Anatomical and Immunocoverage Observations on SuSy, C4H, and Pectate Lyase Family Protein Down-regulated Aspens Genotypes

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This study aims to complement previous studies on down-regulation of sucrose synthase and cinnamate 4-hydroxylase in hybrid aspen (Bjurhager *et al.* 2010; Gerber *et al.* 2014) with a quantitative anatomical comparison (Sandquist *et al.* 2015). The main focus was placed on evaluating quantitative modifications to fiber morphology and lignin composition. This was achieved by combining results from light-, electron-, and UV-microscopy together with histochemical staining. Overall there was good agreement between the morphological results in this study compared to the previously published chemical and physical reports, particularly on cinnamate 4-hydroxylase down-regulated aspen. The previously reported statistical correlations between fiber lumen area and plant diameter, and fiber length and tree height (Sandquist *et al.* 2015) have been further refined. Knock-down of genes coding for biologically important proteins such as sucrose synthase and cinnamate 4-hydroxylase had broad spanning morphological impacts. Down-regulated sucrose synthase genotype showed significantly reduced fiber development, possibly with altered S/G ratio. Down-regulated cinnamate 4-hydroxylase genotype showed an overall reduction of lignin in fibers as indicated from both chemical staining and UV-microscopy measurements. Down-regulated pectate lyase genotype showed no overall significant effects on fiber morphology, ultrastructural, nor lignin staining.

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INTRODUCTION

The increasing world demand for plant lignocellulosic biomass has accelerated in recent years as the competition for conventional use of wood material for pulp and paper and construction has been further extended as a resource for bioenergy and more recently biofuels (Vanholme *et al.* 2008). This competition for lignocellulose sees no sign of plateauing out but rather is likely to accelerate as resources begin to dwindle and pressure from accompanying environmental concerns increase. The ability to "domesticate" trees through genetic engineering has great potential as a means for producing new clones for

producing greater or modified biomass in a shorter time *via* enhanced growth (Hu *et al.* 1999; Pilate *et al.* 2002). Most of the early research on genetic engineering of trees has been conducted using poplar as a model system due to its rapid growth rate and the fact that its genome has been fully sequenced (Tuskan *et al.* 2003), simplifying the production of transgenic plants (Hu *et al.* 1999; Boerjan *et al.* 2003; Li *et al.* 2003).

Wood is composed primarily of cellulose, lignin, and hemicelluloses, as well as other minor components (Sjöström 1993). Of these, cellulose provides load-bearing in trees and products, while lignin gives wood its hydrophobic character, natural resistance to compressive and tension forces, as well as protection against decay (Fengel and Wegener 1989). Lignin also provides rigidity to wood and at the macromolecular level is cross-linked to the hemicelluloses (Sjöström 1993).

Lignin is a recalcitrant component in wood that needs to be removed during chemical pulping, a process that is both energy-consuming (requiring heat and chemicals) and environmentally negative (Hu *et al.* 1999; Chiang 2002; Li *et al.* 2003; Baucher *et al.* 2003; Vanholme *et al.* 2008). The major industrial potential of the modification/reduction of lignin in trees in both content and composition was thus realized quite early on and much research has since been orientated to understanding the biosynthetic pathways of lignin metabolism in trees with this purpose in mind (Boerjan *et al.* 2003; Baucher *et al.* 2003; Vanholme *et al.* 2008). One of the major early findings was demonstration in transgenic poplar that repression of lignin biosynthesis also promotes cellulose accumulation and growth (Hu *et al.* 1999; Li *et al.* 2003).

Despite the intense research on lignin biosynthesis in poplar species, few studies appear however to have concentrated on the morphological effects on wood anatomy, and spatial changes in lignin in relation to ultrastructure and physical properties caused by modifications in lignin biosynthesis (Leple *et al.* 2007; Horvath *et al.* 2010).

The aim of this study was to investigate changes induced in the wood anatomical (tissue) structure, lignin micro-distribution, and ultrastructure of three transgenic aspen trees (*Populus tremula* x *tremuloides*). Each tree has one knocked down gene each, abbreviated as KR112 (sucrose synthase, Sjödin *et al.* 2009), KR139 (cinnamate 4-hydroxylase, Gerber *et al.* 2014) and K213 (pectate lyase coding genes, Sandquist *et al.* 2015) in comparison with their reference wild type control (T89).

