Modified lignification in the cell walls of cad depressed poplars

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MODIFIED LIGNIFICATION IN THE CELL WALLS OF CAD DEPRESSED POPLARS

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SUMMARY

Ultraviolet microscopic spectrophotometry was used to investigate lignification in the secondary cell walls of wood fibers and vessel elements from either wild type or transgenic poplars with depressed activity of cinnamyl alcohol dehydrogenase (CAD). A distinct shoulder at 330 nm was characteristic of the transgenic poplar. Measurements performed after 1) a mild alkali treatment or 2) a reduction with sodium borohydride indicated that this shoulder merely resulted from the occurrence of conjugated carbonyl groups in the lignin polymer rather than alkali-soluble cell wall bound phenolic aldehydes. UV absorbance ratios (A330 / A280) measured in the center of secondary walls in the differentiating xylem clearly showed that the structural changes observed in the lignin polymer of transgenic trees occurred very early during lignin deposition. This suggests that, in poplar trees with low CAD activity, cinnamaldehydes are incorporated into lignin even at the early stages of lignification.

Key words: Cinnamyl alcohol dehydrogenase (CAD), transgenic poplar, UV microscopic spectrophotometry, transmission electron microscopy, lignification, cell wall, wood fibers, vessel elements.

INTRODUCTION

Cinnamyl alcohol dehydrogenase (CAD) catalyzes the reduction of cinnamaldehydes into cinnamyl alcohols, which is the last step in the monolignol biosynthesis pathway. Severe reduction of CAD activity was reported in brown-midrib mutants of maize (Halpin *et al*. 1998) and in a mutant allele of the *cad* gene in loblolly pine (MacKay *et al*. 1999). Genetic engineering with an antisense strategy also led to the production of plants with reduced CAD activity, in tobacco (Halpin *et al*. 1994; Hibino *et al*. 1995; Yahiaoui *et al*. 1998a, b), poplar (Baucher *et al*. 1996) and alfalfa (Baucher *et al*. 1999).

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The chemical structure of lignin from CAD down-regulated plants was analyzed by Fourier-transformed Infrared and Raman spectroscopy (Stewart *et al*. 1997), thioacidolysis (Lapierre *et al*. 1999) and mild alkali hydrolysis (Baucher *et al*. 1996; Lapierre *et al*. 1999, 2004), NMR (Kim *et al*. 2000, 2002, 2003; Akim *et al*. 2001; Ralph *et al*. 2001). When CAD is down-regulated, the cinnamaldehydes instead of cinnamyl alcohols may be incorporated into the polymer by radical coupling. Some model experiments also support the incorporation of cinnamaldehydes into lignin by radical coupling (Higuchi *et al*. 1994; Yahiaoui *et al*. 1998b; Kim *et al*. 2000, 2003; Russel *et al*. 2000). CAD down-regulation also led to an increase in both C6–C1 aldehydes, especially syringaldehyde in mild alkali soluble fraction (Baucher *et al*. 1996; Lapierre *et al*. 1999), and free phenolic units, which may ease lignin solubilization and fragmentation during kraft pulping (Lapierre *et al*. 1999).

The effect of the lignin modifications resulting from low CAD activity were further analyzed with regard to pulping properties (O'Connell *et al*. 2002), decomposition (Hopkins *et al*. 2001) and degradability (Bernard-Vailhé *et al*. 1996, 1998). From the evaluation of field-grown transgenic poplars with low CAD activity, Pilate *et al*. (2002) demonstrated that the lignin modifications were maintained in the transgenic trees over four years and that one of the reduced-CAD lines had improved characteristics, allowing easier delignification during the pulping process, while using smaller amounts of chemicals and yielding more high-quality pulp. But, until now, the changes in lignin structure resulting from CAD down-regulation has been mainly analyzed in lignin purified extract using destructive assays whereas the lignification process in the cell walls of CAD down-regulated poplars has not yet been described. In this study, we investigated the lignification process in the fiber and vessel secondary walls of CAD down-regulated poplars using *in situ* UV microscopic spectrophotometry before and after mild alkali hydrolysis or reduction with sodium borohydride.

MATERIALS AND METHODS

Plant materials

Genetic transformation has been carried out on a hybrid *Populus tremula* × *P. alba* clone (INRA 717-1-B4) as already described (Leplé *et al*. 1992). Biochemical characterizations revealed that ASCAD21 transgenic trees were down-regulated for CAD activity (Baucher *et al*. 1996). In January 1995, poplar microcuttings were acclimatized in the greenhouse and planted in the field (Pilate *et al*. 2002). In June 1999, five centimeter-thick stem slices were collected from the ASCAD21 or from the wild type trees and stored into 80% ethanol. The stems were two years old grown from four-year-old rootstock. The residual CAD activity measured in the ASCAD21 tree used in this study was only 12% of the wild type.

Histochemical staining

In order to detect the occurrence of tension wood, which exhibits unusual lignification pattern particularly in the gelatinous layer, 50-µm-thick transverse sections, sprayed on the whole surface of the wood slices, were cut and stained with phloroglucinol-HCl. Then, small blocks were cut from the slice so that they were devoid of tension wood.

The blocks without tension wood were separated into two categories: 1) the blocks containing differentiating xylem and 2) the blocks containing mature xylem. These two kinds of block from ASCAD21 line and wild type were dehydrated through an ethanol series and embedded in methyl- and butyl-(1 : 1, v/v)methacrylate resin.

