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RESEARCH PAPERS

Role of *PtrXTH1* and *PnXTH1* Genes Encoding Xyloglucan Endo-Transglycosylases in Regulation of Growth and Adaptation of Plants to Stress Factors

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Abstract—The expression level of the gene *PtrXTH1* encoding xyloglucan endo-transglycosylase in the leaves of *Populus tremula* L. of wild-type and in response to exogenous phytohormones treatment was analyzed. The highest level of transcripts of *PtrXTH1* was detected in young, intensively growing leaves of aspen. In young aspen leaves, the expression of *PtrXTH1* was induced by cytokinins, auxins, and brassinosteroids. The content of *PtrXTH1* transcripts increased under the constitutive expression of the *PnARGOS-LIKE* gene. Bioinformatic analysis of *PtXTH1* putative promoter region in *P. trichocarpa* Torr. and A. Gray ex. Hook showed the presence of cis-regulatory elements associated with the regulation of growth and stress resistance. To determine the role of the gene under study, we also created transgenic tobacco plants with constitutive expression of the *PnXTH1* gene (the ortholog of *PtrXTH1* from the black poplar *P. nigra*). Transgenic tobacco plants were characterized by an increase in leaf size and fresh and dry weight of the aboveground part under normal growth conditions. When grown under conditions of salinization and drought, transgenic plants were distinguished by increased stress resistance due to the maintenance of cell expansion in roots and stems at a higher level and the ability to more effectively retain water in leaves compared with wild-type plants.

Keywords: Populus nigra, *Populus tremula*, xyloglucan endo-transglycosylases, auxins, cytokinins, *ARGOS-LIKE*, cell expansion

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INTRODUCTION

The size of the organs, which is regulated by a variety of internal and external factors, is one of the most important growth parameters, and many external factors can be stressful for plants. In general, the growth of plant organs is controlled by two basic mechanisms, namely, the regulation of cell division and cell expansion [1]. The discrepancy of cellulose microfibrils during cell expansion is achieved by three basic mechanisms: hydrolysis of a part of binding glycans with endoglycanases, cutting and new crosslinking of glycans with xyloglucan endo-transglycosylases/hydrolases (XTHs), and the violation of hydrogen bonds between the microfibrils of cellulose and glycan chains, which is carried out by expansins [2–4].

Xyloglucan endo-transglycosylases (XTHs) were characterized almost simultaneously in two laboratories by S. Fry (United Kingdom) and K. Nishitani (Japan) [5, 6]. The enzymes of this group are able to break the glycosidic bond in the core of xyglogucan, connecting the fragments of the two molecules together. The glycosidic bond in the donor molecule is cleaved and combined with the nonreducing end of the acceptor molecule. Moreover, this enzyme can also have hydrolase activity [7]. Dicotyledonous XTHs are encoded by a large multigene family. For example, in the fully sequenced genomes of *Arabidopsis thaliana*, rice (*Oryza sativa*), and poplar (*Populus trichocarpa*), there are 33, 29, and 41 open reading frames, respectively, and all of them presumably encode enzymes with xyloglucan endo-transglycosylase activity (XET) [8–10].

Increased level of XTHs' mRNA content is observed when plants are treated with exogenous auxins, gibberellins, and brassinosteroids, and the stems of experimental plants elongate due to the stimulation of cell expansion [11]. The correlation between the activity of XET and cell expansion was found in the apical segment of tomato hypocotyls, overexpressing the *SlXTH1* gene, where XET specific activity was higher in comparison with the control line [11]. In hypocotyls of transgenic lines with reduced expression of *SlXTH1*, the total extensibility was lower than in wild-type lines. All this indicates that XTHs are involved in the restructuring of the cell wall during the period of intensive organ growth.

Despite the intensive studies of XTHs, the question on the functional significance of a large number of

genes encoding this group of enzymes in plant genomes remains unanswered. Perhaps, each of them performs a specific role in plant organism. Indeed, XTHs are involved in many morphogenetic and destructive processes during the growth and development of plants. The *LeXET4* gene, which is expressed mainly in germinating seeds, to a lesser extent in stems, and is absent in roots, leaves, and flowers, was isolated from tomatoes [12]. The expression of *LeXET4* is stimulated by gibberellins and decreases under the action of ABA. Its protein product plays an important role in rupturing the seed coat and the beginning of seed germination. Other tomato genes, for example *LeXET1*, are expressed in actively growing tissues and activated by auxins [13], and *LeXET2* is expressed in leaves and young fruits [12]. In the literature, there are also data on the participation of XTHs in ensuring the resistance of plants to a water deficit caused by drought and salinity. For example, transgenic tomato plants with constitutive expression of the pepper *CaXTH3* gene were characterized by improved root growth parameters under the action of 100 mM NaCl [14]. Constitutive expression of the *CaXTH3* gene influenced the plasticity of the cell wall of the terminal stomata cells, which led to a decrease in the size of the stomatal pore, which, in turn, contributed to an increase in the drought resistance of transgenic tomato plants. Thus, from the literature data, it follows that the XTHs genes can be used to modify the growth parameters and stress resistance of plants. However, these same literature data do not give an answer to the question on the possibility of using XTHs genes for increasing the productivity of plants.

Studying the XTHs of woody plants is of particular interest, since they can be used in forest biotechnology for creating new arborous species with improved growth parameters. The aspen *Populus tremula* L., which is relatively easily subjected to agrobacterial transformation, is one of the model woody plants. Moreover, the genome of one of the species of poplars, namely the balsamic poplar (*Populus trichocarpa* Torr. and A. Gray ex. Hook) is completely sequenced, which allows carrying out functional studies of XTHs of these woody plants. The closest relative of this species of poplar in the flora of Russia is the black poplar *Populus nigra* L. In this connection, the purpose of this study was to elucidate the possible role of the *PtrXTH1* gene of aspen and its ortholog from the black poplar, *PnXTH1*, during plant growth and adaptation to stress factors. To this end, we determined the level of expression of the gene under study in the leaves of different ages in wild-type aspen during exogenous treatment with phytohormones and at the constitutive expression of the *PnARGOS-LIKE* gene encoding one of the transmembrane proteins with the OSR domain involved in the transduction of phytohormonal signals [15]. The goal was also to generate transgenic tobacco plants with constitutive expression of *PnXTH1* from the black poplar and determine their basic morphometric parameters during growth under normal conditions and under the influence of stress factors.

