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Diverging cell wall strategies for drought adaptation in two maize inbreds with contrasting lodging resistance

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Abstract

The plant cell wall is a plastic structure of variable composition that constitutes the first line of defence against environmental challenges. Lodging and drought are two stressful conditions that severely impact maize yield. In a previous work, we characterised the cell walls of two maize inbreds, EA2024 (susceptible) and B73 (resistant) to stalk lodging. Here, we show that drought induces distinct phenotypical, physiological, cell wall, and transcriptional changes in the two inbreds, with B73 exhibiting lower tolerance to this stress than EA2024. In control conditions, EA2024 stalks had higher levels of cellulose, uronic acids and *p*-coumarate than B73. However, upon drought EA2024 displayed increased levels of arabinose-enriched polymers, such as pectin-arabinans and arabinogalactan proteins, and a decreased lignin content. By contrast, B73 displayed a deeper rearrangement of cell walls upon drought, including modifications in lignin composition (increased S subunits and S/G ratio; decreased H subunits) and an increase of uronic acids. Drought induced more substantial changes in gene expression in B73 compared to EA2024, particularly in cell wall-related genes, that were modulated in an inbred-specific manner. Transcription factor enrichment assays unveiled inbred-specific regulatory networks coordinating cell wall genes expression. Altogether, these findings reveal that B73 and EA2024 inbreds, with opposite stalk-lodging phenotypes, undertake different cell wall modification strategies in response to drought. We propose that the specific cell wall composition conferring lodging resistance to B73, compromises its cell wall plasticity, and renders this inbred more susceptible to drought.

KEYWORDS
cell wall polysaccharides, lignin, stalk-lodging, transcription factors, transcriptome, *Zea mays*

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1 | INTRODUCTION

During their life cycle, plants are exposed to environmental changes that influence their growth and development. In the case of maize (*Zea mays* L.), a worldwide cultivated and strategic crop, drought represents an important abiotic stress that severely impacts yield (Daryanto et al., 2016; Webber et al., 2018). In response to abiotic stressors, plants undergo multiple morphological and physiological changes (Tardieu et al., 2018), including reversible reorganisation and irreversible differentiation of their cell walls. These are responsible for providing structural support to the plant and constitute the first line of defence against environmental stresses (Swaminathan et al., 2022). Cell walls are composed of cellulose, hemicellulose, pectins (homogalacturonans (HG) and type I and type II rhamnogalacturonans (RG-I and RG-II)) and proteins. Furthermore, secondary cell walls contain phenolic compounds like lignin, or polyaliphatic macromolecules as suberin. Maize, as all commelinid monocot species, is characterised by a type II cell wall where the main hemicellulose is arabinoxylan (AX) which is partially substituted with glucuronic acid to generate glucuronoarabinoxylans (GAX) instead of xyloglucan (XG), typically present in type I cell walls, as cited by Penning et al. 2019.

Several studies showed that the expression of cell wall genes (CWGs) is differentially affected by drought in maize inbreds (Hu et al., 2009; Miao et al., 2017; Min et al., 2016; Morari et al., 2015; Zenda et al., 2019; Zheng et al., 2010). Cell wall loosening constitutes a common response to several abiotic stresses and is induced by the increased activity of some xyloglucan endo-transglucosylase hydrolases (XEH) and expansins (Le Gall et al., 2015). Other changes include modifications of the RG-I side chains and the reinforcement of secondary cell walls by increasing hemicelluloses and lignin deposition. In this way, it has been proposed that a common set of cell wall modifying enzymes facilitate plant adaptation to several stresses (Houston et al., 2016).

Lignin content and composition influences the rigidity and water permeability of the secondary cell wall, which in several plants is critical for drought adaptation (Hu et al., 2009; Liu et al., 2018; Liu et al., 2021). Lignin content increases in many plant species under drought conditions (Liu et al., 2021). However, in maize, this nexus is unclear, as drought was observed to either increase or decrease lignin levels in an inbred-dependent manner (Alvarez et al., 2008; Hu et al., 2009; Phillips & Ludidi, 2017). Transcription factors (TFs) described to be involved in drought tolerance, were observed to bind the promoters of different lignin biosynthetic and drought-related genes (Bang et al., 2019; Cao et al., 2021; Sun et al., 2020; Xu et al., 2020).

As drought stress affects cell wall rigidity, it can also influence the mechanical strength of the stalk, leading to lodging and the consequent grain yield losses (Flint-Garcia et al., 2003; Kumar et al., 2021; Wang et al., 2023). In a previous study, our group characterised a collection of maize inbreds and determined that two of them, B73 and EA2024, had a contrasting stalk-lodging resistance, being B73 a lodging-resistant inbred, while EA2024 is lodging-susceptible (Manga-Robles et al., 2021). We also found that these two inbreds have different cell wall compositions, suggesting that the expression of several CWGs may differ between them. Consistent with this notion, the expression of around 70% of CWGs was also reported to differ during normal stem development between B73 and Mo17 maize inbreds (Penning et al., 2019) having different lignin abundance and enzyme digestibility of stem walls (Penning et al., 2014).

At present, very little is known about the impact of drought on maize varieties in relation to their stalk-lodging resistance. Thus, in this work we characterised the biochemical changes in the cell walls of B73 and EA2024 upon drought stress. We also studied the changes occurring in the transcriptome of these two inbreds in response to drought and identified those CWGs that are affected in an inbred-specific manner. Inbred-specific transcriptional regulatory networks were also identified as candidates to coordinate the differential expression of cell wall genes in B73 and EA2024 upon drought. In sum, our results highlight the different strategies undertaken by two maize inbreds with contrasting stalk-lodging resistance to restructure their cell wall metabolism upon drought stress.

2 | RESULTS

2.1 | Phenotypical responses of B73 and EA2024 to drought stress

Maize B73 and EA2024 inbreds were grown in a culture chamber until three leaves stage before being subjected to drought by stopping watering for 10 days. At a macroscopic level, drought affected B73 more severely than EA2024 (Figure 1). To estimate the impact of severe drought on both lines, leaf relative water content (RWC) was determined at the end of 10-days treatment (Figure 1), hence observing that EA2024 leaves tend to retain more water compared to B73, both in control and drought conditions.

Gas exchange and chlorophyll fluorescence were measured to assess plant performance (Supplementary Figure 2). In control conditions, the two inbreds had similar photosystem II quantum yields (ΦPSII), electron transport rates (ETR), CO_2 assimilation rates (A), and intercellular CO_2 concentration (Ci), but EA2024 had a significantly lower transpiration rate (E) than B73. The intercellular CO_2 concentration was increased by drought in EA2024, but was not affected in B73. Twenty-five days after the cessation of watering, plants were rewatered to test the ability of the two inbreds to recover after extreme drought. Remarkably, while B73 did not survive drought stress, EA2024 was able to fully recover its normal phenotype after 1 week of rewatering (Figure 1).

Overall, these results indicate that B73 and EA2024 plantlets display different physiological responses to drought, being the stalk-lodging resistant B73 inbred drought-sensitive, and the lodging susceptible EA2024 more drought-tolerant.

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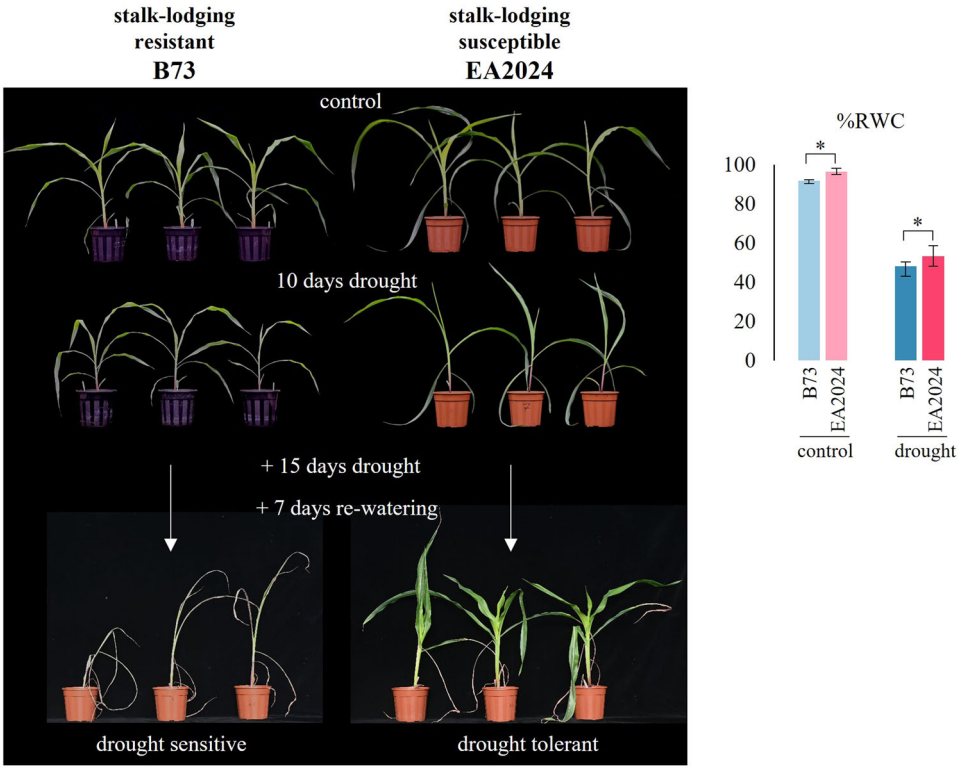

FIGURE 1 Phenotype alterations in B73 and EA2024 submitted to drought. Top: Phenotype of 20-days old B73 and EA2024 plants grown in well-watered (control) or drought conditions during the last 10 days. Bottom: Phenotype of 42-day old B73 and EA2024 plants grown in drought conditions for 15 additional days and then rewatered for 7 days. Right: Relative water content (%RWC) expressed as percentage of water retained by B73 and EA2024 leaves at the end of 10 days drought treatment. Data are expressed as the mean ± SD of the RWC value of two biological replicates, each comprising leaves pooled from five plants, and three technical replicates. Asterisks refer to significant differences ($p < 0.05$).

2.2 | Effect of drought on the cell wall remodelling in B73 and EA2024

To study the impact of drought on cell wall composition, we determined the lignin and polysaccharides content in stems of B73 and EA2024 plants subjected for 10 days to drought conditions. Lignin content was determined by the Klason assay and its composition by thioacidolysis-GC/MS. These studies showed that whereas drought did not affect lignin content in B73, it was reduced by 20% in EA2024 stems (Figure 2). By opposite, drought did not affect lignin composition in EA2024, while a reduction of H monomers and an increase of S monomers and of the S/G ratio was observed in B73 (Figure 2). In both inbreds, drought increased levels of H, G, and S units released by thioacidolysis, suggesting a less condensed state of lignin under drought conditions.

Regarding cell wall polysaccharides, cellulose content was higher in EA2024 than in B73 in control conditions, and it increased by 10% in both inbreds when they were subjected to drought (Figure 3). Total hemicelluloses, quantified as arabinose plus xylose content, were similar in both inbreds and they were unaffected by drought, while a 60% glucose increase was detected in EA2024 cell walls after the drought treatment (Figure 3), as well as a 60% increase of uronic acids in B73 (Figure 3). We also quantified the amount of ester-linked hydroxycinnamates (HCA) in cell walls of these plants (Figure 4). EA2024 cell walls displayed higher levels of esterified *p*-coumarates compared to B73, and no changes were observed under stress conditions. Esterified ferulates, and cross-linked diferulates were similar in both inbreds and no significant changes were observed in the drought-stressed plants (Figure 4). Finally, drought induced a slight but significant increase of the ferulate/*p*-coumarate ratio in EA2024 cell walls (Figure 4).

