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The poplar *ARGOS-LIKE* gene promotes leaf initiation and cell expansion, and controls organ size

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Abstract

We identified a *Populus nigra* auxin-regulated gene involved in organ size (*PnARGOS*)-*LIKE*, encoding one organ size related protein in black poplar. It is homologous to *AtARGOS* and *AtARGOS-LIKE* genes of *Arabidopsis thaliana.* ABRE-like, G-box, GATA and I-box motifs were discovered in the promoter region of the poplar *ARGOS-LIKE* gene. In wild type aspen (*Populus tremula*) plants, an ortholog of the *PnARGOS-LIKE* gene (*PtrARGOS-LIKE*) was noticeably expressed in actively dividing and expanding young leaves and calli, whereas its mRNA content increased in response to exogenous 6-benzylaminopurine, 1-naphthaleneacetic acid, and 24-epibrassinolide. Expression of the *PtrARGOS-LIKE* gene was reduced under a salinity treatment. In addition, we generated transgenic tobacco and aspen plants with an up-regulated expression of the *PnARGOS-LIKE* gene. A constitutive expression of the gene contributed to an increase in size of stems and leaves of the transgenic tobacco plants. In the transgenic aspen, a constitutive expression of the *PnARGOS-LIKE* gene promoted an increase in the frequency of leaf initiations and in leaf length and area. The size of transgenic tobacco and aspen leaves increased due to the enlargement of individual cells. The results show the significance of the *PnARGOS-LIKE* gene for control of leaf initiation and organ growth by cell expansion in poplar.

Additional key words: *Nicotiana tabacum*, phytohormones, *Populus nigra*, *Populus tremula*, transgenic plants.

Introduction

Plant organ size mainly depends on regulation of cell division and cell expansion (Gonzalez *et al*. 2012). In the first place, these mechanisms are carried out by regulation of the number of cells as well as by the rate and duration of cell division in primordia (Mizukami and Fischer 2000). Regulation of such processes as cell expansion, division of meristemoid cells, and endo-reduplication in growing organs also influence the size of the organs (Gonzalez *et al*. 2012). Currently, there are few data concerning interaction of genes that are involved in regulation of cell division and cell expansion. It was suggested that defining role in organ growth regulation belongs to phytohormones and transcription factors (Kim *et al.* 2003, Horiguchi *et al.* 2005, Gonzalez *et al.* 2012). As was recently shown, proteins with an organ size related (OSR)-domain play an important role in coordination of cell expansion and division (Feng *et al.* 2011). Four genes, an auxin-regulated gene involved in organ size (*AtARGOS*), *AtARGOS-LIKE* (*AtARL*), *OSR1*, and *OSR2,* encoding proteins of this group, were discovered and studied in *Arabidopsis thaliana* (Hu *et al.* 2003, 2006, Feng *et al.* 2011, Qin *et al.* 2014). The *AtARGOS* gene of *A. thaliana* encodes a transmembrane protein located in the endoplasmic reticulum and participates in transmission and transduction of a signal from phytohormones to transcription factors (Feng *et al.* 2011). It has been established that signals from the AtARGOS protein are transmitted to the transcription factor AINTEGUMENTA (Hu *et al*. 2003), which is responsible for the maintenance of meristematic competence of the cells in primordia of shoots through regulation of expression of different proteins that participate in cell division (Mizukami and Fischer 2000, Dewitte *et al.* 2003). At the same time, the *OsARGOS*

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Abbreviations: ABA - abscisic acid, *ARGOS* - auxin-regulated gene involved in organ size; *ARL* - *ARGOS-LIKE*; BAP - 6-benzylaminopurine; bZip - basic region/leucine zipper; 2,4-D - 2,4-dichlorophenoxyacetic acid; EBL - 24-epibrassi-nolide; GUS - β-glucuronidase; 2iP - 6-(γ,γ-dimethylallylamino)-purine; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; OSR - organ size related.
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gene of *Oryza sativa* affects both cell division and cell expansion (Wang *et al*. 2009a). Through an example of *A. thaliana*, it was demonstrated that overexpression of the *ARGOS* gene from rice and Chinese cabbage enhances expression of genes that are involved in regulation of cell division (*AINTEGUMENTA*, *CYCLIND3;1*, and *AtGIF1*) along with genes that are involved in regulation and provision of cell expansion, for example, the transcription factor AtGRF1 and expansin AtEXPA10 (Wang *et al.* 2009a,b). Therefore, homologous or heterologous expression of *ARGOS* gene homologs leads to a significant enlargement of organ size in transgenic plants (Hu *et al.* 2003, 2006, Wang *et al.* 2009a,b, Feng *et al.* 2011, Kuluev *et al.* 2011, Qin *et al.* 2014). It was also demonstrated that expression of *ARGOS* gene homologs is regulated by auxins and cytokinins (Hu *et al.* 2003, Wang *et al.* 2009a,b), and *AtARL* gene expression is stimulated mostly by brassinosteroids. The *AtARL* gene is homologous to *AtARGOS* though it encodes a protein that controls cell expansion (Hu *et al.* 2006). Constitutive expression of the *AtARL* gene increases content of *TCH4* (*AtXTH22*) mRNA that encodes one of the xyloglucan endotransglucosylases of *A. thaliana* (Hu *et al.* 2006).

Since it is possible to use genes encoding OSR-proteins for development of transgenic plants with increased productivity, searching and studying homologs of these genes in other agriculturally important plants becomes an essential task (Wang *et al.* 2009a,b, Guo *et al.* 2014). At this point, woody plants are of a special interest due to their high economic value. The genome of *Populus trichocarpa* is completely sequenced, however, the genes encoding OSR-proteins in *Populus nigra* have not been studied yet. In the temperate zone of Russia, another species of poplar, for example black poplar is the most common. In this regard, an important purpose of this investigation was to study the homologs of the poplar *ARGOS* gene and their role in organ growth regulation.

We cloned the *P. nigra ARGOS-LIKE* gene, which was given a name *PnARGOS-LIKE*, and then conducted transformation of aspen trees (*Populus tremula*). Aspen was chosen for development of transgenic trees with overexpression of the *PnARGOS-LIKE* gene because of all poplar species, aspen could be most easily grown *in vitro*.

