Immunolocalization of 8-5′ and 8-8′ linked structure of lignin in plant cell walls

(Summary)

Shingo Kiyoto

2015

Abbreviations

AGP	arabinogalactan-protein
BSA	bovine serum albumin
cCML	corner of compound middle lamella
CML	compound middle lamella
ELISA	enzyme-linked immunosorbent assay
FFCML	CML betwen fibers
FSW	fiber secondary wall
FVCML	CML between fiber and vessel element
G lignin	guaiacyl lignin
HD	high sowing density
IR	irrigation
KM1	Kyoto university monoclonal antibody 1
KM2	Kyoto university monoclonal antibody 2
***p*AHA**	*p*-aminohippuric acid
S lignin	syringyl lignin
SW	secondary wall
TEM	transmission electron microscopy
UV	ultraviolet
VSW	vessel secondary wall

Chapter 1
General introduction

Lignin is one of the main components of woody cell wall and integrated into cell wall framework of vascular plants. It is a complex dehydrogenative polymer of monolignols, namely *p*-coumaryl (H), coniferyl (G), and sinapyl alcohols (S) (Higuchi 1990). In plant cell walls, lignin, and hemicellulose act as a matrix around cellulose microfibrils. Lignification allows plant cell walls to be more hydrophobic, higher pressure-resistant, and more decay-resistant against microorganisms. Better understanding of the complex structure of lignin is also important to produce various value-added chemicals from biomass containing lignified cell walls.

Lignin has various linkages between its monomer units. Linkages of lignin are divided into two main categories, non-condensed (C-O-C ether) structures and condensed (C-C) structures. The non-condensed structures are represented by 8-*O*-4′ linkage whereas condensed structures are represented by 8-5′, 8-8′, and 5-5′ linkage. The structure of lignin is an important factor for industrial use of wood, because condensed structure is resistant to various chemical degradation treatments.

Lignin is considered to be cross-linked matrix in association with cellulose and hemicellulose in cell walls.From an analysis of lignin-carbohydrate complexes (LCCs) in spruce, Lawoko et al. (2005) elucidated the presence of two different types of lignin in LCC and suggested that the more condensed type of lignin is linked to glucomannans, whereas the less condensed type of lignin is associated with xylans. However, the relationship between hemicellulose and lignin are not fully understood.

Since lignin has very complex structures, highly defined monoclonal antibodies against lignin substructures would be effective tools to clarify the mechanism of lignification in the plant cell wall. It is reported that the proportion of 8-5′ and 8-8′ linkage is relatively high among various linkages of lignin (Jouanin et al. 2000; Morreel et al. 2004). Therefore, 8-5′ and 8-8′ linked dimer would be good candidates to produce specific monoclonal antibodies.

The aim of this thesis is to obtain the information on distribution of 8-5′ linked and 8-8′ linked structure of lignin in differentiating xylems of *Chamaecyparis obtusa*, *Betula grossa*, and phloem fibers in *Cannabis sativa* L. by using immunocytochemical techniques with two specific monoclonal antibodies: KM1 and KM2 antibodies.

Characterization of KM1 and KM2 antibodies and distribution of 8-5′ and 8-8′ linked structure in normal wood of *Chamaecyparis obtusa* are described in Chapter 2. The temporal

and spatial distribution of 8-5′ linked structure of lignin in normal and compression wood of *Chamaecyparis obtusa* are shown and then surveyed its relationship with that of xylans in Chapter 3. Chapter 4 describes relationship between lignin, S lignin and 8-5′ or 8-8′ linked structures of lignin in differentiating xylem of *Betula grossa*. Distribution of lignin substructure, hemicelluloses, and arabinogalactan proteins in hemp phloem fibers is shown in Chapter 5. Chapter 6 summarizes the overall results of this thesis.

Chapter 2

Immunolocalization of 8-5′ and 8-8′ linked structures of lignin in cell walls of *Chamaecyparis obtusa* using monoclonal antibodies

