The thickening and modification of the galactan-enriched layer during primary phloem fibre development in *Cannabis sativa*

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Abstract

Primary phloem fibres (PPFs) have higher fibre quality and are economically more important for the textile sector than secondary phloem fibres. Both the chemical composition and mechanical structure of the secondary cell wall mainly influence the quality of bast fibres. We investigated the thickening of the galactan-enriched (Gn) layer and its modification process into a gelatinous (G)-layer, which is the largest portion of the secondary cell wall, during the development of the PPF in *Cannabis sativa*. Stem segments of hemp collected at 17, 29, 52 and 62 days after sowing were comparatively examined using light microscopy, scanning electron microscopy and transmission electron microscopy. The initial cells of PPF started the proliferation and differentiation at 17 days, but the secondary cell wall thickening had already commenced before the 29 days. Both the G- and Gn-layer were rapidly added onto the S-layer of PPFs; thus, the secondary cell wall thickness increased approximately 2-fold at 52 days (from the 29-day mark), and 8-fold at 62 days. The cortical microtubule arrays appeared adjacent to the plasma membrane of PPF cells related to the cellulose synthesis. Additionally, cross-sectioned microfibrils were observed on Gn-layer as the cluster of tiny spots. At 62 days, the specific stratification structure consisting of several lamellae occurred on the G-layer of the secondary cell wall. The secondary cell wall thickened remarkably at 52 days through 62 days so that the mature secondary cell wall consisted of three distinctive layers, the S-, G- and Gn-layer. Cortical microtubule arrays frequently appeared adjacent to the plasma membrane together with cellulose microfibrils on secondary cell wall. The G-layer of PPF at 62 days exhibited the characteristic stratification structure, which demonstrates the modification of the Gn-layer into the G-layer.

Keywords: *Cannabis sativa*; development; G-layer; Gn-layer; hemp; primary cell wall; primary phloem fibre; secondary cell wall; secondary phloem fibre; S-layer; thickening.

Introduction

Hemp fibre is one of the strongest natural fibres and an important raw material due to its high tensile strength and various properties. Thus, hemp bast fibres have been traditionally used in diverse hemp products (Nechwatal and Mieck 2003; Andre et al. 2016). The physical and chemical properties of hemp fibres are influenced by many factors such as type of variety, condition and method of cultivation, stage of plant growth, plant age and fibre processing (Keller et al. 2001; Kostic et al. 2008), whether they are derived from a mono- or dioecious cultivar, and latitude (Sankari 2000). The quality of fibres is mainly depended on...
their softness, fineness, brightness and length uniformity of the fibre bundles. It is primarily determined by genetic factors (Cromack 1998; Petit et al. 2020) as well as various other factors such as irrigation (Fernandez-Tendero et al. 2017), reduced branching (Clarke 1981), seed sowing density (Amaducci et al. 2000; Westerhuis et al. 2009), sex determination and flowering time (Salentijn et al. 2019), and harvest time and retting method (Amaducci et al. 2005, 2008; Liu et al. 2015; Musio et al. 2018). In addition, it is also influenced by the developmental stage at the time of harvest (Salentijn et al. 2019).

In general, fibres are divided into xylary and extraxylary fibres; the latter are of economic importance to the fibre industry owing to their high yield and less effort required to collect them. Also, extraxylary fibres are classified into primary phloem fibres (PPFs) formed from procambium, and secondary phloem fibres originating from cambium (Amaducci 2005; Crônier 2003; Ageeva et al. 2005; Gorshkova et al. 2012). Both types are commonly known as bast fibres. Primary phloem fibre is important in the textile industry because it is softer and has a preferable length and more valuable properties than secondary phloem fibre (van der Werf et al. 1994; Bôcsa and Karus 1998; Snegireva et al. 2015).

