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Involvement of peroxidases in structural changes of barley stem

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ABSTRACT: The aim of this study was to investigate anatomical characteristics of barley (*Hordeum vulgare* L.) internodes and to reveal the connection with activity of guaiacol-peroxidases, their isoenzyme profiles, lignin, and cellulose content. Three lower internodes were sampled at elongation stage and differed in their anatomical features by showing that lignin deposition follows developmental pattern. Epidermis and hypodermis in the first internode were characterized by continuous layer of the lignified cells while in the second and third internode thin walled parenchyma cells could be observed in the hypodermis. These observations were in accordance with increment of peroxidase activity from the first to the third internode. Two acidic and 2 basic isoforms of peroxidases were present. The third internode had higher total soluble protein content and lower dry weight as well as Klason lignin content in comparison to the first and second internode. Studying lignification process in barley stem by using anatomical and physiological approach will provide valuable information regarding developmental changes connected with lignin deposition in the stem.

KEY WORDS: lignin, cell wall, internode, *Hordeum vulgare* L.

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INTRODUCTION

Peroxidases are heme-containing glycoproteins encoded by a large multigene family due to repeated gene duplication during evolution (Passardi et al. 2004). The lack of strict substrate specificity suggests that peroxidases are involved in a number of processes in plants such as auxin metabolism, cell wall modification (e.g. lignification, suberinization), defense against pathogens, senescence, salt and heavy metals tolerance (Passardi et al. 2005). Recently 2 possible modes of peroxidase functioning, named peroxidative and hydroxylic cycles, have been revealed (Passardi et al. 2004). The hydroxylic cycle leads to the production of reactive oxygen species (ROS), such as OH^- and HOO^- radicals, in the presence of hydrogen peroxide (Chen and Schopfer 1999). Such bi-functionality of peroxidase enzymes enables them to play an important role in the regulation of ROS homeostasis, which in turn influences a plethora of cellular processes. In the peroxidative cycle, in turn, peroxidases catalyzes the oxidation of various substrates such as phenolics compounds and lignin precursors coupled with the reduction of hydrogen peroxide (Cosio and Dunand 2009). The role of peroxidases in the lignin polymerization is the most extensively studied function of this class of enzymes. The oxidative coupling of monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohol is catalyzed by class III peroxidases and/or laccases, which leads to the formation of lignin subunits and their subsequent deposition in the cell wall (Vanholme et al. 2010). The involvement of different peroxidases in lignification process, through biochemical and genetic characterization, has been subject of various studies (Herrero et al. 2013; Marjamaa et al. 2009; Shigeto et al. 2015) indicating the requirement for further elucidation of specific role of particular peroxidase isoform in this important process of plant cell differentiation.

Lignin comprises approximately 20% of the secondary cell wall in grasses (Vogel 2008). As a complex aromatic polymer, lignin has many functions such as defense against pathogens and insect as well as it facilitates impermeability of cell wall of the vascular tissues for water and nutrient transport (Boerjan et al. 2003). In crops, such as barley, lignin also plays an important role in resistance to lodging. The mechanical strength of the stem is a very important factor since lodging significantly affects yield and reduces grain quality. Stem strength is determined by the length of the internodes, the thickness of the stem, and stem cortex (van den Berg and Labuschagne 2012). Lignification of the cell wall is spatially and temporally regulated in different tissues and cells (Grabber et al. 2004). Our previous studies have shown that lignin content varies among barley genotypes (Begović et al. 2015) and that lignin deposition in different barley cultivars depends on growth stage. During development, barley stems undergo numerous anatomical and physiological changes that affect their structure and strength and are associated with the process of lignification. The growth of barley stems occurs between nodes and internodes where intercalary meristems can be found. The basal internodes significantly contribute to the mechanical strength of barley stems (Peng et al. 2014). It has been shown by Jung (2003) that developing maize internodes differ in their developmental profile, from bottom to top of the stem due to the different dynamics of secondary cell wall growth and subsequent lignin deposition. At elongation phase in crops, such as barley, wheat, and rice, the first internode is fully developed, while upper internodes are still elongating till anthesis stage. However, it still remains largely unknown how these differences in lignin deposition arise.