Summary

Mouse monoclonal antibodies were generated against dehydrodiconiferyl alcohol- or pinoresinol-*p*AHA-BSA conjugate as probes that specifically react with 8-5′ or 8-8′ linked structure of lignin in plant cell walls. Hybridoma clones were selected that produced antibodies that positively reacted with dehydrodiconiferyl alcohol- or pinoresinol-*p*AHA-BSA and negatively reacted with *p*AHA-BSA and guaiacylglycerol-β-guaiacyl ether *p*AHA-BSA conjugates containing 8-*O*-4′ linkage. Eight clones were established for each antigen and one of each clone that positively reacted with wood sections was selected. The specificity of these antibodies was examined by competitive ELISA tests using various lignin dimers with different linkages. The anti-dehydrodiconiferyl alcohol antibody (KM1) reacted specifically with dehydrodiconiferyl alcohol and did not react with other model compounds containing 8-*O*-4′, 8-8′, or 5-5′ linkages. The anti-pinoresinol antibody (KM2) reacted specifically with pinoresinol and syringaresinol and did not react with the other model compounds containing 8-*O*-4′, 8-5′, or 5-5′ linkages. The antibodies also did not react with dehydrodiconiferyl alcohol acetate or pinoresinol acetate, indicating that the presence of free phenolic or aliphatic hydroxyl group was an important factor in their reactivity. In sections of *Chamaecyparis obtusa*, labeling by the anti-dehydrodiconiferyl alcohol antibody was found in the SWs of phloem fibers and in the CML, and SWs of tracheids. Weak labeling by the anti-pinoresinol antibody was found in SWs of phloem fibers and SWs and CML of developed tracheids. These labelings show the localization of 8-5′ and 8-8′ linked structure of lignin in the cell walls.

Chapter 3

Relative deposition of xylan and 8-5′-linked lignin structure in *Chamaecyparis obtusa*, as revealed by double immunolabeling by using monoclonal antibodies

Summary

Xylan deposition and formation of 8-5′-linked lignin structure in differentiating xylems of normal and compression woods in *Chamaecyparis obtusa* were examined by immunoelectron microscopy. The monoclonal antibodies (LM10 or LM11) were used to detect xylan localization. The 8-5′-linked lignin structure was immunolocalized using KM1 antibody. Xylan and 8-5′-linked lignin double immunolabeling were performed using secondary antibodies labeled with colloidal gold particles of different diameters.

In normal wood, KM1 labeling occurred in the CML and S_1 layer during S_1 layer formation and increased as S_2 and S_3 layers formed, with labeling occurring at the outer part of the previously formed layer. At maturation stage, KM1 labeling appeared on the whole cell wall. In compression wood, mild KM1 labeling occurred in the CML and outer part of the S_1 layer at the later S_1 layer formation stage, with increased labeling as the S_2 layer was formed. Minor labeling occurred in the outer part of the S_2 layer during helical cavity formation. Comparison between KM1 labeling and $KMnO_4$ staining suggested that lignin other than 8-5′-linked structure was formed during early lignification, and the proportion of 8-5′-linked lignin structure increased at later stages of lignification in both normal and compression woods. LM10 and LM11 labeling occurred slightly earlier than KM1 labeling in normal and compression woods, suggesting that xylan deposition preceded the formation of 8-5′-linkage lignin. Less labeling with KM1, LM10, and LM11 occurred in the outer part of the S_2 layer in compression wood, which has abundant lignin. Thus, lignin in this part is composed of lignin substructures other than the 8-5′ linkage.

Chapter 4

Formation and distribution of 8-5′ and 8-8′ linked structure of lignin in differentiating xylem in *Betula grossa*.

Summary

Formation and distribution of 8-5′ and 8-8′-linked lignin structure in differentiating xylem tissues of normal wood in *Betula grossa* were examined by immunoelectron microscopy by using monoclonal KM1 and KM2 antibodies respectively. In addition to the immunolabeling, $KMnO_4$ staining and Mäule reaction were carried out to examine the distribution of lignin and S lignin, respectively.

In wood fibers, both KM1 and KM2 labeling did not appear in the early stage of S_2 layer formation, and increased mainly in the CML during the later stage of cell wall formation. In mature xylem, intense KM1 labeling was seen in the CML and VSWs, especially in the cCML compared to FSW. Comparison between KM1 labeling, $KMnO_4$ staining, and Mäule reaction suggested that the CML and VSWs is abundant of G lignin and also that a large proportion of 8-5' linked substructure of lignin in the CML and VSW especially in the cCML. KM2 labeling was most intense in the FFCML. KM2 labeling density was low and comparable in the VSW and FSW. Interestingly, KM2 labeling density in the FFCML was significantly higher than that in the FVCML. It indicates that 8-8' linked structure of lignin distributes mainly in the FFCML. Overall results showed that 8-5' and 8-8' linked structures of lignin are heterogeneously distributed in relation to the composition of G and S units and to the type of cells.

Chapter 5

Distribution of 8-5′ and 8-8′ linked structure of lignin, hemicelluloses, and arabinogalactan protein in hemp phloem fibers.

