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Bast fibre formation: insights from Next-Generation Sequencing

Gea Guerriero^{a,*}, Marc Behr^{a,b}, Aurélie Backes^a, Claudia Faleri^c, Jean-Francois
Hausman^a, Stanley Lutts^b, Giampiero Cai^c

^aLuxembourg Institute of Science and Technology, Environmental Research and Innovation (ERIN) department, 5 Avenue des Hauts-Fourneaux,
Esch/Alzette, L-4362, Luxembourg.

^bUniversité Catholique de Louvain, Groupe de Recherche en Physiologie Végétale, Earth and Life Institute - Agronomy (ELI-A), 5 (Bte 7.07.13)
Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium.

^cUniversity of Siena, Department of Life Sciences, Via Pier Andrea Mattioli, 4, 53100, Siena, Italy.

Abstract

Bast fibres are extraxylary sclerenchymatous cells characterized by a noteworthy length and by a cell wall composed of crystalline cellulose. Bast fibres support mechanically the phloem and are used for different industrial applications by the textile and biocomposite sectors. Fibre crops like hemp (*Cannabis sativa*), flax (*Linum usitatissimum*), ramie (*Boehmeria nivea*), jute (*Corchorus olitorius*, *C. capsularis*), kenaf (*Hibiscus cannabinus*) are therefore important natural resources which can help develop a sustainable economy. Despite the importance of bast fibres, not all the features related to their initiation and growth are fully explored and understood. In this review we will focus on the current knowledge concerning bast fibre initiation and development by using a transcriptomic angle, in the light of the great advances that Next-Generation Sequencing (NGS) has fostered in the last years. We discuss the results obtained recently on different fibre crops and we conclude our survey with a perspective on future molecular studies aimed at valorising neglected fibre crops, e.g. nettle (*Urtica dioica*).

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* Corresponding author. Tel.: +352-275-888-5023; fax: +352 275 885.
E-mail address: gea.guerriero@list.lu

1. Main text

Bast fibres are unique cells characterized, at maturity, by a thick cellulosic cell wall and by a noteworthy length. Bast fibres show a diffuse (intercalary or global) anisotropic expansion, i.e. the expansion is uniform by apposition of new cell wall components over the whole surface [1]. The mechanism through which bast fibres reach their final length is intrusive (or invasive) growth, a type of growth where the tip of the fibres invades the middle lamellas of the adjacent cells [1-5]. Bast fibres first grow symplastically with the surrounding cells, then their flat ends change to tapered ones, thereby marking the onset of intrusive growth; in the case of hemp primary fibres, the distance from the shoot apical meristem (SAM) at which these structures have been observed is ca. 2 mm [6].

Fibre crops are not only important for bio-economy, but they also represent powerful models for cell wall-related studies: their stems have a woody core and a cortex harboring the cellulosic bast fibres and they show a basipetal lignification gradient where the expression of genes involved in the provision of the precursors for lignin biosynthesis is progressively upregulated [7]. Additionally, an empirically-determined region along the stem and known as snap point (SP), marks the shift from elongation to fibre cell wall thickening [8]. Therefore, sampling stem internodes above and below the SP allows the study of the dynamics in cell wall-related gene expression [7,9].

Next-Generation Sequencing (NGS) has been a real revolution in plant biology [10], because its depth of analysis allows the study of the expression dynamics of thousands of genes, as well as the detection of transcript variants. The application of NGS, i.e. transcriptomics, to fibre crops has provided crucial data that help us better understand the regulation of bast fibre formation and subsequent development.

In this review, we discuss the recent transcriptomic data obtained on different fibre crops, namely flax, ramie, hemp, jute and kenaf. We differentiate the group of plants producing gelatinous fibres (with a G-layer, i.e. flax, ramie and hemp) from those depositing fibres with a xylan-type cell wall (with an S-layer, i.e. jute and kenaf). We conclude our survey by highlighting the importance of future transcriptomics studies on neglected fibre crops, as for example common nettle *U. dioica*.

1.1. Flax

Flax is a member of the Linaceae family and has been the object of several transcriptomic studies. We will here review the most recent transcriptomic data available for this fibre crop.