UV microscopic spectrophotometry on mature xylem

One-μm-thick transverse sections were cut from the embedded mature xylem of ASCAD21 and wild type trees and placed on quartz slides. The slides were soaked in acetone for one hour to remove the resin from the sections. Then, sections were mounted with glycerin and covered with quartz coverslips.

UV absorption was surveyed in the range of 250 to 400 nm in 2.5 nm steps for spot diameter (0.5μm) and a band width of an illuminating monochrometer (5 nm) using a microscopic spectrophotometer (Carl Zeiss MPM 800). UV absorption spectra were measured at the center of the secondary walls of fibers and vessels (noted respectively FSW and VSW) and also at the cell corner middle lamella located between fibers (FF-CC). For each cell type (FSW, VSW, and FF-CC), 14 points were selected in the early-wood side, 11 points were selected in the latewood side, and 5 points were selected in the terminal zone where vessel diameters strongly decreased, at the end of each annual ring. UV photomicrographs were also taken at 280 nm and 330 nm using a microscopic spectrophotometer (Carl Zeiss UMSP-80).

Sections were eventually treated by drop-wise addition of either 1) 2 M sodium hydroxide for two hours at room temperature to remove phenolic aldehydes incorporated into lignin as reported by Lapierre *et al.* (1999) or 2) 0.05 M sodium borohydride in 0.03 M sodium hydroxide for one hour at room temperature to reduce cinnamaldehyde groups incorporated in the lignin polymer. A diluted sodium hydroxide (0.03 M) was used for stabilizing sodium borohydride (Marton 1964). After either of these treatments, sections were washed several times in water, dried, mounted with glycerin, and covered with quartz cover slips. UV absorption spectra were recorded after treatment just as described above except for the measuring conditions that were slightly modified for spot diameter (1.5 μm) and steps (2 nm).

Measurement of UV absorbance in the differentiating xylem

One-μm-thick transverse sections were cut from differentiating xylem of both ASCAD21 and wild type trees. The sections were placed on quartz slides and soaked in acetone for one hour to remove the resin from the sections. The sections were mounted with glycerin, and then covered with quartz cover slips. UV absorption at 280 nm was measured at the center of developing secondary walls of fibers and vessels in the differentiating xylem for spot diameter (0.5 or 1.0 μm) and a band width of an illuminating monochrometer (20 nm) using the same microscopic spectrophotometer. Measurements were done on 29 points for fibers and 18 points for vessels. UV absorption at 330 nm was also measured at the same time to detect potential structural changes in the lignin polymer, which appeared as an additional shoulder around 330 nm in the UV absorption spectrum of the CAD down-regulated poplar samples. Distance from the cambial zone of the individual cell was measured as described in Yoshinaga *et al.* (1997).

Transmission electron microscopy of developing secondary walls

Lignification process of developing cell walls was also observed by transmission electron microscopy after potassium permanganate ($KMnO_4$) staining. Ultrathin sections, about 0.1 μm thick, were cut from differentiating xylem of both ASCAD21 and wild type trees. These sections were mounted on Formvar coated copper grids reinforced by carbon. To remove the resin from the sections, the grids were soaked in acetone for 20 min at room temperature. Then the section were stained with $KMnO_4$ as described in Yoshinaga *et al*. (2004), and observed under a transmission electron microscope (JEOL JEM 1220) at 100 keV.