Flax, a representative bast fibre crop, produces only PPFs (van Dam and Gorshkova 2003; Goudenhof et al. 2019). These extraxylary fibres form bundles much like the bast fibres in many other plant species (McDouggall et al. 1999). Bast fibre plants generally undergo a specific process of development, which comprises an initial elongation through intrusive growth and a subsequent thickening of the secondary cell wall (Guerriero et al. 2017). The thickening of secondary cell wall is a longer process than cell elongation, and usually takes up to 60 days in flax stems (Gorshkova et al. 2003; Ageeva et al. 2005). However, a hemp stem generates both PPFs and secondary phloem fibres (Snegireva et al. 2015; Bourmaud et al. 2017; Fernandez-Tendero et al. 2017). Furthermore, less is known about the thickening of the secondary cell wall than about the elongation process in hemp.

Snegireva et al. (2015) reported that the intrusive elongation of PPFs in hemp stem is completed before the onset of secondary cell wall formation, and the formation of secondary phloem fibres starts long after the beginning of the secondary cell wall thickening. These authors described the development of PPF and secondary phloem fibre in hemp stems at the time of flower formation, at 67 days, and at the beginning of seed maturation, at 117 days. However, they focused on the intrusive elongation of both phloem fibres, and studied the processes at the middle and the late developmental stages.

The bast fibres are currently divided into xylan- and gelatinous-type fibres, depending on the composition of the secondary cell wall. The former such as jute bast fibres are characterized by the presence of xylan and a high degree of lignification, while the latter found in flax, ramie and hemp are rich in crystalline cellulose (Roach et al. 2011; Guerriero et al. 2017). Bast fibres undergo specific developmental stages during the process of differentiation: the fibre cells initially elongate through intrusive growth, and subsequently, the elongation ceases and the secondary cell wall starts to form (Snegireva et al. 2010; Gorshkova et al. 2012).

Depending on the microfibril orientation and the presence of lignin, the secondary cell wall was classified into three layers, S1, S2 and S3 (Haugen and Holst 2013; Sorteil 2016; Jiang et al. 2018), or into four layers if the non-lignified G-layer was included (Saiki and Ono 1971; Clair et al. 2005; Lehringer et al. 2009, Nakagawa et al. 2012; Higaki et al. 2017). The outer layer, S1, is deposited onto the primary cell wall and has microfibrils transversely oriented at a high angle. The other two layers, S2 and S3, with different compositions and structures, are continuously added to the S1 layer during the thickening of the secondary cell wall. The middle layer, S2, usually constitutes the greatest proportion of the wall thickness and has the smallest microfibril angle orientation. Lastly, the inner S3 layer has microfibrils oriented transversely at a high angle, similar to S1 (Brändström 2004; Sorteil 2016).

The G-layer of the secondary cell wall was well defined by Mellerowicz and Gorshkova (2012), and it was recently characterized in hemp fibres by Chernova et al. (2018) as a secondary layer with a large content of crystalline cellulose. Moreover, the modification of the G-layer into the G-layer has been reported on some previous studies; however, these pioneering studies were mainly undertaken at the light microscopic level (Gorshkova et al. 2003, 2004). Due to the limits of the magnification of light microscopy (LM), the microstructure of the Gn- and G-layer was not elucidated in detail and remains largely unknown.

Plant cell wall architecture has been a major issue in cell biology, as well as in other specific fields owing to the complex structures and dynamic functions. One particular issue is the processes of the secondary cell wall thickening of PPFs that basically affects the fibre quality. In addition, hemp is considered an excellent model for observing cell wall thickening because of the distinctive structural cell wall patterns of its stem (Guerriero et al. 2017). The main objectives of this study were to (i) investigate when the thickening of the secondary cell wall commences and completes its formation during development, (ii) examine how the wall materials are deposited on the secondary cell wall and form the G-layer and (iii) clarify how the transformation progresses from the Gn-layer to the G-layer.