The study complements and extends previously published observations on the three genotypes (Table 1), with detailed anatomical structural observations. This has been achieved using quantitative image analysis of transverse sections (Sandquist *et al.* 2015) and macerated fibers, UV-microscopy and histochemical staining of syringyl- and guaiacyl lignin micro-distributions, and ultrastructural observations on changes in fiber wall structure as determined using TEM.

EXPERIMENTAL

Materials
Genetic transformation

Antisense constructs were generated by cloning a partial cDNA (CloneID, popgenie.org, Sjödin *et al.* 2009) as shown in Table 1 in a gateway compatible anti-sense binary vector, pK7GWIWG2(I) (Karimi *et al.* 2002). The construct was then transformed into hybrid aspen (clone T89) (*Populus tremula* x *tremuloides*) as described previously

(Nilsson *et al.* 1992). Kanamycin-resistant lines were clonally propagated *in vitro* and planted in a greenhouse.

Table 1. Aspen Genotype Constructs Used in the Study

Construct	PU No.	POPTR ID	CloneID	Annotation
KR112	PU01211	POPTR_0006s13900.1	A037P68.5pR	SUS4; UDP-glycosyltransferase / sucrose synthase / transferase[a]
KR139	PU01570	POPTR_0013s15380.2	A060P68	C4H (CINNAMATE-4-HYDROXYLASE); trans-cinnamate 4-monooxygenase[b]
KR213	PU00627	POPTR_0015s09930.1	A010P74	Pectate lyase family protein[c]
For further information (such as partial cDNA) please refer to the The Populus Genome Integrative Explorer (http://popgenie.org/) using the CloneID above (Sjödin *et al.* 2009). [a] (Gerber *et al.* 2014); [b](Bjurhager *et al.* 2010); [c](Sandquist *et al.* 2015).				

Plant Material

Eighteen hybrid transgenic aspen (*Populus tremula* x *tremuloides*) were grown in a greenhouse with an 18-h photo-period, a temperature regime of 22/17 ºC (day/night) and relative humidity (RH) of at least 70%. Natural daylight was supplemented with light from HQI-TS 400W/DH metal halogen lamps (Osram, Munich, Germany). Plants were watered daily and fertilized once per week with a 1:100 dilution of SUPERBA S (Hydro Supra AB, Landskrona, Sweden). For each of the three genotypes there were two independent lines, with three replicates. The genotypes were accompanied with six non-transformed reference T89 trees. Trees were planted in February 2010 and harvested at 3 months of age. Plants were measured for height and diameter weekly.

Methods

Sampling, embedding, and sectioning

From each tree at 20 cm above soil height, a 1 cm high section of stem was collected, cut into ~2 mm sectors, and fixed with 3% v/v glutaraldehyde containing 2% v/v paraformaldehyde in cacodylate buffer (0.1 M, pH 7.2) for 9 h at room temperature. Three 1×1 mm samples were collected from the primary fixed sector for each genotype and post-fixed with osmium tetroxide (2% w/v in cacodylate buffer) for 2 h at room temperature. After fixation the samples were washed (2×20 min) with distilled water and dried with an ethanol series consisting of 20 min steps (20, 40, 60, 70, 80, 90, 95, and 99.5%).

For each tree, 1×1 mm primary fixed, samples were cut from the fixed sectors and embedded in Technovit 8100 (Kulzer, Wehrheim, Germany), LR White (London Resin, Basingstoke, UK), and Spurr's resin (Spurr 1969; Agar Scientific, Stansted, UK) according to the manufacturer instructions. The three post-fixed samples were embedded similarly in LR White. For all resins, infiltration solution concentrations were gradually increased over one week.