Summary

Distribution of noncellulosic polysaccharides, lignin, 8-5′ and 8-8′ linked lignin substructure, arabinogalactan protein in hemp (*Cannabis sativa* L.) phloem fibers were surveyed by histochemical and immunological methods. Lignin distribution was determined by UV microscopy, fluorescent microscopy with acriflavine staining and TEM with $KMnO_4$ staining. Xylan, mannan and AGP were immunolocalized with LM11, LM21 and JIM14 antibodies, respectively. The specific lignin substructures, 8-5′ and 8-8′ linked structures were immunolocalized by KM1 and KM2 antibody that recognize 8-5′ and 8-8′ linked structure of lignin, respectively.

UV absorption and $KMnO_4$ staining appeared mainly in the CML and hardly appeared in the SW of phloem fibers whereas acriflavine staining appeared in the whole cell wall. These results suggest that lignin concentration is high at the CML and very low at the SW of hemp fiber and that there is a little amount of lignin-like compounds stained with acriflavine. In addition, some fiber cells showed multi-layered structure. Hemp phloem fibers are characterized as S_1 + nG structure, where n means the number of repetition.

Uniform JIM14 and KM1 labeling appeared on the SWs whereas these labelings did not appear in the CML of hemp fibers. These observations indicate that there are AGP and a little amount of lignin containing 8-5′ linked structure in the SW of the phloem fiber of hemp and that CML of the phloem fiber of hemp has lignin other than 8-5′ linked structure. LM11 labeling appeared in the CML and the S_1 layer. On the other hand, no KM2 labeling was found in the whole cell wall of hemp fibers. It suggests that hemp fibers lignin does not have 8-8′ linked structure with free phenolic hydroxyl groups. Little difference was seen in the polysaccharide and lignin distribution between two growing conditions, high-sowing density and irrigation.

Secondary fibers slightly differ from the primary ones regarding noncellulosic polysaccharides labeling of SW. A faint but detectable xylan labeling and decreased labeling of glucomannan were found in the SWs of secondary fibers.

Chapter 6
Conclusions

This thesis aimed at obtaining information on distribtion of specific lignin structure and its relationship with lignin concentration and distribution of hemicelluloses in plant cell walls by immunocytochemical techniques. Immunolabeling with monoclonal antibodies against specific lignin linkage were applied in combination with histochemical observation and immunolabeling with monoclonal antibodies against noncellulosic polysaccharides.

Chapter 2 described the characterization of the two monoclonal antibodies against lignin specific structures and immunolocalization with these antibodies in mature tracheids and phloem fibers of *Chamaecyparis obtusa*. As a result of the competitive inhibition ELISA, KM1 and KM2 were identified as specifically recognizing dehydrodiconiferyl alcohol with 8-5′ linkage and pinoresinol with 8-8′ linkage, respectively. Interestingly, KM2 antibody also reacted with syringaresinol with 8-8′ linkage. These antibodies should make effective tools for detecting lignin dimers in differentiating xylem if samples are prepared so as to minimize loss of the dimers. In the condition that these dimers are absent, the antibodies should also prove to be useful probes for the immunolocalization of 8-5′ and 8-8′ linked structure of lignin. Competitive inhibition ELISA suggested that the presence of free phenolic or aliphatic hydroxyl groups was an important factor in their reactivity. The immunolabeling of KM1 and KM2 antibodies showed the presence of 8-5′ and 8-8′ linked structure of lignin in the cell wall in the secondary phloem and developed xylem of *Chamaecyparis obtusa*. We suggested that lignin structure of SWs of phloem fibers might be different (richer in 8-5′ structure) from that of SWs of tracheids in xylem.

In Chapter 3, lignin distribution was surveyed with $KMnO_4$ staining in differentiating xylems from normal and compression woods of *Chamaecyparis obtusa*. Xylan deposition and formation of 8-5′-linked lignin structure were also examined with double immunolocalization by combination of KM1 and anti-xylan monoclonal antibodies (LM10 or LM11). In differentiating xylems obtained from normal and compression woods of *Chamaecyparis obtusa*, intense $KMnO_4$ staining occurred earlier than immunolabeling with the KM1 antibody, indicating that the other structure than 8-5′-linkage arises during the early stage of lignification. The KM1 labeling density increased during later stage of lignification, suggesting that the proportion of the 8-5′-linked structure increased during this stage. In compression wood, less KM1 labeling was observed on the outer part of the S_2 layer, despite the fact that this part is rich in lignin. Thus, outer part of the S_2 layer is abundant in lignin

with substructures other than the 8-5′-linkage. In double immunolocalization studies, the labeling with anti-xylan monoclonal antibodies appeared earlier than the labeling with KM1 in both normal and compression wood, suggesting that the deposition of xylan precedes the formation of the 8-5′-linked structure of lignin.

