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Anatomical, Chemical, and Topochemical Characteristics of Transgenic Poplar Down-regulated with *O*-methyltransferase*1

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ABSTRACT

The present work was undertaken to investigate the anatomical and chemical characteristics of transgenic poplar down-regulated with antisense OMT gene. Also the distribution of lignin in transgenic poplar trees was investigated at cellular level. No visible abnormal phenotype was observed in the fibers and vessel elements of transgenic poplar. Any marked differences in the staining intensities of Wiesner and Mäule color reaction were not identified in the transgenic poplar. TEM micrographs did not show any staining intensities in the cell walls stained with $KMnO_4$. Interestingly, the UV spectroscopy of semi-thin sections exhibited a distinct decrease of lignin absorption at 280 nm in the vessel walls, indicating transgenic poplar wood with lower amount of guaiacyl lignin in vessel elements. Chemical composition of antisense OMT poplar was almost identical to that of wild-type poplar. Klason lignin content of transgenic poplar did not show any significant difference from that of the controls. The solid state NMR spectra revealed the transgenic poplar with only slightly more syringyl lignin than the control. The present work showed that antisense OMT gene constructed in the poplar was not enough to reduce the overall content of Klason lignin, and suggested that the expression of transformation was confined to vessel walls.

Keywords : lignin, OMT (*O*-methyltransferase), topochemistry, transgenic poplar

1. INTRODUCTION

Lignin constitutes about 20 to 30% of the dry weight of most woody plants and represents the second most abundant natural polymer after cellulose. It is considered that the ability to synthesize lignin is an important step in the evolution of land plants because lignin is formed only in higher terrestrial plants. Lignin deposits in the walls of plant cells and functions in enhancing the rigidity of cell wall structure, conferring resistance to pathogens and mechanical

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stresses. It also facilitates water transport in the xylem due to its hydrophobicity (Lewis and Yamamoto 1990)

Although lignin plays an important role in the growth and development of plants, lignin is undesirable in the pulp and paper industry since it has to be removed from wood chips during chemical pulping. This pulping process is an energy-intensive, and its by-products are hazardous to the environment, polluting both air and water systems. Because of growing environmental concerns, efforts are being made to find alternative ways to minimize the risk of industrial pollution from chemical pulping. Biological pulping and genetic manipulation of lignin content in trees are the two most important approaches being explored to address this critical issue.

In order to improve the efficiency of chemical wood pulping, 1) higher degree of methoxylation in gymnosperm lignin can be induced through the transfer of bifunctional O-methlytransferase (OMT) and 2) a lower degree of methoxylation in angiosperm lignin can be induced by using OMT antisense constructs. OMT together with cinnamylalcohol dehydrogenase (CAD) is of considerable interest as targets for genetic engineering of lignin biosynthesis in angiosperm.

Park *et al.* (2000) isolated OMT encoding gene from developing secondary xylem of *Populus nigra* × *maximowiczii* and constructed antisense OMT vectors with *Agrobacterium*-mediated transformation. They confirmed that antisense OMT gene was integrated into genomic DNA of *P. nigra* x *maximowiczii*. For better utilization of transgenic poplar wood as a raw material for pulp and paper, its anatomical and chemical properties have to be investigated. Thus, the present work was undertaken to investigate the anatomical and chemical characteristics of the transgenic poplar down-regulated with antisense OMT gene. Also the distribution of lignin in transgenic poplar trees was investigated because the pattern of lignin distribution in cell walls has been suggested to play an important role in determining the qualities of chemical pulp and paper.

2. MATERIALS and METHODS

2.1. Plant Material

One-year old transgenic poplar (*Populus nigra* × *maximowiczii*) trees down-regulated antisense OMT grown at the university forest station were kindly provided by Kyungbuk National University, Daegu, Korea. Transgenic line was produced by depression of OMT activity caused by antisense construct. Both the wild-type and transgenic plants were grown in the field and showed a similar height at a similar growth rate. No visible abnormal growth was found in the transgenic poplars.

2.2. Anatomical Characteristics

Anatomical characteristics were examined from the xylem tissues of control and transgenic plants harvested during the growth season. Diameter, wall thickness, and distribution of vessel element were measured in the transverse sections. The length and width of fibers were measured from maceration.