From the embedded Technovit 8100 samples, 2 µm transverse sections were collected on a Microm microtome (HM 350, Microm, Germany). From the Spurr's resin embedded samples 1 µm transverse sections were collected on a Reichert-Jung Ultracut E ultra-microtome. With the LR White embedded samples, silver and gold ultrathin

transverse sections were collected using a Reichert-Jung Ultracut E ultra-microtome and collected on 100 and 200 mesh nickel grids for TEM.

Wood maceration

Three sectors of wood, approx. 3 mm by 1 cm for each tree were delignified and macerated by treatment in a 1:1 mixture of 100% acetic acid and H_2O_2 at 60 ºC for 18 h to allow fiber length and width measurements (Wise and Murphy 1946).

Chemical staining

Toluidine blue staining was used as an aid for morphological characterization and measurements on transverse Technovit embedded samples (Chaffey 2002). All samples were control stained to verify the absence of tension wood by dual staining with Safranin and Astra Blue (Chaffey 2002). Phloroglucinol and Mäule staining were carried out on fresh 10 µm wood sections and non-resin embedded sections, according to procedures given by Chaffey (2002).

UV-microscopy

Transverse 1 µm Spurr's resin sections were mounted using glycerin on quartz slides and covered with quartz coverslips. UV-absorption was measured at 280 nm using a Carl Zeiss UMSP 80 microscopic spectrophotometer, mainly reflecting guaiacyl absorption (Yoshinaga and Wada 2007). UV-absorption was measured across double fiber-fiber secondary cell walls using ImageJ (Abramoff *et al.* 2004). The mean absorption was taken as the maximum for 50 fiber-fiber cell walls per sample.

Transmission electron microscopy

Ultrathin osmicated sections were post-stained with 2% w/v potassium permanganate solution containing sodium citrate. Sections were observed using a Philips CM/12 TEM operated at 80 kV with images recorded on KODAK 4489 film.

Anatomical characterization

Anatomical characterization was carried out according to the IAWA standard (Wheeler and Gasson 1989). Briefly, one mean was calculated for each tree for each measurement to minimize pseudo replication. These means were based on 300 measurements for fiber lumen area, 100 for cell wall thickness, and a minimum of 60 for the remaining measurements. Fiber lumen area measurements were increased from the IAWA standard of 100 to 300 to account for longitudinal geometrical effects. One complete set of images were taken for the full area of each sector from cambium to pith. Morphological measurements were taken on fully differentiated cells, approximately 1 mm from the cambium. Image analysis was performed with ImageJ (Abramoff *et al.* 2004). Images of macerated fibers were taken of free fully hydrated fibers. Anatomical properties characterized were fiber length, width, lumen area, and double fiber cell wall thickness.

Statistical model for analysis

A multivariate statistical model was applied and evaluated in accordance to the methods outlined in Sandquist *et al.* (2015). The seven variables included in the multivariate model correspond to the measurements given in Table 3, excluding results for guaiacyl lignin. To improve estimates of variance, the model utilized the variance from the equivalent measurements given in Sandquist *et al.* (2015). As there were no equivalents for the UV-measurements of guaiacyl lignin, the guaiacyl measurements were only evaluated with a univariate version of the model composed of measurements from this study. The multivariate model used for the analysis was:

$$
\begin{bmatrix} y_{ijkm}^{(1)} \\ \vdots \\ y_{ijkm}^{(n)} \end{bmatrix} = \begin{bmatrix} \mu^{(1)} \\ \vdots \\ \mu^{(n)} \end{bmatrix} + \begin{bmatrix} \gamma_{ij}^{(1)} \\ \vdots \\ \gamma_{ij}^{(n)} \end{bmatrix} + \begin{bmatrix} \ell_{ijk}^{(1)} \\ \vdots \\ \ell_{ijk}^{(n)} \end{bmatrix} + \begin{bmatrix} e_{ijkm}^{(1)} \\ \vdots \\ e_{ijkm}^{(n)} \end{bmatrix}
$$