Therefore, the objective of this study was to investigate anatomical characteristics of the 3 bottom internodes and to reveal the connection between lignin content and activity of guaiacol-peroxidases and their isoenzyme profiles. We hypothesized that the activity of peroxidase enzymes are associated with the different pattern of lignin deposition in barley stem.

MATERIAL AND METHODS

Plant material and growth conditions

Spring barley (*Hordeum vulgare* L.) cultivar Scarlett was grown under the field conditions at Agricultural Institute Osijek (lat 45°32′N, long 18°45′E). The experiment was set up in a random block design (RCBD) in 4 replications with the area of the basic plot 7.56 m^2 and sowing density of 450 kernels·m^2 per plot. Plants were grown in eutric cambisol pH in 1 M KCl 7.10, humus 3.0% with P_2O_5 = 27.0 mg·100 g^{-1} and K_2O = 25.9 mg·100 g^{-1}. The →

first 3 internodes, starting from the bottom of the plant, were collected at elongation stage (Z 37) according to Zadoks et al. (1974), excised, and leaf sheaths were removed.

Preparation of tissue for microscopy

For all histological preparation middle part of the internode was used. Tissue was fixed in 6% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) at 4 °C. The dehydration was done in 2-methoxyethanol, ethanol, n-propanol and n-butanol (2 changes in each). Then, specimens were embedded in methacrylate resin (Historesin, Leica) according to manufacturer instructions. Three µm thick sections were obtained using the Leica RM 2155 rotary microtome with the glass knife. Sections were stained with 0.05% Toluidine blue O in benzoate buffer pH 4.4 (Lepeduš et al. 2001). Lignin was detected in fresh free-hand sections with phloroglucinol-HCl (Lepeduš et al. 2004).

Proteins extraction and tissue dry weight determination

Prior to all extractions internodes were cut into small pieces and macerated in liquid nitrogen. For soluble proteins analysis, one part of the obtained fine powder was used for the extraction of proteins and the other for the determination of dry weight by drying at 105 °C for 24 h. Soluble proteins were extracted in ice-cold 0.1 M Tris-HCl buffer, pH = 8.0, with the addition of polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 20,000 x *g* and 4 °C, for 20 min (Lepeduš et al. 2005). The obtained supernatant was used for protein quantification, peroxidases activity determination as well as for isoelectric focusing analysis. For SDS-electrophoresis, samples were extracted at 80 °C with buffer containing 0.13 M Tris-HCl (pH 6.8), 16% (v/v) glycerol, 4.6% sodium dodecyl sulphate (SDS, w/v), and 0.59% dithiothreitol (DTT, w/v).

Guaiacol peroxidase assay and soluble proteins content determination

The activity of guaiacol peroxidases (GPOD) in the supernatant was determined spectrophotometrically by measuring the absorbance increase at 470 nm. The reaction mixture contained 5 mM guaiacol and 5 mM H_2O_2 in 0.2 M phosphate buffer, pH 5.8. The enzymatic reaction was started by adding 200 µL of the extract to 800 µL of reaction mixture. The protein content of the extracts was determined according to Bradford (1976) using bovine albumin serum (BAS) as a standard.

SDS-PAGE and isoelectric focusing

Proteins were separated under denaturing conditions by means of discontinuous vertical polyacrylamide gel electrophoresis (PAGE). For PAGE the 1-mm-thick stacking (5%) and separating (12%) polyacrylamide gels were prepared according to (Laemmli 1970). The electrophoresis was carried out at 20 mA of constant current at 4 °C. The visualization of protein bands was done by staining the gel with Coomassie blue R.

The expression of peroxidase isoenzymes was investigated by isoelectric focusing (IEF), which was performed on 5% polyacrylamide gel with ampholytes in the pH range of 3.5 – 10, at 4 °C (Bollag et al. 1991). The running conditions were: 30 min at 100 V, 1.5 h at 200 V followed by 3 h at 400 V. The bands of isoperoxidases were visualized by immersing the gel in the same reaction mixture as the one used for peroxidase activity determination.

