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Cytochemical Evidence on Seasonal Variation of Peroxidase Activities in Cambial Region of *Pinus densiflora*, *Ginkgo biloba*, and *Populus alba*[*1]

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ABSTRACT

The peroxidase activity was localized cytochemically to get an insight into its precise function in lignin biosynthesis. In this work, cerium chloride ($CeCl_3$) was used as a trapping agent for hydrogen peroxide (H_2O_2) generated from peroxidase. Seasonal variation of peroxidase activities in cambial region of *Populus*, *Pinus*, and *Ginkgo* was investigated at subcellular levels. Under transmission electron microscopy, electron dense deposits of cerium perhydroxide formed by reaction with H_2O_2 were observed in cambium and its immediate derivatives. The staining with $CeCl_3$ in cambium varied with growth seasons. The strongest H_2O_2 accumulation, regardless of tree species, appeared in May. Staining pattern of $CeCl_3$ in the cambium of poplar indicated that the production of peroxidase started in March before the opening of buds and reached the highest in May and then declined in August. *Ginkgo* and *Pinus* showed relatively late generation of H_2O_2 production when compared with *Populus*. Although *Ginkgo* and *Pinus* are classified into gymnosperms, however, the generation of peroxidase production and its duration was different from each other. Little staining appeared in all the tree samples collected in September before falling the leaves.

Keywords : Peroxidase, lignin biosynthesis, cambium, cytochemistry, cerium chloride, poplar, pine, *Ginkgo*

1. INTRODUCTION

Lignin is one of the major constituents of the secondary cell walls in the vascular plants. Lignin plays an important role in plant growth and development by improving water conduction through xylem tracheary systems, providing the strength of fibrous tissues and improving the durability of wood against microbial attack by protecting the cellulose microfibrils (Campbell and Sederoff, 1996).

Biosynthesis of lignin has been considered to

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be involved in three compartmental stages; 1) intracellular synthesis of the monolignol precursors (*p*-courmaryl, coniferyl and sinapyl alcohols), 2) transport and secretion of the monolignols, and 3) extracellular polymerization of monolignols in process coordinated with the deposition of other cell wall components (Higuchi, 1997).

Peroxidase has long been regarded as a key enzyme for the turnover of monolignols into lignin polymer (Harkin and Obst, 1973; Higuchi, 1997; Ros Barcelo, 1998). It has been demonstrated that peroxidase oxidizes various substances such as monolignols at the expense of hydrogen peroxide (H_2O_2). Recent studies showed, however, that laccase was equally capable of polymerizing monolignols in the presence of O_2 (Dean and Eriksson, 1996). Consequently the study on the temporal and spatial production of peroxidase will be critical to understand and/or define the function of this enzyme in the lignification process.

Following work has been undertaken to localize the peroxidase cytochemically to get an insight into the precise function of enzyme responsible for lignin biosynthesis. The action of peroxidase was visualized by the cytochemical localization of H_2O_2 which should be produced by the action with peroxidase. In this work, we used the cerium chloride ($CeCl_3$) as a trapping agent for hydrogen peroxide generated from peroxidase (Bestwick *et al.*, 1998). Cerium perhydroxide [$Ce(OH)_2OOH$] precipitated during the reaction can be visualized as an electron-dense products under transmission electron microscopy (TEM). Because of a high resolution with minimal nonspecific deposition of this procedure, the $CeCl_3$ technique has been employed widely in recent year for the localization of H_2O_2 generated in the lignification process and plant-microbial interactions (Czaninski *et al.*, 1983; Kim *et al.*, 2000).

In the present work we focused on the seasonal variation of peroxidase activities in the cambial region. It might be reasonable to assume that the peroxidase activity would vary with the growth seasons if this enzyme would be one of the enzymes involved in lignin biosynthesis. However, it is hard to find any cytochemical studies on the seasonal variation of peroxidase production in the cambium at subcellular levels, although biochemical works have been done on the seasonal changes of peroxidase activities or lignin precursors such as coniferin and β-glucosidase in the cambial zone (Dharmawardhana *et al.*, 1995; Freudenberg and Neish, 1968; Olson and Varner, 1993). In the present work, the site of lignin biosynthesis in the woody plants has been also chased to understand the regulation mechanism of lignification.

2. MATERIALS and METHODS

2.1 Plant materials

Pinus densiflora, *Ginkgo biloba*, and *Populus alba* growing in the University Forest Experimental Station of Chonnam National University at Kwangju, Korea were employed in this work. Two-year old branches of each wood species were collected every second week in March, April, May, June, August, September, and November, 1999.

2.2 Methods

Cytochemical localization of H_2O_2 was performed by cerium chloride as described by Bestwick *et al.* (1997). Fresh pieces of tissues collected from branches were incubated with 5 mM $CeCl_3$ in 50 mM 3-(N-morphino) propanesulfonic acid (MOPS) (pH 7.2) for 2 hours at 4℃.