Materials and methods

Plasmid construction and computer analysis: The *PnARGOS-LIKE* gene was amplified from the black poplar (*Populus nigra* L.) cDNA using primers 5'-CAGAGTTGTTGAATGGATGTGAG-3', 5'-ACAGGT GCATAAGTTTTCT TGA-3' and *Pfu* DNA polymerase (*Bioscreen*, Ufa, Russia). The size of the amplicon was 366 bp. A transgenic vector was constructed by cloning a 35S cassette into the binary vector pCambia 1301 (*CAMBIA*, Canberra, Australia). The expression cassette containing the 35S promoter, polylinker, and cauliflower mosaic virus polyadenylation signal was cut out by the restriction enzyme *Eco*RV from the 35S plasmid and cloned to pCambia 1301 by the site *Sma*I (Kuluev *et al*. 2013). The *PnARGOS-LIKE* gene was cloned into the resulting binary vector. According to colony PCR analysis, the constructs containing an insert of the *PnARGOS-LIKE* gene at the sense orientation relative to the promoter were selected for making transgenic tobacco and aspen plants. We sequenced the final version of the genetic construct and published the nucleotide sequence of the *PnARGOS-LIKE* gene in GenBank (JQ955606).

To elucidate the phylogenetic relationships of *ARGOS*-like genes, 12 predicted *PnARGOS-LIKE* homologs were identified in the *NCBI* database. Alignments of open reading frames of *ARGOS*-like gene homologs were performed by the *CLUSTAL W* method using the program *MegAlign v. 7.1.0* (*DNAStar*, Madison, USA). Protein structure and function prediction were performed by *I-TASSER* (http://zhanglab.ccmb.med. umich.edu/I-TASSER). Transmembrane region predictions were carried out by the server *DAS* (http://www.sbc.su.se/ ~miklos/DAS). In order to identify hormone-responsive elements, we used the DNA sequence of the *P. trichocarpa* genome available *via* http://www. plantgdb.org/PtGDB. For analysis of the poplar *ARGOS-LIKE* promoter region, we used the DNA region of 2 000 bp located before the ATG start codon. In analysis of hormone-responsive elements, we used data from *AtcisDB* (the *Arabidopsis cis*-regulatory element database, http://arabidopsis.med.ohio-state.edu/AtcisDB).

Growth conditions and treatments of wild type aspen plants: *In vitro* grown plants of *Populus tremula* L. were acclimatized and grown in 450 cm^3 pots filled with a universal soil substrate *Terra vita* (*Fart*, St. Petersburg, Russia, Russia) in a greenhouse at day/night temperatures of 27/25 °C, an air humidity of 40/50 %, a photon flux density of 140 μmol m^{-2} s^{-1}, and a 16-h photoperiod. Ninety-d-old plants were sprayed with phytohormones dissolved in deionized water with 0.02 % (m/v) *Silwet Gold* (*Chemtura*, Philadelphia, USA). Plant hormones were used in following concentrations: 10 μmol 6-benzylaminopurine (BAP), 10 μmol 1-naphthalene-acetic acid (NAA), and 1 μmol 24-epi-brassinolide (EBL). Control plants were sprayed with 0.02 % *Silwet Gold* in deionized water. Shoot apices (with leaf primordia and two small youngest leaves) and young leaves were sprayed with BAP, NAA, and EBL; after 6 h they were frozen in liquid nitrogen. Then the total RNA was isolated.

Calli induced from aspen leaf explants were also used for quantitative real time PCR. To generate calli, we used young leaves from two-month-old *in vitro* grown plants. Explants were prepared by transverse cut of leaves using

sterile scalpels and then grown for 23 d on a Murashige and Skoog (MS) medium with 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg dm^{-3} kinetin at environmental conditions mentioned above. Then, the calli were frozen in liquid nitrogen and used for extraction of the total RNA.

For salt stress treatment, aspen plants were germinated in 450 cm^3 pots with the universal soil for 90 d. Then the plants were watered with 50 cm^3 of a 180 or 570 mM NaCl solution. After incubation of plants at these NaCl concentrations for 6 or 24 h, the leaves were frozen in liquid nitrogen and used for isolation of the total RNA.

Tobacco transformation, growth conditions, and analysis of tobacco phenotype: Transgenic tobacco (*Nicotiana tabacum* L.) plants were generated by *Agrobacterium tumefaciens*-mediated transformation of leaf discs detached from three-month-old plants (Gallois and Marinho 1994). An original tobacco plant line, Petit Havana SR1, was used as control. We did not use transgenic plants containing the T-DNA of the pCambia 1301 plasmid without a target gene as control since these plants did not differ from the wild type (Kuluev *et al*. 2013). Control and transgenic seedlings of the T_2 generation were germinated on an MS medium without phytohormones for 20 d, then lengths of roots and hypocotyls were measured and the plants were acclimatized to *ex vitro* conditions. Further, the tobacco plants were grown in 450 cm^3 pots filled with the universal soil (*Terra vita*) in a greenhouse at conditions mentioned above. Morphological parameters were analyzed during the flowering period. We selected five plants of each transgenic line for analysis and measured lengths of three lower leaves (the 2nd, 3rd, and 4th leaves from the top) and stem height. In order to measure an average area of cells in lower epidermis, we used a stripe of the epidermis of about 0.5 - 1 cm^2 from the 7th leaf. A hundred cells were measured in each leaf. The measurements were carried out with a universal fluorescence microscope *Axio Imager M1* (*Carl Zeiss*, Oberkochen, Germany) equipped with the proprietary software. Statistical analysis of all the results was carried out using the *Statistica 6.0* and *Microsoft Excel 2003* programs. Significance of differences was assessed using the Mann-Whitney *U* test.

