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Modification of hemicellulose content by antisense down-regulation of UDP-glucuronate decarboxylase in tobacco and its consequences for cellulose extractability

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Abstract

Extractability and recovery of cellulose from cell walls influences many industrial processes and also the utilisation of biomass for energy purposes. The utility of genetic manipulation of lignin has proven potential for optimising such processes and is also advantageous for the environment. Hemicelluloses, particularly secondary wall xylans, also influence the extractability of cellulose. UDP-glucuronate decarboxylase produces UDP-xylose, the precursor for xylans and the effect of its down-regulation on cell wall structure and cellulose extractability in transgenic tobacco has been investigated. Since there are a number of potential UDP-glucuronate decarboxylase genes, a 490 bp sequence of high similarity between members of the family, was chosen for general alteration of the expression of the gene family. Sense and antisense transgenic lines were analysed for enzyme activity using a modified and optimised electrophoretic assay, for enzyme levels by western blotting and for secondary cell wall composition. Some of the down-regulated antisense plants showed high glucose to xylose ratios in xylem walls due to less xylose-containing polymers, while arabinose and uronic acid contents, which could also have been affected by any change in UDP-xylose provision, were unchanged. The overall morphology and stem lignin content of the modified lines remained little changed compared with wild-type. However, there were some changes in vascular organisation and reduction of xylans in the secondary walls was confirmed by immunocytochemistry. Pulping analysis showed a decreased pulp yield and a higher Kappa number in some lines compared with controls, indicating that they were less delignified, although the level of residual alkali was reduced. Such traits probably indicate that lignin was less available for removal in a reduced background of xylans. However, the viscosity was higher in most antisense lines, meaning that the cellulose was less broken-down during the pulping process. This is one of the first studies of a directed manipulation of hemicellulose content on cellulose extractability and shows both positive and negative outcomes.

Keywords: *Nicotiana tabacum*; Solanaceae; Tobacco; Cell wall; Hemicellulose; UDP-glucuronate decarboxylase; Antisense

Abbreviations: UDPGDH, UDP-glucose dehydrogenase; UDPGlcADCX, UDP-glucuronate decarboxylase; UXS, UDP-xylose synthase.

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1. Introduction

The efficiency of extraction and recovery of cellulose from cell walls influences many industrial processes including fibre production, pulp- and paper-making, and, utilisation of biomass for biofuel. The cell wall is a complex laminate structure, which can be classified in dicots into three distinct zones, the middle lamella, the primary wall, and the secondary wall. The middle lamella of the primary wall is shared by two contiguous cells and is composed almost entirely of pectic substances. The primary cell wall is composed chiefly of interwoven domains, of which the cellulose-xyloglucan framework is the main contributor of the anisotropic fraction. However, the biomass with the highest industrial applicability is mainly derived from secondary walls, which consist of three distinct layers (S1, S2, and S3), easily distinguishable at an ultrastructural level from differences in the orientation of their cellulose microfibrils. The transition from primary to secondary cell wall synthesis is marked by the cessation of pectin deposition and a noted increase in the synthesis and deposition of cellulose/hemicellulose in the secondary wall and of lignin throughout the middle lamella and the primary and secondary wall layers. The non-cellulosic polysaccharides of the secondary cell wall are qualitatively distinct from those found in the primary cell walls. The major differences are in the hemicellulose components of the secondary cell wall, which are primarily xylans and mannans. Like in any complex composite material, the supramolecular organisation between cellulose, hemicellulose and lignin in cell walls determines the properties of the plant fibres. Although there have been many studies identifying structural genes and transcription factors involved in secondary wall formation (Paux et al., 2004; Aspeborg et al., 2005; Brown et al., 2005), understanding of the extent of co-regulation of assembly of these three components is still limited. One of the major questions is the extent to which lignin is coupled to hemicelluloses in dicots; in monocots this has been postulated to occur through feruloylated arabinose residues on side chains of the arabinoxylans (Grabber et al., 2002). One approach to understanding such complexity is study of plants down-regulated in lignin or xylan and analysis of the influence of the changes in the levels of the non-manipulated polymers and the resultant extractability of cellulose.

Some insights into such levels of co-assembly have come about from studies whose primary purpose was manipulation of lignin contents and compositions in plants through genetic engineering. Such studies show that fibre analysis and modelling of pulping processes led to improved cellulose extraction (reviewed in Anterola and Lewis, 2002; Boudet et al., 2003; Boerjan et al., 2003). Many genes of the lignification pathway have now been manipulated in model plants such as Arabidopsis, tobacco and poplar. However, only a limited number of these studies report the consequences of lignin manipulation on the polysaccharide content of walls (Anterola and Lewis, 2002). Some of these revealed putative benefits that outweighed the simple reduction of lignin, since cellulose was claimed to show increased levels in lignin-reduced plants giving positive improvements in the pulping quality of the target species, aspen (Hu et al., 1999; Li et al., 2003). Furthermore, enhanced growth rates were also claimed which would be significant in the use of woody species as short rotation coppice for biomass energy production for example. This type of outcome requires rigorous testing in a number of systems as it is clearly of potential significance. For example, in addition to the influence of lignins on chemical processing, the importance of xylans, was first realised by the pulping industry when it became apparent that delignification during the pulping process was influenced by the hemicelluloses present (Buchert et al., 1996; Viikari et al., 1994; Imai et al., 1997). Recently, the industry has implemented expensive alternatives to purely chemical processing by employing biopulping technologies utilizing hemicellulases (Buchert et al., 1996; Wang et al., 1997; Bajpai, 2004). Modification of hemicelluloses could therefore be an alternative strategy to lignin manipulation as a target for testing effects on plant growth and cellulose content and extractability. Such work is also relevant to biofuel production where microbial digestion substitutes for chemical digestability and similarly depends on the accessability of the cellulose.

The feasibility of hemicellulose modifications depends on knowledge of the genes involved and their regulation. At present this is not as extensively understood as for lignin biosynthesis. Nevertheless, work on Arabidopsis has already shown that modifications of cell wall polysaccharides are possible and tolerated. In the first studies, Arabidopsis lines were generated by mutagenesis and shown to be altered in cell wall fucose, rhamnose or arabinose, while other mutants had complex alterations in the amounts of several monosaccharides (Reiter et al., 1997). Since then there has been considerable progress in gene discovery involved in sugar nucleotide interconversions in plants (reviewed in Seifert, 2004) while relatively less progress has been made for the polysaccharide synthases (Scheible and Pauly, 2004). Gene families coding for enzymes of nucleotide sugar metabolism seem to be distinguished by their large size and probable consequent functional redundancy (Seifert, 2004). With respect to the synthesis of hemicelluloses, the first step of the pathway to UDP-xylose has attracted attention. Two gene families for UDP-glucose dehydrogenase have been cloned in plants (Seifert, 2004), whereas the third possibility for the origin of UDP-glucuronic acid, inositol oxygenase, has recently been characterised at the gene level in Arabidopsis (Kanter et al., 2005). At least five members of the UDP-glucuronate decarboxylase exist in Arabidopsis (Harper and Bar-Peled, 2002), but some of them may adopt other functions such as UDP-apiose synthase (Molhoj et al., 2003). The epimerase family is now well characterised in the Arabidopsis genome. Five UDP-glucose epimerases have been demonstrated functionally (Seifert, 2004), possibly six UDP-glucuronic acid epimerases exist while UDP-xylose epimerase was identified as a wall mutant *mur4* (Burget

et al., 2003). With this gene discovery, a transgenic approach allows determination of the range of permissible wall compositions without deleterious effects on vasculature and plant survival. It is indeed extremely important to evaluate if new tailored plant genotypes more adapted to downstream agro-industrial processes maintain their agronomic characteristics at the same level.

