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REVIEWS

Intrusive Growth of Sclerenchyma Fibers[1]

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Abstract—Intrusive growth is a type of cell elongation when the rate of its longitudinal growth is higher than that of surrounding cells; therefore, these cells intrude between the neighboring cells penetrating the middle lamella. The review considers the classical example of intrusive growth, e.g., elongation of sclerenchyma fibers when the cells achieve the length of several centimeters. We sum the published results of investigations of plant fiber intrusive growth and present some features of intrusive growth characterized by the authors for flax (*Linum usitatissimum* L.) and hemp (*Cannabis sativa* L.) fibers. The following characteristics of intrusive growth are considered: its rate and duration, relationship with the growth rate of surrounding cells, the type of cell elongation, peculiarities of the fiber primary cell wall structure, fibers as multinucleate cells, and also the control of intrusive growth. Genes, which expression is sharply reduced at suppression of intrusive growth, are also considered. Arguments for separation of cell elongation and secondary cell wall formation in phloem fibers and also data indicating diffuse type of cell enlargement during intrusive growth are presented.

Key words: Cannabis sativa - Linum usitatissimum - sclerenchyma fibers - intrusive growth - symplastic growth - diffuse growth - multinucleate cells - immunocytochemistry

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INTRODUCTION

Investigation of cell growth mechanisms is one of fundamental challenges of plant biology. Fibers are the longest plant cells; the length of mature fibers attains hundreds millimeters, which makes them an interesting model for studying elongation growth. The more so that fiber growth is intrusive one; due to this type of growth, the extreme fiber length is mainly achieved [1, 2].

Intrusive growth is one of three types of cell growth differing in the pattern of interpositions of growing cells and coordination of their elongation. These three growth types are symplastic, protrusive, and intrusive growth. At symplastic growth, the areas of contacting surfaces of neighboring cells increase at a similar rate; cell size could differ only due to cell division frequency and the time of its cessation. Contacts between the cells through the middle lamella are not disturbed. This type of growth is characteristic of most cells in the meristem and in the root zone of elongation [1–3]. At protrusive growth, i.e., protrusion formation, an increase in the cell wall surface of the growing cell does not disturb neighboring cells [4]; it is characteristic of epidermal cells. This is the way of trichome formation, such as root hairs, seed hairs, for example in cotton (*Gossypium* sp.), and hairs creating stem and leaf pubescence.

Intrusive growth is cell elongation at the rate exceeding that of surrounding cells; intrusively growing cell intrudes between adjacent cells, which grow at a slower rate, and penetrates along the middle lamella between neighboring cells [1–3, 5]. The examples of intrusive growth in plants are fiber elongation, growth of fusiform cambial initials, laticifers, or pollen tubes within the pistil; fibers, which attain the length of several centimeters, moving between the walls of thousands cells are considered as a classic example.

Intrusive growth is one of three types of plant cell growth and one of the key stages of fiber biogenesis; however, it is poorly characterized. This is mainly explained by difficulties in direct observation of intrusive growth, whereas isolation of intrusively growing cells in amounts sufficient for biochemical and molecular-biological analyses is essentially impossible. There are several reasons for this. Firstly, fiber intrusive growth occurs in the depth of other tissues. Secondly, these cells have prosenchymatous shape (their length may exceed their width more than by 1000 times) together with thin cell walls, which make them extremely vulnerable to damage. Thirdly, fiber intrusive growth was never reproduced in vitro.

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Difficulty in the studying of intrusive growth requires a necessity of nontrivial approaches even for obtaining such basic characteristics as localization in the stem, the growth rate and duration, relationship with the growth rates of surrounding cells, the type of cell elongation, etc. Most data were obtained by microscopy of fixed preparations; in recent years, the methods of molecular genetics and biochemistry were also applied.

The review sums the results of investigations of plant fiber intrusive growth and considers some features of intrusive growth characterized by the authors for flax (*Linum usitatissimum* L.) and hemp (*Cannabis sativa* L.) fibers.

ARGUMENTS FOR FIBER INTRUSIVE GROWTH OCCURRENCE

The occurrence of fiber intrusive growth was debatable for a long time, although arguments for the existence of such stage in fiber development were obvious. The proof of intrusive growth of primary fibers initiated from procambium near apical meristems is an increase in the number of fibers on transverse sections of the organ during plant development [6, 7]. Let us consider this situation using flax primary fibers as an example (Fig. 1). These cells, produced from the procambium [1, 2], are easily identified on flax stem sections made at a distance of approximately 0.3–0.5 mm from the apex. In this region, fibers display symplastic growth, i.e., they grow at the same rate as surrounding cells (Fig. 1b). With increasing distance from the apex, the number of primary phloem fibers on flax stem transverse sections increases from several tens (in the apical zone) to many hundreds (Table 1). Flax phloem fibers are primary fibers; therefore, an increase in their number on the transverse sections could not increase due to cambium activity but results from intrusive growth. Figure 1c illustrates how intrusive cell elongation could result in the increase in the number of fibers on the transverse section. When within the stem segment, the length of particular fibers increases, their number on the transverse section goes up, although the number of cells in a given stem segment remains unchanged.

For secondary fibers produced from the cambium during organ thickening, the argument for intrusive growth occurrence is the greater length of the fibers than that of the cambial initials [1, 8]. Thus, the longest fusiform cambial initial of horse chestnut (*Aesculus hippocastanum* L.) is 329 μm, whereas the length of its derivative, libriform fiber, is 517 μm [9]. In various woody plants, the length of fibers of secondary xylem increases up to 150–400% of the length of cambial initials [10]. The process is still more profound in secondary phloem fibers. Secondary phloem fibers in the hemp stem attain several millimeters in length, whereas the length of fusiform cambial initial is, according to our data, 120 ± 12 μm ($n = 8$). Secondary fibers are formed in the stem region, which has completed its elongation and, thus, they can elongate only intrusively.

Table 1. The number of phloem fibers on transverse sections of flax stem

Distance from apex, cm	No. phloem fibers on transverse section
0.3–0.5	40–90
1.5–2.0	500–600
15–20	700–800
20–40	700–800

INITIATION OF INTRUSIVE GROWTH

The detection of the start of fiber intrusive growth is based on the idea that flat ends characteristic of early stages of fiber formation have to be transformed into sharpened ones required for cell intrusion between other cells (Fig. 1b). Such transformation is accompanied by the formation of so-called knees (Fig. 1c). Knee formation was observed, for example, on longitudinal sections of developing wood [9, 11] and also in the apical part of the flax stem; they are well seen, for instance, after enzymatic isolation of an individual fiber [12]. Knees are formed at both cell ends, indicating that the fiber in the stem elongates both upward and downward. They disappear during further fiber development.