MATERIALS AND METHODS

Bioinformatic analysis of nucleotide sequences. To search for the nearest homologues of *PnXTH1*, a phylogenetic analysis of nucleotide sequences of the open reading frames of the investigated gene and 31 *XTHs* genes of *Arabidopsis thaliana* was performed using MegAlign v. 7.1.0 (DNAStar, United States). The nucleotide sequence of *PtXTH1* from *Populus trichocarpa* (XM_006385804) was used for the alignment. The nucleotide sequences of *XTHs* from *A. thaliana* are deposited in the TAIR database (https://www.arabidopsis.org). To identify the cis-regulatory elements of the putative promoter region of the gene under study, the AtcisDB database was used (http://arabidopsis.med.ohio-state.edu/AtcisDB). In this case, a 3000 bp DNA region located up to the ATG codon of the open reading frame of *PtXTH1* gene from *P. trichocarpa* was used for the analysis. The nucleotide sequence of the putative promoter region of the *PtXTH1* gene was taken from the PtGDB database (http://www.plantgdb.org/PtGDB).

Conditions of aspen cultivation, exogenous treatment with phytohormones, and stress effects. Before the exogenous treatment with phytohormones, the rooted shoots of aspen plants growing in vitro were transplanted into the soil and grown for 90 days under greenhouse conditions at 30°C, illumination of approximately 140 mmol per square meter/second and a photoperiod of 16/8 h (light/dark). Then the entire aboveground part of the plant was sprayed with a solution of phytohormone supplemented with Tween-20 (0.1%). The following concentrations of phytohormones were used: 50 μM 6-benzylaminopurine (BAP), 10 μM α-naphthylacetic acid (NAA), 1 μM 24-epibrassinolide (EB). Control plants were sprayed with 0.1% Tween-20 solution. All experiments were performed in three biological replicates ($n = 3$). Six hours after sprinkling of the shoot tip, young and mature leaves were frozen in liquid nitrogen.

Quantitative real time RT-PCR. Total RNA from the leaves of the studied plants of aspen and tobacco was isolated with trizol, and the first cDNA chain was constructed using oligo(dT) primer and MMuLV-revertase (NEB, United States). Wild-type aspens treated with exogenous phytohormones, three lines of transgenic aspen plants with constitutive expression of *PnARGOS-LIKE* (JQ955606.1) that were generated earlier [15], and the transgenic tobacco plants with constitutive expression of *PnXTH1* were used. The quantitative determination of the mRNA content (after conversion to cDNA) of *PtrXTH1, PnARGOS-LIKE*, and *PnXTH1* genes was carried out using real-time polymerase chain reaction in the presence of the intercalating SYBR Green I dye on

the Rotor-GeneTM 6000 thermal cycler (Corbett Research, Australia). mRNA of actin (EF418792) was used as a standard for working with aspen, and the elongation factor mRNA EF-1α (AF120093.1) was used for tobacco, the expression level of which was taken as 100%. The choice of these reference genes is explained by the fact that, according to the published data, they show the greatest stability of expression level in various tissues of aspen and tobacco under different growth conditions [16–18]. The amplification was carried out in 0.2 mL tubes (AXYGEN, United States) in a volume of 25 μL using a standard kit (Synthol, Russia). To quantitate the content of transcripts of *PtrXTH1* and *PnXTH1*, primers 5'-TTCCAATCAAAAGGTTCATACT-3' and 5'-ATCATCGGCATTCCATAGG-3' were used. RT-PCRs of *PnARGOS-LIKE*, actin and the elongation factor EF-1α were performed with primer pairs 5'-GCAAGTGCAGAGAAAAAGAAA-3'/5'-ATGAAAGCCAAGATTATGAGC-3', 5'-ACTGGTATTGTGTTGGATTCTGG-3'/5'-AGTTGTATGTAGTCTCGTGGATGC-3', and 5'-GAATTGGTACTGTCCCTGTT-3'/5'-TTGCCAATCTGTCCTGAAT-3', respectively. All experiments were carried out in three biological and three analytical replications.

Genetic engineering. *PnXTH1* was amplified from genomic DNA of black poplar using HiFi DNA polymerase (Kapa Biosystems, United States) with primers 5'-CGTCTTATGCGTGTCAAAAA-3' and 5'-TAAAATGTATCCAGCACCAAT-3'. According to theoretical calculations and the results of agarose gel electrophoresis, the size of the DNA amplicon was 2033 bp, of which 51 bp belongs to the 5'-untranslated region, 39 bp to the 3'-untranslated region, and 1058 bp for 3 introns. The theoretically calculated size of the open reading frame of *PnXTH1* is 885 bp, so the target gene potentially encodes a putative protein consisting of 295 amino acid residues. The amplified DNA region was cloned and sequenced from two directions (400 bp each), which unambiguously allowed it to be identified as the *XTH1* gene of the black poplar. The binary vector pCambia 1301 (CAMBIA, Australia) with a selectable marker for resistance to hygromycin, in which an artificially created cassette consisting of a 35S promoter [19] and a polyadenylation site of the cauliflower mosaic virus was cloned, was used to create the genetic engineering constructs. The modified plasmid was digested at the *Sma*I restriction site, and the 2033 bp amplicon of *PnXTH1* was cloned in the resulting binary vector. Based on the results of the PCR analysis, a construct containing the insertion of the target gene in the sense orientation with respect to the promoter was selected. The target genetic engineering design of *35S::PnXTH1* in the binary vector pCambia 1301 was introduced using electroporation into the *Agrobacterium tumefaciens* cells of strain AGL0.