Determination of total lignin and cellulose content

Each internode was ground separately by using 0.5 cm mesh (Retsch, Germany). Samples were dried for 48 h at 65 °C until constant weight. For lignin content analysis tissue was extracted four times with 80% ethanol for 30 min at 80 °C followed by extraction with 100% acetone. Samples were dried overnight at 90 °C. One hundred milligrams was used for determination of Klason lignin (Kirk and Obst 1988) and 70 mg for determination of cellulose content, which was determined according to the procedure of Foster et al. (2010) as follows: 1 mL of Updegraff reagent (Updegraff 1969) was added to the samples, heated at 100 °C for 30 min and centrifuged. Samples were air dried overnight after washing with water and acetone and hydrolyzed with 72% sulfuric acid. Anthrone colorimetric assay was used for determination of cellulose content. A standard curve was prepared using →

glucose (1 mg·mL^{-1}) and anthrone reagent (2 mg·mL^{-1} sulphuric acid). Results for cellulose content are expressed as glucose equivalent (µg of cellulose·g^{-1} DW) while results for Klason lignin are expressed as % of cell wall residue (% CWR).

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze statistical significance between guaiacol peroxidase activity, total soluble protein content, dry weight, Klason lignin, and cellulose content followed by post hoc LSD test (least significant difference). The statistical analyses were conducted with Statistica 12.5 Software (StatSoft Inc., USA). The results are presented as average values of four replicates (n = 4) ± standard deviation (SD). The differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The microscopic inspection revealed differences in anatomical features of the 3 internodes at elongation stage. On transverse sections, the vascular bundles, typical for monocotyledons, were observed. They are arranged in 2 circles with inner bundles larger than the outer (Figure 1). In the first internode, the epidermal and the hypodermal layers formed a continuous ring of cells with the narrow lumen (Figure 1a). On the other hand, in the second internode (Figure 1b), loose parenchyma cells were interpolated below epidermis as well as in the third internode but to a greater extent (Figure 1c). Parenchyma cells were larger with thinner cell walls and occupied a substantial part of the hypodermal layer. These parenchyma cells, called chlorenchyma, can contain chloroplasts and exhibit photosynthetic activity (Matos et al. 2013).

Histochemical evidence of lignin in the 3 barley internodes revealed different lignification pattern in coinciding with anatomical differences between internodes observed after staining with toluidine blue. In the epidermal and hypodermal layer lignified cell walls were found in all 3 internodes (Figure 1). In contrast, interpolated parenchyma cells displayed a reduced amount of lignin in cell walls (Figures 1b,c). Vascular bundles, particularly xylem, were wider in the second and third internode in comparison to the first, (Figure 1) as xylem →