Tobacco has been the model woody plant of choice for much of the work on modifications of lignin (Anterola and Lewis, 2002) and was chosen for this study of the comparative effect of modification of xylan. The probable pathway for the biosynthesis of the xylan precursor, UDP-xylose, has been elucidated in the synthesis of secondary walls in tobacco stems with respect to the induction and expression of the various types of enzymes responsible (Bindschedler et al., 2005). A xylogenic tobacco cell system (Blee et al., 2001) was selected to identify the generic enzyme types responsible for the bulk of this activity by purification to homogeneity of the enzymes involved in the biosynthesis of UDP-xylose (Wheatley et al., 2002; Bindschedler et al., 2005). This has allowed the cloning of monospecific UDP-glucose dehydrogenase (UDPGDH), dual specificity, ADH-like UDP-glucose dehydrogenase (ADH2) and of a number of UDP-glucuronate decarboxylases (UDPGlcADCX) that were expressed in tobacco stems and xylogenic cultures (Bindschedler et al., 2005). The functional identity of some of these clones was established by heterologous protein expression. We now report changes in the cell wall composition and pulping properties of tobacco plants subjected to sense and antisense down-regulation of UDP-glucuronate decarboxylase. Although a reduction in vascular xylan was observed, this occurred without the gross developmental changes seen as a consequence of depletion of UDP-D-apiose/UDP-D-xylose synthases in plants by using gene silencing of NbAXS1 in *Nicotiana benthamiana* (Ahn et al., 2006).

2. Results

2.1. Production of transgenic lines down-regulated for UDP glucuronic acid decarboxylase

M_r, 40 kD polypeptides associated with UDP-glucuronate decarboxylase activity were purified to homogeneity from xylogenic tobacco cell cultures (Wheatley et al., 2002) and had a MALDI fingerprint that gave a high match (Bindschedler et al., 2005) to the pea UDP-glucuronate decarboxylase (BAB40967.1, Kobayashi et al., 2002) and the *Arabidopsis* UXS3 gene (AAK70882.1; Harper and Bar-Peled, 2002). PCR primers were designed and led to isolation of a 490 bp general probe which was used to isolate full length clones designated as UDPGlcADCX1 (AY619950) and UDPGlcADCX2 (AY619951) coding for the soluble isoforms of the enzyme, UDPGlcADCX3 (AY619952) coding for a membrane-bound isoform and a partial sequence UDPGlcADCX4 (AY619953), respectively. Of these, UDPGlcADCX1 was shown to encode a functional UDP-glucuronate decarboxylase by expression in *E. coli* (Bindschedler et al., 2005). We use this nomenclature to avoid the biochemical inaccuracy of UXS (UDP-xylose synthase) since the reaction is a decarboxylation and not a synthase.

In order to generally down-regulate the potential UDP-glucuronate decarboxylase encoding genes, transgenic plants expressing a fragment of UDP-glucuronate decarboxylase in the sense or antisense orientation were produced. The PCR product DCX7 with highest similarity of 82% to the known functional UDP-glucuronate decarboxylase (AtUXS2 ; Pattathil et al., 2005) was chosen. It was subsequently found to be most similar to the full length tobacco membrane bound UDPGlcADCX3 with 90% similarity. With respect to the other full length tobacco clones, it has a stretch of 176 nts with a similarity of 73% or a stretch of 68 nts with 85% similarity for UDPGlcADCX1, whose enzyme activity has been demonstrated, and of 71% over 329 nts and 82% over 80 nts similarity with UDPGlcADCX2. However, exact identity stretches of 21–23 nucleotides, which is required for definite silencing phenomenon as a length of exact bases of 19–25 is sufficient to enable RNAi (Semizarov et al., 2003), were only found for the membrane bound isoform UDPGlcADCX3 in addition to the UDPGlcADCX7 for which there is only partial sequence (Fig. 1a).

The DCX7 PCR insert was transferred into the *Agrobacterium* binary vector pBNPDV35S in the sense or antisense orientation between the 35S promoter and the 35S terminator (Fig. 1b). Twenty-seven control plants transformed with the β-glucuronidase (GUS) gene (10040), 77 sense (10642) and 77 antisense (10643) T_0 transgenic plants survived selection on kanamycin and were grown and harvested together with 27 non-transformed control plants (non-co-cultivated; NCC) after about 2.5 months, just before flowering. Seventy-two sense (10642) plants and 74 antisense (10643) plants were confirmed to be transgenic, as the gene conferring resistance to kanamycin could be amplified by PCR. No obvious morphological phenotype could be detected in the transgenic lines harbouring either the sense (10642) or antisense (10643) construct when compared to non-transformed or transgenic controls.

Hand-cut sections from internode 13 were stained with phloroglucinol–HCl for a visual estimation of lignin content to test the hypothesis that if plants are down-regulated for the biosynthesis of xylan, more lignin might be produced for compensation. No obvious variation could be detected in the intensity of the staining between controls, transgenic controls and antisense plants. Following this initial screen five lines of each of the transgenics were selected for in-depth analysis.

2.2. Confirmation of UDP-glucuronate decarboxylase down-regulation in transgenic lines

Since UDP-glucuronate decarboxylase mRNA expression was only detected in mature stems (Bindschedler

