Lignin biosynthesis in wheat (*Triticum aestivum* L.): its response to waterlogging and association with hormonal levels

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**Abstract**

**Background:** Lignin is an important structural component of plant cell wall that confers mechanical strength and tolerance against biotic and abiotic stressors; however, it affects the use of biomass such as wheat straw for some industrial applications such as biofuel production. Genetic alteration of lignin quantity and quality has been considered as a viable option to overcome this problem. However, the molecular mechanisms underlying lignin formation in wheat biomass has not been studied. Combining molecular and biochemical approaches, the present study investigated the transcriptional regulation of lignin biosynthesis in two wheat cultivars with varying lodging characteristics and also in response to waterlogging. It also examined the association of lignin level in tissues with that of plant hormones implicated in the control of lignin biosynthesis.

**Results:** Analysis of lignin biosynthesis in the two wheat cultivars revealed a close association of lodging resistance with internode lignin content and expression of 4-coumarate:CoA ligase 1 (*4CL1*), *p*-coumarate 3-hydroxylase 1 (*C3H1*), cinnamoyl-CoA reductase 2 (*CCR2*), ferulate 5-hydroxylase 2 (*F5H2*) and caffeic acid O-methyltransferase 2 (*COMT2*), which are among the genes highly expressed in wheat tissues, implying the importance of these genes in mediating lignin deposition in wheat stem. Waterlogging of wheat plants reduced internode lignin content, and this effect is accompanied by transcriptional repression of three of the genes characterized as highly expressed in wheat internode including *phenylalanine ammonia-lyase 6* (*PAL6*), *CCR2* and *F5H2*, and decreased activity of PAL. Expression of the other genes was, however, induced by waterlogging, suggesting their role in the synthesis of other phenylpropanoid-derived molecules with roles in stress responses. Moreover, difference in internode lignin content between cultivars or change in its level due to waterlogging is associated with the level of cytokinin.

**Conclusion:** Lodging resistance, tolerance against biotic and abiotic stresses and feedstock quality of wheat biomass are closely associated with its lignin content. Therefore, the findings of this study provide important insights into the molecular mechanisms underlying lignin formation in wheat, an important step towards the development of molecular tools that can facilitate the breeding of wheat cultivars for optimized lignin content and enhanced feedstock quality without affecting other lignin-related agronomic benefits.

**Keywords:** Gene expression, Plant hormone, Lignin, Lodging, Waterlogging, Wheat

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Background

Lignin is a complex phenolic polymer closely linked with cellulose and hemicellulose, forming an important structural component of plant secondary cell wall. It provides plants with mechanical strength and vascular integrity [1], and also plays important roles in conferring tolerance against biotic and abiotic stresses [2, 3]. Recently, increased concerns about climate change and the need to reduce carbon emissions have triggered a growing interest in producing renewable fuels and bioproducts from lignocellulosic biomass [4]. Wheat is one of the most economically important crops globally, and its straw represents an abundant source of biomass that can be used as a feedstock for sustainable production of biofuel and bioproducts [5, 6].

Efficient conversion of lignocellulosic biomass to biofuels is hindered by lignin, which limits the accessibility of plant cell wall polysaccharides to chemical, enzymatic and microbial digestions [7, 8]. Genetic alteration of the quantity and quality of lignin in plant biomass has been considered as a viable alternative to mitigate this problem [9–11]. However, lignin content in cereal crops such as wheat has been shown to be closely associated with resistance to lodging [12], one of the major impediments of wheat production leading to harvestable yield loss by up to 80% [13]. Therefore, it is imperative to design tools and approaches that can alter the quantity and quality of lignin in wheat biomass without affecting its functions in conferring structural support for normal growth and development, and field performance. This, however, requires detailed dissection of the molecular mechanisms underlying the formation of lignin in wheat biomass.

It has been established that lignin in plants is formed by the oxidative coupling of three monolignols that serve as building blocks; coniferyl, sinapyl and p-coumaryl alcohols [1]. These monolignols are synthesized from phenylalanine through the general phenylpropanoid pathway (A) and monolignol-specific pathway (B) [1, 10]. For example, suppression of genes early in the pathway such as PAL, C4H, HCT, C3H and CCoAOMT in alfalfa and tobacco leads to reduction in total lignin content [9]. Genetic repression of CAD, encoding an enzyme that catalyzes the last step in the synthesis of monolignols, the PAL and CAD enzymes, respectively, are believed to play critical roles in regulating lignin accumulation in plants [15–17]. Given that most of the lignin biosynthetic enzymes have isoforms, they appear to be encoded by gene families [18, 19], and previous studies have demonstrated the physiological roles for most of these genes in determining lignin deposition and composition [1, 10]. For example, repression of genes early in the pathway such as PAL, C4H, HCT, C3H and CCoAOMT in alfalfa and tobacco leads to reduction in total lignin content, while repression of FSH or COMT, which are involved in the synthesis of the S unit of lignin, results in alteration of the S/G ratio of lignin with only minimal effect on total lignin content [9]. Genetic repression of CAD, encoding an enzyme that catalyzes the last step in the biosynthesis of...
monolignols, also causes reduction in the S/G ratio with limited effect on total lignin content. Similarly, the fsh1 and cont mutants of Arabidopsis are characterized by decreased amounts of S unit containing oligolignols with no effect on the total lignin content, whereas the c4h, 4cl1, ccoaomt1, and ccr1 mutants contain lower amount of total lignin as compared to the wild-type [20].

Although lignin biosynthesis pathway has not been elucidated in wheat to date, previous studies have characterized the expression patterns of the wheat homologs of lignin biosynthetic genes. The transcripts for most of these homologs appeared to be highly abundant in the stem tissue as compared to the leaf sheath and leaf blade, and the expressions of PAL6, C4H, 4CL1, C3H1, CCR2, F5H1 and F5H2 in particular are found to have a significant correlation with lignin content [3]. Furthermore, the stem exhibits higher abundance of CCR1 and CAD1 transcripts and higher activity of the corresponding enzymes than that detected in other tissues, while COMT1 is expressed constitutively across the different tissues including stem, leaf and root [12, 21, 22]. Comparative analysis between wheat cultivars with varying degree of resistance to lodging showed the presence of higher transcript abundance of CCR1, COMT1 and CAD1 genes and higher activities of the corresponding enzymes in the stem of lodging resistant cultivar following the heading stage, and these factors have been shown to be closely associated with stem lignin content and mechanical strength [21]. Despite these results, the molecular mechanism underlying the synthesis of lignin in wheat tissues is yet poorly understood.

