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Role of Class III Peroxidases in Stem Lignification of *Zinnia elegans* Jacq. †

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Abstract: Class III peroxidases (EC 1.11.1.7) have use a wide range of substrates and perform numerous functions, including synthesis of monolignols, lignin precursors. The activity and tissue localization of cationic guaiacol (GPOX) and anionic benzidine peroxidases (BPOX) were studied in the first internode of zinnia plants of different age. The lignin was staining in cross section by phloroglucinol-HCl; and lignin content was determined by Klason. Enzyme activity and H_2O_2 amount were determined spectrophotometrically. The hypocotyl grew for 40 days and the lignin content for 60 days. In 20 days-old plants lignin was detected in protoxylem, and in 60 days-old–in sclerenchyma, protoxylem and metaxylem. Enzyme histochemistry revealed that BPOX was localized in endoderm, phloem, and protoxylem, while GPOX–in the metaxylem and sclerenchyma. A moderate increase of GPOX activity during internode growth was shown. In contrast, BPOX activity was high at the initial growth stage, and declined to 60-th day. Thus, the most intense lignification in mechanical tissue and xylem occurred during the period from 20 to 40 days of plant growth. BPOX is likely involved in the process at the early stages of growth, while GPOX is responsible for sclerenchyma and metaxylem lignification at the later stages.

Keywords: peroxidases; plant development; hydrogen peroxide; lignin; zinnia

1. Introduction

The III class peroxidases (EC 1.11.1.7) includes enzymes localized in vacuoles and apoplast. They oxidize a wide spectrum of phenolic substrates using hydrogen peroxide as electron donors [1]. The oxidase activity of peroxidases leads to the formation of reactive oxygen species, such as OH and HOO radicals. The dual function of peroxidases, their affinity for many phenolic compounds, provide numerous functions, as protection against pathogens [1], healing of wound surfaces, catabolism of auxin and anthocyanins, and porphyrin metabolism [2,3].

Apoplastic peroxidases oxidize monolignols (coumaric, coniferyl, and sinapyl alcohols) with the formation of radicals–lignin precursors [4]. According to the isoelectric point, class III peroxidases are divided into acidic, neutral and basic [4,5]. It is known that basic peroxidases (isoelectric point > 7.0) oxidize para-coumaric, coniferyl, and synapyl alcohols [5], while acidic peroxidases (isoelectric point < 7.0) have a low affinity for the oxidation of synapyl alcohol [6], therefore the role of basic and acidic peroxidases in cell wall lignification may be different. Much attention is paid to the study of the role of the main peroxidases in the lignification of the cell walls of herbaceous plants, including zinnia. It was revealed that ZePrx peroxidase oxidize sinapyl alcohol [7]. Purified peroxidase ZPO-C from *Z. violacea* in vitro was shown to use both synapyl and coniferyl alcohols as a substrate [8]. It was reported that the activity of class III peroxidase isoforms is tissue-specific and changes during

plant growth [9]. It was shown that the specific activity of class III peroxidases is involved in regulation of cell growth and differentiation in various tissues through the lignification of cell walls. Since the lignin provide strength and hydrophobicity of cell walls, it limits cell growth by stretching. So, studying of lignification dynamics during plant development is essential for better understanding of plant growth and adaptation to the biotic and abiotic stress factors.

In our study, the histochemical staining of BPOX (optimum pH < 5.0) and GPOX (optimum pH in the range of 7.0–7.5) on cross sections of the **z**innia stem was done, and the determination of their activity was fulfilled spectrophotometrically. We assume that the activity of these peroxidases is specific for different stages of lignification and tissue development in the first internode of zinnia.

2. Experiments

Growth condition. Plants of zinnia (*Zinnia elegans* Jacq.) Were cultivated on a pre-autoclaved substrate–a mixture of soil: coconut substrate in a ratio of 3: 1. Plants were grown under conditions of a 16/8 photoperiod and a temperature of 23 °C for 60 days. When they reached the age of 20 (juvenile), 40 days (vegetative) and 60 days (flowering), the length of the first internode (above the cotyledons) was measured.

Lignin staining. The first internodes of plants were fixed in Clarke's solution (glacial acetic acid and ethanol 1:3) at room temperature. Then samples were washed and stored in ethanol at +4 °C. For histochemical studies, the cross-sections of 20, 40 and 60-day-old plants were prepared with a freezing microtome MZ-2 (Medpribor, Russia). The samples were stained with 1% phloroglucinol (*w*/*v*) in 12% HCl for 5 min, washed in distilled water, stored in 50% glycerin [10]. Sections were studied by a Meiji MT 4300L light microscope («Meiji Techno», Japan).