Materials and Methods

Plant material and cultivation

A fibre hemp cultivar (Cannabis sativa cv. Chungsam) grown in Andong, South Korea (36.5°N, 128.7°E; 140 m above sea level, and with natural daylight and watering) was used in this study. Andong is known to provide favourable environmental conditions for high-quality hemp products in South Korea, which has an average temperature of 13.2 °C and year-round precipitation of 934 mm. According to the three developmental stages of hemp, proposed by Bôcsa and Karus (1998), we slightly modified and established four developmental stages: 17 days (slow-growth stage I), 29 days (slow-growth stage II), 52 days (fast-growth stage) and 62 days (pre-flowering stage) after sowing. However, the plant samples harvested at 17 days were studied with LM only because of their juvenile cell wall structure. However, all samples of the other three stages were examined by LM, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Light microscopy

The stem samples of 17 days were collected from the first internode, but three developmental stages (29, 52 and 62 days) were collected between the third and fourth nodes, which have a higher rate of maturation during the early stage of development (Amaducci et al. 2005). The samples were fixed with formalin–ethyl alcohol–acetic acid for 12 h at 4 °C. The fixed sections were dehydrated in a graded ethanol series. They were replaced with xylene, and then embedded in hard paraffin. Paraffin blocks
were cut into 8 µm in thickness using a rotary microtome (Leichert Jung 2030, Leica, Germany), and the dewaxed sections were stained with safranin and fast green F. They were observed and photographed using a light microscope (Axiophot II, Zeiss, Germany).

**PPF extraction**
Five grams of fresh plant material at 17 days were hydrolysed in 50 mL of 1 N HCl for 1 h at 65 °C. The extracts were centrifuged at 22 000 × g for 5 min (MF 600, Hanil Sci, Korea) and rinsed with distilled water. The supernatant was then stained with 1 % safranin, and observed with a light microscope (Axiophot II, Zeiss, Germany).

**Scanning electron microscopy**
The stem segments of the three developmental stages (29, 52 and 62 days) were collected between the third and fourth nodes. The stem segments were fixed for 2 h in 2 % glutaraldehyde in 25 mM phosphate buffer, pH 7.2. After being rinsed with deionized water, they were post-fixed for 1 h in 2 % osmic acid. Following dehydration in a graded ethanol series, they were transferred into isoamyl acetate. The samples were subjected to critical point drying with pressurized liquid carbon dioxide (Bioradical E3000, Bio-Rad, USA). The dried specimens were mounted on aluminium stubs, coated with gold–palladium in a sputter coater (JFC-1100E, JEOL, Japan) and photographed in a FE-SEM (JSM-6700F, JEOL, Japan) at 15 kV.

**Transmission electron microscopy**
The segment samples were treated with the SEM fixation method described above. The materials were dehydrated with a graded ethanol series and replaced with propylene oxide. Then, the samples were embedded in Spurr resin. Semithin sections of 0.4 µm thickness were cut with a glass knife and stained with toluidine blue-basic fuchsin. Subsequently, ultrathin sections of 70 nm thickness were cut with a diamond knife (Micro Star SU-30, Ted Pella, USA) using an ultramicrotome (Reichert Ultracut S, Leica, Germany) and collected sections on 300 mesh copper grids. The sections were stained for 20 min with 1 % uranyl acetate and for 10 min with 1 % lead citrate. Image acquisition was performed with a transmission electron microscope (JEM-2000 EX II, JOEL, Japan) at 80 kV.