Generation of transgenic tobacco plants, morphological characteristics, and growing conditions of plants. Transgenic tobacco plants of *Nicotiana tabacum* L. of the Petit Havana strain SR1 were obtained by the agrobacterial transformation of leaf discs carved from the leaves of 3-month-old plants [19]. The selection of transgenic plants was carried out by histochemical analysis of the activity of the reporter *GUS* gene and PCR analysis for the presence of the 35S promoter, marker genes, and the target gene. After obtaining the seeds of transgenic plants, they were plated on the selective MS medium. After three weeks, the seedlings were stable or unstable against the selective agent and the splitting was determined by the inheritance of the selective marker gene. The results were processed by the χ-square method according to a standard procedure and the lines with one integrated copy of transgenes were isolated for further work. Morphological analysis was carried out on transgenic second-generation plants pregrown on a selective medium with hygromycin to eliminate nontransgenic variants. For the analysis, the lines of transgenic plants characterized by a relatively high mRNA content of the *PnXTH1* transgene were selected. Plants of transgenic lines and control plants were cultivated in 450 mL filled vegetation vessels filled with a common soil (Terra vita, Russia) under conditions of a greenhouse at a temperature of 30°C with an illumination of approximately 140 mmol per square meter in sec and a photoperiod of 16/8 h (light/dark). Nontransgenic tobacco plants of Petit Havana strain SR1 grown on MS medium without antibiotics and acclimatized to soil conditions were used as control. The length and area of the leaves were determined during the flowering period. Five plants were selected for each line, in which three lower leaves were measured (the second, third, and fourth leaves). The stem length and fresh and dry weight of the aboveground part was also determined during flowering, the length of five flowers was also measured for each plant.

Estimation of growth parameters of transgenic plants under the influence of salinity, drought and cold. Transgenic plants were germinated in Binder (Germany) climate chambers at a temperature of 25°C, illumination of approximately 140 mmol per square meter/second and a photoperiod of 16/8 h (light/dark) on the MC medium. After 10 days of germination on a selective medium, seedlings with the same root sizes were transferred to vertically oriented Petri dishes and, after 10 days, the root growth was determined under normal conditions and the action of 100 mM NaCl. The 20-day transgenic seedlings that continued to grow on horizontally oriented Petri dishes were transferred to the soil and grown in a greenhouse. To determine the salt tolerance, transgenic plants were grown under normal conditions in a greenhouse in vessels with a volume of 450 mL for 20 days. Then the experimental plants were poured four times during 2 weeks (twice a week) with 50 mL of a 2% solution of NaCl, while the concentration of NaCl of 150 mM in the soil was achieved. Control plants were watered with a similar volume of distilled water. Morphological analysis

was performed 30 days after the start of the salt addition test. To determine the growth parameters of transgenic plants under the influence of low positive temperatures, the plants were grown on soil for 12 days in 250 mL vegetation vessels in a greenhouse, then some of the plants were transferred to the Sanyo MIR-154 (Japan) incubator with a temperature of 12°C. All plants were watered with distilled water three times a week (the volume was 20 mL). After 20 days, the fresh and dry weight of the aboveground part of the transgenic plants was determined. Experiments on the effects of drought began after 22 days of growing in a greenhouse under normal conditions in vegetation vessels with a soil volume of 450 mL. Control plants continued to be watered three times a week, while the experimental plants were watered only once a week (the water volume was 50 mL). After 30 days, the fresh and dry weight of the aboveground part of the transgenic plants was determined. A portion of the transgenic plants was not watered for 20 days and their relative water content (RWC) was determined [14]. To assess the rate of water loss by the leaves, the seventh leaf was cut out in transgenic plants, placed in a thermostat at 45°C, and weighed every hour during 4 h; the results were expressed as a percentage from the original mass of the leaf. The results of the studies were presented in the form of histograms with average sample values. Bars denoted the standard error of the mean. The reliability of the differences in all the experiments was estimated using the Mann–Whitney *U*-test.

RESULTS

Search for Arabidopsis thaliana Genes Homologous to PnXTH1 Gene and the Features of Regulation of Their Expression According to NCBI GEO

GenBank contains the nucleotide sequences of the orthologues of *PnXTH1* gene from *P. trichocarpa* (XM_002304589), *P. alba* (JX431932), *P. tomentosa* (KM267529), and the hybrid aspen *P. tremula* × *P. tremuloides* (*PttXET16A*, AF515607), however, the gene under study remains poorly investigated in these plants. Only *PttXET16A* is known to encode XTH, the activity of which in the formation of secondary cell walls is found in xylem and phloem [20]. In addition, the genes of other plants that are closest to *PnXTH1* in their nucleotide sequence that were determined by BLAST are the genes of other plants: *Prunus mume* (XR_001677370), *Phoenix dactylifera* (XM_008780219), *Malus domestica* (XM_008371365), *Gossypium raimondii* (XM_012581830), *Theobroma cacao* (XM_007032031), *Daucus carota* (XM_017371651), *Camelina sativa* (XM_010493866), *Cucumis melo* (XM_008466945), *Actinidia deliciosa* (L46792), *Medicago truncatula* (XM_003610093), *Jatropha curcas* (XM_012233283), *Ricinus communis* (XM002512657), *Vitis vinifera* (XM_002274484), *Betula pendula* (DQ235254), and *Rosa chinensis* (GU320707). Of all these genes, only two studies have been initiated: EF194052 and L46792; however, their role in regulating growth and stress resistance remains unexplored.

In various biological databases, such as EMBL (http://www.ebi.ac.uk) and NCBI GEO (http://www.ncbi.nlm.nih.gov), the results of the expression analysis of various *A. thaliana* genes was performed using DNA microarrays. These databases contain information on the regulation of the expression of many XTHs of *A. thaliana*. This information is very useful in the study of homologous XTHs genes in other plants. In connection with this, the task was to determine the closest homologues of *PnXTH1* in the genome of *A. thaliana*. Phylogenetic analysis of the nucleotide sequences of the open reading frames of the XTHs genes of *A. thaliana* and poplar *PnXTH1* showed that this gene is the closest to genes *AtXTH5* (*ENDOXYLOGLUCAN TRANSFERASE A4*, *EXGT-A4*) and *AtXTH4* (*ENDOXYLOGLUCAN TRANSFERASE A1*, *EXGT-A1*) (Fig. 1a).