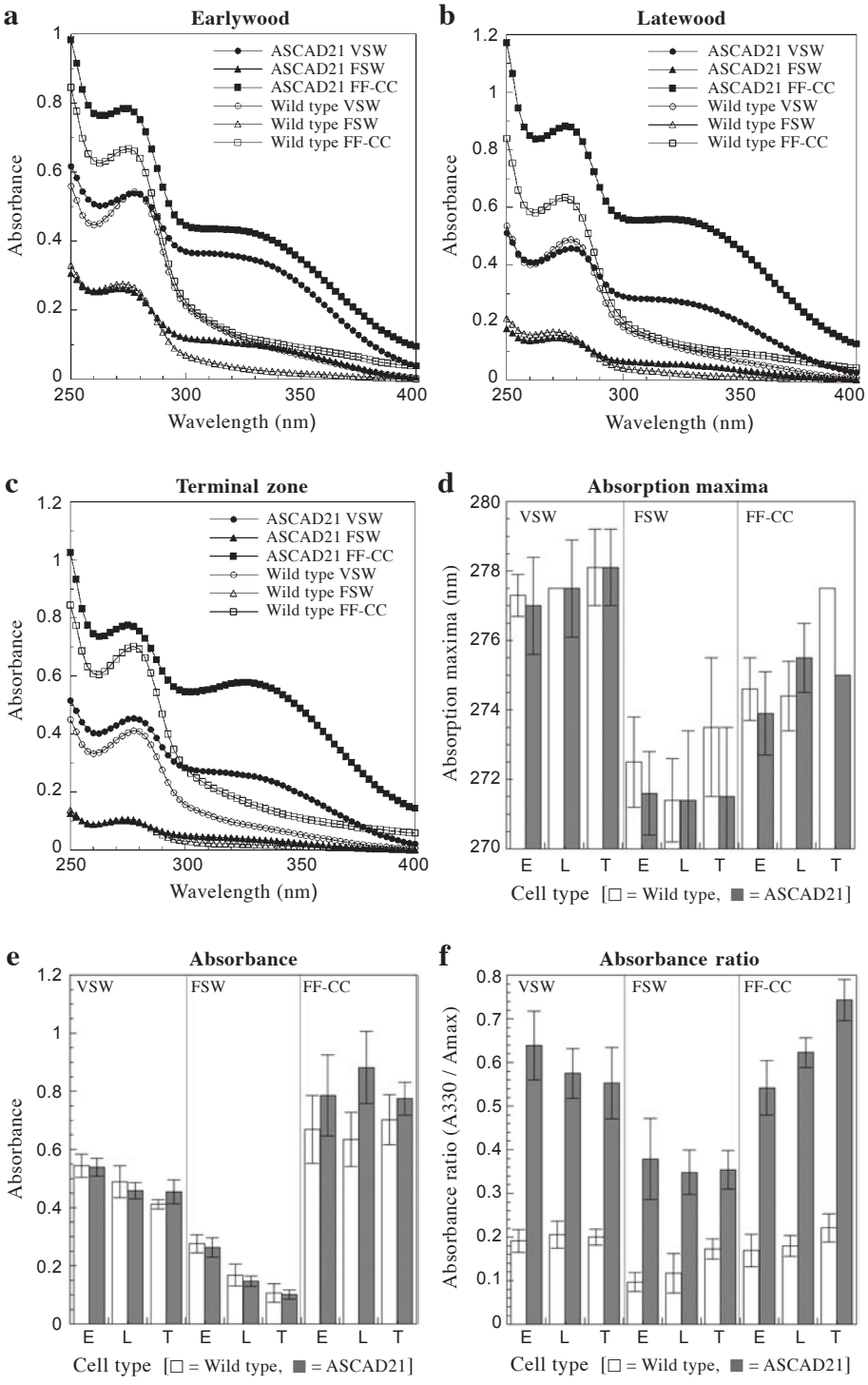
RESULTS AND DISCUSSION

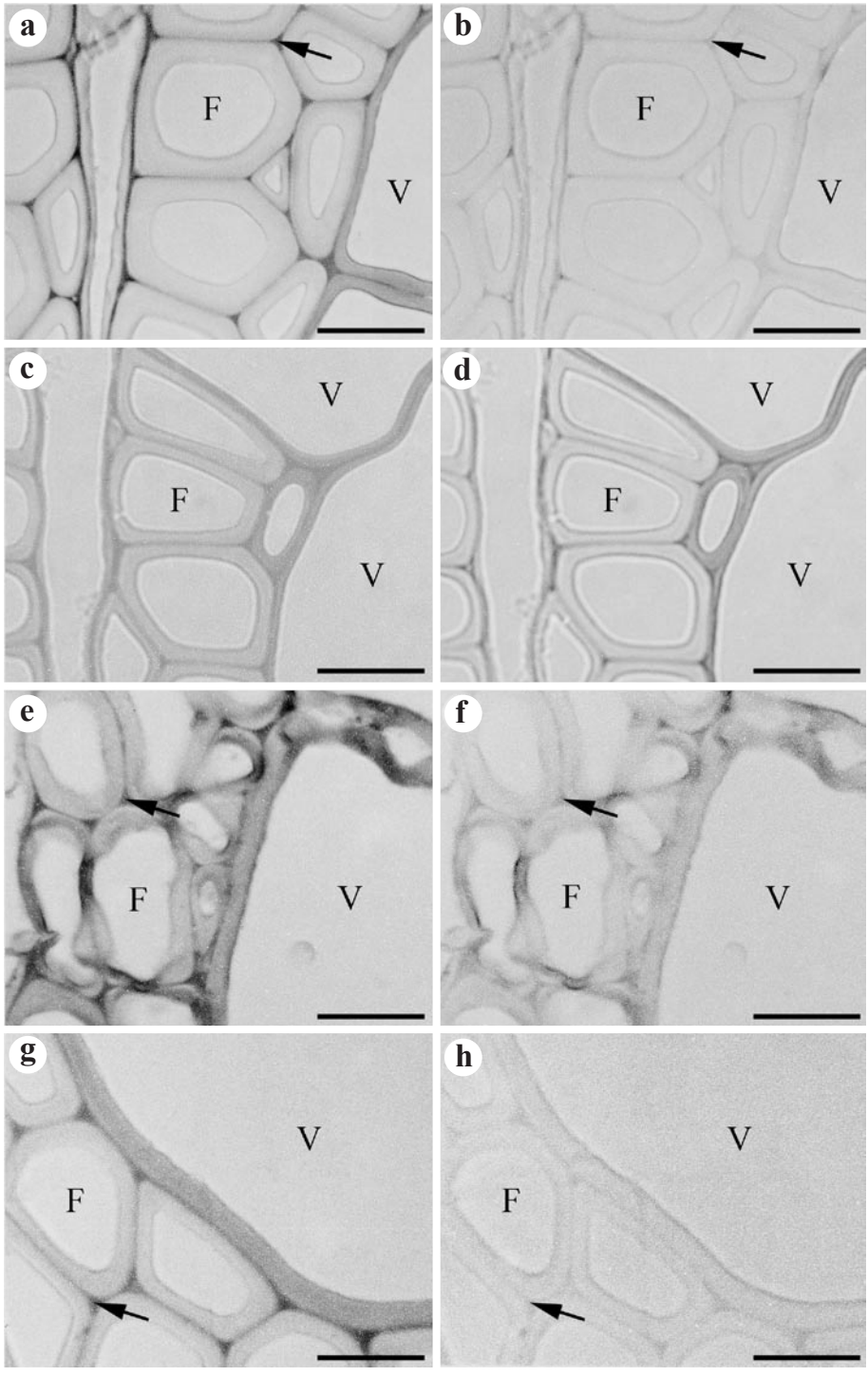
UV absorption spectra are different in ASCAD21 and wild type trees