Using the appearance of knees on the ends of fibers as a marker of the start of intrusive growth, it was established that it begins earlier that symplastic growth of surrounding cells finishes. Thus, in flax stem, the formation of such knees in the primary phloem fibers begins at a distance of 1–2 mm from the stem apex (Fig. 1), whereas elongation of other cells occurs in the region up to 10–20 mm from the apex [12]. Before the start of intrusive growth, primary fiber growth is symplastic [1, 2], but such growth does not provide for the size of phloem fibers greater than 70 ± 18 μm in length and 6 ± 1 μm in diameter ($n = 9$).

Initiation of intrusive fiber growth must be coupled with an increase in the turgor pressure in them. Expansion of each plant cell is known to be determined by cell-wall extensibility and the value of turgor pressure [13]. In the cells with symplastic or protrusive growth, cell enlargement could be determined by changes in both their turgor pressure and the properties of their cell walls. It is obvious that initiation of fiber intrusive growth occurring between symplastically growing, i.e., turgescent cells, could occur only due to the preceding rise in the turgor pressure because primary cell wall loosening would obligatory result in compression and mashing fibers by neighboring cells. Fiber turgor pressure was never measured and also it is unknown what the osmolyte provides for it. Elucidation of this osmolyte nature might help understanding

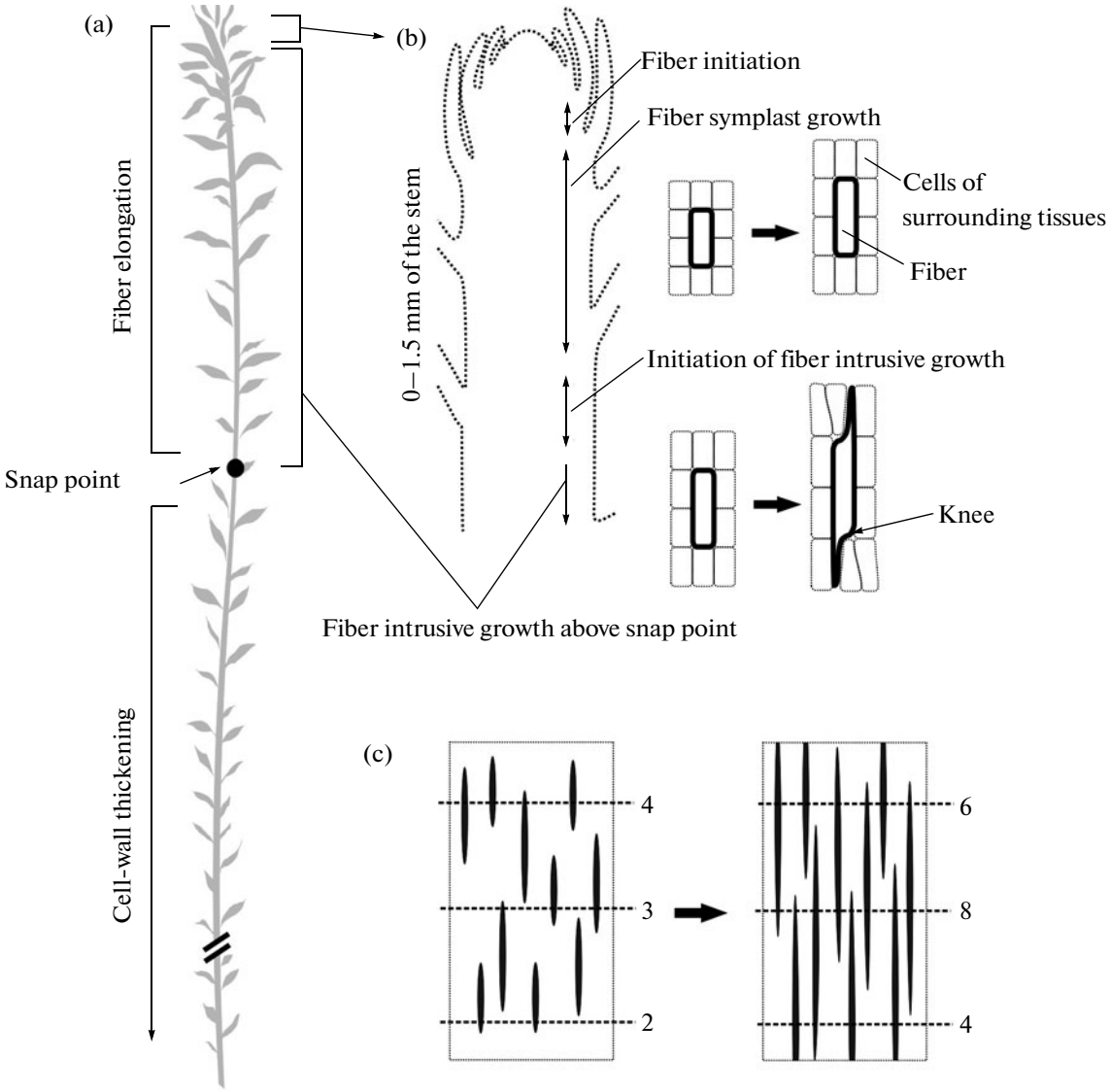

Fig. 1. Some characteristics of intrusive growth of primary fibers in flax stem.
(a) Flax plant during rapid growth period; the positions of fibers at different developmental stages are indicated; intrusive growth could continue down to the snap point [7, 12]; (b) the scheme of the longitudinal section of stem apex illustrating the initiation of fiber intrusive growth with indication of symplastic and intrusive growth stages; (c) scheme of the increase in the number of fibers on transverse stem sections occurring due to intrusive growth; an increase in the length of particular fibers (at remaining unchanged of their total number within the stem segment) results in the increase in the number of fibers on transverse section (indicated by figures).

the mechanisms of intrusive growth regulation because the control of this compound metabolism and/or transport could be an immediate signal for intrusive growth. The importance of fiber water balance maintenance during intrusive growth becomes evident from active expression at this stage of some genes encoding aquaporins, membrane proteins involved in water transport [14] (see below).