Cell wall fractionation was also performed to determine whether drought affected polysaccharide distribution among the calcium-bridged pectins (CDTA fraction), loosely crosslinked hemicelluloses (KI fraction) and strongly crosslinked hemicelluloses (KII fraction) (Figure 5). In B73, drought increased the total sugars content (extractable hemicelluloses) in the KII fraction without affecting CDTA and KI fractions, thus resulting in an increase in the KII/KI ratio. In EA2024, drought caused a slight decrease in the CDTA extractable pectins, but no alteration in hemicellulose partitioning. Also, B73 and EA2024 cell walls responded differently to drought regarding uronic acids content and distribution (Figure 5). EA2024 cell walls had higher levels of crosslinked uronic acids in control conditions compared to B73 (Figure 3), even if its content in the CDTA fraction was lower in EA2024 as compared to B73 (Figure 5). Upon drought, the uronic acids level in this fraction increased in EA2024, while it decreased in B73 (Figure 5). However, the most prominent changes in uronic acids content upon drought occurred in the B73 KI and KII fractions, with a 1.5- and 3.5-fold increase, respectively (Figure 5).

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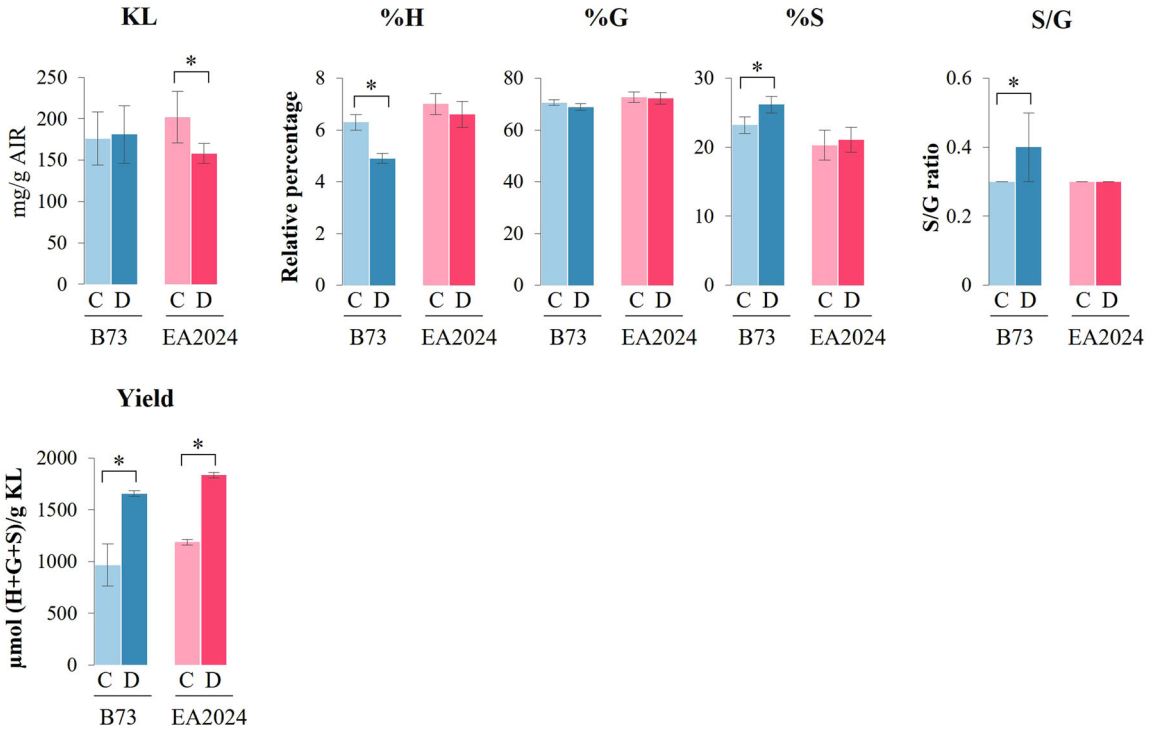

FIGURE 2 Lignin content and composition of B73 and EA2024 grown in control and drought conditions. Lignin content was determined by the Klason assay and the relative amounts of the three main subunits was determined by thioacidolysis GC-MS. "KL" refers to Klason Lignin. "Yield" refers to thioacidolysis yield. "AIR" refers to Alcohol Insoluble Residue. "C" and "D" refer to control and drought conditions, respectively. Data are presented as the mean of two biological replicates, each comprising the entire aerial part pooled from five plants, and three technical replicates. Asterisks refer to significant differences of plants grown in drought conditions compared to the control group, determined by a Student's-*t* test with $p < 0.05$.

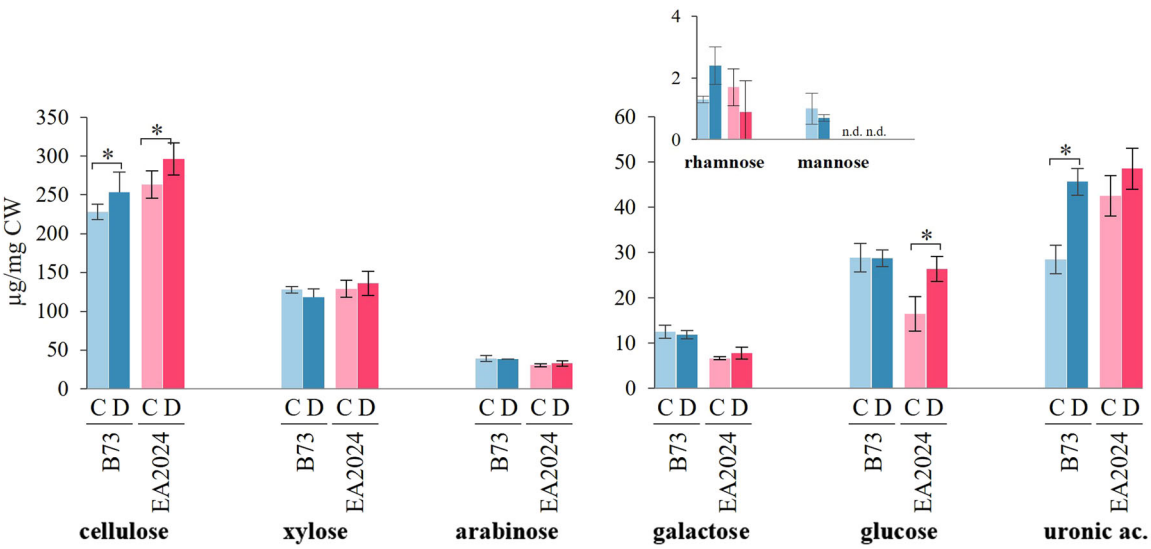

FIGURE 3 Cell wall polysaccharide composition in B73 and EA2024 grown in control and drought conditions. "HC" refers to hemicellulose. "n.d." means not detected. "C" and "D" refer to control and drought conditions, respectively. Data are presented as the mean ± SD of two biological replicates, each comprising the entire aerial part pooled from five plants, and three technical replicates. Asterisks refer to a significant difference of plants grown in drought conditions compared to the control group, determined by a Student's-*t* test with $p < 0.05$.

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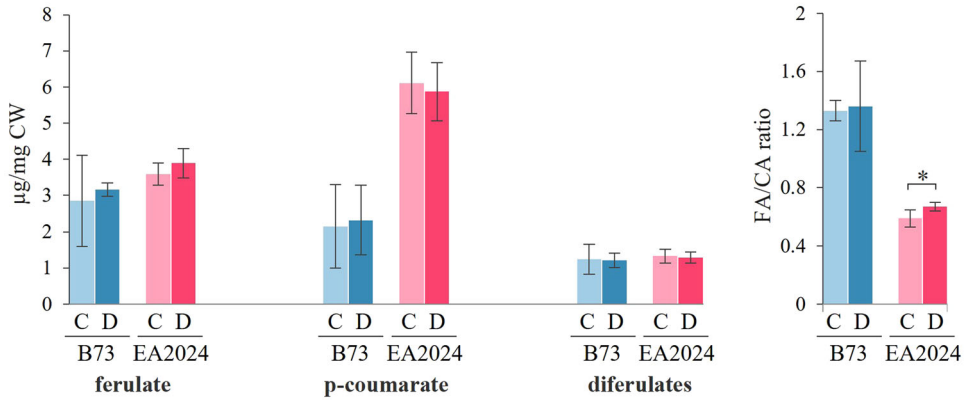

FIGURE 4 Cell wall hydroxycinnamate composition in B73 and EA2024 grown in control and drought conditions. Diferulates were calculated as the sum of 5-5, 8-O-4, 8-5-open and 8-5 benzofurans forms. "FA/CA" refers to the ferulate/*p*-coumarate ratio. "C" and "D" refer to control and drought conditions, respectively. Data are presented as the mean ± SD of two biological replicates, each comprising the entire aerial part pooled from five plants, and three technical replicates. Asterisks refer to a significant difference in plants grown in drought compared to the ones of the control plants, determined by a Student's-*t* test with $p < 0.05$.

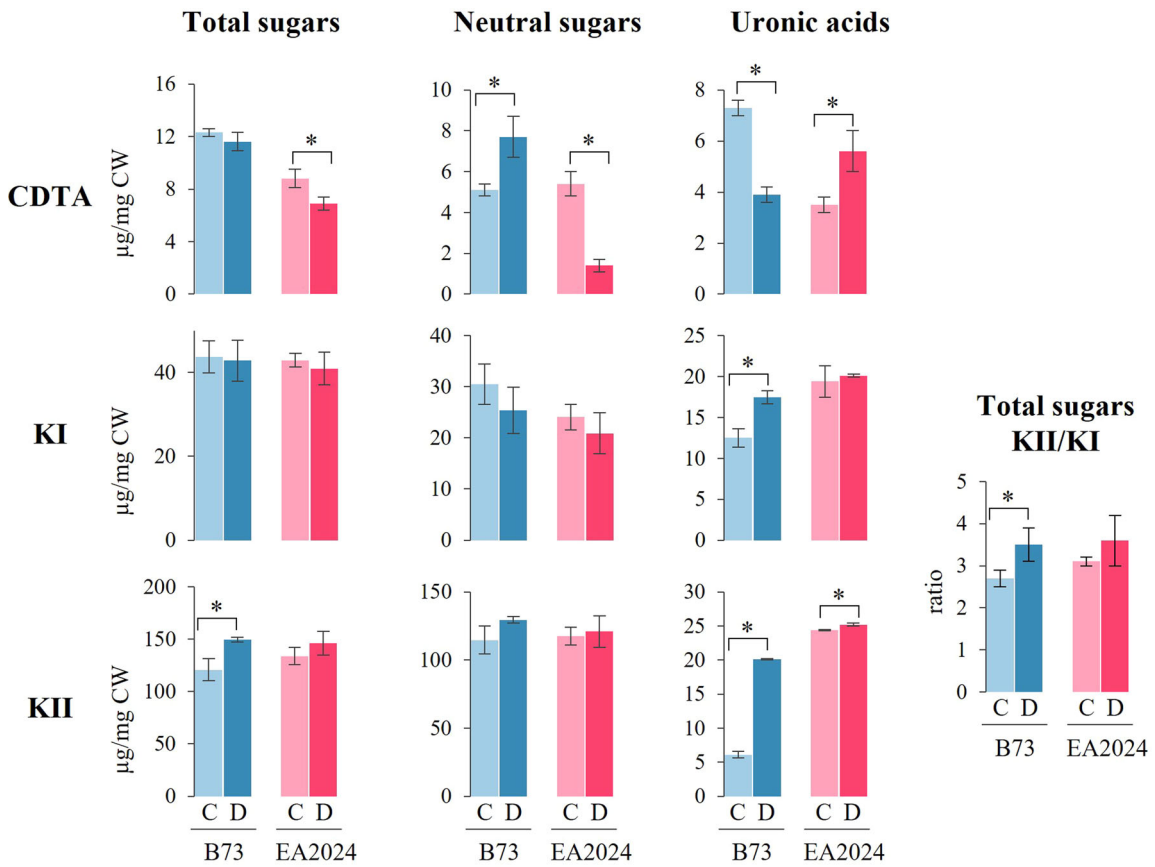

FIGURE 5 Total sugar content in hemicellulose fractions from cell walls B73 and EA2024 grown in control and drought conditions. "CDTA" refers to Ca^{2+}-bridged pectins extracted with CDTA. "KI" refers to loosely-crosslinked hemicelluloses extracted with 0.1 N KOH-0.1 N. "KII" refers to strongly-crosslinked hemicelluloses extracted with 4 N KOH. The ratio of total sugars present in KII fraction to the ones in KI fraction is shown on the right side of the figure. "C" and "D" refer to control and drought conditions, respectively. Data are presented as the mean ± SD of two biological replicates, each comprising the entire aerial part pooled from five plants, and three technical replicates. Asterisks refer to a significant difference of plants grown in drought conditions compared to the control group, determined by a Student's-*t* test with $p < 0.05$.

