Transcriptome profiling of *Brassica napus* stem sections in relation to differences in lignin content

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**Abstract**

*Background:* Brassica crops are cultivated widely for human consumption and animal feed purposes, and oilseed rape/canola (*Brassica napus* and *rapa*) is the second most important oilseed worldwide. Because of its natural diversity and genetic complexity, genomics studies on oilseed rape will be a useful resource base to modify the quantity and quality of biomass in various crops, and therefore, should have a positive impact on lignocellulosic biofuel production. The objective of this study was to perform microarray analysis on two variable lignin containing oilseed rape cultivars to target novel genes and transcription factors of importance in Brassica lignin regulation for applied research.

*Results:* To gain insight into the molecular networks controlling cell wall biosynthetic and regulatory events, we conducted lignin and microarray analysis of top and basal stem sections of brown seeded *Brassica napus* DH12075 and yellow seeded YN01–429 cultivars. A total of 9500 genes were differentially expressed 2-fold or higher in the stem between the cultivars, with a higher number of expressed genes in the basal section. Of the upregulated genes, many were transcription factors and a considerable number of these were associated with secondary wall synthesis and lignification in *B. napus* and other plant species. The three largest groups of transcription factors with differential expression were C2H2 and C3HC4 zinc fingers and bHLH. A significant number of genes related to lignin and carbohydrate metabolism also showed differential expression patterns between the stem sections of the two cultivars. Within the same cultivar, the number of upregulated genes was higher in the top section relative to the basal one.

*Conclusion:* In this study, we identified and established expression patterns of many new genes likely involved in cell wall biosynthesis and regulation. Some genes with known roles in other biochemical pathways were also identified to have a potential role in cell wall biosynthesis. This stem transcriptome profiling will allow for selecting novel regulatory and structural genes for functional characterization, a strategy which may provide tools for modifying cell wall composition to facilitate fermentation for biofuel production.

**Keywords:** Biomass, Brassica, Cell wall, Stem lignin, Microarray, Transcription factors

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**Background**

Limiting sources of fossil energy resources and potential climate change underlie increasing worldwide economic interest and scientific focus on renewable biofuel. Lignocellulosic biomass is abundant in nature and can be used as sustainable and renewable feedstocks for biofuel production. Grasses, such as maize, sorghum, switchgrass and Miscanthus, and fast-growing trees, such as poplar and willow are potential sources of lignocellulosic biomass. Crop residues collected from the production of major food crops such as canola, maize, rice and wheat can also be used as raw material for biofuel. Brassica crops are among the oldest cultivated plants, and oilseed rape/canola (*Brassica napus* and *rapa*) is currently the second most important oilseed worldwide [1]. Because of its wide spread cultivation, natural diversity and genetic complexity [2, 3], genomic studies on oilseed rape are a useful resource base from which to modify the quantity and quality of biomass...
in various crops, and hence, should have a positive impact on lignocellulosic biofuel production.

Crop residues are a rich source of plant biomass, which is mainly composed of plant cell walls. The major components of plant cell walls are cellulose, hemicellulose, pectin and lignin. Cellulose is the world’s most abundant biopolymer [4] and can be used as a sustainable and renewable feedstock for biofuels [5, 6]. However, this process is inhibited by the presence of lignin [7], a major component of secondary cell walls, which provides structural support to plants but also acts as an obstacle to glucose fermentation by increasing the recalcitrance of cell wall digestibility [8]. Moreover, lignin can adsorb hydrolytic enzymes that are used to generate monosaccharides from lignocellulose, and some lignin degradation products inhibit subsequent fermentation steps [9]. Novel strategies to reduce lignin and/or modify its composition in the plant cell wall are therefore needed, and a clear understanding of cell wall biogenesis-related gene expression with emphasis on lignin biosynthesis and factors regulating its accumulation and polymerization is essential to addressing cell wall recalcitrance.

Although many of the structural genes in lignin biosynthesis have been characterized, additional knowledge on transcription factors controlling this pathway is still needed. Studies have shown developmental regulation of the phenylpropanoid pathway genes by various classes of trans-acting factors [10]. Although, most of the genes encoding lignin biosynthetic enzymes have been targets for modification of lignin biosynthesis, only a few studies have taken advantage of the possibility of modulating the trans-acting factors involved in phenylpropanoid metabolism. Some promising findings on the role of transcription factors with phenylpropanoid end products, including lignin biosynthesis, have been reported through mutant analysis [11–15], but additional work is still needed in this area. Presumably, altering the expression level of appropriate transcription factors would coordinately affect a group of genes in the pathway. By doing so, it might be possible to avoid deleterious phenotypes associated with the accumulation of pathway intermediates due to single gene/enzyme manipulations [16].

Microarrays have been an important technology for the global analysis of gene expression in plants, including in cell walls, although more recently RNA sequencing has risen in importance. Hertzberg et al. [17] used poplar cDNA arrays to profile changes in gene expression at various stages of secondary xylem differentiation. Ko et al. [18] used short oligonucleotide arrays to identify genes that display preferred expression in secondary xylem and during the transition from primary to secondary growth in Arabidopsis stems. Ehling et al. [19] studied the metabolic, developmental and regulatory events at different stages of vascular and interfascicular fiber differentiation in inflorescence stems of Arabidopsis using oligo arrays.

The yellow seeded B. napus YN01–429 cultivar was recently developed through classical breeding approaches. It was found to contain significantly reduced seed lignin content relative to the more conventional brown seeded cultivar DH12075 [20, 21]. The yellow-colored trait of Brassica seeds is valuable to Brassica breeders since it is associated with a thinner seed coat and reduced dietary fiber content [21]. Light seed color and low fiber content are believed to share precursors and biochemical pathways leading to lignin and pigment synthesis [22, 23]. Since plant stems represent the major contribution to Brassica plant residue, we analyzed the total lignin content of stem sections of these two unique cultivars. On the basis of differential lignin content, we performed microarray experiments to determine variations in the transcription profiles of different sections of the stem. Analysis of transcript profiles allowed us to categorize genes into specific sets involved in various regulatory, developmental and metabolic processes including transcriptional regulation, carbohydrate metabolism, and lignin and cellulose biosynthesis. By correlating changes in gene expression with changes in the levels of lignin, we hypothesized that it should be possible to derive insights into the regulatory mechanisms of cell wall biosynthesis and to find target transcription factors of importance in Brassica lignin regulation for practical purposes.

**Methods**

**Plant materials and growth conditions**

Brown seeded B. napus DH12075 (designated DH), yellow seeded B. napus YN01–429 (designated YN), alfalfa (Medicago sativa L.) and Arabidopsis thaliana ecotypes and mutant lines were grown in soil in a controlled greenhouse environment (16 h light/8 h dark, 20 °C/17 °C). For B. napus stem samples, plants with well-developed siliques were harvested and 40 cm of the stem was selected (excluding the lower most 10 cm). Collected stem samples were divided into 4 segments, 10 cm each, and apical (section 1) and basal sections (section 4) were used for lignin, microarray and qPCR analyses. For Arabidopsis lignin analysis, Arabidopsis plants (42-day-old) were cut at the base of the inflorescence stem and leaves and thin branches were removed, keeping the stem and main/robust branches for analysis.