Waterlogging is one of the abiotic factors adversely affecting crop productivity; it causes a reduction in gas diffusion and thereby affects the availability of oxygen in the rhizosphere, inducing changes in biochemical and metabolic processes [23]. One of the main metabolic changes involves a shift from aerobic to anaerobic respiration, impairing ATP production. Compensation of the resulting energy deficit requires accelerated glycolysis via increased activities of glycolytic and fermentative enzymes, leading to the depletion of carbohydrate reserves, a phenomenon referred to as the "Pasteur effect" [24, 25]. Consistently, transcriptional activation of glycolytic and fermentative genes has been reported in rice coleoptiles under anoxic conditions [26]. In contrast with this, root hypoxic conditions have been shown to result in accumulation of soluble carbohydrates such as sucrose, and those with storage function such as fructan in both root and shoot tissues of wheat seedlings [27]. Under long-term hypoxic conditions, a large amount of sucrose in wheat roots partitions to the synthesis of cell wall components, mainly cellulose, leading to changes in cell wall structure [28]. It is therefore likely that such alteration in the structure of the cell wall serves as one of the strategies used by the root tissue to compensate the progressive dissolution of cortical cells for aerenchyma formation, thereby contributing to the maintenance of cell wall function under low O2 stress conditions [29]. However, little is known to date about the molecular features mediating the response of lignin biosynthesis to waterlogging conditions.

In addition to environmental factors, cellular signaling molecules such as plant hormones regulate lignin biosynthesis [30, 31]. For example, auxin and cytokinin induce the expression of the lignin biosynthetic gene, peroxidase (Prx), in Zinnia elegans, and secondary growth/lignification [30]. Consistently, the promoter of Prx consists of motifs that mediate the response of Prx to these hormones and thereby act as targets for transcription factors regulating secondary growth [30, 32]. Furthermore, hypergravity-induced auxin accumulation leads to enhanced expression of selected lignin biosynthetic genes, and in turn lignification in the inflorescence stem of Arabidopsis [33]. It has been shown previously that the level of salicylic acid (SA) is inversely related to lignin content in plants where lignin content is reduced through downregulation of specific lignin biosynthetic genes, and SA mediates growth supression in these plants; however, genetic reduction of SA level was found to restore growth but not lignin content [34].

To gain insights into the molecular mechanisms underlying the regulation of lignin formation in wheat, we performed comparative analysis of lignin biosynthesis in the internode tissue of wheat cultivars with varying degree of resistance to lodging, a trait closely associated with stem lignin content and mechanical strength. Furthermore, the study identified candidate genes that mediate the response of lignin biosynthesis in wheat tissues to waterlogging, and examined the association between the levels of lignin and selected plant hormones that have been implicated to have roles in the regulation of lignin biosynthesis.

**Methods**

**Plant material**

Two hexaploid wheat cultivars, Harvest and Kane, were used in this study, and seeds for these cultivars were kindly provided by Dr. Stephen Fox of Agriculture and Agri-Food Canada (AAFC)-Cereal Research Center (Winnipeg, Manitoba, Canada). Both are hard red spring wheat cultivars developed by AAFC [35, 36], and were selected for the present study on the basis of their agronomic quality in terms of lodging resistance, which is considered as a measure of stem mechanical strength, and their close genetic relationship. Mature dry seeds of the two cultivars were imbibed in Petri dishes for 3 days, after which the germinated seeds were transplanted to 1-gallon plastic pots (one seed per pot) containing Super Mix supplied with 18 g of fertilizers (ACER’nt 13-12-12 consisting of 13 % N, 12 % P2O5, 12 % K2O and micro
Additional family members from wheat (derived from a database (http://www.ncbi.nlm.nih.gov/nuccore/) for as a query to blast search the NCBI GenBank nucleotide release 12 (when a complete CDS is not available) was used to obtain the respective unigenes, which consist of a wheat UniGene dataset containing 56,943 unigenes (http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=4565). The GenBank IDs corresponding to the newly identified TCs were used to search for homologous genes from other species using the criteria of at least 50% coverage of the query sequence and E-value of ≤10−20. Sequences meeting these criteria were collected for each lignin biosynthetic gene family and used to generate phylogenetic trees. The sequences were collected mainly from rice (Oryza sativa, taxid 4530), sorghum (Sorghum bicolor, taxid 4558), barley (Hordeum vulgare, taxid 4513), switchgrass (Panicum virgatum, taxid 38727), maize (Zea mays, taxid 4577), and other species for which candidate lignin biosynthetic gene sequence information is available. Sequences of the Arabidopsis lignin biosynthetic genes were collected based on the protein sequences reported in Xu et al. [19]. The nucleotide sequences of the genes were then aligned using ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2) [40] and the phylogenetic tree was generated with the Molecular Evolutionary Genetic Analysis (MEGA, version 6) software (http://www.megasoftware.net) [41] using a Tamura-Nei model and a 500 replicate bootstrap method of phylogeny test.

**Waterlogging treatment**

For waterlogging treatment, plants of cv. Harvest were grown as described above except that the soil mixture consisted of clay and sand (2:1, v:v). Four weeks after transplanting, a set of 48 plants was subjected to waterlogging by submerging each pot containing a plant in a 5 L containers filled with water in order to maintain the water level at 2 cm above the surface of the soil. The water in the bigger container was replenished as required. Concurrently, another set of 48 plants was subjected to regular watering (0.5 L water per pot every other day). At heading stage, which occurred at 20 to 22 days after the start of waterlogging in both treated and untreated control plants, the flag leaf and IN-2&3 tissues were harvested in liquid nitrogen and stored at −80 °C until further use.