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We used a comprehensive immunodetection assay (IDA) and heatmap analysis to characterise the cell wall polysaccharides fine structure (substitution pattern and degree of methylation) of the CDTA, KI, and KII fractions (Figure 6 and Table S1). In drought conditions, B73 showed an increase in low methyl-esterified homogalacturonans (HG) (detected using JIM5 antibodies) and a reduction of arabinan side chains in rhamnogalacturonan I (RG-I) (LM6 antibodies) of the KI fraction as compared to control plants. In the KII fraction, drought decreased the levels of RG-I arabinan side chains, while increased the levels of galactan side chains (LM5 antibodies).

Regarding EA2024, more pronounced alterations in its cell walls were observed in response to drought compared to those noted in B73. The CDTA fraction had higher levels of low methyl-esterified HG, while their levels in the KI fraction were lower than in controls. The KI fraction also had higher levels of partially methyl-esterified HG (JIM7 antibodies), arabinogalactan proteins (LM2 antibodies) and non-fucosylated xyloglucans (LM15 antibodies). In the KII fraction, the arabinan side chains of RG-I were increased by drought. In sum, although following drought treatment differences were observed in the three cell wall fractions of both inbreds, strongest alterations were detected in the EA2024 KI fraction.

2.3 | Changes in CWGs gene expression in response to drought

To comprehensively explore the transcriptional dynamics during drought in B73 and EA2024 inbred lines, samples were collected at three distinct time point – 7, 9 and 10 days after cessation of watering. Subsequently, the extracted RNA from these samples underwent pair-end RNA-Sequencing, with reads mapped to the B73 reference genome. The reads exhibited similar mapping pattern for both inbreds (Table S2). Principal Component Analysis (PCA) of the read data revealed that 94% variance of these datasets could be attributed to two main factors: 66% due to inherent differences between inbreds (PC1), and 28% associated with the drought response (PC2) (Figure S3).

Utilising DESeq2 package, we identified genes as differentially expressed in plants subjected to 10 days drought with respect to control when the $\log_2$ Fold Change ($\log_2$FC) was higher than 1 or lower than −1, with an adjusted *p*-value of less than 0.05. At the full transcriptome scale, drought affected the expression of 29% B73 genes and 21% EA2024 genes (Figure 7). To assess the impact of drought on cell wall-related gene expression, we gathered a list of 1485 genes related to cellulose, hemicellulose, pectins, genes encoding membrane-bound and cell wall proteins, lignin, suberin, callose, proteases and transcription factors (TFs)

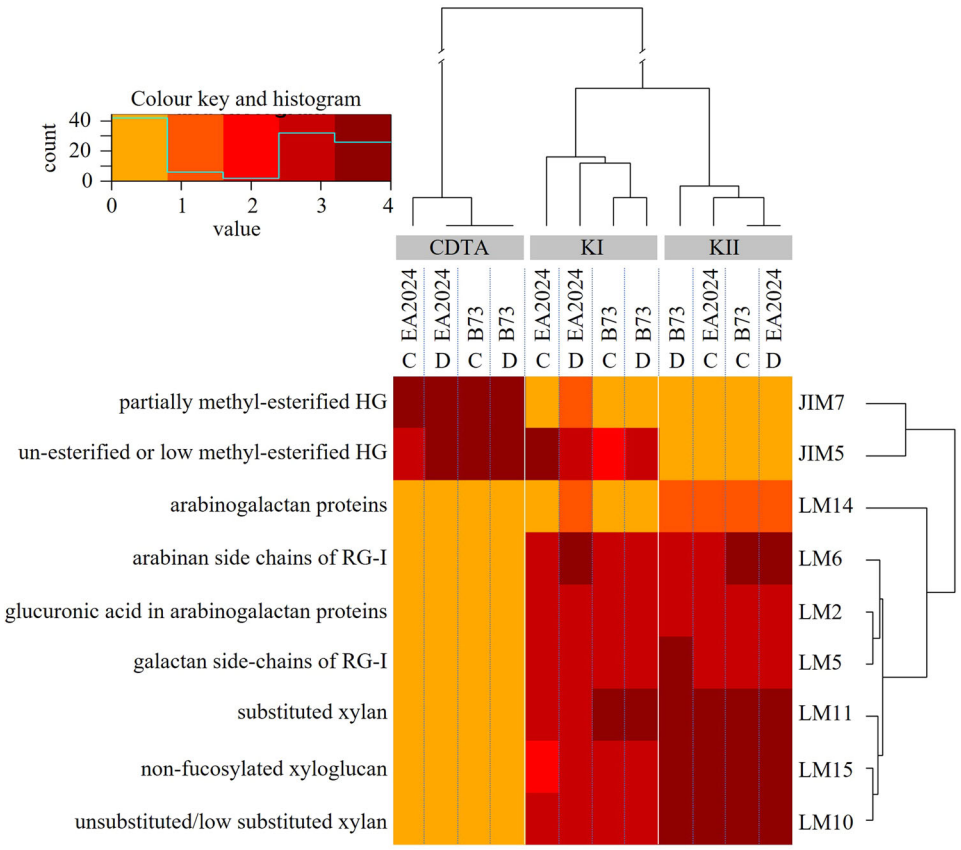

FIGURE 6 Heatmap of the immunodetection of cell wall epitopes. Analysis of the calcium-bridged pectins (CDTA), the loosely-crosslinked hemicelluloses (KI), and the strongly-crosslinked hemicelluloses (KII) cell wall fractions of B73 and EA2024 grown in control and drought conditions. "C" and "D" refer to control and drought conditions, respectively. The commercial antibodies used are indicated on the right side of the heatmap. The epitope recognised by each antibody is shown on the left side of the heatmap. "HG" refers to homogalacturonan and "RG-I" refers to rhamnogalacturonan I.

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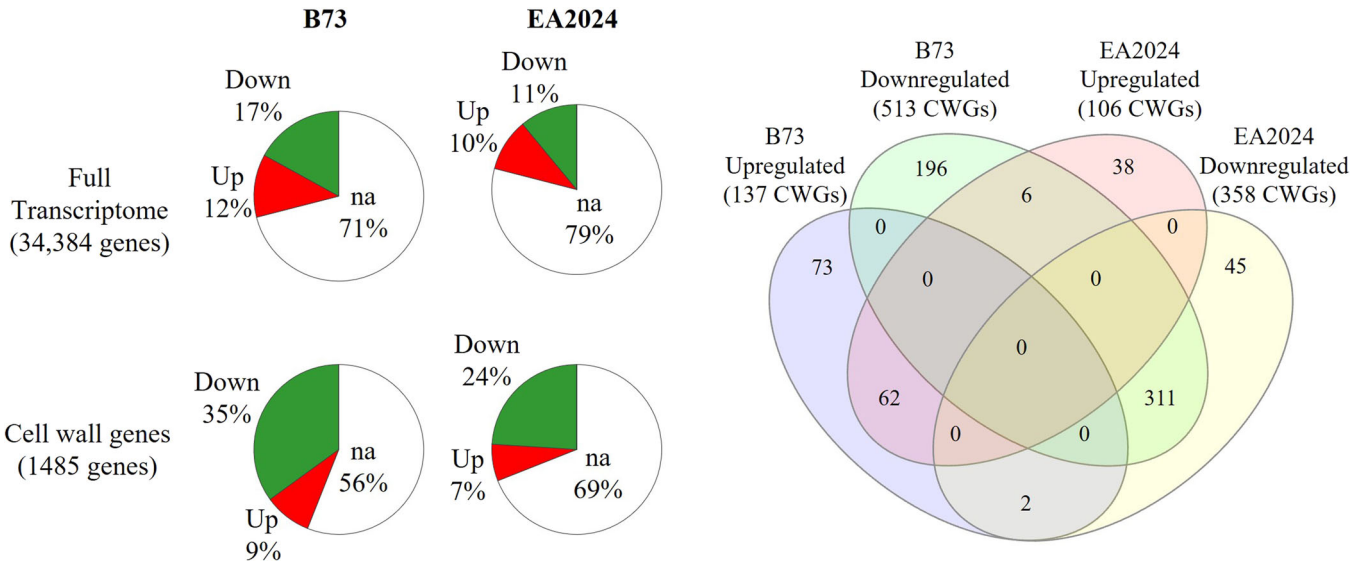

FIGURE 7 Percentage of genes whose expression is altered by 10 days of drought in the full genome and in the cell wall gene catalogue. Left: in white, percentage of genes whose expression is not affected (na). In green, the percentage of genes that are downregulated (Down). In red, the percentage of genes that are upregulated (Up). The whole transcriptome and Cell Wall Genes (CWGs) catalogue correspond, respectively, to 34,384 and 1485 genes. Right: Venn diagram distribution of the cell wall genes. The total number of cell wall genes whose expression is affected at 10 days of drought in B73 and/or EA2024 inbred lines is indicated in parentheses.

known or predicted to be involved in cell wall biosynthesis (Data S1). Out of this CWGs list, 44% were affected by drought in B73 and 31% in EA2024 (Figure 7). For both B73 and EA2024, the percentage of genes affected by drought was higher in the CWG subset than in the total genome.

Venn diagram analyses showed that several CWGs were differentially expressed in both B73 and EA2024, while others were altered only in one inbred (Figure 7), in addition to underscore that the number of drought repressed CWGs was higher than those induced. Of the 1485 genes in the CWGs catalogue, 513 (34.5%) were repressed in B73 and 358 (24.1%) in EA2024. Furthermore, 311 were repressed in both inbreds, hence accounting for 60.6% and 87% of the repressed CWGs in B73 and EA2024, respectively. In contrast, 196 CWGs (38.2%) were downregulated only in B73, while 45 (12.6%), only in EA2024. Concerning the upregulated genes, 137 CWGs (9.2%) were induced in B73 and 106 (7.1%) in EA2024. As before, 62 of those were induced in both inbreds, representing 45% and 58.5% of all induced CWGs in B73 and EA2024, respectively, whereas 73 (53.3%) were induced only in B73 and 38 (35.8%) only in EA2024 (Figure 7).

Interestingly, eight CWGs displayed an opposite response in both inbreds upon 10 days of drought (Figure 7). Specifically, two genes – a cinnamate 4-hydroxylase, C4Hb, of the lignin/phenylpropanoid pathway, and a sucrose synthase, putatively involved in the synthesis of cell wall polysaccharides – were induced in B73 and repressed in EA2024. Conversely, three cell wall peroxidases involved in lignin biosynthesis or reactive oxygen species (ROS) generation, one polygalacturonase involved in pectin degradation, one glycosyl-transferase 14 responsible of transferring glucuronic acid to galactans of arabinogalactans (Xuan et al., 2021), and one metallo-endoproteinase putatively involved in cellulose and callose deposition, were found to be downregulated in B73, while induced in EA2024.

