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Expression of Cellulose Synthase Genes During the Gravistimulation of Flax (*Linum usitatissimum*) and Poplar (*Populus alba* × *tremula*) Plants

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Abstract—Plant cellulose is synthesized on the plasma membrane by the cellulose synthase complex and a number of coenzymes. Different cellulose synthases are thought to be involved in the primary and secondary cell wall biosynthesis. Plant fibers, such as flax phloem fibers and xylem fibers of poplar tension wood, produce a tertiary cell wall with increased cellulose content and a lack of xylan and lignin. The composition and types of cellulose synthase complexes involved in the tertiary cell wall biosynthesis have not yet been established. Based on transcriptome data for flax (*Linum usitatissimum*) and poplar (*Populus alba* × *tremula*) plants, we evaluated the expression of genes encoding cellulose synthases during the development of a gravitropic response with the participation of the phloem and/or xylem fibers producing the tertiary cell wall. Changes in the expression of cellulose synthase genes characteristic of both primary and secondary cell walls in various model systems indicate the mobility of an ensemble of different cellulose synthases during the gravistimulation, which can affect both an individual cell type and a set of tissues with different types of cell walls. For the isolated flax phloem fibers, the involvement of both types of cellulose synthases in the formation of the tertiary cell wall at all stages of graviresponse was demonstrated.

Keywords: cellulose synthases, tertiary cell wall, plant fibers, transcriptome analysis

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INTRODUCTION

Cellulose, being an indispensable component of cell walls of any plant cell, is believed to be the most widely spread biopolymer on Earth. Cellulose is a β-1,4-D-glucan, the elementary unit of which is β-cellobiose (β-D-glucopyranosyl-(1,4)-β-D-glucopyranose). Despite its quite simple chemical structure, biosynthesis of cellulose molecules in plants is provided by a complex multicomponent enzymatic system. The central role in this system is played by cellulose synthases (CESA), which belong to the glycosyltransferase 2 family (GT2, EC 2.4.1.12, according to the CAZy). The CESA are assembled into complexes, so called "rosettes," in which each enzyme synthesizes an individual glucan chain. Presently, it is believed that a rosette consists of six petals, and each petal of the rosette consists of three individual CESA proteins. Hence, one complex is responsible for synthesis of one cellulose microfiber, which consists of 18 glucan chains [1, 2]. For example, the genome of the rockress (*Arabidopsis thaliana*) contains 10 CESA encoding genes [3]. It is known that the set of CESA isoforms is different for the primary and secondary cellular walls, which are formed at different stages of cell development and characterized by different properties and functions. For example, cellulose of primary cellular walls is synthesized by the isoforms CESA1, 3 and 6 (further PCW CESA, PCW—the primary cell wall), while cellulose of secondary cellular walls is synthesized by the isoforms CESA4, 7 and 8 (further SCW CESA, SCW—the secondary cell wall) [4, 5]. The CESA10 is the close homolog of the CESA1; CESA2, 5 and 9 are close homologs of the CESA6 [6]. Interestingly, in vitro, each of the CESA isoforms may interact with any of the others [7]. Moreover, it has been reported that expression of the *PCW CESA* genes proceeds in cells of xylem vessels that form secondary cellular walls. This may be due to the switch of the cells from biosynthesis of primary cellular wall to biosynthesis of secondary cellular wall, which they need to undergo, and, for some time, both complexes may

Abbreviations: CESA, cellulose synthases; PCW, primary cellular wall; SCW, secondary cellular wall; PCW CESA and SCW CESA, cellulose synthases, which synthesize cellulose of primary and secondary cellular walls respectively; PUL, pulling side of a gravistimulated plant stem; OPP, side of the stem opposite to the pulling one; TW, tension wood; OW, wood located oppositely to the tension wood; NW, normal wood; TGR, total gene reads.

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function in plasmalemma simultaneously [8]. Some researchers hypothesize that both complexes may be involved in the synthesis of secondary cellular walls [9].

The behavior of cellulose synthase complexes during the formation of the tertiary cellular wall, which is found in fibers only and characterized by high cellulose content of about 85–90%, is still poorly studied [10]. Fibers of flax (*Linum usitatissimum* L.) and tension wood of poplar (*Populus* sp.) are popular models to study the formation of tertiary wall [11, 12]. The phloem fibers of flax, when cease growing by stretching (intrusive growth), form thickened tertiary cellular wall. It is noteworthy that this process is preceded by accumulation of a thin layer of the secondary cellular wall, which may be identified by immune labeling only [13]. The tertiary cell wall is constitutively formed in the flax phloem fibers during the normal development of plants. Its high mechanical strength and accumulation into fibers allows fast isolating of flax phloem fibers, which are developed in planta. This allows one to analyze a certain cell type at a certain developmental stage and avoid problems with interpreting data that may occur during the analysis of heterogenic samples, consisting of different cell types.

It was shown that flax (*L. usitatissimum*) genome contains 16 genes, encoding cellulose synthases (*LusCESA*), 11 of which encode the PCW CESA and 5—SCW CESA, as considered from the homology with the genes of *A. thaliana* [14]. The first data on the expression of cellulose synthase genes in flax fibers [14] were obtained soon after the sequencing of flax genome [15]. Data of quantitative PCR (qPCR) showed that the expression of the *SCW LusCESA* was higher at the stage of tertiary cellular wall formation as compared with tissues, which formed the primary cellular wall. The transcriptome analysis allowed us to assess the expression of all LusCESA isoforms in fibers at different developmental stages, as well as to find out that fibers, which formed the primary cellular wall, expressed the *PCW LusCESA* genes only, whereas fibers, which formed tertiary cellular wall, expressed both the *PCW LusCESA* and *SCW LusCESA* genes [16].

The xylem fibers of poplar (*Populus* sp.) tension wood also form the tertiary cellular wall (the alternative name is the G-layer of the secondary cellular wall [17]). Its composition and structure is similar to that of the tertiary cellular wall of flax fibers [18]. However, its formation is induced by gravistimulation (the stem slope), in contrast to the phloem fibers of flax, in which the tertiary cellular walls are formed constitutively. During the response to gravistimulation, the development of the stem in the place where a curve is formed proceeds asymmetrically: tension wood and fibers with tertiary cellular walls as its characteristic element are formed on only one, pulling side of the stem; on the opposite side such changes do not occur. The poplar (*Populus trichocarpa*) genome contains 17 genes, which encode the PtiCESA [19], and their phylogeny is similar to that of flax cellulose synthases [14]. The tertiary cellular wall may be induced in xylem fibers of flax plants, which underwent gravistimulation [20, 21]. Its mode of formation should be similar to that of the formation of the tertiary cellular wall in the xylem fibers of poplar tension wood. Gravitropic response induces significant changes in the constitutively formed tertiary cellular walls of phloem fibers [20, 22]. In 8 h after gravistimulation, the expression of some LusCESA isoforms is activated in phloem fibers located at the upper (pulling) side of the stem: LusCESA1-A, LusCESA3-B,3-C, LusCESA4, LusCESA7-B and LusCESA8-B [16].

Accumulation and systematization of transcriptomic data for flax and poplar allowed the creation of the on-line FIBexDB platform (https://ssl.cres-t.org/fibex/, [23]), which makes it possible to analyze and compare the expression of cellulose synthase genes in different stem tissues of flax and poplar during the graviresponse development.

The aim of this study was to reveal the expression features of cellulose synthase genes in fibers of different origin, which form a thickened tertiary cellular wall rich with cellulose. The study considers three different systems: 1) phloem fibers of flax under gravistimulation conditions, which normally (constitutively) form the tertiary cellular wall; 2) xylem part of flax plants (*L. usitatissimum*), in xylem fibers of which the formation of tertiary cellular wall is induced by gravistimulation; 3) poplar (*Populus alba* × *tremula*) tension wood, in xylem fibers of which the tertiary cellular wall is formed (Fig. 1). The comparison of expression levels of different cellulose synthases in these systems helps understanding how cellulose biosynthesis works in the tertiary cellular wall, which remains unclear.