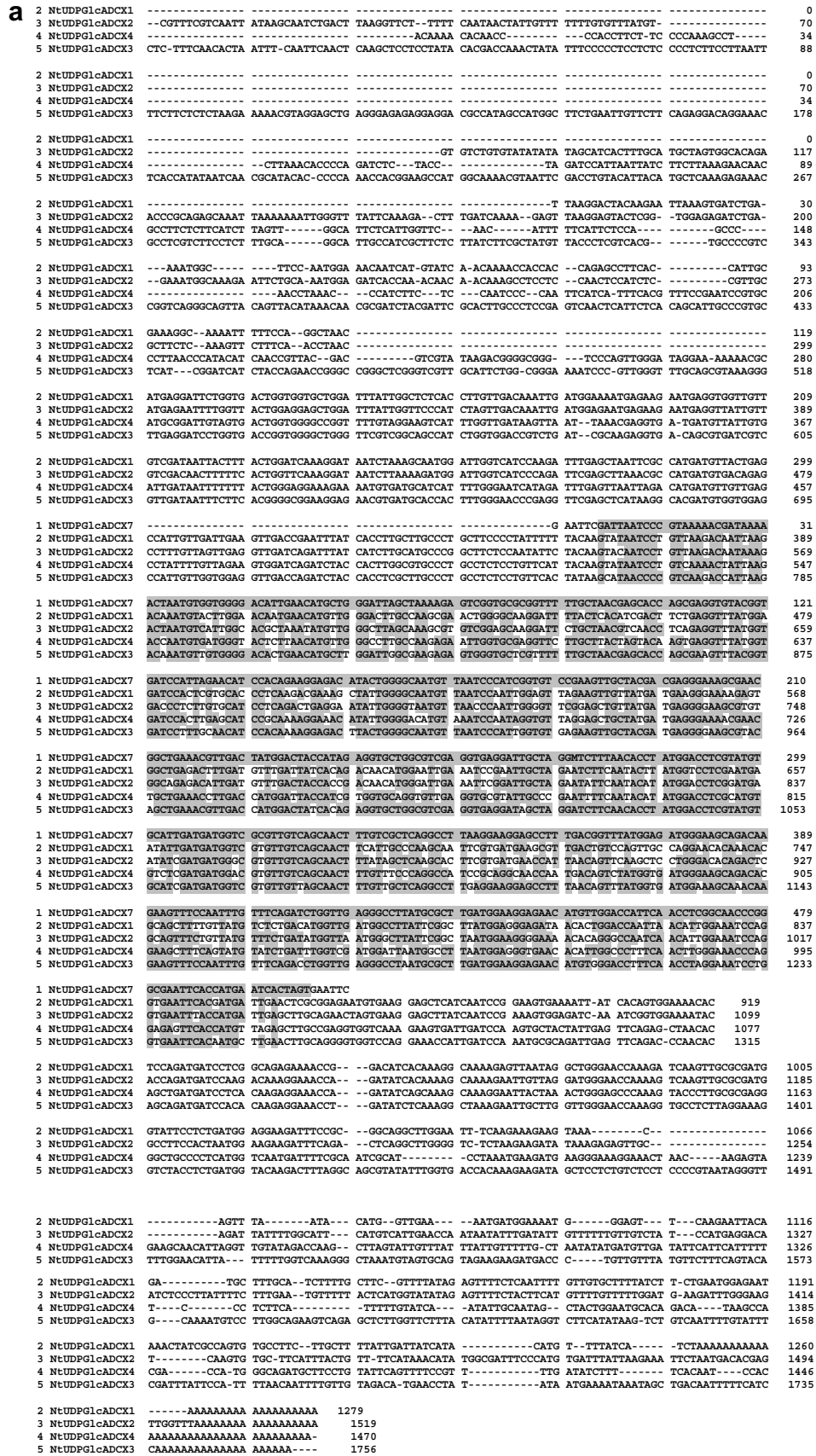

Fig. 1. (a) 490 bp sequence used in sense and antisense constructs aligned to show stretches of similarity with full length cloned tobacco UDP-glucose decarboxylase NtUDPGlcADCX 1–4 (AY619950–AY619953, respectively). (b) Diagram of construct used for transformation.

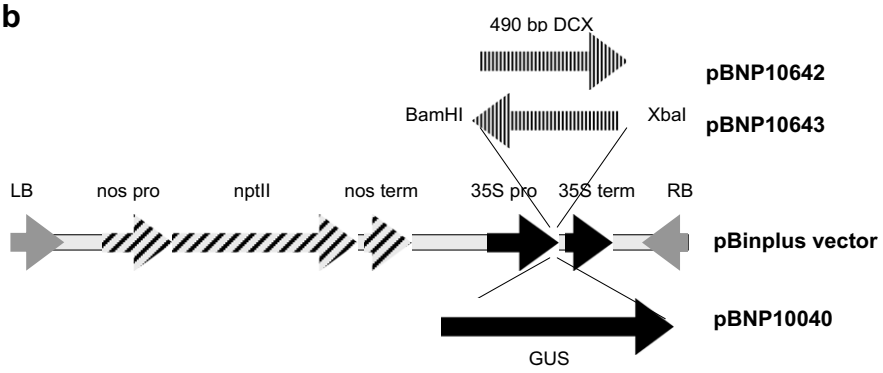

Fig. 1 (*continued*)

et al., 2005), most of the analysis of transgenic plants was confined to these tissues rather than the meristematic internodes. UDP-glucuronate decarboxylase could be detected in these tissues using an anti-tobacco UDP-glucuronate decarboxylase antibody in tissue prints and Western blots (Wheatley et al., 2002; Bindschedler et al., 2005). Cross-reactivity was obtained on the outer and inner side of the cambium in tissue prints of freshly cut sections from internodes 13 and 14, as previously shown in Bindschedler et al. (2005). However, no decrease of expression in transgenic lines could be observed using this technique. Clear differences were observed though by western blotting for some of the 89 T_0 sense and antisense lines tested when compared to 42 non-transformed and transgenic controls. For example, Fig. 2 shows the antisense lines (construct 10643) 21, 25 and 59 were confirmed to be clearly down-regulated but not the lines 16 or 52.

In an attempt to correlate this protein expression with enzyme activity, UDP-glucuronate decarboxylase was first assayed radioactively according to Wheatley et al. (2002). For quantification, products were separated by either paper chromatography or by measuring the radioactivity corresponding to the $^{14}CO_2$ freed by decarboxylation. Neither of these methods were reproducible when using crude extracts unlike either purified or expressed protein (Wheatley et al., 2002; Bindschedler et al., 2005). Therefore, a new method was established by separating UDP-sugars by capillary electrophoresis, adapting methods used to separate UDPGlcNAc from UDPGalNAc, UDPGlc and UDPGal (Lehmann et al., 2000; Xu et al., 1998) or for enzyme assays such as UDPGlcNAc–UDPGalNAc epimerase (Kowal and Wang, 2002). This method was much more reproducible. Unlike the other methods, it did not require an acid hydrolysis to remove the UDP moiety from the UDP-sugars prior to separation of the substrate from the product, thus avoiding lactone formation that co-migrated with xylose standards. Initial screening of T_0 lines showed that some had reduction in UDP-glucuronate decarboxylase enzyme activity of up to 30% less than the average transgenic control. For instance, the estimated activity levels of lines 42–20, 42–23, 43–16 and 43–59 were less than any control transgenic line (Fig. 3a). When a much larger range of T_1 lines were tested, some of them showed an acceptable decrease in enzyme activity, for example T_1 lines 902 (derived from T_0 line 43 to 59) and 823 (derived from T_0 line 42 to 20) showed a decrease of 52% and 53%, respectively (Fig. 3b). Overall therefore, immunodetection of the level of UDP-glucuronate decarboxylase protein did not correlate well with reduction of measurable enzyme activity except, for example, 42–20 and 43–59. This may be as a result of functional redundancy amongst the UDP-glucuronate decarboxylase gene family.

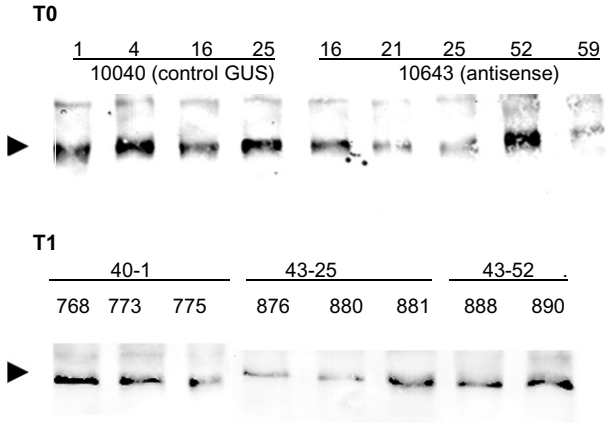

Fig. 2. Confirmation of lines down regulated for UDP-glucuronate decarboxylase by Western blotting. The intensity of the band detected (arrowed) is much less for several antisense lines.

2.3. Cell wall analysis of down-regulated lines

Since the latter method of screening appeared the most reliable, only T_0 lines that demonstrated decreased UDP-glucuronate decarboxylase activity were selected for saccharide analysis of cell walls. This screen had highest priority, as the desired outcome was to generate plants with an increase in cellulose levels relative to xylans. Thus, xylem, which is rich in secondary cell wall was excised from lyophilised internodes 13 and 14 of control and transgenic plants. Quantitative polysaccharide composition was evaluated from cell walls, which were obtained following

