A wheat caffeic acid 3-O-methyltransferase TaCOMT-3D positively contributes to both resistance to sharp eyespot disease and stem mechanical strength

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Plant caffeic acid 3-O-methyltransferase (COMT) has been implicated in the lignin biosynthetic pathway through catalyzing the multi-step methylation reactions of hydroxylated monomeric lignin precursors. However, genetic evidence for its function in plant disease resistance is poor. Sharp eyespot, caused primarily by the necrotrophic fungus *Rhizoctonia cerealis*, is a destructive disease in hexaploid wheat (*Triticum aestivum* L.). In this study, a wheat COMT gene *TaCOMT-3D*, is identified to be in response to *R. cerealis* infection through microarray-based comparative transcriptomics. The *TaCOMT-3D* gene is localized in the long arm of the chromosome 3D. The transcriptional level of *TaCOMT-3D* is higher in sharp eyespot-resistant wheat lines than in susceptible wheat lines, and is significantly elevated after *R. cerealis* inoculation. After *R. cerealis* inoculation and disease scoring, *TaCOMT-3D*-silenced wheat plants exhibit greater susceptibility to sharp eyespot compared to unsilenced wheat plants, whereas overexpression of *TaCOMT-3D* enhances resistance of the transgenic wheat lines to sharp eyespot. Moreover, overexpression of *TaCOMT-3D* enhances the stem mechanical strength, and lignin (particular syringyl monolignol) accumulation in the transgenic wheat lines. These results suggest that *TaCOMT-3D* positively contributes to both wheat resistance against sharp eyespot and stem mechanical strength possibly through promoting lignin (especially syringyl monolignol) accumulation.

Hexaploid wheat (*Triticum aestivum* L., namely bread wheat or common wheat, genome AABBDD) is one of the most widely cultivated and consumed food crops worldwide. Stem lodging in wheat is a major agronomic problem that has far-reaching economic consequences1. Crop lodging is a complicated phenomenon that is influenced by many factors from physiology and genetics to husbandry and environment2,3. Green Revolution has effectively increased lodging resistance and the harvest index through the use of semi-dwarf trait. However, a reduction in plant height for lodging resistance may reduce the photosynthetic capacity of a canopy which would then limit the final yield4. A paper reported that the optimum plant height for maximum photosynthetic capacity is between 70 cm and 100 cm in wheat5. Thus, alternative strategies for further improvement in lodging resistance of crops must be developed. Increasing the stem/culm mechanical strength has been shown to be efficient for improving crop lodging resistance6,7. Sharp eyespot is a devastating soil-borne fungal disease globally, which results in yield losses of 10–40%8,9. The necrotrophic fungus *Rhizoctonia cerealis* Van der Hoeven is a major causal pathogen of sharp eyespot. The pathogen can infect the basal stems and sheath, destroy the transport tissues of infected plants and block transportation of substances required for nutrition, leading to lodging and dead spikes. Isolation and application of important resistance-related genes make it possible to generate wheat materials with resistance to both this disease and lodging.

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Lignin is a phenolic cell wall polymer, which is composed of guaiacyl, syringyl and p-hydroxyphenyl units derived from the monolignol precursors (p-coumaryl, coniferyl, and sinapyl alcohols), respectively (Supplemental Fig. S1)10–12. Lignin is mainly deposited in the walls of certain specialized cells such as the tracheary elements, sclerenchyma and phloem fibers11. It plays pivotal roles in cell wall structural integrity, stem strength, water transport, mechanical support, and responses to various environmental and biotic stresses12–18. During plant-pathogen interactions, the deposition of lignin is thought to play a role as a physical barrier against infection. Lignification also can chemically modify cell walls to be more resistant to cell wall-degrading enzymes, increase the resistance of cell walls to the diffusion of toxins from the pathogens to the hosts and of nutrients from the hosts to the pathogens, produce toxic precursors and free radicals, and entrap the pathogens11,19. Monolignol biosynthesis genes contribute to local resistance to biotrophic and hemibiotrophic pathogens. For example, in diploid wheat (Triticum monococcum), transient knock-down of monolignol pathway enzymes [phenylalanine ammonia-lyase (PAL), caffeoyl-CoA O-methyltransferase (CCoAOMT), caffeic acid 3-O-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD)] individually and pair-wise led to decreased basal immunity or penetration resistance to the fungal pathogens Blumeria graminis f. sp. tritici and Blumeria graminis f. sp. Hordei, respectively11. In rice (Oryza sativa L.), the cinnamoyl-CoA reductase (CCR) contributed to R-mediated immunity against the hemibiotrophic fungal pathogen Magnaporthe grisea20,21. In Arabidopsis and tobacco, loss or down-regulation of PAL led to decreased basal immunity to the hemibiotrophic bacterial pathogen Pseudomonas syringae22 and to the biotrophic viral pathogen tobacco mosaic virus23,24, respectively. In Arabidopsis, through assessment on double mutant (cad-c cod-d), the genetic and functional analysis indicated that the CAD-C (At3g19450) and CAD-D (At4g34230) were the primary genes being involved in lignin biosynthesis25,26, and acted as essential components of defense response against virulent and avirulent strains of the bacterial pathogen Pseudomonas syringae pv. tomato22. Recently, Rong et al reported27 that TaCAD12 positively contributed to host defense response to a necrotrophic fungal pathogen R. cerealis27. More recent paper reported that a maize (Zea mays L.) CCoAOMT named ZmCCoAOMT2 was isolated18. ZmCCoAOMT2 has been shown to confer quantitative resistance to both southern leaf blight and gray leaf spot through altering lignin level and metabolites of the phenylpropanoid pathway and regulation of programmed cell death18. However, defense role of COMT in plant species is poorly understood.

