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962). These cultures were maintained under continuous light. Shoot apical meristems of between 8 to 10 cm plantlets were decapitated, and 48 hours later, stem sections with axillary buds were placed into sterile colchicine solution (0.05% or 0.1% [w/v]) and incubated for 48 hours in the dark. After colchicine treatment, these stem sections were rinsed three times in sterile distilled water and placed on hormone-free 0.6% (w/v) agar medium without colchicine. Two to three cm long shoots grown from the treated axillary buds were cut and placed in agar-solidified culture medium and used for further *in vitro* propagation. The differentiated roots were used for ploidy analyses. During the years, the tetraploid plantlets were maintained and propagated by nodal cuttings *in vitro*, and between 8 to 10 cm high rooted plantlets were transferred to soil in the greenhouse.

Under these conditions, these willow plants developed green woody stems that can be used as a propagation material for both greenhouse and field studies.

2.2.1. Identification of energy willow variants with duplicated genome size

The autotetraploid willow variants from treated buds were screened by both chromosome counting and flow cytometry analysis. The flow cytometric analysis was carried out according to Galbraith et al. (1983). Root tips (approximately between 5 to 10 mm) of 2-week-old cuttings were excised from the plants grown either in agar medium or in water used for rooting willow cuttings. Determination of ploidy levels was conducted by flow cytometry (BD FACSCalibur) equipped with a 532-nm green solid-state laser operating at 30 mW. Identical instrument settings were used in order to have comparable relative fluorescence intensity values while analyzing diploid and tetraploid samples. For the determination of chromosome numbers in mitotic willow cells, the protocol described later for FISH studies was used.

2.2.2 Phenotyping of autotetraploid willow plants shoot and root growth

Phenotyping based on digital imaging has allowed to use the number of green and white pixels to characterize the aboveground and root biomass mass each plant. A detailed description of the method is given in our publication (Dudits et al. Plant Physiol. 2016). Thus, it was possible to compare the growth rate of each genotype.

2.3. Cytogenetic characterisation of *Salix viminalis* L.

2.3.1. Cytological preparation of willow

Due to the lack of any described standard protocol for the preparation of chromosome spread of willow, first, we optimised the procedure. Briefly, the mitotic events were synchronized by cold treatment at 4°C for 4 days. After 22 hours of incubation at room temperature, root tips were collected and fixed in Carnoy's solution (ethanol:acetic acid, 3:1 [v/v]). Cell walls of the fixed roots were digested in 1% enzyme mixture: 0.3% (w/v) cellulase, 0.3% (w/v) pectolyase, and 0.3% (w/v) cytohelicase, and squash preparations were made in 45% acetic acid. Glass slides were exposed to liquid nitrogen, and after removal of coverslips, cells were stained with DAPI and observed with an Olympus FV1000 confocal microscope.

2.3.2. Karyotyping of *Salix viminalis* L. chromosomes using FISH method with labelling repetitive DNA probes

In our experiments we used a fluorescence *in situ* hybridisation (FISH) protocol as a new approach to analyse the genomic constitution of *Salix viminalis* using the heterologous DNA clones: pSc119.2, pTa71, pTa794, pAs1, Afafamily, pA11, HT100.3, ZCF1 and the GAA microsatellite marker. DNA probes were labelling with nick translation and Digoxigenin-16-dUTP and biotin-11-dUTP, these were detected using anti-digoxigenin-rhodamine Fab fragments (Roche) and streptavidin-FITC (Roche), respectively. Probe labelling and the FISH procedure were carried out according to Linc et al. (1999; 2012) with minor modificatons.

2.4. Statistical analyses

For all statistical analyses, R statistical analysis software was used (R Core Team; <https://www.R-project.org/>). For all genotypes, the traits of individual plants were measured, and the distribution of data was displayed by box and whisker plots (Spitzer et al. 2014). The plots were generated with the Web tool BoxPlotR (<http://boxplot.tyerslab.com/>) and edited with CorelDraw Graphics Suite X7.