UV absorption spectra were determined in the secondary walls of wood fibers (FSW) and vessel elements (VSW) and also in the cell corner middle lamella between wood fibers (FF-CC) in ASCAD21 and wild type trees (Fig. 1a–c). These spectra were taken from the earlywood (a), the latewood (b), and the terminal zone (c) of mature xylem and expressed as the average of the different measuring points. In ASCAD21 line, an additional shoulder around 330 nm was consistently detected in FSW, VSW and FF-CC, whereas this shoulder was not detected in wild type trees. Second derivatives calculated on UV absorption spectra in ASCAD21 line revealed that the absorption maxima were in the range of 330 to 335 nm. Further analyses revealed that there were no obvious differences between ASCAD21 and wild type trees in the absorption maxima (Fig. 1d) and the absorbance at the absorption maxima (Fig. 1e). On the contrary, the absorbance ratios (A330/Amax) in Figure 1f were consistently higher in the ASCAD21 tree (in the range of 0.5–0.6 in VSW, 0.3–0.4 in FSW, and 0.5–0.8 in FF-CC) than in the wild type tree (in the range of 0.1–0.2 in VSW, FSW and FF-CC). It should be noted that the ratio (A330/Amax) was higher in VSW and FF-CC than FSW. This suggests larger structural lignin modifications in VSW and FF-CC than in FSW.

UV photomicrographs taken at 280 nm and 330 nm in wild type (Fig. 2a, b) and ASCAD21 line (Fig. 2c, d) confirm UV absorption spectra measurements. Indeed, in the wild type, there was almost no absorption in all cell walls at 330 nm (Fig. 2b), whereas in ASCAD21 line, the absorption at this wavelength was important in the secondary walls of wood fibers and vessel elements and compound middle lamella (Fig. 2d). Chabannes *et al*. (2001) also observed such an increased absorption around

Figure 1. UV absorption spectra and evaluation of lignification in the mature xylem of ASCAD21 and wild type trees. – a: spectra from the earlywood side. – b: spectra from the latewood side. – c: spectra from the terminal zone. – d: absorption maxima (mean and SD) of the spectra. – e: absorbance at the absorption maxima (mean and SD) of the spectra. – f: absorbance at 330 nm/absorbance at the absorption maxima (mean and SD) of the spectra. — E: earlywood; L: latewood; T: terminal zone; VSW: vessel secondary walls; FSW: wood fiber secondary walls; FF-CC: cell corner middle lamella between wood fibers. The spectra in a–c were expressed as the average of the different measuring points.


a
Earlywood
ASCAD21 VSW
ASCAD21 FSW
ASCAD21 FF-CC
Wild type VSW
Wild type FSW
Wild type FF-CC
Absorbance
Wavelength (nm)
b
Latewood
c
Terminal zone
d
Absorption maxima
VSW
FSW
FF-CC
Absorption maxima (nm)
E L T E L T E L T
Cell type [□ = Wild type, ■ = ASCAD21]
e
Absorbance
f
Absorbance ratio
Absorbance ratio (A330 / Amax)


a
F
V
b
F
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c
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f
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Table 1. The absorption maxima (λmax) and absorbance ratios (A330/Amax) in ASCAD 21 and wild type trees.

Cell type (treatment)	Wild type				ASCAD 21			
	EW		LW		EW		LW	
	λmax (nm)	A330 / Amax	λmas (nm)	A300 / Amax	λmax (nm)	A330 / Amax	λmax (nm)	A330 / Amax
VSW (no treatment)	278	0.202	278	0.134	278	0.435	276	0.635
VSW (NaOH)	274	0.134	278	0.235	278	0.453	276	0.605
VSW ($NaBH_4$)	278	0.127	276	0.148	278	0.102	278	0.056
FSW (no treatment)	276	0.083	274	0.053	274	0.335	274	0.641
FSW (NaOH)	274	0.038	272	0.064	270	0.489	276	0.619
FSW ($NaBH_4$)	276	0.092	274	0.062	272	0.098	272	0.108
FF-CC (no treatment)	278	0.365	280	0.259	276	0.465	278	0.672
FF-CC (NaOH)	278	0.384	282	0.431	278	0.616	274	0.566
FF-CC ($NaBH_4$)	278	0.158	278	0.218	276	0.103	280	0.287

330 nm in the xylem of CAD down-regulated tobacco and proposed it resulted from an accumulation of wall bound phenolics whose absorption maximum is in the range of 330 nm.