The COMT is an O-methyltransferase that can potentially act in various branches of the phenylpropanoid pathway12,13. In gymnosperms, the COMT is believed to catalyze caffeic acid rather than 5-hydroxyferulic acid. In angiosperms, COMT can catalyze caffeic acid and 5-hydroxyferulic acid as well flavone18,28,29. The highly conserved S-adenosyl methionine (SAM) binding domain in COMT proteins indicates the use of SAM as the methyl group donor to the hydroxyl group of a methyl acceptor molecule30. COMT catalyzes the multi-step methylation reactions of hydroxylated monomeric lignin precursors31,32,33. COMT is involved in the methylation of caffeic acid to ferulic acid, which is then hydroxylated at position five by ferulate-5-hydroxylase. The subsequent methylation by COMT at this position yields sinapic acid. Similarly, COMT catalyzes the 3-O-methylation of caffeoyl aldehyde and conifer-aldehyde/coniferyl alcohol in the process of guaiacyl and syringyl monolignol biosyntheses13,12. For example, Alfalfa COMT (MsCOMT) exhibits the higher catalyzing efficiency towards 5-hydroxyconiferaldehyde and caffeoyl aldehyde rather than caffeic acid33. Interestingly, TaCM (GenBank accession number EF413031), a wheat COMT gene, was reported to locate on wheat chromosome 3BL and to encode an authentic COMT enzyme TaCM13. Kinetic analysis indicated that the TaCM protein exhibited the highest catalyzing efficiency towards caffeoyl aldehyde and 5-hydroxyconiferaldehyde as substrates, suggesting a pathway leading to syringyl lignin via aldehyde precursors13. Antisense TaCM transgenic tobacco assay indicated that the down-regulation of TaCM resulted in reduction in COMT activity and a sharp reduction in the syringl monolignol 13. It has been demonstrated that in maize, rice and sorghum (Sorghum bicolor L.), the O-methyltransferase responsible for the production of syringyl lignin was also involved in the synthesis of lignin-linked tricin13. Several groups aimed to improve the production of biofuel and generated transgenic plants with deficit/reduction of syringyl lignin unit through knock-out/down of the lignin-related COMT genes in plants13,36–39. However, no genetic evidence for defense roles of COMT genes has yet been reported in plants.

In this study, the probe with Agilent GeneChip number A_99_P198406, being homologous to certain plant COMTs, was identified to be in response to R. cerealis R0301 through comparative transcriptomic analysis on the sharp eyespot-resistant wheat line CI12633 and the susceptible wheat line Wenmai 6. Subsequently, this COMT gene located on wheat chromosome 3DL, designated as TaCOMT-3D, was cloned from the resistant wheat line CI12633. Importantly, through generation and evaluation of TaCOMT-3D- silencing and overexpressing wheat transplants, we dissected roles of TaCOMT-3D in resistance response to R. cerealis, lignin accumulation, and stem mechanical strength in wheat. The functional characterization proved that TaCOMT-3D positively contributed to syringyl monolignol biosynthesis, wheat resistance response to R. cerealis, and the stem mechanical strength.

Results
Identification, cloning, and chromosomal location of TaCOMT-3D. Microarray-based comparative transcriptomic assay was used to identify differentially expressed probes between sharp eyespot-resistant wheat line CI12633/Shanhhongmai and the susceptible wheat line Wenmai 6 inoculated with R. cerealis R0301 (microarray raw data, GEO accession number GSE69245). Among them, the probe A_99_P198406, 100% matching to 3′-terminal sequence of a wheat cDNA sequence with accession number AK332908, displayed significantly transcriptional increase in sharp eyespot-resistant wheat lines (CI12633 and Shanhhongmai) than in the susceptible wheat line Wenmai 6 at 4, 7, and 21 dpi (day post inoculation) with R. cerealis R0301 (Fig. 1A), respectively. For example, the probe A_99_P198406 showed 2.89-, 7.00-, and 18.22-fold transcriptional increase in CI12633 at 4, 7, and 21 dpi, respectively. The full-length cDNA sequence of the gene, containing the...
A complete open-reading frame (ORF), was cloned from cDNA of CI12633 stems inoculated with *R. cerealis* R0301 using nested PCR method and two pairs of primers designed based on the sequence of AK332908. The pairwise alignment showed that only one nucleotide difference exists between the cloned cDNA sequence of the gene from CI12633 and the corresponding sequence of AK332908. The gene promoter sequences were also cloned from CI12633 and Wenmai 6, respectively. The sequence analysis indicated that no difference existed between the gene promoter sequences from these two wheat lines (Supplemental Fig. S2).

To clarify the chromosome assignment of this gene, the cloned genomic sequence was subjected to alignment with the chromosome-based draft sequence of the common wheat (Chinese Spring) genome (http://www.wheat-genome.org/) (IWGSC). The alignment result displayed that this gene sequence shared 95.33%, 95.42%, and 100% identities with the matched sequences of the TGACv1_scaffold_195106_3AL, TGACv1_scaffold_220646_3B, and TGACv1_scaffold_249256_3DL, respectively. The data suggested that the gene should locate on the long arm of the wheat chromosome 3D. To verify this alignment on the chromosomal localization, genomic DNAs extracted from Chinese Spring Nulli-tetrasomic lines (NTs) were used as template of PCR using the gene-specific primers to confirm the chromosome location of the gene. The results proved that the gene was indeed located on the long arm of chromosome 3D (3DL) (Fig. 1B). Blast search against the nucleotide acid sequence database in the National Center for Biotechnology Information (NCBI) indicated that the gene sequence is homologous to COMT. Thus, this gene cloned from CI12633 was designated as *TaCOMT-3D*.

**Sequence characteristics of TaCOMT-3D.** The full-length cDNA and genomic sequences of *TaCOMT-3D* was cloned from cDNA and genomic DNA of CI12633 stems inoculated with *R. cerealis* R0301, respectively. The comparison of the cDNA and genomic sequences indicated that *TaCOMT-3D* genomic sequence with 1480 bp-length was comprised of one intron (161-bp length) and 2 exons (Fig. 2A). The *TaCOMT-3D* cDNA sequence with 1319-bp contains an ORF with 1071-bp (from 62 to 1132 nt), the 5′-untranslated region (UTR) with 61-bp, and 3′-UTR with 187-bp (Fig. 2A,B). Blast results showed that *TaCOMT-3D* was the closest to the previously reported wheat caffeic acid 3-O-methyltransferase encoding gene *TaCM* (GenBank accession no. EF413031; Ma and Xu, 2008)\(^1\) and flavonoid O-methyltransferase encoding gene *TaOMT2* (GenBank accession no. DQ223971).\(^2\) A pairwise alignment indicated that the *TaCOMT-3D* cDNA sequence shared 95.24% and 95.33% identities with *TaCM* and *TaOMT2*. The predicted protein *TaCOMT-3D* is consisted of 356 amino acids with a molecular weight of 38.697 kD and a theoretical isoelectric point of 6.042. As shown in Fig. 2B, the *TaCOMT-3D* protein contains one dimerization domain (27–78 aa) and one methyltransferase domain (121–341 aa). The comparison of amino acid sequences showed that the *TaCOMT-3D* protein shared a 98.03% identity with the wheat flavonoid O-methyltransferase *TaOMT2* and caffeic acid 3-O-methyltransferase *TaCM* (GenBank accession no. ABB03907.1). It was reported that only one amino acid difference existed between *TaCM* and *TaOMT2* proteins, in which E89 in *TaCM* was replaced by D89 in *TaOMT2*.\(^3\) As shown in Fig. 3, seven amino acid differences existed between the protein sequences of *TaCOMT-3D* and *TaCM*. Fortunately, *TaCOMT-3D* and...
TaCM proteins shared 100% identity in all the biochemically functional sites, including the SAM binding motif (LVDVGGVG), catalytic residues (H269, E297 and E329), and active site substrate binding/positioning residues (M123, N124, L129, A155, H159, F165, F169, M173, H176, V309, I312, M313, and N317) (Fig. 3). In the previous paper, both enzymatic in vitro and in vivo (transgenic tobacco) results revealed that TaCM was an authentic COMT enzyme and participated predominantly in syringl monolignol biosynthesis 13. Thus, TaCOMT-3D should be an authentic COMT enzyme, too.