**Identification of candidate lignin biosynthetic genes and phylogenetic analysis**

The tentative consensus (TC) sequences of candidate lignin biosynthetic genes from the Dana-Farber Cancer Institute (DFCI) wheat gene index (TaGI) release 10 and 11 [3] were used to search for the corresponding TCs in TaGI release 12 (ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Triticum_aestivum/) [37]. The GenBank IDs corresponding to the newly identified TCs were used to search the National Center for Biotechnology Information (NCBI) wheat UniGene dataset containing 56,943 unigenes (http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=4565) [38] to obtain the respective unigenes, which consist of a cluster of sequences representing a unique lignin biosynthetic gene family member. A complete coding sequence (CDS) from each sequence cluster or a TC sequence from release 12 (when a complete CDS is not available) was used as a query to blast search the NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore/) [39] for additional family members from wheat (derived from a different unigene) or for homologous genes from other species using the criteria of at least 50% coverage of the query sequence and E-value of ≤10−20. Sequences meeting these criteria were collected for each lignin biosynthetic gene family and used to generate phylogenetic trees. The sequences were collected mainly from rice (Oryza sativa, taxid 4530), sorghum (Sorghum bicolor, taxid 4558), barley (Hordeum vulgare, taxid 4513), switchgrass (Panicum virgatum, taxid 38727), maize (Zea mays, taxid 4577), and other species for which candidate lignin biosynthetic gene sequence information is available. Sequences of the Arabidopsis lignin biosynthetic genes were collected based on the protein sequences reported in Xu et al. [19]. The nucleotide sequences of the genes were then aligned using ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2) [40] and the phylogenetic tree was generated with the Molecular Evolutionary Genetic Analysis (MEGA, version 6) software (http://www.megasoftware.net) [41] using a Tamura-Nei model and a 500 replicate bootstrap method of phylogeny test.

**RNA extraction and cDNA synthesis**

Total RNA was isolated from tissues using TRIzol Reagent following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). The RNA samples were digested with DNase (DNA-free Kit; Ambion, Austin, TX, USA) to eliminate genomic DNA contamination. The first strand cDNA was synthesized from 1 μg of total RNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) in a total reaction volume of 20 μl. The resulting cDNA samples were diluted 20× before use as a template for real-time quantitative PCR (qPCR).

**Real-time quantitative PCR assay**

The real time qPCR assays for the candidate lignin biosynthetic and the reference β-actin genes were performed with the gene specific primers described previously [3] except for the new candidate genes identified in this study and the genes for which redesigning the primers was required due to their lack of target gene specificity (Additional file 1: Table S1). Gene specificity of all the primers was verified first by blast searching the target amplicons against GenBank database and then by RT-PCR. The qPCR assay was performed on the CFX96 Real-Time PCR system (Bio-Rad) and the reaction consists of 5 μL of the diluted cDNA as a template, 10 μL of SsoFast EvaGreen Supermix (Bio-Rad), 1.2 μL of 5 μM forward primer (300 nM final concentration), 1.2 μL of 5 μM reverse primer (300 nM final concentration) and 2.6 μL diethylpyrocarbonate treated water. The samples were subjected to the following thermal cycling conditions: DNA polymerase activation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C
for 15 s, annealing at 50–66 °C (depending on the melting temperature of the primer set) for 30 s and extension at 72 °C for 30 s in duplicate in 96-well optical reaction plates (Bio-Rad). Transcript levels of the target genes were expressed after normalization with β-actin using the Livak and Schmittgen method [42].

Measurement of enzyme activity
Protein concentrations and enzyme activities were determined spectrophotometrically (Ultrospec 3100 pro, Artisan Scientific, Champaign, IL, USA). Total protein extraction, and the assays for determining the activities of coniferyl aldehyde recognizing CAD (CAD-CA) and sinapyl aldehyde recognizing CAD (CAD-SA) were carried out as described in Zhang et al. [43]. Briefly, frozen plant tissue (~200 mg of flag leaf or ~300 mg of internode) was ground into fine powder using mortar and pestle, mixed with protein extraction buffer and then incubated at 4 °C for 2.5 h. Protein concentration in the extract was measured using Quick Start™ Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). The CAD-CA and CAD-SA activities were examined by using total protein extract (100 μg) in 500 μL reaction buffer and monitoring the conversion of coniferyl alcohol, a substrate for CAD-CA, and sinapyl alcohol, a substrate for CAD-SA, to their respective products at 400 nm. The activity of PAL in the protein extract was assayed as described in Edwards and Kessmann [46] except that the initial extraction was performed with 80 % (v/v) acetonitrile containing 1 % (v/v) acetic acid and elutions of the IAA and SA extracts from the respective columns were performed with 80 % (v/v) acetonitrile containing 1 % (v/v) acetic acid while the IPA and t-zeatin extracts were eluted with 60 % (v/v) acetonitrile containing 5 % (v/v) aqueous ammonia. Quantification of the IAA, IPA and t-zeatin, and SA levels was performed using liquid chromatography-tandem mass spectrometry system (Agilent 6430) (Agilent, Santa Clara, CA, USA) according to the protocol described in Yoshimoto et al. [47].

Statistical analysis
Significant difference between sample means was tested using either LSD test or t-student test at P < 0.05. Statistical analysis was performed with GenStat version 12 [48].

Results
Update on the wheat homologs of lignin biosynthetic genes
Using the TaGI release 10 and 11, Bi et al. [3] identified 32 candidate lignin biosynthetic genes belonging to 10 gene families; however only two of them, COMT1 and COMT2, were found to have sequences that represent complete CDS in the respective cluster of sequences. Since the GenBank database has been updated with more wheat gene sequences and the TaGI release 12 consisting of 221,925 TCs has been made available since the report of Bi et al. [3], we performed further database mining and updated the list of the candidate wheat lignin biosynthetic gene (Table 1). Our search revealed that specific ESTs assigned by Bi et al. [3] to different gene family members appear to originate from the same unigene, thus represent the same gene family member instead of different members. ESTs designated in Bi et al. [3] as 4CL1 and 4CL2 are found to belong to the same unigene (Ta. 45532), thus represent the same 4CL gene designated hereafter as 4CL1; ESTs designated as CCoAOMT1 and CCoAOMT4 come from the same unigene (Ta. 18653), thus represent the same CCoAOMT gene designated hereafter as CCoAOMT1; ESTs designated as CCoAOMT3 and CCoAOMT5 originate from the same unigene (Ta. 48354), thus represent the same CCoAOMT gene designated hereafter as CCoAOMT3, and ESTs designated as PAL2, PAL3 and PAL4 derive from the same unigene (Ta. 47240), thus represent the same PAL gene designated hereafter as PAL2. Accordingly, the ESTs identified by Bi et al. [3] as PAL5, 6, 7 and 8 are redesignated as PAL3, 4, 5 and 6, respectively. The selection of a particular EST for the designation in each family member was based on their coding region.
coverage, and these specific ESTs were considered for our gene expression analysis (Table 1). Furthermore, the EST assigned by the same authors as CAD2 is found to be a homolog of the CCR gene, thus it is re-designated hereafter as CCR5 (Table 1 and Fig. 2a). Our search also led to the identification of new candidate lignin biosynthetic genes belonging to the C3H (designated as C3H3), CCR (designated as CCR6) and COMT (designated as COMT3) gene families (Table 1).