2.4 | Impact of drought on the expression of different CWGs functional categories

Since the impact of drought on CWGs expression was higher in B73 than in EA2024, we investigated whether drought affected different CWGs categories. We grouped CWGs into five categories: genes involved (or putatively involved) in (1) cellulose and hemicellulose biosynthesis (C/HC), (2) pectin biosynthesis, (3) encoding membrane-bound and cell wall proteins (MB/CWP), (4) lignin biosynthesis (including cell wall peroxidases and laccases), and (5) suberin and callose deposition (Data S1, sheet 1). Genes in each category affected by drought were around 50%, but this ratio was the highest (55%) for genes belonging to the category "lignin" (Figure 8). We then separated genes in each category among those affected in both inbreds, affected only in B73, or only in EA2024. In the categories "C/HC" and "lignin", the number of genes affected specifically in one inbred was higher than 50%. For "C/HC", 90 and 21 genes were affected only in B73 and only in EA2024, respectively. In fact, the number of genes affected only in B73 was even higher than those affected in both inbreds (83 genes). A similar situation held true for genes belonging to the category "lignin" where of the 170 drought-affected genes, 64 and 30 were affected only in B73 and only in EA2024, respectively (Figure 8). This was in clear contrast with the rest of categories (pectins, MB/CWP, suberin and callose, and others), for which most Differentially Expressed Genes (DEGs) were common to both inbreds. Together, these studies revealed that genes involved in cellulose, hemicellulose, and lignin biosynthesis were mainly affected by drought in B73, whereas those involved in the biosynthesis of pectins, membrane-bound and cell wall proteins (and "others") were similarly affected in both inbreds.

We thus studied more in detail the effect of drought on genes belonging to the different functional categories. Concerning cellulose biosynthesis (Figure 9), although cell wall cellulose levels were

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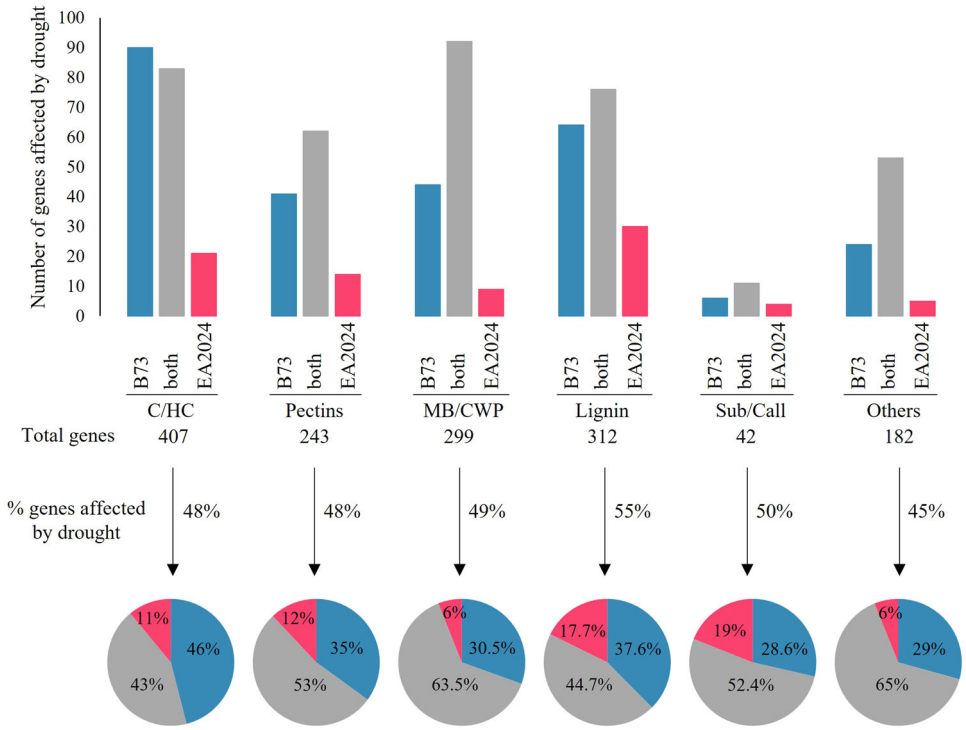

FIGURE 8 Cell wall genes (CWGs) whose expression is affected by 10 days of drought. Top: CWGs are distributed in five specific functional categories plus "others". "C/HC" refers to the cellulose and hemicellulose genes. "MB/CWP" refers to membrane-bound and cell wall proteins. "Sub/Call" refers to suberin and callose genes. The total number of CWGs in each category is indicated at the bottom. Bottom: the percentage of genes whose expression is affected by drought in each category is indicated on the upper side of the pie charts. The percentage of genes that is affected only in B73 (blue), only in EA2024 (pink) and in both inbreds (grey) with respect to the total number of genes that are affected by drought in each category is indicated within pie charts.

elevated by drought, most cellulose synthase A (CesA) genes were downregulated in both inbreds, and only CesA2 was induced in B73. Drought effects on the expression of genes involved in hemicellulose biosynthesis were however different in the two inbreds, with the most remarkable differences observed for genes of the glycosyl transferase (GT) family. Even though repression was generally more frequent than induction, six out of the nine differentially regulated GTs in EA2024 were upregulated, while they did not change in B73. By contrast, a different set of GTs were differentially regulated only in B73, of which 10 were induced and 24 repressed (Figure 9).

As compared to EA2024, B73 was more affected in genes involved in pectin biosynthesis (Figure 9). This was the case for polygalacturonases (PG), pectin methyl esterases (PME) removing methyl esters from pectins, and pectin lyase (PL), which, together with GT8 family genes, are involved in pectin degradation. With respect to the glycosyl/galactosyl transferase (G/GalT) gene family, a higher number of genes were affected by drought in EA2024 than in B73. On the other side, only GH35, which mediates hydrolysis of terminal nonreducing β-D-galactose residues of pectins, was repressed by drought in EA2024. Drought also affected expression of several families of membrane-bound and cell wall proteins (Figure 9), with those specifically affected in B73 being always higher than those of EA2024.

In the case of suberin, multiple GDSL esterase/lipases (GELPs), implicated in the polymerisation or degradation of suberin, were repressed in both inbreds (Figure 9). In addition, some GELPs were repressed only in B73, while a single one was repressed only in EA2024. Similarly, a feruloyl-CoA transferase (FCoAT) and several HCT genes, with a role in suberin biosynthesis, were repressed in both inbreds. Several callose synthase genes were induced only in EA2024, while none was induced or repressed in B73 upon drought.

Most steps of the lignin biosynthetic pathway were found to differ between B73 and EA2024 upon drought stress (Figure 10). Drought repressed three phenylalanine ammonia-lyases (PALs) only in B73, while other three were induced only in EA2024, including the PAL2 gene that is the closest homologue to the PTAL enzyme described in *Brachypodium distachyon* (Barros et al., 2016). Curiously, no other genes in the lignin pathway were induced by drought only in EA2024 (Figure 10). A cinnamate 4-hydroxylase, C4Ha, was induced only in B73 and C4Hb was induced in B73 but repressed in EA2024. The 4-coumarate 3-hydroxylase/ascorbate peroxidase (C3H/APX), which converts *p*-coumaric acid into caffeic acid (Barros et al., 2019), and two *O*-methyltransferase genes were induced only in B73, suggesting that, in this inbred, drought triggered the flux from *p*-coumaric acid to ferulic acid.

Drought also affected the expression of other genes of the lignin pathway in an inbred-specific manner. Curiously, several cinnamyl alcohol dehydrogenase (CAD) and BAHD

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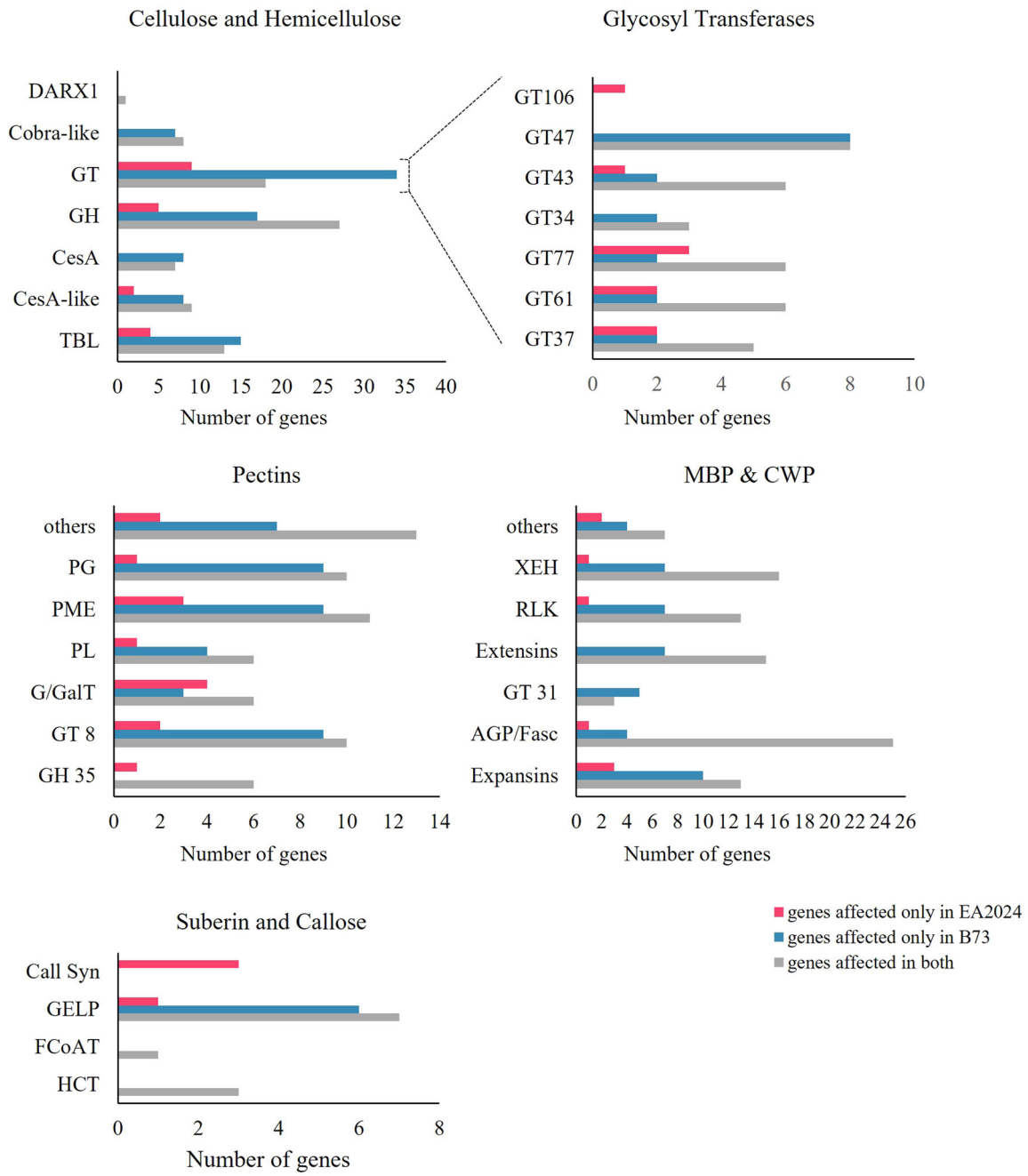

FIGURE 9 Distribution of genes affected by drought in B73 and/or EA2024 among functional CWG categories. Top left: distribution of genes involved in the biosynthesis of cellulose and hemicelluloses. Top right: detail of GT genes. Middle left: distribution of genes involved in the biosynthesis of pectins. In "others" are included genes encoding GH3, GT106, *O*-fucosyl transferases, pectin acetyl transferases, pectinesterases, pectinesterase inhibitors, PG inhibitors and rhamnogalacturonan-I lyases. Middle right: distribution of genes encoding membrane-bound proteins (MBP) and cell wall proteins (CWP). In "others" are included genes encoding germin and yeldin proteins. Bottom left: distribution of genes involved in the biosynthesis of suberin and callose. The grey columns refer to genes affected in both inbreds; blue columns refer to genes affected only in B73; and the pink columns refer to genes affected only in EA2024.

acyltransferases closely related to *p*-coumaroyl-CoA monolignol transferase (PMT) (Marita et al., 2014) were downregulated only in B73. Finally, several cell wall peroxidases and laccases exhibited inbred-specific regulation in B73 and EA2024. Notably, three peroxidases were repressed in B73 but induced in EA2024 (Figure 10). In addition, a set of lignin genes were also found to be affected by drought in both inbreds (Figure S4).