The hypolignification of flax gelatinous bast fibres has been studied in detail by Chantreau and colleagues [11] in a work reporting ethyl methanesulfonate mutagenized flax lines. We believe it is important to describe the results of this work, although a microarray approach was used (and not a NGS strategy), as it allows a clear understanding of the mechanisms regulating flax bast fibre hypolignification. Mutant flax lines (called *lignified bast fibers*, *lbf*) showing lignified bast fibres were identified in this study (93 M2 families in total) and subdivided into 8 different groups, depending on the type of altered lignification profile (i.e. lignification in bast fibres only, or in the surrounding cells too). Chemical analyses of the lignin content allowed the identification of one mutant line, *lbf1*, showing a significant increase in lignin content in the outer stem tissues (350% increase). The microarray analysis of this mutant identified 959 transcripts which were more abundant and 806 less expressed in the outer tissues of the mutant with respect to the wild-type: transcripts involved in monolignol biosynthesis (*CCR*, *COMT*, *CAD*) and polymerization (orthologs of *Arabidopsis* *PRX52*, *PRX53*, *PRX71*) were more expressed in *lbf1*. These results demonstrate that the hypolignification observed in flax bast fibres is linked to the transcriptional regulation of genes involved in monolignol oxidation [11].

The intrusive growth of bast fibres is a fascinating process that awaits characterization. For example, key players involved in this process are still to be identified (e.g. transcription factors, TFs). The characterization of marker genes of intrusive growth would open the way to important functional studies, as for example done for the TFs regulating secondary growth.

The availability of the transcriptome of the SAM of flax, reported by Zhang and Deyholos in 2016 [12] is in this respect an important resource. In this study the authors analyze the transcriptome of the apical-most 0.5 mm of the stem (AR) and the whole stem region excluding the apical-most 1 cm (BR) and provide a rich list of candidates. These genes clearly deserve further investigation to be able to shed light on the molecular factors determining the

bast fibre identity. The comparison between the AR and BR highlighted 4405 genes which were expressed at least 2-fold more in the apical part [12]. Among these genes, transcripts encoding *PDF1* (*PROTODERMAL FACTOR 1*), *CUC* (*CUP-SHAPED COTYLEDON*) and *STM* (*SHOOT MERISTEMLESS*) were found.

Very recently, an RNA-Seq study of isolated bast fibres and different stem parts in flax was carried out to understand the mechanisms regulating fibre differentiation [9]. The transcriptome analysis revealed that in bast fibres at advanced stages of tertiary cell wall formation (bottom region of the stem) there was an increase in the expression of genes coding for metallothioneins, lipid transfer proteins, as well as candidates involved in protein synthesis/translation and ubiquitin-mediated degradation. These data show that an active metabolism, reflected in the rich transcriptome profile, is present in mature bast fibres and therefore these cells should not be considered as dead [9]. As compared to the top stem region, isolated bast fibres at advanced stages of differentiation showed the upregulation of 156 genes, among which several cell wall-related genes, namely a xyloglucan endotransglucosylase/hydrolase (XTH), β -galactosidases, chitinases, rhamnogalacturonate lyase-related genes. TFs involved in secondary cell wall deposition decreased in expression in the bast fibres, as compared to other stem regions, while members of the G2-like, bZIP, bHLH and MYB-related families were upregulated in isolated bast fibres [9]. Genes involved in lignin and xylan biosynthesis were downregulated in isolated fibres, which is in agreement with the known hypolignification of bast fibres.

1.2. Hemp

Textile hemp belongs to the Cannabaceae family and it is a multi-purpose crop [13] which provides cellulosic bast fibres used in both the textile and biocomposite industries [14]. Hemp bast fibres grow symplastically when they are young and then intrusively and can reach a length of 50 mm [6]. Differently from flax, the hemp stem and hypocotyl produce both primary and secondary bast fibres (Fig. 1a) originating from the procambium and vascular cambium, respectively. Ultrastructural microscopy with transmission electron microscopy (TEM) shows the presence of different layers in the bast fibre cell wall (Fig 1b): an outermost dark primary cell wall, followed by a gray outer secondary cell wall and an inner thick region composed of crystalline cellulose.