Table 1 Updated list of the candidate lignin biosynthesis gene family members of wheat

| Name       | TC ID         | UniGene ID   | GenBank ID   |
|------------|---------------|--------------|--------------|
| PAL1       | TC383948      | Ta.70840     | CIJ56144     |
| PAL2       | TC414011      | Ta.47240     | CIJ873958    |
| PAL3       | TC385013      | Ta.70842     | CIJ963737    |
| PAL4       | TC369518      | Ta.48480     | CIJ954700    |
| PAL5       | TC405175      | Ta.71604     | CIJ964755    |
| PAL6       | TC385356      | Ta.21253     | CIJ728010    |
| C4H1       | TC384685      | Ta.253       | CIJ57495     |
| 4CL1       | TC395589      | Ta.45352     | CIJ962785    |
| HCT1       | TC396512      | Ta.37502     | CIJ93498     |
| HCT2       | TC396512      | Ta.5332      | CIJ99765     |
| C3H1       | TC372953      | Ta.24789     | AJ583530.1, AJ583531.1 |
| C3H2       | TC368628      | Ta.31019     | AJ585988.1, AJ585990.1, AJ585991.1 |
| C3H3       | NA            | Ta.48868     | AJ583352.1   |
| CCoAOMT1   | TC374467      | Ta.18653     | CD939543     |
| CCoAOMT2   | TC373325      | Ta.39255     | CJ928722     |
| CCoAOMT3   | TC398408      | Ta.48354     | CIJ966710    |
| CCR1       | TC401546      | Ta.12690     | CV066123     |
| CCR2       | TC378424      | Ta.1183      | DJQ449508.1  |
| CCR3       | TC374055      | Ta.13990     | AY771357.1   |
| CCR4       | TC377614      | Ta.71133     | CJ803490     |
| CCR5       | TC373421      | Ta.48506     | BJ226457     |
| CCR6       | NA            | Ta.58638     | AF307997     |
| FSH1       | TC410836      | Ta.73421     | CA68885      |
| FSH2       | TC383087      | Ta.70215     | CJ962608     |
| COMT1      | TC369087      | Ta.70123     | DJQ223971.1, EF413031.1 |
| COMT2      | TC368870      | Ta.336       | AY226581.1   |
| COMT3      | NA            | Ta.41808     | EF423611.1   |
| CAD1       | TC445835      | Ta.32577     | CJ710661     |
| CAD2       | TC374092      | Ta.70656     | CJ723808     |
| CAD3       | TC379496      | Ta.28562     | GUS63724.1   |

*TC ID: tentative consensus sequences ID (DFCI TaGI 12.0) [37]
*UniGene ID: NCBI UniGene ID [38]
*GenBank ID: NCBI GenBank ID [39]
*GenBank IDs in bold represent complete coding sequences
*NA not available

Phylogenetic analysis of the candidate lignin biosynthesis genes

Phylogenetic analysis of the wheat candidate lignin biosynthetic genes along with the representative homologs from other species (9 to 34 genes depending on the gene family) revealed that each wheat candidate gene is clustered with more than one homolog derived from the other species except that all the three wheat COMT genes formed their own group (Fig. 2; Additional file 2: Figure S1, Additional file 3: Figure S2, Additional file 4: Figure S3 and Additional file 5: Figure S4). In contrast, the six CCR genes were distributed among five different clades (Fig. 2b). Overall, most of the wheat lignin biosynthetic genes are grouped mainly with their homologs that originate from monocot species instead of those derived from Arabidopsis and other dicot species (Fig. 2; Additional file 2: Figure S1, Additional file 3: Figure S2, Additional file 4: Figure S3 and Additional file 5: Figure S4).

Lodging behavior of the wheat cultivars

Harvest and Kane are among the cultivars widely grown in the wheat growing regions of the Canadian Prairies [49]. These two cultivars are closely related genetically as they are developed by a cross that involved a common parental line; Kane is derived from a cross between AC Domain and McKenzie [35], and Harvest from a cross between AC Domain*2 and ND640 [36] (*2 denotes two doses of AC Domain; one backcross after the original cross). With respect to their resistance to lodging, Kane and Harvest are rated as ‘very good’ and ‘good’ respectively [50, 51]. It can also be inferred from the registration data of Harvest and Kane, which compared each cultivar against the same check cultivar over three years at multiple locations, that Harvest exhibits lower lodging score than Kane; lodging rated on a 1–9 scale, 1 = vertical and 9 = flat [35, 36]. Kane exhibited consistently similar lodging scores to the check cultivars AC Barrie (average lodging score of 1.7 in year I, 2.1 in year II and 2.5 in year III for Kane, and 1.9 in year I, 2.2 in year II and 2.5 in year III for AC Barrie) [35]. In contrast, Harvest exhibited lower lodging score than the check cultivar AC Barrie (on average 2.0 in year I, 1.4 in year II and 1.7 in year III for Harvest, and 2.6 in year I, 2.4 in year II and 2.5 in year III for AC Barrie) [36]. As the studies that compared each cultivar against the common check cultivar were conducted at different times, the results also suggest that the severity of lodging was worse in years when the performance of Harvest was tested.