2.5 | Dynamics of CWGs expression in B73 and EA2024

To investigate the CWGs drought responsive pattern over the course of 7-, 9- and 10-days, we used the maSigPro package with a cutoff of R > 0.6 (see Materials and Methods for details). These analyses grouped 1000 out of the 1485 CWGs into nine clusters based on different expression

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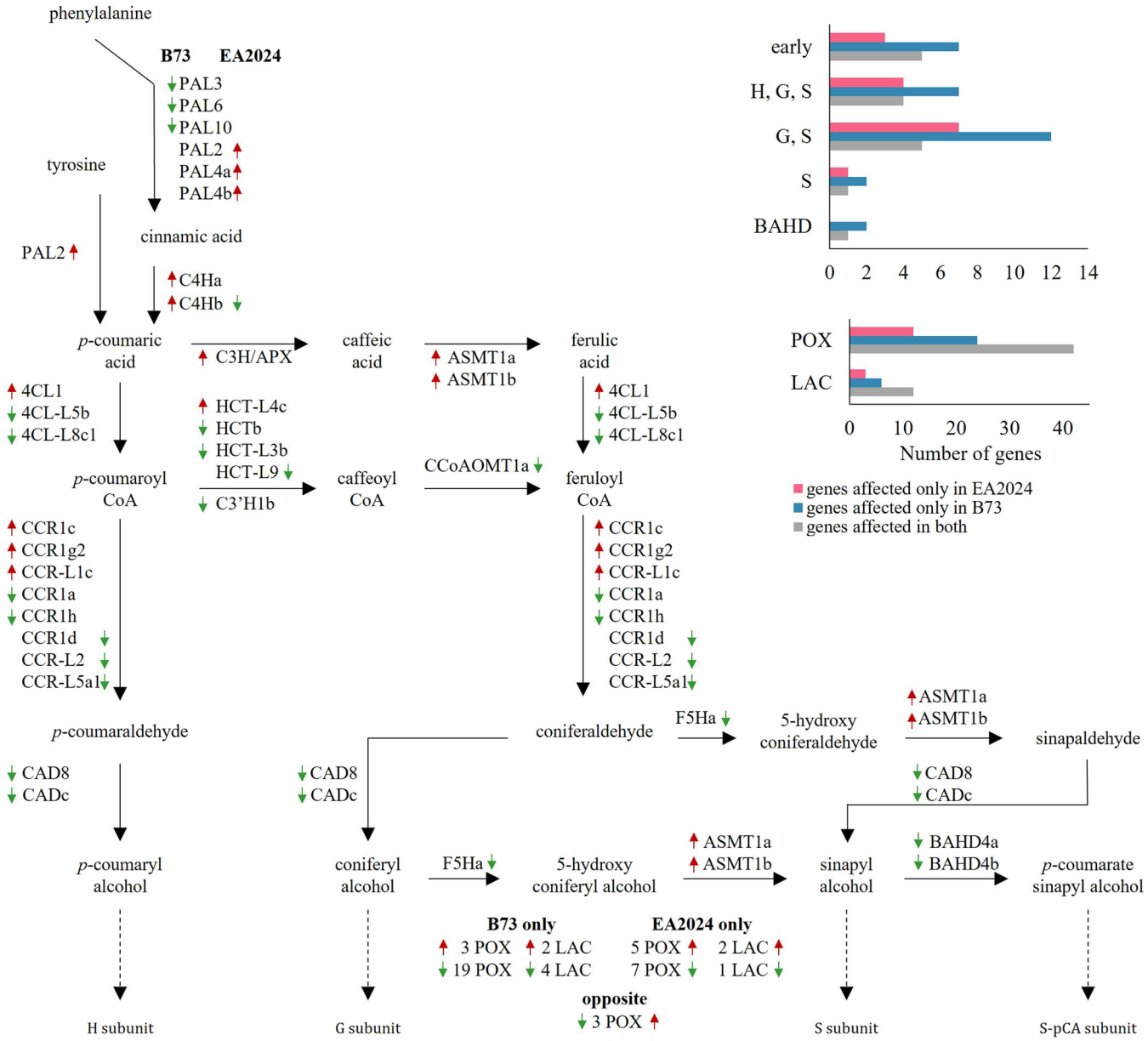

FIGURE 10 Schematic representation of the lignin biosynthetic pathway in maize. Lignin genes that are affected by 10 days drought only in one inbred are shown. The green and red arrows refer to gene repression and induction, respectively. Arrows at the left side of the gene indicate changes in gene expression in B73, while arrows at the right side correspond to alterations in EA2024. Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate CoA ligase (4CL), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), hydroxycinnamoyl transferase (HCT), 4-coumaroyl shikimate/quinate 3′-hydroxylase (C3′H), 4-coumarate 3-hydroxylase/ascorbate peroxidase (C3H/APX), caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H), caffeic acid *O*-methyltransferase (COMT) genes, *p*-coumaryl-CoA monolignol transferase (PMT family of BAHD), cell wall peroxidases (POX) and laccases (LAC). Broken arrows mean monolignol transport to the cell wall. PAL2 is the closest homologue to the PTAL enzyme described in *Brachypodium distachyon* (Barros et al., 2016). The cell wall localisation of peroxidases was performed using the DeepLoc 2.0 website (Thumuluri et al., 2022). Broken arrows refer to the transport of the monolignols to the cell wall, where they will be polymerised to generate the *p*-hydroxyphenyl (H), guaiacyl (G), sinapyl (S), and sinapyl *p*-coumarate (S-pCA) subunits of the lignin polymer. On the right side (top): distribution of lignin genes affected by 10 days of drought in B73 and/or EA2024. "Early" corresponds to the PAL, C4H and 4CL genes. "H, G and S" correspond to the CCR and CAD genes. "G, S" corresponds to the HCT, C3H, and CCoOAMT genes. "S" corresponds to F5H and COMT genes. BAHD refers to PMT. The grey columns refer to genes affected in both inbreds; the blue columns refer to genes affected only in B73; and the pink columns refer to genes affected only in EA2024.

patterns (Figure 11 and Data S1, sheet 1). Cluster 1 contains CWGs showing a similar transcript abundance and similar repression kinetics in the two inbreds upon drought. The same expression pattern is observed for genes in cluster 2, but in this case, genes were induced. Thus, these two clusters include CWGs that behave similarly in the two inbreds. In contrast, clusters 3–9 include CWGs whose mRNA abundance and/or expression pattern was different between inbreds, either in control and/or drought conditions, and therefore act in an inbred-dependent manner.

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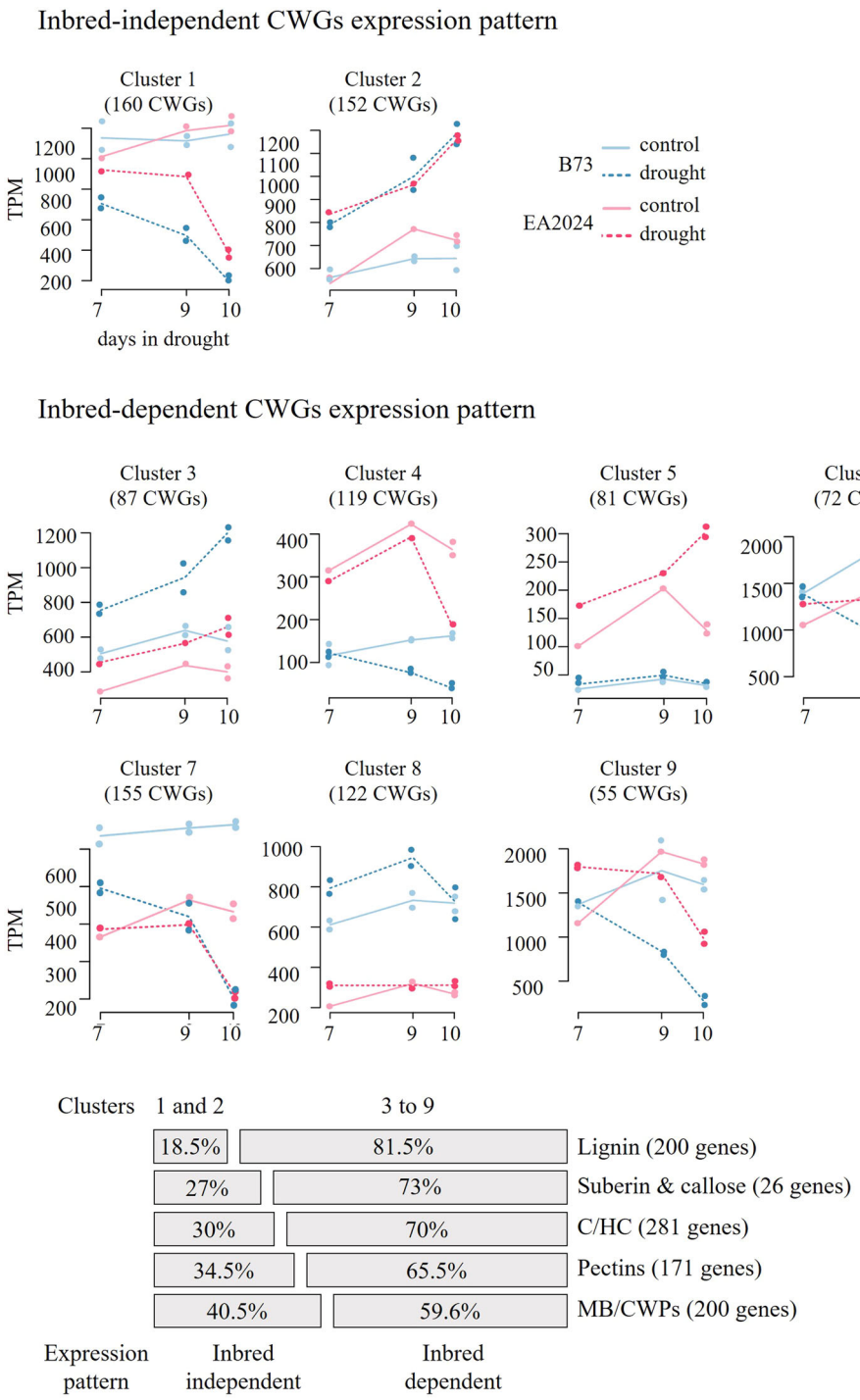

FIGURE 11 Cell wall gene expression profiles of B73 and EA2024 plants grown in control conditions or under 7, 9 and 10 days of drought. Top: The expression pattern of 1000 out of 1485 cell wall genes (CWGs) was clustered in 9 expression profile clusters using the maSigPro package (see methodology for details). TPM refers to transcripts per million. The number of CWGs that belong to each cluster is presented in parentheses. B73 genes are represented in solid (control) and dashed (drought) black lines and the ones of EA2024, in solid (control) and dashed (drought) grey lines. Clusters 1 and 2 contain CWGs having an inbred-independent expression pattern, while clusters 3–9 contain CWGs having an inbred-dependent expression pattern. Bottom: percentage of CWGs has an inbred-independent or inbred-dependent expression pattern within each functional category. The name of each category is shown at the right side of each bar. "C/HC" refers to the Cellulose and Hemicelluloses gene category. "MB/CWP" refers to membrane-bound proteins and cell wall proteins.