Cis-regulatory Elements of the Putative Promoter Region of PnXTH1 Gene Ortholog from P. trichocarpa

The transcription of the XTHs genes, like any other genes, is controlled primarily by the promoter. In this regard, useful information on the mechanisms of regulation of expression of the studied genes can be obtained by analyzing the cis-regulatory elements of the promoter region. In aspen and black poplar, promoters of *XTH1* gene are not sequenced. Among the poplars, only the genome of *P. trichocarpa* was completely sequenced; therefore, the putative promoter site of the *P. trichocarpa XTH1* gene was analyzed. Six GATA elements [21], six BS IN AG elements [22], three W-box elements [23], and three AtMYC2 elements [24] were detected in the analyzed DNA region. In the promoter region of *XTH1*, cis elements for AtMYB2, SORLIP1, Ibox, BOXII, ABRE-like, and ATB2 (one for each) were also detected (Fig. 1b).

Content of PtrXTH1 Transcripts of Wild-Type Aspen in Leaves of Different Age and under Exogenous Treatment with Phytohormones

After the bioinformatics analysis, we conducted a study of the expression profile of *PtrXTH1* of wild-type aspen in leaves of different ages and under exogenous treatment with phytohormones. The maximum level of *PtrXTH1* expression was recorded in the youngest intensively growing aspen leaves (Fig. 2a). A relatively low level of *PtrXTH1* transcripts was found in the apex of the shoot, consisting of an apical meristem and leaf primordia. Under the exogenous treatment with phytohormones, the level of transcript content did not increase at the top of the shoot, while brassinosteroids contributed to a decrease in the expression level of *PtrXTH1* (Fig. 2a). In the youngest leaves, cytokinins significantly increased the expression level of *PtrXTH1*, and brassinosteroids, on the contrary,

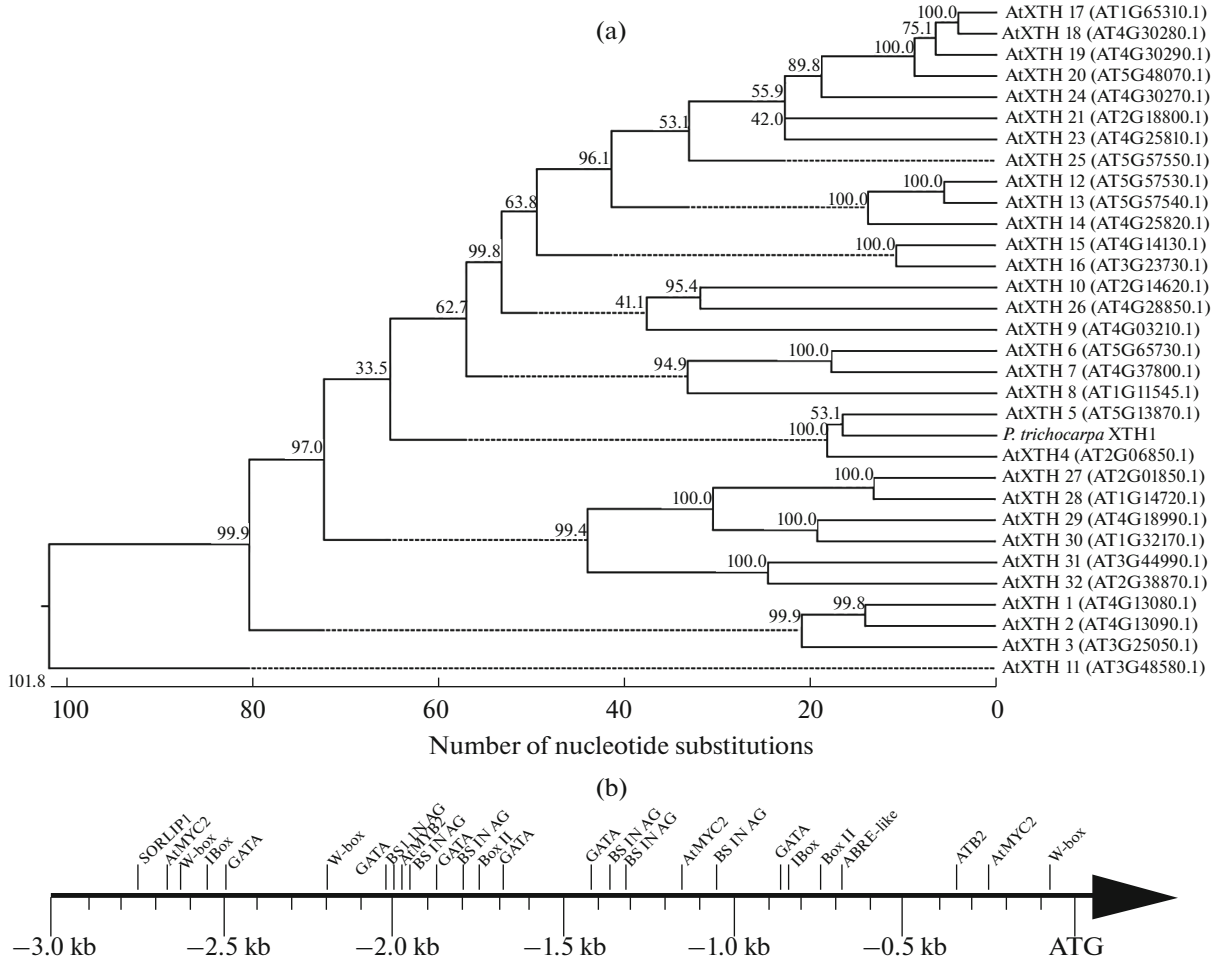

Fig. 1. Bioinformatic analysis of poplar *XTH1* gene and its putative promoter region. (a) Phylogenetic tree of the similarity between *PtXTH1* of *Populus trichocarpa* and the homologous *Arabidopsis thaliana* genes (bootstrap trials = 1000, seed = 111); (b) results of search for the cis-regulatory elements in the putative promoter region of *P. trichocarpa PtXTH1* gene. Kb—kilobase (1000 bp).

significantly reduced the content of the transcripts of the gene under study (Fig. 2a). In the young leaves of older age, all three phytohormones that were used for exogenous treatment contributed to an increase in the content of *PtrXTH1* transcripts. The greatest impact was exerted by auxins, which increased the expression level of *PtrXTH1* by eight times compared to the control (Fig. 2a).