3. RESULTS

3.1. Production and identification of autotetraploid willow variants by flow cytometric analysis and chromosome counting methods of root cells

Colchicine inhibits the mitosis of cells in the axillary meristems, which can cause the chromosome duplication in these cells. Plantlets were recovered from the treated shoots. For early screening of ploidy level, nuclei were isolated from root tips of stem cuttings for flow cytometric determination. As shown by the histograms of flow cytometric analysis, plants of autotetraploid ENERGO (PP-E) lines have doubled DNA content in their root cells. These results were confirmed by chromosome counting using fluorescence microscopy (Figure 1.).

Diploid ENERGO plants have a karyotype with $2n=2x=38$ chromosomes. Sixteen lines with $2n=4x=76$ chromosomes were identified by these tests.

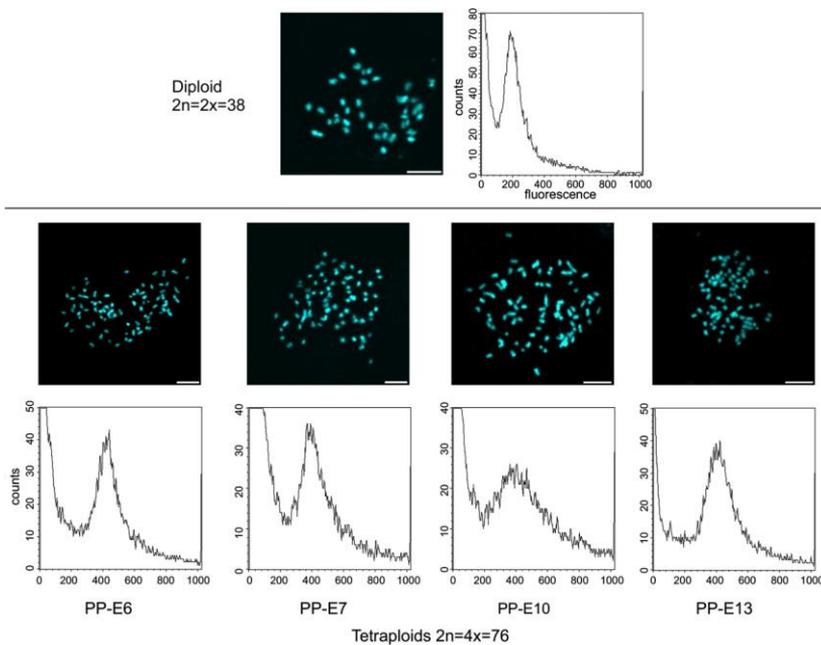


Figure 1. Identification of autotetraploid willow genotypes by chromosome counting (using 4',6-diamidino-2-phenylindole [DAPI] stain) and flow cytometric analysis of relative DNA content (using propidium iodide).

3.1.2. Morphological characterisation of autotetraploid willow genotypes

The genom duplication in autotetraploids willow plants resulted in a very complex and multiple changes in the anatomical and morphological levels. The main changes were observed at the leaf size, wider stem diameter and increasing root-system density.

3.1.2.1. Duplication of the willow genome affects leaf size, morphology and functions

Comprehensive characterization of several independent autotetraploid lines of energy willow revealed substantial changes in leaf structure and functions as consequences of genome size duplication. Increase of leaf biomass is accompanied by alterations in leaf shape and extended lamina length and width. The experimental findings presented here indicate that the enlargement of foliage size generated by autotetraploidy was accompanied by improvement of the photosynthetic productivity of tetraploid willow plants. Increases in both the stomatal conductance and the CO₂ assimilation rate can be a prerequisite for the potential improvement of biomass yield. In tetraploid willow leaves, fewer but larger palisade parenchyma cells were detected.

3.1.2.2. Effects of autopolyploidy in primary and secondary growth of stem

Growth intensity of shoots was characterized by primary and secondary growth analyses. The growth intensity of tetraploid genotypes was different from that of the diploid genotype. The PP-E2 line produced higher growth rate but the PPE-13 line produced lower growth intensity in the green biomass production than the diploid "ENERGO" plants. The diploids had higher average stem length compared to tetraploids. It was a significant difference between diploid and tetraploid lines. In contrast, the stem diameter thickening was improved in plants from the tetraploid genotypes.