Aspen transformation, growth conditions, and analysis of its phenotype: For *Agrobacterium*-mediated transformation, sterile aspen stems were pre-cultured for two days on an MS medium with 2 mg dm^{-3} NAA and 1 mg dm^{-3} 6-(γ,γ-dimethylallylamino)-purine (2iP, *Sigma*, St. Louis, USA). The overnight culture of the *A. rhizogenes* strain A4 containing the binary vector pCambia 1301 with the *35S::PnARGOS-LIKE* construct was centrifuged and resuspended in 10 cm^3 of a sterile *MinA* medium, and 3',5'-dimethoxy-4'-hydroxyacetophenone (*Sigma*) was added to the medium to achieve a 50 μM concentration. Cultivation of *Agrobacterium* was carried out at 28 °C for 1 h. Then, prepared aspen explants were treated with *Agrobacterium* and a silicon carbide suspension in a 1 % (m/v) concentration and shaked for a few minutes. Co-cultivation of stem explants with *A. rhizogenes* was carried out for three d on an MS medium with 2 mg dm^{-3} NAA and 1 mg dm^{-3} 2iP. Afterwards, the explants were transferred to an MS medium without phytohormones with addition of 250 mg dm^{-3} cefotaxime and 250 mg dm^{-3} carbenicillin and cultured in the dark at 25 °C. One and a half month later, hairy roots of aspen were checked for reporter β-glucuronidase (*GUS*) gene expression. Blue stained roots were selected and transplanted to a new MS medium and cultured in a *Binder* climatic chamber (Tuttlingen, Germany) at 25 °C under a photon flux density of 200 μmol m^{-2} s^{-1}, and a 16-h photoperiod. After 2.5 months, shoots developed on hairy roots were cut and transplanted for rooting on a woody plant medium with 20 mg dm^{-3} hygromycin without growth regulators. After root formation, the transgenic plants were acclimatized for one month in sterile *Vermiculite* with a 10 % (m/v) Hoagland-Arnon solution. Then, the transgenic plants were grown in 450 cm^3 pots filled with the universal soil substrate in a greenhouse. After four months of cultivation, we performed morphological analysis of the transgenic aspen plants. We measured lengths of 10 upper and 10 lower leaves and petioles. We also measured stem height, the total number of leaves and lengths of internodes. The aspen *in vitro* culture generated from hairy roots free of target and reporter genes passed a similar procedure of acclimatization and served as control.

Quantitative real time PCR: The total RNA was isolated from different organs of wild type *P. tremula*, young leaves of *35S::PnARGOS-LIKE* transgenic tobacco, and aspen transgenic plants using a *Trizol* reagent (*Invitrogen*, Waltham, USA) in accordance with the manufacturer's instructions. The first strand cDNA was synthesized using *oligo (dT)* primers and *M-MuLV*-reverse transcriptase (*New England Biolabs*, Ipswich, USA). Real time quantitative PCR (qPCR) was performed with gene specific oligonucleotide primers: 5'-GCAAGTGCAGAGAAAAAGAAA-3' and 5'-ATG AAAGCCAAGATTATGAGC-3' for the *PnARGOS-LIKE* gene and its aspen ortholog (the *PtrARGOS-LIKE* gene). For aspen, we used the mRNA of the actin gene (EF418792) as internal standard and primer pairs 5'-ACTGGTATTGTGTTGGATTCTGG-3'/5'-AGTTGT ATGTAGTCTCGTGGATGC-3'. In experiments with transgenic tobacco, elongation factor *EF-1α* gene expression was used as reference (Schmidt and Delaney 2010) with the following primers: 5'-GAATTG GTACTGTCCCTGTT-3' (forward) and 5'-TTGCCA ATCTGTCCTGAAT-3' (reverse). Real time qPCR reactions were performed on a *Rotor-Gene™ 6000* thermocycler (*Corbett Research*, Mortlake, Australia) using *SYBR Green I* under the following conditions: 30 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s. Calculations were made using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Results and discussion

We conducted a phylogenetic analysis of 12 putative orthologs and paralogs of the *PnARGOS-LIKE* gene from some representatives of angiosperms. As expected, *ARGOS*-like genes from *P. trichocarpa* (XM_006372243.1), *Vitis vinifera* (AM475052.1), *Ricinus communis* (XM_002533293.1), *Solanum lycopersicum* (NM_001247750.1), and *S. tuberosum* (XM_006342385.1) have a high sequence similarity to the *PnARGOS-LIKE* gene of *P. nigra*. *AtARGOS* and *AtARL* are located at about the same distance from *PnARGOS-LIKE* on a phylogenetic tree (Fig. 1*A*). The alignment of the predicted amino acid sequences of PnARGOS-LIKE homologs shows that it is most similar to the PtARGOS-LIKE protein of *P. nigra*. However, seven amino acid substitutions were observed between the two proteins (data not shown).

Analysis of predicted PnARGOS-LIKE protein was carried out using the *I-TASSER* server (Roy *et al.* 2010). It is shown that the PnARGOS-LIKE protein contained three putative α-helices, and the remainder of the protein molecule mainly consisted of random coils. A part of the protein between positions 40 and 96 consisted mainly of hydrophobic amino acids, which may indicate a transmembrane location of this region (Cserzo *et al.* 1997). Using the *DAS* program, we performed a search for a possible transmembrane domain in the studied