**Lignin analysis**

Lignin content of stem material was determined by thioglycolic acid (TGA) method [24]. Fifty mg of stem tissue ground in liquid N2 from 3 different plants per cultivar
DNA microarray
Total RNA from stem sections of both DH and YN were extracted as described in Carpenter and Simon [25] followed by clean-up using the commercial RNaseasy mini kit (Qiagen, Valencia, CA, USA). Two biological replicates were collected for each sample. RNA amplification, labeling with Cy3- or Cy5-dCTP dyes (GE Healthcare, Buckinghamshire, UK), and probe fragmentation was carried out using Ambion AminoAllyl MessageAmp II RNA amplification kit according to the manufacturer’s instructions (Ambion, Austin, TX, USA). A spotted 15K 50-mer B. napus oligo array, previously developed at the Saskatoon Research and Development Centre, Agriculture and Agri-Food Canada [26], was hybridized with the Cy5- and Cy3-labelled probe pairs. Cy3 dye was used for labeling the YN RNA and Cy5 for DH RNA. A dye swap (Cy3/Cy5) labelling experiment was performed for each biological replicate. Labeling, hybridization and post-hybridization washing were conducted according to the protocol for Corning epoxide slides (Corning Inc., Lowell, MA, USA). Hybridization was carried out at 37 °C for 17 h in a MAUI hybridization station (BioMicro Systems, Salt Lake City, UT, USA). Following the post-hybridization washes, slides were scanned with a VersArray ChipReader laser scanner (Bio-Rad Laboratories, Hercules, CA, USA).

Image files were transferred to ArrayPro Analyzer software (Media Cybernetics Inc., Bethesda, MD, USA) for image analysis, spot verification, normalization, filtering and feature extractions. A standard statistical program GeneSpring GX (Agilent Technologies, Santa Clara, CA, USA) was used to determine that the data set would fit a normal distribution (log 10) and to check the validity of expression data by assessing the variation between control spots. Data files for the duplicates on individual slides and the dye swap data files for each experiment were merged and average spot intensities used to reduce experimental bias. Normalization was performed on merged data using the Lowess (sub-grid) method, and the local background was subtracted from the values of each spot. The intensity of each spot at λ549 nm (Cy5) and λ647 nm (Cy3) was analyzed using a BASE plug-in and finally transformed into a DH/YN ratio value that denoted the most upper (section 1) and lowest stem sections (section 4), including DH1:YN1, DH4:YN4, DH1:DH4 and YN1:YN4. Additional data processing was performed using tools available in BASE (http://base.thep.lu.se). Gene ontology (GO) analysis was conducted using the TAIR database [27]. Microarray ratios with transcript changes > 2-fold upregulated or downregulated between two different B. napus stem sections were considered for all ratios where p ≤ 0.05 (Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: Table S1A and B to S6A and B). Data in these 12 Additional files were screened using either Excel or Microsoft Access to identify individual or groups of B. napus genes expressed in multiple DH and YN ratios in the main categories of cell wall, other carbohydrate genes and transcription factors, and in additional sub-categories. Ten major transcription factor families, Arabidopsis homologue IDs and corresponding mutant lines for selected B. napus genes were retrieved from http://www.arabidopsis.org/browse/genefamily/index.jsp [28].

Quantitative real-time RT-PCR
For quantitative real time reverse transcription PCR (qRT-PCR) experiments, total RNA was extracted from stem sections using a TRIzol reagent (http://www.invitrogen.com) following the manufacturer’s instructions. Three biological replicates (independent RNA preparations) were collected and used with three technical reps for qRT-PCR analysis. RNA concentration was determined using a nanodrop spectrophotometer (Fisher Scientific, Canada). Twenty micrograms of total RNA (per sample in each replicate) was treated with Turbo DNase (www.ambion.com) as per the manufacturer’s instructions to eliminate trace amounts of genomic DNA. Reverse transcription reactions were performed with Superscript™ III Reverse Transcriptase (http://www.invitrogen.com) according to the manufacturer’s instructions using 1.5 μg RNA per reaction. Two RT reactions per sample were done and reactions were pooled after 10-fold dilution with nuclease-free water. Polymerase chain reactions were carried out using 96-well plates in a LightCycler® 480 II (http://www.roche-applied-science.com/lightcycler-online) using SYBR® Green. Reactions contained 10 μl of 2× SYBR® Green Master Mix (Roche), 5 μl of diluted cDNA and 200 nM of gene/EST-specific primer (Additional file 13: Table S7) in a final volume of 20 μl. For data normalization, five reference genes (actin, adenine phosphoribosyl transferase, β-tubulin, cyclophilin and elongation factor 1-a) were included in the experiment and two genes with stable expression, namely β-tubulin and actin, were selected using geNorm software [29]. PCR efficiency, in the range of 85 to 100%, was determined from amplification plots using the program LinRegPCR [30].

Plant transformation
RNAi and over-expression constructs were developed using either the closest Arabidopsis homologue according to Hossain et al. [31, 32] or the closest alfalfa stem cDNA according to Li et al. [33]. Alfalfa primers were identified by Laberge (personal communication). Floral bud transformation was conducted in Arabidopsis according to Hossain et al. [31, 32]. Alfalfa transformation was conducted using Agrobacterium tumefaciens according to Aung et al. [34].
Results

DH and YN stems differ in lignin and exhibit different gene expression profiles

To validate our introductory hypothesis, we first determined the stem lignin content of DH and YN plants. Total lignin content showed higher levels in both apical (section 1) (DH1) and basal sections (section 4) (DH4) of DH compared to their counterparts in YN cultivar sections (YN1 and YN4) (Fig. 1a). Within stem sections of each cultivar, we found the highest lignin content to be in the basal sections and the lowest in the apical sections. Increased lignin accumulation is the result of increased enzyme activity and/or increased gene expression. Hence, we compared global gene expression levels between the two cultivars and also between these sections using a 15 K B. napus microarray that was used earlier to investigate gene expression in developing B. napus seed [35] and diseased stem sections [26]. In detail, we related lignin levels with transcriptome changes between DH and YN (both apical and basal sections for each cultivar (Fig. 1b; Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: Tables S1A and B to S6A and B). The greater upregulation of genes in the apical stem sections compared with basal stem section expression pattern is reasonable because the apical region of the plant is developmentally more active compared to the basal region.

Functional classification of genes up-regulated in high lignin stems of B. napus

Gene annotation of sequences differentially expressed using the 15 K B. napus array was conducted using The Arabidopsis Information Resource (http://www.arabidopsis.org). Annotated genes were grouped into three major functional categories: cellular component, molecular function and biological process, and then divided into subcategories (Fig. 2; Table 1). For ease of presentation, data was expressed as the average of genes in each subcategory in DH vs. the average in YN. Within the category of biological processes in DH4 vs. YN4, genes involved in defense responses were 7.6% of the total, whereas stress responsive genes were 7.4%; genes involved in developmental processes were 4.9 and 4.7% were involved in transport; 2.35% of genes were involved in transcription and 0.6% in DNA or RNA metabolism. In the cellular component category, 6.4% genes were related to plasma membrane, and nucleus genes accounted for 6.02%; cell wall genes were 2.12 and 1.6% genes were related to cytoplasm functions. In the molecular function category, 7.4% genes were involved in protein binding, and 5.7% genes accounted for DNA or RNA binding activity; 4.8% genes were involved in transporter activity, and 3.4% showed transcription factor activity (Fig. 2; Table 1).

Annotation for genes differentially expressed in DH1 vs. YN1, DH1 vs. DH4 and YN1 vs. YN4 pairwise comparisons was also carried out. In all three functional categories, genes belonging to the above three groupings showed a similar pattern of distribution to those of DH4 vs. YN4.