The involvement of hormones or any other regulators in the induction of intrusive growth is unclear. Thus, the questions concerning stimuli initiating intrusive growth and mechanisms of their action remain open.

SEPARATION OF CELL ELONGATION AND SECONDARY CELL WALL FORMATION

For most types of plant cells, it was established that their expansion precedes cell-wall thickening. For a long time, the fiber, which length may attain tens of centimeters, was believed an exclusion from this rule. According to Esau [1, 2], deposition of the secondary cell wall in the middle part of the fiber occurs simulta-

Table 2. The number of nuclei per primary fiber in flax and hemp

Fiber length, mm	No. nuclei	Fiber length, mm	No. nuclei	Fiber length, mm	No. nuclei
*Linum usitatissimum**					
6	29	11	100	20	55
6	38	11	74	20	85
6	44	12	68	22	108
7	37	12	50	23	53
7	39	13	65	23	115
7	63	13	74	23	164
8	61	14	80	24	113
8	49	16	92	24	142
9	70	16	140	26	79
10	58	17	33	36	175
11	40	18	74	48	98
11	66	20	29	52	103
*Cannabis sativa***					
8	19	15	27	20	30
8	30	15	41	20	66
9	23	15	51	20	74
10	29	15	44	21	69
10	31	16	54	22	38
10	35	17	39	23	71
10	60	17	54	27	109
12	25	18	45	28	88
12	58	18	54	35	137
13	49	19	67		
14	68	19	80		

Notes: The number of nuclei was determined in mature fibers stained with DAPI (4',6-diamide-2-phenylindol); fiber integrity and individuality was checked under microscope.

* $n = 36$; average fiber length is 16 ± 8 mm; the average number of nuclei is 77 ± 29; the coefficient of correlation between fiber length and the number of nuclei in it is +0.6.

** $n = 31$; average fiber length is 17 ± 5 mm; the average number of nuclei is 54 ± 20; the coefficient of correlation between fiber length and the number of nuclei in it is +0.9.

neously with elongation of its thin-walled ends. Until now, this point of view is accepted by most researchers for long phloem fibers. However, it contradicts to some experimental data obtained at the analysis of flax and hemp fibers [6, 7, 12].

Thus, during rapid flax plant growth, in the upper part of its stem, there is a region easily identified at an accuracy of several millimeters, where mechanical properties of stem fibrous part change sharply. This site was called the snap point. Above this snap point, the stem is easily broken, whereas below it, a great effort is required to this end. The assessment of the number of fibers on transverse sections on a definite stem height during flax plant ontogeny demonstrated that this number increased only above the snap point but not along the entire stem, as it is expected in the case of continued intrusive growth [7]. Thus, all fibers elongate only above the snap point (Fig. 1a), and thereafter their number remains unchanged until the end of plant ontogeny. NMR analysis also demonstrated the absence of intrusive growth below the snap point [15]. Serial transverse sections of particular flax fibers demonstrated the cell wall uniformly thickened along the entire cell length, which indicates uniform deposition of the secondary cell wall on the fiber inner surface. Like in flax, hemp elongating fibers are also positioned

Table 3. The list of genes, which expression reduced to a greatest degree after intrusive growth cessation in fiber-type flax

Closest homologue, accession no. in GenBank	Significance of determination, score	Description	Decline in expression, folds
gbEEE86933.1	149	predicted protein	13.9
gbEEF47006.1	627	conserved predicted protein	12.1
gbEEF47365.1	153	factor of peptide chain release (probable)	6.9
gbEDP36122.1	53	predicted protein	6.9
gbAAD15398.1	137	ribosomal protein S12	5.6
gbAAU95415.1	71	protein from ATPase family	5.2
gbEEE91209.1	210	predicted protein	4.5
gbEEF04671.1	329	TIP aquaporin	4.5
gbAAB04557.1	327	δTIP aquaporin	4.5
gbEEF05675.1	160	predicted protein	4.5
gbAAL60048.1	84	60S ribosomal protein L17A (RPL18aC)	4.2

Note: Closest homologues and score values are determined using BLAST algorhythm (www.ncbi.nlm.nih.gov/BLAST/tBLASTx).

only in the upper part of the stem; the period of their elongation is not long and separated from the stage of secondary cell wall formation [16].

In the case of secondary fibers, separation of their elongation from thickening is impossible to confirm only by their number counting. The number of hemp secondary fibers on transverse sections on a definite stem height increased by several times during plant development [16]. However, this increase is related not only to fiber elongation by intrusive growth but also to new fiber development due to cambium activity. Nevertheless, like for flax fibers, on the serial transverse sections of hemp secondary fibers, uniform cell wall thickening along the entire cell length was demonstrated. Thin-wall ends never were observed in the cells with secondary cell wall thickening. This indicates that the stages of elongation and secondary cell wall thickening are separated in the secondary phloem fibers as well.

It seems likely that these rules are also true for fibers in other tissues. Thus, it is known that the formation of the secondary cell wall in the case of fibers associated with conducting vessels in bamboo (*Phyllostachys pubescens* (Carrière) J. Houz.) [17] and hypodermal fibers of melinjo (*Gnetum gnemon* L.) [18], the formation of the secondary cell wall starts after ceasing elongation of these cells.

For wood fibers, separation of stages of intrusive growth and deposition of secondary cell wall is a question resolved long ago. Using serial sections, Wenham and Cusick [11] analyzed stem anatomy of several woody plant species in detail. They distinguished several zones in the wood: (1) cambial zone, (2) zone of elongation, and (3) zone of secondary cell wall formation. It is established that intrusive elongation of xylem fibers starts already in the cambial zone, occurs mainly in the zone of elongation, and is absent from the zone of secondary cell wall deposition. Thus, intrusive elongation of wood fibers and thickening of their secondary walls are also separated in time [8, 11].

Thus, various fibers of sclerenchyma, despite the extreme length of these cells, develop according to the common rule saying that cell elongation and secondary cell wall formation occur successively. Separation of stages of intrusive growth and cell wall thickening opens wide opportunities for studying stage-specific processes.