where *n* is the number of dimensions/variables, $\mu = [\mu^{(1)}, \ldots, \mu^{(7)}]'$ = the vector of overall means, $\gamma_{ij} = [\gamma_{ij}^{(1)}, \ldots, \gamma_{ij}^{(7)}]'$ = the vector of genotype effects, and $j = 0, \ldots, n_i$, $n_1 = 9$, $n_2 = 3$ are the fixed effects (genotypes in group A, B and C, respectively). The vectors of random effects for line and residual variation $\ell_{ijk} = [\ell_{ijk}^{(1)}, \ldots, \ell_{ijk}^{(7)}]'$ and $e_{ijkm} = [e_{ijkm}^{(1)}, \ldots, e_{ijkm}^{(7)}]'$ are assumed to follow multivariate normal distributions with mean vectors 0 and covariance matrices:

$$
\Sigma_\ell = \begin{bmatrix} \sigma_\ell^2(1) & \sigma_\ell(1,2) & \ldots & \sigma_\ell(1,n) \\ \sigma_\ell(2,1) & \sigma_\ell^2(2) & \ldots & \sigma_\ell(2,n) \\ \vdots & \vdots & \ddots & \vdots \\ \sigma_\ell(n,1) & \sigma_\ell(n,2) & \ldots & \sigma_\ell^2(n) \end{bmatrix}
$$

$$
\Sigma_e = \begin{bmatrix} \sigma_e^2(1) & \sigma_e(1,2) & \ldots & \sigma_e(1,n) \\ \sigma_e(2,1) & \sigma_e^2(2) & \ldots & \sigma_e(2,n) \\ \vdots & \vdots & \ddots & \vdots \\ \sigma_e(n,1) & \sigma_e(n,2) & \ldots & \sigma_e^2(n) \end{bmatrix}
$$

The vectors ℓ_{ijk} and e_{ijkm} are assumed independent.

RESULTS AND DISCUSSION

Group and Line Effects

Corresponding to previous studies (Sandquist *et al.* 2015), each genetic transformation was carried out twice in two independent lines. Trees were grown under similar conditions as described earlier (Sandquist *et al.* 2015). To acquire better estimates of variance, measurements in the present study were evaluated together with those previously reported (Sandquist *et al*. 2015) as an additional (third) group.

No statistically significant line variations were found over the extended population of three groups, except for fiber width (Table 2). Plots of line effects and residuals did not show any essential deviations from the assumption of normal distributions.

Overall Statistical Observations

Despite being down regulated in significant molecular pathways, we have been impressed with how well overall developed the three genotypes appear to be. Statistical relationships over the extended population of three- instead of two groups showed only minor variations to the previously reported correlations (Table 2) (Sandquist *et al.* 2015). The only differences were the indication of statistical significant relationships between fiber lumen area and plant diameter, and between plant height and fiber length. From previously reported tree growth patterns (Kollmann 1968) these indications are in accordance with expected correlations.

The main reason for utilizing estimates of variation from previous measurements was to improve the error estimation. Evaluation of the new population on its own (with 24 samples divided into 4 groups with two lines and three replicates) generates results that are over sensitive to line variations. This effect was observed in the ultraviolet guaiacyl lignin evaluation, which had no previous equivalent measurements. If line variations are neglected, however, the statistical model is reduced to genotypes with six replicates, and then genotypes KR112 and KR139 show a statistical significant decrease in UV-absorption (*p*-values ≤0.0001 and 0.0001, respectively).

### Morphological Effects of Sucrose Synthase Down-regulation

Genotype KR112 with a relative reduction in density due to knock-down of the sucrose synthase gene (Gerber *et al.* 2014) showed reduced fiber development, with a strong overall significant difference compared to wild type (Table 3). This was mainly manifested in reduced fiber cell wall thickness (Fig. 1D and 2A *vs.* 2D), and subsequently increased fiber lumen area (Table 3). There were also indications of reduced phloroglucinol staining across the fiber – middle lamella – fiber cell wall compared to the vessels, as shown in Figs. 1E *vs.* 1H. There were also indications of reduced UV-absorption across the fiber-fiber cell walls (Table 3 and Fig. 1) although this was more difficult to discern visually in the UV-images (Figs. 1M *vs.* 1P). However, the Mäule staining remained unchanged (Figs. 1I *vs.* 1L), and intensity of staining at the ultrastructural level did not appear to be affected (Figs. 2A *vs.* 2D). The Mäule reaction is considered to be selective towards syringyl-lignin (Chaffey 2002), and this may therefore indicate a shift in the S/G ratio for genotype KR112, with a possible reduction of guaiacyl content. This

observation corroborates previously reported observations in a shift in the S/G ratio (Gerber *et al.* 2014). Furthermore, the reported shrinkage upon drying (Gerber *et al.* 2014) has been observed to an even greater extent in these samples due to the embedding procedure, which further corroborates the observed change in cell wall architecture.