The tissues were then thoroughly washed with the same buffer, followed by fixation in the mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde in cacodylate buffer (pH. 7.4) for 2 hours at 4 °C and post-fixation in 1% osmium tetroxide for 1 hour. Prior to embedding in London Resin White, tissues were dehydrated using an ethanol series. Ultrathin sections of 80 nm thickness prepared with diamond knife were mounted on a uncoated nickel grid of 300 mesh and examined with a Jeol 1200 TEM at an accelerating voltage of 80 kV without counter-staining. For the confirmation of specificity of $CeCl_3$, tissues were treated with catalase for decomposition of H_2O_2 and sodium azide for inhibition of peroxidase before the incubation with $CeCl_3$ as described elsewhere (Kim *et al.*, 2000). Some samples were also fixed directly without $CeCl_3$ incubation.

To assess the accumulation of H_2O_2 objectively, a 4-point scale developed by Bestwick *et al.* (1997) was employed to examine the appearance of deposits of cerium perhydroxides within the cell walls as follows: O, none; 1, faint and patchy; 2, dense but patchy; 3, dense and confluent deposits. The degree of deposits assigned to the categories is shown in Figure 4A to 4D. The accumulation of H_2O_2 was examined after the assessment of a total of at least 20 reaction sites per category.

3. RESULTS

Electron dense deposits of cerium perhydroxide formed by reaction with H_2O_2 were localized to cambium and its immediate derivatives in wood species of *Pinus*, *Ginkgo* and *Populus*. However, the staining with $CeCl_3$ in cambium appeared only in specific annual cycle of growth season. As revealed in Figures 1 to 3, the staining intensity of $CeCl_3$ was dependent upon the growth season in trees examined.

In *Populus*, the most dense and confluent staining of $CeCl_3$ at cambium appeared in May (Figure 1C). However, the faint staining of $CeCl_3$ was also confirmed even in the samples collected in March (Figure 1A) and staining increased with on-going growth season, showing dense and patchy staining in April (Figure 1B). Faint and patchy staining of $CeCl_3$ appeared also in the samples collected in August (Figure 1D). Any staining could not be observed, however, in the samples collected in November (Figure 1E).

Fig. 1. Localization of H_2O_2 accumulation in cambial region of *Populus alba*. (A) March: faint staining in cambium; (B) April: dense but patchy staining in cambium; (C) May: dense and confluent staining of cerium perhydroxyides localized to cambial area; (D) August: faint and patchy staining; (E) November: no staining in cambial area. Bar = 1 μm.

Fig. 2. Localization of H_2O_2 accumulation in cambial region of *Ginkgo biloba*. (A) April: faint staining and the vesicles stained with $CeCl_3$ which attached to cambium; (B) May: dense but patchy staining of cambium; (C) August: no staining in cambium. Bar = 1 ㎛.

Staining pattern of $CeCl_3$ in the cambium of *Populus* indicated that the production of H_2O_2 started in March before the opening of buds and reached the highest in May. The production of H_2O_2 declined in summer season and shut-downed in September. The cessation and/or decrease of H_2O_2 production in poplar occurred before falling the leaves.

Same trend was observed in *Ginkgo biloba*. In Figure 2, vesicles stained with $CeCl_3$ were about to accumulate in the cells of cambial region in April, however, it is hard yet to observe the staining in the primary wall of cambial derivatives (Figure 2A). The most dense staining in *Ginkgo* occurred in May (Figure 2B). In contrast, the staining with $CeCl_3$ in cambial derivatives in the samples collected in August was nearly nil (Figure 2C), although *Ginkgo* still holds its foliages. *Ginkgo* showed relatively late generation of H_2O_2 production and ceased H_2O_2 generation earlier than *Populus*.

In *Pinus densiflora*, the production of H_2O_2 at cambium were detected from April (Figure 3A); faint and patchy staining occurred in the primary walls and the strongly stained vesicles appeared at the cambial region. The most strong staining in *Pinus* occurred in May (Figure 3B). The declined staining could be observed in August (Figure 3C) and no staining occurred in September (Figure 3D). Although *Ginkgo* and *Pinus* have been classified usually into coniferous trees, the generation time of H_2O_2 production and its duration was different from each other. Furthermore, *Pinus* did not show any staining with $CeCl_3$ in autumn season although pine trees still hold their needles.

Overall intensity of staining with $CeCl_3$ was also different between tree species as shown in Figures 1 to 3. The most dense staining appeared in *Populus*, followed by in turn of *Pinus* and *Ginkgo*. It is not determined in the present work, however, that the dense staining with $CeCl_3$ would be related with the content and/or composition of lignin.