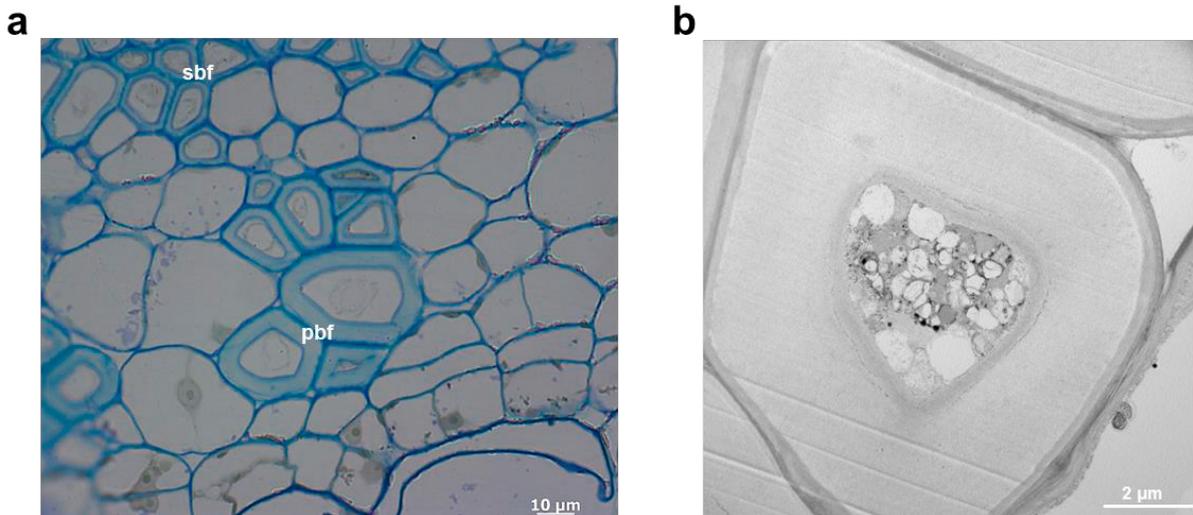


Fig. 1. (a) cross section of hemp hypocotyl stained with toluidine blue, showing primary and secondary bast fibres (pbf and sbf, respectively); (b) transmission electron microscopy picture of a primary bast fibre from hemp hypocotyl showing a thick gelatinous cell wall.

We have recently shown that the hemp hypocotyl is a suitable model to study the processes accompanying secondary growth, because it shows an initial stage of active elongation (until day 9 after sowing) and then it thickens and forms both primary and secondary bast fibres [15]. We will here review our recently published NGS

data on the hemp hypocotyl, as they provide an overview of the key processes characterizing primary and secondary growth.

Secondary growth in the hemp hypocotyl was shown to be associated with the up-regulation of the TFs *NST1*, *MYB46* and *WLIM1* [15]. *NST1* is the major regulator of fibre differentiation [16]. *MYB46* is involved in secondary cell wall biogenesis and xylan biosynthesis [17]. *WLIM1* favors fibre extension by bundling the actin filament but also fibre lignification by promoting the lignin/lignin-like biosynthetic genes via binding to the PAL-box [18]. Bast fibre extension depends on the activities of XTH such as orthologs of *AtXTH5* and *AtXTH8*, which were more expressed in the elongating hemp hypocotyl [15]. Other isoforms of XTHs were more expressed in old hemp hypocotyls undergoing secondary growth (15 and 20 days after sowing), such as orthologs of *AtXTH15* and *AtXTH22/TOUCH4*. *TOUCH4* may participate in the targeting of xyloglucan to the primary-secondary cell wall junction in the secondary cell wall S1 layer.

Two genes of the COBRA family involved in cellulose biosynthesis, *COB* and *COBL4*, as well as secondary cell wall cellulose synthase genes (*CesA4*, 7 and 8) were highly expressed in hypocotyls undergoing secondary growth [15].