Validation of microarray data by quantitative RT-PCR

To validate the results of microarray experiments, transcript levels of seven genes were examined by qRT-PCR using total RNA prepared from stem sections. Emphasis was placed on functional diversity in selecting the following genes for qRT-PCR experiments; ANAC062 (Accession # ES901271), PHYTOCHROME INTERACTING FACTOR 4 (PIF4; EV181356), GLYCINE-RICH RNA BINDING PROTEIN (RBP/CAA78513.1), DIMINUTO 1, LIPASE 1 (AY866419), FIBRILLIN (AF084554.1) and GDSL-MOTIF CONTAINING LIPASE/HYDROLASE (CN730052) (Fig. 3a and b; Additional file 13: Table S7). From the microarray data, it was determined that these selected genes were up-regulated in the DH cultivar with high lignin content. The expression profiles of most of the genes tested by qRT-PCR were consistent with our microarray data, although the difference in expression was less in the latter data set. Expression of DIMINUTO 1 was significantly higher in the basal section of DH (DH4). Expression of ANAC062 was also higher in the basal section of DH4, whereas PIF4 showed higher expression in both apical (DH1) and basal sections (DH4). Expression of LIPASE1 was also higher in both sections of DH compared to those of YN. Transcripts of RBP exhibited slight numerical increase in both sections of DH, although the difference was not statistically significant. Because of their extremely low expression levels relative to other tested genes, the two genes encoding FIBRILLIN and GDSL-MOTIF PROTEIN were assessed separately (Fig. 3c).
Both of the latter genes showed significantly higher expression in the two sections of DH relative to their counterparts in YN.

**Upregulation of monolignol biosynthetic genes**

Genes involved in monolignol biosynthesis were retrieved from the TAIR database (www.arabidopsis.org) and used to identify *B. napus* biochemical genes that were upregulated at least 2-fold in high lignin-containing stem material using the microarray data (Table 2). Tissue comparisons showed that several genes encoding enzymes involved in monolignol biosynthesis were upregulated in the DH cultivar relative to the YN cultivar. Among these genes, caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT1) was upregulated in the top and basal sections of DH relative to the top and basal sections of YN (Table 2). In addition, 3-adenosyl methionine synthase (SAMS) and CCoAOMT enzymes that methylate lignin precursors [36] were coded by strongly upregulated genes in our microarray experiments (Table 2). SAMS catalyzes the transfer of an adenosyl group from ATP to the sulphur atom of methionine, resulting in the synthesis of SAM, a common methyl group donor. In the microarray experiments, *SAMS3* was one of the stronger upregulated genes in the top and basal sections of DH relative to those of YN and in the basal section relative to the top section of DH (Table 2).

Cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) also play important roles in monolignol biosynthesis. The cinnamoyl-CoA esters, precursors of monolignol biosynthesis, are generated by the general phenylpropanoid pathway and then converted into monolignols by CCR and CAD [7]. *CCR1* was among the most upregulated genes in the top and basal sections of DH relative to those of YN, and the same gene was upregulated in the basal section relative to the top section of YN in the array (Table 2). *CCR2* was also upregulated in the top section of DH relative to the top section of YN. Another major gene change in the phenylpropanoid pathway was coded by *p*-coumarate 3-hydroxylase (C3H), also known as REDUCED EPIDERMAL FLUORESCENCE (*REF8*) was upregulated in the top and basal sections of DH relative to those of YN (Table 2).

The *DE-ETIOLATED 3* (*DET3*), At1g12840, was upregulated in the top and basal section of DH relative to the top and basal section of YN (Table 2). This gene encodes the C-subunit of the V-type ATPase [37] implicated in the maintenance of pH homeostasis in plants [38]. Moreover, the *FAH1* gene was upregulated in the basal section of DH4 relative to the basal section of YN4, and the same gene is upregulated in the basal section relative to the top section of both cultivars (Table 2). The basal section is
### Table 1 Total number of changed stem transcripts in three major functional categories of B. napus DH12075 (DH) and YN01–429 (YN)

| Cellular Component                      | DH4:YN4 | DH1:YN1 | DH1:DH4 | YN1:YN4 |
|-----------------------------------------|---------|---------|---------|---------|
|                                        | Up      | Down    | Up      | Down    | Up      | Down    | Up      | Down    |
| Other intracellular components          | 754     | 693     | 668     | 580     | 475     | 396     | 377     | 205     |
| Chloroplast                            | 602     | 567     | 581     | 462     | 413     | 320     | 308     | 159     |
| Other cytoplasmic components           | 636     | 469     | 531     | 403     | 361     | 321     | 280     | 161     |
| Other membranes                        | 531     | 482     | 466     | 483     | 374     | 290     | 284     | 156     |
| Unknown cellular components            | 607     | 607     | 513     | 373     | 463     | 352     | 347     | 187     |
| Plastid                                | 291     | 343     | 260     | 303     | 174     | 151     | 246     | 122     |
| Plasma membrane                        | 438     | 381     | 421     | 326     | 262     | 240     | 133     | 79      |
| Nucleus                                | 392     | 227     | 331     | 200     | 247     | 203     | 181     | 107     |
| Other cellular components              | 284     | 285     | 184     | 153     | 225     | 162     | 163     | 103     |
| Mitochondria                           | 198     | 191     | 210     | 188     | 125     | 94      | 97      | 55      |
| Cell wall                              | 127     | 123     | 115     | 88      | 75      | 66      | 64      | 40      |
| Cytosol                                | 121     | 102     | 106     | 80      | 66      | 66      | 56      | 31      |
| Extracellular                          | 110     | 82      | 82      | 62      | 66      | 62      | 44      | 33      |
| ER                                     | 90      | 76      | 81      | 60      | 60      | 48      | 44      | 33      |
| Golgi apparatus                        | 49      | 43      | 40      | 37      | 31      | 26      | 18      | 12      |
| Ribosome                               | 23      | 23      | 23      | 18      | 16      | 13      | 10      | 8       |
| Molecular Function                      |         |         |         |         |         |         |         |         |
| Other enzyme activity                  | 515     | 695     | 439     | 383     | 492     | 295     | 383     | 216     |
| Unknown molecular functions            | 650     | 471     | 578     | 515     | 341     | 376     | 259     | 156     |
| Other binding                          | 525     | 469     | 455     | 393     | 327     | 274     | 248     | 150     |
| Transferase activity                   | 295     | 308     | 243     | 229     | 211     | 186     | 188     | 100     |
| Hydrolase activity                     | 341     | 328     | 275     | 299     | 240     | 199     | 151     | 98      |
| Protein binding                        | 351     | 260     | 306     | 230     | 223     | 199     | 176     | 115     |
| Nucleotide binding                     | 279     | 132     | 231     | 217     | 131     | 96      | 139     | 53      |
| Kinase activity                        | 137     | 245     | 114     | 110     | 175     | 143     | 85      | 88      |
| DNA or RNA binding                     | 257     | 182     | 160     | 183     | 83      | 125     | 121     | 73      |
| Transporter activity                   | 164     | 201     | 197     | 131     | 153     | 93      | 70      | 56      |
| Other molecular functions              | 185     | 157     | 143     | 110     | 122     | 93      | 103     | 45      |
| Transcription factor activity          | 165     | 149     | 130     | 115     | 117     | 93      | 79      | 47      |
| Nucleic acid binding                   | 119     | 111     | 98      | 102     | 76      | 64      | 61      | 28      |
| Structural molecule activity           | 35      | 22      | 28      | 25      | 26      | 16      | 20      | 8       |
| Receptor binding or activity           | 15      | 18      | 17      | 12      | 16      | 10      | 9       | 5       |
| Biological Process                     |         |         |         |         |         |         |         |         |
| Other cellular processes               | 1254    | 1145    | 1066    | 943     | 803     | 710     | 641     | 398     |
| Other metabolic processes              | 1139    | 1016    | 974     | 860     | 754     | 664     | 597     | 364     |
| Unknown biological processes           | 763     | 760     | 637     | 575     | 583     | 418     | 444     | 233     |
| Response to abiotic or biotic stimulus | 387     | 373     | 350     | 279     | 221     | 197     | 200     | 115     |
| Response to stress                     | 381     | 342     | 337     | 263     | 206     | 188     | 181     | 115     |
| Other biological processes             | 353     | 334     | 325     | 269     | 215     | 197     | 183     | 105     |
| Protein metabolism                     | 375     | 359     | 333     | 317     | 247     | 229     | 130     | 127     |
| Developmental processes                | 251     | 261     | 222     | 181     | 197     | 158     | 189     | 83      |
more mature and richer in lignin content relative to the top section (Fig. 1a), suggesting the involvement of this gene with lignin accumulation in Brassica. The ferulate-5-hydroxylase (F5H) enzyme is a key regulatory point in the determination of lignin monomer composition [39] by catalyzing the conversion of ferulic acid to sinapic acid and syringyl lignin.