RESULTS AND DISCUSSION

Expression of cellulose synthase genes in flax (*L. usitatissimum*) stem tissues at gravistimulation. The comparative analysis of cellulose synthase gene expression during the gravitropic response of flax plants has been carried out on the basis of previous experiments, the primary results of which have been deposited in the NCBI (PRJNA631357) and FIBexDB databases. Method of transcriptome analysis is described in [16, 21, 24]. In these studies, gravistimulation was reached by tilting the flax plants; further development of graviresponse samples of isolated phloem fibers (tFIB) and xylem part of the stem (sXYL) from the pulling (PUL) side and from the opposite side (OPP) were collected in 8, 24, and 96 h after tilting (Fig. 1). In 96 h, the stem of the plant upwards from the curve (formed 3–5 cm above the cotyledonous leaves) returned to the upright position. Previously, it was shown that gravistimulation led to the occurrence of visible morphological changes in

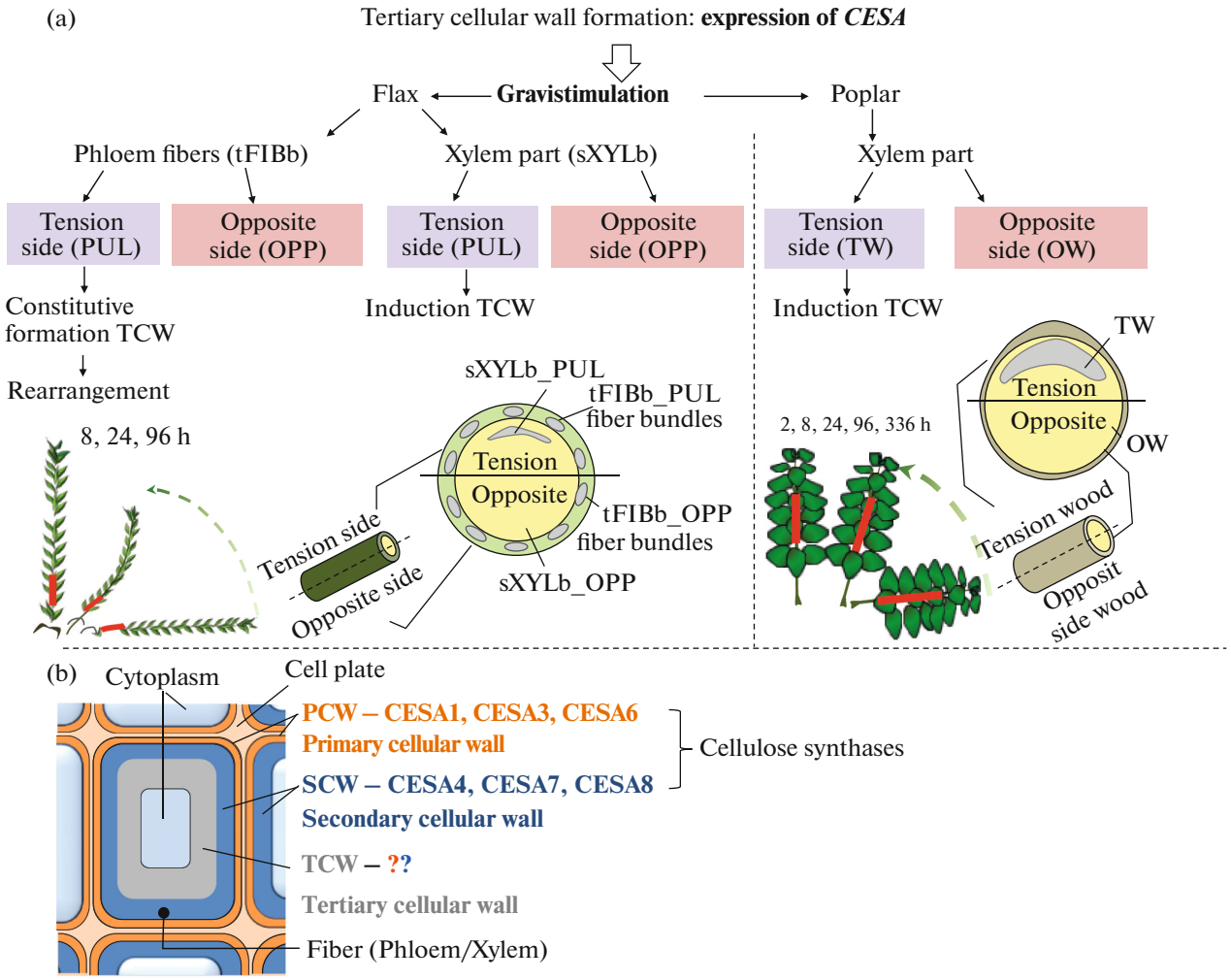

Fig. 1. (a) Models for the studying tertiary wall formation and the scheme of selection of samples of flax [16, 20, 21, 24] and poplar [25]; (b) cellular wall layers and isoforms of cellulose synthases (CESA) involved into the cellular wall formation [4, 5].

phloem fibers (widening of the fiber lumen and formation of constrictions [20]), as well as to changes in expression of several genes, the products of which are involved into the formation of the cellular wall [24], and modifications in intermolecular interactions between polysaccharides of the cellular wall [22]. It was also shown that gravistimulation induced the formation of the tertiary cellular wall in xylem fibers [21].

We analyzed the expression of the *LusCESA* genes during the graviresponse development in flax tissues analyzed. Both phloem fibers and xylem parts of the stem were shown to express the *LusCESA*, the products of which synthesize cellulose in both primary and secondary cellular walls (Fig. 2a). The *SCW LusCESA* were expressed more actively in the xylem part of the stem in comparison with the phloem fibers, whereas the *PCW LusCESA* demonstrated almost equal expression in all samples analyzed. The xylem part of the stem is represented by a mixture of tissues, in which cells with primary cellular walls are present. However, the main part of these samples was represented by vessels and fibers, which formed the secondary cellular wall. In this case, if rely on classical principles of cell wall formation, we may associate the expression of the *PCW LusCESA* genes in the xylem part of the stem with the formation of cellular walls of parenchyma, young vessels and fibers, while increased expression of the *SCW LusCESA* genes may be associated with the formation of secondary cellular walls in mature vessels and fibers, which prevail in this part of the stem. Moreover, it is known that during the switch from the synthesis of the primary cellular wall to the secondary, simultaneous function of both types of complexes is possible for some time [8].

The expression of the *PCW LusCESA* genes in phloem fibers, which form the tertiary cellular wall, was almost same as in the xylem, and in the tension parts of the stem, for some isoforms, even more effective at early stages of graviresponse (8 and 24 h). Moreover, samples of isolated fibers were enriched with

fibers with a tertiary cellular wall, and do not act as a mixture of tissues as it was in xylem. This means that at least in one type of cell, i.e., in the fibers that form the tertiary cellular wall, both *PCW LusCESA*, and *SCW LusCESA* genes are expressed simultaneously. Moreover, this effect is observed in the control plants as well at different stages of graviresponse development.

The comparative analysis of the *LusCESA* in phloem fibers and xylem of gravistimulated and control plants revealed obvious changes in the *LusCESA* expression in isolated phloem fibers (Fig. 2b). These changes were observed in both the tension part of the stem and in the opposite side of the stem, though at a different time. The expression of the majority of the *PCW* and *SCW LusCESA* genes was increased in the fibers of the tension part of the stem in 8 h after tilting, whereas in the opposite side of the stem changes in expression was observed later (24 h after tilting) (Fig. 2b).