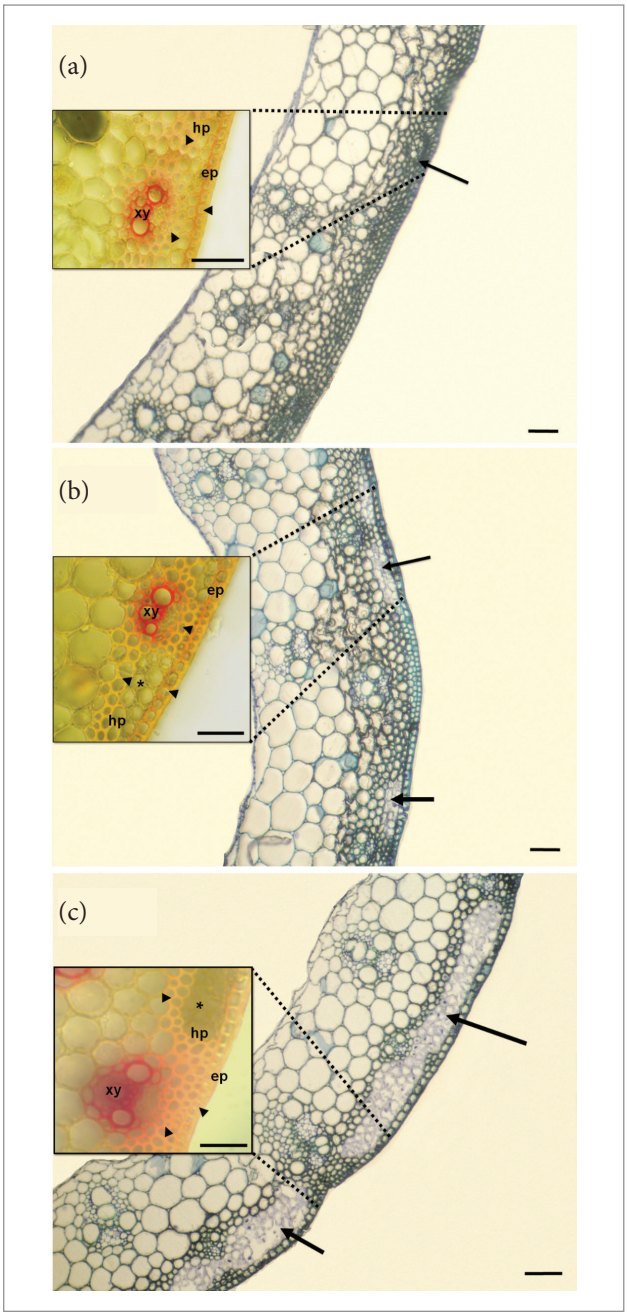

Figure 1. Transverse 3 µm thin sections of the 3 barley internodes stained with Toluidine blue O (a, b, and c) and free-hand sections stained with phloroglucinol-HCl (inserts). The first internode (a) had continuous lignified epidermis and hypodermis (arrow), while loose parenchyma cells (arrow) were interpolated in the second (b) and the third (c) internodes below the epidermis. Based on the red coloration, the highest lignin deposition in all internodes was found in the xylem (xy). Certain lignification of the cell walls (inserts) was present in the epidermal (ep) and hypodermal (hp) layers in all 3 internodes (arrow head), while there was no lignin deposition in parenchyma cells below the epidermis (asterisks). Scale bar = 500 µm.

cells go through apoptosis faster in older internodes. In developmentally older internodes, secondary cell wall thickening and deposition of lignin mark the completion of elongation stage (Jung and Casler 2006) whereas younger parts still elongate and undergo subsequent lignin deposition process. This pattern was also observed in the present study, as the extent of the lignified tissue decreased from the first to the third internode (Figure 1).

The observed anatomical features went along with dry weight contents in the internodes (Figure 2a). Our results showed that the first internode had significantly higher dry weight content in comparison to the second and third, which had the lowest dry weight content. The increase of the cell wall material (i.e. cellulose, hemicellulose, and lignin) deposited in the primary and secondary cell walls significantly contributes to the dry weight content (Vogel 2008). This statement was further corroborated by the Klason lignin analysis, which showed higher lignin content in the first and second, more mature, internodes (Figure 2b). The result of this study also confirmed that, at elongation stage, lignin deposition occurred earlier in the lower internodes (the first and second) than in the third. Also, similar to maize (Jung 2003), barley internodes demonstrated different developmental profiles from the bottom to the top of the stem with lower internodes being older and more lignified. On the other hand, cellulose content was not significantly different between internodes (Figure 2c). The reason for such result may be explained by the fact that cellulose deposition precedes lignin deposition. Cellulose serves as a network of microfibrils and thus, enables lignin deposition and consequential stiffening of the secondary cell wall (Cosgrove and Jarvis 2012). Therefore, we assume that the observed increase in dry weight content from the first to the third internode could be mainly attributed to the lignin deposition in the secondary cell wall, indicating the completion of the elongation stage of particular internode and maturation of the cells.