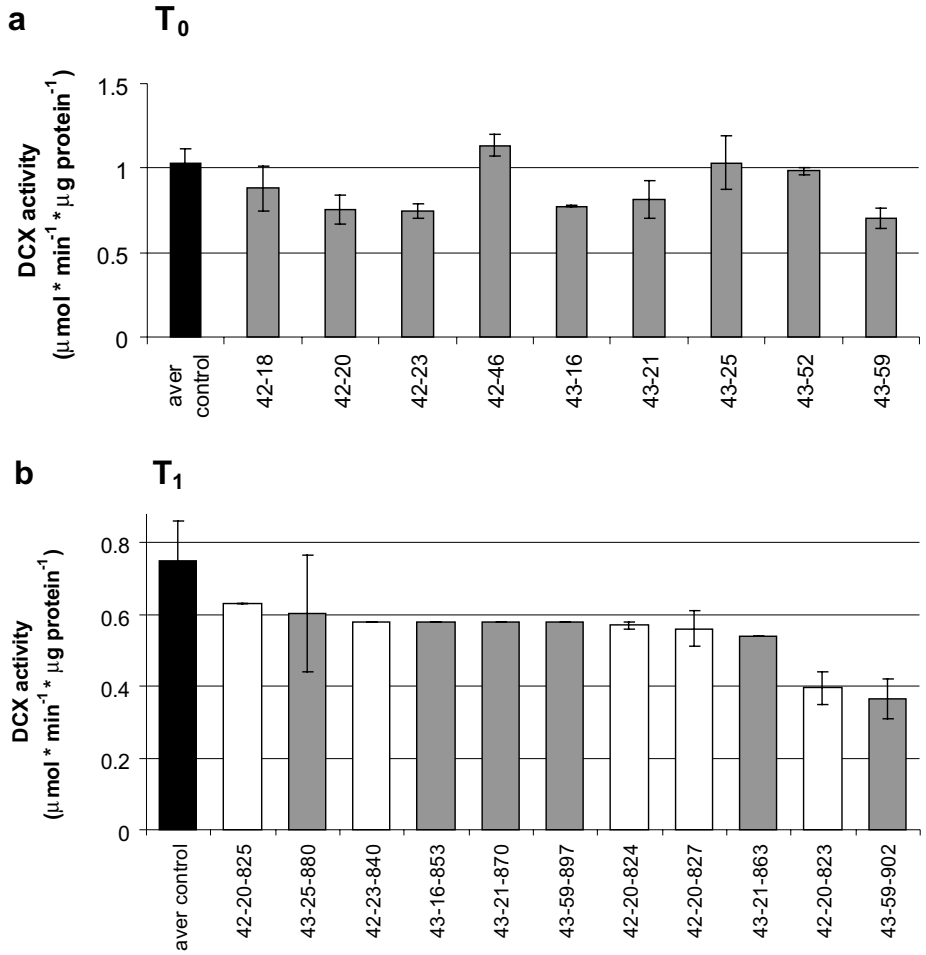

Fig. 3. Identification of tobacco lines down regulated in UDP-glucuronate decarboxylase activity. (a) T_0; (b) T_1 lines.

sequential extractions, including removal of starch, so that the glucose levels measured following hydrolysis are entirely derived from cellulose. Sugar analysis revealed that the Glc/Xyl ratio in xylem was more affected in all antisense plants (construct 10643) than sense plants. The change in the Glc/Xyl ratio was mostly due to a reduction in level of xylose present in the antisense plants, while the glucose amount remains relatively constant (Table 1). Particularly striking lines with low enzyme activity such as 43–59 at 68%, 42–23 (72%), 42–20 (74%), 43–16 (75%) and 43–21 (79%) compared with control values (Fig. 3a), had resultant higher Glc/Xyl ratios. Thus the antisense plants showed a modification of the cell wall carbohydrate status that was consistent in a reduction of xylose (xylan) content (from ca. 22% to ca. 18%). This was seen in all lines including those not obviously down-regulated at the protein level such as 43–16 and 43–52, further substantiating functional redundancy amongst the UDP-glucuronate decarboxylase gene family. Other cell wall carbohydrates were present in minor proportions and the variations in arabinose, galactose and mannose were not significant. Uronic acid estimation did not show significant change either, remaining at ca. 4.5% (data not shown). Following this screening of T_0 lines, pBNP10642 lines 10, 18, 20, 23, 46 and pBNP10643 lines 16, 21, 25, 52, 59 were taken forward to the T_1 generation.

2.4. Morphological analysis of T_0 and T_1 lines

Amongst the lines taken forward to the T_1 generation, four control WT lines, five GUS-constructs, four sense and five antisense transgenic lines were analysed by light and transmission electron microscopy and by immunolocalisation. Some deviation from normal vascular structure was observed. For example, confocal laser scanning microscopy (CLSM) observation showed that the xylem of the T_1 antisense line 889, derived from the T_0 43–52 (construct 10643), had a reduced number of vessels (Fig. 4a–c). TEM using the PATAg reaction did not reveal modifications in the cell wall ultrastructural organisation although there was some difference observed in the reactivity of fibres to the PATAg silver staining reaction in many antisense lines, giving rise to an irregular silver grain distribution. Fig. 4d and e shows, for example, that the same T_1 antisense line 889, is altered for its reactivity to silver staining in the S2 layer of the secondary wall of xylem vessels.

Table 1
Polysaccharide analysis of the xylem from T_0 transgenic lines

Tobacco line	Yield %	Ara	Man	Gal	Xyl	Glc	Glc/Xyl
NCC-3	79.1	0.5	1.6	0.5	22.1	48.6	2.4
NCC-9	81.6	0.7	1.9	0.7	22.3	51.3	2.3
NCC-13	80.3	0.6	1.4	0.7	21.8	48.3	2.2
NCC-27	82.0	0.8	1.6	0.9	23.2	48.6	2.1
GUS 40-1	81.5	1.0	2.2	0.8	23.5	50.1	2.2
GUS 40-3	79.5	0.5	2.0	0.7	21.6	50.8	2.3
GUS 40-4	81.0	0.6	1.9	0.7	22.0	50.7	2.3
GUS 40-16	81.5	0.4	2.1	0.8	22.6	51.1	2.4
GUS 40-17	75.0	0.6	1.9	0.8	22.2	41.8	1.9
Av. NCC/GUS	80.17	0.63	1.84	0.73	22.37	49.03	2.23
Sense 42-18	80.0	0.7	2.5	1.0	21.1	50.5	2.4
Sense 42-20	80.5	0.7	2.6	1.0	20.5	50.2	2.4
Sense 42-23	79.5	0.6	2.2	1.0	20.1	49.2	2.5
Sense 42-46	79.0	0.7	2.0	1.0	20.3	48.9	2.4
Av sense	79.74	0.68	2.33	1.0	20.5	49.7	2.43
T-test	0.611	0.536	0.028	0.000	0.000	0.542	0.008
Antisense 43-16	73.0	0.6	1.9	0.8	16.8	45.5	2.7
Antisense 43-21	76.5	0.7	1.8	0.8	18.3	47.6	2.6
Antisense 43-25	79.5	0.8	1.7	0.8	17.6	52.0	2.9
Antisense 43-52	77.0	0.7	1.6	0.6	17.1	48.1	2.8
Antisense 43-59	81.5	1.2	1.5	0.8	20.1	52.4	2.6
Av antisense	77.5	0.8	1.7	0.76	17.98	49.12	2.72
T-test	0.149	0.212	0.222	0.636	0.001	0.960	0.000

Non-co-cultivated NCC (non-transformed control) plant lines represent WT tobacco and GUS (β-glucuronidase) lines numbered as (40-) are vector controls. Both sense (42-) and antisense (43-) lines showed similar yields of polysaccharide material. Of the two transgenic types, the antisense lines showed consistently higher glucose to xylose content indicating a reduction of hemicellulose relative to cellulose. Analyses were performed in duplicate and variance was <10%. *T*-tests are shown for both sense and antisense lines for changes in each sugar relative to WT.

This could suggest a slight difference in the accessibility of hemicelluloses to the reagents and therefore as an indication of alteration in the wall assembly. However there was no indication of the irregular xylem phenotype seen during mutation of CesA or CCR genes (Jones et al., 2001; Persson et al., 2005) or CCR manipulation by antisense (Chabannes et al., 2001).