The expression profile of TaCOMT-3D. To investigate if the gene expression is related with sharp eyespot-resistance, quantitative real-time PCR (qRT-PCR) technique was used to analyze the transcriptional levels of TaCOMT-3D in stems of five wheat lines/cultivars with different resistance degrees, including sharp eyespot-resistant lines CI12633 and Shanhongmai, moderately-resistant line Shannong 0431, moderately-susceptible wheat cultivar Yangmai 158, and susceptible cultivar Wenmai 6 at 21 dpi with R. cerealis R0301. The results showed that the expression levels of TaCOMT-3D were highest in Shanghongmai, slightly decreased in CI12633, gradually declined in Shannong 0431 and Yangmai 158, and reached the lowest in Wenmai 6 (Fig. 4A). The data indicated that the TaCOMT-3D transcriptional level is associated with the resistance degree of wheat against sharp eyespot caused by R. cerealis. Additionally, qRT-PCR analysis suggested that the R. cerealis biomass (represented by the expression level of R. cerealis Actin gene) increased with the pathogen inoculation duration (Fig. 4B), and the expression level of TaCOMT-3D in CI12633 was markedly elevated after infection of R. cerealis (Fig. 4C). Furthermore, the tissue expression profile of TaCOMT-3D at inflorescence stage was investigated in wheat cultivars Yangmai 16 and CI12633. As shown in Fig. 4D, at the inflorescence stage, the expression
The level of TaCOMT-3D was the highest in the inflorescence and stem tissues in both the wheat lines Yangmai 16 and CI12633, respectively, and the lowest in the leaf tissue in both Yangmai 16 and CI12633 at 21 dpi with R. cerealis R0301. These results suggested that TaCOMT-3D might participate in wheat defense response to R. cerealis infection.

Knock-down of TaCOMT-3D suppresses host resistance to sharp eyespot. The barley stripe mosaic virus (BSMV)-based virus-induced gene silencing (VIGS) technique was used to analyze rapidly the functions of TaCOMT-3D in wheat. To knockdown TaCOMT-3D expression in the resistant wheat line CI12633 through BSMV-VIGS method, a 3′-terminal fragment (233-bp) specific to TaCOMT-3D was inserted in an antisense orientation into Nhe I restriction site of the BSMV RNAγ chain to generate BSMV:TaCOMT-3D recombinant construct (Fig. 5A). When the sharp eyespot-resistant wheat CI12633 plants had been infected with BSMV for 10 d, BSMV-infected symptom was present in both BSMV:GFP- and BSMV:TaCOMT-3D-inoculated CI12633 plants (Fig. 5B), and the expression of BSMV CP gene could be detected from stems of these plants (Fig. 5C). The results indicated that these BSMV:GFP and BSMV:TaCOMT-3D viruses successfully infected these viruses-inoculated wheat plants. qRT-PCR analysis indicated that the TaCOMT-3D transcriptional level was substantially reduced in stems of BSMV:TaCOMT-3D-infected CI12633 plants (Fig. 5D), and the expression of BSMV CP gene could be detected from stems of these plants (Fig. 5C). The results indicated that these BSMV:GFP and BSMV:TaCOMT-3D viruses successfully infected these viruses-inoculated wheat plants. qRT-PCR analysis indicated that the TaCOMT-3D transcriptional level was substantially reduced in stems of BSMV:TaCOMT-3D-infected CI12633 plants, revealing that TaCOMT-3D was successfully knock-downed in BSMV:TaCOMT-3D-infected CI12633 plants (Fig. 5D). Then, these plants were further inoculated with R. cerealis isolate WK207 to evaluate the defense role of TaCOMT-3D. At 45 dpi with R. cerealis WK207, more serious symptom of sharp eyespot was present on the stems of BSMV:TaCOMT-3D-infected CI12633 plants (infection types: 3.5, 3.2, 4.3), compared with that on the stems of BSMV:GFP-treated CI12633 plants (average infection type: 2.1) (Fig. 5E). These results proved that the silence of TaCOMT-3D in CI12633 suppressed host resistance to sharp eyespot caused by R. cerealis, suggesting that TaCOMT-3D is required for the wheat resistance against R. cerealis infection.