2.3. Mäule Reaction and Phloroglucinol Staining for Lignin

For Mäule reaction, transverse sections, 20 μm thick, were treated with 1% potassium permanganate and 3% hydrochloric acid. For phloroglucinol staining, the fresh free-hand cross-sections were stained with 1% phloroglucinol in 6N HCl for 15 min and thoroughly washed with distilled water.

2.4. Ultraviolet (UV) Microscopy and TEM

For the determination of lignin concentration in secondary cell walls of transgenic poplars, semi-thin sections, 1 μm thick, were obtained from Spurr-embedded specimens with a diamond knife on an ultramicrotome. The sections were mounted on quartz slides, covered with quartz cover-slips, and analyzed with UV-VIS microscopic photometer (MPM 800, Zeiss). UV-absorption spectra were taken as point analysis (spot diameter 1 μm) in the center of fiber cell walls at wavelengths from 240 to 400 nm in 1 nm step. Ten measurements were taken for each cell wall area. For transmission electron microscopy (TEM), samples were fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 4 hrs at room temperature and subsequently dehydrated in ethyl alcohol series and embedded in London resin white (LRW). Ultrathin sections were stained with 1% potassium permanganate in 1% sodium citrate for 5 min at room temperature and examined in a TEM (JEM-1010, JEOL).

2.5. Chemical Characterization of Transgenic Poplar Wood

Air-dried wood meal of 40~60 mesh was extracted with benzene:alcohol (2:1, v/v) for 8 hrs in a Sohxlet apparatus and again air-dried. Holocellulose was prepared by delignification of extractive-free wood with acidified sodium chlorite at 75°C for 4 hrs. Lignin content was measured using the Klason method. In addition, extractive-free wood was treated with 1% NaOH solubility for 1 hr at 80°C.

2.6. FTIR and 13C-NMR Spectroscopy

Wood meals were mixed with KBr to make a pellet (1 mg wood meal in 300 mg KBr), and then used to obtain infrared spectra with a Bio-Rad FT-IR spectrometer. Each sample was replicated three times. Solid-state cross-polarization/ magic angle spinning (CP/MAS) NMR (DSX 400 MHz, Bruker) analysis, was used to obtain the carbon spectrum of transgenic poplar wood at a spin rate of 6.8 kHz. The carbon spectra of the solid-state ^{13}C CP/MAS NMR spectroscopy were obtained at 100 MHz. The Hartmann-Hahn match was done by tunning ^{1}H and ^{13}C channel with adamantine. The wood powder was packed into a 7-mm zirconium oxide rotor sealed with Ke-F cap. The rotor was spun at a MAS speed of 12 kHz, contact time of 1 ms, and recycle delay of 20 s for spectra acquisitions.

3. RESULTS

3.1 Anatomical Characteristics

The xylem in the transgenic poplar with repression of OMT displayed a whitish color, suggesting that transformation did not cause any chemical changes in the lignin. Transgenic plants exhibited red coloration in their lignified tissues. Such red brown color in xylem was not expressed in the OMT transgenic poplar. Tsai *et al.* (1998) provided the evidence that the presence of an abnormal amount of coniferyl aldehyde residues in lignin could be the common cause of the coloration observed in transgenic plants with inhibited CAD activities.

Fibers in both transgenic and control did not show any marked differences in their dimension (Table 1). The dimension of vessel elements in the transgenic was also similar to that of vessel elements in control poplar. No abnormal phenotype was observed in the vessel elements of OMT antisense plants. Zhong *et al.* (1998) observed deformed vessel walls in the transgenic plants with a reduction in CCoAMT and CAOMT. Similar alteration was also noted in CCR antisense tobacco plants (Piquemal *et al.* 1998). In