The comparative analysis of the *LusCESA* genes in isolated fibers collected from the tension and opposite side of the stem of the same plants (PUL/OPP) revealed obvious upregulation in phloem fibers collected from the tension side at early stages of graviresponse (8 h) (Fig. 2c). Fibers located on the tension side of the stem were the very place where significant morphological modifications were observed during the graviresponse [20]. Changes in the expression in the xylem part of the stem were insignificant, though some of the *PCW LusCESA* genes were upregulated in the tension part of the stem and slight upregulation of the *SCW LusCESA* genes was also observed at later stages of graviresponse (96 h after tilting) (Fig. 2c). As the formation of the tertiary cellular wall is induced in xylem fibers obtained from the tension part of the stem [20, 22], absence of obvious variations in expression of some of the *LusCESA* genes may be due to heterogeneity of xylem samples, in which the number of cells that form the tertiary cellular wall is much lower than cells that form the secondary cellular wall. The data obtained suggest that in the latter case the expression of the *LusCESA* genes did not change significantly in response to gravistimulation. It is also possible that the high level of *SCW LusCESA* expression, which is typical for xylem cells that form the secondary cellular wall, is sufficient to initiate the formation of the tertiary cellular wall during graviresponse (Fig. 2a).

Clusterization of the *LusCESA* genes by the character of changes in expression, which was carried out for the phloem fibers and xylem samples of flax, revealed several groups of genes in both cases (Fig. 3). It was shown that in some of these clusters the *PCW LusCESA* and *SCW LusCESA* genes were mixed with each other. For example, in phloem fibers, the *LusCESA8-A* gene in the second cluster occupied neighboring position with the *LusCESA3* and c *LusCESA6-B* genes, while the third cluster contained several variations of the *LusCESA6* and *LusCESA4* genes (Fig. 3a). Similarly, the second cluster of xylem samples contained several variations of the *SCW LusCESA* and *LusCESA3-C* genes (Fig. 3b). This may point at a similar principle of regulation of some of the *PCW* and *SCW LusCESA* genes during the formation of the tertiary cellular wall that is not typical for other types of cellular walls. The consequences of such a combination for the properties cellulose microfibers formed are a matter for future studies.

Clusterization clearly reveals differences in the expression of the *LusCESA* genes in samples collected from different sides of the stems of gravistimulated flax plants (PUL and OPP). These differences are especially obvious in isolated phloem fibers. Although study of the structural features of cellulose of gravistimulated flax plants by solid-phase NMR did not reveal significant differences in the size of cellulose microfibers of PUL and control samples, it did demonstrate significant differences in their organization, as well as in the parameters of microfibers, which formed those macrofibers. Gravistimulation led to the increase in the level of cellulose crystallization due to the increase in the size of the crystal core of microfibers, a decrease in the $I\alpha/I\beta$ ratio, primarily because of the increase in the portion of β-allomorph, a decrease in the portion of paracrystal cellulose as well as in the portion of chains of the unreachable surface of microfibers, which is submerged inside a macrofiber. The increase in regularity was followed by the decrease in cellulose mobility that was demonstrated by SP/MAS ^{13}C-NMR experiments [22].

The expression of cellulose synthase genes in poplar (*P. alba* × *tremula*) wood with gravistimulation. The most well-known example of induced formation of tertiary cellular walls is the tension wood of poplar (*Populus* sp.), in which the tertiary cellular wall is formed in xylem fibers in the upper part of the trunk (stem) under tilting conditions. Accumulation of the tertiary cellular wall occurs in xylem fibers, which already contain expressed layers (one or more) of the secondary cellular wall [10]. It was formerly shown that formation of a gelatinous layer of the tertiary cellular wall in the tension wood of aspen (*Populus tremula*) is followed by the increase in expression of the *PtaCESA8-B* gene. Other *SCW PtaCESA* genes were shown to be downregulated as compared with normal wood [26]. However, neither analysis of the expression of genes, which encode all CESA isoforms, during the graviresponse development in the tension wood (TW) nor comparison with normal wood (NW) and the wood on the opposite side of the trunk (opposite wood, OW) was carried out. In the present study we used data obtained for the hybrid of abele and aspen (*P. alba* × *tremula* INRA 717-1B4) available in FIBexDB in order to analyze the expression of the *Pa×tCESA* genes [25]. To perform the RNA-seq-analysis, Zinkgraf et al. collected the TW and OW samples in 2, 8, 24, 48, 96 and 336 h after plant tilting. The genome of polar (*P. trichocarpa*) was used as the refer-