Histochemical peroxidase localization. Hand-made cross sections (~0.5 mm thickness) of fresh internode were stained. For the detecting BPOX–in the solution, composed of 0.85% NaCl, 0.1% ammonium molybdate and benzidine with 20% hydrogen peroxide. The stem sections were incubated for 5 min at 4 °C [11]. For GPOX–in 0.7% guaiacol with 0.03% hydrogen peroxide solution [12]. Stem sections were incubated for 10 min at 20 °C.

Biochemical studies. The activity of GPOX (EC 1.11.1.7) and BPOX (EC 1.11.1.7), and the hydrogen peroxide content were determined spectrophotometrically using Shimadzu UV-1800 (Shimadzu, Japan) in three biological and five analytical replications.

The fresh plant material was homogenized on ice with 0.05 M Tris-HCl buffer (pH 7.0 The homogenate was centrifuged at 12,000× *g* for 30 min at 4 °C. The supernatant was used for enzyme assays and determination of hydrogen peroxide. The BPOX activity was determined based on the rate of the benzidine oxidation by H_2O_2 at pH 5.0 and was expressed Units/mg protein*min [13]. The GPOX activity–based on the rate of the guaiacol oxidation by H_2O_2 as an electron donor, at pH 7.0 and was expressed mM guaiacol/mg protein*min [14]. The protein content was determined according to Bradford using bovine serum albumin as a standard [15,16].

The amount of H_2O_2 was determined by the method based on the oxidation of xylenol orange chelates with iron (III) ions with hydrogen peroxide and was expressed in μM hydrogen peroxide/g dry weight [17].

Lignin content. The dry stem biomass (500 mg) was treated by 72% H_2SO_4 (5 mL) at 20 ± 1 °C over 2.5 h. The material was diluted to 3% H_2SO_4 and then refluxed at 80 °C for 2 h. The Klason lignin content was determined after recovery by filtration and drying of acid-insoluble residue at 110 ± 2 °C over 16 h [18].

Statistical Analysis. Statistical data processing was carried out in the STATISTICA 10 program for Windows 10 using Student's *t*-test for morphometric parameters and Mann-Whitney U-test for biochemical parameters.

3. Results

In zinnia the length of the first internode increased for 40 days from seed germination (vegetative stage of growth), and did not change for the next period of plant growth (flowering stage, 60-days). So, the elongation period for this internode last for several weeks.

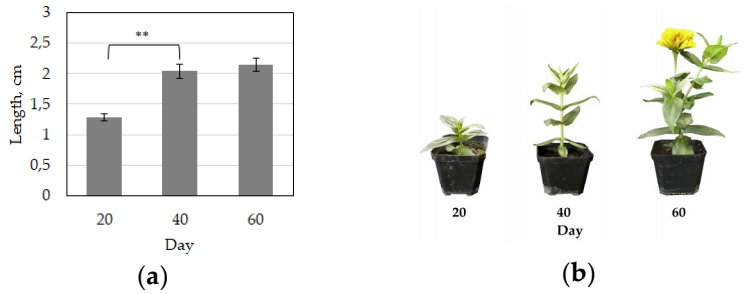

Figure 1. The length of the first internode (**a**) and plants height (**b**) in zinnia plants of different age. The statistical significance of differences was determined by Student's *t*-test (** $p < 0.01$).

According to the histochemical analyses and Klason lignin assay the content of total lignin in stem tissues increased during all studied period. Phloroglucinol-HCl staining revealed that in juvenile plants (20 days) lignin was found mainly in protoxylem and differentiating vessels of metaxylem, and in adult plants (40 and 60 days-old)–in sclerenchyma, protoxylem and metaxylem (see Figure 2). In a cross section, the staining of lignin revealed the increase of lignified tissues during plant development.

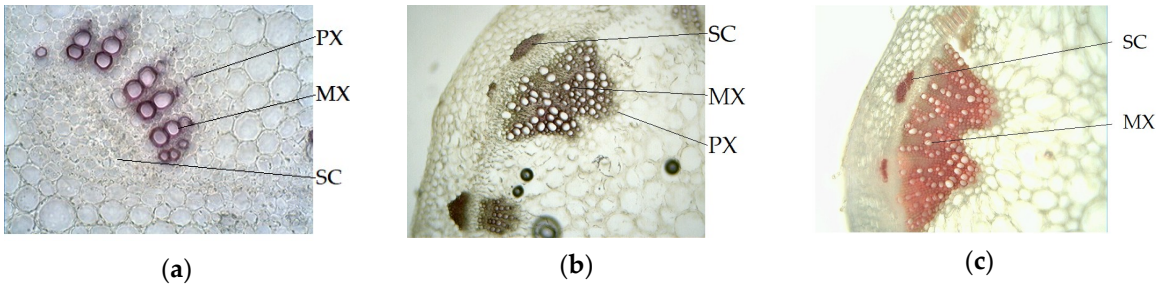

Figure 2. Lignification of xylem and sclerenchyma in *Zinnia* plants. First internode cross section of 20, 40 and 60-days old plants were stained with phloroglucinol-HCl. PX—protoxylem, MX—metaxylem and SC–sclerenchyma.