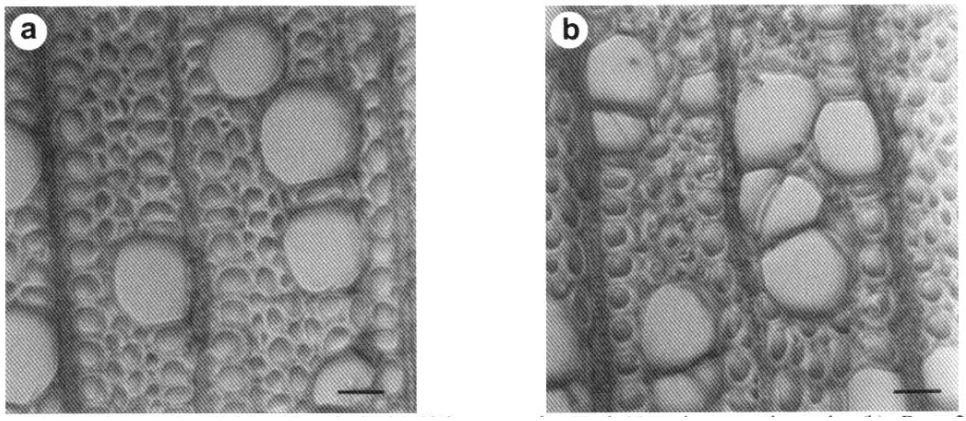

Fig. 1. Cross sections stained with phloroglucinol-HCl in stem of control (a) and transgenic poplar (b). Bar = 20 μm.

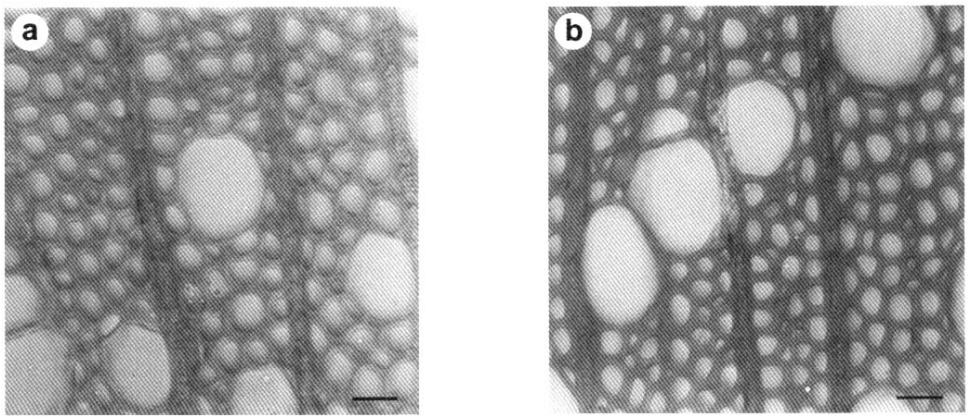

Fig. 2. Cross sections stained with Mäule color reaction in stem of control (a) and transgenic poplar (b). Bar = 20 μm.

both cases, a reduction in lignin content resulted in the collapse of vessel elements. Since lignin provides mechanical strength to the walls of conducting cells, the lack of lignin weakens the vessel walls, so that they can no longer withstand the negative stresses. However, such an anomaly was not identified in the transgenic poplar down-regulated in OMT activities in the present work.

3.2 Histochemical Characteristics of Transgenic Poplar

Stem-sections from both wild-type and transgenic plants were stained with phloroglucinol-HCl. Any makred changes in color intensities were not observed in the down-regulated OMT poplar (Fig. 1). Similarly, sections subjected to the Mäule reaction showed purple-red color, which is specific for S lignin unit in hardwood (Fig. 2). The pink staining of lignified xylem cell walls from the phloroglucinol-HCl represents the 4-O-linked hydroxycinnamyl aldehyde structures contained in lignins (Pomar *et al.* 2002).

3.3. Distribution of Lignin Determined by TEM and UV Microscopy

Potassium permanganate has been widely

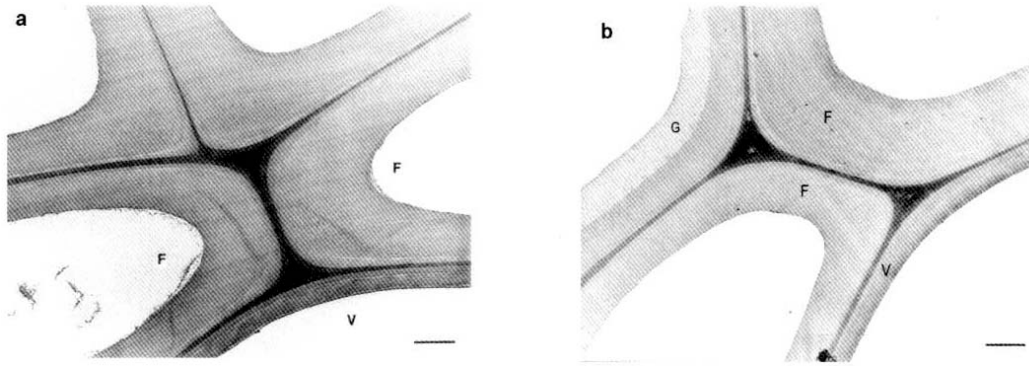

Fig. 3. TEM micrographs of fibers (F) and vessel (V) wall in cross section of control (a) and transgenic poplar (b). Note the relatively weaker staining intensity in secondary walls of transgenic poplar, stained with potassium permanganate. G: galatin layer Bar = 1 μm.