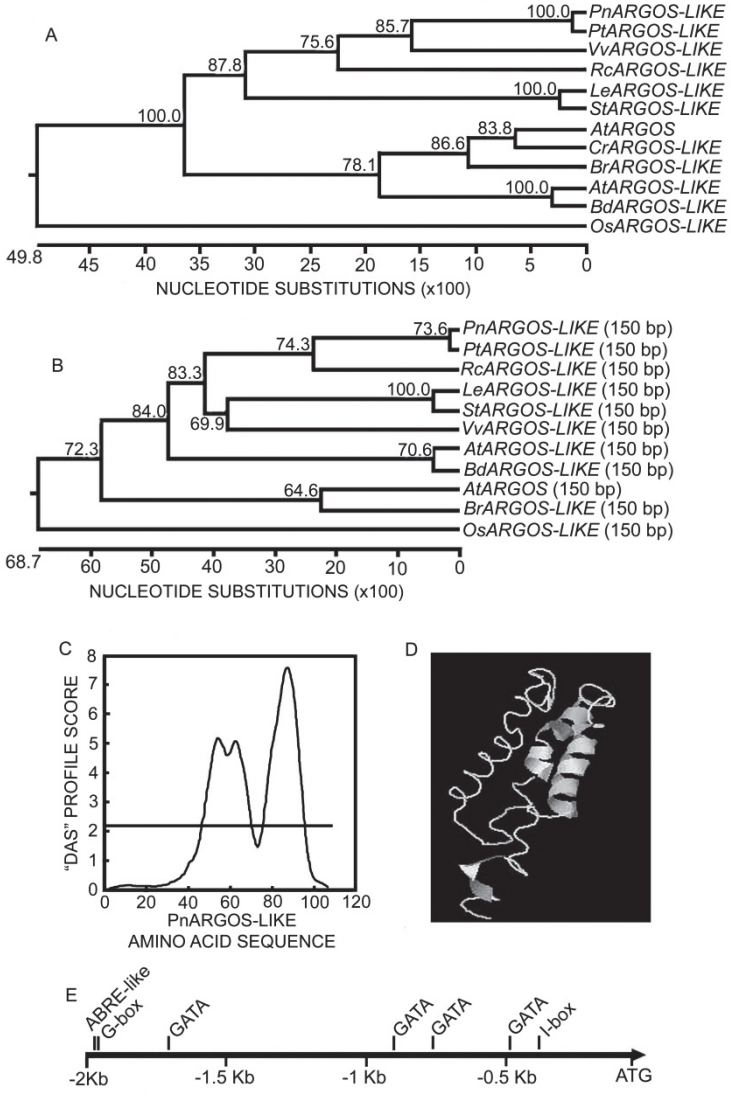

Fig. 1. Bioinformatic analysis of the poplar *ARGOS-LIKE* gene and the predicted amino acid sequence of its protein. *A* - A neighbor-joining phylogenetic tree showing relationships among *PnARGOS-LIKE* homologs; *B* - a phylogenetic tree constructed on the basis of comparison of 150 bp 5'-ends of *PnARGOS-LIKE* homologs; *C* - results of search for predicted transmembrane domains in the PnARGOS-LIKE protein using the *DAS* program; *D* - one of the variants of the predicted secondary structure of the PnARGOS-LIKE protein; *E* - *cis*-regulatory elements of the 2 kb putative promoter region of the *PtARGOS-LIKE* gene of *P. trichocarpa*.

protein; two domains of this type were found in the predicted PnARGOS-LIKE protein, *i.e.*, from 47th to 70th amino acid and from 76th to 95th amino acid (Fig. 1*C*). The predicted secondary structure of PnARGOS-LIKE is shown in Fig. 1*D*. Based on the analysis of the predicted secondary structure, it can be suggested that two α-helices were located in the membrane (Stangl and Schneider 2015), and one α-helix was probably located outside the membrane (Fig. 1*D*).

Since transmembrane domains of the ARGOS homologs are highly conserved, it is possible that the basis of their functional differences, which were found in *A. thaliana* (Hu *et al.* 2003, Feng *et al.* 2011), is the variation of the N- or C-termini. Therefore, we carried out alignment of short 150 bp 5'-regions of *ARGOS-LIKE* homologs. According to the results of the short gene regions alignment, *PnARGOS-LIKE* is closer to *AtARL* than to *AtARGOS* (Fig. 1*B*). Based on these data, we can expect that the functions of PnARGOS-LIKE may be closer to *AtARL* of *A. thaliana* than *AtARGOS*.

In a study of *ARGOS* homolog functions, the search for *cis*-regulatory elements in the promoter can be viewed as useful (Feng *et al.* 2011). However, the *P. nigra* genome has not been sequenced yet. Therefore, we analyzed a putative 2 kb promoter region of the *PtARGOS-LIKE P. trichocarpa* gene. Within the analyzed region of the DNA, four GATA motifs, G-box, J-box, and ABRE-like motifs were found (Fig. 1*E*).

The highest amount of *PtrARGOS-LIKE* transcripts was detected in aspen stem tips with leaf primordia and two youngest small leaves (Fig. 2*A*). Also, a high expression was detected in young intensively growing leaves (Fig 2*A*). In fully grown leaves, the *PtrARGOS-LIKE* gene was also expressed but at a lower level than in young leaves. In the calli, the amount of *PtrARGOS-LIKE* transcripts was four times higher than in the stem tips (Fig. 2*A*). Thus, *PtrARGOS-LIKE* expression was closely associated with a high rate of organ growth by cell division and cell expansion.

Due to the fact that plant hormones have a key role in regulation of plant growth and development, we examined changes in abundance of the *PtrARGOS-LIKE* mRNA in response to different phytohormones in the stem tips and young leaves since *PtrARGOS-LIKE* expression was stable and relatively high in these organs. In the stem tips and young leaves, the amount of *PtrARGOS-LIKE* transcripts noticeably increased in response to NAA (Fig. 2*B*). In addition, it was possible to find a slight increase of the *PtrARGOS-LIKE* mRNA level in the stem tips in response to BAP, but in the young leaves, BAP had no significant effect on *PtrARGOS-LIKE* expression (Fig. 2*C*). It is notable that in the young leaves, *PtrARGOS-LIKE* expression increased in response to EBL (Fig. 2*C*).