Participation of the PnARGOS-LIKE Gene in the Regulation of PtrXTH1 Gene Expression

Earlier, we cloned and studied the *PnARGOS-LIKE* gene encoding one of the proteins with an OSR domain that is involved in the initiation of leaf primordia and leaf growth due to cell expansion [15]. Unlike *PtrXTH1*, the highest level of expression for this gene was found not in young leaves but in the tip of the shoot. Expression of *PnARGOS-LIKE*, as well as *PtrXTH1*, was induced by cytokinins and auxins (using the same phytohormone concentrations). Since OSR-domain proteins are involved in the transduction of the phytohormonal signal and are likely to mediate the transmission of the signal from phytohormones to XTHs [25], studying the expression level of *PtrXTH1* upon overexpression of *PnARGOS-LIKE* is of great interest. To carry out this analysis, we used earlier generated transgenic aspen plants with constitutive expression of *PnARGOS-LIKE* [15]. The experiments were performed on young leaves of transgenic aspen, in which the level of expression of *PnARGOS-LIKE* was four times higher than in wild type plants (Fig. 2b). At the same time, the levels of *PnARGOS-LIKE* transgenic mRNA of black poplar and its homologue of aspen *PtrARGOS-LIKE* host gene were detected simultaneously, since the primers were suitable for both species of poplar. In the same leaves of aspen during the overexpression of *PnARGOS-LIKE*, a sevenfold increase in the content of transcripts of *PtrXTH1* was found (Fig. 2c). Thus, we have shown

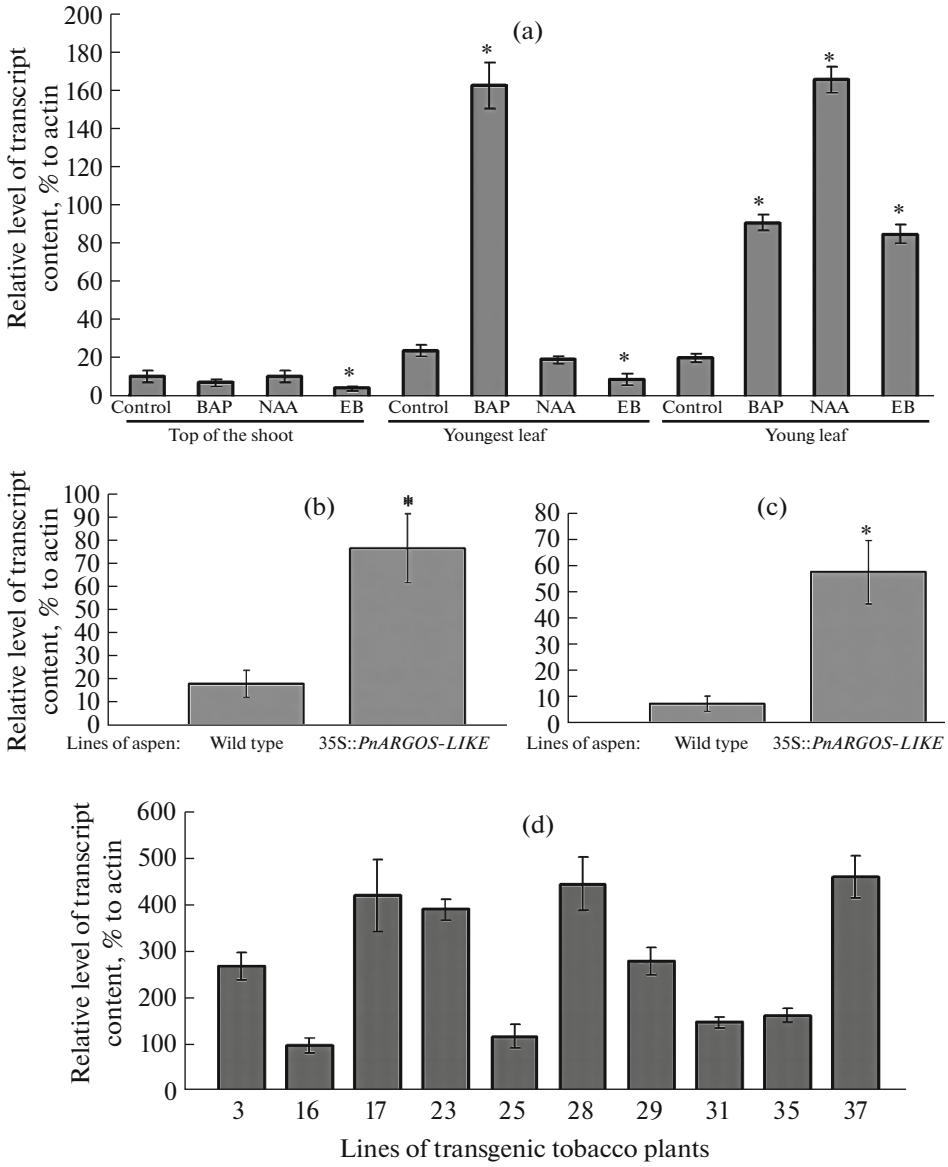

Fig. 2. Expression profile of *XTH1* in aspen and transgenic tobacco plants. (a) Results of the analysis of the content of aspen *PtrXTH1* transcripts in the leaves of different ages and under the treatment with 6-benziaminopurine (BAP), α-naphthylacetic acid (NAA), and 24-epibrassinolide (EB) in wild-type aspen plants; (b) content of *PnARGOS-LIKE* transcripts in young leaves of wild-type aspen and transgenic *35S::PnARGOS-LIKE* plants of aspen; (c) content of *PtrXTH1* transcripts in young leaves of wild-type aspen and transgenic *35S::PnARGOS-LIKE* plants of aspen; (d) results of the analysis of the content of *PnXTH1* transcripts in young leaves of transgenic tobacco plants transformed with *35S::PnXTH1* construction. $n = 3$. * $P < 0.01$.

that the overexpression of *PnARGOS-LIKE* promotes a significant increase in the content of transcripts of *PtrXTH1.*

Molecular and Morphological Analysis of Transgenic Tobacco Plants with Constitutive Expression of the PnXTH1 Gene