**Table 1** Total number of changed stem transcripts in three major functional categories of *B. napus* DH12075 (DH) and YN01–429 (YN) (Continued)

|                        | DH4:YN4 Up | DH4:YN4 Down | DH1:YN1 Up | DH1:YN1 Down | DH1:DH4 Up | DH1:DH4 Down | YN1:YN4 Up | YN1:YN4 Down |
|------------------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|
| Transport              | 266        | 232          | 232        | 193          | 165        | 124          | 139        | 85           |
| Cell organization and biogenesis | 171        | 154          | 150        | 137          | 127        | 110          | 97         | 56           |
| Transcription          | 159        | 146          | 130        | 119          | 104        | 88           | 73         | 42           |
| Electron transport or energy pathways | 81         | 74           | 61         | 54           | 51         | 31           | 45         | 17           |
| Signal transduction    | 75         | 52           | 66         | 46           | 39         | 38           | 37         | 22           |
| DNA or RNA metabolism  | 35         | 27           | 24         | 21           | 25         | 20           | 18         | 14           |

NB data was ranked in descending order using the DH4:YN4 pair wise comparison. Up, upregulated. Down, down regulated

The importance of cellulose and its integration with lignin and pectin in plant cell walls led us to determine transcript levels for genes coding for cellulose biosynthetic enzymes in the microarray experiment. Genes involved in cellulose biosynthesis in Arabidopsis were downloaded from www.arabidopsis.org and were compared with our microarray data to identify genes that were upregulated by 2-fold or more in high lignin-containing material (Table 5, Additional file 14: Table S8). Comparisons showed that several genes encoding enzymes involved in cellulose biosynthesis were upregulated in the basal section of the cultivars. **CELLULOSE SYNTHASE A1** (*BnCESA1;* BN12852) was strongly upregulated in the basal section of DH relative to its counterpart in YN (Table 5). **CESA2** (BN17389) was also upregulated in the basal section of both cultivars relative to the top sections, while **CESA6** was upregulated in the top and basal sections of DH relative to the top and basal sections of YN in the array experiments. **DEFECTIVE GLYCOSYLATION (DGL1)** (BN19297) was upregulated in the top and basal sections of DH relative to the top and basal sections of YN (Table 5). **DGL1** encodes a plant ortholog of human SOT48 or yeast WBP1 and is an essential protein subunit of the oligosaccharyltransferase (OST) complex.

Other genes involved in carbohydrate synthesis were also upregulated in DH stems (Table 5). One of these, **RADIAL SWELLING 10** (**RSW10;** BN17967), was upregulated in the top section of DH relative to the top section of YN (Table 5). RSW 10 encodes a ribose 5-phosphate isomerase involved in the formation of uridine used for the synthesis of UDP-sugars.

Plant primary carbohydrate metabolism is complex, yet flexible, and is regulated at many levels. To gain molecular insights into the carbohydrate metabolic process in *B. napus* stem, we retrieved genes involved in cellular carbohydrate metabolic process from www.arabidopsis.org. A total of 419 loci were retrieved and compared to identify upregulated genes in the arrays. In total, 85 genes were found to be upregulated of which 52, 33, 35 and 29 belonged to DH4:YN4, DH1:YN1, DH4:DH1 and YN4:YN1, respectively (Additional file 14: Table S8).
Targets for transcriptional regulation of cell wall formation

Transcriptional regulation plays a key role in the complex series of events leading to cell wall formation [41]. To identify transcription factors (TFs) that were upregulated in the array, a search of our data was performed using 10 major transcription factors from the *A. thaliana* Transcription Factor Database [42]. The search revealed that a wide range of TFs were upregulated and downregulated in DH vs. YN stems. Several gene families, zinc finger (C2H2 and C3HC4), basic/helix-loop-helix (bHLH), basic region/leucine zipper motif (bZIP), AP2/EREBP and NAC (NAM/ATAF1/2/CUC2), were over-represented among the differentiated TFs (Table 6; Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: Tables S1A and B to S6A and B).

The largest group of transcription factors with differential expression (25 gene loci; 13 upregulated and 12 downregulated) consisted of Cys2His2-like (C2H2) zinc finger genes. C2H2 zinc fingers are well known and display a wide range of functions, from DNA and RNA binding to the involvement in protein-protein interactions. In our array data, the numbers of differentially expressed members (> 2-fold) for this family in DH4:YN4, DH1:YN1, DH1:DH4 and YN1:YN4 were 38, 25, 11 and 11, respectively (Table 6; Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: Tables S1A and B to S6A and B). BN21811 related to *At1g14580*, a member of this family, was over-expressed in all 4 groups whereas the other 3 family members were upregulated in 3 groups. Another gene, BN15654 related to *At5g04240*, was upregulated in the basal section of DH relative to the basal section of YN. This latter gene is a member of the jumonji group of C2H2 transcription factors (Table 6; Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: Tables S1A and B to S6A and B).

C3HC4 zinc finger genes formed the next largest gene category (Table 6). One of these genes BN22477 (related to *At5g05830*) was strongly upregulated in three of the four tissue comparisons and another in two comparisons. In contrast, BN17974 (related to *At3g09770*) was strongly downregulated in the DH4:YN4 and DH4:DH1 comparisons. B helix loop helix transcription factors were the third most differentially expressed genes in DH1:YN1, DH1:DH4 and YN1:YN4, but to a lesser extent than in DH4:YN4 (Table 6). In more detail, BN155636 related to *At2g18300* (*AtbHLH64*) and BN12702 related to *At1g05805* (*AtbHLH128*) were over-expressed in three groups of our array (Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: Tables S1A and B to S6A and B). *AtbHLH64* is known to play a role in Arabidopsis cytokinin signaling [43]. Though the role of bHLH TFs in lignin regulation is correlative rather than firmly established, we cannot rule out their possible involvement considering the common precursors of the lignin and flavonoid pathways and the known influence of bHLH on the flavonoid pathway genes, as well as the known interaction between bHLHs and MYB TFs in other physiological and developmental systems such as Brassica trichome development [44].