Effect of a mild alkali treatment on UV absorption spectra

UV absorption spectra at FSW, VSW and FF-CC in ASCAD 21 earlywood and latewood after a mild alkali treatment indicated that the typical absorption shoulder found at around 330 nm remained mostly unaffected (Fig. 3a, c, e). The absorbance at 280 nm became higher in FSW and slightly lower in VSW and FF-CC after the treatment. This suggests a different reactivity of FSW against alkali treatment compared to VSW and FF-CC. As illustrated in Figure 2e & f, deformation appeared more important in FSW than in VSW and FF-CC. This suggests that FSW is more heavily affected by alkali treatment. The increase in the absorbance at 280 nm in FSW may be due to the removal of some hemicelluloses and/or a change in absorptivity of lignin with alkali. Alternatively, as it is known that the lignin from ASCAD poplar wood is easily removed with alkali, it is also possible that, together with hemicelluloses, some lignin was removed during the alkali treatment in VSW and FF-CC. UV photomicrographs taken at 280 nm and 330 nm on ASCAD 21 sections after a mild alkali treatment showed that there is still an increased absorption at 330 nm (Fig. 2d) as compared with wild type (Fig. 2b)

Figure 2. UV photomicrographs of mature xylem in ASCAD21 and wild type trees. – a: wild type at 280 nm. – b: wild type at 330 nm. – c: ASCAD21 line at 280 nm. – d: ASCAD21 line at 330 nm. – e & f: ASCAD21 line after a mild alkali treatment; e: at 280 nm; f: at 330 nm. – g & h: ASCAD21 line after a reduction with sodium borohydride; g: at 280 nm; h: at 330 nm. — F: wood fibers; V: vessel elements; Arrows: cell corner middle lamella between wood fibers. — Scale bars = 20 μm.

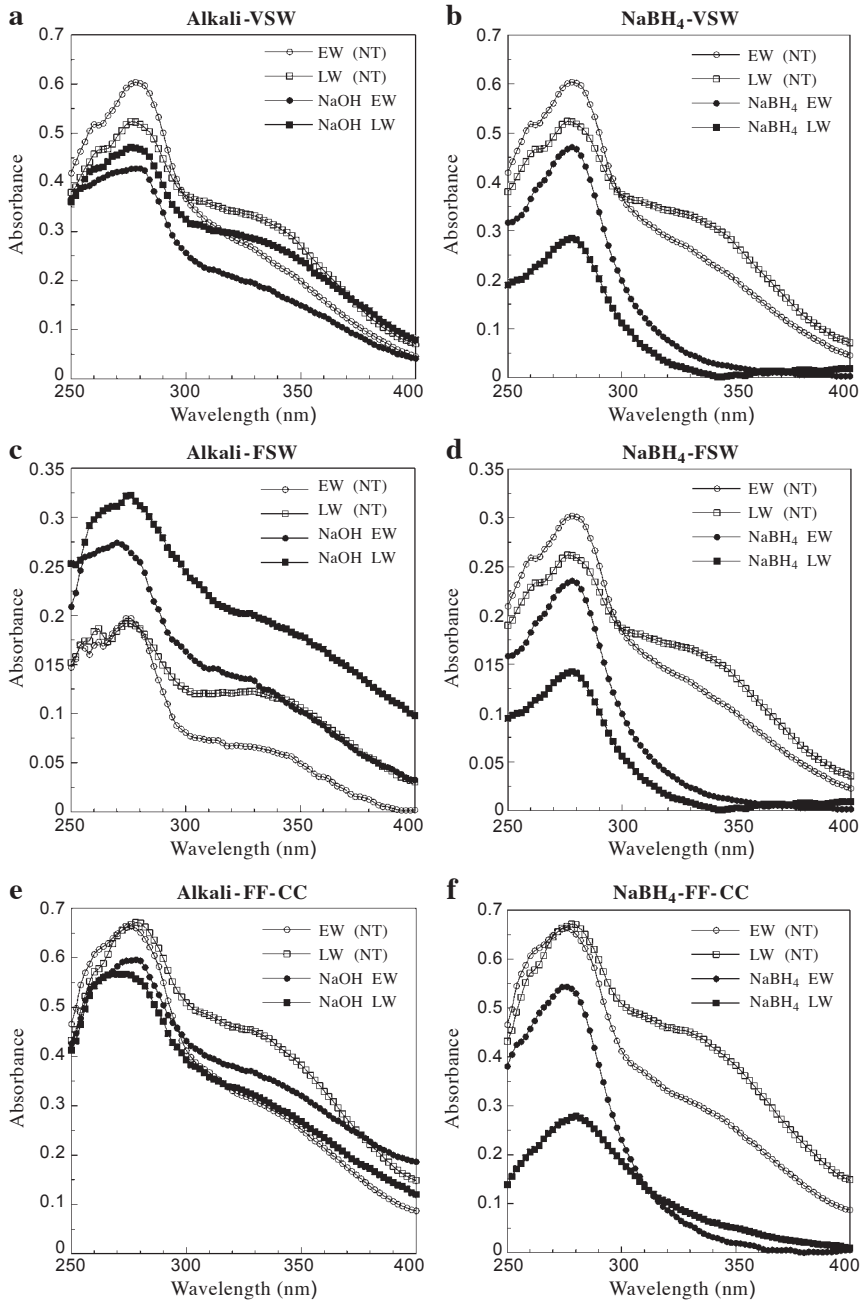

Figure 3. UV absorption spectra of mature xylem in ASCAD21 trees before and after a mild alkali treatment (a, c & e) or a reduction with sodium borohydride (b, d & f). – NT: no treatment. – NaOH: after a mild alkali treatment. – $NaBH_4$: after a reduction with sodium borohydride. – EW: earlywood. – LW: latewood. – VSW: vessel secondary walls. – FSW: wood fiber secondary walls. – FF-CC: cell corner middle lamella between wood fibers.

after a mild alkali treatment in the secondary walls of wood fibers, vessel elements and compound middle lamella (Fig. 2f). Interestingly, the absorbance ratio (A330/Amax) of VSW, FSW and FF-CC in ASCAD21 poplar (Table 1) increased in earlywood when it slightly decreased in latewood after a mild alkali treatment. In wild type tree, the ratio decreased in VSW and FSW in earlywood, but increased in FF-CC in earlywood as well as in VSW, FSW and FF-CC in latewood. This suggests that the reactivity against alkali treatment may be different in early- and latewood. An increased ratio may result from a change in the absorptivity of lignin (decrease in Amax), whereas a decreased ratio may result either from the removal of substances absorbing at 330 nm (decrease in A330) or from a change in the absorptivity of lignin (increase in Amax).