INTRUSIVE GROWTH DURATION AND RATE

The establishment of markers of intrusive growth initiation and termination permits us to evaluate the rate and duration of this process. We calculated these indices for flax primary phloem fibers. The stages of fiber elongation and secondary cell wall formation are separated spatially, and fiber growth occurs only above the snap point, which is positioned at a distance of 7–10 cm from the apex. During rapid flax plant growth, the rate of stem growth is 1–2 cm/day. The higher growth rate the longer a distance from the apex to the snap point. On the basis of all these values, we can conclude that intrusive growth duration of a particular flax fiber does not exceed 3–5 days.

At a distance of 2 cm from the apex, the number of fibers on the transverse sections of the flax stem comprises 80% of the number of fibers after complete cessation of intrusive growth [15] (Table 1). This part of the stem is characterized by the completing of symplastic growth [12]. Most fibers have the length about 2 cm [6, 19]. From all these data, we can conclude that primary fibers grow mainly in the period of symplastic growth of surrounding cells.

One of the basic indices characterizing cell growth is the rate of the cell size increase with distinguishing the linear (absolute) growth rate, expressed as the

increment of the cell length per time unit, and relative growth rate depending on the initial cell length and showing the proportion of length increment in the initial cell length per time unit [20].

To measure the growth rate of particular cells, their lengths are measured under microscope and their changes for definite time periods are assessed. In the case of fibers, the task is rather complicated because it is impossible to measure their lengths inside the tissue already soon after the start of intrusive growth.

The following reasoning permits for evaluation of the rate of fiber intrusive elongation. By the start of intrusive elongation, fibers are about 0.1 mm in length, which is achieved due to growth symplastic with surrounding cells. The average length of the mature fiber is about 20 mm [6]. The stem part, where intrusive growth occurs mainly, is its upper 20 mm. The average rate of stem growth is 10 mm/day. Thus, duration of fiber intrusive growth in this stem region is 48 h. Using these indices, we can calculate [20] the average V(av) (formula 1) and relative k (formula 2) rates of fiber intrusive growth:

$$V(\text{av}) = (L - l)/\Delta t, \quad (1)$$

$$k = 1/\Delta t \times \ln(L/l), \quad (2)$$

where Δt is time of fiber growth. l is the initial fiber length, and L is final fiber length.

The average rate of fiber intrusive growth is 0.8 mm/h, i.e., approximately 2 cm/day. The relative rate of fiber intrusive growth is 0.22/h. For comparison, the relative growth rate of the cells in poplar (*Populus deltoids* Bartr. ex Marsh.) shoot is 0.02/h, Japanese morning-glory (*Pharbitis nil* L.) shoot is 0.027/h [21], ryegrass (*Lolium perenne* L.) leaf epidermis is 0.06/h [22], and cucumber (*Cucumis sativus* L.) hypocotyl is 0.06/h [23]. These values of the relative growth rates of aboveground organ cells (even very high values for intrusively growing fibers) are incomparable with those for root cells of various plant species. Thus, in maize (*Zea mays* L.) roots, this rate is 0.4/h, in cucumber—0.38/h [23], in timothy grass (*Phleum pretense* L.)—1.27/h [21].

In flax plants, there are fibers, which are much longer than 20 mm. At the average distance from stem apex to the snap point equal to 80 mm, fibers up to 80 mm in length are met. This implies that some fibers continue to elongate up to the snap point; at the indicated rate of plant growth, this process takes further 3 days. In this case, the relative elongation rate of these fibers is 0.02/h, whereas the average rate is 0.8 mm/h. Thus, linear rate of intrusive fiber elongation is maintained at the constant level after ceasing symplastic growth of surrounding cells, whereas the relative rate decreases markedly, which is explained by the large cell length.

The period of intrusive elongation of hemp secondary phloem fibers could be evaluated by the time of the appearance of a new layer of secondary fibers with thickened cell walls. According to our data, the new layer of secondary fibers with thickened cell walls appeared in 8 days; thus, the period of their intrusive elongation could not be longer than 8 days. Thin-walled secondary hemp fibers were observed only in immediate vicinity to the cambium, at a distance of 3–4 cell layers.

THE TYPES OF FIBER INTRUSIVE ELONGATION

Cell growth could be apical (tip) when elongation occurs only at the expense of cell tips on the background of the rest nongrowing cell part or diffuse (intercalary) when elongation and insertion of new cell-wall components occurs over the entire cell surface. Symplastic cell growth is always diffuse one. In relation to fibers, there is no common opinion about the type of their intrusive elongation. The elucidation of this question is important because apical and diffuse growth differ markedly in their mechanisms and the ways of regulation.

Detail analysis of flax phloem fibers at the stage of intrusive elongation [7, 12] allowed making the following observations: (1) there is no cytoplasm zonality and other signs of the cell ultrastructure, characteristic of apical growth; (2) in the cell ends, cortical microtubules are oriented strictly transversely (like during symplastic diffuse growth) but not longitudinally (like at apical growth); (3) in pulse-chase experiments, the label (radioactive or fluorochrome Calcofluor White) incorporates evenly in the cell wall along the entire length of intrusively growing fiber; (4) plasmodesmata in the fiber cell walls are present only at the stage of coordinated growth; at the stage of intrusive growth, they are disrupted, and, at consequent stages of fiber development, they are absent from the entire cell surface. When the fiber would grow apically, plasmodesmata could be retained in the middle parts of fibers because there were no shifts of these cell parts relative to surrounding cells. All these data indicate that fiber intrusive elongation occurs by diffuse growth.

So far, it is not clear whether this statement is true for other fibers. Moreover, some researchers believe that just apical growth underlies the development of various fiber types. Such opinion was given for poplar [8] and horse chestnut [9] xylem fibers, hypodermal fibers in the leaves of melinjo [18], gelatinous phloem fibers of melinjo [24], and fibers associated with conducting vessels in bamboo (*Dendrocalamus asper* Back.) stems [25]. In these works, fiber shape with sharpened ends and the presence of the knees are presented as the basic proof of apical growth occurrence. However, such shape, really characteristic of fibers on a definite developmental stage (Fig. 1b), is explained rather by a specificity of the initial steps of fiber development, but does not prove the apical type of their elongation.

The analysis of cortical microtubule orientation in the cambial cells and their derivatives during wood differentiation in horse chestnut showed that, close to the cambial zone, spiral microtubule orientation was observed in initiating fibers (i.e., long before deposition of the secondary cell wall) [9]. It is characteristic that this occurs along the entire cell length, which argues for elongation along the entire cell surface but not only cell ends, although Chaffey et al. [9] did not draw such conclusion.