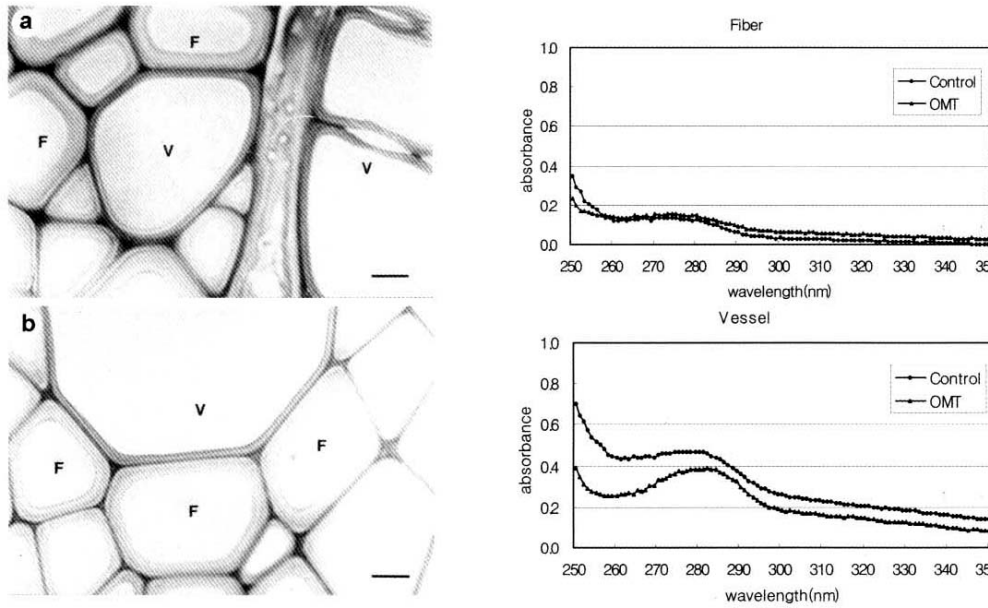

Fig. 4. Cross-sectional micrographs of UV microscopy in control (a) and transgenic poplar (b) and UV absorption spectra. Bar = 10 μm. F, fiber; V, vessel.

used in electron microscopy to contrast lignin. A qualitative estimation of the lignin distribution across the cell walls of transgenic poplar was obtained by $KMnO_4$ staining. In the TEM micrographs, no detectable differences of staining intensities in the transgenic and wild type poplar appeared although the staining intensity of transgenic poplar was in general slightly weaker than the control (Fig. 3).

The concentration of lignin in the cell wall was analyzed by the absorbance values at 280 nm. The UV spectroscopy of semi-thin sections showed little differences between transgenic poplar and control in the absorbance value at 280 nm in fiber walls. Fig. 4 gives the corresponding extinction values in S_2 wall regions

Table 1. Dimension of vessel elements and fibers in wild-type (control) and transgenic poplar

	Vessel		Fiber		
	Diameter (μm)	Wall thickness (μm)	Length (mm)	Width (μm)	Wall thickness (μm)
Control	29.1±8.6	0.7±0.2	0.7±0.1	16.4±3.0	1.6±0.5
Transgenic	32.1±6.6	0.7±0.1	0.6±0.1	18.4±3.8	1.6±0.4

Table 2. Chemical composition of wild-type (control) and transgenic poplar

	Lignin (%)	Holocellulose (%)	Extractives (%)	
			1% NaOH	Hot Water
Control	17.5±1.7	76.0±0.7	29.8±1.3	11.2±0.9
Transgenic	17.2±1.0	74.0±1.1	30.0±1.0	9.5±0.3

of transgenic and wild poplar.

However, a distinct decrease in absorption was observable in the vessel walls of transgenic poplar. A reduction in the absorbance value of about 15% was found in secondary walls as compared to control. Vessel secondary walls were known to consist mainly of G lignin (Musha and Goring 1975). Lower absorption value of vessel walls at 280 nm indicated transgenic poplar wood with lower amount of G lignin in vessel elements in comparison with control plant.