Expression of the candidate lignin biosynthetic genes in different wheat tissues

The expression patterns of candidate lignin biosynthetic genes in different tissues and stages of wheat have been characterized previously [3]. Given that the list of the
candidate genes has been updated with the latest databases (Table 1) and our experimental materials involve wheat cultivars different from that used by Bi et al. [3], we decided to re-examine the spatiotemporal expression pattern of all the candidate genes. Our analysis revealed that all the candidate genes showed higher expression in the peduncle and/or internode tissues than in the flag leaf blade/sheath except that CCR1 and CCR5 genes exhibited significantly higher expression in the flag leaf blade than in the other tissues analyzed in this study, and COMT2 and CAD2 in their respective gene family are found to be highly expressed in both flag leaf sheath and internode tissues (Additional file 6: Table S2). The C3H2, CCoAOMT2 and CCR4 genes are found to be expressed at very low/undetectable level across all the tissue analyzed.
Specific members in each gene family including PAL6, C4H1, 4CL1, HCT1, C3H1, CCoAOMT1, CCR2, F5H2 and COMT2 appeared to be highly expressed in the peduncle/internode (Table 2). From the CAD gene family, the transcripts of both CAD1 and CAD2 are highly abundant in the internode, although the transcript level of CAD2 is slightly higher than that of CAD1. Comparative analysis across the different internode sections revealed that most of these genes are highly expressed in the IN-2&3 and/or IN-4 sections (Table 2). As a result, their expression in IN-2&3 was further compared between the two cultivars with varying degree of resistance to lodging, a trait closely associated with stem lignin content. It appeared from our analysis that 4CL1, HCT1, C3H1, F5H2 and COMT2 genes exhibit significantly higher transcript level in the internode of cv. Harvest than in cv. Kane (Fig. 3). Although not statistically significant, higher expression of CCR2 was also evident in the internode of cv. Harvest. The other four genes, PAL6, C4H1, CCoAOMT1 and CAD2, exhibited similar expression level between the two cultivars.

Transcriptional response of candidate lignin biosynthetic genes to waterlogging

In order to gain insights into the transcriptional regulation of lignin biosynthesis genes by waterlogging, we analyzed the expression of all the candidate genes in the internodes of waterlogged plants. Waterlogging led to a significant transcriptional repression of PAL6, 4CL2, CCR2, CCR6, F5H1, F5H2 and COMT1 genes (Fig. 4a). On the other hand, all the PAL genes, except PAL4 and PAL6, were upregulated in response to waterlogging; the expression of PAL1 in particular was induced by ~200-fold. However, the basal expression level of this gene in the internodes of untreated control plants was relatively low (Additional file 6: Table S2). Other genes with ≥2-fold upregulation in response to waterlogging include C4H1, CCoAOMT1, CCoAOMT2, CCoAOMT3, CCR3 and CCR4. The waterlogging treatment also induced upregulation (~1.8-fold) of CAD1 and CAD3 genes, although not ≥2-fold. However, the expressions of PAL4, 4CL1, C3H1, C3H3, COMT2, CCR5 and CAD2 remained unaffected by the waterlogging treatment.

To better understand the effects of waterlogging on lignin synthesis, we also analyzed the expression of lignin biosynthesis genes in the flag leaf (blade plus sheath) of waterlogged plants. Waterlogging led to ≥2-fold upregulation of PAL6, C4H1, 4CL1, CCoAOMT1, CCoAOMT2, CCR2, CCR3, F5H2, COMT1, COMT2, CAD1 and CAD3 genes (Fig. 4b), of which PAL6, C4H1, 4CL1, CCoAOMT1, F5H2 and COMT2 are found to be the predominant genes in the leaf tissue in their respective family (Table 2; Additional file 6: Table S2). Although CCoAOMT2 and CCR3 showed drastic induction (over 7-fold) in response to waterlogging, their basal expression in the flag leaf of the control untreated plants was very low (Additional file 6: Table S2).

Waterlogging induced ≥2-fold upregulation of PAL1, PAL2, PAL3, PAL5 and CCR4 was evident in the internode but not in the flag leaf, where their expression appeared not to be affected by waterlogging (Fig. 4). In contrast, waterlogging led to ≥2-fold upregulation of PAL6, 4CL1,

| Gene       | FB    | FS    | PE    | IN-1  | IN-2&3 | IN-4   |
|------------|-------|-------|-------|-------|--------|--------|
| PAL6       | 0.83 ± 0.07d  | 9.05 ± 0.80c | 89.25 ± 19.53a | 54.50 ± 6.71b | 54.57 ± 11.98b | 33.66 ± 6.98b |
| C4H1       | 0.38 ± 0.03d  | 0.91 ± 0.04c | 2.68 ± 0.40b   | 3.67 ± 0.61b  | 9.57 ± 1.20a  | 11.22 ± 3.71a  |
| 4CL1       | 1.00 ± 0.09d  | 2.04 ± 0.08c | 7.61 ± 1.10b   | 7.13 ± 0.57b  | 12.51 ± 2.02a | 16.84 ± 2.83a  |
| HCT1       | 0.58 ± 0.03d  | 0.70 ± 0.06d | 2.53 ± 0.54b   | 1.54 ± 0.20c  | 3.90 ± 0.40b  | 5.42 ± 0.60a   |
| C3H1       | 0.12 ± 0.03e  | 0.21 ± 0.03de| 1.24 ± 0.06a   | 0.87 ± 0.16b  | 0.55 ± 0.12c  | 0.46 ± 0.03d   |
| CCoAOMT1   | 1.52 ± 0.17e  | 3.32 ± 0.58d | 32.73 ± 5.28a  | 11.98 ± 2.51c | 19.56 ± 3.51bc| 21.89 ± 4.77b  |
| CCR1<sup>+</sup> | 11.11 ± 0.21a | 6.64 ± 0.36b | 0.29 ± 0.03f   | 1.59 ± 0.31c  | 0.78 ± 0.02d  | 0.45 ± 0.07e   |
| CCR2       | 0.17 ± 0.02e  | 1.56 ± 0.07d | 3.77 ± 0.78c   | 5.93 ± 0.29b  | 8.44 ± 0.85a  | 7.82 ± 1.09a   |
| CCR5<sup>+</sup> | 13.49 ± 1.10a | 9.33 ± 0.21b | 0.93 ± 0.09d   | 2.76 ± 0.66cd | 2.85 ± 0.36cd | 3.54 ± 0.63c   |
| F5H2       | 0.84 ± 0.04e  | 1.39 ± 0.04d | 9.20 ± 1.67a   | 6.09 ± 0.97ab | 3.38 ± 1.05c  | 4.23 ± 0.84bc  |
| COMT2      | 15.51 ± 1.13b | 36.04 ± 4.20a| 16.09 ± 2.86b  | 31.56 ± 4.40a | 14.18 ± 1.64b | 12.63 ± 0.67b  |
| CAD2       | 1.32 ± 0.07b  | 2.05 ± 0.08a | 0.16 ± 0.00c   | 0.95 ± 0.22b  | 2.02 ± 0.17a  | 2.55 ± 0.29a   |