Important information on the role of *PtrXTH1* and *PnXTH1* genes can be obtained by generating transgenic plants and analyzing them morphologically. We carried out the transformation of tobacco and obtained 37 lines of transgenic plants, the transgenicity of which was proven by PCR, histochemical analysis of the activity of the reporter protein β-glucuronidase, and the analysis of the cleavage of resistance to the hygromycin. Further, the content of *PnXTH1* transcripts in transgenic plants was analyzed. Its expression level was stably detected in a series of experiments and found in lines 3, 16, 17, 23, 25, 28, 29, 31, 35, and 37. The highest mRNA levels of the transgene were typical for lines 3, 17, 23, 28, 29, and 37 (Fig. 2d). All ten lines of transgenic tobacco plants with stable expression of the transgene were selected for further morphological

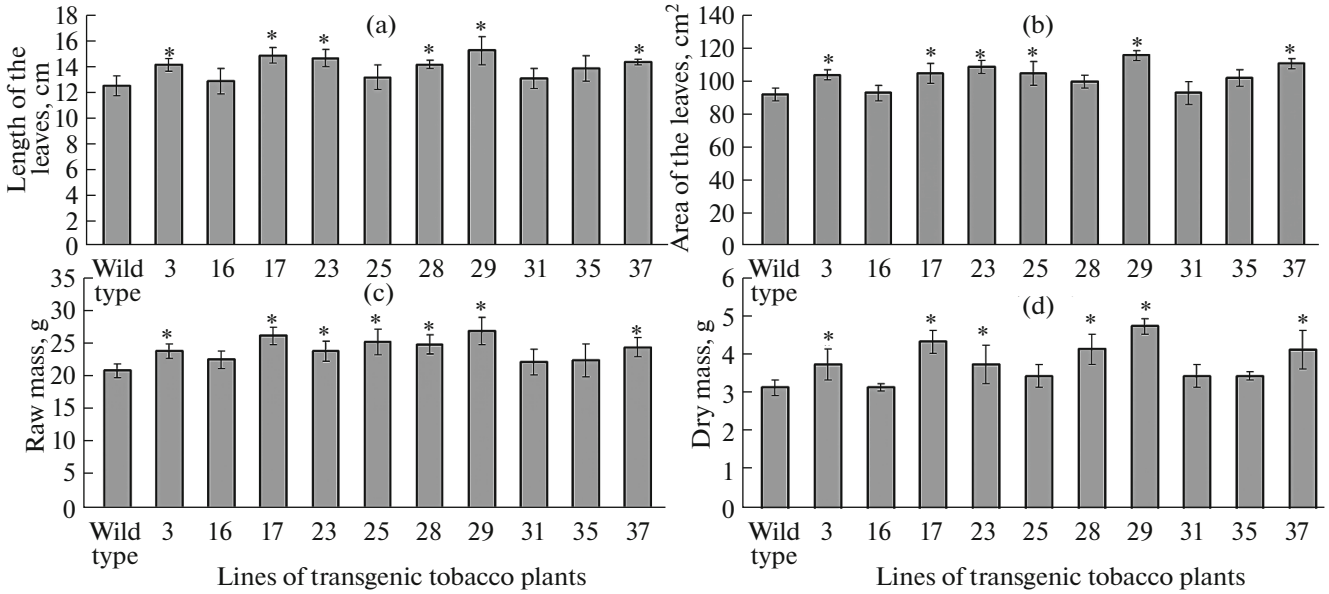

Fig. 3. Morphometric parameters of transgenic tobacco plants overexpressing *PnXTH1* under normal growth conditions. (a) Length of the leaves; (b) area of the leaves; (c) raw mass of the aboveground part; (d) dry mass of the aboveground part. $n = 5$. * $P < 0.01$.

analysis. During the flowering period, a significant increase in leaf length was typical for lines 3, 17, 23, 28, 29, and 37 (Fig. 3a). The degree of increase in the length of the leaves reached an average of 22% compared to the control for line 29. In the area of the leaves, some lines of transgenic plants also showed a significant increase on average from 13% for line 3 to 26% for line 29 (Fig. 3b). The control and transgenic plants did not differ significantly by the height of the stem and the length of the flowers. For lines 3, 17, 23, 25, 28, 29, and 37, an increase in the fresh weight was also characteristic, and the increase in this parameter was 25% on average for line 17 compared to the control (Fig. 3c). Constitutive expression of *PnXTH1* also contributed to an increase in dry weight in six lines of transgenic plants, with an increase in the line 29 for an average of 50% (Fig. 3d). Thus, the constitutive expression of *PnXTH1* promoted an increase in the fresh and dry weight of the aboveground part of the plants mainly due to stimulation of leaf growth. Moreover, the degree of morphological changes in transgenic plants correlated with the expression level of *PnXTH1* (Fig. 2d).

Growth Parameters of Transgenic Tobacco Plants with PnXTH1 Constitutive Expression under the Action of Stress Factors

Root length of plants grown in vertically oriented Petri dishes was analyzed in seven lines of transgenic tobacco plants. Under normal conditions, the length of the roots of transgenic plants in all the lines increased in comparison with the wild type. However, an even greater difference in root length between control and transgenic plants was detected under the action of 100 mM NaCl (Fig. 4a). For example, in line 37, the root length during salinity was, on average, 142% greater than in wild-type plants. In control plants grown on a medium with 100 mM NaCl, the root length remained the same as in the absence of salt. At the same time, the root length increased substantially with the action of salt in all transgenic plants, for example, in line 37, on average, by 55% (Fig. 4a). When growing on soil in saline conditions, the height of the stem decreased both in control and in most part of the ten analyzed lines of transgenic plants. The degree of decrease in stem height during salinization was the greatest in wild-type plants and amounted to 41%, whereas, for example, in line 28, the stem height decreased, on average, by only 19% than that in plants of the same line grown under normal conditions. It should be noted that the height of the stem did not decrease in lines 35 and 37 under salinization in comparison with plants of the same lines growing under normal conditions. The length and area of the leaves under the action of NaCl did not significantly change either in the control or in the transgenic tobacco plants. In general, the size of the leaves of wild-type plants and *35S::PnXTH1* tobacco plants during salinity did not differ from each other. The increase in fresh weight of plants growing under saline conditions was typical for lines 28, 35, and 37 (Fig. 4b), which was most likely due to improved growth parameters of the transgenic plants compared to the control. The dry mass at salinity significantly increased in lines 17, 28, 31, and 35 (Fig. 4d).