**Statistical analyses**
For statistical analyses on the thickness of the PPF walls, a one-way ANOVA with Tukey’s multiple comparisons test (n = 17–18, ****P < 0.0001, *P < 0.05) was used. All the statistical analyses were performed using the GraphPad Prism software package (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

**Histology of PPF**
The height of the stem was on average 40 cm at 17 days (Fig. 1A), 110 cm at 29 days, 146 cm at 52 days and 155 cm at 62 days. The

![Image of Cannabis sativa plants with three internodes](https://academic.oup.com/aobpla/article-figures/13/4/plab044/6321332)

**Figure 1.** (A) External feature of Cannabis sativa plants with three internodes (IN) at 17 days. (B and C) LM micrographs of two internodes of a hemp stem illustrating the different stages of fibre maturation. (B) Internode 2 demonstrates the PPFs of stems arranged in bundles inwardly from the cortical cells. (C) Internode 1 showed that PPFs were more developed, but the cortical region was largely collapsed due to the expansion of PPFs and secondary xylem. Sections were stained with fast green F and safranin. Cortex (CT), primary phloem fibre (PPF), pith (PH), secondary phloem (SP), secondary xylem (SX) and vascular cambium (VC).
The stem of the plants consisted of 3, 5, 7 and 10 internodes at 17, 29, 52 and 62 days, respectively. The structure of each internode was visibly different depending on the developmental stage. At 17 days, Internode 2 showed that PPFs were tightly aggregated in groups and exhibited a banded appearance beneath the compact cortical cells (Fig. 1B). In contrast, Internode 1 showed most of the cortical cells were crushed, and the PPFs were more developed and appeared as multifaceted cells in either a rectangular or heptagonal shape (Fig. 1C).

At the initial stage of stem development, the cortical region formed a multilayered region composed of the specific lamellar collenchyma with four to five cell layers thick at the ridged region of the stem. The initial cells of the PPFs, which were multiplied by anticlinal division, appeared adjacent to the endodermis at 17 days. These initial cells were elongated and at first had irregular oval shapes, then later became four- or seven-angled in a cross-section. Thus, PPFs were readily identified from the cortical cells and lamellar collenchyma by this characteristic feature (Fig. 2A).

Lignin deposition in the secondary cell wall of the PPFs was less detected by the phloroglucinol staining. This histochemical staining showed lignin localization on the inner xylem cells, but either no or less deposition in the outer region of stem, including the PPF cells at 29 days (Fig. 2B). At the 17-day stage the PPFs extracted by the maceration method were younger and elongated, tapering towards the ends. These PPFs were approximately 23 μm in diameter and 350 μm in length (Fig. 2C).

Early in ontogeny at 29 days, the undifferentiated cells of the PPFs were compactly associated with the cortical cells without intercellular spaces between the phloem and the endodermis layer (Fig. 3A). At 52 days the peripheral groups of PPFs tended to mature centripetally. These PPFs were inwardly differentiated and became mature fibres with a thick secondary cell wall, but this process was partially commenced in some PPFs of the inner region. Additionally, some young secondary phloem fibre cells were present in the phloem region at this stage. The prominent endodermis clearly separated PPFs from the cortical cells and the lamellar collenchyma cells (Fig. 3B). As the maturation process continued, the outer PPFs were fully matured and had thicker walls compared to the neighbouring cells (Fig. 3C).

The secondary cell wall thickness of PPFs in hemp was determined at three developmental stages: 1.3 μm at 29 days, 3.1 μm at 52 days and 11.2 μm at 62 days. The thickness of the secondary cell wall in PPFs at 62 days specifically increased compared to the thickness at 29 days by approximately 8-fold (Fig. 4).

Microstructural changes at 29 days

At 29 days, the cortical region showed a compact organization with a prominent endodermis adjacent to the PPFs. A single PPF was initially four- to seven-angled, but became more compressed during stem development, taking on a somewhat distorted shape (Fig. 5A). In a cross-section, the long axis of the PPF ranged from approximately 23 to 37 μm, and the short axis of