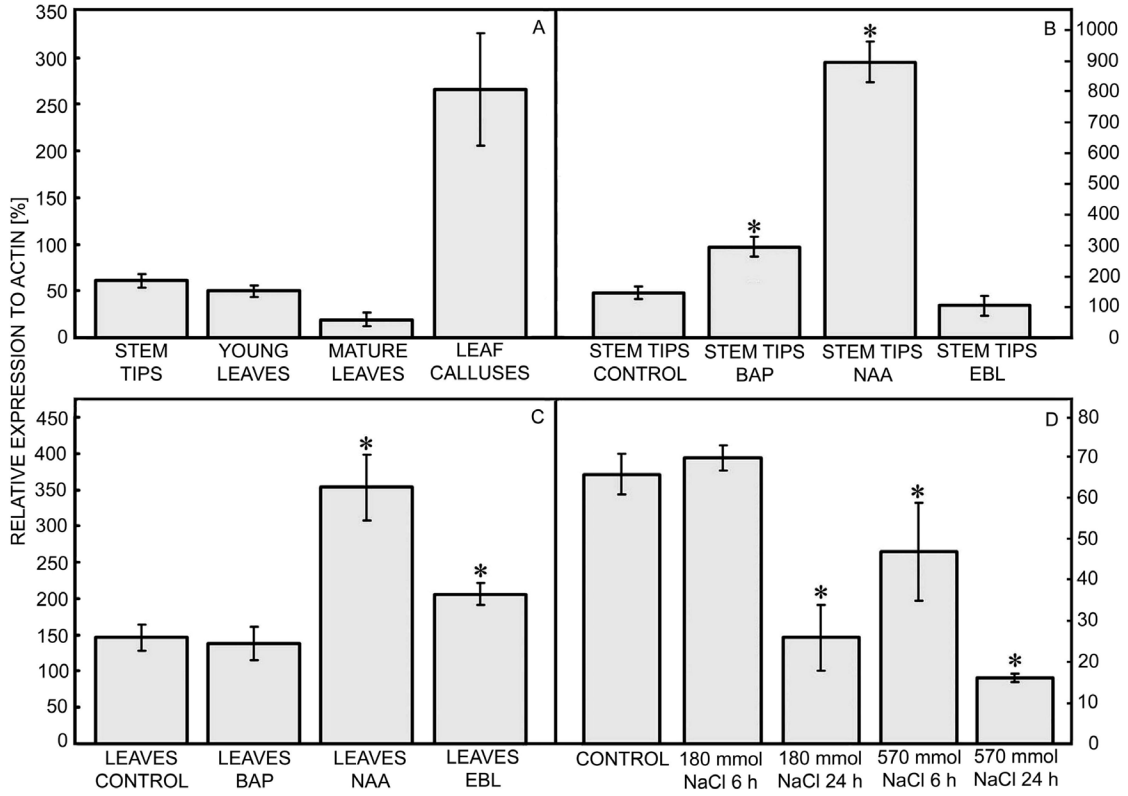

Fig. 2. Expression of *PtrARGOS-LIKE* in wild type aspen plants. *PtrARGOS-LIKE* transcript abundance in different organs (*A*), in stem tips under the influence of phytohormones (*B*), in young leaves under the influence of phytohormones (*C*), and in young leaves under the influence of NaCl (*D*). Means ± SEs (n = 3; * - significant differences from a control at $P < 0.05$).

ARGOS-LIKE proteins are members of the OSR group involved in regulation of cell division and cell expansion (Feng *et al.* 2011). The possibility of involvement of cell division and cell expansion regulation in abiotic stress responses is discussed in literature (Huang *et al.* 2008, Choi *et al.* 2011, Han *et al.* 2012). In order to determine whether *PtrARGOS-LIKE* gene expression is affected by salinity stress, we studied transcription of *PtrARGOS-LIKE* in young aspen leaves treated with different concentrations of NaCl for 6 and 24 h. *PtrARGOS-LIKE* expression did not increase in response to NaCl. In contrast, *PtrARGOS-LIKE* expression was reduced within 6 h of incubation in 570 mM NaCl, and after 24 h, it was significantly reduced under both 180 mM NaCl and 570 mM NaCl (Fig. 2*D*).

We produced 20 transgenic *N. tabacum* lines overexpressing the *PnARGOS-LIKE* gene. Seven lines (2, 4, 6, 7, 14, 19, and 20) had a single copy of the transgene in the genome and a relatively high expression (Fig. 3*A*), so they were selected for morphological analysis. An increased leaf length was typical for lines 2, 4, 14, and 20 (Fig. 3*B*). Lines 2, 14, and 21 showed a statistically significant ($P < 0.05$) enlargement both of leaf length and of leaf area compared with the control (Fig. 3*B*,*C*). A significant enlargement of stem height was typical for lines 2, 4, and 20 (Fig. 3*D*). Organ size of transgenic plants could be larger due to increasing number of cells (Hu *et al.* 2003) and/or enlargement of cell size (Hu *et al.* 2006). All lines of the *35S::PnARGOS-LIKE* plants were characterized by a significant increase in size of leaf epidermal cells (Fig. 3*E*). At the same time, as expected, the number of cells in the *35S::PnARGOS-LIKE* plants decreased. Therefore, it can be suggested that the product of the *PnARGOS-LIKE* gene in tobacco affected cell expansion primarily and thus could contribute to longer leaves.

ARGOS homolog overexpressions mainly affect size of above-ground organs (Hu *et al.* 2003, 2006). However, there are data in literature about the influence of the *OsARGOS* rice gene product on root growth of *A. thaliana* (Wang *et al.* 2009a). In this connection, it seems important to study the effect of *PnARGOS-LIKE* overexpression on root length. In this regard, we studied 20-d-old seedlings of 7 *35S::PnARGOS-LIKE* tobacco lines and control plants. It is shown that root length of all the lines of the test plants was not significantly different from length of the roots of the control plants. At the same time, lines 2, 4, 6, and 20 were characterized by an increase in hypocotyl length as compared with the control plants (data not shown).