A linear, unsubstituted xylan chain was prepared from Esparto grass xylan by selective acid hydrolysis of the arabinofuranosyl substituents. The specificity of action of the antiserum raised in rabbits was tested against various wall polysaccharides and xylan model compounds. The antibody (anti-xylan-L) showed specificity for xylans with low substitution pattern and had no recognition for any of the usual wall polysaccharides such as cellulose, glucomannan, galactomannan, pectic polymers, galactan and arabinan (Ruel et al., 2006). Its application on tobacco plants

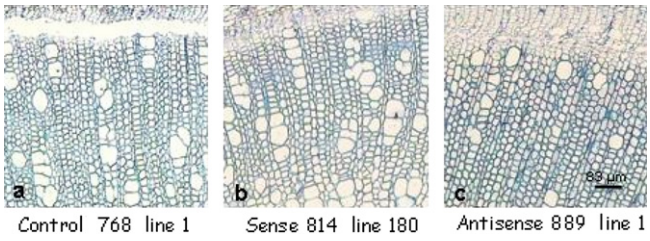

Fig. 4. (a–c) Histological phenotypes of down-regulation of UDP-glucuronate decarboxylase in Tobacco. Toluidine blue-stained transverse sections of xylem ring from WT (a), sense line 42-23-814 (b) and antisense 43-52-889 (c). Note the strong reduction in vessels number in antisense 889 line. (d–g) Ultrastructural distribution of the polysaccharides in cell walls. (d, e) General staining of polysaccharides with PATAg. In WT, no distinct zones are visible in the different sublayers, particularly in S2 (d). More reactive concentric zones are visible in S2 (arrows) of the transformed line 889. (f, g) Immunocytochemical labeling of linear xylans with the anti-xyl-L. WT linear xylans are essentially located in S2 (f). In down-regulated line 889, there is a reduction in the number of gold particles in S2.

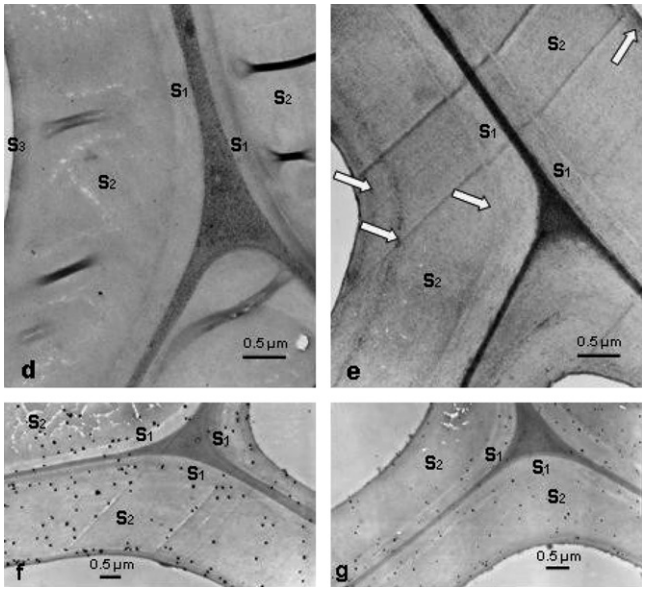

Fig. 4 (*continued*)

provided a general visualisation of xylans in the secondary walls of tobacco. This was also visible after immunolabelling with the anti-linear xylan antiserum, which suggested that the homoxylans were more evenly distributed (Fig. 4f and g). Cell walls were also immunolabelled with an antibody directed against substituted xylans. For example, the antisense line 52–889 labelling was much weaker than the control transgenic line 1–768. Overall, this data is indicative of a change in xylan amount and distribution or organisation as a result of UDP-glucuronate down-regulation.

2.5. Lignin and cellulose extractability of T_1 transgenic lines

Following screening of T_0 lines, pBNP10642 lines 10, 18, 20, 23, 46 and pBNP10643 lines 16, 21, 25, 52, 59 were taken forward to the T_1 generation together with control lines pBNP10040 lines 1, 2, 3, 4, and 6. Ten kanamycin resistant plants from all lines were grown in the greenhouse and material was collected just before flowering. In order to have sufficient biomass, T_1 plants were analysed in pools according to their T_0 origin (Table 2). The different tobacco stem batches were subjected to a laboratory-scale kraft pulping process as previously published for CCR and CAD antisense lines (O'Connell et al., 2002) and the main pulping parameters were quantified to determine the chemical pulping behaviour.

The Kappa number is an indication of the efficiency of the delignification process and is a quantitative parameter for the level of the residual lignin present in the pulp after processing. Compared to the control lines, there was a tendency for a lower delignification for both sense and antisense lines, particularly, pBNP10642–18, pBNP10643–16 and pBNP10643–52. Taken together, the overall average kappa value for the respective lines, pBNP10040 of 32.2, pBNP10642 of 39.1 and pBNP10643 of 42.3 suggests that both sense and antisense lines were less amenable to kraft pulping, i.e. less amenable to alkali treatment.

Paradoxically, the lines with higher Kappa number showed less residual alkali than the control (Table 2). Normally, the high Kappa number coincides with high residual alkali indicating lower delignification. However, the Kappa measurement can be affected by conversion of glucuronic acid residues in xylans to hexenuronic acids by the alkaline treatment, which interferes with the integral permanganate oxidation as part of the assay. Therefore, an acidic treatment (pH 3.0 with sulphuric acid, 90 °C, 2 h) was carried out on two pulps in order to remove glucuronic acid residues although total uronic acids were not determined as increased in abundance in the transgenic lines and was constant at about 4.5%. Following this treatment, the impact on the Kappa number was only a slight reduction of 3.5 U for the control line pBNP10040-1 and 1.3 U for the antisense line pBNP10643-25 confirming that delignification was indeed less efficient in the modified lines and not an artifact. One possible explanation for this is that the down-regulation of UDP-glucuronate decarboxylase led to less xylan and modified the lignin-carbohydrate complexes, rendering the lignin less amenable to alkaline treatment and some linkages between lignin and cellulose/hemicelluloses could have been strengthened.

The pulp yield is an equally important economic parameter for the chemical pulp industry where the objective is to produce a pulp with the highest yield in the most efficient way. Filtering using screens is employed to remove chemically resistant particles, which normally in an industrial process would be subject to kraft pulping again. This pulp yield after filtering is the most significant parameter for the pulp quality. For the transgenic lines, screened pulp yield was lower with the following averages: sense lines BNP10642, 23.0% and antisense lines pBNP10643, 22.5% compared to control transformants pBNP10040, at 26.0%. Furthermore, the unextracted particle content was higher for the pBNP10643 lines (data not shown), confirming that the antisense plants were more difficult to process under alkaline conditions.

Finally, the degradation of the cellulose was determined by measuring the pulp viscosity and cellulose degree of polymerisation. On average, the pulp viscosity was slightly lower for the control lines compared to the transgenic ones,

Table 2
Pulping analysis of T_1 pools of selected transgenic tobacco

T1 line	Kappa number	Residual alkali (g/l)	Screened pulp yield (%)	Pulp viscosity (mPa)	Cellulose (DP)
40–1	36.5	2.1	25.1	46.2	1260
40–2	30	3.04	25.8	36.4	1160
40–3	31.2	2.66	28.0	40.4	1210
40–6	31.2	2.34	26.1	38.8	1190
Average 40	32.2 ± 2.5	2.5 ± 0.3	26.2 ± 1.0	40.5 ± 3.6	1205 ± 42
42–23	36	1.67	24.2	42.6	1230
42–46	34.5	2.28	28.1	43.8	1240
43–16	40.6	1.3	22.4	51.1	1305
43–21	39.1	1.92	23.6	45.7	1260
43–25	38.2	1.42	23.9	45.1	1250
43–52	59.6	0.69	17.2	51.5	1310
43–59	34.2	1.79	25.1	43.7	1240

Lines expressing UDP-glucuronate decarboxylase in sense (42-) or antisense (43-) orientation are compared to the average of control transgenic plants expressing GUS (constructs 40-). Lines were tested as described in O'Connell et al. (2002). DP, Degree of polymerisation.

respectively pBNP10040, 40.9, pBNP10642, 43.7 and pBNP10643, 47.4. This result was expected because of the higher kappa number of the transgenic lines. When delignification is less, the cellulose matrix would be much more protected and consequently the pulp viscosity greater. Similarly, the cellulose had a higher degree of polymerization. This type of result has a precedent in high xylan pulps where xylans can be dissolved during pulping but subsequently absorbed to a considerable extent on cellulose fibres by co-crystallisation of xylan segments with cellulose and by formation of strong xylan-cellulose hydrogen bonds (Mora et al., 1986; Buchert et al., 1996; Viikari et al., 1994). This could have a direct effect on pulp viscosity by an increase in molar mass.