Table 2. Estimated Variances of Morphological Residuals and Correlations

		Plant diameter	Fiber length	F. lumen area	Fiber width	Plant height	Radial fiber thickness	Tangential fiber thickness
		0.73	*1.2E-3*	*865.0*	*2.43*	*179.2*	*0.87*	*1.09*
Plant diameter	*0*		0.15	**0.18***	0.09	**<u>0.71</u>**	0.03	0.04
Fiber length	*0*	-		<u>0.23</u>	0.12	**0.19***	0.04	0.18
F. lumen area	*0*	-	-		0.05	0.05	**<u>-0.30</u>**	**<u>-0.27</u>**
Fiber width	*<u>**1.95**</u>*	-	-	-		0.12	-0.08	-0.13
Plant height	*31.5*	-	-	-	-		0.02	0.01
Radial fiber thickness	*0.09*	-	-	-	-	-		**<u>0.82</u>**
Tangential fiber thickness	*0.13*	-	-	-	-	-	-	

Estimated variances of residuals (*first row*) and lines (*first column*). Residual correlations shown above the diagonal. Bold underlined values are statistically significant at $p \leq 0.05$ compared with control. Bold values show a statistical tendency of significance with $p \leq 0.10$ compared with control. *Differences compared to earlier report (Sandquist *et al.* 2015). Abbreviation: F., Fiber.
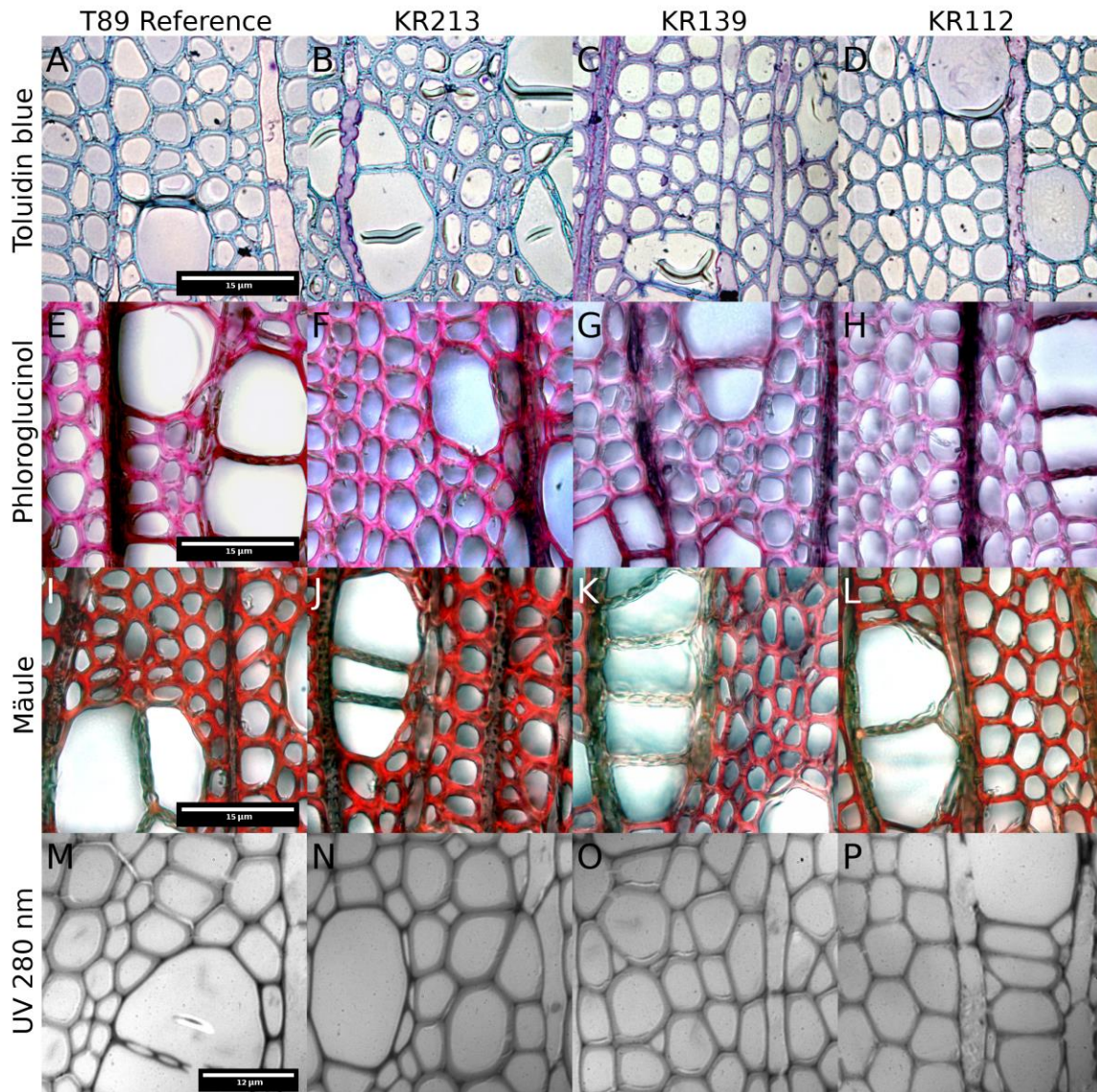
Fig. 1. Representative images of reference wildtype T89, genotypes KR213, KR139 and KR112. Staining was with toluidine blue (first row), phloroglucinol (second row) and Mäule reaction (third row). Bottom row shows UV-microscopy images at 280 nm. Scale is the same within each row. Top 3 rows show images at the same magnification to allow visual comparisons. The images reflect observations on a minimum of 3 sections, with multiple images per sample. Genotype KR213 shows no statistical difference compared with the reference, whereas KR112 shows reduced staining with phloroglucinol and genotypes KR139 shows reduced staining with both phloroglucinol and the Mäule reaction.