In the heterologous tobacco system, overexpression of *PnARGOS-LIKE* promoted an increase of leaf and stem sizes. However, more valuable data about a homologous expression system could be obtained after defining the functions of various genes (Wang *et al.* 2009a). Therefore, we carried out work on making transgenic aspen plants that overexpress the *PnARGOS-LIKE* gene. In the first place, with the help of *A. rhizogenes*, we induced hairy roots in aspen (Fig. 1 Suppl.). Transgenic

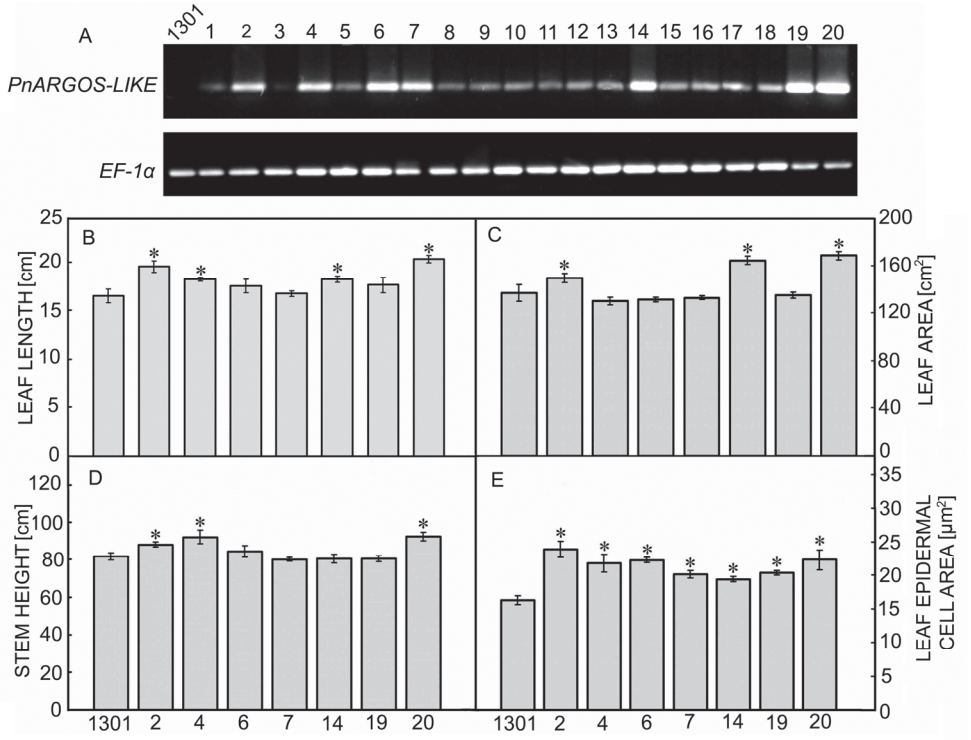

Fig. 3. Molecular and morphological analysis of transgenic *35S::PnARGOS-LIKE* tobacco plants. *A* - Transcript abundance of *PnARGOS-LIKE*, *B* - leaf length, *C* - leaf area, *D* - stem height, and *E* - leaf epidermal cell area in young leaves of transgenic tobacco plants (1301 - control plants, 1 to 20 - lines of *35S::PnARGOS-LIKE* tobacco plants).

hairy roots with *GUS* and *PnARGOS-LIKE* genes were selected using a histochemical method of determining β-glucuronidase activity (Fig. 1 Suppl.). Shoots that appeared on transgenic roots (Fig. 1 Suppl.) were transplanted to an MS medium for rooting (Fig. 4*E*). Then we selected transgenic aspen plants without the hairy root phenotype that were also characterized by a high *GUS* expression (Fig. 1 Suppl.). As result, we generated 28 *in vitro* grown transgenic aspen plants. Each of these plants was propagated up to two copies, so we prepared 56 transgenic plants. From these plants, we managed to acclimate to soil conditions only 19 plants that were grown in a greenhouse under artificial lighting (Fig. 2 Suppl.). For morphological analysis, we used 8 plants from four typical lines of transgenic aspen characterized by a high *PnARGOS-LIKE* expression (Fig. 4*A*).