The amount of lignin increased linearly while internode grew (see Table 1). Its content was 1.24 times less at the 20-th day then at 40-th, and 1.16 times higher at the 60-th day compared to 40-th. The intense lignification evidenced on the formation sclerenchyma in vascular bundle, mechanical tissues, and xylem differentiation. Thus, at the stage of intensive elongation the lignin content was lower than in old stem tissues.

Table 1. Klason lignin in stem tissues, hydrogen peroxide content and peroxidases activities in the first internode of zinnia on different growth stages.

Plant Age, Days	Klason Lignin, %	H_2O_2, μM g^{-1} dry weight	BPOX, Units mg^{-1} protein * min	GPOX, mM guaiacol mg^{-1} protein * min
20 (juvenile)	14.30 ± 0.56	114.2 ± 6.8	1.03 ± 0.045	0.21 ± 0.03
40 (vegetative)	17.71 ± 0.61 [a]	171.2 ± 2.6 [a]	2.99 ± 0.12 [a]	0.42 ± 0.01 [a]
60 (flowering)	20.58 ±1.62 [b]	179.2 ± 5.3	0.27 ± 0.01 [b]	0.61 ± 0.04 [b]

[a,b] statistical significance of differences was determined by U-test ($p < 0.01$).

It was shown that anionic as well as cationic peroxidases are involved in the lignification, but they use different phenolics as substrates [4,5,19]. H_2O_2 is also the substrate for peroxidases, involved

in lignin synthesis, and its content increased in internode tissues for 40 days by 33.2%, and then did not change up to 60 days (see Table 1).

In our research a moderate increase of GPOX activity was shown during internode growth, and it positively correlated with Klason lignin content in stem (see Table 1). The high activity of the enzyme was observed in flowering plants. In contrast, BPOX activity was high at the initial growth stage (20 and 40 days), and declining to 60 days. The maximal activity was found on vegetative stage of growth (40 days-old plant).

Figure 3. BPOX and GPOX localization in the first internode of juvenile (20 days-old) *Zinnia* plants. PX–protoxylem, MX–metaxylem and SC–sclerenchyma.

Histochemistry of enzymes in plant tissues revealed that BPOX was localized in endoderm, phloem, and protoxylem in juvenile zinnia plants, while GPOX–in the metaxylem and sclerenchyma (see Figure 4). So, anionic and cationic peroxidases may be localized in different tissues and cold be involved in their differentiation.

4. Discussion

It is known that accumulation of high amount of lignin led to the thickening of the secondary cell walls and marked the end of the cell elongation. In juvenile cells the elongation lasts before the lignin deposition process begins. Hydrogen peroxide is a marker of lignification; together with phenolic compounds, it is used by class III peroxidases to form monolignol radicals–the precursores of lignin. According to Ros Barcelo and Novo-Uzal, in juvenile plants H_2O_2 is localized mainly in non-lignified cells of stem parenchyma and protoxylem, and in adult–in mechanical tissues, metaxylem, and phloem [20,21]. In our study the H_2O_2 amount was lower in the young internode and higher in the mature one.

It was shown, that in barley stem in mature internode cationic isoforms of peroxidases were active, while in young internode–anionic isoforms [19]. Our data confirm this: we have found the high BPOX activity at the early stages of zinnia development, and GPOX was active in mature tissues. Probably, high activities of anionic and cationic peroxidases at the vegetative stage (40 days) of zinnia growth may provide the intensive increase of lignin content and the amount of lignified tissues in the first internode.

5. Conclusions

The cell wall lignification is an important process in cell development. The use of histochemical and biochemical approaches in studying cell lignification in zinnia internode provided valuable information about the lignin deposition and peroxidases functions during plant development. The most intense lignification in the first internode of zinnia was revealed in mechanical tissue and xylem during the period from 20 to 40 days of plant growth. Class III peroxidases had a different tissue localization. Anionic BPOX is likely involved in the processes at the early stages of growth, and localized in endoderm, phloem, and protoxylem, while cationic GPOX was responsible for sclerenchyma and metaxylem lignification at the later stages of zinnia development.

Author Contributions: A.T., A.E., H.W. and I.K. conceived and designed the experiments; A.T. and D.P. performed the experiments; A.T., A.E. and I.K. analyzed the data; A.T. and I.K. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BPOX	Benzidine peroxidase
GPOX	Guaiacol peroxidase
PX	Protoxylem
MX	Metaxylem
SC	Sclerenchyma

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