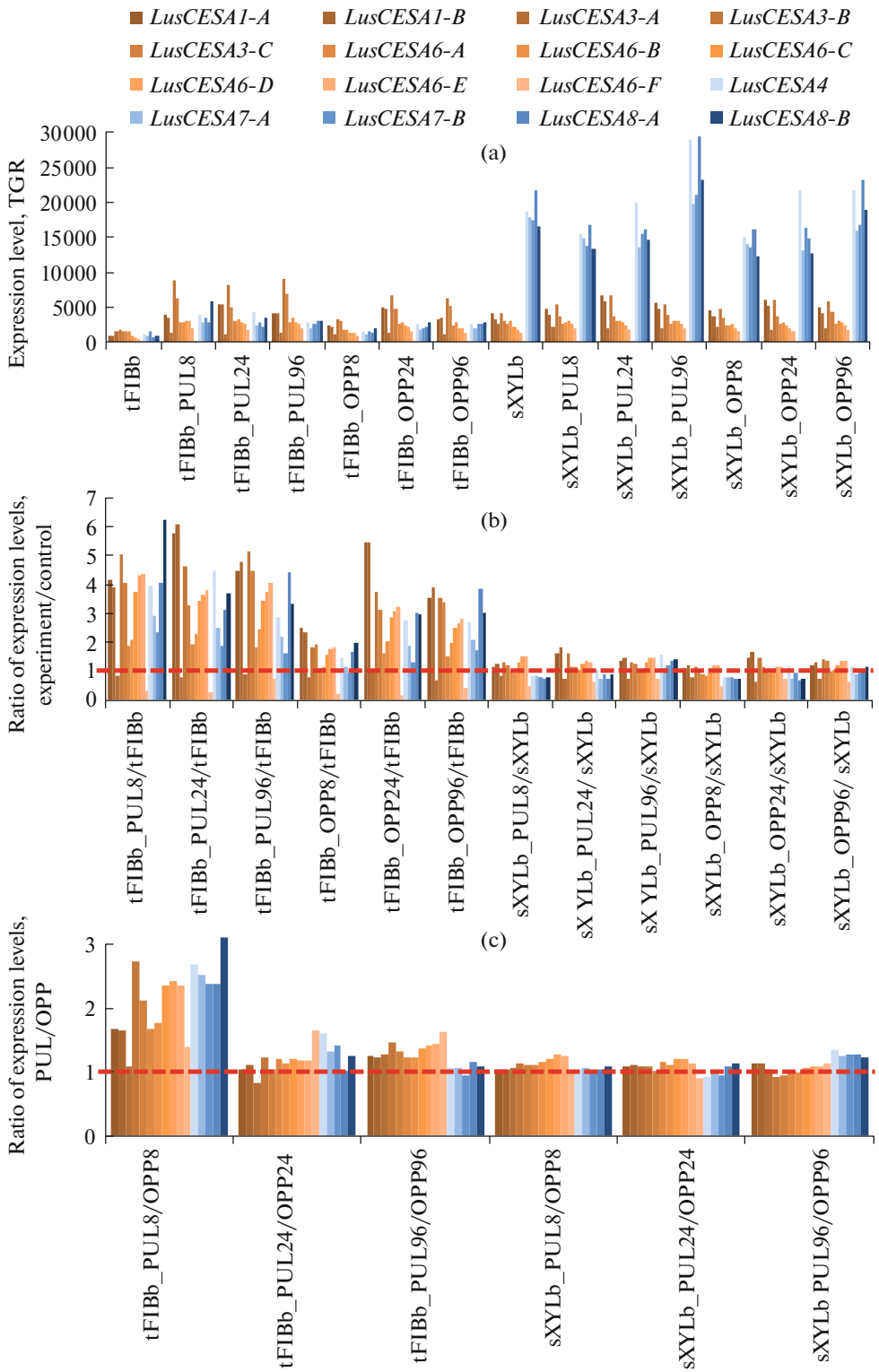

Fig. 2. (a) Expression of the *LusCESA* genes in the phloem fibers (tFIB) and in the xylem part (sXYL) on different sides of flax stem (pulling, PUL, and the opposite, OPP) during the development of graviresponse (8, 24 and 96 h after tilting); (b) the *LusCESA* gene expression ratio in flax tissues (tFIB_PUL, tFIB_OPP, sXYL_PUL and sXYL_OPP) during the development of graviresponse (8, 24 and 96 h after tilting) with respect to the expression of genes in the corresponding tissues of control plants, which did not undergo gravistimulation (tFIBb, sXYLb); (c) ratio of the *LusCESA* gene expression in flax tissues (tFIB, sXYL) collected from the pulling (PUL) side of the stem to the expression of genes in the corresponding tissues of stem collected from the opposite side of the same plants (OPP) during the development of graviresponse (8, 24 and 96 h after tilting). Orange columns correspond to the *LusCESA* genes, the products of which are involved into the formation of the primary cellular wall (PCW); blue columns correspond to the *LusCESA* genes, the products of which are involved into the formation of the secondary cellular wall (SCW).

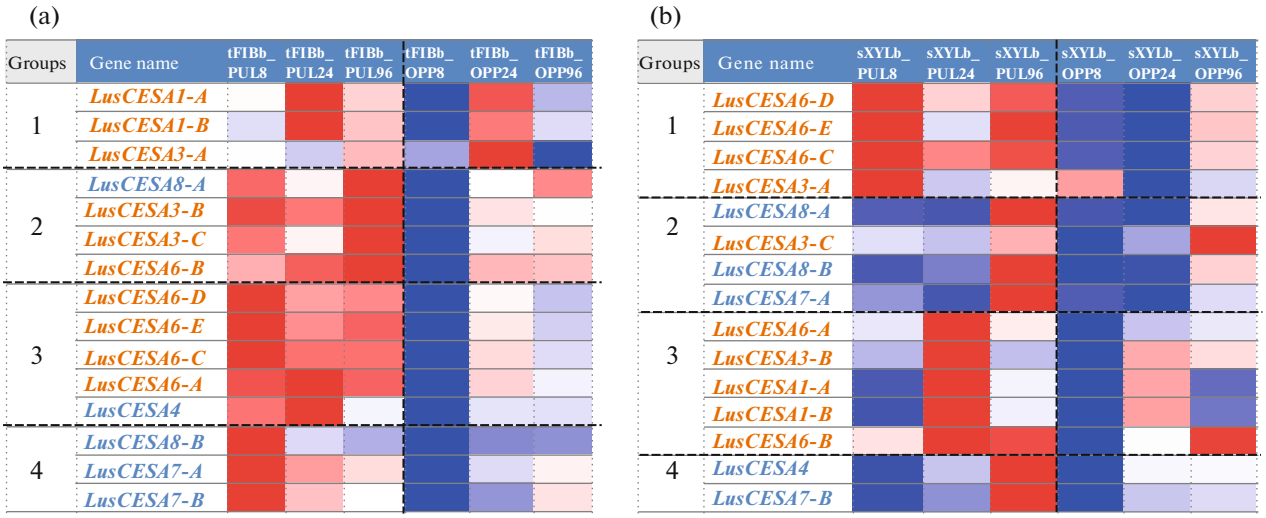

Fig. 3. The clusterization of the *LusCESA* genes in phloem fibers (a) and xylem part of the flax stem (b) during gravistimulation (8, 24 and 96 h after tilting). tFIB_PUL and sXYL_PUL are isolated phloem fibers and xylem parts from the pulling side of the flax stem, respectively; tFIB_OPP and sXYL_OPP are isolated phloem fibers and xylem part from the opposite side of the flax stem, respectively. Colors of the heat map demonstrate the expression level in the horizontal row (samples) from the maximal (red) to minimal (blue).

ence for the RNA-seq-analysis of the transcriptome data of the hybrid poplar (*P. alba* × *tremula*) [25]. The nucleotide sequences of the genes of these species demonstrated minimal differences, and the efficacy and acceptability of such an analysis is also reported by Liu et al. [27]. The analysis of the *Pa*×*tCESA* gene expression in the wood of hybrid poplar expectedly revealed higher level of the *SCW Pa*×*tCESA* genes expression in comparison with the *PCW Pa*×*tCESA* genes in all tissues (Fig. 4a). The expression of the *SCW Pa*×*tCESA* genes (with averaging of expression of genes of all isoforms of PCW and SCW) was ~12.7 times higher in comparison with the *PCW Pa*×*tCESA* genes: 28000 and 2200 total gene reads (TGR), respectively. We observed a difference in the expression of genes, encoding different isoforms of PCW and SCW in different samples of wood, as well as changes in the expression of genes of different isoforms during the development of the gravitropic reaction. For example, all samples of wood demonstrated high expression of the *Pa*×*tCESA4* gene, whereas the *Pa*×*tCESA8-A* was expressed minimally among the *SCW Pa*×*tCESA* in all samples of wood.

The comparative analysis of the *Pa*×*tCESA* gene expression in the tension wood (TW) formed in response to gravistimulation and in the normal wood (NW) (Fig. 4b) showed that expression of some of the *PCW Pa*×*tCESA* and all the *SCW Pa*×*tCESA* genes was downregulated in the opposite wood (OW), whereas some of the *PCW Pa*×*tCESA* genes were conversely upregulated significantly in these samples in comparison with the control samples. In the TW this difference was less significant as compared with the control samples (Fig. 4b), and upregulation of the *SCW Pa*×*tCESA* genes was observed beginning from 48 h after plant tilting. Based on these data, it is impossible to correlate upregulation of some isoforms with the formation of a certain type of cellular wall, as wood samples are represented by a mixture of tissues in which both primary and secondary cellular walls are formed. The TW differs from the NW and OW mainly by the presence of the tertiary cellular wall in xylem fibers.

The comparative analysis of the transcription profiles of the *Pa*×*tCESA* genes in samples collected from the same plants (TW and OW) revealed upregulation of some of the *PCW Pa*×*tCESA* genes (*Pa*×*tCESA1-A*, *3-A*, *3-B*, *6-A*, *6-B*, *6-D*, *6-E*) and downregulation of other *PCW Pa*×*tCESA* genes in the TW samples (Fig. 4c). Maximal differences in expression of the *PCW Pa*×*tCESA* genes were observed in 48 and 96 h after gravistimulation. These differences decreased gradually over 336 h after gravistimulation, whereas the expression of some of the *SCW Pa*×*tCESA* genes (*Pa*×*tCESA7-A*, *8-A*, *8-B*) was increased in the TW in comparison with the OW and reached maximum at 336 h after gravistimulation (Fig. 4c). Clusterization of genes revealed groups represented by either the *PCW Pa*×*tCESA* or *SCW Pa*×*tCES* genes only (Fig. 5).