Although a signal specific for β -1,4-galactan has never been detected in hemp bast fibres [15,19], it is not possible to rule out its existence. In the fibre crop flax, the formation of the gelatinous, crystalline cellulose layer is associated with the maturation of a galactan (Gn) layer. A hemp ortholog of *Arabidopsis* β -galactosidase 3 and flax β -galactosidase 1 may play a role in this remodeling, as they are involved in secondary cell wall deposition in flax and were highly expressed in old hemp hypocotyls.

Fasciclin-like arabinogalactan proteins (FLAs) may play a role in the deposition of the G-layer. Orthologs of *AtFLA11* and *AtFLA12* were more expressed in old hemp hypocotyls, a time point at which both primary and secondary bast fibres are present [15]. FLA members are known to impact cellulose, arabinose and galactose content in plant cell walls [20] and may therefore also contribute to determine the bast fibre cell wall composition.

Our RNA-Seq analysis highlighted the up-regulation of several genes involved in the biosynthesis of glucuronoxylan during secondary growth in older hypocotyls: the analysis showed, in particular, genes involved in the elongation of the backbone (*IRX10*, *IRX10L*, *IRX14* and *IRX15L*), backbone acetylation (*ESK1*, *RWA3*) and substituent methylation (*GXM3*) [15].

1.3. Ramie

Ramie (a.k.a. Chinagrass) belongs to the Urticaceae family and provides smooth fibres with an excellent tensile strength [21]. The Illumina paired-end sequencing technology was used to characterize the ramie transcriptome (from a pool of tissues) and identified genes belonging to the cellulose synthase superfamily [21]: this work led to the identification of 43990 new genes in *B. nivea* and 36 genes of the cellulose synthase superfamily highly expressed in bast tissues. Of these 36 genes, 33 were expressed at higher levels in the bark than other tissues.

A subsequent study analyzed, separately, the transcriptomes of the phloem and xylem tissues and identified an enrichment of RNA and galactose metabolism-related genes in the phloem [22]. Pyrosequencing (which led to the assembly of 58369 unigenes, of which 13386 contigs and 44983 isotigs) was used to study the transcriptome of ramie bast fibres at different developmental stages and identified members of three gene families, i.e. cellulose synthase, xyloglucan endotransglucosylase/hydroxylase and expansin gene families, upregulated during young stages of fibre development [23].

An additional study worth mentioning is that by Liu and colleagues [24] where the transcriptomes of a wild type and domesticated ramie variety were compared. Interestingly, the results identified a *WATI*-related gene (*WALLS ARE THINI*, which is involved in secondary cell wall biosynthesis) among the genes shown to undergo significant purifying and positive selection. This shows that domestication of varieties with higher fibre content may be linked to the positive selection of this gene which, therefore, likely regulates the higher fibre yield trait.

1.4. Jute

Jute belongs to the Malvaceae family and to the *Corchorus* genus [25]. Together with kenaf, it produces bast fibres with a xylan-type cell wall; hence molecular analyses via NGS are very valuable to understand the dynamics of formation of these fibre types.

Several high-throughput studies are available for jute. A *de novo* transcriptome on *C. capsularis* (a.k.a white jute) has allowed the identification of major bast-related genes involved in cellulose biosynthesis [26]. More specifically, the *de novo* assembly gave 48914 unigenes with an average length of 903 bp. The comparison of the bast fibre transcriptome with that from a pool of tissues (leaves, roots, stem bast and stem stick) revealed an enrichment of genes related to cellulose biosynthesis: sucrose synthase, UDP-glucose pyrophosphorylase, cellulose synthases were expressed at higher levels in the bast fibres [26].