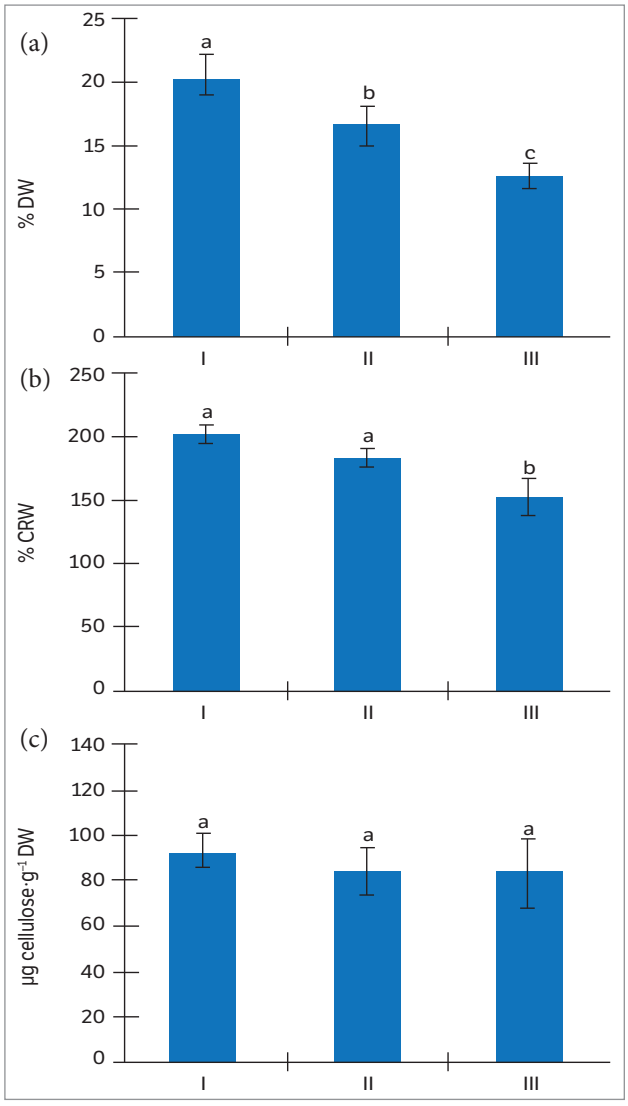

Figure 2. (a) Dry weight (DW), (b) Klason lignin expressed as percentage of cell wall residue (%CWR) and (c) cellulose content expressed per DW in the first (I), second (II), and third (III) internodes of barley at elongation stage. Bars denote mean values of 4 replicates (n = 4) ± standard deviation. Different letters indicate significance at $p \leq 0.05$.

Previous findings showed that simultaneously with the formation of the secondary cell wall autolytic factors in vacuole accumulate and indicate the beginning of the cell death. In investigated barley internodes, the measurement of total soluble protein content showed that the third (youngest) internode had the highest content, oppositely to the first and second which had similar, but decreased content (Figure 3). These results demonstrated that, in developmentally younger tissues, higher demand for soluble proteins was present as a consequence of growth process unlike in older internodes (the first and second) where cell death process was more pronounced considering increased lignin content (Figure 2b) and subsequent deposition in the cell wall (Figure 1).

Previous studies have shown that anionic as well as cationic peroxidases are involved in lignification (Cesarino et al. 2012; Lin et al. 2016) and that they differently contribute to the lignin polymer formation →

(Li et al. 2003). In addition, *Arabidopsis* plants with a disrupted function of three peroxidase isoforms showed lower lignin content (Shigeto et al. 2015). Due to the fact that investigated barley internodes differed in lignin content (Figure 2b), we analyzed guaiacol-oxidizing peroxidases by IEF gel. Our results showed that 4 isoforms were present in all 3 internodes (Figure 4a). Two isoforms of cationic peroxidases, namely POD 1 and POD 2, and 2 of anionic peroxidases, POD 3 and POD 4, were detected. Interestingly, the intensity of the cationic bands (POD 1 and POD 2) was higher in the first than in the second and third internodes as opposed to anionic isoforms (POD 3 and POD 4) that were similar in all 3 internodes. However, analyses by SDS-PAGE did not reveal any specific variation in protein patterns between the 3 internodes (Figure 4b) indicating that the observed differences were more quantitative than qualitative. Since it has been shown that transgenic aspen lines had 20% lower lignin content as a consequence of anionic peroxidase down-regulation (Li et al. 2003) and that down-regulation of cationic peroxidase decreased lignin by 50% in tobacco (Blee et al. 2003) we assume that higher intensity, and therefore activity, of the 2 cationic isoforms, might be related to the higher lignin content (Figure 2b) and observed more lignified epidermal and hypodermal layer in the first internode (Figure 1a). These results also coincided with analyses of peroxidase activity using guaiacol as a substrate, which showed that activity was the highest in the first, more lignified internode, and significantly decreased in the second and third internode (Figure 5).

The lignification of the cell wall is a part of development and differentiation processes of distinct cell types in plants (Wang et al. 2013). In crops, lignin is important for stem strength enabling mechanical support for the spikelet, especially during grain filling. The dynamics of lignin synthesis and deposition are essential for proper function as well as for adaptation of plant to the environmental changes (Barros et al. 2015).