*Transcript levels in different tissues of cv. Harvest were expressed relative to that of 4CL1 in flag leaf, which was arbitrarily set to value of 1
*Data are means of 2 to 3 independent biological replicates ± SE
*Means followed by different letters within each gene show statistically significant difference at P < 0.05
*Genes highly expressed specifically in the leaf tissues
FB flag leaf blade, FS flag leaf sheath, PE peduncle, IN-1 the first internode, IN-2&3 the second and third internode, IN-4 the fourth internode (see the Methods section for numerical designation of internodes)
CCR2, F5H2, COMT1 and COMT2 in the flag leaf but not in the internode, where their expression was either repressed or remained unaffected.

Analysis of the activity of lignin biosynthesis enzymes
To gain insights into the association between transcriptional and post-transcriptional regulations of genes encoding key lignin biosynthetic enzymes, we measured the activities of PAL catalyzing the first committed step in the phenylpropanoid pathway and CAD catalyzing the last step in the monolignol pathway (Fig. 1). Our data showed slightly higher activity of internode-derived PAL in cv. Kane than in cv. Harvest (Fig. 5c). The activity of CAD-SA and CAD-CA in the internode was found to be similar between the two cultivars. Waterlogging led to significant reduction in the activities of internode-derived PAL and CAD-SA enzymes although no effect was evident on the activity of CAD-CA (Fig. 6a). However, it caused significant increases in the activities of flag leaf-derived PAL, CAD-CA, and CAD-SA enzymes (Fig. 6b).

Analysis of the major lignocellulosic constituents
To determine the association of stem resistance to lodging with the level of lignin and other cell wall components including cellulose and hemicellulose, we profiled their levels in the internode tissue of cvs. Harvest and Kane. Internode lignin content of cv. Harvest was over 23 % higher than that observed in cv. Kane (Fig. 7a). Similarly, the internode cellulose content of cv. Harvest was over 9 % higher than that of cv. Kane. With respect to hemicellulose, the internode of cv. Kane exhibited ~6 % higher level than that of cv. Harvest. Waterlogging caused significant reduction in the amounts of both lignin (36 %) and cellulose (20 %) contents in the internode tissues; however, no effect was evident on the content of hemicellulose (Fig. 7b). It also led to 60 % decrease in the flag leaf lignin content, while causing 5 and 30 % increases in the contents of cellulose and hemicellulose, respectively (Fig. 7c).

Hormone analysis
To examine the association between the levels of lignin and plant hormones that are implicated in the regulation of lignin biosynthesis, we measured the amounts of IAA, IPA and t-zeatin, and SA in the internode tissues. The levels of IPA, t-zeatin and SA in the internode of cv. Harvest were found to be higher than that observed in the internode of cv. Kane while the IAA content did not show any difference (Fig. 8a). Waterlogging, which decreased lignin content (Fig. 7c), led to a significant reduction in IPA and t-zeatin levels. In contrast, the level of IAA increased in response to waterlogging while no change in SA content was evident (Fig. 8b).

Discussion
In order to gain insights into the molecular basis for the regulation lignin biosynthesis in wheat tissues, this study first examined the expression of lignin biosynthetic genes in different wheat tissues and then compared the expression patterns of selected genes in the internode tissue between two different wheat cultivars exhibiting different degree of resistance to lodging. The study also investigated the effect of waterlogging on the expression of lignin biosynthetic genes and the level of major lignocellulosic
constituents. Furthermore, we assessed if lignin content is associated with the level of plant hormones implicated in regulating lignin biosynthesis in other species.

Our gene expression data indicated high transcript abundance of PAL6, C4H1, 4CL1, HCT1, C3H1, CCoAOMT1, CCR2, CCR5, F5H2, COMT2 and CAD2 genes in wheat tissues (Table 2; Additional file 6: Table S2), thus, it is more likely that these genes play important roles in the regulation of lignin biosynthesis in wheat. These results are in agreement with the expression patterns of lignin biosynthetic genes reported previously in both wheat and Arabidopsis [3, 18]. Within the stem, older internodes appeared to have higher expression for most of the candidate genes than the younger internode sections. Consistently, the expressions of selected lignin biosynthetic genes and lignin level have been shown to increase with internode age [3]. Although a specific member in each gene family exhibits predominance in expression across the different wheat tissues, two members of the CCR gene family showed tissue specificity; CCR5 is predominantly expressed in the flag leaf while CCR2 in the internode (Table 2). It has also been shown previously that the gene we designated as CCR2 exhibits higher expression in the internode [21].

We further compared the expression of the candidate genes in two wheat cultivars exhibiting varying degrees of resistance to lodging, namely Harvest and Kane; cv. Harvest is designated agronomically as more resistant to lodging than cv. Kane. The higher internode lignin content of cv. Harvest than that of cv. Kane (Fig. 7a) suggests a close association between stem mechanical strength/lodging resistance and stem lignin content [12, 22]. The association of internode lignin content of cv. Harvest with the expression of 4CL1, CCR2, F5H2 and COMT2 genes reflects the significance of these
specific genes in regulating internode lignin content, which contributes to stem mechanical strength/lodging resistance in wheat. A close association between wheat stem lignin content and the expression of \( CCR \) and \( COMT \) genes and activities of the corresponding enzymes has also been reported previously [12, 21]. However, the difference in lodging resistance between the two cultivars may also be at least partly due to other factors that contribute to stem stiffness and thickness than internode lignin content, such as densities of cellulose and hemicellulose, structural properties of the secondary cell wall, and structural traits associated with cortical fiber tissues [52–54]. Consistently, the enhanced culm mechanical strength in a lignin deficient rice variety a has been shown to be associated with high densities of cellulose and hemicellulose, well developed secondary cell walls and thick layer of cortical fiber tissues [53].