The *de novo* transcriptomes of a white jute mutant, *deficient lignified phloem fibre* (characterized by a reduced growth and undulated phenotype, lower lignin content, but higher cellulose amount) and the wild-type JRC-212 allowed the discovery of isoforms of several genes. For example, this study identified 37 isoforms of genes involved in the shikimate-aromatic amino acid and 43 in the monolignol pathways; additionally, it detected a mutant isoform of *PAL1* (phenylalanine ammonia-lyase) which was down-regulated at early growth stages [27]. Interestingly, this study also identified a gene coding for a FLA protein (*FLA6*) co-regulated with the cellulose synthase gene *CesA7* in mutant fibres; this indicates that FLA6 may orchestrate with *CesA7* the deposition of the S-layer in xylan-type fibres. *FLA6* is the ortholog of *FLA11* from thale cress, which is in its turn the closest paralog of a poplar tension wood-specific FLA; hence FLA6 may be required for the deposition of both S- and G-layers [27].

The recent genome sequencing of *C. olitorius* and *C. capsularis* has shed light on the molecular mechanisms taking place during bast fibre differentiation in these important crops [25]. This study has shown that, in the fibres, the expression of the TFs *MYB83*, *WOX4* (*WUSCHEL RELATED HOMEBOX 4*), *APL* (*ALTERED PHLOEM DEVELOPMENT*) and the homeobox gene *HAT22* were high in the fibres. The work by Islam and colleagues [25] also highlighted the expansion of lignin-biosynthetic genes in jute with respect to the other fibre crop *L. usitatissimum*, namely 4-coumarate:CoA ligase (*4CL*), cinnamoyl-CoA reductase (*CCR*), *trans*-caffeoyl-CoA 3-O-methyltransferase (*CCoAOMT*) and caffeic acid O-methyltransferase (*COMT*). This study also confirmed the role of *CesA7* (and *CesA4*) in secondary cell wall biosynthesis. This high-throughput genome/transcriptome analysis represents an important addition to the study of fibre crops, because it compares two jute varieties with different characteristics (*C. olitorius* fibres contain more lignin and less cellulose compared to white jute) and distinct responses to (a) biotic stress (white jute is more tolerant to pests, but also more susceptible to flood, drought and salt stress as compared to *C. olitorius*).

1.5. Kenaf

Kenaf belongs to the Malvaceae and is grown for its bast fibres which are used for paper, rope, cloth [28]. It produces xylan-type bast fibres as mentioned above for jute [29], characterized by a higher abundance of xylan and lignin with respect to gelatinous cellulosic fibres. The recent *de novo* transcriptome of the elite cultivar ‘Fuhong 952’ has resulted in 90175 unigenes with an average length of 700 bp and has highlighted sequence similarity with cotton genes [28]. The *de novo* transcriptome also enabled the identification of 92 MYB TFs, whose members are known to coordinate important phases of secondary cell wall biosynthesis. Additionally, the transcriptome revealed 317 unigenes involved in aspects of the primary metabolism linked to cell wall formation, i.e. sucrose and starch-related pathways. Importantly, the analysis of the kenaf transcriptome allowed the identification of 11083 EST-SSRs (expressed sequence tags/simple sequence repeats), which are a resource for future genetic mapping and marker-assisted breeding [28]. In a more recent study, Illumina paired-end sequencing was used to analyze the transcriptome of another cultivar, “Zhong hong ma 16”: 71318 unigenes with an average length of 1143 nt were assembled and 9324 EST-SSRs were developed [30].

1.6. Future perspectives

In order to better understand the mechanisms of development and differentiation of bast fibres, fibre crops other than those previously described should also be studied using high-throughput approaches. One such example is stinging nettle, *Urtica dioica*. Awakening so far little interest in the scientific community, stinging nettle produces cellulosic bast fibres just like hemp, jute and ramie. Common nettle develops indeed long and silky hypolignified phloem fibres with high tensile strength, a characteristic that is attractive to the textile industry. In the bottom region of the nettle stem, the cellulose content of the fibres exceeds 80%, while lignin is around 3.5–4.4% [31]. Apart from its use in the textile sector, common nettle has multiple applications in different agri-food, cosmetic and pharmaceutical sectors too [32]. The use of *U. dioica* in such diverse areas is justified by the presence of many active compounds (flavonoids, tannins and sterols), mainly located in the leaves. The economic potential of nettle is currently underexploited and future studies centered on this neglected but multi-purpose plant may not only increase our understanding of bast fibre formation, but also contribute to its industrial valorization.

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