Together with that, Schoch-Bodmer and Huber [26] had long ago reported that xylem fibers consist of wider middle parts corresponding in their lengths to the cambium cell and two thin ends appeared during intrusive growth. In such fibers, bordered pits are present only in the middle parts [27]. The authors present these data concerning the cell shape and plasmodesmata distribution in the favor of the theory of xylem fiber apical growth. Just these long protrusions (starting from the knees) are usually considered as the regions of fiber elongation [28]. The size of these regions (20 μm and more) exceeds markedly the size of the growing zone during classical apical growth (3–5 μm) characteristic of root hairs and pollen tubes [29, 30]. By now, the presence of key feature of apical growth—zonality of the cytoplasm structure—has not been demonstrated for the xylem fiber.

Taking into account contradictory opinions, let us make clear criteria of apical and diffuse growth. When to consider the presence of non-elongating cell surface regions as a key feature of apical growth, xylem fiber growth should be referred to the apical type. However, when to use the classical signs of apical growth, e.g., the small size of the growing region and the presence of zones of organelle distribution, then we should accept xylem fiber diffuse growth, although some part of their surface is tightly connected with neighboring cells and could not elongate. It should be noted that if we even believe that xylem fiber growth is apical one, the pattern of their elongation would differ markedly from the scheme suggested by K. Esau for phloem fibers. According to this scheme, apical intrusive growth occurs in a particular cell simultaneously with cell wall thickening in the cell middle part. Both in phloem [6] and xylem [8, 11] fibers, cell elongation precedes cell wall thickening.

Fibers of different type grow under substantially different conditions. Thus, most active intrusive growth of primary phloem fibers, which length increases by thousands times relative to the initial one, occurs in the zone of symplastic growth of surrounding cells. In this zone, none of the fiber surface regions could remain nongrowing. Before intrusive growth starts, fiber growth is also symplastic; in this time period, all cells elongate by diffuse growth. Thus, at the initiation of intrusive growth, diffusely growing fiber simply starts to grow at a higher rate.

Secondary xylem fibers show intrusive growth in the stem region completed its elongation. The degree of lengthening of xylem and primary phloem fibers differ markedly: their lengths increase by 1.5–4.0 and hundreds–thousands times, respectively. Nevertheless, so far there is no sufficient basis to believe that the mechanisms of these fibers growth differ substantially. The ultimate conclusion relative to the type of wood fiber elongation is complicated by the fact that all studies of their intrusive growth were regretfully performed on relatively short fibers, which elongated not more than by 1.5 times as compared to cambial initials. Usually the region above the knee comprised not more than 10–15% of the length of the middle cell part [11, 28]. It would be important to analyze intrusive growth resulting in the cell lengthening by several times; the occurrence of such xylem fibers was reported in some reviews [8, 10].

FIBER CELL WALL AT THE STAGE OF INTRUSIVE GROWTH

Like in other cells, a necessary condition for the fiber size increase is expansion of already deposited layers of the cell wall and the synthesis of additional portions of polymers required for cell wall building. Intrusive nature and the high growth rate of fibers could be associated with specific composition of their primary cell walls. Since it is essentially impossible to isolate fibers at this developmental stage because of their easy damaging, until now immunocytochemistry served a basic approach for fiber cell wall characterization. In recent time, gene-engineering modification of enzymes and other cell-wall proteins is also applied.

It was supposed that, in the cells of various types, expansins are primary agents inducing cell wall expansion. It is believed that they disrupt hydrogen bonds between cellulose microfibrils connected in the united network by cross-linking glycans, xyloglucans primarily [13]. In correspondence with these notions, in cambium and expansion zones of developing wood in hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.), very active expression of the *PttEXPA1* expansin gene was observed [28]. In growing xylem fibers, mRNA of this gene was accumulated just in the cell ends (to be exact, in the regions beyond knees), which was demonstrated in situ using real-time PCR. However, experiments with genetic transformation have cast doubt on the role of this expansin in fiber intrusive growth. In transgenic plants with overexpression of the *PttEXPA1* gene, fibers were wider by 9–16% than in wild type but their length remained unchanged. Vessel diameters were not changed, although in some lines, they were longer by 2–7%, which was evidently explained by some enhancement of internode elongation and the increase in the length of cambial initials [31, 32]. In other fiber types, strong expansin gene expression was not demonstrated; but the reason may be the scarcity of detailed studies.

The key role in expansion of various cells belongs to a specific polysaccharide, xyloglucan, and the enzyme

xyloglucan endotransglucosylase/hydrolase modifying this polysaccharide. During cell expansion, xyloglucan endotransglucosylase/hydrolase fulfills two main functions: (1) insertion of new xyloglucan molecules in the cell wall, and (2) cell wall restructuring during cell expansion. Using a CCRC-M1 monoclonal antibody, xyloglucan was detected in developing wood; this immunochemical reaction was more pronounced in fibers than in vessel elements [33]. The xyloglucan endotransglucosylase/hydrolase content is also higher in fibers than in surrounding cells; this was assessed after the level of its mRNA, the content of corresponding protein, and its activity [34].

During intrusive growth, elongating fiber intrudes between neighboring cells along the middle lamella. It is likely (although nobody demonstrated this) that partial digestion of the middle lamella facilitates this intrusion. The main component of the middle lamella is pectic compounds, polygalacturonic acid primarily; this is a homopolymer of galacturonic acid residues connected by α-(1⟶4) bonds. Polygalacturonan is secreted into the cell wall in a highly methylated form, which is relatively resistant to endopolygalacturonases and pectin-lyases, i.e., enzymes cleaving α-(1⟶4) bonds between nonmethylated residues of galacturonic acid in the backbone of pectin molecules. Methyl groups are removed by a specific enzyme present in the cell wall, pectin methylesterase (PME).

During fiber intrusive growth, PME could exert double effect. On the one hand, demethylation of polygalacturonic acid could result in the enhancement of its hydrolysis by polygalacturonases or pectin-lyases and thus facilitate fiber intrusion between neighboring cells. On the other hand, the appearance of demethylated regions creates a possibility for the formation of the Ca^{2+}–pectate gel, which is produced due to the interaction of Ca^{2+} ions with nonmethylated residues of homogalacturonan. Such gel could additionally tighten neighboring cells and affect cell wall plasticity, the degree of cell connection between themselves, and thus the growth rate [35, 36].