TaCOMT-3D overexpression improves wheat resistance to sharp eyespot. The TaCOMT-3D overexpressing transgenic wheat lines were generated and used to further investigate the role of TaCOMT-3D in resistance response to R. cerealis. By using the primers specific to the transformation vector pWMB-TaCOMT-3D (Fig. 6A), PCR analysis results showed that the introduced TaCOMT-3D transgene was present in four wheat lines (OM1, OM2, OM3, and OM4) in T0-T2 generations (Fig. 6B), suggesting that the transgene could be inheritable in these four lines. qRT-PCR analyses indicated that the transcriptional levels of TaCOMT-3D in these four transgenic lines (OM1, OM2, OM3, and OM4) were markedly elevated than in wild-type (WT) Yangmai 16 (recipient) (Fig. 6C), and the introduced TaCOMT-3D was overexpressed in these four lines. The western blotting results proved that the introduced TaCOMT-3D-His was translated into the fusion protein in these four overexpressing lines, but not in WT Yangmai 16 (Fig. 6D). The sharp eyespot severity assessments in two successive (T1–T3) generations showed that compared with WT Yangmai 16, all these four TaCOMT-3D-overexpressing wheat lines (OM1, OM2, OM3, and OM4) displayed significantly enhanced resistance to sharp eyespot caused by R. cerealis R0301 (Fig. 6E, Table 1). For example, the average infection types and disease indices of these four TaCOMT-3D-overexpressing lines in T1 generation were 1.33, 1.34, 1.34, and 1.82, and 27.66, 26.70, 26.70, and 34.62, respectively, whereas the average infection type and disease index of WT Yangmai 16 were 2.08 and 41.54, respectively (Table 1). These results indicated that TaCOMT-3D overexpression improved resistance of the transgenic wheat to R. cerealis infection, and TaCOMT-3D positively participates in wheat resistance to R. cerealis infection.
Figure 4. Transcription of TaCOMT-3D in wheat (*Triticum aestivum*). (A) Expression patterns of TaCOMT-3D in five wheat cultivars with different degrees of resistance to *Rhizoctonia cerealis*. The expression level of TaCOMT-3D in the Wenmai 6 plants inoculated with *R. cerealis* for 21 days was set to 1. DI indicates disease index of sharp eyespot. (B) qRT-PCR analysis of *R. cerealis Actin* (*RcActin*) gene in CI12633 plants inoculated with *R. cerealis* at the indicated time. (C) qRT-PCR analysis of TaCOMT-3D induction by *R. cerealis* inoculation in CI12633 plants. The expression level of TaCOMT-3D at 0 day post inoculation (dpi) with *R. cerealis* is set to 1. (D) Transcription of TaCOMT-3D in roots, stems, leaves, sheaths, and spikes of Yangmai 16 and CI12633 plants. The transcriptional level of TaCOMT-3D in leaves was set to 1. Statistically significant differences are derived from the results of three independent replications (*t*-test: **P < 0.01).
TaCOMT-3D overexpression promotes lignin accumulation in stems of wheat. To explore if TaCOMT-3D affects the lignin biosynthesis in wheat, lignin content and composition in the second basal internode at milky stage were investigated. The examination data showed that the lignin content in TaCOMT-3D-overexpressing transgenic wheat lines was higher than that in WT Yangmai 16 plants (Table 2). Furthermore, hand-cut sections from the transgenic and WT plants were subjected to Wiesner and Mäule stains. When subjected to lignin-specific Wiesner reagent, cross sections of the TaCOMT-3D-overexpressing wheat lines displayed increased staining (red-brown) relative to the WT samples (Fig. 7A, B), which is indicative of a higher lignin level. When subjected to Mäule staining specific for syringyl monolignol, compared with WT samples, the TaCOMT-3D-overexpressing wheat lines displayed higher frequency of syringyl monolignol (Fig. 7C, D). These results suggested that TaCOMT-3D overexpression enhanced lignin biosynthesis, especially syringyl monolignol accumulation.

TaCOMT-3D overexpression enhances wheat stem mechanical strength. Previous study showed that TaCM expression participated in lignin synthesis and consequently contributed to stem strength and the lodging-resistant trait in wheat lodging-resistant line C6001 and lodging-susceptible wheat H45642. Here, to evaluate the effect of TaCOMT-3D on stem lodging resistance in wheat, the stem mechanical (breaking) strength of the second basal internode of TaCOMT-3D-overexpressing and WT wheat Yangmai 16 lines was measured using a Texture Analyser (TA) with a three-point bend test setup at milky and harvest stages, respectively. As shown in Table 3, comparing with WT Yangmai 16 plants, these four TaCOMT-3D-overexpressing wheat lines (OM1, OM2, OM3, and OM4) exhibited significantly increased mechanical strength of the second basal internode at both milky and harvest stages. These data suggested that overexpression of TaCOMT-3D enhanced the stem mechanical strength of the transgenic wheat.

*Figure 5.* Silencing of TaCOMT-3D by barley stripe mosaic virus (BSMV)-induced gene silencing impairs CI12633 resistance to *Rhizoctonia cerealis.* (A) Scheme of genomic RNAs of BSMV construct and the construct of the recombinant virus expressing the wheat (*Triticum aestivum*) gene TaCOMT-3D, BSMV:TaCOMT-3D. The orientation of the TaCOMT-3D insert is indicated by dark boxes. (B) Mild chlorotic mosaic symptoms were observed on leaves at 10 days post inoculated (dpi) with BSMV: GFP or BSMV:TaCOMT-3D. (C) RT-PCR analysis of the transcription levels of BSMV coat protein encoding gene CP in the wheat plants infected by BSMV: GFP or BSMV:TaCOMT-3D at 10 dpi with *R. cerealis.* (D) qRT-PCR analysis of the transcription levels of wheat TaCOMT-3D gene in the wheat plants infected by BSMV: GFP or BSMV:TaCOMT-3D at after infection with *R. cerealis.* The relative transcript level of TaCOMT-3D in BSMV:TaCOMT-3D-infected (TaCOMT-3D-silencing) wheat CI12633 plants is relative to that in BSMV: GFP-infected (control) plants (set to 1). Significant differences were analyzed based on three replications (t-test: **P < 0.01). Error bars indicate standard deviation. (E) Sharp eyespot symptoms of the control and TaCOMT-3D-silencing CI12633 plants at 45 dpi with *R. cerealis.* IT indicates the infection type of wheat plant response to *R. cerealis.*
Discussion

Plant COMT catalyzes the multi-step methylation reactions of hydroxylated monomeric lignin precursors, consequently is involved in the lignin biosynthesis. However, little is known about COMT participating in defense responses to necrotrophic fungal phytopathogens. In this study, the wheat caffeic acid O-methyltransferase encoding gene TaCOMT-3D was identified through microarray-based comparative transcriptomic analysis. The chromosome-based draft sequence alignment and PCR analysis indicated that TaCOMT-3D located on the long arm of wheat chromosome 3D. Previously, two genetic studies reported that two QTLs on 3D locating in the intervals Xgwm61~Xgwm172–240 and Xgwm314~Xgwm38341 for sharp eyespot resistance, respectively, the latter of which explained 7% of the phenotypic variation. Here, qRT-PCR analysis showed that the transcriptional
level of TaCOMT-3D was higher in sharp eyespot-resistance wheat lines than in susceptible wheat lines/cultivars. To explain the differences in the expression profile of TaCOMT-3D between resistant and susceptible wheat, the gene promoter sequences were also cloned from CI12633 and Wenmai 6, respectively. However, no difference existed between the gene promoter sequences from these two wheat lines. Thus, we deduced the different

| Lines  | Lignin content (mg/g) | P value | Significance test |
|--------|-----------------------|---------|------------------|
| OM1    | 199.53 ± 11.01        | 0.048089108 | *                |
| OM2    | 227.06 ± 22.63        | 0.039334573 | *                |
| OM3    | 228.10 ± 21.30        | 0.035968636 | *                |
| OM4    | 218.75 ± 8.81         | 0.013258457 | *                |
| WT     | 172.79 ± 6.24         | —       | —                |

Table 2. Comparison of lignin content of basal second internodes in TaCOMT-3D overexpressing transgenic and wild-type wheat lines. Significant difference between each transgenic lines and WT wheat at P < 0.05 (t-test). OM1, OM2, OM3, and OM4 indicate TaCOMT-3D-overexpressing wheat lines; WT indicates untransformed wild-type Yangmai 16.