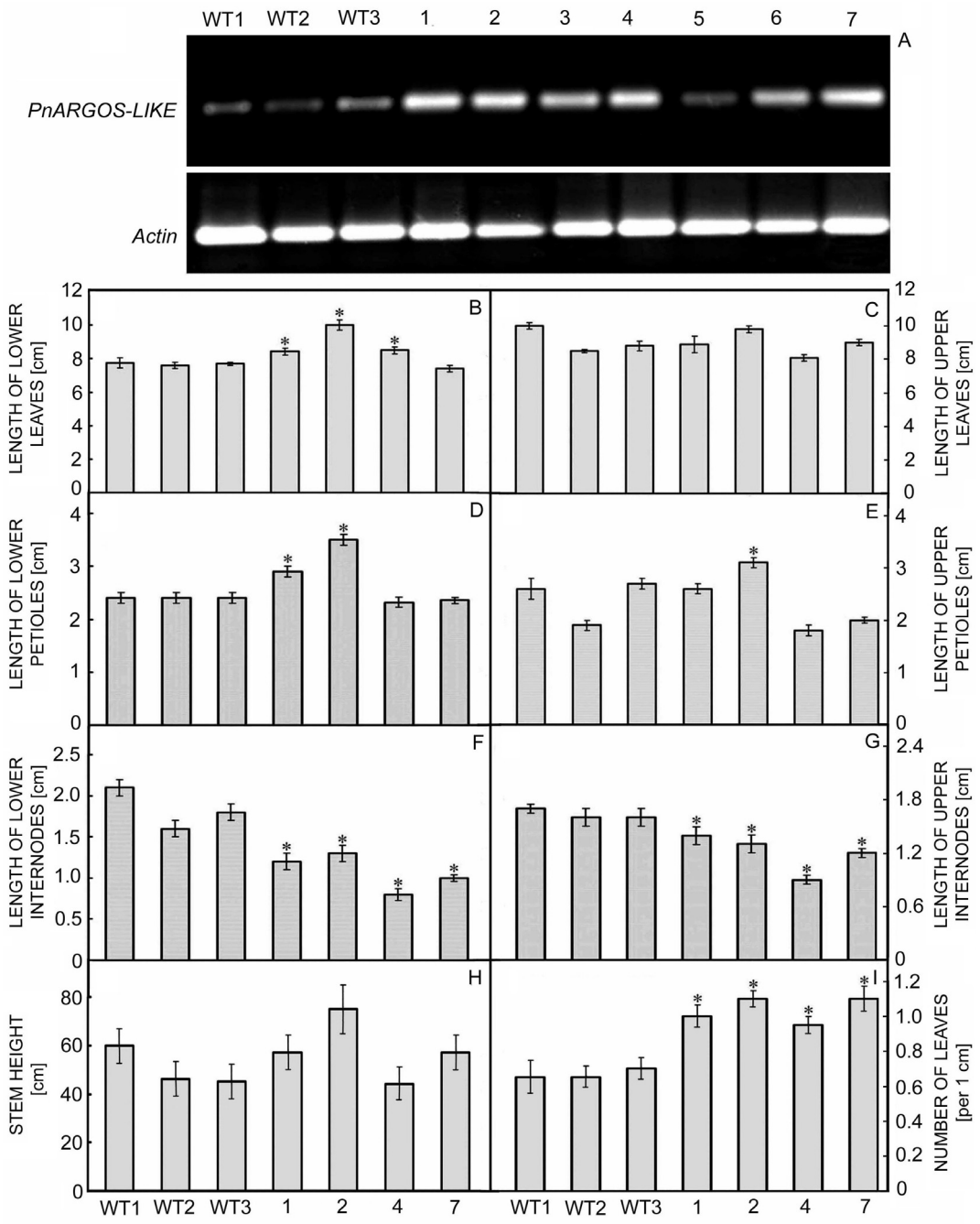

Fig. 4. Molecular and morphological analysis of transgenic *35S::PnARGOS-LIKE* aspen plants. Transcript abundance of *PnARGOS-LIKE* in young leaves (*A*), length of lower leaves (*B*), petioles (*D*), and internodes (*F*); length of upper leaves (*C*), petioles (*E*), and internodes (*G*); stem height (*H*), number of leaves per 1 cm of stem of aspen plants (*I*). WT1 to WT3 - control plants, 1 to 7 - *35S::PnARGOS-LIKE* aspen plants.

Three of the four transgenic aspen lines were characterized by an increase in length of lower leaves (Fig. 4*B*). Two transgenic aspen lines were characterized by elongation of lower petioles (Fig. 4*D*). At the same time, sizes of upper leaves and petioles in the transgenic plants remained in the normal range (Fig. 4*C*,*E*). In general, stem height of the transgenic plants did not significantly change (Fig. 4*H*). All analyzed transgenic aspen lines were characterized by a decrease in length of internodes in both a lower part and an upper part of shoots (Fig. 4*F,G*). So, all four analyzed transgenic aspen lines had an increased number of leaves compared to the control group (Fig. 2 Suppl. and Fig. 4*I*). Since the size of leaves of the three *35S::PnARGOS-LIKE* aspen lines increased, it was of a great interest to determine size of cells in leaves of all analyzed aspen lines. It has been shown that at least two transgenic aspen lines with enlarged leaves were characterized by an increase in epidermal cell size (Fig. 5*A*). At the same time, the number of cells in the three lines of *35S::PnARGOS-LIKE* aspen decreased (Fig. 5*B*). Thus, it can be suggested that the product of *PnARGOS-LIKE* in the homologous system also affected cell expansion.

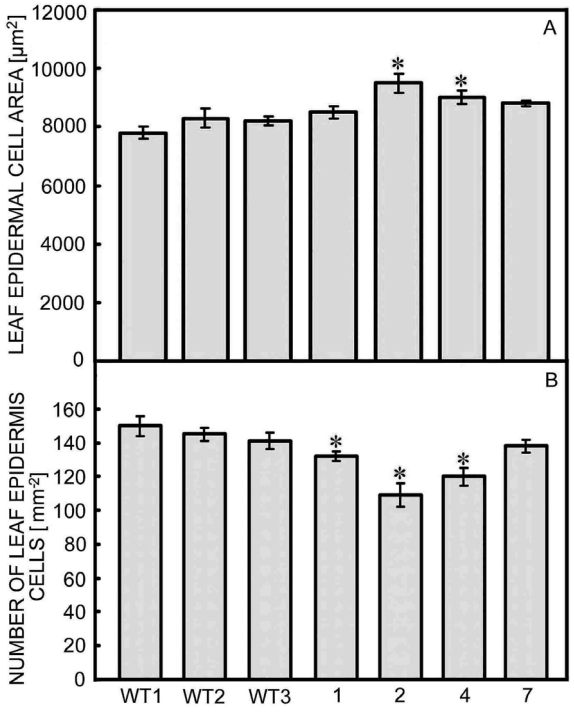

Fig. 5. Leaf epidermal cell area (*A*) and number of leaf epidermis cells per 1 mm^2 of control (WT1 to WT3) and transgenic (1 to 7) aspen plants.

We identified the *PnARGOS-LIKE* gene that encodes one of the black poplar OSR-proteins. It is homologous to *AtARGOS* and *AtARL* genes of *A. thaliana*. It is known that AtARGOS and AtARL proteins have different functions (Hu *et al.* 2003, 2006), so we suggested that the same homologous proteins with similar functions could be found in poplar. Therefore, we investigated which *OSR*-gene of *A. thaliana* (*AtARGOS* or *AtARL*) is most close to the *PnARGOS-LIKE* gene. However, the phylogenic analysis of the nucleotide sequences of the *OSR*-genes showed that only *PnARGOS-LIKE* is similar to both *AtARGOS* and *AtARL*. It should be viewed as evidence that the divergence of *OSR*-gene functions occurred individually in each plant group (Feng *et al.* 2011). Coincidentally, the analysis of 5'-ends of the *OSR*-genes shows that the *PnARGOS-LIKE* gene and *AtARL* gene were most alike though this similarity cannot be only the result of the same origin, but also of performing identical functions.