Plant basic region/leucine zipper motif (bZIP) transcription factors regulate processes including pathogen defense, light and stress signaling, seed maturation and flower development in Arabidopsis [45]. The numbers of
bZIP family members differentially expressed more than 2-fold in DH4-YN4, DH1-YN1, DH1-DH4 and YN1-YN4 were 24, 11, 11 and 11, respectively in the array data (Table 6). BN15088 related to At2g18160 (ATB-ZIP2) were upregulated in 3 groups.

AP2 (APETALA2) and EREBPs (ethylene responsive element binding proteins) are a family of transcription factors unique to plants and share a highly conserved region of about 60 to 70 amino acids (the so-called AP2 domain) with no apparent similarity outside this domain. The numbers of differentially expressed members (> 2-fold) for this family in DH4-YN4, DH1-YN1, DH1-DH4 and YN1-YN4 were 16, 10, 7 and 2, respectively, in the array data (Table 6). In detail, BN16694 related to At1g68550 (ATERF118), was upregulated in 3 groups in our microarray experiments. Another of these genes, BN16341 related to At5g67190 (DREB2) was upregulated in the basal section relative to the top section of DH stems. In addition to the six classes of over-represented transcription factors, MYB factors were more than 2-fold upregulated in our microarray comparisons of lignified and less lignified tissues. MYB factors represent a family of proteins with a conserved R2R3 (stimulating) or R3 (inhibiting) MYB DNA-binding domain. They often bind to bHLHs. In our array data, the numbers of over-expressed members of this family in DH4-YN4, DH1-YN1, DH1-DH4 and YN1-YN4 were 16, 10, 7 and 5, respectively (Table 6). One member of this family, BN13387 related to At1g61110, was over-expressed in all 4 groups of our array and was previously reported to be expressed in stamens [47]. Other NAC transcription factors were only upregulated in 3 groups (Table 6). BN24136 was highly upregulated and is related to At3g49530 (ANAC062), a gene that plays an integrative role in plant responses to stress [48].

Table 2 Differential upregulation of monolignol biosynthetic genes ≥ two-fold in stem sections of B. napus DH12075 (DH) and YN01–429 (YN)

| Probe name | Upregulated expression | Corresponding Arabidopsis Loci | Description |
|------------|------------------------|--------------------------------|-------------|
| BN18476    | 2.04 2.13              | AT1G09500                      | Similar to Eucalyptus gunnii alcohol dehydrogenase |
| BN10302    | 3.64 4.25              | AT1G12840                      | De-etiolated 3 (DET3) |
| BN21410 BN21411 | 6.45 2.18 2.25 | AT1G15950                      | Cinnamoyl CoA reductase (CCR1) |
| BN26995    | 2.27                   | AT1G33030                      | O-methyltransferase family 2 protein |
| BN17817    | 2.54                   | AT1G67980                      | Caffeoyl-CoA 3-O-methyltransferase |
| BN24319 BN24320  | 2.43                  | AT1G77520                      | Cinnamyl-CoA reductase family |
| BN23842    | 2.5                    | AT1G80820                      | Cinnamyl-CoA reductase (CCR2) |
| BN25563    | 2.14 2.19              | AT2G02400                      | Cinnamyl-CoA reductase family |
| BN15586    | 8.73 2.64              | AT2G40890                      | Caffeoyl-CoA reductase family |
| BN26731    | 2.99                   | AT2G33600                      | S-Adenosylmethionine synthetase 3 (SAM3) |
| BN14781 BN14782 BN14783  | 8.79 4.72 2.9  | AT3G17390                      | S-Adenosylmethionine synthetase 3 (SAM3) |
| BN22074    | 4.24                   | AT3G19450                      | Cinnamyl-Alcohol Dehydrogenase (CAD4) |
| BN18553 BN18554  | 3.09 3.51 2.48  | AT4G36220                      | Ferulic acid 5-hydroxylase 1 (FAH1) |
| BN13060    | 2.18                   | AT5G14700                      | Cinnamyl-CoA reductase-related |
| BN19086 BN19087  | 3.0 3.26              | AT5G49830                      | Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) |
| BN13706    | 5.22 5.01              | AT5GS4160                      | Caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT1) |
| BN20498    | 2.49                   | AT5GS8490                      | Cinnamyl-CoA reductase family |

NAC proteins constitute one of the largest families of plant-specific transcription factors, and are expressed in various developmental stages and tissues [46]. The NAC domain was originally characterized from consensus sequences from petunia NAM and from Arabidopsis ATAF1, ATAF2 and CUC2. In our array data, the number of differentially expressed members (> 2-fold) for this family in DH4-YN4, DH1-YN1, DH1-DH4 and YN1-YN4 were 16, 10, 7 and 5, respectively (Table 6). One member of this family, BN13387 related to At1g61110, was over-expressed in all 4 groups of our array and was previously reported to be expressed in stamens [47]. Other NAC transcription factors were only upregulated in 3 groups (Table 6). BN24136 was highly upregulated and is related to At3g49530 (ANAC062), a gene that plays an integrative role in plant responses to stress [48].
(AtMYB34) was expressed in all 4 groups of the array data while 2 other members of this family were upregulated in 3 groups (Table 6). BN26939 related to At2g47460 (AtMYB12) was also strongly upregulated in the array data.

In order to highlight the promising genes for future functional characterization from these elaborated sets, 25 TFs with representation from families mentioned above, plus six biochemical or physiological genes, were selected and their Arabidopsis knockdown mutants retrieved from TAIR (Additional files 14, 15, 16 and 17: Tables S8 to S11). In our analysis on a subsample of these lines, 4 TF lines and 4 non-TF lines showed lower lignin content than the Col-0 control line, while 2 TF lines and 3 non-TF lines showed enhanced lignin content (Additional files 16 and 17: Tables S10 and 11).

### Discussion

The yellow seeded *B. napus* YN01–429 cultivar was recently developed through classical breeding, and was found to contain significantly reduced seed lignin content relative to seeds of the more conventional brown seeded cultivar DH12075 [20, 21]. Here, we determined that the stems of the brown-seeded cultivar were more lignified than those of the yellow-seeded cultivar and that basal stem sections were more lignified than apical stem sections for both cultivars.

### Biochemical genes

Biochemical genes specifying monolignol formation, such as *COMT* [10], *CcAOtM1* [49], *SAMS* [50], *CAD* [51], *REF8 (C3H)* [52] and *F5H (FAHS)* [53], were more highly expressed in the more lignified stem tissues we examined. Upregulation in more lignified tissue also