Lignin biosynthesis in plants is affected by both biotic and abiotic stress factors [55]. Waterlogging is among the abiotic stress factor that triggers reduction of leaf photosynthetic capacity [56, 57] and accumulation of soluble carbohydrate in plant biomass [27]. Furthermore, it triggers increased demand for soluble sugars, particularly glucose [58, 59], which might lead to the channeling of more phosphoenol pyruvate to glycolysis rather than to the shikimate pathway that produces phenylalanine, an important lignin precursor [20]. These changes have important implications on the level and/or composition of structural carbohydrate polymers such as cellulose, hemicellulose and lignin [60, 61]. Consistently, the level of lignin in both internode and flag leaf tissues was substantially reduced in response to waterlogging (Fig. 7b, c). In the internode, waterlogging also caused a significant reduction in cellulose content; leading to a decrease in the total amount of structural carbohydrates. Since the synthesis of structural carbohydrate components such as lignin is an energy-consuming and irreversible process [62, 63], reduction in their level might be one of the energy/carbon saving strategies employed by plants under waterlogging conditions [24, 25]. In the flag leaf, the decrease in lignin content appears, however, to be compensated by significant increases in the levels of cellulose and hemicellulose (Fig. 7b).
Among the genes identified as highly expressed in wheat biomass, transcriptional repression of PAL6, CCR2 and FSH2 was apparent specifically in the internode of waterlogged wheat plants (Fig. 4b), suggesting the importance of these genes in modulating lignin formation in the internode under such stress conditions. Consistent with the lignin profile and expression of the PAL6 gene, waterlogging caused reduction in the activity of internode derived PAL (Fig. 6a), an enzyme catalyzing the first committed step in the general phenylpropanoid pathway, and thereby controlling lignin synthesis [15, 16]. In contrast, waterlogging mediated reduction of lignin content in both internode and leaf tissues is accompanied by upregulation of PAL1, 2, 3 and 5 in the internode and PAL6 in the leaf; upregulation of PAL6 in the leaf is associated with increased activity of the PAL enzyme. Given that individual PAL genes respond differentially to abiotic stressors [64] and some PAL genes have been implicated in the synthesis of other phenylpropanoid-derived molecules such as tannins and anthocyanin [65, 66], which are known to function in diverse stress responses [67, 68], it is likely that the PAL genes upregulated in the tissues of waterlogged plants play a role for enhanced production of molecules that provide protection against waterlogging stress. Since the PAL1, 2, 3 and 5 genes of waterlogged internodes are upregulated while the PAL activity is substantially reduced, we cannot rule out the possibility that these genes are subjected to post-transcriptional regulation. In support of this, Kelch repeat F-box (KFB) proteins mediated ubiquitination and degradation of the PAL isozymes have been shown to occur in Arabidopsis [69]. Furthermore, mapping of phosphopeptides in the PAL of Zea mays and Medicago truncatula has led to the speculation that PAL activity can be regulated post-transcriptionally by phosphorylation [70].

Waterlogging, which markedly reduced lignin content, also induced ≥2-fold upregulation of several genes downstream of PAL including C4H1, HCT1 and CCoAOMT1 in the internode and C4H1, 4CL1, HCT1, CCoAOMT1, FSH2, COMT2, CAD1 and CAD3 genes in the leaf (Fig. 4); and the induction of CAD1 and CAD3 in the leaf is associated with increased activity CAD (Fig. 6b). It has been shown in previous studies that phenylpropanoid/monolignol pathway intermediates can serve as precursors not only for lignin but also for a range of secondary metabolites such as flavonoids, coumarins, stilbenes, hydroxycinnamic acid conjugates and lignans that play important roles in stress-related processes [64, 66]. Under stress conditions, when photosynthesis is sub-optimal, plants may re-direct the phenylpropanoid/monolignol pathway intermediates towards the production of these secondary metabolites [64]. Therefore, waterlogging-induced upregulation of the several lignin biosynthesis genes downstream of PAL in both internode and leaf tissues while lignin level is reduced might suggest the role of these genes for increased synthesis of anti-stress secondary metabolites at the expense of lignin to help the plants overcome the adverse effects of waterlogging. In support of this hypothesis, some isoforms of the enzymes or family members of the genes downstream of PAL have been implicated in the synthesis of phenylpropanoid-derived secondary metabolites [66, 71]. However, the possibility for post-transcriptional regulation of these downstream monolignol pathway genes can not be excluded as a recent study in poplar has shown that the activity of 5-hydroxyconiferaldehyde O-methyltransferase (AldOMT)/COMT can be regulated
by phosphorylation [72], and the CAD and CCR of some species contain phosphopeptides that act as sites for phosphorylation mediated post-transcriptional regulation [70].

Previous studies have provided important insights into the role of different plant hormones in regulating the synthesis of lignin in plant tissues. For example, the levels of SA and lignin have been shown to have inverse relationship in plants where lignin content is reduced through down regulation of lignin biosynthetic genes, and SA was reported to mediate growth reduction in these plants [34]. However, genetic reduction of SA level restores growth but not the lignin content. The presence of similar amounts of SA between the internodes of cvs. Harvest and Kane (Fig. 8a) or between those derived from waterlogged and control plants (Fig. 8b) irrespective of differences in their lignin content might implicate that SA does not directly affect lignin formation. Close association between IAA and lignin levels has been reported previously in which IAA induces the expression of lignin biosynthetic genes and activity of the corresponding enzymes, and thereby lignification [30, 32]. However, internodes of the two cultivars with varying lignin content exhibit similar IAA level (Fig. 8a). Furthermore, internodes of waterlogged wheat plants, which exhibit reduced lignin content (Fig. 7b), contain higher amount of IAA than the internodes of control plants (Fig. 8b). These results might imply the significance of IAA signaling rather than IAA level in the control of lignin formation in wheat stem. Consistent with their role in enhancing lignin synthesis [30, 32], the levels of two bioactive cytokinins, IPA and t-zeatin, are directly associated with internode lignin content, suggesting that the level of lignin in wheat biomass can be optimized through manipulation of the level of cytokinin, IPA and t-zeatin. Since these plant hormones can also influence other plant developmental processes that potentially affect cell wall properties and composition [73], further studies are needed to examine their specific physiological roles in determining lignin formation in wheat tissues. A recent report has also implicated other hormones such as abscisic acid, brassinosteroids, jasmonates and ethylene in regulating cell wall properties and
compostion [73, 74]. Given that plant hormones interact either synergistically or antagonistically in regulating a wide range of plant developmental processes [75], it is important to elucidate molecular mechanisms underlyng the role of hormonal interactions in the control of lignin synthesis in wheat tissues.