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We analysed the distribution of the CWGs among the 9 clusters and we observed that in some cases they were not randomly distributed. In particular, lignin genes constitute the functional category having the highest percentage of genes with an inbred-dependent expression (81.5%). In contrast, only 59.5% of genes belonging to the MB/CWP category had an inbred-dependent expression pattern.

2.6 | Identification of transcription factors controlling CWGs expression in an inbred-specific manner

The identification of a set of differentially expressed CWGs in B73 and EA2024 prompted us to search for transcription factors (TF) that might be responsible for these expression differences. Thus, we performed TF enrichment analyses in PlantRegMap (Tian et al., 2020) using the list of CWGs affected by 7-, 9- and 10-days of drought stress in B73 and/or in EA2024 to identify enriched motifs for predicting putative TFs involved. The regulatory interactions obtained from these analyses were represented using Cytoscape. We identified the 28 most significant inbred-specific TFs displaying an over-representation among the CWGs affected by drought. Twelve of these TFs grouped into four "B73-specific" subclusters whose genes were affected by drought in the two inbreds (represented in beige color) or only in B73 (blue color) (Figure 12 and Data S2). Sixteen TFs grouped into five "EA2024-specific" subclusters including genes affected in both inbreds (beige color) or in EA2024 only (pink color). Although many of such subclusters contained CWGs of all cell wall categories, subcluster 9 (EA2024-specific) and subcluster 43 (B73-specific) were mainly composed of CWGs involved in the biosynthesis of cellulose and hemicelluloses. Interestingly, all TFs in subcluster 9 belong to the WRKY family, while the TFs of subclusters 40, 41 and 42 (B73-specific) belong to the MYB, IDD and ABI families, respectively.

Three TFs associated with the phenylpropanoid pathway also generated inbred-specific subclusters. This is the case of P1 of subcluster 42 (B73-specific), involved in flavonoid regulation (Grotewold et al., 1994), MYB19 of subcluster 40 (B73-specific), involved in phenylpropanoid biosynthesis in *Arabidopsis thaliana* (Zhou et al., 2009) and maize (Yang et al., 2017), and MYB31 of subcluster 6 (EA2024-specific), proposed to be involved in abscisic acid (ABA)-dependent response to drought (Fornalé et al., 2010; Vélez-Bermúdez et al., 2015; Zhu et al., 2023). We identified 5 additional TFs associated with ABA signalling, in maize or other species. This is the case of ABI3/VP1 and ABI19 (Cao et al., 2007; Luerßen et al., 1998) of subcluster 42 (B73-specific), MYB162 of subcluster 43 (B73-specific) (Zhang, Qiu, et al., 2021), GBF1 of subcluster 4 (EA2024-specific), and HB128 of subcluster 8 (EA2024-specific) (Pehlivan, 2019; Pruthvi et al., 2014; Valdés et al., 2012). Arabidopsis ABI3/VP1 gene expression has been related with the circadian clock regulatory machinery (Legnaioli et al., 2009) and interestingly, the homologues of two more EA2024-specific TFs, PIF3 of subcluster 4 and MYBST1 of subcluster 8, are also controlled by the circadian clock (Lu et al., 2014; Soy et al., 2016). In sum, our TF enrichment and regulatory network analyses of drought responsive CWGs differentially expressed in B73 and EA2024, identified several TFs that may act as inbred-specific CWGs regulators in maize.

3 | DISCUSSION

In maize, as other crops, drought severely impacts yield and affects the morphological and physiological fitness of the plant (Tardieu et al., 2018; Webber et al., 2018). We had previously reported that two maize inbred lines, B73 and EA2024, display different stalk-lodging phenotypes, being B73 resistant and EA2024 susceptible (Manga-Robles et al., 2021), and reported on cell wall compositional differences between these two inbred lines. As in maize stalk-lodging and drought are two associated stresses (Ren et al., 2020), we studied here the cell wall dynamics of B73 and EA2024 when subjected to drought conditions. The phenotypic changes, biochemical modifications and differences in the level and intensity of changes in gene expression indicate that B73 is more severely affected by drought than EA2024, which had the ability to fully recover after rewatering.

In fact, although both inbreds displayed a similar photosynthetic efficiency decrease upon drought, EA2024 had lower transpiration rates than B73, suggesting that the reduction in its photosynthetic efficiency caused by CO_2 restrictions upon drought can be better compensated as it increases the concentration of intercellular CO_2.

Cell wall cross-linked phenolic compounds such as *p*-coumarates have been associated with drought adaptation in wheat plants, suggesting that they act as hydrophobic stabilisers and prevent apoplastic water loss (Hura et al., 2012). In our study, drought did not alter *p*-coumarate levels, but EA2024 had around threefold more *p*-coumarates than B73 in control conditions and this could contribute to the higher water retention of its leaves.

Drought affects the full transcriptome of B73 more severely than that of EA2024. This difference is even greater when we compare changes in the expression of cell wall genes (CWGs). It has been reported that up to 70% of the CWGs of two other maize lines displayed at least a two-fold change in transcript levels in response to drought, which correlated with different lignin abundance and digestibility of stem walls (Penning et al., 2019). Our data is in line with these results, as the expression profile of around 69% of the CWGs analysed differed between B73 and EA2024 when they were grown under control and/or drought conditions. However, our data showed that this trend varies significantly among the different categories of CWGs. While only 59.5% of genes involved in the biosynthesis of membrane bound and cell wall proteins had a different expression between B73 and EA2024 in control versus drought conditions, this percentage increased to more than 80% for lignin genes. This suggests that the contribution of lignin genes to B73 stalk-lodging resistance has been higher than that of other cell wall genes. This observation is consistent with previous studies where genes involved in lignin biosynthesis and the general

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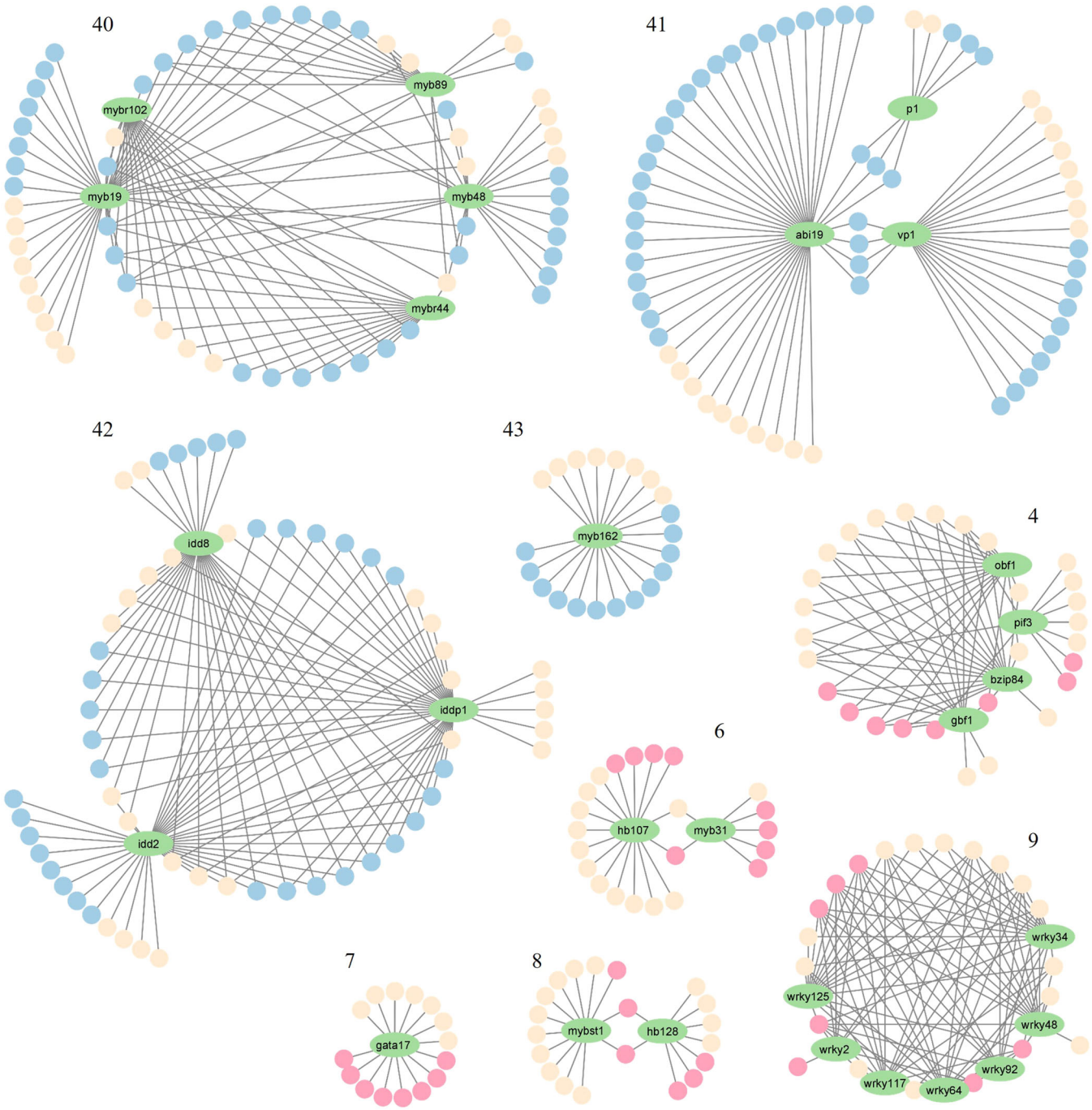

FIGURE 12 Inbred-specific regulatory networks of transcription factors and CWGs in B73 and EA2024 inbreds. Blue circles refers to CWGs with the expression affected only in B73. Pink circles refers to CWGs with the expression affected only in EA2024. Beige circles refers to CWGs whose expression is affected in both inbred. TFs are shown in green colour. The number next to each subcluster corresponds to the subcluster generated by Cytoscape. Node table of the regulatory network of B73 and EA2024 exported from Cytoscape is presented in the Data S2 (sheet 1).

phenylpropanoid pathway were identified as QTLs candidates for lodging resistance (Flint-Garcia et al., 2003; Sun et al., 2018; Wang et al., 2023). The different alterations of the lignin polymer occurring upon drought in these two inbreds can be attributed to inbred-specific expression of lignin genes. This is the case for PAL genes, which catalyse the entry point to the synthesis of monolignols. Three members of this family were repressed only in B73, and three other PAL genes were induced only in EA2024. These include PAL2, a putative PTAL that has been described to convert tyrosine into *p*-coumaric acid (Barros et al., 2016), a reaction that does not require the involvement of the C4H enzymes (see Figure 10 for details). Our data reveal that the C4Hb gene was repressed in EA2024 (while it

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was induced in B73), thus reinforcing the idea that drought increases the production of *p*-coumaric acid by tyrosine deamination in EA2024. On the other hand, drought induced the expression of C3H/APX and two *O*-methyltransferases (OMTs) only in B73, suggesting an increase in the flux that converts *p*-coumaric acid into caffeic acid and ferulic acid in B73. The induction of these two OMTs only in B73 can also explain why this inbred produced an S-enriched lignin in drought conditions, as these enzymes are also likely involved in synthesis of sinapyl alcohol.