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**Table 3** Differential upregulation of glucosyltransferase genes ≥ two-fold in stem sections of *B. napus* DH12075 (DH) and YN01–429 (YN)

| Probe name | DH4:YN4 | DH1:YN1 | DH4:DH1 | YN4:YN1 | Corresponding Arabidopsis loci | Description |
|------------|---------|---------|---------|---------|-------------------------------|-------------|
| BN18392    |         |         | 2.44    |         | AT1G05530 UDP-GLUCOSYL TRANSFERASE 75B2 |
| BN25565    |         | 2.12    |         |         | AT1G07250 UDP-GLUCOSYL TRANSFERASE 71C4 |
| BN19757    | 2.83    |         |         |         | AT1G23870 Arabidopsis thaliana TREHALOSE-PHOSPHATASE/SYNTHASE 9 |
| BN18181    |         |         | 2.28    |         | AT1G55850 CELLULOSE SYNTHASE LIKE E1 |
| BN20190    | 5.55    | 2.19    | 2.09    |         | AT1G70290 TREHALOSE–6-PHOSPHATASE SYNTHASE S8 |
| BN17018    | 3.95    |         |         |         | AT2G31750 UDP-GLUCOSYL TRANSFERASE 74D1 |
| BN11938    | 2.24    |         |         |         | AT2G31960 GLUCAN SYNTHASE-LIKE 3 |
| BN13391    | 2.12    | 2.39    |         |         | AT2G36850 GLUCAN SYNTHASE-LIKE 8 |
| BN18400    | 2.05    |         |         |         | AT2G43820 UDP-GLUCOSYLTRANSFERASE 74F2 |
| BN19396    | 2.01    |         |         |         | AT3G03050 CELLULOSE SYNTHASE LIKE D3 |
| BN25630    | 2.35    |         |         |         | AT3G0740 UDP-GLUCOSYL TRANSFERASE 72E1 |
| BN16340    | 2.26    |         |         |         | AT3G55830 ECTOPICALLY PARTING CELLS |
| BN19754    |         |         | 12.95   |         | AT4G01070 UDP-GLUCOSE-DEPENDENT GLUCOSYLTRANSFERASE 72 B1 |
| BN18305    | 2.23    |         |         |         | AT4G02280 SUCROSE SYNTHASE 3 |
| BN17034    | 2.09    |         |         |         | AT4G03550 GLUCAN SYNTHASE-LIKE 5 |
| BN16416    | 2.33    |         |         |         | AT4G10120 ATSP54F |
| BN26358    | 2.56    |         |         |         | AT4G15490 UGT84A3 |
| BN25190    | 2.69    |         |         |         | AT4G17770 TREHALOSE–6-PHOSPHATASE SYNTHASE S5 |
| BN20567    | 3.81    | 2.87    |         |         | At4g31590 CELLULOSE-SYNTHASE LIKE C5 |
| BN12852    | 7.67    | 2.04    |         |         | At4g32410 CELLULOSE SYNTHASE 1 |
| BN22038    | 4.45    | 2.19    |         |         | AT4G34135 UDP-GLUCOSYLTRANSFERASE 73B2 |
| BN23469    | 3.48    | 2.43    |         |         | AT4G39350 CELLULOSE SYNTHASE A2 |
| BN20784    | 2.13    |         |         |         | AT5G05170 CELLULOSE SYNTHASE 3 |
| BN26739    | 2.21    |         |         |         | AT5G05870 UDP-GLUCOSYL TRANSFERASE 76C1 |
| BN12647    | 4.39    | 2.08    |         |         | AT5G20830 SUCROSE SYNTHASE 1 |
| BN17387    | 2.38    | 2.32    |         |         | AT5G64740 CELLULOSE SYNTHASE 6 |
occurred for genes specifying monolignol glucosylation, and monolignol polymerization, including peroxidases and laccases, when pairs of tissue sections were compared. Cellulose biosynthesis, the entire cell wall and plant development appeared to be affected by some of these differentially expressed Brassica genes. For example, we found that Brassica CES genes \[54, 55\] and peroxidases \[56\] were also more upregulated in more lignified tissues. Passardi et al. \[56\] showed that two highly homologous Arabidopsis peroxidases, AtPpx33 and Atprx34, were involved in cell elongation, and the knockout mutant, cesa2, also had severe defects in cell wall formation and microtubule orientation \[57\]. However, the numbers of cellulose biosynthetic genes upregulated in DH were fewer than lignin biosynthetic genes. Hence, our comparative transcriptome analyses of lignified and less lignified material could serve as a source of differentially expressed biochemical genes that impact

| Probe name | DH4:YN4 | DH1:YN1 | DH4:DH1 | YN4:YN1 | Corresponding Arabidopsis loci | Description |
|------------|---------|---------|---------|---------|-------------------------------|-------------|
| Peroxidase genes |         |         |         |         | AT1G05260 | RARE COLD INDUCIBLE GENE 3 |
| BN24392    | 2.23    |         |         |         | AT1G09500 | similar to Eucalyptus gunnii alcohol dehydrogenase |
| BN18476    | 2.04    |         |         |         | AT1G12840 | ARABIDOPSIS THALIANA VACUOLAR ATP SYNTHASE SUBUNIT C |
| BN10302    | 4.25    |         |         |         | AT1G15950 | CINNAMOYL COA REDUCTASE 1 |
| BN21410    | 3.73    |         |         |         | AT1G20620 | CATALASE 3 |
| BN14306    | 2.16    | 2.54    |         |         | AT1G20630 | CATALASE 1 |
| BN18084    | 6.60    | 2.27    |         |         | AT1G271695 | Peroxidase 12 |
| BN16003    | 2.56    |         |         |         | AT1G44970 | Peroxidase, putative |
| BN27576    | 2.14    |         |         |         | AT1G48130 | ARABIDOPSIS THALIANA 1-CYSTEINE PEROXIREDOXIN 1 |
| BN11140    |         | 2.02    |         |         | AT1G71695 | Peroxidase 12 |
| BN23842    | 2.50    |         |         |         | AT1G80280 | CINNAMOYL COA REDUCTASE |
| BN25563    | 2.41    |         |         |         | AT2G02400 | Cinnamoy-CoA reductase family |
| BN13322    | 2.48    |         |         |         | AT2G25080 | GLUTATHIONE PEROXIDASE 1 |
| BN11700    | 2.0     |         |         |         | AT2G30860 | GLUTATHIONE S-TRANSFERASE PHI 9 |
| BN19562    | 3.68    |         |         |         | AT2G33830 | Peroxidase 22 (Per22) |
| BN15586    | 3.54    |         |         |         | AT2G40890 | CYP98A3, encodes coumarate 3-hydroxylase (C3H) |
| BN24228    | 2.77    |         |         |         | AT3G03670 | Peroxidase, putative |
| BN13555    | 2.09    |         |         |         | AT3G11630 | Encodes a 2-Cys peroxiredoxin (2-Cys PrxA) |
| BN14781    | 4.72    |         |         |         | AT3G17390 | S-ADENOSYL METHIONINE SYNTHETASE 3 |
| BN23842    | 2.50    |         |         |         | AT3G49110 | PEROXIDASE 33 |
| BN16513    | 4.57    | 2.62    | 2.02    |         | AT4G21960 | PEROXIDASE PRXR1 |
| BN24317    |         | 2.03    |         |         | AT4G26010 | Peroxidase, putative |
| BN23955    | 2.67    |         |         |         | AT4G30170 | Peroxidase, putative |
| BN27325    | 4.35    | 2.17    | 2.17    |         | AT4G35000 | ASCORBATE PEROXIDASE 3 |
| BN13535    | 2.63    |         |         |         | AT4G36430 | Peroxidase, putative |
| BN22581    | 3.69    |         |         |         | AT4G37530 | Peroxidase, putative |
| BN14948    | 3.30    |         |         |         | AT5G06290 | 2-CYSTEINE PEROXIREDOXIN B |
| BN19087    | 2.29    |         |         |         | AT5G48930 | HYDROXYCINNAMOYL-COA SHIKIMICATE/QUINATE HYDROXYCINNAMOYL TRANSFERASE |
| BN13706    | 5.01    |         |         |         | AT5G54160 | O-METHYLTRANSFERASE 1 |
| Laccase genes |         |         |         |         | AT2G38080 | LACCASE-LIKE MULTICOPPER OXIDASE 4 |
| BN23268    | 2.17    |         |         |         | AT5G66020 | LACCASE 17 |
on cell wall recalcitrance and could be useful to enable plant molecular breeders to fine tune crop residue composition for industrial applications.