Morphological Effects of Cinnamate 4-hydroxylase Down-regulation

Genotype KR139 with a relative reduction of lignin due to knock-down of the cinnamate 4-hydroxylase coding gene (Bjurhager *et al.* 2010), showed only an indication of difference in fiber morphology compared to the wild type (Table 3). A visual comparison can be made between Figs. 1A *vs.* 1C. The reduced fiber length was the only individually significant observation (Table 3). Less intense staining was observed for both phloroglucinol and the Mäule reaction, as seen in Figs. 1E *vs.* 1G, and 1I *vs.* 1K. There was also an indication of reduced lignin absorption in UV in the fiber-fiber cell

wall (Fig. 1 and 1M *vs.* 1O) which was also reflected at the ultrastructural level (Figs. 2A *vs.* 2C). Together, this suggests normal wood development, with an overall reduction of lignin concentration in the fiber-fiber cell wall, including the middle lamella region. No shift in S/G ratio can be inferred. These observation confirm previously reported observations by Bjurhager *et al.* (2010) in the studied aspen genotype, Sewalt *et al.* (1997) in tobacco, and Reddy *et al.* (2005) in alfalfa. This further emphasizes the key role played by this enzyme in lignin biosynthesis.

Morphological Effects of Pectate Lyase Coding Gene Down-regulation

Genotype KR213 was previously reported with reduced fiber development (Sandquist *et al.* 2015), but in this extended study it did not show any overall significant differences. The previously reported results reflected aspen grown under summer conditions, whereas the present results reflect spring conditions (Sandquist *et al.* 2015). Despite having been grown under otherwise similar conditions in the green house, these results may indicate a difference between spring and summer development. Alternatively, this change could be caused by more accurate interpretation from a larger statistical population.