With a 20-day growth under conditions of hypothermia (12°C), the fresh weight of wild-type plants decreased by an average of 62% compared to the same

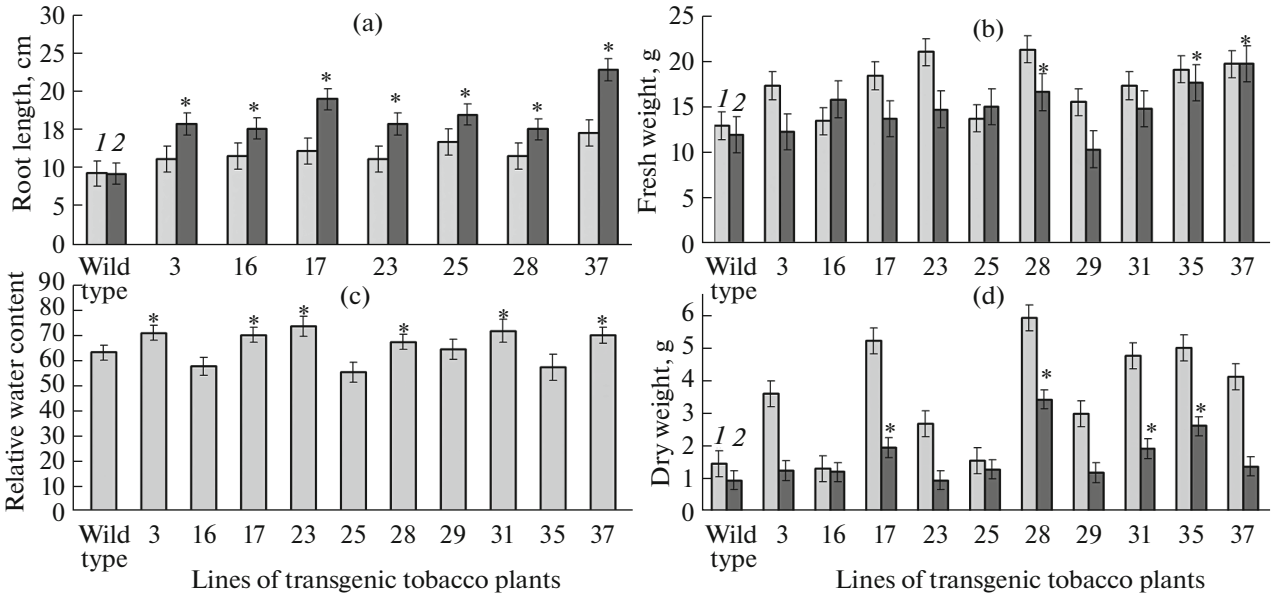

Fig. 4. Morphometric parameters of transgenic tobacco plants overexpressing *PnXTH1* in response to stress factors. (a) Root length of plants grown in vertically oriented Petri dishes in response to 100 mM NaCl; (b) fresh weight of the aboveground part of plants grown in soil under saline conditions (150 mM NaCl); (c) relative water content (RWC) in the shoots after a 20-day drought; (d) dry weight of the aboveground part of plants grown in soil under saline conditions (150 mM NaCl). *1*—control plants; *2*—plants grown under conditions of salinity. $n = 5$. * $P < 0.01$.

plants grown in the absence of stress factors. In the analyzed transgenic plants under cold conditions, the fresh weight also decreased by 60–70% depending on the line. A reduction in fresh weight by 38% was observed only for line 37. The dry weight of the control plants decreased by an average of 50% at 12°C compared with normal conditions. In the case of transgenic plants, the dry weight under these conditions decreased from 50% in line 37 to 75% in line 35. Thus, none of the lines of transgenic tobacco plants showed increased productivity in response to cold, and only line 37 showed an increase in the fresh weight in comparison with wild-type plants.

Further, we evaluated the growth parameters of transgenic plants during prolonged drought action. It was shown that the fresh weight of both control and transgenic plants under the 30-day action of drought reduced by approximately two times compared to the same plants grown in the absence of stress factors. The dry weight of all the analyzed plants decreased approximately to the same degree. In general, the degree of change in fresh and dry weights in response to drought in transgenic plants did not differ significantly from wild-type plants. At the same time, it should be noted that the transgenic plants of lines 17, 23, 28, and 37 gained more dry weight under drought conditions than wild-type plants under the same conditions. This means that the listed lines of transgenic plants are more productive under drought than control plants.

The ability of transgenic plants to grow under drought conditions may be due to more effective water retention in tissues and organs [14]. To test this suggestion, we estimated the relative water content (RWC) in the analyzed plants. A significant increase in this indicator compared to wild-type plants was recorded in lines 3, 17, 23, 28, 31, and 37 (Fig. 4c), which largely coincides with the data on the increase in dry weight during drought. Plants lose water primarily via transpiration through the stomata. In order to estimate the rate of transpiration water loss, we measured the changes in the mass of the cut leaves under the action of heat. The leaves of transgenic plants of lines 17, 23, 28, and 29 lost mass slower than the leaves of wild-type plants (Fig. 5), which largely coincides with the data on the accumulation of dry weight during drought and an increase in the RWC.

DISCUSSION

PnXTH1 gene encodes one of the xyloglucan endotransglycosylases of the black poplar, whose orthologs are found in the genomes of aspen and balsamic poplar. Among the genes encoding XTHs of *A. thaliana*, the nucleotide sequences of the genes *AtXTH4* and *AtXTH5* are the most similar to *PnXTH1*/*PtrXTH1*. In the promoter region of poplar *XTH1*, we found six binding elements with GATA transcription factors, which play an important role in the development, differentiation, and regulation of cell proliferation [21]. In the promoter of poplar *XTH1*, 6 BS IN AG cis-regulatory elements were also detected, which are associated with the homeodomain transcription factor BELLRINGER involved in the regulation of vascular