The present UV microscopical work suggested that the main transformation by antisense OMT construct was expressed in vessel walls but not in fiber walls. Despite transformation in the vessel walls, the total lignin content in the transgenic poplar did not change, suggesting that slight decrease of G lignin in transgenic poplars vessel walls might be compensated by the corresponding increase in S lignin in other tissues or cells.

3.4. Chemical Characteristics

The results of chemical analysis of transgenic poplar are summarized in Table 1. It is apparent that the chemical composition of antisense OMT poplar is almost identical to that of wild-type poplar. Klason lignin content of transgenic poplar did not show significant difference from that of the controls. These data, together with histochemical reactions, suggested that transformation did not affect the degree of lignification significantly in the transgenic plant. Additionally, there was little or no difference even in the amount of polysaccharides.

3.5. FT-IR

The FT-IR spectra of OMT and wild-type poplar are essentially same because of sharing similar maxima (e.g. 1740, 1650, 1600, 1500, 1460, 1430, 1380, 1335, 1250, 1050, 900 cm^{-1}). FT-IR spectral characteristics showed that no fundamental changes in cell-wall structure occurred, and that manipulation of OMT expression had not discriminately influenced cell-wall structure or composition (Fig. 5).

3.6. Solid-State ^{13}C CP/MAS NMR Spectroscopy

In general, the spectra of both the control and modified poplar xylems were similar when solid-state ^{13}C CP/MAS NMR was used. The chamical shifts and their structural assignments

Table 3. Chemical shifts and their structural assignments of the control and transgenic poplar (Ralph *et al.* 2001)

Chemical shift (ppm)		Structural assignment
Control	OMT	
171.8	171.8	COOH in aliphatic acid
152.8	152.9	C_3/C_5 ether-linked syringyl
	137.1	C_4 in syringyl, not ether-linked
133.8		C_1 of G and S, not ether-linked C_4 of S
104.1	104.4	C_1 of cellulose
74.1	74.4	C_2, C_3, C_5 of 4-linked polysaccharides
62.8	63.1	C_6 of crystal-surface cellulose
56.5	56.2	$-OCH_3$
21.5	21.0	$-CH_3$

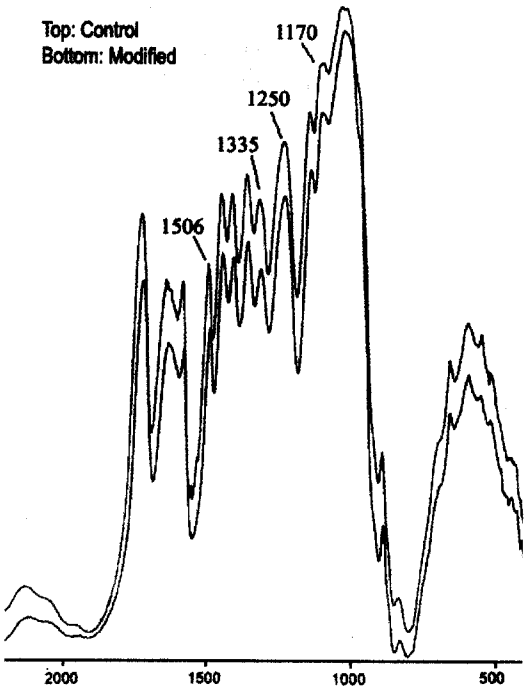

Fig. 5. FT-IR spectra of wild-type and transgenic poplar.

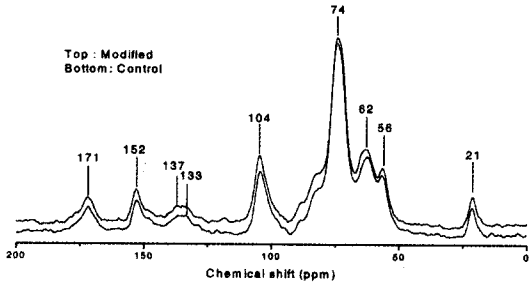

Fig. 6. Solid-state ^{13}C NMR spectra of control and transgenic poplar.

are shown in Table 3. The spectra consisted of carbohydrate region from 60 to 110 ppm and aromatic regions of typical lignin from 120 to 155 ppm as well as some aliphatic carbons at around 20 ppm region.