| Lines  | Milky stage Stem strength (Newton, N) | P value | Harvest stage Stem strength (Newton, N) | P value |
|--------|---------------------------------------|---------|----------------------------------------|---------|
| OM1    | 11.85 ± 3.03*                         | 0.045244| 15.04 ± 3.51*                          | 0.010972|
| OM2    | 15.38 ± 1.94*                         | 0.043641| 13.27 ± 3.87*                          | 0.043407|
| OM3    | 14.08 ± 4.05*                         | 0.032308| 14.04 ± 3.05*                          | 0.036096|
| OM4    | 14.89 ± 2.78**                        | 0.002739| 15.39 ± 3.61**                         | 0.003181|
| WT     | 10.43 ± 1.78                          | —       | 10.44 ± 2.59                           | —       |

Table 3. Comparison of stem breaking strength of basal second internodes in TaCOMT-3D overexpressing transgenic wheat lines and wild-type wheat lines. * or ** significant difference between each transgenic lines and WT wheat at P < 0.05 or 0.01 (t-test). OM1, OM2, OM3, and OM4 indicate TaCOMT-3D-overexpressing wheat lines; WT indicates untransformed wild-type Yangmai 16.

Figure 7. Lignin staining of TaCOMT-3D-overexpressing wheat plants and wild-type (WT) wheat Yangmai 16. (A,B) Transverse sections stained using the Wiesner method. (C,D) Transverse sections stained using the Mäule method. OM1, TaCOMT-3D-overexpressing transgenic line. Bar = 200 μm.
expression profile of TaCOMT-3D between resistant and susceptible wheat may be due to the differences in regulatory pathways that activate/suppress TaCOMT-3D expression in wheat. The gene expression level was obviously elevated after infection of R. cerealis. These results imply that TaCOMT-3D may participate in defense response of wheat to R. cerealis infection. BSMV-based VIGS technique has been shown to be an effective reverse genetic tool for rapidly investigating the defense functions of interesting genes in barley and wheat. In this report, the BSMV-VIGS results show that silencing of TaCOMT-3D in the resistant wheat line CI12633 significantly impairs host resistance to R. cerealis. The data suggest that TaCOMT-3D is required for resistance response of wheat, at least the resistant wheat line CI12633, to R. cerealis infection. To further confirm the defense function of TaCOMT-3D, transgenic wheat plants overexpressing TaCOMT-3D were generated and characterized. Taken molecular assay together disease resistance assessments for T_{0}-T_{4} generations, these results indicate that TaCOMT-3D-overexpressing transgenic wheat lines display significantly enhanced resistance to sharp eyespot during whole growth stages. Interestingly, TaCOMT-3D overexpression in wheat did not obviously affect major agronomic traits (including plant height, spike number, spike length, grain number per spike, and days to heading) in wheat (Supplemental Table S1). These results reveal that TaCOMT-3D positively contributes to host resistance to sharp eyespot caused by R. cerealis infection. To the best of our knowledge, this is the first report about a COMT that participates positively in plant resistance response to a necrotrophic fungal pathogen R. cerealis. However, the TaCOMT-3D-silencing and overexpressing wheat plants at seedling and adult stages showed typically susceptible phenotype to mixed strains (E9, E21, and E23) of Blumeria graminis f. sp. tritici, suggesting that TaCOMT-3D had no obvious effect on the wheat resistance phenotype to the mixed strains of powdery mildew. It is also important and interesting to further assay responses of TaCOMT-3D silenced and overexpressing wheat plants to infection of additional pathogens in future. Anyway, the current results broaden the current knowledge of plant defenses against pathogens.

The sequence alignment of amino acid sequences showed that the TaCOMT-3D protein shares a 98.03% identity with the wheat caffeic acid 3-O-methyltransferase TaCM. Sequence analyses reveal that SAM binding motif, catalytic residues, and active site substrate binding/positioning residues of TaCOMT-3D are the same as those in TaCM, even though seven amino acid differences exist between the protein sequences of TaCOMT-3D and TaCM. Interestingly, TaCOMT-3D and TaCM proteins shared 100% identity in all the biochemically functional sites, including the SAM binding motif, catalytic residues and active site substrate binding/positioning residues. The functional studies showed that TaCM protein exhibited a strong catalyzing activity towards caffeoyl aldehyde, caffeoyl-CoA, 5-hydroxyferuloyl-CoA and 5-hydroxyconiferaldehyde, revealing that TaCM is a typical COMT involved in lignin (syringyl monolignol) biosynthesis. Additionally, among the functional sites, 13 amino acid residues being involved in the active site substrate binding/positioning residues have only one amino acid difference between TaCOMT-3D (M123, N124, L129, A155, H159, F165, F169, M173, H176, V309, I312, M313, and N317) and the alfalfa COMT (M123, N124, L129, A155, H159, F165, F169, M173, H176, V309, I312, M313, and N317; GenBank accession no. AAB46623)15,16, with V instead of I in the position 312. The crystal structure of COMT in complex with S-adenosyl-L-homocysteine, ferulic acid, and 5-hydroxyconiferaldehyde provides a structural understanding of the observed substrate preferences. And these crystal structures identify residues lining the active site surface that contact the substrates. These results suggest that TaCOMT-3D should possess the COMT activity and may participate in lignin (especially syringyl monolignol) biosynthesis.

In previous studies, assays of knock-out/down mutants/transgenic plants indicated that COMT participated predominantly in syringyl monolignol biosynthesis and can change lignin content and composition12,16-19. For example, the Brachypodium Bd5139 mutant with a single nucleotide mutation in the caffeic acid O-methyltransferase encoding gene BdCOMT6 displayed a moderately altered lignification in mature stems. Ma and Xu reported that the expression of antisense TaCM in transgenic tobacco specifically reduced the COMT enzyme activity and marginally decreased lignin content, especially sharply reduced the syringl lignin monomer. Ma reported that TaCM expression participated in lignin synthesis and was positively associated with stem strength and the lodging-resistant trait in wheat lodging-resistant line C6001 and lodging-susceptible wheat H45642. However, little has been known about effect of COMT overexpression on lignin biosynthesis and stem mechanical strength in crop plants. Here, our results indicated that overexpression of TaCOMT-3D increased lignin level, especially syringyl monolignol content. Furthermore, TaCOMT-3D overexpression enhanced the basal stem mechanical strength, and the content of lignin and syringyl monolignol had a significantly positive correlation with the culm mechanical strength, which were in line with the effect of TaCM expression on lignin synthesis and stem strength in two wheat cultivars. Previous studies documented that lignin accumulation and its composition (i.e., hydroxy-phenyl-, guaiacyl-, and syringyl-type monomers) are important factors influencing the breaking strength of wheat culm15,19. Zheng et al. showed that syringyl monolignol accumulation provided significant mechanical advantages to angiosperm species. In this report, these assays suggest that TaCOMT-3D primarily enhances syringyl monolignol accumulation, leading to the increased the basal stem breaking strength. Lignin has been implicated in plant disease resistance12,16,19. Recently, the lignin biosynthesis-related enzyme ZmCCoAOMT2 in maize has been reported to confer quantitative resistance to southern leaf blight and gray leaf spot through increasing levels of lignin and other metabolites of the phenylpropanoid pathway18. Taken together, we can deduce that TaCOMT-3D enhances wheat disease response to R. cerealis and stem mechanical strength possibly through promoting lignin, especially syringyl monolignol accumulation.