We cannot establish a relation between the increased content of phenolic aldehydes (especially syringaldehyde) in the ASCAD21 tree observed by Baucher *et al.* (1996) and Lapierre *et al.* (1999) and the typical absorption shoulder that we observed at 330nm in the ASCAD tree, since the conditions of mild alkali treatment used in these studies are different.

Changes in UV absorption spectra after reduction with sodium borohydride

UV absorption spectra after sodium borohydride treatment at FSW, VSW and FF-CC on ASCAD21 sections does not reveal anymore the typical absorption shoulder around 330 nm and these spectra became very comparable to those recorded on wild type sections (Fig. 3b, d, f). There was no obvious difference in spectral shape in wild type after the treatment (data not shown).

UV photomicrographs taken at 280 nm and 330 nm in ASCAD21 line after sodium borohydride treatment confirmed the disappearance of the absorption at 330 nm in the secondary walls of wood fibers, vessel elements and compound middle lamella (Fig. 2g, h). This disappearance was also confirmed by the absorbance ratios (A330/Amax) summarized in Table 1. Sodium borohydride has been used to evaluate the conjugated carbonyl groups in lignin (Chen 1992). The time required for reduction varies according to the type of conjugated carbonyl structures and also with the occurrence of an etherification at the C4 (Adler & Marton 1959). Our results suggest that there is an increase in conjugated carbonyl groups (cinnamaldehyde or α- or β-carbonyl groups) in the lignin of ASCAD21 secondary walls of both wood fibers and vessel elements.

There were quite large differences in absorbance at maxima between early- and latewood after the treatment in VSW, FSW and FF-CC (Fig. 3b, d, f). Owing to the fact that lignin from ASCAD poplar is more easily removed with alkali (Baucher *et al.* 1996), this suggests that weak amounts of lignin may have been removed during the reduction with borohydride thanks to the simultaneous addition of 0.03 M sodium hydroxide. Alternatively, the lignin absorbance may decrease with the reduction of aldehyde groups. Notably, considering that absorbance ratio (A330/Amax) in ASCAD21 (untreated samples) are always lower in earlywood as compared to latewood, one can suggest that the overall decreasing absorbance of lignin in latewood after sodium borohydride treatment would rely on a higher contribution of aldehyde groups as compared to earlywood. Finally, there was no obvious difference in the position of absorption maxima between treated and control samples (Table 1). G-lignin exhibits a maximum