2.6. Increased lignin is not responsible for the change in pulping behaviour

Since kraft pulping showed differences, increased lignin content might be the sole contributor. Therefore, lignin content was analysed in a second batch of T_1 plants, which were pooled together. Also these lines were subjected to kraft pulping and behaved similarly to the first batch with an increase in their Kappa number and a reduced residual alkali value. Lignin content for 4 control and 8 transgenic lines is shown in Fig. 5a. In most of these no differences were observed between the controls and the down-regulated lines and only lines 880, 881 (derived from 43 to 25) and 889 (derived from 43 to 52) contained more lignin than the controls. Comparing this data, increased lignin content cannot be the sole explanation for the high Kappa values. Interestingly, line 880 was confirmed to have reduced UDP-glucuronate decarboxylase expression (Fig. 5b), and also behaved as the parent line in the pulping. Line 889 was also shown to have the same pulping behaviour as its parent and had less xylans in xylem vessels (Fig. 4). Other lines deserve future testing on the basis of their residual enzyme activity. For example, line 902 derived from 43 to 59 and not shown in Fig. 5a, is also of interest as it had the lowest recorded activity resulting in higher Glc/Xyl ratio and consequently decreased residual alkali in pulping. Similarly, line 42-20-823 showing the next lowest activity, also had a higher kappa number but less residual alkali (data not shown). In summary the data shows that lines with a Glc/Xyl less than 2.5 have a Kappa value of 36 and below and those with a Glc/Xyl higher than 2.5 have

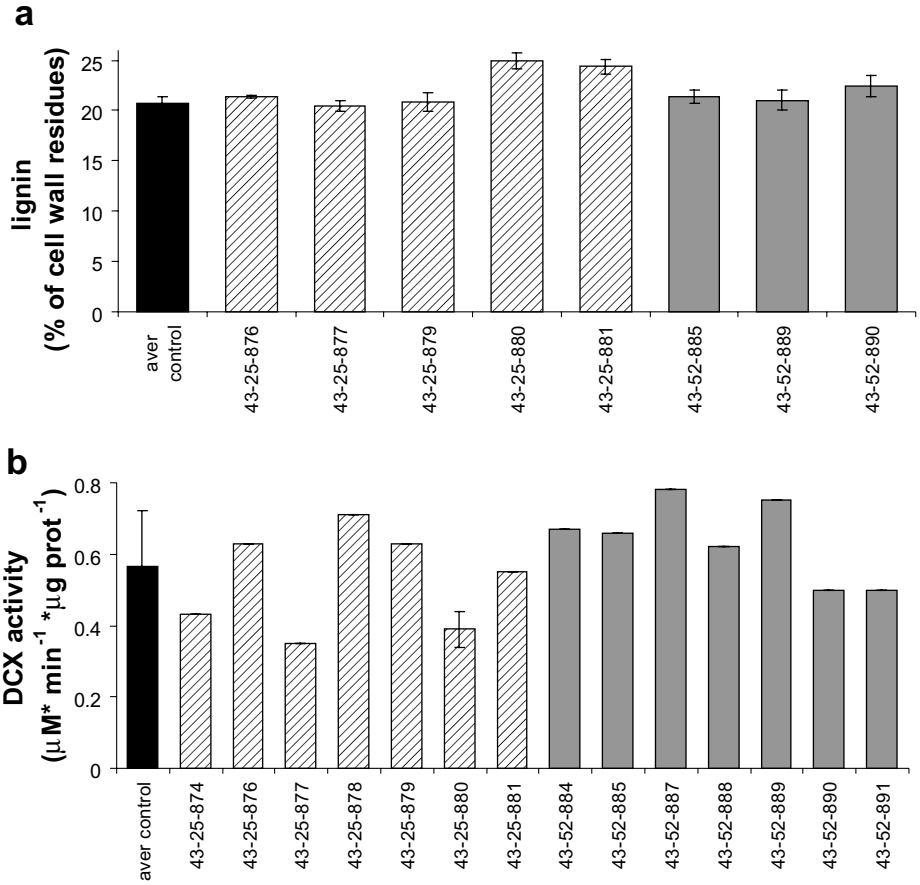

Fig. 5. (a) Lignin estimation (% of cell wall residues) of T_1 selected transgenic tobacco expressing UDP glucuronate decarboxyase in antisense orientation compared with (b) extractable enzyme activity. Lines are control lines, shown as the average of five lines, antisense lines 876, 877, 879, 880, 881 (derived from 43 to 25) and 885, 888, 889 (derived from 43 to 52), respectively. Lines 880 and 881 have up to 20% more lignin while other have equivalent levels to WT. Therefore, the data indicates that changes in cellulose extractability (Table 2) cannot be due to less lignin in the stems.

Table 3
Correlation between lignin in pulps and polysaccharides in the control and transgenic tobacco lines

T1 line	Kappa number	Glucose/xylose
GUS 40–1	36.5	2.2
GUS 40–3	31.2	2.3
Sense 42–23	36.0	2.5
Sense 42–46	34.5	2.4
Antisense 43–16	40.6	2.7
Antisense 43–21	39.1	2.6
Antisense 43–25	38.2	2.9

a Kappa number greater than 36 (Table 3). Such data can give insights into the relationship between xylan synthesis and lignification, and the interaction between xylans and lignins and cellulose. It suggests that the level of xylan relative to lignin is an important factor in delignification. Possibly as a result of reduction of xylan without comparable reduction in lignin, a portion of lignin normally associated with xylan may become attached as lignocellulose complexes to account for increased residual lignin in the transgenic lines.

3. Discussion

3.1. Synthesis and modification of secondary wall xylans

Interest in newer uses for plant cell walls as industrial fibre feedstocks and as biomass for energy has further highlighted deficiencies in our knowledge of the biosynthesis of this resource. Although progress has been made in identifying the genes involved in the synthesis of cell wall components such as cellulose and lignin, identifying those involved in the synthesis and modification of other components, such as hemicelluloses, is still relatively less complete. Such genetic and biochemical analysis has also modified prior assumptions and this is best exemplified when lignin is considered where the pathway or metabolic grid has undergone considerable modification recently (Anterola and Lewis, 2002; Boudet et al., 2003; Boerjan et al., 2003). Similarly, in the case of the cellulose synthase gene family, a complexity of gene expression has also been revealed in relation to development (Scheible and Pauly, 2004; Persson et al., 2005). However, no clear picture has yet emerged as to the co-regulation of these two components even without the additional consideration of an integrated role for the hemicelluloses. The extent of any co-regulation is important to establish to begin to address these added complexities and the influence of lignin and hemicellulose on cellulose content, availability and quality after extraction.