**Targets for transcription regulation**

Microarray analysis of differentially lignified tissues resulted in six transcription factor families being more prominent in expression changes than other major TFs. Transcription factors have recently received attention from plant molecular breeders because of their ability to modify entire biochemical pathways or developmental families of genes. For example, overexpression of the Arabidopsis GLABRA3 (AtGL3) bHLH transcription factor in B. napus enhanced trichome coverage and insect resistance [44, 58].

Here, we screened our microarray data for the top 10 TF families present in lignified B. napus stems with the intent of beginning the process of characterizing the function of a subset of regulatory genes using Arabidopsis knockdown mutants. The most modified TF family in our stem microarrays was the zinc finger family. Several zinc finger proteins (ZFPs) in plants, e.g. Arabidopsis and petunia, have already been found to be involved in a variety of processes such as the regulation of floral organogenesis, leaf initiation, lateral shoot initiation, gametogenesis and stress response [59]. The C2H2 group was the most highly upregulated set of zinc finger families in our analyses. This latter group is known to be involved in the regulation of Arabidopsis flowering time [60]. Prigge and Wagner [61] reported that SERRATE (At2g27100), another member of the C2H2 family, plays a role in embryogenesis and is transcribed in shoot meristem and in emerging organ primordia throughout development. Dong and co-workers [62] also showed that SERRATE is required for the accurate processing of microRNA (miRNA) precursors in the plant cell nucleus. Moreover, some zinc finger genes highly expressed in trichomes have been reported to play a role in cell wall biosynthesis [63]. Thus, selections from among these differentially expressed zinc finger genes could target B. napus regulatory genes that can modify entire developmental programs.

BHLH TFs are one of the largest TF families in Arabidopsis. Phylogenetic analysis, divergence in binding site specificity and their varied ability to engage in homodimerization and heterodimerization events strongly support their roles in a multiplicity of transcriptional programs [64, 65]. BHLH TFs are known to play important roles in the flavonoid branch of the phenylpropanoid pathway; hence it makes sense that we would find them so plentiful in the closely related lignified tissue. In Arabidopsis seeds, the MYB transcription factor TRANSPARENT TESTA 2 (TT2) forms a complex together with the bHLH transcription

| Table 5 | Differential upregulation of carbohydrate biosynthetic genes by ≥2-fold in stem sections of B. napus DH12075 (DH) and YN01–429 (YN) |
|---------|--------------------------------------------------------------------------------------------------|

| Probe name | Upregulated expression | Corresponding Arabidopsis Loci | Description |
|------------|------------------------|--------------------------------|-------------|
| BN18181    | 2.28                   | AT1G55850                       | CELLULOSE SYNTHASE LIKE E1 |
| BN17967    | 3.43                   | AT1G71100                       | RADIAL SWELLING 10 (RWS10) |
| BN12852    | 7.67                   | AT4G32410                       | CELLULOSE SYNTHASE 1 (CESA1) |
| BN23469    | 3.48 2.43              | AT4G39350                       | CELLULOSE SYNTHASE 2 (CESA2) |
| BN20787    | 2.44                   | AT5g05170                       | Constitutive Expression of VSP1 (CEV1) |
| BN18701    | 6.43                   | AT5G19220                       | ADP GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1 (APL1) |
| BN19160    | 2.66 4.97 2.21         | AT5G49720                       | ARABIDOPSIS THALIANA GLYCOSYL HYDROLASE 9A1 (ATGH9A1) |
| BN17389    | 3.1 2.22               | AT5G64740                       | CELLULOSE SYNTHASE 6 (CESA6) |
| BN19297    | 8.49 5.27              | AT3G66680                       | DEFECTIVE GLYCOSYLATION (DGL1) |

| Table 6 | Ten major transcription factors differentially expressed 2-fold or more in stem sections of B. napus DH12075 (DH) and YN01–429 (YN) |
|---------|--------------------------------------------------------------------------------------------------|

| Transcription factor | Sample groups in microarray | Up | Down | Up | Down | Up | Down | Up | Down |
|----------------------|-----------------------------|----|------|----|------|----|------|----|------|
| C2H2                 | 16 22 14 14 7 6 5           |    |      |    |      |    |      |    |      |
| C3HC4                | 13 15 12 9 3 4 2 2         |    |      |    |      |    |      |    |      |
| bHLH                 | 13 11 11 7 7 3 3 1         |    |      |    |      |    |      |    |      |
| bZIP                 | 9 15 4 7 6 5 4             |    |      |    |      |    |      |    |      |
| NAC                  | 8 7 8 4 6 2 4 1           |    |      |    |      |    |      |    |      |
| AP2-EREBP            | 8 8 4 6 4 3 1 1           |    |      |    |      |    |      |    |      |
| Homeobox             | 6 8 6 3 4 3 3 4           |    |      |    |      |    |      |    |      |
| MYB                  | 4 2 3 0 4 4 5 2           |    |      |    |      |    |      |    |      |
| MADS                 | 4 4 1 3 3 3 3 3           |    |      |    |      |    |      |    |      |
| WRKY                 | 4 6 2 5 2 3 2 1           |    |      |    |      |    |      |    |      |
factor TT8 and a WD40 scaffold protein to control the expression of BANYULS, a proanthocyanidin biosynthetic gene [66]. In a leaf study, TT8 and its closest homolog GL3 and ENHANCED GLABRA (EGL3) regulate flavonoid biosynthesis through interaction with two homologous MYB proteins PRODUCTION OF ANTHOCYANIN PIGMENTS 1 (PAPI, MYB75) and PAP2 (MYB90) [67]. In our stem microarrays, several bHLH genes were also differentially expressed along with a number of MYB genes. These genes should be paired and tested to determine whether any are binding partners and whether their modification affects lignin in transgenic plants or mutants. Already, the alfalfa bHLH TT8 homologue has been shown in RNAi studies to downregulate carbohydrate and dry matter accumulation, but not lignin, in alfalfa forage [68].

AP2/EREBP genes represent the fifth most differentially expressed genes in the B. napus stem microarrays. These genes play a variety of roles throughout the plant life cycle; from being key regulators of several developmental processes, like floral organ identity determination or control of leaf epidermal cell identity, to forming part of the mechanisms used by plants to respond to various types of biotic and environmental stresses [69]. NAC genes ranked sixth among our most differentially expressed TFs in the stem microarrays. Mitsuda et al. [70] showed that NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST3 are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. Lu et al. [71] showed that ATAFl mRNA expression is strongly induced by dehydration and abscisic acid (ABA) treatment and they suggested a general role of this protein as a repressor in drought stress responses. Wu et al. [72] also implicated ATAFl in diverse biotic and abiotic stress responses including drought, high-salinity, ABA, methyl jasmonate, mechanical wounding and Botrytis cinerea infection. Considering the expression pattern of NAC transcription factors in the array data and their role in lignin biosynthesis as well as in diverse biotic and abiotic stress responses, this family is a promising target for lignin modulation of the plant cell wall.