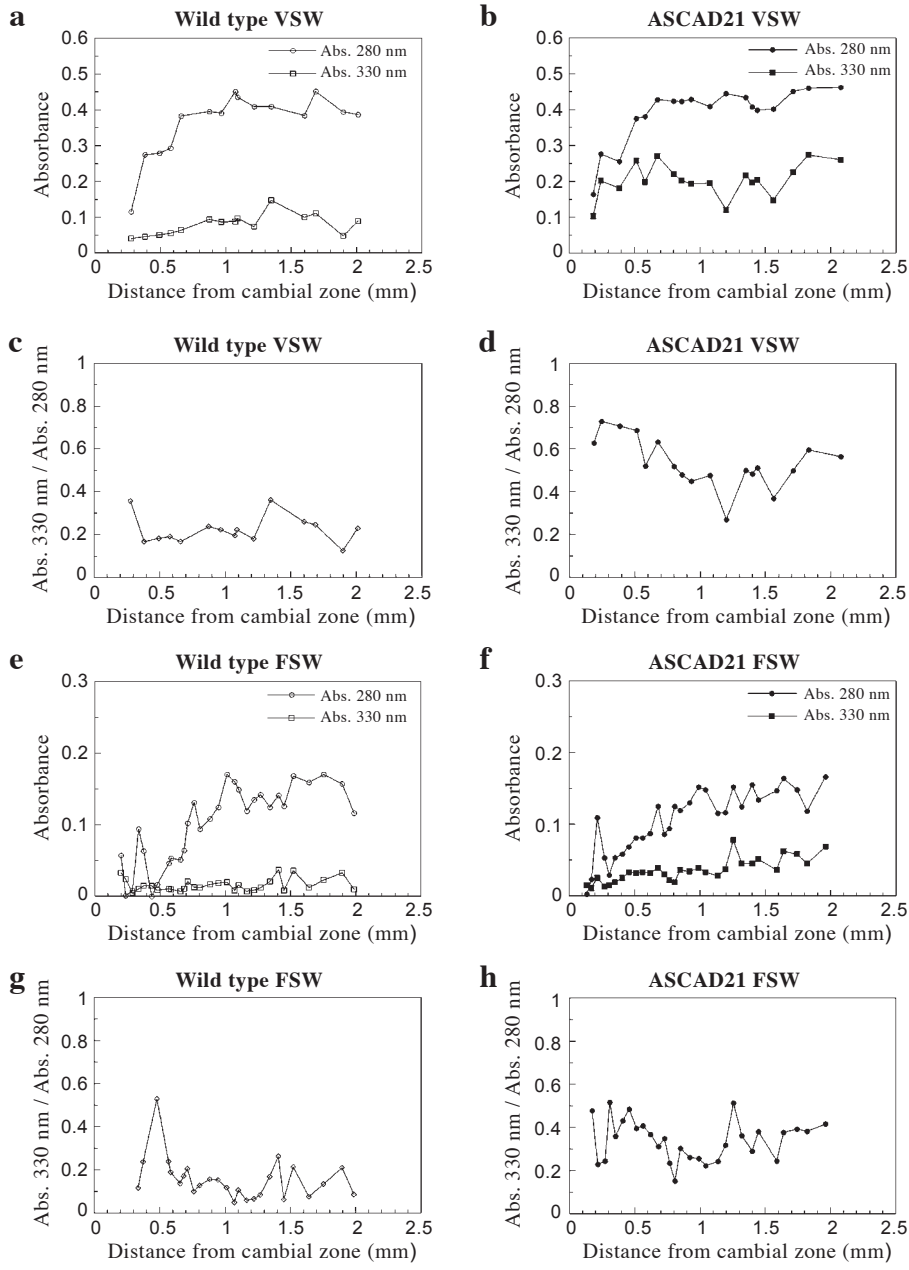

Figure 4. Changes during xylem differentiation in the absorbance at 280 nm and 330 nm measured at the center of developing secondary walls and the absorbance ratio (absorbance at 330 nm/ absorbance at 280 nm) in ASCAD21 and wild type trees. – a & c: vessel secondary walls (VSW) in wild type. – e & g: wood fiber secondary walls (FSW) in wild type. – b & d: VSW in ASCAD21 line. – f & h: FSW in ASCAD21 line.

absorbance at 280 nm, whereas the maximum shifts, by 7–10 nm, to a shorter wavelength in syringyl lignin (Fergus & Goring 1970). Therefore, this result indicates that the content in guaiacyl and syringyl lignins was not affected by the treatment.

Lignification in the differentiating xylem of ASCAD21 and wild type trees

UV absorbance at 280 nm and 330 nm in developing secondary walls of wood fibers and vessel elements in ASCAD21 line and wild type was plotted according to the distance from the cambial zone (Fig. 4). The absorbance at 280 nm is indicative of the lignification process in the secondary walls whereas the absorbance at 330 nm is indicative on the extent of the structural differences caused by CAD down-regulation. In the wild type vessel secondary walls (Fig. 4a), the absorbance at 280 nm increased rapidly to reach a maximum and then remained constant. The absorbance at 330 nm was also slightly increased to become constant, as well. The absorbance ratio (A330/A280) was in the range of 0.2–0.3 (Fig. 4c). In ASCAD21 vessel secondary walls (Fig. 4b), the absorbance at 280 nm and at 330 nm evolved much like what was observed in the wild type if it was the higher absorbance ratio (in the range of 0.4–0.6) constantly recorded even in the early stage of lignification (Fig 4d).

In the wild type fiber secondary walls (Fig. 4e), the absorbance at 280 nm increased and became constant whereas the absorbance at 330 nm remains quite low. In accordance, the absorbance ratio (A330/A280) was in the range of 0.1–0.2 (Fig. 4g). In the ASCAD21 fiber secondary walls (Fig. 4f), the absorbance at 280 nm and at 330 nm during xylem differentiation are much comparable to what occurred in the wild type fiber secondary cell walls, with again a higher absorbance at 330 nm, as was generally observed in the samples with low CAD activity. In consequence, the absorbance ratio (A330/A280) was higher than for the wild type (in the range of 0.2–0.4) and almost constant even during the early stage of lignification (Fig. 4h). However, the absorbance ratio for fibers was lower than what was recorded in the vessel secondary walls. In conclusion, the lignification pattern in VSW and FSW appeared similar in wild type and ASCAD21 tree, whereas the lignin structural changes resulting from CAD down-regulation were already present in VSW and FSW at the very early stages of lignification.

UV and TEM observation of differentiating xylem did not reveal any obvious difference in the lignification pattern of ASCAD21 and wild type trees (Fig. 5). UV absorption and contrast in TEM were almost similar between ASCAD21 and wild type trees both in lignifying cells (Fig. 5a–d) and in lignified cells (Fig. 5e–h). This is clearly different from what has been previously observed in the fibers of CAD down-regulated tobacco (Takabe *et al.* 1996) although the residual CAD activity in ASCAD21 poplar

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Figure 5. UV and TEM micrographs of differentiating xylem in ASCAD21 and wild type trees. – a–d: lignifying cells. – e–h: lignified cells. – a, c, e & g: wild type. – b, d, f & h: ASCAD21. – a, b, e & f: UV photographs taken at 280 nm. – c, d, g & h: TEM micrographs of $KMnO_4$ stained sections. — F: wood fibers; V: vessel elements. — Scale bars are 20 μm (a, b, e & f) and 1 μm (c, d, g & h).