Several approaches have been adopted to identify the genes involved in the short pathway leading to the synthesis of xylan in Arabidopsis, tobacco and other species. Some steps such as UDP-glucose dehydrogenases and UDP-glucuronate decarboxylases have been initially cloned by homology to known candidates in other species (Seifert, 2004). Functional redundancy amongst these gene families also probably exists in tobacco (Bindschedler et al., 2005; Molhoj et al., 2003). Proteomic approaches confirmed the identity of UDP-glucuronate decarboxylase and also led to identification of a second UDP-glucose dehydrogenase with alcohol dehydrogenase activity. Provision of UDP-glucuronate can also take place through the *myo*-inositol pathway through inositol oxygenase as identified in Arabidopsis (Kanter et al., 2005). Xylan synthase itself remains to be identified but may well prove to be a member of the Csl family, possibly CslD (Samuga and Joshi, 2004). In comparison, CslA codes for mannan synthase (Dhugga et al., 2004; Liepman et al., 2005) while CslE and CslG in tobacco are unlikely to be candidates from expression analysis (M. Compier, J.-P. Vincken, R. Visser, L.V. Bindschedler, G.P. Bolwell, unpublished data). Alternatively, a GT43 family glycosyltransferase may prove to be xylan synthase (Pena et al., 2007). Added complexities may also occur through xylan remodelling. Such remodelling has been indicated already for cellulose where recent work has indicated a role for β-glucanases in initial synthesis and assembly (Scheible and Pauly, 2004). Since xylanase has been shown to be expressed in differentiating tobacco cells, both by cell wall proteomics (Blee et al., 2001) and the presence of two forms of high similarity in the ESTs of these xylogenic cells (L.V. Bindschedler, D.J. Millar, G.P. Bolwell, unpublished data), it may perform a role in regulating xylan structure and content. These xylanases have high similarity to the Arabidopsis xylanase cDNA shown to have a role in secondary cell wall metabolism and plant development (Goujon et al., 2003). Therefore, faced with such complexities, initial forays into engineering hemicelluloses have focussed in the present study on the UDP-glucuronate decarboxylase step as the least likely functionally redundant step coded for by known gene sequences. In contrast, antisense expression of the two distinct enzyme systems capable of UDP-glucose dehydrogenase activity in tobacco (Bindschedler et al., 2005) did not produce striking changes in the cell wall (L.V. Bindschedler, G.P. Bolwell, J. Kossmann, U. Schutler, unpublished data), although over-expression of UDP-glucose dehydrogenase in alfalfa did result in changes in the xylose content of wall but not the uronic or pectin content (Samac et al., 2004).

3.2. Reduction of xylan by antisense expression of UDP-glucuronate decarboxylase

Expression of the UDP-glucuronic acid decarboxylase in an antisense orientation, in particular, led to a lower recovery of enzyme activity and detection of protein by Western blotting. Although only partial, these values are comparable to the levels shown for many down-regulated enzymes involved in lignin biosynthesis (Anterola and Lewis, 2002). In these cases, this decrease in enzyme activity was still sufficient to lead to substantial reduction of lignin deposition, and thus these reduced lignin plants are of potential biotechnological use (Boudet et al., 2003).

The variable down-regulation detected in the lines by a combination of both screening methods may be due to functional redundancy of the large gene family and also the ability of other enzymes to be involved in UDP-xylose provision such as the generically different 87 kDa UDP-glucuronate decarboxylase (Wheatley et al., 2002) and the xylose/apiose biosynthetic enzyme (Molhoj et al., 2003; Ahn et al., 2006). It may be that some of these genes are turned on to compensate gene silencing generated by sense/antisense expression. Indeed, any depletion of UDP-xylose, which is predicted to be a key feedback regulator of the entire sugar nucleotide network (Seifert, 2004), would add complexity to regulation, understanding of which is beyond the present study.

Nevertheless, the antisense plants in particular were found to have a consistent reduction in xylan content relative to cellulose. Considering that UDP-D-xylose is the direct precursor of UDP-L-arabinose via the corresponding epimerase, it is also interesting that the cell wall carbohydrate analysis did not reveal significant variation of arabinose proportions in the down-regulated lines. Since the proportion of xylose in the wall decreased by about 20% in the transgenic lines, a similar reduction on the low arabinose proportion of the walls (<1%) would remained insignificant. Xylose reduction may affect both secondary wall xylans as well as xyloglucans from primary walls. Due to the known interaction between xyloglucans and cellulose, essential to primary wall formation and structure, an alteration in xyloglucan synthesis would be expected to have noticeable impact on the further secondary wall development. Since histochemical and ultrastructural alterations observed in the walls of the transgenic plants were only minor and localized in the S2 layer of the secondary wall (Fig. 4), this suggests that xyloglucans, and thus primary walls, were not significantly affected.

3.3. Alteration of cellulose extractability from antisense lines

Overall, the net result of engineering lines was a consistent lower xylose and higher glucose content in the polysaccharides and consequent changes to pulping properties. The lignin content was mostly unchanged so these pulping changes are more likely to be due to a property of less/modified xylan. The comparison of control lines and UDP-glucuronate decarboxylase-down-regulated lines indicated that the latter seemed to be more difficult to delignify due to an effect on the lignin-carbohydrate complexes and generating kraft pulps with higher Kappa number. Consistent with this observation is the fact that the glucose/xylose ratios correlate to the Kappa number values in two main groups (Table 3). The normal lines exhibited Glc/Xyl ratios lower than 2.5 and had Kappa values below 36, whereas the transgenic lines exhibited Glc/Xyl ratios above 2.5 corresponding to Kappa values higher than 36. Since Kappa values essentially measure the residual lignin content in kaft pulps, these results show that the reduction of xylans in the transgenic lines leads to an increase in the lignin remaining after processing. It suggests that the level of xylan relative to lignin is an important factor in delignification; there may be a portion of lignin not associated with xylan, when this is reduced, and which becomes attached as lignocellulose complexes to account for increased residual lignin in the transgenic lines. This might not be predicted and may indicate important clues as to the molecular interactions and proximity of lignin or xylan to cellulose, which is not understood at present, as well as putative cross-talk between the xylan and the lignin pathways. In addition, the pulp yield was lower and the pulp viscosity slightly higher, due to the lower delignification. With respect to cellulose, the degree of polymerisation was similar for all the pulps produced from control or transgenic lines. Although hemicellulose mutants have been identified in Arabidopsis, this is the first example to our knowledge of the consequences of directed manipulation of xylans in woody plants to test for alterations in pulping properties.

In comparison, transgenic tobacco plants, severely suppressed in the activity of cinnamoyl-CoA reductase produced by introduction of a partial sense transgene and subjected to direct Kraft pulping trials identical to the methods used in the present study, showed more lignin could be removed from the transgenic wood than from wild-type wood at the same alkali charge (O'Connell et al., 2002). This was also seen for tobacco plants expressing an antisense cinnamyl alcohol dehydrogenase gene where the modified lignin was more easily removed during pulping without any adverse effects on the quality of the pulp or paper produced. However, there were no changes to cellulose degree of polymerisation.

With respect to future prospects of engineering xylans, there was some variability in the strength of the down-regulation amongst individual plants and due to the size of the UDP-glucuronate decarboxylase gene families there is likely to be functional redundancy amongst the family members (Harper and Bar-Peled, 2002; Bindschedler et al., 2005). It also cannot be excluded that gene silencing of the transgene in some of the T_1 plants might lead to a weaker difference regarding the Glc/Xyl ratio when compared to the T_0. Nevertheless because some T_1 plants showed a significant decrease in enzyme activity decrease, these lines should be further analysed in search of stronger phenotype. It is not known whether xylan, being the main secondary cell wall hemicellulose, is required for viability of the plant or not. Although down regulation of xylan content has been demonstrated here it is more likely to be made more efficient in future by targeting a gene specifically expressed in vascular xylem, for instance by specifically targeting the UDP-xylose involved in the xylan formation without affecting the UDP-xylose pool that will enter the primary cell wall xyloglucan biosynthetic pathway, thus avoiding the undesirable side effect of manipulated xyloglucan as well.

In summary, although less xylan might be predicted to lead to improvements in cellulose extractability this would

not appear to be the case. It appears that increased lignification, perhaps in compensation, does not occur extensively but it does lead to closer association with the cellulose so that delignification is less efficient. However, this does lead to the benefit of increased degree of polymerization with a slight protective effect upon the cellulose.

4. Experimental

4.1. Growth of Tobacco

Nicotiana tabacum plants cultivar K326 were grown in the greenhouse under illumination (1000 lx) on an 8 h dark – 16 h light cycle. Plant positions were randomised in the greenhouse.