Comparative analysis of expression of different CESA isoforms in flax and poplar stem tissues during gravistimulation. All systems analyzed in the present study were represented by the samples of pulling part (PUL, TW) and the opposite part of the stem (OPP, OW) of a gravistimulated plant that allowed us to compare the dynamics of the *CESA* expression during graviresponse. This comparison is based on the data of former phylogenetic analysis of amino acid sequences

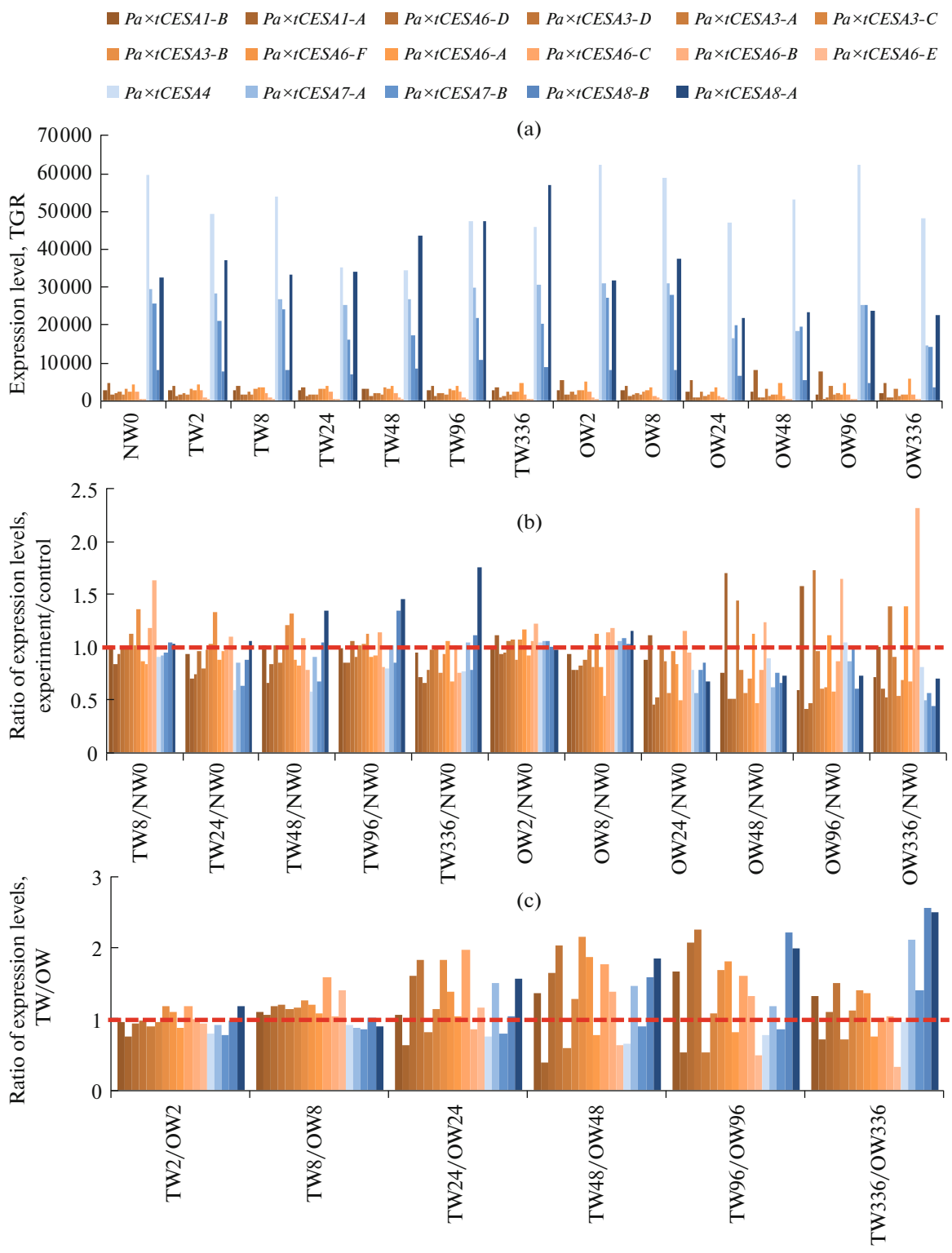

Fig. 4. (a) Expression of the *Pa×tCESA* genes in normal wood (NW), in the tension wood (TW) and in the opposite side wood (OW) of the hybrid poplar (*P. alba* × *tremula*) during the development of gravireponse (2, 8, 24, 48, 96 and 336 h after tilting); (b) expression ratio of the *Pa×tCESA* in wood tissues (OW, TW) during the development of gravireponse (2, 8, 24, 48, 96 and 336 h after tilting) with respect to the expression of these genes in control plants (NW); (c) ratio of the *Pa×tCESA* gene expression in the tension wood (TW) to the expression of these genes in the opposite side wood (OW) during the development of gravireponse (2, 8, 24, 48, 96 and 336h after tilting). Orange columns correspond to the *Pa×tCESA* genes, the products of which are involved into the formation of primary cellular wall; blue columns correspond to the *Pa×tCESA* genes, the products of which are involved into the formation of secondary cellular wall.

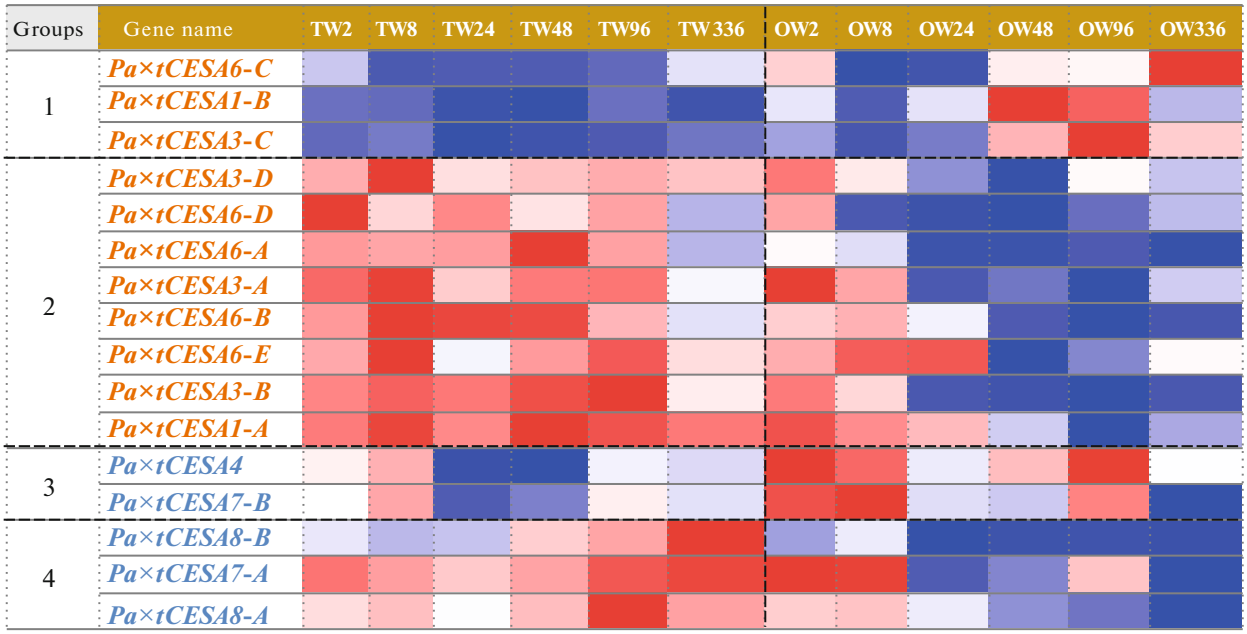

Fig. 5. Clusterization of the *Pa×tCESA* genes in the tension wood (TW) and in the wood of the opposite side of the stem (OW) during gravistimulation of poplar (after 2, 8, 24, 96 and 336 h after tilting). Colors of the heat map show the expression level in horizontal row (samples) from maximal (red) to minimal (blue).

of cellulose synthases of *Arabidopsis* (*A. thaliana*), flax (*L. usitatissimum*) and poplar (*P. trichocarpa*) that allowed us to compare different CESA isoforms and designate them taking into account the level of their relationships [14].The genomes of flax and poplar were characterized by an increased number of CESA isoforms in comparison with *Arabidopsis*, and the majority of orthologs in the flax genome possessed the corresponding orthologs in the poplar genome, except the PtiCESA3-D isoform, which was absent in flax. The expression of paralogs of one of the genes may be characterized by either similar dynamics, indicating their similar functions, or different dynamics, indicating different functions. For the CESA we observed the first scenario more often, especially in case of obvious duplication of genes, such as, for example *LusCESA1-A/B* and *LusCESA7-A/B*, whereas differences in expression were observed for the isoforms from clades with a large number of representatives, such as LusCESA3 and LusCESA6.