For the confirmation of the specificity of $CeCl_3$, cerium chloride staining was examined in the presence of catalase and potassium cyanide. All the tissues treated with the catalase or potassium cyanide did not show any accumulation of

Fig. 3. Localization of H_2O_2 accumulation in cambial region of Pinus densiflora.
(A) April: dense but patchy staining; (B) May: dense and confluent staining; (C) September: weak or no staining; (D) November: no staining. Bar = 1 μm.

deposits of cerium perhydroxides in the cell walls, indicating the specificity of cerium chloride staining for the localization of H_2O_2.

The appearance of cerium perhydroxides in the cell walls was assessed with a four-point scale by categorizing into 0, 1, 2, and 3 (Bestwick *et al.*, 1997). The results presented in Table 1 clearly showed that H_2O_2 accumulation was the strongest in May in all the tree species examined. Table 1 also indicated that the peroxidase activities varied between the tree species and the degree of H_2O_2 accumulation was also different between the tree species.

4. DISCUSSION

Peroxidase has been thought to be involved in the final stages of lignin biosynthesis by polymerizing the monolignols into lignin (Freudenberg and Neish, 1968; Harkin and Obst, 1973; Higuchi, 1997). Since H_2O_2 is required for the action of peroxidase, H_2O_2 production was examined cytochemically in the present work to elucidate the function of peroxidase in the lignification and the seasonal variation of peroxidase activities. TEM work showed that staining of cambial regions with $CeCl_3$ (Figures 1-3) varied between growth season, indicating the production of peroxidase in cambial zone changed with annual cycle of growth season. It is well established that the annual cycle of growth is reflected in the degree of lignification (Freudenberg and Neish, 1968; Savidge, 1991). Wadrop (1971) showed that the cambial activity of *Fraxinus* was initiated before the opening of the bud, indicating the direct and indirect influence of light in the lignification of xylem.

Relatively little work has been conducted cytochemically on the influence of environmental factors on lignification. Our TEM works showed clearly that the activities of peroxidase at cambial regions varied with the annual cycle of growth season. Regardless of tree species examined, the highest activity of peroxidase appeared in May and its activity declined even before September, indicating the different degree of lignification with the annual cycle of growth, and the time of H_2O_2 production was also different. The initiation of H_2O_2 generation in cambial region of *Populus* occurred in March before the opening of buds as in *Fraxinus* (Wadrop, 1971). However, *Ginkgo* and *Pinus* did not show any H_2O_2 production even after the opening of buds (Table 1). We have not determined in the present work the factors for the initiation of peroxidase activities in woody plants. Our results suggest,

Table 1. Staining density of $CeCl_3$ at cambial region during growth season.

Species	March	April	May	June	August	September	November
Pinus	1	2	2	1	1	0	0
Ginkgo	0	1	2	1	0	0	0
Populus	1	2	3	2	1	0	0

(0) None; (1) weak; (2) dense but patch; (3) dense and confluent.

however, that not only the environmental factor but also other physiological factors may influence the initiation of lignification. Lignification in the stem was thought to be influenced by auxin and its production to be associated with the activity of the buds (Kozlowski and Pallardy, 1997; Savidge, 1991). Further studies are needed to chase the environmental and hormonal factors for the initiation of lignification in woody plants.

In addition to seasonal variation of peroxidase activities in various wood species, the staining of cambial regions with $CeCl_3$ (Figures 1-3) is also to be discussed. Staining with $CeCl_3$ in the cambium revealed in the present work was somewhat unexpected observation because the main lignification has long been thought to occur in the thickening secondary cell walls (Freudenberg and Neish, 1968; Higuchi, 1997). It is not known yet fully whether peroxidase is involved in lignification process at cambium or not. Previous histochemical works showed that occurrence of lignin precursors such as coniferin or β-glucosidase was restricted to the zone of differentiation and lignifying xylem in the actively growing pine stem but not in cambium (Dharmawardhana *et al.*, 1995; Freudenberg and Neish, 1968; Higuchi, 1997; Wadrop, 1971). Freudenberg and Neish (1968) demonstrated that peroxidase and laccase were present in the zone of differentiating xylem and that the amount of these enzymes decreased in the more heavily lignified cells. Goldberg *et al.* (1985) reported also that peroxidase was restricted to lignifying tissues.