Bioinformatic analysis of the *PnARGOS-LIKE* gene promoter region revealed only ABRE-like, G-box, GATA, and Ibox motifs. The presence of the ABRE-like motif in the promoter can evidence participation of the PnARGOS-LIKE protein in response to abiotic factors leading to water deficit (Shinozaki and Yamaguchi-Shinozaki 2000). It is known that water deficit can be caused by drought, salinization and cold (Koshkin 2010). Besides, in response to these stress factors, content of abscisic acid (ABA) in plants increase. Abscisic acid itself stimulates expression of basic region/leucine zipper (bZip) transcription factors that specifically interact with the ABRE-like motif regulating activity of different genes (Shinozaki and Yamaguchi-Shinozaki 2000). The majority of transcription factors involved with the G-box motif also turn up to be bZip proteins containing the leucine zipper (Ishige *et al.* 1999). It is known, that G-box proteins are involved in regulation of gene expression in response to light, oxygen deficiency, and ABA (Ishige *et al.* 1999), methyl jasmonate (Figueroa and Browse 2012), and salt and drought (Liu *et al.* 2014). Our experimental data concerning the change in *PtrARGOS-LIKE* expression due to the salt treatment also provide evidence that it may be involved in the response to water deficit. According to *NCBI GEO*, salt might increase expression of the *AtARGOS* gene (GDS3918/251436_at/ARGOS; GDS3927/251436_at/ARGOS), as well as *AtARL* gene (GDS3927/267230_at/ARL). However, we observed only a reduction in *PtrARGOS-LIKE* expression in response to the salt treatment. It is notable that overexpression of the *OSR*-gene *ZAR1* of *Zea mays* positively affect not only yield but also drought tolerance of transgenic maize (Guo *et al.* 2014). Based on these data, it is of interest to continue investigation of involvement of *OSR*-genes in plant growth regulation under stress conditions.

The GATA factors have been shown to play a critical role in development, differentiation, and control of cell proliferation in plants (Behringer and Schwechheimer 2015). The Ibox motif and its associated transcription factors are still poorly investigated though it is known that this motif is connected to genes, expressions of which are regulated by radiation (Giuliano *et al.* 1988).

The *PnARGOS-LIKE* gene is mostly expressed in young growing organs where rates of both cell expansion and cell division are high. The highest *PnARGOS-LIKE* expression was observed in calli. It is well known that the

callus consists of undifferentiated but actively dividing cells, *i.e.*, callus growth occurs primarily by increase in cell number. However, initiation of cell expansion also takes place in calli so their cells grow for a short time until they reach an optimum size (Blackman and Overall 1995). From the analysis of the spatial expression, it was concluded that the product of the *PnARGOS-LIKE* gene is associated with both intensive cell division and initial stages of cell expansion.

We show that the expression of the *ARGOS-LIKE* gene of poplar was stimulated by auxins. However, in stem tips with young dividing leaves, BAP had also a positive effect on expression of this gene (Fig. 2*C*). In older organs, expanding leaves in particular, we observed an increase in *PtrARGOS-LIKE* gene expression in response to the EBL treatment. Therefore, we can suggest that expression of poplar *ARGOS-LIKE* gene is controlled by cytokinins, auxins, and brassinosteroids, which represents another similarity with the *AtARL* gene of *A. thaliana* (Table 1 Suppl.). It is well known, that cytokinins, auxins, and brassinosteroids are involved in regulation of both cell expansion and cell division (Tsukaya and Beemster 2006). These phytohormones affect expression of *OSR*-genes in different ways (Feng *et al.* 2011). Besides, according to our data, a response to a certain phytohormone may depend on the age of an organ. Overall, based on our results and data from *NCBI GEO*, we suggest that on the grounds of expression regulation, the *ARGOS-LIKE* gene of poplar is closer to the *AtARL* gene of *A. thaliana* than the *AtARGOS* and *OSR1* genes (Table 1 Suppl.).

The expressions of some *ORS*-genes are observed in roots (Qin *et al.* 2014), and their overexpression may take a positive effect on root growth (Wang *et al.* 2009a, Qin *et al.* 2014). However, according to our data, over-expression of *PnARGOS-LIKE* gene did not significantly affect the length of roots like *AtARGOS* and *AtARL* genes (Hu *et al.* 2003, 2006). In the transgenic aspen, the *PnARGOS-LIKE* gene promoted an enlargement of leaves and petioles but not stems. In summary, it can be stated that the product of the *PnARGOS-LIKE* gene in poplar participated in regulation of leave and petiole growths in the first place. The *OSR*-genes control plant growth by influencing cell expansion and division (Feng *et al.* 2011). Some *OSR*-genes may affect both processes at once (Wang *et al.* 2009, Feng *et al.* 2011), whereas the others affect only one of them selectively (Hu *et al.* 2003, 2006, Qin *et al.* 2014). The *PnARGOS-LIKE* gene overexpression promoted leaf epidermis cell enlargements in tobacco and aspen, so we suppose that *PnARGOS-LIKE* is involved mostly in regulation of cell expansion. Considering that expression of the investigated gene was the most high in the callus, it can be suggested that the protein product of the *PnARGOS-LIKE* gene participated in the early stages of cell expansion. However, in the transgenic aspen plants, increases in length of lower mature leaves and petioles were observed. At the same time, there was no difference in size of upper leaves of the control and transgenic trees. It could be caused by the product of *PnARGOS-LIKE*, which did not increase the rate of cell expansion but prolonged it like the product of the *AtARL* gene (Hu *et al.* 2006). Prolongation of cell expansion appears to be achieved due to a longer maintenance of a high expression of *PnARGOS-LIKE* gene under control of the constitutive 35S promoter.

Overexpression of the *PnARGOS-LIKE* gene led to a decrease in length of internodes and to an increase in number of leaves per unit of stem length. This may indicate that the product of the *PnARGOS-LIKE* gene affected initiation of new leaf primordia. It is known that initiation of leaf primordium can be induced by placing sephacryl beads, containing cucumber hypocotyl expansin, on the shoot apex (Fleming *et al.* 1997). It means that new leaf primordia appear in the places with a local weakening of cell walls, which can be caused by expansins. In the literature, there is information about *ARGOS* gene homologs overexpression being able to induce expression of expansins (Wang *et al.* 2009a, Kuluev *et al.* 2013, Kuluev *et al.* 2014) So it can be suggested that overexpression of the *PnARGOS-LIKE* gene also induces expression of certain proteins influencing the weakening of the cell wall. However, to identify the molecular mechanisms of signal transmission from phytohormones to expansins through OSR-proteins, further investigation must be conducted. It should be noted that a decrease in length of internodes could also be related to *RolA* gene expression (Roychowdhury *et al.* 2013), which could continue in some selected transgenic aspens.

Overall, our data show the significance of the *PnARGOS-LIKE* gene for the control of leaf primordium initiation and organ growth through regulation of cell expansion.

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