The comparative analysis of transcriptome data obtained for different model systems allowed us to reveal several principles in the dynamics of *CESA* expression during graviresponse formation. It was shown that in the xylem samples the reaction developed later than that in the phloem fibers; this is most probably due to the fact that the formation of a tertiary cellular wall in phloem fibers is already fine-tuned, whereas in xylem samples it is initiated from the very beginning (Figs. 6a, 6b). This, apparently, partly explains one order lower expression of the *SCW LusCESA* genes in the flax phloem fibers even on the pulling side of the stem in comparison with xylem samples obtained from both plant species (Figs. 7a, 7b). This effect is believed to be caused mainly from the active formation of the secondary cellular wall in many tissues of heterogenic xylem samples, whereas in homogenous phloem fibers this process is completely absent. The *PCW* and *SCW CESA* genes are obviously expressed at the same level during the formation of the tertiary cellular wall, whereas during the formation of the secondary cellular wall this balance is turned towards the expression of *SCW CESA*. Changes in expression of the *Pa×tCESA* genes in the tension wood of poplar are more variable than that in flax. For example, it was found that the graviresponse was associated with upregulation of some of the *Pa×tCESA* genes in xylem tissues located on the OW-side as compared with the TW (*Pa×tCESA1* and *Pa×tCESA4*; Figs. 6c, 7c). In similar samples of flax, the expression of none of the *LusCESA* isoforms in the OPP samples exceeded that in the PUL. It was suggested that cellular wall biosynthesis in phloem fibers of poplar may be different from those in xylem fibers with a tertiary cellular wall, as knockout of the *SCW PtiCESA* genes affected the morphology of these cells differently [28]. It is noteworthy that in flax all basic characteristics of the tertiary cellular wall, which is constitutively formed in all phloem fibers, were similar to those induced in the xylem fibers during the graviresponse formation [21].

Different genes, which encoded different isoforms of the same variation of cellulose synthase (for example, CESA3), were characterized by different expres-

sion patterns, and some of the principles were revealed in all model systems analyzed. For example, the level of the *CESA6-F* gene expression was significantly lower in all samples (flax and poplar) in comparison with other genes of primary isoforms (by an order/orders) that allowed us to suggest functional differences in the product encoded by these genes from the products of other CESA6. Diversification of expression of different isoforms of the *LusCESA3* gene was clearly shown: we observed upregulation of the *LusCESA3-B* and *LusCESA3-C* genes in the fibers located on the pulling side of the stem and, conversely, low expression of the *LusCESA3-A* gene in all tissues analyzed (Figs. 6a, 6b). Apparently, the product of the *LusCESA3-A* is, similarly to the *LusCESA6-F*, functionally different from other members of the considered clade and is not associated with implementation of graviresponse.

During the development of the graviresponse, the dynamics of expression of some of the *CESA* genes was similarly different in the samples obtained from the pulling and opposite side of the stem in all model systems. For example, the expression of the *CESA8-B* gene was upregulated in all samples obtained from the pulling side of the stem (Fig. 7). A similar effect was observed in the tension wood of aspen (*P. tremula*). It was also suggested that the tertiary cellular wall is synthesized in the tension wood by a special complex, which consists of the CESA8-B only [26]. This suggestion was disproved in a recently published study, in accordance with which the knockout of any of the *SCW CESA* genes (*PtiCESA4* or *PtiCESA7A/B*, or *PtiCESA8A/B*) leads to the absence of the tertiary cellular wall formation in both xylem and phloem fibers of gravistimulated plants [28].

The experimental studies of the functioning of cellulose synthase complexes sometimes raise the question of stoichiometry of isoforms included in these complexes. It is generally accepted that the SCW CESA are involved in the complexes in equimolar ratio 1 : 1 : 1 for the CESA4, 7, and 8 as it was shown for the formation of secondary cellular walls in the xylem of *Arabidopsis* (*A. thaliana*) [29] and in the wood of whitewood (*Picea abies*) [[26]]. The stoichiometry of the isoforms of CESA1, 3 and 6 during the formation of primary cellular wall of *Arabidopsis* was also shown to be equimolar, i.e., 1 : 1 : 1 [30]. However, mass-spectrometric analysis of peptide fragments, which are specific for certain isoforms, in the wood of aspen (*P. tremula*) showed that the SCW CESA ratio was 3 : 2 : 1 (PtaCESA8a/b : PtaCESA4 : PtaCESA7a/b). Moreover, in the tension wood this ratio changed to 8 : 3 : 1 [26]. According to the data of our studies, which were obtained as a result of analysis of the dynamics of the level mRNAs specific for the corresponding genes, the stoichiometry of both PCW and SCW CESA is flexible and may be different since not all isoforms are equally activated. For example, the *LusCESA3-B* and *LusCESA3-C* genes are activated more effectively in phloem fibers located on the pulling side of the stem. It is also a matter for discussion whether the CESA stoichiometry is different in fibers of different origin (phloem or xylem).

The question of the isoform stoichiometry is linked with the question of their functional differences, particularly with the isoform distribution pattern in the complex, as well as their interaction with one another and other auxiliary proteins. The analysis of functional features of the SCW CESA revealed differences in site-specificity of the protein interactions that may be due to their location in the complex. For example, it was shown that CESA7 is characterized by the highest site-specificity, and that the location of this protein is most crucial for the functioning of the whole complex, whereas the CESA8, conversely, demonstrated the lowest specificity in interaction with other proteins [31]. Interestingly, the AtCESA7 is the very cellulose synthase which can partially recover the cellulose biosynthesis in the primary cellular wall after transformation of its gene into the *cesa3* mutants of Arabidopsis, while the AtCESA1 cellulose synthase may partially recover the biosynthesis of the secondary cellular wall of the *cesa8ko* mutant [7]. Moreover, according to the data of phylogenetic analysis, the clade of genes which encode the CESA7 is different from the clades which encode the other SCW CESA (Fig. 7d).

Therefore, gravistimulation leads to upregulation of some LusCESA isoform genes. In phloem fibers of flax this activation may be more likely associated with the remodeling of fibers in the tertiary cellular wall, whereas in xylem tissues and tension wood the activation of CESA may be connected with remodeling and biosynthetic processes in different types of cells. On the whole, the data obtained indicate the role of different isoforms of cellulose synthases in the formation of the tertiary cellular wall. However, further studies are necessary in order to find out which parameters of cellulose biosynthesis are most affected by these specific features.

MATERIALS AND METHODS

Normalized transcriptome data deposited in the FIBexDB database, the subdatabase of flax transcriptomes (https://ssl.cres-t.org/fibex/flax/, [23]), were used to assess the expression of *CESA* genes. For the flax plants the transcriptome data (in two repeats) were previously obtained for isolated fibers of the xylem part of the stem of upright plants (tFIBb, sXYL), as well as for the pulling (tFIBb_PUL, sXYLb_PUL) and opposite side (tFIBb_OPP, sXYLb_OPP) of the stem during the development of the gravitropic reaction after 8, 24, and 96 h after tilting of the plant (NCBI project no. PRJNA631357) (Table 1). The transcriptome data were verified by qPCR via paired comparison of expression of some LusCESA isoforms, as well as genes of other proteins, in fibers from different sides of the stem with the fibers collected from control plants