Figure 2. Light micrographs of Cannabis sativa stem collected at 17 days. (A) Initial cells of PPFs are distinctively visible (double-pointed arrow). The sample was stained with toluidine blue and basic fuchsin. (B) Histochemical detection of lignin in hemp stems stained by phloroglucinol–HCl at 29 days. The staining shows normal lignin deposition in the walls of the xylem but not in the fibre cells (double-pointed arrow). (C) Isolated PPFs at 17 days showing slight elongation with tapering ends. Endodermis (Ed), primary phloem fibre (PPF), lamellar collenchyma (LC), secondary phloem (SP), secondary xylem (SX) and vascular cambium (VC).
PPF was measured 11 to 24 μm (Fig. 5B). In a longitudinal section of the stem, the endodermal cells were clearly distinguished from the PPFs beneath them (Fig. 5C). High-magnified SEM images revealed the PPF wall consisting of the middle lamella, primary cell wall and secondary cell wall including the S-layer. The secondary cell wall of the PPF was frequently seen detached from the primary cell wall. The process of secondary cell wall thickening progressed unevenly during development; thus, the thickness of the secondary cell wall at the corner of the cell was thicker (Fig. 5D). A large number of cellulose microfibrils were observed across the secondary cell wall, and they were considered to contribute to the deposition of cell wall materials on the developing S-layer (Fig. 5E).

Gn- and G-layer formation at 52 days

At 52 days, the cortical cells became extremely flattened due to the maturation activity of the PPFs in the cortex. The PPFs in the outer region were almost matured, while the other PPFs in the inner region were still developing. The secondary cell wall of the PPFs was continuously thickened until the next stage (Fig. 6A). The maturation of the PPFs progressed centripetally; the outermost PPFs had fully thickened walls, whereas walls of the inner PPFs were less thickened and their lumen was large reaching a diameter of 1.5–8.5 μm (Fig. 6B). The S and other layers of the secondary cell wall, such as the G-layer and Gn-layer, were distinctly identified at higher SEM magnifications. The newly deposited Gn-layer was more distinguishable in the PPF. Characteristically, a border line between the S- and G-layer was clearly observed in a cross-section of the PPF (Fig. 6C). In addition, the middle lamella and the primary cell wall were both more strongly stained than the secondary cell wall at this stage. As the process of secondary cell wall formation continued, the cytoplasm was concentrated into a small space close to the plasma membrane (Fig. 6D).

Concentrated cytoplasm was frequently observed in PPF cells at 52 days. Although the cytoplasm gradually disintegrated in the...
peripheral region of the fibre cell at this stage, some organelles involved in the synthesis of cellulose were actively adjacent to the plasma membrane (Fig. 7A–D). The wall thickness of the PPF increased significantly from this stage to the 62-day stage. The PPF wall consisted of primary cell wall, a thin S-layer and a thick G-layer (Figs 7A, B and 8). Cortical microtubules were arrayed close to the plasma membrane. There was some variation of the distance between cortical microtubules, ranging from 50 to 200 nm apart from each other. The cellulose microfibrils were seen as the clusters of tiny spots with high electron densities on Gn-layer of the secondary cell wall (Fig. 7A). The endoplasmic reticulum often appeared in the peripheral cytoplasm (Fig. 7C). The endomembrane system of the cytoplasm was concentrated into a smaller space. The endoplasmic reticulum, various vesicles, ribosomes, mitochondria and chloroplast were often visible in the cytoplasm (Fig. 7D and E).

Modification of the deposited cell wall layers at 62 days

At 62 days, the PPFs were completely differentiated and formed a fibre bundle composed of 12 or 15 fibre cells with a fully thickened secondary cell wall. The secondary phloem fibres were conspicuous in the region of the secondary phloem at this stage. As these fibres gradually matured, they suppressed the cortical cells and led to their deformation (Fig. 8A). The lumen of each fibre was drastically reduced, resembling a slit ranging from 1.0 to 2.5 μm in diameter (Fig. 8B). The secondary cell wall of the PPF appeared relatively homogeneous under SEM. The two contiguous structures, the middle lamella and primary cell wall, were tightly combined as a compound middle lamella which was often detached from the secondary cell wall (Fig. 8B and C). The major wall layers of the PPFs were not easily discernable. In particular, the stratification structure with several layers on the inner parts of the G-layer was observed (Fig. 8D).