The role of PME in providing of intrusive growth was confirmed to some degree by modification of corresponding gene expression using gene-engineering approaches. In hybrid aspen plants, an elevation by approximately 50% of *PttPME1* gene expression, encoding the main PME of the wood, resulted in some decrease in the degree of polygalacturonic acid methylation and small but significant reduction of the length (by 5%) and width (by 10%) of xylem fibers [37]. Transgenic lines with suppressed (by 50%) level of this gene expression had fibers by 10–15% longer than in wild type. In this case, diameters of both fibers and vessels increased substantially. The authors concluded that homogalacturonan containing many methyl groups (at low PME activity) was less prone to the formation of Ca^{2+}–pectate gel, which facilitated easier movement of the ends of growing fibers. Thus, PME plays the role of a negative regulator of xylem fiber intrusive growth in hybrid aspen. Small effects observed could be partially explained by the presence of numerous PME isoforms in the developing wood [36].

Differences in the degree of polygalacturonan methylation in the cell walls of intrusively growing cells and surrounding tissues were also shown using corresponding antibodies. In the stem apex of showy milkweed (*Asclepias speciosa* Torr.), a difference between binding of JIM5 antibody recognizing homogalacturonans with a low degree of methylation and JIM7 antibody against homogalacturonans with a high degree of methylation was more pronounced in the cell walls of intrusively growing nonarticulated laticifers than in the cell walls of surrounding cells [38].

When we studied flax fibers at the stage of intrusive growth using antibodies LM19 and LM20 [39], which are close but not identical to antibodies JIM5 and JIM7, respectively, we also observed differences in the pattern of label distribution between fibers and surrounding cells. The LM19 antibody, recognizing homogalacturonans with a low degree of methylation, cross-reacted with the cell walls of intrusively growing fibers more actively than with cell walls of neighboring parenchymal cells (Figs. 2a, 2b). The target of LM20 (homogalacturonans with the higher degree of methylation) were weakly represented in all tissues above the snap point (Figs. 2c, 2d).

Peculiarities of fiber cell walls were also revealed when LM13 antibody was used; the epitop of this antibody is the fragment of α-(1⟶5)-arabinan of unknown length, which is sensitive to endoarabinase [40]. The LM13 antibody was bound well in almost all stem tissues, including vascular bundle sheath, but it was completely absent from the fiber cell walls (Figs. 2e, 2f). It is of interest that fibers cross-reacted with the LM6 antibody, which epitop is oligomer α-(1⟶5)-arabinan [41], i.e., the fragment of the same polymer, which cross-reacts with LM13. It might be that LM13 and LM6 recognize not only polymer composition but also its conformation (J.-P. Knox, personal communication). It should be noted that the absence of LM13 binding could not be considered as a marker of the cell walls of intrusively growing fibers because this antibody was also absent from other, less abundant tissues of primary vascular bundles (Figs. 2e, 2f). The difference in LM13 binding between fibers and vascular bundles, on the one hand, and surrounding tissues, on the other, was manifested already at early developmental stages of these structures, and this can help in their identification, when characteristic secondary cell wall is still absent, and in understanding specificity of primary cell wall organization in different tissues.

Intruding of fiber ends between neighboring cells during intrusive growth results in the disruption of middle lamellae and resembles penetration of fungal hyphae in plant tissues, i.e., the process when a pronounced wound effect is observed. It is known that, at wounding, especial polysaccharide, callose, appears in the cell walls; callose is often called wound polysac-