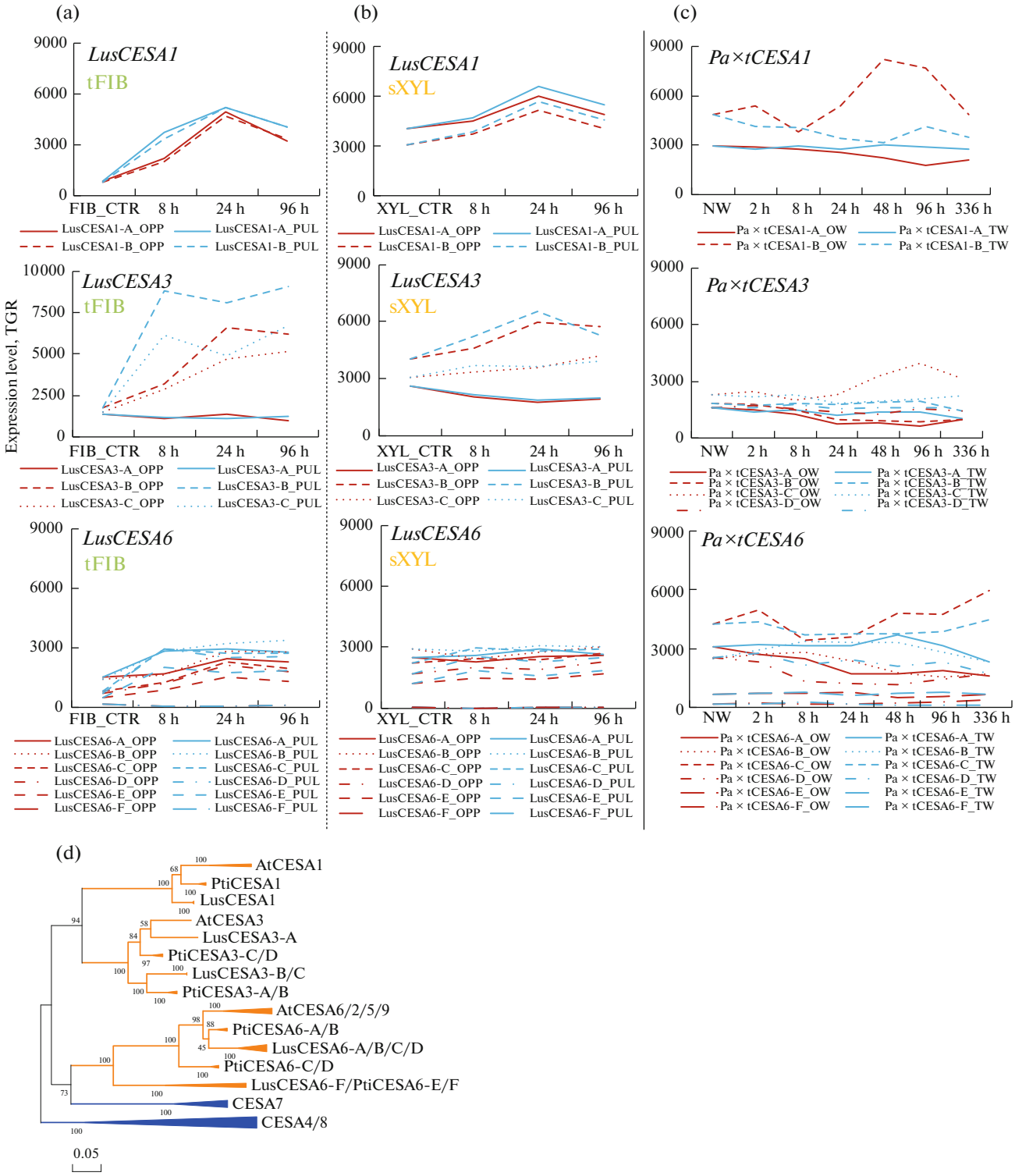

Fig. 6. Expression of the *PCW CESA* genes in isolated flax fibers (a), xylem part of the flax stem (b) and in the wood of poplar (OW, TW) (c) during graviresponse development. Data for the control plants, which did not undergo gravistimulation, are shown as the first point; (d) the phylogenetic tree of CESA of Arabidopsis, poplar (*P. trichocarpa*) and flax composed for the amino acid sequences of CESA [14] using the MEGA7 program (Maximum Likelihood approach, the JTT+G model). Clades, which carry the PCW CESA isoforms (orange) are combined; clades for the SCW CESA isoforms (blue) are combined for different plants.

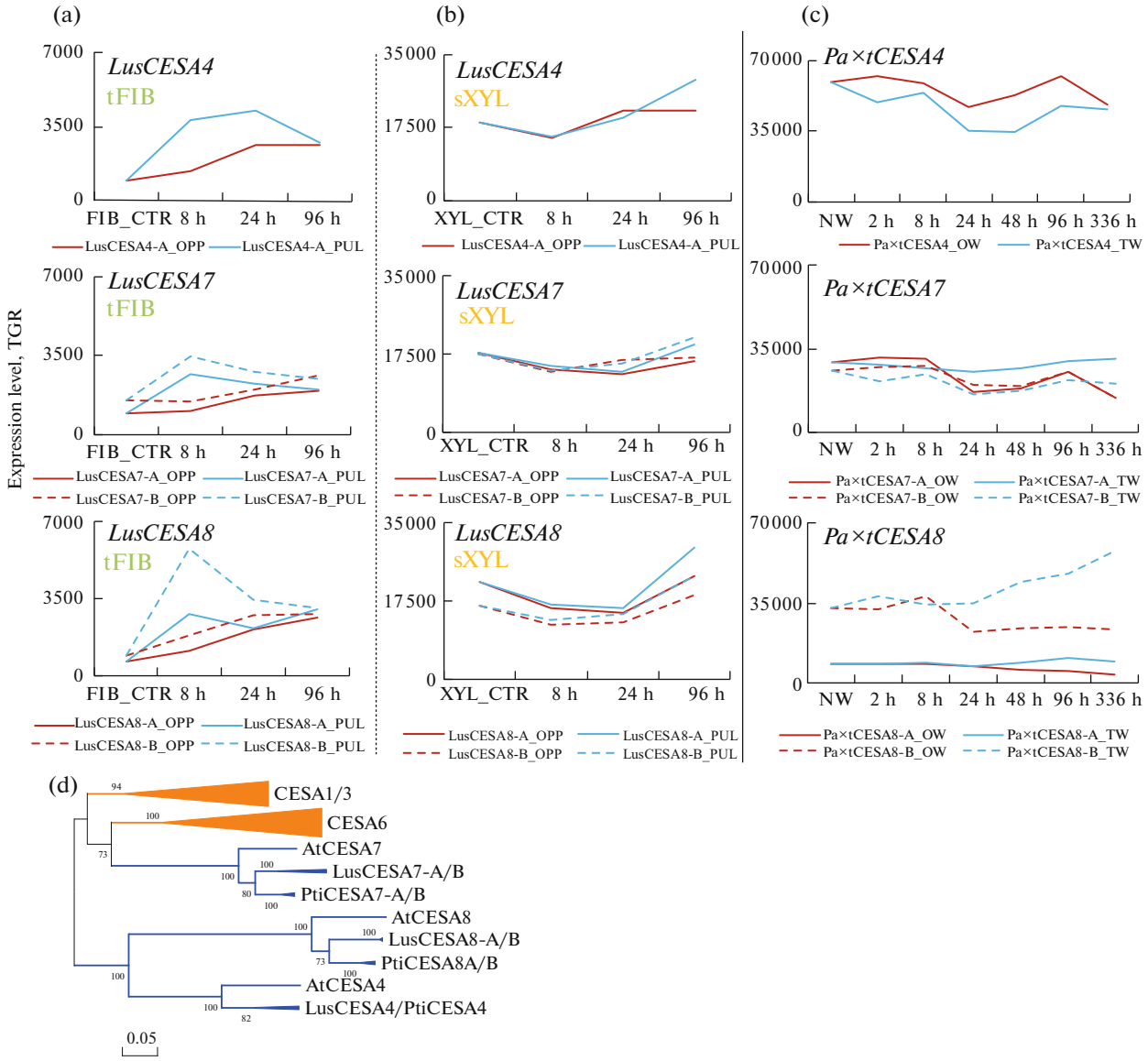

Fig. 7. Expression of the *SCW CESA* genes in isolated flax fibers (a), xylem part of the flax stem (b) and in the wood of poplar (OW, TW) (c) during the graviresponse formation. Data for the control plants, which did not undergo gravistimulation, are shown as the first point; (d) the phylogenetic tree of CESA of Arabidopsis, poplar (*P. trichocarpa*) and flax composed for the amino acid sequences of CESA [14] using the MEGA7 program (Maximum Likelihood approach, the JTT+G model). Clades, which carry the SCW CESA isoforms (blue) are combined; clades for the PCW CESA isoforms (orange) are combined for different plants.