Apart from the PAL genes, and some peroxidases and laccases, no other genes involved in the production of lignin were induced only in EA2024, which partially explains the reduction in lignin content occurring in this inbred upon drought. Induction of the CCR genes has been described as an important mechanism for drought tolerance in rice (Bang et al., 2022). Our data reveal that the expression of the maize CCR1a gene, involved in lignin biosynthesis (Tamasloukht et al., 2011), was lower in B73 than in EA2024 in control conditions. Moreover, this gene was repressed upon drought only in B73, thus contributing to explain why this inbred is less tolerant to drought than EA2024.

It has been described that an increase in cellulose deposition under drought could help to maintain cell wall integrity and turgor pressure, hence allowing continued cell growth (Le Gall et al., 2015). In line with this, we observed that drought increases cellulose levels in the cell walls of both B73 and EA2024, although EA2024 already accumulated more cellulose than B73 in control conditions. At present, the molecular mechanisms controlling the biosynthesis of cellulose are far for being clearly understood (Zhang, Gao, et al., 2021). Intriguingly, our data revealed that most CesA genes are repressed in both inbreds upon drought despite the increase in cellulose. We observed that only CesA2 was induced in B73, suggesting that this gene is involved in the biosynthesis of cellulose in an inbred-specific manner upon drought.

A remarkable difference between B73 and EA2024 was that the amount of negatively charged uronic acids linked to the hemicellulose-enriched fractions was lower in B73 than in EA2024 under control (well-watered) conditions. However, they strongly increased upon drought only in B73, achieving similar content as in EA2024. This result can suggest an increase in the amount of glucuronic acid substitutions in the arabinoxylans of the hemicellulose-enriched fractions of B73. There is evidence that glucuronic acid residues of xylan polysaccharides can participate in the crosslinking of hemicelluloses to lignin, making cell walls less degradable (Lyczakowski et al., 2017; Terrett & Dupree, 2019; Tryfona et al., 2023). Our results support this role of glucuronic acids, as cell walls of B73, having less uronic acids in the hemicellulose-enriched fractions (in control conditions), are more degradable than the ones of EA2024 (Manga-Robles et al., unpublished results). Our immunodetection assays reveal an increased abundance of arabino-furanosyl residues in the side chains of xylans in B73 compared to EA2024. The presence of these residues have been associated with mechanical properties of the cell wall (Konishi and Ishii, 2012). Thus, the reduction of glucuronic acid in B73 (compared to EA2024), along with increased amounts of arabinofuranosyl residues substitutions, could participate in increasing the stiffness of its cell wall aimed to generate a stalk-lodging resistant inbred.

As mentioned previously, drought increases the amounts of uronic acids in the hemicellulose-enriched fractions in the B73 inbred. We propose that increasing the uronic acids substitutions in arabinoxylans (upon drought) can favour the separation between arabinoxylan backbones, thus generating spaces to store and retain water, as observed in pectins (Leucci et al., 2008). As we detected that B73 can retain less water than EA2024, we can propose that one strategy of B73 to increase cell wall stiffness and become a stalk-lodging resistant inbred (in well-watered conditions), has been achieved by decreasing the amount of negatively charged glucuronic acid substitutions to reduce the distance between arabinoxylan backbones, even though it negatively affects its water storage capacity. In sum, our findings suggest that the high selection pressure to increase stalk-lodging resistance led to a reduced cell wall plasticity of B73, which requires a deep hemicelluloses rearrangement when subjected to drought.

The shift of uronic acids from the weakly bound to the strongly bound cell wall fractions induced by drought in B73 cell walls (but not in EA2024) may also indicate a displacement of pectins to the strongly bound cell wall fractions in this inbred. This behaviour may represent another strategy of B73 to increase cell walls capacity to retain more water, as pectins hold important roles in controlling the hydration of the wall by modifying their branching substitutions (Thompson and Islam, 2021). In this respect, we observed an increase of arabinan substitutions in the rhamnogalacturonan-I (RG-I) side chains of EA2024 and a decrease in B73 upon drought. It has been reported that the amount of RG-I and RG-II lateral chains increases in drought tolerant wheat lines during water stress, and this is a determinant factor in protecting plants from water stress (Leucci et al., 2008). Moreover, studies in plants surviving extreme dehydration, such as resurrection fern *M. caffrorum* and the basal angiosperm *M. flabellifolia*, revealed that arabinose-rich pectins, and also arabinogalactan proteins (AGPs), allow these plants to readily rehydrate their cell walls after drought (Moore et al., 2013). Hence, the increase in these two arabinan-rich polymers in EA2024 could contribute to a more efficient water usage of this inbred compared to B73 during drought. Interestingly, the increase of AGPs in the KI fraction of EA2024 can be explained by our finding that the AGP glucuronosyl-transferase GT14-2 gene was upregulated in EA2024 while it was repressed in B73.

It is well documented that the degree of methyl-esterification of pectins, and particularly of homogalacturonans (HG), determines mechanical properties of the cell wall (Lionetti et al., 2007; Peaucelle et al., 2008; Wu et al., 2018). A reduced methylation of pectins may result in higher water holding capacity of the cell wall (Willats et al., 2001) and stress tolerance, as suggested for salt-resistant maize inbreds (Uddin et al., 2013). This data is in line with the fact that EA2024 is more tolerant to drought than B73, as our immunodetection assays showed that EA2024 had an increase of un-esterified HG (or low esterified) upon drought in the CDTA fraction, while the

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partially methyl-esterified increased in the KI fraction. It has been described that increasing the methylation of HG can be indicative of either the *de novo* deposition of newly synthesised HG and/or a decrease in the activity of pectin methyl-esterases (PME) (De la Rubia et al., 2021). Our transcriptomics data revealed that drought induced the GATL4b and GS'GT5 pectin glycosyl transferase genes only in EA2024, while pectin esterases and polygalacturonases were generally not affected or repressed in both inbreds. Thus, this supports the idea that drought induced the *de novo* deposition of newly synthesised HG in EA2024. Our results show that EA2024 can modify pectins more intensively than B73 in response to drought, indicating, once more, that EA2024 has a more dynamic, loose, and plastic cell wall matrix than B73. Thus, the CWGs involved in the synthesis of pectins and their side-chain modifications identified in this work represent promising targets for future research as they may play pivotal roles in the dynamic restructuring of cell walls under drought conditions.

As the expression of numerous CWGs differed between B73 and EA2024 inbreds exposed to drought, we searched for TF that could regulate these genes in an inbred-specific manner. Regulatory network analysis using Cytoscape identified several inbred-specific subclusters including CWGs of all functional categories. However, two of these subclusters were enriched in genes involved in cellulose and hemicellulose biosynthesis, and were found to be affected by drought only in B73 (90 genes) or only in EA2024 (21 genes). These results further indicate that B73 needs a deeper reprograming of these cell wall genes (especially hemicelluloses) than EA2024 to readapt its cell wall metabolism to drought conditions.

We also identified a few TFs that appear to be key for CWGs regulation. This is the case of MYB31, that was previously shown to bind to the promoters of several lignin pathway genes in maize (Fornalé et al., 2010; Vélez-Bermúdez et al., 2015). Heterologous expression of MYB31 in Arabidopsis was shown to lead to reduced levels of lignin without affecting its composition (Fornalé et al., 2010). Interestingly, here we show that similar lignin alterations take place in EA2024 upon drought, indicating that MYB31 (detected in an EA2024-specific cluster) is an important inbred-specific regulator of lignin biosynthesis. Furthermore, we propose that the role of MYB31 may be extended beyond lignin biosynthesis, as CWGs from other functional categories were also included in its regulatory node. We also identified MYB19 as another TF that is involved in lignin pathway regulation. This finding is in line with the reported role as a regulator of phenylpropanoid biosynthesis in maize (Yang et al., 2017) and the known function of its Arabidopsis homologue as transcriptional activator of lignin pathway during secondary cell wall formation (Zhou et al., 2009). Hence, MYB19 would be the first positive regulator of lignin biosynthesis identified in maize. Interestingly, several TF nodes identified had been earlier implicated in ABA- and auxin-dependent activation of drought responsive genes in other species. For instance, MYB162 (B73-specific cluster) has been reported to mediate the response to water deprivation, wax biosynthesis, and seed dormancy by positively regulating the ABA- and auxin-signalling pathways (Castorina et al., 2020; Zhang, Qiu, et al., 2021). On the other hand, MYB44, MYB102 and P1 (B73-specific clusters) are the maize homologues of AtMYB11, which in Arabidopsis is shown to respond to auxin and activate transcription of the flavonoid F3H gene (Mehrtens et al., 2005). We also identified HB128 (EA2024-specific cluster), whose heterologous expression in Arabidopsis promotes drought tolerance by activating ABA signalling (Qiu et al., 2022). The two VP1/ABI3-like proteins, ABI3-VP1 and ABI19 (B73-specific clusters), may also be responsible for the differences between the two inbreds, as they are induced by ABA and abiotic stresses in maize (Cao et al., 2007). Thus, the regulatory network analysis identified a set of eight TFs coordinating CWGs in maize in an inbred-specific manner, serving as promising candidates for subsequent research.

In sum, our study reveals that adjusting cell wall metabolism is critical for plants to grow in drought conditions. We have been able to associate changes in cell wall gene expression with biochemical alterations in the cell wall components in response to drought. Table 1 summarises the main phenotypic, biochemical, and genotypic differences observed between B73 and EA2024 in both control and drought conditions. We discovered that B73, a stalk-lodging resistant inbred, is more severely affected by drought than the lodging-susceptible EA2024 inbred, which leads us to propose that the stalk-lodging resistance of B73 occurred at expenses of a decrease in cell wall plasticity. Based on gene expression changes and the biochemical effects on cell wall composition, we provide a set of cell wall genes and transcription factors that could be useful to improve drought tolerance in stalk-lodging resistant maize plants.

4 | MATERIALS AND METHODS

4.1 | Plant material and sampling

Seeds of the maize B73 and EA2024 inbred lines were kindly provided by Dr. Rosana Malvar (Misión Biológica de Galicia, Spain). The maize plants used for RNA-Seq experiment were grown in culture chambers (25°C day and 22°C night, 60% humidity and 16/8 h photoperiod) until the stage of three leaves. Then, for drought treatment, irrigation was completely stopped for a different period of time (7, 9 or 10 days). Control plants were irrigated for the same period. At this time-point, the expression of the water stress-inducible gene Rab17 (Vilardell et al., 1990) exhibited a strong increase in both inbreds (Supplementary Table 3). For relative water content (RWC), gas exchange, chlorophyll fluorescence and cell wall characterisation, plants were grown in a greenhouse (26°C day and 22°C night, 60% humidity and 14/10 h photoperiod) and sampled after 10 days of drought. For the drought-rewatering experiment, plants were rewatered after 25 days of drought and survival was assessed 1 week later.

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TABLE 1 Summary of the main phenotypic, biochemical and genotypic differences in B73 and EA2024 maize inbred lines in control and drought conditions.