Fig. 6 exhibits that the chemical shift at around 137 ppm occurred for the transgenic plant while its intensity was very weak for the control. This shift could be interpreted as the C_4 of S lignin (Chen and Robert 1988). The presence of this peak for the transgenic poplar indicates the modified poplar possibly having more S lignin than control. In addition, the presence of 133 ppm for the control sample indicates the control sample with G lignin as well as S lignin. NMR spectra data suggested strongly that genetic modification in lignin biosynthetic pathways occurred in the transgenic poplar.

4. DISCUSSION

OMT has been suggested to play an important role in mediating the synthesis of S lignin (Higuch 1997). OMT uses caffeic acid or 5-hydroxyferulic acid as substrates, which serve as the starting point for the *in vivo* manipulation of lignin monomers in transgenic plants. OMT has been characterized in a number of species.

Our work showed that the expression of antisense OMT gene constructed in the poplar was not sufficient to reduce the overall the content of Klason lignin. The anatomical features also appeared to be similar. Transformed poplar with antisense poplar OMT did not exhibit any changes in the lignin content. Solid state NMR spectra indicated that the genetically modified poplar contained slightly more S lignin as compared to control.

Dwivedi *et al.* (1994) observed no alteration in the lignin content, but a change in lignin monomeric composition (reduction in syringyl aldehyde content) in the transformed with an antisense aspen COMT cDNA. In spite of 50% reduction of OMT activity in stems, the monomer composition of lignin was only slightly modified. This result seems to demonstrate that a strong reduction of OMT activity (greater than 60%) is necessary to produce significant changes in lignin composition (Boudet *et al.* 1995; Lapierre *et al.* 1999). Grünwald *et al.* (2002) also observed that the transgenic poplars did not show any distinctive differences in the structure and chemical composition of xylem cells as compared with the wild type trees.

However, substantial decrease in lignin content has been observed in some transgenic plants from modification of the expression of OMT enzymes (Sewalt *et al.* 1997; Kajita *et al.* 1997; Piquemal *et al.* 1998). A reduction in lignin content (15 to 57%) was found in the transformed tobacco with an antisense alfalfa COMT cDNA (Ni *et al.* 1994). Marita *et al.* (2003) also found that the alfalfa deficient in caffeoyl CoA-3-O-methytransferase exhibited a dramatic decrease in lignin content even though lignin of transgenic plant was structurally similar to that of control.

The differences obtained with different transgenic plants might be related to the heterologous expression or to the analytical method used by the different researchers, or various promoters used. Recently, an alternative methylation pathway in the lignin biosynthetic process has emerged (Parvathi *et al.* 2001). In addition to COMT and CCoAMT activities, other OMT activities may occur in the lignin biosynthetic pathway with various substrate specificities (Li *et al.* 1997). The occurrence of several OMT activities with various specificities and spatial or temporal expression patterns makes it difficult to predict the effect of selectively targeting bifunctional OMT.

Our work used just 1-year-old poplar trees regenerated from OMT-down regulated poplar. Wood quality of one-year-old trees is known to be quite different from that of mature woods, for example short fiber cells. Hence the data obtained in this study can not be representative of mature fiber cells because all tissues belong to juvenile wood. Further studies with transgenic mature trees are needed to determine the effect of down regulation in OMT on wood characteristics of transgenic poplar.

5. CONCLUSION

Anatomical and chemical characteristics of the transgenic poplar down-regulated with antisense OMT gene were investigated. The color of xylem, the dimensions of fiber and vessel element in the transgenic poplar were similar to those of control poplar. No visible abnormal phenotype was observed in the vessel elements of transgenic poplar. No marked differences

occurred in the staining intensities in Wiesner and Mäule color reaction, and no detectable difference was observed in $KMnO_4$-staining intensities in the TEM micrographs in the transgenic poplar. The UV spectroscopy of semi-thin sections showed a distinct decrease in the vessel walls in absorption maxima at 280 nm. Chemical composition of antisense OMT poplar was almost identical to that of wild-type poplar. Even little or no differences were found in the amount of polysaccharides. The solid state NMR spectra showed that a chemical shift at around 137 ppm for the transgenic plant, indicating the transgenic poplar possessing more S lignin as compared to control. In conclusion, the present work showed that antisense OMT gene constructed in the poplar was not sufficient to reduce the overall the content of Klason lignin.

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