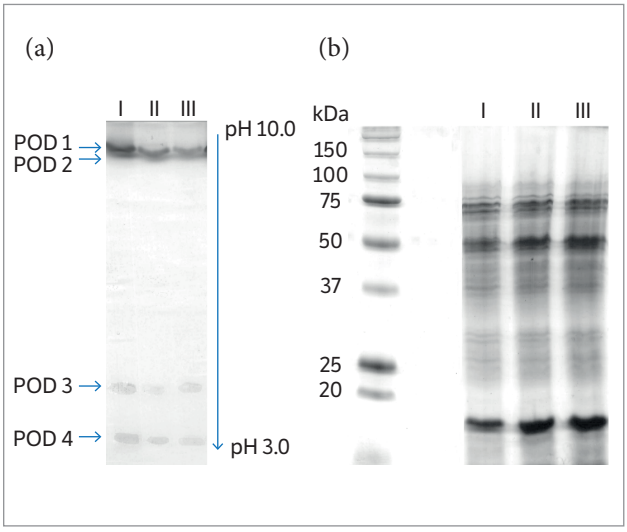

Figure 4. (a) Isoenzyme pattern in the first (I), second (II), and third (III) internodes after isoelectric focusing. Arrows indicate the position of 4 isoforms of peroxidase (POD) stained with guaiacol; (b) SDS-PAGE of total protein extracts from the first (I), second (II), and third (III) internodes.

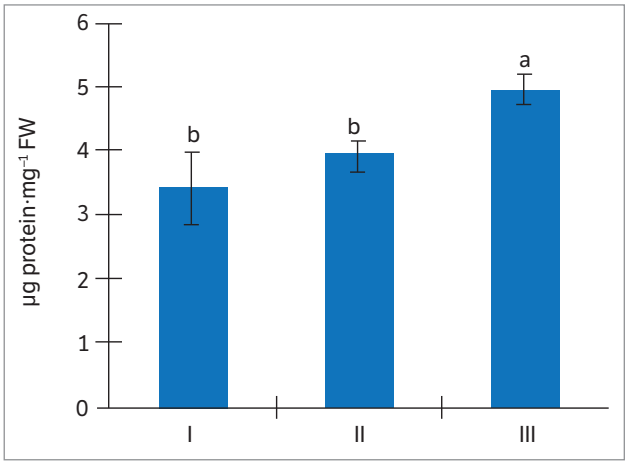

Figure 3. Total soluble protein content in the three internodes of barley at elongation stage expressed as µg of protein per g of fresh weight (FW). Bars denote mean values of four replicates (n = 4) ± standard deviation (SD). Different letter indicate significance at $p \leq 0.05$.

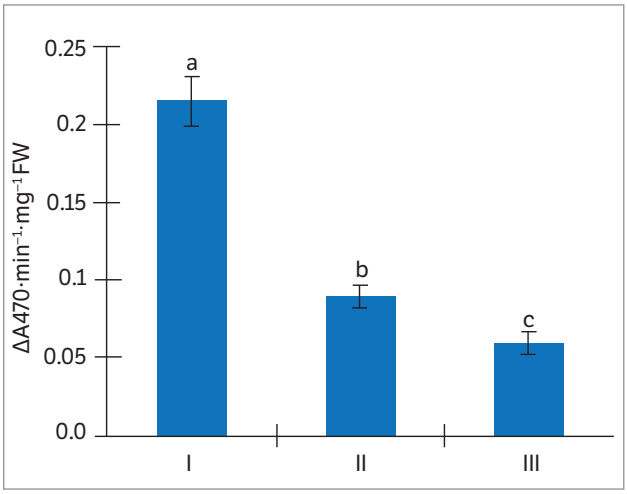

Figure 5. Total activity of guaiacol peroxidases in the 3 internodes of barley at elongation stage. Bars denote mean values of 4 replicates (n = 4) ± standard deviation (SD). FW = Fresh weight. Different letters indicate significance at $p \leq 0.05$.

CONCLUSION

The results presented in this study demonstrate that structural changes associated with barley internode development are related to the lignin deposition in the secondary cell wall and connected to the peroxidase activity. Results showed that more mature internodes, the first and the second, had significantly higher lignin content and peroxidase activity. Isoelectric focusing showed that 2 observed cationic peroxidase isoforms were to some extent more pronounced in the first, also more lignified, internode compared to the anionic once. However, the potential involvement of particular peroxidase isoforms in lignin polymer formation during barley stem development should be explored in more detail in future research.

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