Discussion

Histology and development of PPF

The cross-sections of young hemp stems at 29 days after sowing consisted of two main regions: the phloem fibre region and the woody core region. The former is composed of epidermis, lamellar collenchyma, cortical parenchyma, endodermis, secondary phloem and phloem fibres (primary and secondary phloem fibres, both referred to as bast fibres). The phloem fibres are arranged in bundles and localized between the endodermis and vascular cambium (Bonatti et al., 2004; Crônier et al., 2005). Primary phloem fibres undergo differentiation just beneath the
endodermis; later, in ontogeny, secondary phloem fibres develop within the phloem region. Although the PPFs were obviously identified near the zone of the endodermis at each growth stage, secondary phloem fibres were either not observed or were difficult to find at the earlier stages of development under the LM. However, they were clearly observed in SEM images at 62 days.

Internode 1 exhibited a comparatively older stage of fibre development, whereas Internode 2 showed a younger developmental stage with compact cortical tissues including the ribs, which were rich in collenchyma and PPFs (Ordina 1978).

The most prominent changes in early development included changes in the external region: they were flattened, partially collapsed and with large intercellular spaces. At 52 days, the gradual expansion of secondary xylem cores progressively suppressed the cortical cells, leading to deformation of their features. This is thought to be caused by the rapid expansion of PPFs and a vigorous increase of secondary xylem. After the bidirectional elongation of phloem fibres, they join each other and form the bundle (Snegireva et al. 2015).

The PPFs commenced the process of proliferation and differentiation at 17 days, and ceased the process of differentiation at 62 days, although the thickening of the secondary cell wall continued in secondary phloem fibres located in the periphery of phloem. The PPFs of hemp are 10–40 mm long and originate from the procambium. In contrast, the secondary phloem fibres are shorter, 2–3 mm in length and are derived from the vascular cambium (Linger et al. 2002; Snegireva et al. 2010; Gorshkova et al. 2012; Snegireva et al. 2015). In the present study, the PPFs were approximately 23 μm in diameter and 350 μm in length at 17 days, which was nearly the 5 % maturation rate of the PPF. Sankari (2000) reported that phloem fibres account for 20–30 % of volume of each hemp stem at 10 days before harvest. However, the PPFs of hemp stems at the early development ranged from 4 to 9 % volume in this study.

**Microstructure and composition of the secondary cell wall**

Throughout the entire developmental period, the thickness of the cell wall of the fibres was uniform except for the greater thickness in the corners, and contained several electron-dense layers. This implies that the composition of the secondary cell wall varies. The S-layer in electron micrographs of flax was difficult to discern (Andème-Onzighi et al. 2000), but the layers of the secondary cell wall in hemp phloem fibres were distinctive. Xyloglucan, which reinforce the links between the primary cell wall and secondary cell wall in the S1 layer junction, seems to become easily disjointed (Bourquin et al. 2002). The thickness
of secondary cell wall in the PPF was approximately 1.3 μm at 29 days. However, the secondary phloem fibres were not discernible at this stage. In multilayered fibre cell walls, there seems to be no relationship between the layering structure and the thickness of the cell wall (Gritsch et al. 2004).

The different microfibril orientation of the secondary cell wall is such a characteristic feature in species that it was often used as a key in identification. It is designated as either a Z- or S-twist, which means a right- or left-handed fibre. In flax and hemp, S1 is a Z-twist and S2 is a S-twist in both species; however, S3 is a Z-twist in flax, but is almost parallel to the fibre axis in hemp (Lewin 2006). Additionally, the microfibril angles in flax and hemp are 6.5° and 7.5°, respectively (Haugan and Holst 2013). This difference in the microfibril orientation provides mechanical durability to cell walls through various mechanisms, for example, in cone opening and wheat awn movement (Burgert and Fratzel 2009), and the creation of tension in tension wood (Goswami et al. 2008). However, little is known about the biophysical mechanism of the cell wall structures caused by the orientation of microfibrils.