Our investigation is not fully in agreement with those works (Freudenberg and Neish, 1968; Goldberg *et al.*, 1995) in that cambium was positively stained with $CeCl_3$. The reason of this difference should be investigated in detail. However, it should be mentioned that those histochemical methods did not allow anatomical details to specify the exact cellular location of enzyme activities at cellular levels because of the diffusion of reaction products into other tissues and cells in the histochemical procedures. One advantage of cytochemistry, compared with the histochemistry, is that in the cytochemical procedures it is possible to differentiate the exact enzyme activities between the different cell types. The strong staining of $CeCl_3$ in cambial areas of *Populus, Pinus* and *Ginkgo* observed in the present work may be attributed to the production of peroxidases in cambium, suggesting the beginning of lignin biosynthesis at the cambium prior to the thickening of secondary cell wall layers. Savidge (1996) pointed out that cambium itself, at least in conifers, possessed an element of commitment to lignification. Further studies are necessary to elucidate the lignification process in cambium, using more refined methods and techniques. Immunocytochemical methods would be one of the feasible candidates in chasing the lignin biosynthesis at cellular level.

5. CONCLUSION

Hydrogen peroxide production in cambial regions was examined cytochemically with

Fig. 4. Variable intensity of staining used for qualification of H_2O_2 accumulation as detected by the formation of cerium perhydroxides at reaction sites.
(A): no staining; (B) category 1: faint and patchy staining; (C) category 2: dense but patchy staining; (D) category 3: dense and confluent staining. Bar = 1 ㎛.

cerium chloride procedures for better understanding the function of peroxidase in lignin biosynthesis. Electron dense deposits of cerium perhydroxide formed by reaction with H_2O_2 were localized to cambium and its immediate derivatives. The staining intensity of $CeCl_3$ was, however, dependent upon the growth season of trees. Staining pattern of $CeCl_3$ in the cambium of *Populus* indicated the initiation of peroxidase activities in March before the opening of buds and the highest activities in May. Peroxidase activities appeared to be declined in summer season and shut-downed in autumn season before falling the leaves. In *Pinus* trees, the production of peroxidase at cambium was begun to be detected from April and the dense staining continued to May. But *Ginkgo* showed relatively late generation of peroxidase and earlier cessation its activities than *Pinus*. The appearance of cerium perhydroxides within the cell walls may indicate the activities of peroxidase in woody plants to be different with the annual cycle of growth season and the time of peroxidase production to be different between tree species.

REFERENCES

1. Bestwick, C. S., I. R. Brown, M. H. R. Bennet, and J. W. Mansfield. 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv *phaseolicola*. Plant Cell. 9: 209-221.
2. Campbell, M. M. and R. R. Sederoff. 1996. Varia-

tions in lignin content and composition. Mechanism of control and implications for the genetic improvement of plants. Plant Physiol. 110: 3-13.

3. Czaninski, Y., R. M. Sachot, and A. M. Catesson. 1983. Cytochemical localization of hydrogen peroxide in lignifying cell walls. Ann. Bot. 72: 547-550.
4. Dean, J. F. D. and K.-E. L. Eriksson. 1994. Laccase and the deposition of lignin vascular plants. Holzforschung 48. Supplement. 21-33.
5. Dharmawardhana, D. P., B. E. Ellis, and J. E. Carlson. 1995. A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. Plant Physiol. 107: 331-339.
6. Freudenberg, K. and A. C. Neish. (ed.). 1968. Constitution and biosynthesis of lignin. Springer Verlag, New York.
7. Goldberg, R., T. Le, and A. M. Catesson. 1985. Localization and properties of cell wall enzyme activities related to the final stages of lignin biosynthesis. J. Exp. Bot. 36: 503-510.
8. Harkin, J. M. and J. R. Obst. 1973, Lignification in trees: indication of exclusive peroxidase participation. Science. 180: 296-297.
9. Higuchi, T. 1997. Biochemistry and molecular biology of wood. Springer Verlag, Berlin.
10. Kim, Y. S S. G. WI, and K. H. Lee. 2000. Cytochemical lo 'ization of hydrogen peroxide production in br n rot fungus *Tyromyces palustris* and *Coniophora puteana*. Holzforschung (in review).
11. Kozlowski, T. T. and S. G. Pallardy. 1997. Physiology of woody plants, Academic Press, San Diego.
12. Olson, P. D. and J. E. Varner. 1993. Hydrogen peroxide and lignification. Plant J. 4:887-892.
13. Ros Barcelo, A. 1998. Hydrogen peroxide production is a general property of the lignifying xylem from vascular plants. Ann. Bot. 82: 97-103.
14. Savidge, R. A. 1991. Seasonal cambial activity in *Larix larcina* saplings in relation to endogenous indol-3-acetic acid, sucrose and coniferin. Forest Sci. 37: 953-958.
15. Savidge, R. A. 1996. Xylogenesis, genetic and environmental regulation - A review. IAWA J. 17: 269-310.
16. Wadrop, A. B. 1971. Occurrence and formation of lignin in plants. In: K. V. Sarkanen and C. H. Ludwig, Eds. Lignins: Occurrence, formation, structures and reactions. Wiley-Intersci. New York, pp. 19-41.