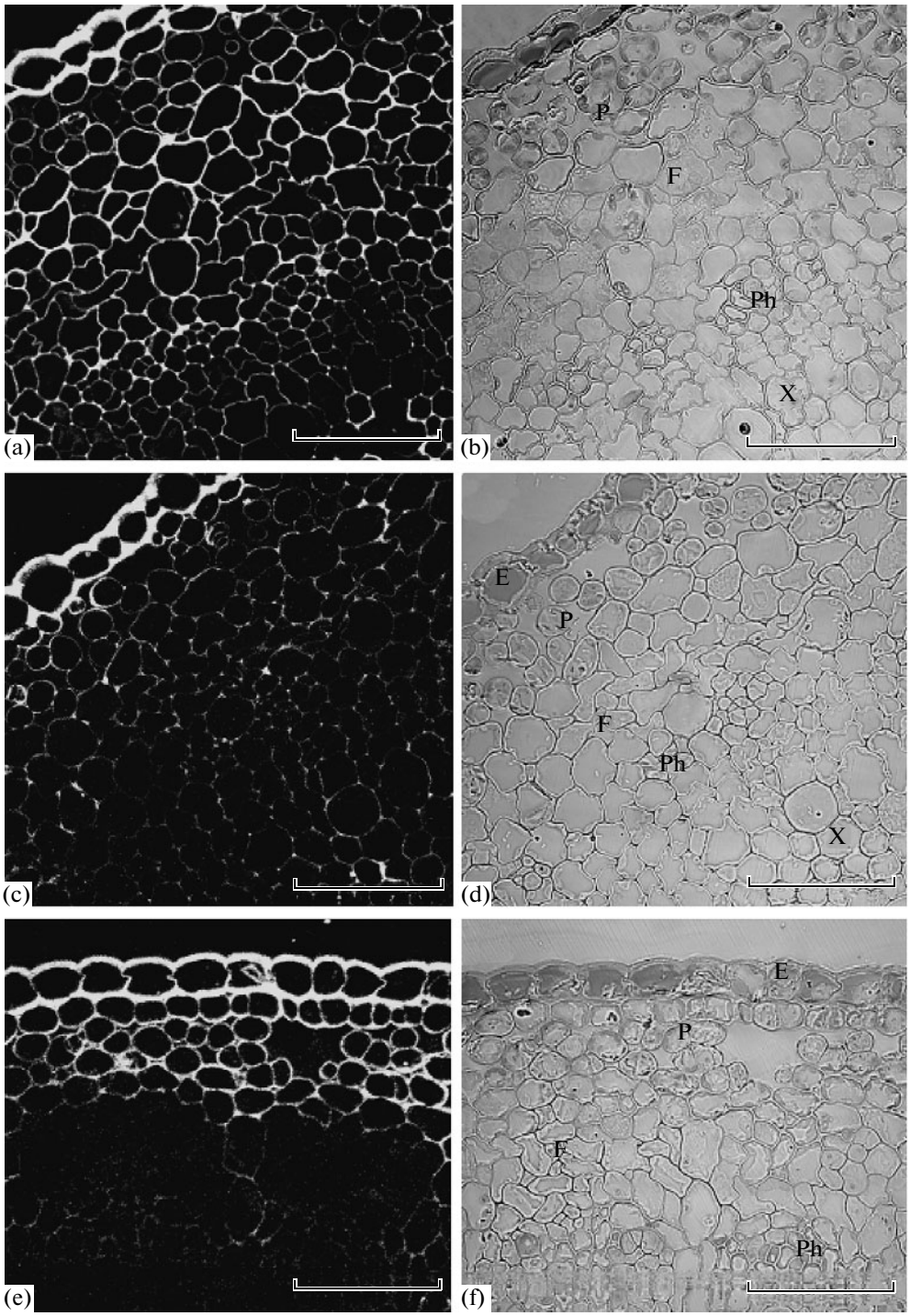

Fig. 2. Detection of antibodies LM19 (a, b), LM20 (c, d), and LM13 (e, f) epitops in tissues of long-fibered flax stem in the region containing intrusively growing fibers.

LM19 and LM20 epitops are fragments of homogalacturanans with low and high degree of methylation, respectively [41]; the LM13 epitop is the fragment of α-(1⟶5)-arabinan [42]. Epitop localization is shown in the left row of photos, whereas the right row demonstrates the structure of corresponding stem region. F—fibers; P—parenchyma; E—epidermis, Ph—phloem; and X—xylem. Scale bar is 50 μm.

charide because it is involved in injured cell wall "mending" [42]. Following this logic, the level of callose was analyzed in intrusively growing fibers. Judging from our data obtained by flax stem staining with aniline blue, the dye revealing callose, fiber intrusive growth does not induce the synthesis of this polysaccharide. Similar results were obtained at investigation of the cell walls of intrusively growing laticifers of showy milkweed [38]. Thus, callose absence during intrusive growth indicates that this process does not induce the wound response.

CONTROL OF FIBER ELONGATION

At present, the data concerning fiber elongation driving forces and control are fragmentary. The conditions of plant growth, such as temperature, humidity, and water content in soil affect the rate and duration of fiber intrusive growth. Osmotic stress induced by short-term drought was shown to retard growth of fibers positioned above the snap point; therefore, they were shorter and remained such until the end of plant development [43].

Little is known about hormonal regulation of fiber elongation. In jute (*Corchorus capsularis* L.) and hemp plants treated with GA, fibers were fourfold longer than in nontreated plants [44]. Similar effect of GA was described for pea (*Pisum sativum* L.) but only in combination with auxin [45]. The analysis of data about changes in the fiber length, entire internode length, and the number of fibers on transverse sections presented by Aloni [45] allowed us to evaluate what type of growth, symplastic or intrusive, was more affected by various concentrations of exogenous hormones (GA and auxin). At most combinations of hormones, fiber length was changed similarly to the internode length, i.e., coordinated growth of all tissues was affected, and only at 20-fold exceeding of auxin over GA, fibers elongated more rapidly than the internode as a whole.

Fiber growth could be related to specific features of ionic transport in these cells. Thus, a potassium channel (PTORK) was detected immunocytochemically in young fibers of poplar differentiating wood [46]. At membrane depolarization, this channel could withdraw potassium from the cell and thus control its electro-physiological properties. An increase in the potassium concentration was shown to result in the increase of fiber but not vessel diameter [47]. Regretfully, there is no similar data concerning fiber length.

Specific features in the control of fiber intrusive growth were observed in experiments using NMR [15]. Its application allowed a conclusion that the damage to the part of intrusively growing fibers stopped intrusive elongation of fibers positioned at a significant distance from the site of damage. Such fiber behavior indicates that the signal transduction between these cells should occur. So far, the nature of such signal could be only supposed. It is clear that it is not transferred along the symplast because flax intrusively growing phloem fibers become symplastically isolated [12].

Nothing is known about the mechanisms of intrusive growth suppression. We only could mention that it is not directly related to the start of secondary cell wall formation because nongrowing fibers located below the snap point could for a long time (judging the stem height where they occur, about two weeks) be thin-walled [12]. They do not produce secondary cell wall, including its outer xylan layers, which is well seen at application of the LM11 antibody.

FIBERS ARE MULTINUCLEATE CELLS

During development of primary phloem fibers, the protoplast becomes multinucleate. This fact was observed long ago. in particular, Esau noted that tobacco (*Nicotiana tabacum* L.) and flax phloem fibers were multinucleate cells [48, 49]. We studied this phenomenon in detail for flax and hemp fibers and found that the number of nuclei in fibers increased mainly during their intrusive elongation [12].

Nuclei in fibers have characteristic elongated shape, whereas in the cell of adjoining parenchyma, they are oval or round (Fig. 3b). The number of nuclei in fibers starts to increase still at the stage of symplast growth, when nuclei divide synchronously; however, there are no signs of the cell plate formation during division of nuclei [1, 2, 49]. Thus, there is obvious break between karyokinesis and cytokinesis, which is rarely observed in plant vegetative cells. When the fiber turns to intrusive growth, division of nuclei becomes asynchronous; as a result, their number in mature cells differs from the value $2n$ [12]. At the stage of cell wall thickening, division of nuclei was not observed. At the average length of primary fibers of about 20 mm, the average number of nuclei in flax fiber is 80 and in hemp—54 (Table 2). The highest reported number of nuclei in studied fibers was 175 (at the cell length of 36 mm). Such a great number of nuclei per cell was not detected in any other plant vegetative cell. A positive correlation ($r = 0.6$ for flax and $r = 0.9$ for hemp) exists between the fiber length and the number of nuclei. However, other cell types are known (protrusively growing trichomes of cotton seeds, in particular), which attain similar length but have only a single nucleus [50]. According to the literature [3] and our data, secondary fibers, which also grow intrusively and attain a great length, have only a single nucleus (Fig. 3d). It might be that nuclei of secondary fibers are polyploid; however, this supposition yet to be checked.