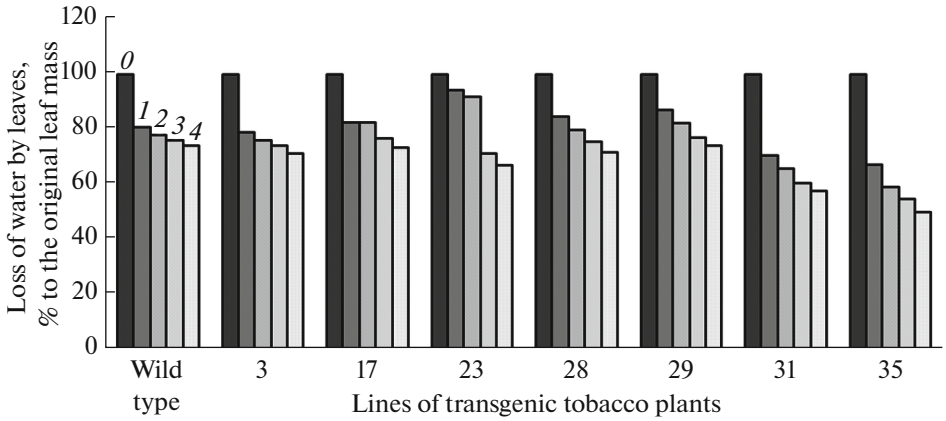

Fig. 5. Results of the analysis of the rate of water loss by the leaves of tobacco. *PnXTH1*/3-35—lines of transgenic tobacco plants. *0*—leaf mass immediately after cutting (accepted as 100%); *1*—leaf mass as a percentage from the original mass after 1 h of incubation at +45°C; *2*—after 2 h of incubation; *3*—3 h after incubation; *4*—4 h after incubation.

differentiation, phyllotaxis, and development of flowers and fruits. This trans-factor has been shown to control the expression of a variety of proteins involved primarily in the regulation of cell wall enlargement [26], but its effect on XTHs expression has not yet been reported. W-box cis element involves a large family of WRKY transcription factors involved in the regulation of plant growth and development, as well as in response to biotic and abiotic stress factors [27]. We also found the cis elements of AtMYC2 and AtMYB2 transcription factors that control the expression of drought and ABA-induced genes [24] in the promoter of the gene under study, which may indicate the involvement of poplar *XTH1* in response to stress effects. The presence of the ABRE motif in the promoter also indicates the possible involvement of the XTH1 protein in response to abiotic stress factors that cause a water deficit [28]. It should be noted that a comparative analysis of the expected promoter site in several species of poplars is required to obtain more reliable data on cis-regulatory elements; however, its nucleotide sequence in other species of the genus Populus is not sequenced at the moment.

An increase in the mRNA content of *XTHs* in response to the treatment of plants with exogenous auxins, gibberellins, and brassinosteroids is well known [11]. We also obtained data showing that the level of *PnXTH1* transcription in the youngest leaves increases only under the influence of cytokinins. Cytokinins participate in the regulation of cell expansion [29] and have a positive effect on the expression of expansins [30], but there is less information on the effect of this group of phytohormones on the expression level of *XTHs*. In the older leaves, exogenous auxins exerted the greatest influence on the expression of the gene under study. Based on NCBI GEO data, *AtXTH4* and *AtXTH5* genes homologous to *PnXTH1* were also induced by auxins (GDS744/250214_at; GDS744/266215_at; GDS671/17540_s_at; GDS668/250214_at; GDS668/266215_at). However, it should be noted that a particular role in regulating the expression of *PtrXTH1* is apparently played by cytokinins, which increased the level of transcripts of the gene under study precisely in those leaves in which the level of its expression was initially high. Brassinosteroids contributed to a decrease in the level of expression of the gene under study at the shoot tip and in the youngest leaves, whereas the expression level of *PtrXTH1* was increased in the older leaves. It should be noted that the treatment with a brassinozol, brassinosteroid biosynthesis inhibitor, contributed to a decrease in the expression level of *AtXTH4* and *AtXTH5* (GDS3823/250214_at; GDS3823/266215_at). It can be suggested that brassinosteroids, in contrast to other phytohormones, more finely regulate the expression of *XTHs* and, depending on the stage of cell development, they can both increase and decrease the level of transcription of the *PtrXTH1* gene. Earlier, it was also shown on tobacco that exogenous brassinosteroids can both increase and decrease the expression level of the expansins depending on the age of the leaf [30]. Signals from phytohormones to *XTHs* can come from proteins with an OSR domain [25]. Earlier, we showed that the expression level of *PtrARGOS-LIKE*, which encodes one of the proteins with the OSR-domain of aspen [15], increases in response to auxins, cytokinins, and brassinosteroids. In this paper, we showed that an increase in the expression level of *PnARGOS-LIKE* of the black poplar by four times contributes to a sevenfold increase in the content of *PnXTH1* transcripts. Based on these data, it can be suggested that the product of *PnARGOS-LIKE* serves as a mediator between phytohormones and XTHs, and the enhancement of the phytohormonal signal is probably one of the functions of this protein with the OSR domain.

Constitutive expression of the *PnXTH1* gene in transgenic tobacco plants facilitated the increase in leaf size and the fresh and dry weight of the aerial parts

of the plants. The degree of increase in growth parameters correlated with the level of transcripts of the target gene. From the data obtained, it follows that, under normal growth conditions, the product of the gene takes part to a greater extent in the processes of leaf growth, while no increase in the size of other organs in the transgenic tobacco plants has been recorded.

Transgenic *35S::PnXTH1* plants were characterized by higher parameters of root and stem growth under the action of salt than wild-type plants. In the literature, there is a wealth of data on the positive effects of overexpression of proteins involved in providing cell expansion for salt tolerance, but most of the information relates to expansins. As for *XTHs*, it is known that transgenic tomato plants overexpressing the *CaXTH3* gene were characterized by increased salt tolerance due to a longer preservation of chlorophyll from destruction and the maintenance of root extension at normal levels when exposed to NaCl [14]. The drought resistance of these transgenic tomato plants was ensured by reducing the width of the stomatal pore, which contributed to a decrease in the loss of transpiration water. Transgenic *35S::PnXTH1* plants of tobacco also showed an increased ability to retain water in the leaves. However, the mechanism of this feature of transgenic plants remains unknown. According to the literature, the increased ability of transgenic plants to retain water in the leaves can be achieved by reducing both the size of the stomatal pore and the number of stomata per unit area of the leaf [14].

Thus, the *XTH1* gene of poplar encodes the xyloglucan endo-transglycosylase induced by cytokinins and auxins and involved in leaf and stem growth as well as adaptation of plants to salinity and drought.

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