Currently, an immunocytochemical study of hemp revealed that only the S-layer of both PPFs and secondary phloem fibres was labelled with an antixylan antibody, whereas the remaining layers of the thickened cell wall did not contain epitopes for this antibody (Petit et al. 2019). This result confirmed that lignin was present only in the S-layer of the secondary cell wall in phloem fibres in hemp.

In this study, a test for the presence of lignin in PPFs was conducted by a histochemical staining. Phloroglucinol is the most common dye used for lignin detection in plant tissues. This staining produced a brilliant pink or red colour in the xylem, where the aldehyde groups are present. The intensity of the colours varies based on the lignin content (Adler 1977; Yang et al. 2013). As shown in Fig. 2B, phloroglucinol staining was negative in PPFs but very strong in xylem tissues. This result confirmed that almost no lignin deposited on the secondary cell wall of hemp PPF at 29 days or not detected at the light microscopic level.

In hemp PPFs, immunofluorescence studies using some antilignin monoclonal antibodies demonstrated that only the outer layer of the fibre cell walls was labelled, for example, LM10 and LM11 specifically for xylan (McCarteny et al. 2005), and LM15 for xyloglucan (Behr et al. 2016). Additional immunocytochemical studies also proved the occurrence of cell wall epitopes for
xylan in S1 (Gorshkova et al. 2015), for crystalline cellulose in all cell layers (Blake et al. 2008) and for galactans in the G-layer (Gorshkova and Morvan 2006) of flax PPFs. These specific techniques provided insight into the wall architecture of PPF and secondary phloem fibre in hemp.

**Modification of the Gn-layer into the G-layer**

The cell wall is a dynamic compartment in which the composition undergoes massive modification during development (Behr et al. 2016). The cell wall architecture is partially controlled and organized by the intracellular cytoskeleton (Bashline et al. 2014). Many studies have reported on the G-layer within phloem fibres of diverse taxa (Gorshkova et al. 2004; Gierlinger and Schwanninger 2006; Bowling and Vaughn 2008; Schreiber et al. 2010; Guerriero et al. 2013). However, the ontogeny of the G-layer is a matter of debate and some researchers have proposed the term-tertiary cell wall (Clair et al. 2018; Gorshkova et al. 2018). In this study we followed the terminology described by Tatyana et al. (2018). The galactan-enriched layer (Gn-layer), which is a newly deposited portion of the secondary cell wall, appears to be loosened and obtains a larger amount of electron-dense substances than the mature G-layer (Tatyana et al. 2018). The Gn-layer is a transitional layer that transforms into a G-layer as new portions of the Gn-layer are deposited. Therefore, the transition of the Gn-layer into the G-layer is a sequential process of secondary cell wall formation during fibre development (Roach et al. 2011).

As shown in Fig. 8D, the layering structure with several lamellae was characteristically observed on the G-layer of the secondary cell wall. As the stratification structure translocated into the outer region of the G-layer, these gradually lost their electron densities and became homogeneous. A similar result in flax bast fibres was previously reported by Gorshkova et al. (2010). Interestingly, the Gn-layer also consisted of a minute stratification with high electron densities. This result suggests that the previously formed lamellae on the Gn-layer gradually move on the outer region when additional lamellae laid down newly onto Gn-layer.

At 52 days after sowing, the Gn-layer was loosely packed and showed a heterogeneous structure, with parallel bands of electron-dense substances separated by a more transparent matrix. In contrast, the G-layer was much more homogeneous and lacked electron-dense bands. The cellulose microfibrils in thick G-layers are almost parallel to each other and the longitudinal cell axis (Rüggeberg et al. 2013). Specifically, the most active period of secondary cell wall thickening in PPFs occurred between 52 days and 62 days.