**Conclusions**

This study showed that among the candidate lignin biosynthetic genes that are highly expressed in wheat tissues, the expressions of 4CL1, C3H1, CCR2, FSH2 and COMT2 appear to be associated with internode lignin content, which contributes at least partly to lodging resistance. Reduction of the lignin content in the internode of waterlogged wheat plants appeared to be mediated by transcriptional repression of PAL6, CCR2 and FSH2 genes, implying the significance of these genes in modulating lignin level under such stress conditions. Furthermore, changes in internode lignin content are shown to be accompanied by changes in the level of cytokinin, IPA and t-zeatin, suggesting the role of cytokinin in the regulation of lignin deposition in wheat biomass. Given that mechanical strength/resistance to lodging, tolerance to biotic and abiotic stressors and feedstock quality of wheat straw are closely associated with lignin content, the findings of this study provides important insights into our understanding of the molecular mechanisms underlying lignin formation in wheat biomass. This is an important initial step towards the development of molecular tools that can facilitate the breeding of wheat cultivars with optimized lignin content, enhancing the feedstock quality of wheat straw without affecting lignin-associated agronomic traits.

**Availability of supporting data**

The datasets supporting the conclusions of this article are included within the article and its additional files.

**Additional files**

- **Additional file 1: Table S1.** Primer sequences used for qPCR assays of specific lignin biosynthetic genes. Primer sequences were designed for the newly identified candidate lignin biosynthetic genes or redesigned in cases where previously reported primers [3] do not exhibit target gene specificity. (PDF 12 kb)

- **Additional file 2: Figure S1.** Phylogenetic relationships of wheat 4CL and C4H genes with the homologs from other species. Phylogenetic trees of 4CL (A) and C4H (B) were generated based on nucleic acid sequence similarity of the wheat genes with 28 4CL and 9 C4H genes, respectively, of other monocot and dicot species identified from the NCBI nucleotide database [59] using MEGA program [41], and the trees were inferred using Maximum Likelihood method based on the Tamura-Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replicates is shown next to the branches. ●, wheat candidate gene; ▲, genes from dicot species other than Arabidopsis; *, wheat sequence used for the analysis. (PDF 127 kb)

- **Additional file 3: Figure S2.** Phylogenetic relationships of wheat PAL and HCT genes with the homologs from other species. Phylogenetic trees of 4CL (A) and C4H (B) were generated based on nucleic acid sequence similarity of the wheat genes with 28 4CL and 9 C4H genes, respectively, of other monocot and dicot species identified from the NCBI nucleotide database [59] using MEGA program [41], and the trees were inferred using Maximum Likelihood method based on the Tamura-Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replicates is shown next to the branches. ●, wheat candidate gene; ▲, genes from dicot species other than Arabidopsis; *, wheat sequence used for the analysis. (PDF 176 kb)

- **Additional file 4: Figure S3.** Phylogenetic relationships of wheat C3H and CCoAOMT genes with the homologs from other species. Phylogenetic trees of C3H (A) and CCoAOMT (B) were generated based on nucleic acid
sequence similarity of wheat genes with 15 C3H and 19 CoAOMT genes, respectively, of other monocot and dicot species identified from the NCBI nucleotide database [39] using MEGA program [41], and the trees were inferred using Maximum Likelihood method based on the Tamura-Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replications is shown next to the branches. ▲, wheat candidate gene; ●, genes from dicot species other than Arabidopsis; *, wheat sequence used for the analysis. (PDF 175 kb)

Additional file 5: Figure S4. Phylogenetic relationships of wheat FSH and CAD genes with the homologs from other species. Phylogenetic trees FSH (A, B) and CAD (C) were generated based on nucleic acid sequence similarity of wheat genes with 14 FSH and 22 CAD genes, respectively, of other monocot and dicot species identified from the NCBI nucleotide database [39] using MEGA program [41], and the trees were inferred using Maximum Likelihood method based on the Tamura-Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replications is shown next to the branches. Two separate phylogenetic trees were generated for wheat FSH (A) and FSH2 (B) genes as MEGA could not find common sites between FSH1 and FSH2. ●, wheat candidate gene; ▲, genes from dicot species other than Arabidopsis; *, wheat sequence used for the analysis. (PDF 178 kb)

Additional file 6: Table S2. Relative transcript levels of candidate ligin biosynthesis genes in different tissues of wheat. Transcript levels in different tissues of cv. Harvest were expressed relative to that of 4CL1 in flag leaf, which was arbitrarily set to value of 1. Data are means of 2 to 3 independent biological replicates ± SE. Means followed by different letters within each gene show statistically significant difference at P < 0.05. nd, not detected; FB, flag leaf blade; FS, flag leaf sheath; PE, peduncle; IN-1, the first internode; IN-2&3, the second and third internode; IN-4, the fourth internode (see the Methods section for numerical designation of internodes). (PDF 106 kb)

**Abbreviations**

ADF: acid detergent fiber; CAD: cinnamyl alcohol dehydrogenase; CAD-CA: coniferyl aldehyde recognizing CAD; CAD-SA: sinapyl aldehyde recognizing CAD; IN: internode; NDF: neutral detergent fiber; G: guaiacyl unit of lignin; H: hydroxyphenyl unit of lignin; S: syringyl unit of lignin.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

BTA conceived the experiments. BTA and T-NN designed the experiments and wrote the manuscript. BTA, T-NN and SHS performed the experiments and analyzed the data. MJC and DBL contributed materials and provided input into the projects direction. All authors read and approved the final manuscript.

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