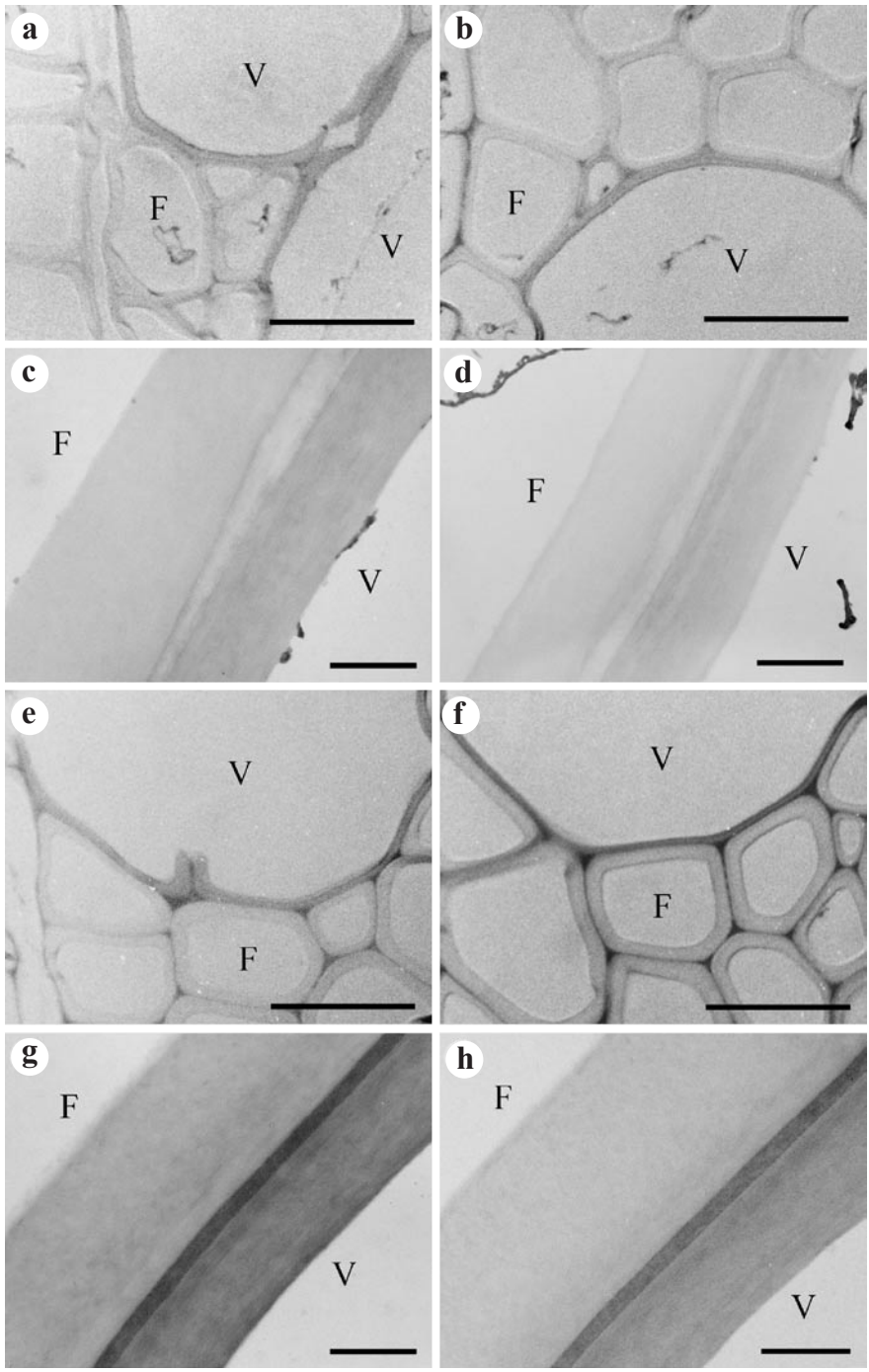

Figure 5; for the legends, see the previous page.

was lower than that in their CAD down-regulated tobacco. Indeed, these authors have detected strong UV-absorptive substances (electron-opaque materials under TEM) both at the inner surface of the fibre cell walls and in the lumen of vessels. Although we used similar techniques, we were not able to find such substances in ASCAD21 xylem fibers.

CONCLUSION

In this study, we have demonstrated that the change associated with low CAD activity results in a shoulder at 330 nm in mature and differentiating xylem in VSW, FSW, and FF-CC. In addition, a higher absorbance ratio has been recorded in ASCAD21 compared to wild type tree during the early stage of lignification in both vessel and fiber secondary walls. Results from sodium borohydride reduction with mature xylem are indicative of an increase in conjugated carbonyl groups in the lignin of the ASCAD21 tree. All these results show that the shoulder is most likely associated to an incorporation of cinnamaldehydes in the lignin polymer, in agreement with Ralph *et al.* (2001), Kim *et al.* (2002) and Lapierre *et al.* (2004).

According to Terashima (1990), lignin from vessels is richer in G units as well as middle lamellae and cell corners that are the area where lignification begins. However, the changes associated with low CAD activity were consistently detected in FF-CC, FSW and VSW, even during the early steps of secondary wall development. This is not in favor of a differential effect of CAD down-regulation on guaiacyl and syringyl lignin synthesis. Spectral comparison before and after sodium borohydride reduction using coniferyl aldehyde and sinapaldehyde and their polymerization products will be necessary to elucidate any potential differential effect of CAD down-regulation on guaiacyl and syringyl lignin synthesis in ASCAD trees.

In this study, *in situ* UV microspectrophotometric analysis of cell walls of ASCAD poplar tree demonstrate the incorporation of cinnamaldehydes in the lignin of VSW, FSW and FF-CC, whereas the lignification process in vessel and wood fiber walls did not seem different from what occurs in wild type trees.

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