GENES EXPRESSED DURING FIBER INTRUSIVE GROWTH

Keeping in mind extreme shortage of approaches for studying of fiber intrusive growth, it seems especially interesting to analyze the transcriptome characterizing this fiber developmental stage. There are two

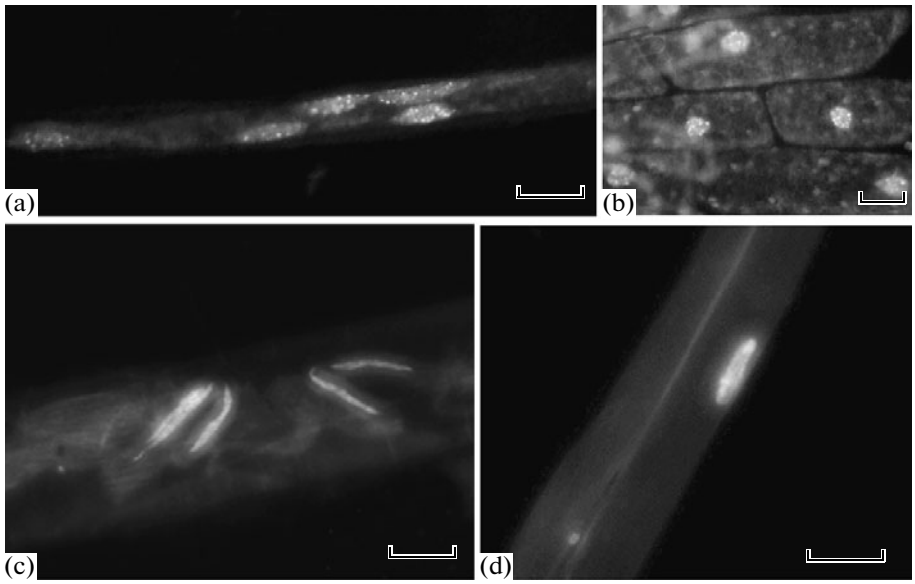

Fig. 3. Nuclei stained with DAPI.
(a) Flax fiber; (b) flax stem epidermis; (c) hemp primary fiber; (d) hemp secondary fiber. Scale bar is 20 μm.

limitations for performing such studies. Firstly, such data were obtained only for few cases, when it was possible to sample material during intrusive growth initiation or after its cessation. Secondly, it is well known that changes in gene expression during process triggering are easier interpreted than profiles obtained during process stoppage. However, it is essentially impossible to collect material sufficient for RNA isolation from stem regions where fibers are already present but their intrusive growth does not yet start. The researchers meet this problem when study various types of growth. In this case, they attempt to reveal genes, which expression is suppressed after completion of elongation. Such approach was used for the studying of protrusive elongation of cotton seed hairs [51, 52] and symplastic cell elongation in the root apex [53].

At present, we have the results of transcriptome analysis for intrusively growing fibers in the two cases. First, it is radial wood sections at different development stages. Thus, in the stem of hybrid aspen (*Populus tremula* × *P. tremuloides*), several zones differing in xylem development stages could be distinguished: (a) meristematic cells (cambium); (b) early expansion; (c) late expansion; (d) formation of the secondary cell wall; and (e) maturation [54]. Expression of genes involved in fiber intrusive growth could be expected in the zones (b) and (c). However, this system gives knowledge of changes in gene expression in the xylem as a whole, which in hybrid aspen consists of fibers by only 53–55% [8]. From 2874 genes tested in this study, 386 genes were expressed constitutively at all stages of xylem development, whereas 1246 genes changed their expression markedly (more than fourfold) [54].

Another type of material for studying intrusive growth is stem regions of fiber crops containing primary phloem fibers. Their usage is based on the separation of the stages of intrusive growth and secondary cell wall formation [6, 7]. Expression profiles for stem regions containing fibers at different developmental stages were obtained for oil-seed flax [55], hemp [56], and long-fibered flax (Table 3). Sharp changes in the content of definite gene transcripts associated with the switch in the fiber developmental stage allow us to relate their expression just with this type of cells.

A common feature of all these samples is high expression of genes for aquaporins of the tonoplast (TIP) and plasmalemma (PIP) involved in the maintenance and elevation of turgor pressure. Such high expression was also observed for other elongating cells, for example, protrusively growing trichomes of cotton seeds [51, 52, 57] or symplastically growing cells in the root elongation zone [53].

For hemp fibers and aspen developing wood, genes encoding expansins were found, which expression reduced after cessation of intrusive growth. It is of importance that, for flax phloem fibers, such genes were not identified either in our work or in the work of Roach and Deyholos [55]. Expression of some other genes encoding proteins involved in the cell wall formation and modification, glycosylhydrolases and glycosyltransferase, and also genes belonging to the *CESA* family encoding, for example, cellulose synthase and cellulose synthase-like (CSL) proteins reduced as well. The flax gene encoding cellulose synthase is homologous to the *AtCesA3* gene of arabidopsis, which was shown to participate in the synthesis of the primary cell wall, and the flax gene of CSL protein is homologous to the gene of arabidopsis protein of CSLD group [58]. However, *CESA* are not present in

the list of genes, which expression changes were most pronounced.

Along with genes of proteins modifying the cell wall, this list comprises some other genes. For example, high expression was reported for genes of the photosynthetic apparatus, in particular genes of reaction center proteins and Rubisco. In all samples containing fibers at the stage of intrusive growth, high expression of genes encoding some ribosomal proteins was detected. This possibly indicates some peculiarity of protein synthesis regulation at this fiber developmental stage. Elevated expression was characteristic for genes of DUF-domain-containing proteins, which was observed for flax [55] and hemp [56] fibers. Proteins of this family are characteristic only for plants and could be related to the cell wall formation [59].

It should be noted that, at suppression of fiber intrusive elongation, changes in gene expression for such proteins as vacuolar invertase and tonoplast and plasmalemmal H^+-ATPases were not observed, although these proteins play an important role in the turgor pressure maintenance and/or in the mechanisms of cell wall expansion. High expression of these genes was characteristic of the stage of rapid elongation of cotton trichomes [51, 52, 57] and the zone of root elongation [53].

Undeniably, the results of cDNA microassays are important, but they could not give complete pattern of fiber intrusive growth. It should be noted that genomes of flax, hemp, and aspen are not sequenced completely so far, and gene nucleotide sequences discussed in some papers [55, 60] are, as a rule, published only partially. Most genes, which expression decreased with cessation of intrusive growth, belong to the groups of "unknown proteins" or "proteins with unknown function." This confirms once more a requirement for studying physiological processes underlying fiber intrusive growth.

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