4.2. Production of tobacco line transgenic for UDP glucuronate decarboxylase

Sequences from plant UDP-glucuronate decarboxylases (Arabidopsis UXS1, UXS2, UXS3 and the pea UXS1) were aligned. Degenerate primers were designed as primer 5′-AAY CCN GTN AAR CAN ATH AAR AC-3′ and reverse 5′-CAT NGT RAA YTC NCC NGG RTT NCC-5′. RNA from 3-day-old xylogenic cultures was reverse transcribed and amplified. A PCR product of 490 bp was cloned into pGEMT-easy vector (Promega). The same PCR product was then used to screen the cDNA library to isolate UDPGlcADCX family members from tobacco (Bindschedler et al., 2005). The 490 bp PCR product (DCX7) was cloned in both directions into TopoII PCR vector (Invitrogen). PCR DCX7.1 (sense, pBNP10642) and DCX7.7 (antisense, pBNP10643) for the UDP-glucuronate decarboxylase were then subcloned into the pBinplus vector between a CaMV35S promoter and terminator. The construct pBinplus containing a CaMV35S-GUS (β-glucuronidase) cassette was used as a control (pBNP10040). *Nicotiana tabacum* var. K326 was used for transformation. Twenty-seven non-transformed controls designated non-co-cultivated controls (NCC), 27 transgenic controls containing the GUS gene (10040), 77 sense (10642), and 77 antisense (10643) T_0 transgenic plants were regenerated, grown and harvested. Plants were analysed by PCR for the presence of the transgenes. Plants showing single inserts, GUS controls and non-transformed controls (NCC) were moved into the greenhouse. The plants were harvested after about 2.5 months just before flowering and tested.

Seed of the following lines were sown for analysis of T_1 progeny plants: pBNP10040 lines 1, 2, 3, 4, and 6, pBNP10642 lines 10, 18, 20, 23 and 46 and pBNP10643 lines 16, 21, 25, 52 and 59. Ten Kanamycin resistant plants from all lines and 10 plants each from pBNP10040 line 1 and from pBNP10642 line 20 were grown in the greenhouse. Material was collected just before flowering. Internode 15 was fixed for microscopy for analysis, internodes 13 and 14 were lyophilised and used for polysaccharide analysis. A part of internode 13 was stained with phloroglucinol. A fragment of internode 12 was flash frozen in a microfuge for enzyme activities. Internodes 11 and 12 were lyophilised for Western blot analysis. The rest of the stems were lyophilised for pulping property analysis. Internodes were counted from apex to base, starting with internode 1 immediately underneath the flower buds. The plants were grown up to flowering and seed collected from all plants. Leaf material was collected from all plants to confirm the genotype by PCR for the UDP-glucuronate decarboxylase transgene.

4.3. Analysis of selected lines for UDP glucuronate decarboxylase down-regulation

For UDP-glucuronate decarboxylase activity assays, a section from internodes 11 and 12 were flash frozen when harvested and kept at −70 °C until used. Samples were ground in liquid N_2. Just before the assay, proteins were extracted from 20 mg sample on ice with 200 μl of 100 mM sodium phosphate buffer pH 7.0 containing 1 mM EDTA and 5 mM β-mecaptoethanol. The enzyme assay was performed by mixing 20 μl of 0.6 mM UDPGlcA in 100 mM sodium phosphate buffer at pH 7.0 containing 1 mM EDTA and 5 mM β-mecaptoethanol with 20 μl protein extract in the same buffer and incubating for 10 min at 37 °C. The assay was terminated by incubating the mix at 95 °C for 5 min. After centrifugation at 14,000*g* for 5 min, 30 μl was transferred to a fresh tube containing 30 μl of 90 mM sodium borate buffer pH 9.0. The enzyme substrate UDPGlcA and product UDPXyl were separated by capillary electrophoresis, using a modified protocol adapted from Lehmann et al. (2000) and Xu et al. (1998). UDPGlcA and UDPXyl were detected by absorbance at 262 nm. Optimised capillary electrophoresis conditions to separate UDPGlcA from UDPXyl using instrument ABI 270A-HT (Applied Biosystems, Foster City, CA, USA) with a fused bore silica capillary of 72 cm × 75 μm inner diameter. Separation was performed in 90 mM Na borate buffer pH 9.0 at 30 °C with 20 kV. Elution time for UDPXyl was typically 10 min and 11.5 min for UDPGlcA. UDPGlc was also assayed and eluted at 8.5 min. 0.1 mM UDPXyl and 0.3 mM UDPGlcA were run as standards. Before each series of determinations the capillary was reconditioned by a 30 min wash with 0.1 M NaOH and 30 min water. Between each sample, the capillary was washed for 3 min with 0.1 M NaOH injected followed by a 3 min equilibration with 90 mM Na borate buffer pH 9.0.

For expression analysis, proteins from internode 11 were extracted in a sodium phosphate buffer (0.1 M, pH 7.0, 1 mM EDTA, 0.5 ml/l 2-mercaptoethanol and 2% PVP). Proteins were separated on 10% acrylamide gels and transferred on nitrocellulose for western blotting. Nitrocellulose membranes were blocked for 16 h in TBST with 2% BSA. Anti-(UDP-glucuronate decarboxylase) was used at 1:3000 dilution and incubated for 1 h at 25 °C in the blocking solution.

4.4. Morphological and chemical analysis of tobacco lines

4.4.1. Polysaccharide analysis

The samples for carbohydrate analyses were lyophilised tobacco stem internodes 13 and 14. Xylem tissues were isolated by hand dissection and the xylem yield was expressed as % of dry weight of the whole internode. Ground xylem tissues were sequentially extracted with a mixture of toluene and EtOH (2:1, v/v), followed by hot H_2O and then EtOH. The insoluble cell wall material was freeze-dried and polysaccharides were then hydrolysed in 72% H_2SO_4 (6 h, 30 °C) followed by 1 M H_2SO_4 (4 h, 100 °C) in the presence of *myo*-inositol as internal standard. After neutralisation with $BaCO_3$, the monosaccharides were converted to their alditol acetate derivatives and analysed by gas–liquid chromatography.

4.4.2. Immunocytochemistry

A linear, unsubstituted, xylan chain was prepared from Esparto grass xylan by selective acid hydrolysis of the arabinofuranosyl substituents. The specificity of action of the antiserum raised in rabbits was tested against various wall polysaccharides and xylan model compounds. The antibody (anti-xylan-L) showed specificity for xylans with low substitution pattern and had no recognition for any other of the usual wall polysaccharides such as cellulose, glucomannan, galactomannan, pectic polymers, galactan and arabinan (Ruel and Joseleau, 2005). Immunolabelling in TEM was done on ultra-thin transverse sections (500 Å) using first antibody at 1:100 and secondary marker (protein A-gold, pA 5, Amersham) diluted 1:25 using the method of Billosta et al. (2006).

4.4.3. Lignin analysis

Internodes 13 and 14 were harvested from 2.5-month old tobacco plants (just before flowering) and frozen in liquid N_2 and freeze-dried. Cell wall residue was prepared as described previously (Chabannes et al., 2001) and Klason lignin content was determined using a micro-Klason technique (Whiting et al., 1981). Two plants from each line were pooled for extraction of the walls and measurements were performed in triplicate for each combined residue.

4.4.4. Pulping analysis of transgenic lines

A method established for the pulping of tobacco was utilised (O'Connell et al., 2002). Essentially, the lyophilised stems were cut into small pieces for a better impregnation with the chemicals before pulp processing. Kraft pulping was then simulated into pressurised reactors immersed into an oil bath with the following conditions applied to the chips: active alkali 28%, sulfidity 25%, with a liquor to wood ratio of 4.25/1. The cooking temperature of 170 °C was raised in 90 min and kept for 90 min. After kraft pulping, the pulps were washed with tap H_2O and screened with a 150 μm slotted screen to remove the uncooked particles. Kappa number, pulp viscosity, cellulose degree of polymerisation, unscreened and screened yields and alkali consumption were also evaluated as described by O'Connell et al. (2002).

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