Even though the trends remain the same, the previously reported individual indications of reduced fiber lumen area and increased fiber cell wall thicknesses (Sandquist *et al.* 2015) were absent in the spring developing fibers (Figs. 1A *vs.* 1B). The earlier reported fiber length reduction are now significant (Table 1). No change in lignin composition were observed with the two lignin stains (Figs. 1E *vs.* 1F and 1I *vs.* 1J), UV-microscopy (Fig. 1, 1M *vs.* N) nor at the ultrastructural level (Fig. 2A *vs.* 2B).

Despite being grown in a greenhouse under the same climate and (compensated) light conditions, an overall significant increase was observed in fiber lumen area from spring to summer compared to what was observed for the wild type. In addition, we noted a significant decrease in overall fiber length and width for the same period.

Ultrastructural TEM Observations

At the ultrastructural level, no major differences were observed in the secondary cell wall architecture of fibers and vessels (*i.e.* S1, S2) in any of the three genotypes in comparison to the reference plants using TEM at the magnification used. This contrasts with previous studies, in which changes in the secondary wall, particularly the S2 layer (*e.g.* concentric loosening of S2 wall layer), has been reported in poplar (Leple *et al.* 2007), *Arabidopsis* (Ruel *et al.* 2009), and flax (Day *et al.* 2009) in knock-down mutants affecting enzymes of lignin biosynthesis. The TEM observations were however consistent with the histochemical and UV-observations of fibers and vessel wall architecture, suggesting that any changes were likely to be on a supermolecular rather than ultrastructural level. The reduced UV-absorption for lignin at 280 nm of the double fiber wall (Reference > KR213 > KR139 > KR112, Table 3) was also consistent with TEM staining where the transgenic trees showed less staining of the fiber and vessel S2 and middle lamella regions using OsO_4 and $KMnO_4$ (Fig. 2).

Table 3. Summary of Properties

Construct	Fiber length	Fiber width	Fiber lumen area	Radial fiber thickness	Tangential fiber thickness
	mm	*µm*	*µm2*	*µm*	*µm*
Ctrl	1.03	38.5	36.7	2.0	1.8
KR112	1.02	39.1	**51.9**	**1.0**	**1.1**
KR139	**0.97**	38.8	40.0	1.7	1.8
KR213	**0.98**	37.2	33.6	2.2	2.2
Mean	1.00	38.4	40.5	1.7	1.8
Literature		34.9[b]		2.06[a],2.01[b]	2.00[a],1.94[b]

Construct	Overall multivariate analysis	Plant height	Plant diameter	UV Guaiacyl	
	p-values	*cm*	*mm*	*max. intensity*	
Ctrl	-	105.0	7.5	38500	
KR112	**<0.001**	105.0	7.6	30100	
KR139	**0.025**	90.2	7.4	30900	
KR213	0.056	92.3	7.0	35900	
Mean	-	98.2	7.4	33900	