Immunolabeling with KM1 and KM2 antibodies in chapter 4 revealed that the proportion of the 8-5′ and 8-8′-linked structure increases during later stage of cell wall formation. Both in vessel elements and wood fibers, KM1 labeling distributes mainly in the CML especially in cCML. It indicates the abundance of 8-5′ substructure there. Histochemical staining and KM1 labeling indicates that VSW has higher concentration of G lignin and 8-5′-linked structure of lignin than FSW. KM2 labeling suggests that the CML between wood fibers may have not only different proportion of S lignin but also different proportion of 8-8′ linked structure of lignin from the CML between wood fibers and vessel elements.

In chapter 5, lignin distribution of hemp fibers was investigated by the histochemical method such as TEM observation with $KMnO_4$ staining and UV microscopic observation. 8-5′ and 8-8′ linked structure of lignin were shown by immunological techniques with KM1 and KM2 antibodies, respectively. Strong KM1 labeling occurred on the G-layer with low lignin concentration, whereas almost no labeling was found on the CML. These results indicate that there is a little amount of lignin in the SW of phloem fibers of hemp containing 8-5′ linked structure and that CML of the phloem fiber of hemp has lignin other than 8-5′ linked structure. It also suggests that KM1 antibody can detect 8-5′ linked structure of lignin in the cell wall regardless of the low lignin concentration. Immunolabeling also revealed the uniform distribution of AGP in the whole SW of hemp fibers. On the other hand, almost no KM2 labeling appeared in the phloem fiber. However, KM2 labeling appeared almost uniform in the xylem fiber.

To summarize immunolabeling with KM1 antibody, 8-5′ substructure is present mainly in the CML of xylem cells such as tracheid of softwood, wood fiber, and vessel element of hardwood. However, the difference of KM1 labeling density between CML and SW of xylem fiber of hardwood is more marked than that of tracheid of softwood. In addition, this structure is more localized in the VSW than in the FSW in hardwood. On the other hand, the structure showed almost uniform localization in xylem fiber of hemp. These results suggest that distribution of 8-5′ linked structure of lignin differs not only by plant species but also types of cells, possibly having different function. In addition, it is interesting that phloem fibers of softwood and hemp have 8-5′ linked structure mainly in the SWs in spite of different plant species.

To summarize immunolabeling with KM2 antibody, a little KM2 epitopes consist in the SW of phloem fiber in softwood whereas there was almost no KM2 labeling in the hemp phloem fiber. These results indicates that there may be different lignin substructure in phloem fibers between softwood and hemp. In wood fiber of hardwood, KM2 labeling was weak and a little strong in the CML. In vessel element of hardwood and xylem fiber of hemp, KM2 labeling was almost uniform. These results suggest that also distribution of 8-8′ linked structure of lignin differs not only by plant species but also types of cells, possibly having different function.

In conclusion, this thesis firstly clarified that the two kinds of monoclonal antibodies recognize 8-5′ and 8-8′ linked structure of lignin, respectively. Then this thesis clearly showed that 8-5′ linked structure of lignin increases during later stages of lignification, preceded by xylan deposition, in differentiating secondary xylem of normal wood and compression wood of the *Chamaecyparis obtusa*. In differentiating secondary xylem of normal wood of *Betula grossa*, 8-5' and 8-8' linked structures of lignin increase during the later stage of cell wall formation and are heterogeneously distributed in relation to the composition of G and S units and to the type of cells. The SW of phloem fiber of hemp at grain maturity stage contains a little amount of lignin with 8-5′ linked structure. In researches of this thesis, distribution of these structures did not necessarily correspond with that of lignin observed by histochemical methods. It suggests that 8-5′ and 8-8′ linked structures do not necessarily localize in the place where lignin concentration is high and that these monoclonal antibodies can detect the specific structures of lignin in the cell wall where lignin concentration is low.

References

Lawoko M, Henrikisson G, Gellerstedt (2005) Structural differences between the lignin-carbohydrate complexes present in wood and in chemical pulps. Biomacromolecules 6:3467-3473

Higuchi T (1990) Lignin biochemistry: biosynthesis and biodegradation. Wood Sci Technol 24: 23-63

Jouanin L, Goujon T, de Nadaï V, Martin MT, Mila I, Vallet C, Pollet B, Yoshinaga A, Chabbert B, Petit-Conil M, Lapierre C (2000) Lignification in transgenic poplars with extremely reduced caffeic acid *O*-methyltransferase activity. Plant Physiol 123:1363-1373

Morreel K, Ralph J, Kim H, Lu F, Goeminne G, Ralph S, Messens E, Boerjan W (2004) Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem. Plant Physiol 136:3537-3549