At 62 days after sowing, the S-, G- and Gn-layer were distinctly evident, and the secondary cell wall of PPF was almost homogeneous under both SEM and TEM observations. The middle lamella and primary cell wall are contiguous structure and become indiscernible (Sorieul et al. 2016). The PPF in hemp frequently showed the compound middle lamella when they were isolated from the secondary cell wall as shown in Figs 5 and 6.

Considering that most of the Gn-layer of the secondary cell wall was modified into a mature G-layer, it was slightly difficult to discriminate between two layers. The transition
from the Gn-layer to G-layer involves modification of a tissue-specific galactan. β-Galactosidase is specifically required for the hydrolysis of galactans during the formation of cellulosic G-layers (Roach et al. 2011). However, this process may not be completed during fibre development, because a very thin layer of Gn-layer remains in the innermost region of secondary cell wall in a fully developed fibre.

The microtubules lie underneath the plasma membrane during secondary cell wall synthesis (Watanabe et al. 2015). Meents et al. (2018) reported that numerous secondary cell wall cellulose synthase complexes produced cellulose microfibrils, which consist of high glucan chains in the plasma membrane; these microfibrils are extruded into a cell wall matrix rich in the secondary cell wall under the guidance of a cortical microtubule. Other studies have suggested that microtubules become bundled and line the plasma membrane in the region of the secondary cell wall formation (Fujita et al. 2011; Watanabe et al. 2015). Furthermore, transverse microtubules are mostly correlated with a rapid growth in plants (Bashline et al. 2014). In the PPF cells of hemp, cortical microtubules were also regularly arrayed adjacent to the plasma membrane. Moreover, the cortical microtubules affect the orientation of cellulose microfibris in the cell wall during plant growth and development (Murata et al. 2013; Bashline et al. 2014). The cellulose microfibrils in thick G-layers are almost parallel to each other and the longitudinal cell axis (Rüggeberg et al. 2013). At 52 days, a large number of cellulose microfibrils with a high electron density were deposited parallel to the cell axis on the Gn-layer of the secondary cell wall. Specifically, these microfibrils were seen as clusters of tiny spots due to the transverse-sectioned images. Similarly, a schematic of cell wall architecture depicting the relationship between the microtubules and wall complexes was proposed (Cyr 2005).

According to the multi-net growth hypothesis, new layers of cellulose microfibrils are deposited in a transverse orientation due to the transverse-sectioned images. At 62 days, the G-layer exhibited the stratification structure with several lamellae. This specific structure seems to be related to the modification process of the Gn-layer to the G-layer. The 62-day stage seems to be a critical point of programmed cell death in PPF.

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Conflict of Interest
None declared.

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Contributions by the Authors
W.C. performed the experiments. E.S.K. analysed the image data and drafted manuscript. S.H.P. helped in statistical analysis and reviewed manuscript. All the authors have read and approved the final version of this article.

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Conclusions
The thickening and modification process of PPFs during the early development of stem in C. sativa was observed using optical microscopy, SEM and TEM. The initial cells of PPF started their proliferation and differentiation at 17 days, although the secondary cell wall thickening had already commenced before the 29-day stage. At 29 days, the PPF consisted of a thin primary cell wall and an S-layer, while the PPF in the other two stages added a G-layer and Gn-layer on the secondary cell wall. At 52 days, the process of the secondary cell wall thickening actively progressed. An array of cortical microtubules appeared close to the plasma membrane, and cellulose microfibrils on the Gn-layer of the secondary cell wall were also observed. The microfibrils were seen as clusters of tiny spots on the Gn-layer due to the transverse-sectioned images. At 62 days, the G-layer exhibited the stratification structure with several lamellae. This specific structure seems to be related to the modification process of the Gn-layer to the G-layer. The 62-day stage seems to be a critical point of programmed cell death in PPF.
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