Bold underlined values are statistically significant at $p \leq 0.05$ compared with control. Bold values show a statistical tendency of significance with $p \leq 0.10$ compared with control. *P*-values are adjusted for multiple comparisons within groups. [a](Roach *et al.* 2012). [b](Sandquist *et al.* 2015).
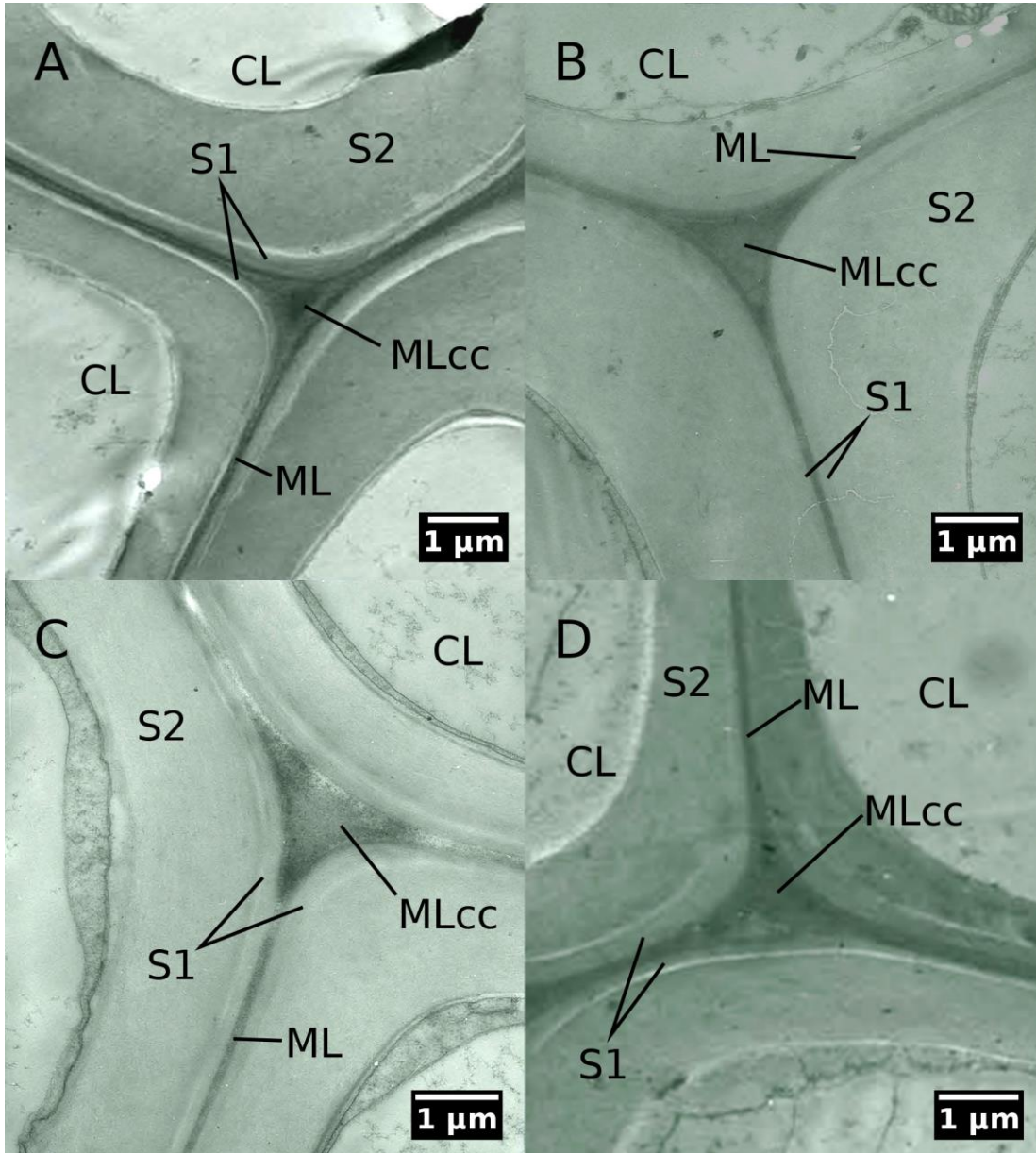
Fig. 2. TEM images showing reference wildtype T89 (A), KR213 (B), KR139 (C) and KR112 (D). Samples were post-stained with OsO_4 and sections post-stained with $KMnO_4$. They are representative selections from multiple images per sample and line. The images confirm previous observations with no major morphological changes except for the apparent reduction in cell wall thickness in genotype KR112. Genotype KR139 shows less intense staining of the primary cell wall and particularly of the middle lamella cell corners. This corroborates both the UV and histochemical observations. Abbreviations: S1, S2, secondary cell wall layers; ML, middle lamella; MLcc, middle lamella cell corner; CL, cell lumen.

CONCLUSIONS

1. Knock-down of the sucrose synthase (SUS4) coding gene in aspen significantly decreased fiber development. There were also indications that this decrease affects the S/G lignin ratio.

2. Knock-down of the cinnamate 4-hydroxylase coding gene showed from the multivariate evaluation only an overall indication of modified fiber morphology. Chemical staining and UV-microscopy measurements indicate an overall reduction of lignin in the fibers, with no shift in S/G ratio.

3. Knock-down of the pectate lyase family coding gene did not contribute to any overall observed changes in fiber morphology, nor lignin content.

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