In conclusion, the wheat gene TaCOMT-3D was identified through microarray-based comparative transcriptomics. The transcriptional level of TaCOMT-3D is higher in resistant wheat lines and significantly elevated after R. cerealis infection. TaCOMT-3D positively contributes to both resistance response to R. cerealis and adult stem mechanical strength possibly through promoting accumulation of lignin (especially syringyl monolignol). This study provides novel insights into the biological roles of COMT members in plants. TaCOMT-3D is a potential gene for improving resistance to both sharp eyespot and lodging.
Materials and Methods

Plant and fungal materials. Six wheat lines/cultivars, including sharp eyespot-resistant lines CI12633 and Shanhongmai, moderately-resistant cultivar Shannong 0431, moderately-susceptible cultivars Yangmai 158 and Yangmai 16, and susceptible cultivar Wenmai 6, were used in this research. A major Jiangsu virulent strain of the pathogen _R. cerealis_ isolate R0301, was kindly provided by Profs. Huaigu Chen and Shibin Cai (Jiangsu Academy of Agricultural Sciences, China). A North China high-virulence strain of the pathogen _R. cerealis_ isolate WK207 was provided by Prof. Jinfen Yu (Shandong Agricultural University, China).

DNA or RNA extraction and cDNA synthesis. Genomic DNA for each sample was isolated from the wheat leaves using the CTAB method. Total RNA was extracted using TRizol (Invitrogen, America), and then subjected to Rnase-free Dnase I (Promega, America) digestion and purification.

The purified RNA sample (2 μg) was reverse-transcribed to cDNA using the FastQuant RT Kit with gDNase (TransGen Biotech, China).

Isolation and characterization of the _TaCOMT-3D_ sequence. The microarray analysis using the Agilent wheat microarray indicated that a probe with Agilent GeneChip number A_99_P198406, corresponding to 3′-terminal sequence of a wheat cDNA sequence with accession number AK332908, was expressed at a significantly higher level in sharp eyespot-resistant wheat lines CI12633 and Shanhongmai than in the susceptible wheat Wenmai 6. This gene cloned from stems of the resistant wheat CI12633, based on AK332908 sequence, was designated as _TaCOMT-3D_. The 1319-bp full-length cDNA sequence of _TaCOMT-3D_ was amplified by nested PCR from cDNA of CI12633 stems inoculated with _R. cerealis_ R0301 for 4 d. The primers for the first round PCR were _TaCOMT-3D-F1_ (5′-GAAAGTAGTACGAGAGGGAGTT-3′) and _AUAP_ (5′-GGCCACGGCGTCGACTAGTAC-3′). These for the second round PCR were _TaCOMT-3D-F2_ (5′-CTCGCTACACTCAACGCGC-3′) and _TaCOMT-3DL_ (5′-GGTGGATTHTTATACACACAA-3′). The deduced protein sequence was analyzed by the Compute pl/Mw tool (http://web.expasy.org/compute_pi/) to determine the theoretical iso-electric point and molecular weight, Pfam database (http://pfam.xfam.org) and smart software (http://smart.embl-heidelberg.de/) to predict conserved motifs, DNAMAN software to analyze sequence identity.

BSMV-VIGS assay for _TaCOMT-3D_ function. To generate the BSMV:TaCOMT-3D recombinant construct, a 233-bp sequence specific to _TaCOMT-3D_ (from 1022 to 1254 nucleotides in _TaCOMT-3D_ cDNA sequence) was sub-cloned into an antisense orientation into the _Nhe_ I restriction site of the RNA-γ of BSMV (Fig. 5A). Following the protocols described by Holzberg et al., the tripartite cDNA chains of BSMV:TaCOMT-3D or the control BSMV:GFP virus genome were separately transcribed into RNAs, mixed, and used to inoculate the leaves of wheat CI12633 plants at the two-leaf stage. Then, the plants were grown in a 14 h light (22 °C)/10 h dark (12 °C) regime. To investigate if BSMV successfully infected CI12633 plants, and to test if _TaCOMT-3D_ transcript was down-regulated, at 10 dpi with the BSMV virus, the fourth leaves of the inoculated seedlings were collected and subjected to analysis on the transcription of the BSMV coat protein gene (CP) by RT-PCR and the transcription of _TaCOMT-3D_ by qRT-PCR. At elongation stage, the BSMV-infected CI12633 plants were further inoculated with _R. cerealis_ isolate WK207 mycelia according to Wei et al. The infection types (ITs) of these plants were scored at 45 dpi with _R. cerealis_ WK207 following the protocol described by Chen et al.7.

_TaCOMT-3D_-overexpressing construct and transformation into wheat. The _TaCOMT-3D_ ORF plus a 6 × His epitope tag sequence was sub-cloned into monocot transformation vector pWMB122. In the resulting overexpression transformation vector pWMB-TaCOMT-3D (Fig. 6A), the transcript of the _TaCOMT-3D-His_ fusion gene is driven by a maize ubiquitin (Ubi) promoter, and terminated by 3′-non-transcribed region of _Agrobacterium tumefaciens_ nopaline synthase gene (Tnos). A total of 274 immature embryos of the wheat cultivar Yangmai 16 were transformed with through _Agrobacterium_ harbouring the pWMB-TaCOMT-3D vector according to the protocol described by Wang et al.32.