	In control conditions	In drought conditions
Physiological parameters		
Survival after drought		B73 sensitive EA2024 tolerant
Leaf relative water content	B73 < EA2024	B73 < EA2024
Transpiration rate	B73 > EA2024	both ↓
Photosystem II quantum yields	similar levels	
Electron transport rates		
CO_2 assimilation rates		
Intercellular CO_2 concentration		EA2024 ↑
Cell wall alterations		
Lignin		
Total lignin content	similar amounts	EA2024 ↓
H subunits		B73 ↓
S subunits		B73 ↑
S/G ratio		B73 ↑
Thioacidolysis yield		both ↑
Polysaccharides		
Total sugars (KII)	similar amounts	B73 ↑
Neutral sugars (CDTA)		B73 ↑ EA2024 ↓
Arabinan substitutions of RG-I (KI, KII)		B73 ↓ EA2024 ↑
Arabinogalactan proteins (KI)		EA2024 ↑
Cellulose	B73 < EA2024	both ↑
Uronic acids (total)		B73 ↑
p-coumarate		
Uronic acids (KI)		B73 ↑
Uronic acids (KII)		B73 ↑
Unesterified homogalacturonans (KI)		B73 ↑ EA2024 ↓
Total sugars (CDTA)	B73 > EA2024	EA2024 ↓
Glucose		EA2024 ↑
FA/CA ratio		EA2024 ↑
Uronic acids (CDTA)		EA2024 ↑
Unesterified homogalacturonans (CDTA)		EA2024 ↑

Transcriptomic alterations upon drought		
Full transcriptome (34,384 genes)	B73 29%	EA2024 21%
Cell wall genes (1485 genes)	B73 44%	EA2024 31%
Cell wall genes affected only in one inbred		
Cellulose and hemicellulose	B73 46%	EA2024 11%
Lignin	B73 38%	EA2024 18%
Pectin	B73 35%	EA2024 12%
Membrane-bound and cell wall proteins	B73 31%	EA2024 6%

Note: Top (in orange): physiological and biochemical differences in control condition and changes occurring upon drought. Significant increases and reductions compared to drought conditions are denoted by red and green arrows, respectively. Bottom (in blue): global and cell wall genes transcriptomic alterations upon drought. The percentages of the different functional categories of cell wall genes affected by drought are shown for B73 and EA2024 maize inbreds.

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4.2 | Relative leaf and soil water content, gas-exchange, and chlorophyll fluorescence measurements

Soil water content for both B73 and EA2024 was monitored to ensure uniform stress severity across both genotypes (Figure S1). The initial soil weight was recorded before sowing, and after a 10-day drought period, the soil weight was measured again, following the removal of the plants, to determine the weight of the dry soil.

Leaf relative water content (RWC) was determined as already described (Wang et al., 2016). Briefly, leaves fresh weight (FW) was measured immediately after harvesting and after rehydration in water for 24 h at 4°C until fully turgid to obtain the turgid weight (TW). Samples were then dried at 60°C for 48 h to record the dry weight (DW). Relative water content (RWC) was then calculated using the formula: RWC (%) = (FW−DW)/(TW−DW)*100. The CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (gsw), intercellular CO_2 (Ci), photosystem II PSII quantum yield (ΦPSII) and electron transport rate (ETR) were measured using a portable photosynthesis system (Li-6800; Li-COR Inc.). The measurements were made on the youngest fully expanded leaf between 10:00 am and 1:00 pm. Two technical measurements on four individual plants were taken for each inbred and treatment. The ambient CO_2 concentration was set at 400 µmol mol^{-1}, air flow at 400 µmol s^{-1}, relative humidity at 50% and PAR at 1500 µmol m^{-2} s^{-1}.

4.3 | Lignin analysis

Dried whole stems from both inbred lines were ground to a fine powder, extracted with 20 volumes of methanol, and filtered through Whatman GF/A microfiber filters. This alcohol insoluble residue (AIR) was dried at 60°C for 4 days. AIR was then de-starched, treated with acidified phenol and washed with organic solvents to obtain the cell wall residue, as previously described (Manga-Robles et al., 2021).

Total lignin content was gravimetrically determined by the Klason procedure, whereas lignin composition was determined by thioacidolysis (followed by GC-MS), as already reported (Fornalé et al., 2015). Lignin content and composition were determined in two biological replicates, each comprising the entire aerial part pooled from five plants for both treatment and control conditions. The experimental procedure was repeated with three technical replicates for each biological replicate.

4.4 | Cell wall polysaccharides, hydroxycinnamate and fractionation analyses

Neutral sugar analysis was performed according to Albersheim et al. (1967). Dried cell walls were hydrolysed with 2 M TFA (trifluoroacetic acid) for 1 h at 121°C and the resulting sugars were derivatized to alditol acetates and analysed by gas chromatography (GC). Uronic acid contents were determined by the m-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen 1973), with galacturonic acid as a standard. Cellulose was quantified in crude cell walls by the Updegraff method (Updegraff, 1969) with the hydrolytic conditions already described (Saeman et al., 1963) and the quantification of the glucose released was performed by the anthrone method (Dische, 1962) using glucose as a standard.

For cell wall fractionation, dried cell walls were extracted (1 mg/2.5 mL) with 50 mM cyclohexane-trans-1,2-diamine-N,N,N′,N′-tetraacetic acid sodium salt (CDTA) at pH 6.5 for 8 h and washed with boiling distilled water. The residue was incubated with 0.1 N KOH + 20 mM $NaBH_4$ (KOH I) for 8 h and washed with distilled water. Then, 4 N KOH + 20 mM $NaBH_4$ (KOH II) was added to the residue for 8 h and washed again with distilled water. The extracts and their washings were combined and acidified to pH 5.0 with glacial acetic acid, representing CDTA, KI and KII fractions, respectively.

Ester-linked ferulate and *p*-coumarate monomers and ferulate dimers (diferulates) were extracted from the AIR with 2 M NaOH at room temperature and quantified by HPLC based on a procedure previously described (Santiago et al., 2006). Total diferulates were calculated as the sum of four identified and quantified dimers: 8-5-open (noncyclic), 8-5-benzofuran (cyclic), 8-O-4, and 5-5. The assays were conducted on two biological replicates, each comprising the entire aerial part pooled from five plants for both treatment and control conditions. The experimental procedure was repeated with three technical replicates for each biological replicate.

4.5 | Cell wall immuno-dot assays

Immuno-dot assays (IDAs) were performed as described by García-Angulo et al. (2006). A 1 µL aliquot from each cell wall fraction was spotted onto nitrocellulose-membranes (Scheicher & Schull, Dassel, Germany) as replicated 1/5 dilution series of the preceding one. After drying, the nitrocellulose membranes were blocked with 4% (m/v) fat-free milk powder in phosphate-buffered saline (M-PBS: 0.14 M NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4 12 H_2O, 1.5 mM KH_2PO_4, pH 7.2) and then incubated with a 1/5 dilution of primary antibodies (Plant Probes, Leeds, UK) (Supplementary Table 1) in M-PBS for 2 h at room temperature. The membranes were further washed under running tap water followed by PBS and incubated with the secondary antibody (anti-rat horseradish peroxidase conjugate, Sigma®) diluted to 1/1000 in M-PBS for 2 h at room temperature. The membranes were washed as described above before color development in the revealing solution (for membrane: 5 mL distilled H_2O + 1 mL of methanol with 5 mg/mL 4-chloro-1-naphthol + 6 µL 30% (w/v) H_2O_2). Colour development was stopped by washing the-membranes with running tap water. Samples of commercial pectin (P25,-P59,-P94, Sigma-Aldrich®, Germany), arabic gum (Sigma-Aldrich®) and xyloglucan (kindly donated by Dr. T. Hayashi) were used as reference compounds.

Immunolabelling semi-quantification for each fraction was performed by scoring the number of dilutions labelled. Thus, a value ranging from 0 to 4 was assigned according to the number of colored spots appeared after the color development. Finally, the IDA

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results were used to generate a heatmap that was carried out by RStudio (v.4.0.2) (R Core Team, 2013) using factoextra (Kassambara & Mundt, 2020), FactoMineR (Lê et al., 2008) and gplots (Warnes et al., 2015) packages. The assays were conducted on two biological replicates, each comprising the entire aerial part pooled from five plants for both treatment and control conditions. The experimental procedure was repeated with three technical replicates for each biological replicate.

4.6 | RNA extraction, sequencing, and differential gene expression analyses

Total RNA was extracted from control and drought-stressed plants using the Maxwell® RSC RNA Purification Kit (Promega) following the manufacturer's instructions. Approximately 1 µg of total RNA was reverse-transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen) according to manufacturer's instructions. RNA sequencing was performed at the Centre for Genomic Regulation (CRG, Barcelona, Spain). Libraries preparation and sequencing were performed according to the V4 Illumina protocol. About ~60 million paired-end strand-specific reads of 76 bp were produced per sample.

Adaptors, unpaired reads and sequences shorter than 21 were removed with Trim Galore! V0.4.5 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Clean reads were mapped against version 4 of *Zea mays* reference transcriptome (ENSEMBL: Zea_mays. AGPv4. cdna. all. fa) using Salmon programme v0.8.2 (Patro et al., 2017).

The differential expression analysis was done using the raw counts calculated by Salmon as the input for the DESeq. 2 analysis. Genes with less than 10 counts in all samples were removed. A Principal Components Analysis (PCA) plot was generated with DESeq. 2 to visualise the overall patterns of variation across all the biological replicates of the RNA-Seq of B73 and EA2024 submitted at 7, 9 and 10 days of drought. The expression data of the top 500 genes with the highest row variance across samples was employed.

Then, the differential gene expression analysis at 10 days of drought was conducted on the normalised data from two biological replicates, each comprising a pool of 5 individual plants for both treatment and control conditions. Genes were considered differentially expressed in drought with respect to control conditions when the $\log_2$ Fold Change ($\log_2$FC) was higher than 1 or lower than −1, with an adjusted *p*-value of less than 0.05. The results were visualised in a Venn diagram constructed using the tool available at http://www.interactivenn.net/.

qRT-PCR assays were conducted to assess the expression of 16 cell wall genes (or to validate RNA-seq data), and water stress-inducible gene Rab17 (Vilardell et al., 1990), utilising gene-specific primers detailed in Table S4. Three technical replicates were conducted for each of the two biological replicates, and qPCR reactions were run on a Light Cycler 480 (Roche) using SYBR Green dye (Roche). The maize Leuning (LUG) gene was used for data normalisation, as established by Manoli et al. (2012). The PCR conditions comprised an initial denaturation step at 95°C for 10 min, followed by 45 cycles consisting of a denaturation step at 95°C for 10 s, an annealing step at 60°C for 30 s and an extension step at 72°C for 30 s.

Subsequently, after selecting the group of 1485 cell wall-related genes, their differential expression was evaluated to discern significant differences between B73 and EA2024 inbreds over the course of drought stress (7, 9, and 10 days). This time-course analysis was performed using the R package maSigPro (Conesa et al., 2006), with each time-point considered as an individual replicate in the analysis. The design matrix was constructed according to four time-series: B73-Control, B73-Drought, EA2024-Control and EA2024-Drought of three time-points each: 7, 9, and 10 days. The stepwise regression was set to R-squared >0.6 and the multiple test correction to FDR ≤ 0.05 to identify time-course differentially expressed genes. Hierarchical clustering using *hclust* function was performed based on the correlation coefficient distance with a default number of clusters = 9.

Normalised data were used as input to create a multidimensional scaling plot with plotMDS function from edgeR package (Robinson et al., 2010) and the data set was represented on a two-dimensional scatterplot so that distances approximate the largest 500 $\log_2$-fold changes between each pair of samples.

4.7 | Transcriptional regulatory network of CWGs

Using the maSigPro package, we obtained the cell wall differentially expressed genes of B73 and EA2024 over the course of drought stress (7, 9, and 10 days). These genes were used to construct a transcriptional regulatory network of CWGs. We used the "TF Enrichment" tool, available at the Plant Transcriptional Regulatory Map website http://plantregmap.gao-lab.org/ (Tian et al., 2020) to infer the transcription factors having significantly overrepresented targets among the promoter regions of the differentially expressed CWGs in B73 and in EA2024, separately (Data S2). The putative regulatory interactions of TFs and cell wall target genes were visualised and further analysed using Cytoscape. The networks obtained separately for B73 and EA2024 differentially expressed CWGs were then merged and subclustered with the community clustering (GLay) algorithm. Finally, TFs that were common between the two lines and subclusters with fewer than six interactions were removed.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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