The thickened stem diameter of tetraploid willow plants results from an anatomical change that is probably caused the doubled genome size. Significant growth of primary and secondary xylem rings and development of thicker bark compared to the diploid genotype can be seen in the cross sections of the stem of tetraploid willow genotypes.

3.1.2.3. Effects of the autotetraploid willow genome on the root system

Using the root phenotyping platform, growth of the root system was monitored by digital imaging from both side and bottom views. Despite the fact that the cumulative white pixel counts generated cannot represent the whole-root biomass, this approach could be used for the detection of genotypic differences in root growth rate. During the first 3 weeks of root development, stem cuttings from tetraploid genotypes analyzed produced significantly higher root densities than cuttings from the diploid variant. As the cultivation period proceeded, differences in root formation between tetraploid and diploid plants were increased considerably. After 7 weeks of growth at the end of growing period the wet root weight data indicated that the autotetraploid willow plants produced a larger root biomass than the diploid plants. On the other hand, dry weight measurements showed that the differences between the genotypes were less pronounced, which may be due to different water contents.

Analysis of cross sections also revealed significant differences in anatomy between diploid and tetraploid roots. Root cortex cells were found to be larger in plants with duplicated genome size.

3.2. Karyotyping of *Salix viminalis* L. chromosomes using molecular cytogenetic method

In breeding for high biomass productivity, limited knowledge is available on the molecular cytogenetics of willow, which could be combined with genetic linkage mapping. In the present study we described a standard protocol for the FISH method and preparation of chromosome spread of willow, and we have optimised the procedures.

3.2.1. FISH-based chromosome karyotyping of *Salix viminalis* L. using heterologous DNA probes

In contrast to the progress in the molecular genetic mapping of the willow genome, the chromosomal structure of this species has not been studied extensively. Therefore, the adaptation of the fluorescence *in situ* hybridisation (FISH) protocol is presented as a new approach in the characterisation of willow chromosomes. FISH is based on labelled heterologous DNA probes allowing the identification of chromosomes or chromosome pairs as a first step in the prediction of the chromosome partial karyotype.

In our experiment describes the adaptation of a fluorescence *in situ* hybridisation (FISH) protocol as a new approach to analyse the genomic constitution of *Salix viminalis* using the heterologous DNA clones: pSc119.2, pTa71, pTa794, pAs1, Afafamily, pA11, HT100.3, ZCF1 and the GAA microsatellite marker. Three of the nine probes showed unambiguous signals on the metaphase chromosomes. FISH analysis with the pTa71 probe detected one major 18S-5.8S-26S rDNA locus on the short arm of one chromosome pair, however, the pTa794 rDNA site was not visible. One chromosome pair showed a distinct signal around the centromeric region after FISH with the telomere-specific DNA clone HT100.3. Two chromosome pairs were found to have pAs1 FISH signals. Among the woody species, this is the first result that shows two chromosome pairs with pAs1 FISH signals.

3.3. New scientific results

1. Production of 16 independent autotetraploid lines (Poli Plus ENERGO variants) from activated lateral buds of willow after colchicine treatment. The genome size was identified by using flow cytometry and chromosome counting. These autotetraploid willow lines were propagated first *in vitro* and then characterized in the greenhouse after transplantation into soil.
2. The digital phenotyping system was used to characterize the growth parameters of each willow genotype and to identify tetraploid lines with higher growth rates compared to the diploid variant.
3. The PP-E plants developed wider leaves with thicker midrib and enlarged palisade parenchyma cells. In addition to the morphological changes, the tetraploid leaves showed significantly increased efficiency of CO₂ fixation relative to units of the leaf area.
4. Autotetraploidy slowed down primary growth during early shoot development and increased the shoot diameter as parameter of secondary growth. The duplicated genome size enlarged bark and wood layers in twigs.
5. Autotetraploidization increased the biomass of the root system of PP-E plants relative to diploids.
6. We described a new cytological protocol for the willow (*Salix viminalis* L.) chromosome counting.
7. We described of two putative pair of *Salix viminalis* ENERGO chromosomes, and characterized of the willow chromosomes partial karyotype using heterologous non-specific DNA probes.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Identification of energy willow variants with doubled genome size