PCR detection of introduced _TaCOMT-3D_ transgene. The presence of the _TaCOMT-3D_-overexpressing transgene in the transformed wheat plants was monitored by PCR using the specific primers, _TaCOMT-3D-F_ (5′-GAGAGGTACGAGAGGGAGTT-3′, located in _TaCOMT-3D_ coding sequence) and _Nos-R_ (5′-TAAATGTGATAATGGCGGAG_3′-5′ located in _Tnos_ of the transformation vector). PCR was performed in a 25 μl volume containing ~200 ng genomic DNA, 12.5 μl 2 × Taq MasterMix (TransGen Biotech, China), 1 μl each primer (10 mM). The amplified product (318-bp size) specific to the introduced _TaCOMT-3D_ transgene was resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Analysis of transcriptional levels of target genes. qRT-PCR analysis with specific primers _TaCOMT-3D-QF_ (5′-AGAAGGTGCCCTCGGGAT-3′) and _TaCOMT-3D-QR_ (5′-GATGTAGTGATGGATG-3′) was used to investigate the relative transcriptional levels of _TaCOMT-3D_ in various wheat plants. qRT-PCR was performed using SYBR Green I Master Mix (TaKaRa, Japan) in a volume of 25 μl on an ABI 7500 RT-PCR system (Applied Biosystems). Reactions were set up using the following thermal cycling profile: 95 °C for 15 min, followed by 40 cycles of 95°C for 10 s, 56°C for 30 s, and 72°C for 32 s. The relative transcriptional levels of the target genes was calculated using the 2−ΔΔCT method, where the wheat _Actin_ gene _Actin_ was used as the internal reference. The relative transcriptional levels of the tested genes in the _TaCOMT-3D_-overexpressing wheat lines or in BSMV:TaCOMT-3D-infected wheat plants were relative to those in WT recipient or in BSMV:GFP-infected wheat plants.
The transcriptional level of BSMV CP gene in wheat plants inoculated with BSMV, an indicator of BSMV infection, was investigated by semi-quantitative RT-PCR method with the BSMV-CP gene-specific primers (BSMV-CP-E: 5′-TGACTGCTAAGGTTGGAGGA-3′, BSMV-CP-R: 5′-CGGGTGAACATCAGCAAGAT-3′).

**Western blotting analysis for TaCOMT-3D-overexpressing protein.** The TaCOMT-3D-His fusion protein in the overexpressing wheat lines was visualized via western blotting analysis. Total proteins were extracted from ~0.2 g of stems inoculated with *R. cerealis* R0301 for 20 d by using the tissue protein extraction kit (CWBio, China). Total soluble proteins (~20 μg) for each line were separated on 12% SDS-PAGE and transferred to polyvinyl difluoride membranes (Amersham). The blotting membranes were incubated with 2500-fold diluted Anti-His Mouse Monoclonal Antibody (TransGen Biotech, China) at 4°C overnight, then incubated with 4,000-fold diluted Goat Anti-Mouse IgG (H + L), HPR conjugated secondary antibody (TransGen Biotech, China) at 22–23°C for 1 h. The TaCOMT-3D-His fusion protein was visualized using the Pro-light HRP Chemiluminescent Kit (TransGen Biotech, China).

**Assessment of response of transgenic wheat plants to *R. cerealis*.** At the active tillering stage, at least 30 plants in *T1* and 110 plants in *T2* generations for each line of the TaCOMT-3D-overexpressing wheat lines and non-transgenic wheat *Yangmai 16* (recipient) were inoculated with sterilized grains harboring the well-developed mycelia of *R. cerealis* isolate R0301 following the protocol of Zhu et al.54. According to the modified protocol described by Chen et al.7, at harvest stage (about 60 dpi), the infection type (IT) of each plant was scored based on the disease lesion squares54, and disease index (DI) for each wheat line were categorized.

**Examination of stem mechanical strength.** Stem strength testing of the material was carried out using a Texture Analyser (TA) with a three-point bend test setup as described by Miller et al.55. Briefly, the TA was fitted with a load cell with maximum loading capacity of 5 kg. The support stands were set at 2.5 cm apart (across which the 5 cm stem sample was placed) and the testing probe was set to move at a constant speed of 2 mm/s. The TA, connected to a computer, produces a real-time graphical output, representing the mechanical profile of the stem sample being tested. From this graph, F max, the absolute resistance of the stem sample to break under-load, were obtained.

**Examination of lignin content and syringyl monolignol.** Lignin content was quantitatively measured by using the lignin ELISA Kit (ChemFaces, China) according to the manufacturer’s protocols. For histochemical analysis, fresh hand-cut sections were prepared from basal second internodes of wheat. Wiesner and Mäule staining methods were performed as previously described26. Briefly, Wiesner staining was performed by incubating sections in KMnO4. After 10 min, sections were washed and acidified with HCl for 1 min, washed again, and then incubated in NaHCO3. Cross sections were immediately observed under an optical microscope.

**References**

1. Acreche, M. M. & Slaper, G. A. Lodging yield penalties as affected by breeding in Mediterranean wheats. *Field Crop Res.* 122, 40–48 (2011).
2. Ma, Q. H. The expression of caffeic acid 3-O-methyltransferase in two wheat genotypes differing in lodging resistance. *J. Exp. Bot.* 60, 2763–2771 (2009).
3. Zheng, M. et al. Manipulation of lignin metabolism by plant densities and its relationship with lodging resistance in wheat. *Sci. Rep.* 7, 41805 (2017).
4. Wu, T. Y., Xie, L. Q., Yang, X. J., Zhang, C. Y. & Chen, R. F. Study on the correlations between the components of the plant height and the yield and other traits of wheat. *J. Agric. Univ. Hebei* 25, 10–18 (2002).
5. Flintham, J. E., Borner, A., Worland, A. J. & Gale, M. D. Optimizing wheat grain yield: effects of Rht (gibberellin-insensitive) lines and non-transgenic wheat *Yangmai 16* (recipient) were inoculated with sterilized grains harboring the well-developed mycelia of *R. cerealis* isolate R0301 following the protocol of Zhu et al.54. According to the modified protocol described by Chen et al.7, at harvest stage (about 60 dpi), the infection type (IT) of each plant was scored based on the disease lesion squares54, and disease index (DI) for each wheat line were categorized.
6. Boerjan, W., Ralph, J. & Baucher, M. Lignin biosynthesis. *Annu. Rev. Plant Biol.* 59, 515–524 (2008).
7. Trabucco, G. M. Improvement of multiple agronomic traits by a disease resistance gene via cell wall reinforcement. *Nat. Plants* 3, 17009 (2017).
8. Hu, K. et al. Improvement of multiple agronomic traits by a disease resistance gene via cell wall reinforcement. *Nat. Plants* 3, 17009 (2017).
9. Chen, L. et al. Overexpression of TiERFI enhances resistance to sharp eyespot in transgenic wheat. *J. Exp. Bot.* 59, 4195–4204 (2008).
10. Hu, K. et al. Mapping of QTl conferring resistance to sharp eyespot (*Rhizoctonia cerealis*) in bread wheat at the adult plant growth stage. *Theor. Appl. Genet.* 126, 2865–2873 (2013).
11. Boerjan, W., Ralph, J. & Baucher, M. Lignin biosynthesis. *Annu. Rev. Plant Biol.* 54, 519–546 (2003).
12. Tronchet, M., Balague, C., Kroj, T., Jouanin, L. & Roby, D. Cinnamyl alcohol dehydrogenases-C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in Arabidopsis. *Mol. Plant Pathol.* 11, 83–92 (2010).
13. Jia, X. L. et al. De novo assembly, transcriptome characterization, lignin accumulation, and anatomic characteristics: novel insights into lignin biosynthesis during celery leaf development. *Sci. Rep.* 5, 8259 (2015).
14. Yang, Q. et al. A gene encoding maize caffeoyl-CoA O-methyltransferase confers quantitative resistance to multiple pathogens. *Nat. Genet.* 49, 1364–1372 (2017).
15. Nicholson, R. L. & Hammerschmidt, R. Phenolic compounds and their role in disease resistance. *Annu. Rev. Phytopath.* 30, 369–389 (1992).
16. Ono, E. et al. Essential role of the small GTPase Rac in disease resistance of rice. *Proc. Natl. Acad. Sci. USA* 98, 759–764 (2001).
21. Kawasaki, T. et al. Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. *Proc. Natl. Acad. Sci. USA* **103**, 230–235 (2006).