[24]. Genes of cellulose synthases were annotated in accordance with Mokshina et al. [14].

For the wood of poplar the transcriptome data from [25] (NCBI project no. PRJNA398515) deposited in the FIBexDB, poplar transcriptome subdatabase (https://ssl.cres-t.org/fibex/poplar/, [23]) were used. Zinkgraf et al. [25] tilted the 6-month old poplar plants (*P. alba* × *tremula* INRA 717-1B4) to the horizontal position and collected samples from the 20–40 internodes of the stem after removal of bark 2, 8, 24, 48, 96 and 336 h after gravistimulation. The xylem part was collected with the blade from the corresponding parts of the stem (TW, OW) (Table 1) and the RNA-seq-analysis was carried out [25]. The *CESA* genes were annotated as described in Kumar et al. [19] using the Popgenie service (https://popgenie.org/) (Table 2). According to the nomenclature proposed by Kumar et al. [19], the prefix of the *CESA* genes of *P. trichocarpa* was shortened to *Pti*, *P. tremula* to *Pta*, and *P. canescens* (*alba* × *tremula*) to *Pa*×*t*. For the genes of flax and poplar the ID was indicated in accordance with the Phytozome database (https://phytozome-next.jgi.doe.gov/), in which prefixes Lus and Potri were used to indicate the flax and poplar genes respectively.

Clusterization was performed with the FIBexDB database option, pasting the gene numbers into the "search by multiple queries" window. When the data on the expression of the selected genes and samples were

Table 1. The description of samples, the transcriptome data of which were used in this study

No.	Name of the sample	Plant	Description	Reference
1	tFIBb	*L. usitatissimum*	Isolated fibers with tertiary cellular wall (tFIBb). The bottom part of the stem, 5 cm, without tilting	[32]
2	tFIBb_PUL8/24/96	*L. usitatissimum*	Isolated fibers with tertiary cellular wall obtained from the pulling side of the stem (PUL) after 8, 24 and 96 h after tilting	[21, 24]
3	tFIBb_OPP8/24/96	*L. usitatissimum*	Isolated fibers with tertiary cellular wall (tFIB) obtained from the opposite side of the stem (OPP) after 8, 24 and 96 h after tilting	[21, 24]
4	sXYLb	*L. usitatissimum*	Xylem part of the stem with secondary cellular wall (sXYL). Bottom part of the stem, 5 cm, without tilting	[32]
5	sXYLb_PUL8/24/96	*L. usitatissimum*	Xylem part of the stem with secondary cellular wall (sXYL) obtained from the pulling side (PUL) of the stem after 8, 24, and 96 h after tilting	[21, 24]
6	sXYLb_OPP8/24/96	*L. usitatissimum*	Xylem part of the stem with secondary cellular wall (sXYL) obtained from the opposite part (OPP) of the stem after 8, 24 and 96 h after tilting	[21, 24]
7	NW_0	*P. alba* × *tremula*	Normal wood without tilting	[25]
8	TW_2/8/24/48/96/336	*P. alba* × *tremula*	Tension wood after 2, 8, 24, 48, 96 and 336 h of gravistimulation	[25]
9	OW_2/8/24/48/96/336	*P. alba* × *tremula*	Wood obtained from the opposite side of the stem after 2, 8, 24, 48, 96 and 336 h of gravistimulation	[25]

Table 2. Numbers of *CESA* genes and their crossings

Flax gene (Phytozome)	Gene name	Poplar gene (Phytozome)	Gene name*
		PCW CESA	
Lus10018902	*LusCESA1-A*	*Potri.018G029400*	*PtiCESA1-A/Pa×tCESA1-A*
Lus10028597	*LusCESA1-B*	*Potri.006G251900*	*PtiCESA1-B/Pa×tCESA1-B*
Lus10039607	*LusCESA3-A*	*Potri.006G052600*	*PtiCESA3-A/Pa×tCESA3-A*
Lus10007538	*LusCESA3-B*	*Potri.016G054900*	*PtiCESA3-B/Pa×tCESA3-B*
Lus10012198	*LusCESA3-C*	*Potri.009G060800*	*PtiCESA3-C/Pa×tCESA3-C*
		Potri.001G266400	*PtiCESA3-D/Pa×tCESA3-D*
Lus10006161	*LusCESA6-A*	*Potri.005G087500*	*PtiCESA6-A/Pa×tCESA6-A*
Lus10041063	*LusCESA6-B*	*Potri.007G076500*	*PtiCESA6-B/Pa×tCESA6-B*
Lus10003526	*LusCESA6-C*	*Potri.005G194200*	*PtiCESA6-C/Pa×tCESA6-C*
Lus10002939	*LusCESA6-D*	*Potri.002G066600*	*PtiCESA6-D/Pa×tCESA6-D*
Lus10002940	*LusCESA6-E*	*Potri.013G019800*	*PtiCESA6-E/Pa×tCESA6-E*
Lus10022449	*LusCESA6-F*	*Potri.005G027600*	*PtiCESA6-F/Pa×tCESA6-F*
		SCW CESA	
Lus10008226	*LusCESA4*	*Potri.002G257900*	*PtiCESA4/Pa×tCESA4*
Lus10043485	*LusCESA7-A*	*Potri.006G181900*	*PtiCESA7-A/Pa×tCESA7-A*
Lus10043486	*LusCESA7-B*	*Potri.018G103900*	*PtiCESA7-B/Pa×tCESA7-B*
Lus10007296	*LusCESA8-A*	*Potri.011G069600*	*PtiCESA8-A/Pa×tCESA8-A*
Lus10029245	*LusCESA8-B*	*Potri.004G059600*	*PtiCESA8-B/Pa×tCESA8-B*

* Names of the *PtiCESA* and *Pa×tCESA* genes coincide because the genome of *P. trichocarpa* was used as the reference [25] to analyze the *P. alba* × *tremula* transcriptomes.

shown on the screen, the algorithm for the *k*-means clusterization (Cluster3) (the number of clusters is 4), and the Pearson correlation was chosen.

The phylogenetic tree was composed on the basis of amino acid sequences of CESA of flax (*L. usitatissimum*), poplar (*P. trichocarpa*) and Arabidopsis (*A. thaliana*) [14], using the MEGA7 program (Maximum Likelihood method, model JTT+G).

CONCLUSIONS

It was shown that gravistimulation induces rearrangements in cellular wall of phloem fibers of flax (*L. usitatissimum*), which are followed by a short-time activation of biosynthetic processes at early stages of graviresponse and involves a variety of isoforms of cellulose synthases. In the xylem part of the flax stem gravistimulation leads to the formation of the tertiary cellular wall in xylem fibers that is followed by upregulation of cellulose synthase genes involved in the formation of the secondary cellular wall (SCW LusCESA). Gravistimulation of poplar (*Populus* sp.) wood was also followed by upregulation of some *PCW* and *SCW Pa×tCESA* genes in the tension wood. Activation of expression of some of the *PCW Pa×tCESA* genes and downregulation of the other *PCW Pa×tCESA* genes was observed in the wood located on the opposite side of the tension wood side of the stem. A special role in the graviresponse development in different systems (phloem fibers, tension wood) may belong to the CESA8-B, as upregulation of this isoform was shown in both tension wood and phloem fibers on the pulling side of the flax stem. The stoichiometry of the SCW CESA may be similar in flax fibers and in the tension wood, whereas the stoichiometry of the PCW CESA is supposed not to be equimolar.

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COMPLIANCE WITH ETHICAL STANDARDS

The present article does not contain any studies carried out with people or animals as objects.

Conflict of Interests

The authors declare no conflict of interests.

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