Although not among the most differentially expressed B. napus stem TFs, MYB-related transcription factors are an important group since they regulate different branches of secondary metabolism, in addition to the identity and fate of plant cells [73]. Studies on MYB transcription factors in Arabidopsis, pine and eucalyptus confirmed regulatory roles that MYB TFs play in lignin biosynthesis [14, 15, 74, 75]. MYB58 and MYB63 are transcriptional regulators specifically activating lignin biosynthetic genes during secondary wall formation in Arabidopsis [75]. Dominant repression of their functions led to a reduction in secondary wall thickening and lignin content, while overexpression of MYB58 and MYB63 resulted in specific activation of lignin biosynthetic genes and concomitant ectopic deposition of lignin in cells that are normally un lignified [75]. Newman and co-workers [13] suggested a role for another Arabidopsis MYB gene, AtMYB61, in cell wall deposition in normal vascular development [76]. However, transcription of these three MYB genes was unaltered in our array experiments. Instead, MYB12 and MYB34 were differentially regulated in the arrays. MYB34 plays a key role in the regulation of indole glucosinolate homeostasis in Arabidopsis [77], and MYB12 acts as a flavonol-specific activator of flavonoid biosynthesis [78]. Luo et al. [79] showed that MYB12 can also activate the caffeoyl quinic acid (CQA) biosynthetic pathway when expressed in a tissue-specific manner in tomato. The role of these two MYB TFs in secondary metabolism pathways and the correlation of their expression pattern with stem lignification establish this family as an important target in the modulation of lignin biosynthesis.

Analysis of Arabidopsis mutants for lignin, eight mutant lines showed lower lignin content than the Col-0 control line, while five showed enhanced lignin content. These verified lignin genes can now be used to modify Brassica lignin using our microarray oligonucleotides. Moreover, the Brassica and Arabidopsis sequences can be used to find homologues in other species. In fact, DIM1 and eEF-1β1 have already been shown to modify lignin and carbohydrate in Arabidopsis [31, 32] and TT8 and HBl2 have been shown to modify carbohydrate structure in alfalfa [34, 68]. In addition, several other genes (HBS, DIM1, ZFP2, eEF-1β1, ANAC62) have also been used to modify alfalfa (Amyot, personal communication).

Conclusions

Most of the structural genes in the lignin biosynthetic pathway in plants have been characterized; however, more investigation is needed in the areas of understanding transcriptional regulation and unraveling the mechanism of monolignol polymerization. Here, we examined the expression pattern of genes in stem sections of two Brassica cultivars with differential lignin content. In doing so, we identified and established expression pattern of many new genes likely involved in cell wall biosynthesis and regulation. This transcript profiling allowed for the identification of novel gene candidates with strong potential at playing key roles in cell wall construction during stem development. The genes represent valuable tools to make cell walls more amenable to hydrolysis. More important, we were able to identify the 10 most important transcription factor families and listed 25 promising TF genes for future characterization. Elucidation of
their precise mechanisms of transcriptional activation or repression of the cell wall biosynthetic process will not only shed light on the effects of these transcriptional regulators in several plant species, but may also make it possible for the molecular breeding community to modify entire biosynthetic pathways of cellulose or lignin by altering one or a few transcription factors.

Additional files

Additional file 1: Table S1A. Differential expression of DH1 vs DH4 less than point 5 (microarray). (XLSX 360 kb)
Additional file 2: Table S1B. Differential expression of DH1 vs DH4 greater than 2 (microarray). (XLSX 442 kb)
Additional file 3: Table S2A. Differential expression of DH1 vs YN1 less than point 5 (microarray). (XLSX 496 kb)
Additional file 4: Table S2B. Differential expression of DH1 vs YN1 greater than 2 (microarray). (XLSX 565 kb)
Additional file 5: Table S3A. Differential expression of YN1 vs YN4 less than point 5 (microarray). (XLSX 226 kb)
Additional file 6: Table S3B. Differential expression of YN1 vs YN4 greater than 2 (microarray). (XLSX 417 kb)
Additional file 7: Table S4A. Differential expression of DH4 vs YN4 less than point 5 (microarray). (XLSX 728 kb)
Additional file 8: Table S4B. Differential expression of DH4 vs YN4 greater than 2 (microarray). (XLSX 718 kb)
Additional file 9: Table S5A. Differential expression of DH2 vs YN2 less than point 5 (microarray). (XLSX 567 kb)
Additional file 10: Table S5B. Differential expression of DH2 vs YN2 greater than 2 (microarray). (XLSX 585 kb)
Additional file 11: Table S6A. Differential expression of DH3 vs YN3 less than point 5 (microarray). (XLSX 512 kb)
Additional file 12: Table S6B. Differential expression of DH3 vs YN3 greater than 2 (microarray). (XLSX 556 kb)
Additional file 13: Table S7. qRT-Primers used in this study. (DOC 47 kb)
Additional file 14: Table S8. Differential upregulation of genes involved in cellular carbohydrate metabolic processing. (DOC 143 kb)
Additional file 15: Table S9. Ten categories of TFs with BN orgonucleotides retrieved from stem section comparisons of B. napus DH12075 (DH) and YN01~429 (YN). (XLSX 100 kb)
Additional file 16: Table S10. Targeted transcription factors selected for lignin validation using Arabidopsis mutants. (DOCX 25 kb)
Additional file 17: Table S11. Selected non-TF genes for lignin validation using Arabidopsis mutants. (DOCX 17 kb)

Abbreviations

AHA: Abscisic acid; ADP-Glc PPase: ADP-glucose pyrophosphorylase; AP2: APETALA2; BHLH: Basic/ helix-loop-helix; bZIP: Basic region/leucine zipper; C3H: p-coumarate 3-hydroxylase; CAD: Cinnamyl alcohol dehydrogenase; CCoAOMT: Caffeoyl-coenzyme A O-methyltransferase; CCR: Cinnamoyl-CoA reductase; cDNA: Complementary DNA; CESAs: CELLULOSE SYNTHASE; DET3: DE-ETIOLATED 3; DGL: DEFECTIVE GLYCOSYLATION; DMIN1: DIMINO1; DNA: Deoxynucleobasic acid; EQL: ENHANCED GLABRA; ERF: Ethylene responsive element binding protein; F5H: Ferulate-5-hydroxylase; GO: Gene ontology; miRNA: microRNA; NST: NAC SECONDARY WALL THICKENING PROMOTING FACTOR; OMT1: Caffeic acid/5-hydroxyferulic acid O-methyltransferase; OST: Oligosaccharinyltransferase; PAP: PRODUCTION OF ANTHOCYANIN PIGMENTS; qRT-PCR: Quantitative real time reverse transcription PCR; REF: REDUCED EPIDERMAL FLUORESCENCE; RNA: Ribonucleic acid; RSW: RADIAL SWELLING; SAMS: S-adenosyl methionine synthase; TAIR: The Arabidopsis Information Resource; TF: Transcription factor; TGA: Thiglycolic acid; TT: TRANSPARENT TESTA; ZFP: Zinc finger protein

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Availability of data and materials

The data sets supporting the results of this article are included within the article and as Additional files.

Authors’ contributions

ZH, BVSP, MY, and LA conducted experiments, analyzed data and drafted the manuscript. AH and MYG planned the project, secured funding, analyzed data, and edited the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Plant materials such as Brassica napus and alfalfa germplasm/cultivars were obtained from an on-going research program within Agriculture and Agri-Food Canada. Arabidopsis ecotypes were procured from the Arabidopsis Biological Resource Center (ABRC) located at the Ohio State University, Columbus, OH, USA for academic research.

Competing interests

The authors declare that they have no competing interests.

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