22. Huang, J. L. et al. Functional analysis of the arabidopsis PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiol.* **153**, 1526–1538 (2010).

23. Elkind, Y. et al. Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. *Proc. Natl. Acad. Sci. USA* **87**, 19057–19061 (1990).

24. Pallada, J. A. et al. Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. *Plant J.* **10**, 281–296 (1996).

25. Sibout, R. et al. Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in Arabidopsis. Isolation and characterization of the corresponding mutants. *Plant Physiol.* **132**, 848–860 (2003).

26. Sibout, R. et al. Cinnamyl Alcohol Dehydrogenase-C and -D are the primary genes involved in lignin biosynthesis in the floral stem of Arabidopsis. *Plant Cell* **17**, 2059–2076 (2005).

27. Rong, W. et al. Wheat cinnamyl alcohol dehydrogenase TaCAD12 contributes to host resistance to the sharp eyespot disease. *Front. in Plant Sci.* **7**, 1723 (2016).

28. Zhou, J. M. et al. Sequential O-methylation of tricetin by a single gene product in wheat. *BBA-Gen Subjects* **1760**, 1115–1124 (2006).

29. Zhou, J. M. et al. Structure-function relationships of wheat flavone O-methyltransferase: Homology modeling and site-directed mutagenesis. *BMC Plant Biol.* **10**, 156 (2010).

30. Joshi, C. P. & Chiang, V. L. Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. *Plant Mol. Biol.* **37**, 663–674 (1998).

31. Bugos, R. C., Chiang, V. L. & Campbell, W. H. cDNA cloning, sequence analysis and seasonal expression of lignin-bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase of aspen. *Plant Mol. Biol.* **17**, 1203–1215 (1991).

32. Zubieta, C., Kotta, P., Ferrer, J. L., Dixon, R. A. & Noel, J. P. Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase. *Plant Cell* **14**, 1265–1277 (2002).

33. Parvathi, K., Chen, F., Guo, D. J., Blount, J. W. & Dixon, R. A. Substrate preferences of O-methyltransferases in alfalfa suggest new pathways for 3-O-methylation of monolignols. *Plant J.* **25**, 193–202 (2002).

34. Wu, X. et al. Phylogenetic, molecular, and biochemical characterization of caffeic acid o-methyltransferase gene family in *Brachypodium distachyon*. *Int. J. Plant Genomics* **2013**, 423389 (2013).

35. Edes, A. et al. ScbOMT (Bm12) is involved in the biosynthesis of tricon-lignin in sorghum. *Plos One* **12**, e0178160 (2017).

36. Jouanin, L. et al. Lignification in transgenic poplars with extremely reduced caffeic acid O-methyltransferase activity. *Plant Physiol.* **123**, 1363–1373 (2000).

37. Pincon, G. et al. Repression of O-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth. *Phytochemistry* **57**, 1167–1176 (2001).

38. Ho-Yue-Kuang, S. et al. Mutation in *Brachypodium* caffeic acid O-methyltransferase *sli* alters stem and grain lignins and improves straw saccharification without deteriorating grain quality. *J. Exp. Bot.* **67**, 227–237 (2016).

39. Shafrin, F. et al. Modification of monolignol biosynthetic pathway in jute: different gene, different consequence. *Sci. Rep.* **7**, 39984 (2017).

40. Ren, L. et al. SSR markers linked resistance QTLs to sharp eyespot (Rhizoctonia cerealis) in wheat. *Journal of Yangzhou University (Agricultural and Life Science Edition)* **33**, 1363–1373 (2017).

41. Christensen, A. H. & Quail, P. H. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res.* **5**, 213–218 (1996).

42. Wang, K., Liu, H., Du, L. & Ye, X. Generation of marker-free transgenic hexaploid wheat via an Agrobacterium-mediated co-transformation strategy in commercial Chinese wheat varieties. *Plant Biotechnol. J.* **15**, 614–623 (2017).

43. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402–408 (2001).

44. Zhou, J. M. et al. Functional analysis of the arabidopsis *PAL* gene family in plant growth, development, and response to environmental stress. *Proc. Natl. Acad. Sci. USA* **81**, 8014–8018 (1984).

45. Holzberg, S., Bressos, P., Gross, C. & Pogue, G. P. Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J.* **30**, 315–327 (2002).

46. Wei, X. et al. The wheat calcium-dependent protein kinase TaCPK7-D positively regulates host resistance to sharp eyespot disease. *Mol. Plant Pathol.* **17**, 1252–1264 (2016).

47. Christensen, A. H. & Quail, P. H. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res.* **5**, 213–218 (1996).

48. Wang, K., Liu, H., Du, L. & Ye, X. Generation of marker-free transgenic hexaploid wheat via an Agrobacterium-mediated co-transformation strategy in commercial Chinese wheat varieties. *Plant Biotechnol. J.* **15**, 614–623 (2017).

49. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402–408 (2001).

50. Miller, C. N. et al. Functional analysis of the arabidopsis PAL gene family in plant growth, development, and response to environmental stress. *Proc. Natl. Acad. Sci. USA* **81**, 8014–8018 (1984).

Acknowledgements

Authors are grateful to Professor Xingguo Ye and Dr. Demei Wang for help in generation of transgenic wheat and examining stem mechanical strength. This study was supported by the programs of the National “Key Sci-Tech” Project in China (2016ZX08002001-4) and the Chinese Key Research & Development Plan Project (2016YFD0101802).
Author Contributions
Zengyan Zhang designed the research and wrote the paper. Minxia Wang, Xiuliang Zhu, Meiying Luo, and Tianlei Shan performed the cloning, sequencing and transcriptional analyses, vector construction, molecular characterization and functional assays. Chungui Lu analyzed the data. Ke Wang conducted wheat transformation.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-24884-0.

Competing Interests: The authors declare no competing interests.

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