Shoots regenerated from colchicine-treated buds showed a marked change in leaf and root morphology already in the *in vitro* cultures. Wider, rounded leaves and thicker root systems can serve as early markers in the selection of polyploid plants, that confirmed by the results of chromosome number determination and flow cytometry.

According to the current EU directive, these techniques of modifying the chromosome number in plant breeding is not a GMO procedure. Therefore, this method of increasing biomass yield can be used in any country or region where the cultivation of transgenic plants is prohibited by law, despite of the fact that incorporation of isolated genes can safely improve the biological performance of plants.

4.2. Effect of autotetraploidy on primary and secondary growth

As a result of autopolyploidization, in several tetraploid willow genotypes showed a decrease in stem length, and wider stem diameter.

Due to the higher leaf mass, the yield of tetraploid green biomass is higher than that of the diploid varieties. In order to further increase the mass of woody shoots, it is appropriate to produce triploid variants. The breeding value of the tetraploid variants presented here is that they can serve as a crossing partner in diploid x tetraploid combinations.

The expected utilization of tetraploid genotypes will not be in short rotation plantations, but rather will be directly utilized in the production of cylindrical wood.

4.3. Direct effect of doubling the willow genome on foliage and increasing biomass yield

Comprehensive characterization of the independent autotetraploid energy willow lines revealed that the structure and function of the leaves have changed significantly as a result of changes in genome size. The larger leaf surface of autotetraploid willow plants is capable of fixation twice as much CO₂ per unit leaf area. Increased carbon dioxide assimilation rate and improved photosynthetic efficiency of these types of short-rotation energy willow can play significant role in reduction of the negative effects of climate change. The positive effects of such plantations are increased by the fact that both green and woody willow biomass are suitable for biogas production.

4.4. Relation of the size of the autotetraploid willow genome and the increased root system

The increased root biomass is noticeably noteworthy, which improves a detoxification efficiency and thus makes tetraploid genotypes can more suitable for heavy metal contaminated soils to bioremediation.

The utilization of Poli Plus ENERGO breeding materials is only possible if a variety is included in a variety certification system after breeding and evaluation. DUS testing of tetraploid lines has begun.

4.5. Molecular cytological characterization and partial karyotyping of energy willow

In breeding for high biomass productivity, limited knowledge is available on the molecular cytogenetics of willow, which could be combined with genetic linkage mapping. The present study describes the adaptation of a fluorescence *in situ* hybridisation (FISH) protocol as a new approach to analyse the genomic constitution of *Salix viminalis* using the heterologous DNA probes.

The further experiments based on the characterization of the willow chromosome's FISH pattern and set may serve as a based for karyotyping willow species.

It will be necessary to isolate and use species-specific DNA clones from the *S. viminalis* genome. Furthermore will need to re-optimization of FISH method too.

5. PUBLICATIONS

Publications in international scientific journals:

Dénes Dudits, Katalin Török, András Cseri, Kenny Paul, **Anna V. Nagy**, Bettina Nagy, László Sass, Györgyi Ferenc, Radomira Vankova, Petre Dobrev, Imre Vass and Ferhan Ayaydin (2016): Response of organ structure and physiology to autotetraploidization in early development of energy willow *Salix viminalis* L. Plant Physiology, March 2016, Vol. 170, pp.1504-1523, Impact Factor: 6.456

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Publications in Hungarian scientific journals:

Dudits Dénes, **Nagy Anna Viktória (2017)**: A fás szárú energianövények nemesítése hagyományos és géntechnológiai módszerekkel. Növénytermelés. Crop Production. 66. évfolyam, 1. szám (2017 március), 97-118

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