Maize WI5 encodes an endo-1,4-β-xylanase required for secondary cell wall synthesis and water transport in xylem

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Abstract Water transport from roots to leaves through xylem is important for plant growth and development. Defects in water transport can cause drought stress, even when there is adequate water in the soil. Here, we identified the maize (Zea mays) wilt5 (wi5) mutant, which exhibits marked dwarfing and leaf wilting throughout most of its life cycle under normal growth conditions. wilt5 seedlings exhibited lower xylem conductivity and wilted more rapidly under drought, NaCl, and high temperature treatments than wild-type plants. Map-based cloning revealed that WI5 encodes an active endo-1,4-β-xylanase from glycosyl dehydration family 10, which mainly functions in degrading and reorganizing cell wall xylan. Reverse-transcription polymerase chain reaction and β-glucuronidase assays revealed that WI5 is highly expressed in stems, especially in internodes undergoing secondary wall assembly. RNA sequencing suggested that WI5 plays a unique role in internode growth. Immunohistochemistry and electron microscopy confirmed that wi5 is defective in xylan deposition and secondary cell wall thickening. Lignin deposition and xylan content were markedly reduced in wi5 compared to the wild-type plants. Our results suggest that WI5 functions in xylem cell wall thickening through its xylanase activity and thereby regulates xylem water transport, the drought stress response, and plant growth in maize.

INTRODUCTION

The cell wall functions not only as a physical barrier and the first line of defense between the plant cell and the external environment, but it is also a flexible, responsive structure involved in regulating plant growth (Seifert and Blaukopf 2010; Wolf et al. 2012). The plant cell wall is a composite structure consisting of cellulose microfibrils embedded in a matrix of hemicelluloses, pectins, and proteins. The cell walls of typical grass plants contain approximately 55% hemicellulose, 25% cellulose, and only 10% pectin. Glucuronoarabinoxylan is the major component of hemicellulose, comprising 20%–40% of the primary cell wall and 40%–50% of the secondary cell wall (Lerouxel et al. 2006; Vogel 2008). Plant cell walls have the remarkable property of extreme tensile strength combined with extensibility. Genes associated with cell wall thickening often contribute to the mechanical properties of the cell wall in structures such as the xylem of vascular bundles and in epidermal cells. Arabidopsis thaliana KNAT7 regulates secondary cell wall biosynthesis and thickening through directly activating the promoters of xylan...
biosynthetic genes, an essential process in the development of xylem cells with normal morphology (Liu et al. 2014; He et al. 2018). Mutants in some kinds of endo-beta-1,4-glucanases resulted in changes in crystallinity of cellulose, which affect secondary cell wall development (Glass et al. 2015).

The upward growth and transpiration of terrestrial plants present challenges to water transport capacity. Water is transported from roots to leaves through xylem, and xylem impairment impedes water transport (Yuan et al. 2007; Venturas et al. 2017). The water transport capacity of xylem is determined by the unique structure of its cell wall, especially the secondary cell wall. Increased cellulose synthesis and cell wall elasticity contribute to the maintenance of cell turgor and ensure normal water transport in plants. Several Arabidopsis mutants with interrupted cell wall synthesis show increased sensitivity to abiotic stress. The lew3 mutant, with impaired cellulose synthesis and collapsed xylem, is more sensitive to salt stress and loses less water through transpiration than the wild-type (Zhang et al. 2009a). The sos6 mutant, containing a mutation in a cellulose synthase-like protein gene, is hypersensitive to salt and oxidative stress (Zhu et al. 2010). Cellulose synthase CESA6 and cellulose synthase-interactive protein CS1t are involved in cellulose deposition and essential for salt stress (Zhang et al. 2016). By contrast, disrupting the cellulose synthesis gene, AtCESA8, results in enhanced drought and osmotic stress tolerance in Arabidopsis (Chen et al. 2005). Several cellulose synthesis-associated genes that affect water transport have been reported in rice (Oryza sativa). MicroRNA166 (miRNA166) regulates cellulose synthesis genes by targeting OsBH4. Knockdown of miRNA166 resulted in plants with xylem vessels with reduced diameters and stems with reduced water conductivity (Zhang et al. 2018). Rice CLD1/SRL1 encodes a glycosylphosphatidylinositol (GPI)-anchored membrane protein; the loss-of-function of CLD1/SRL1 leads to significant decreases in cellulose and lignin contents in the secondary cell walls of leaves, resulting in rapid water loss and leaf rolling (Li et al. 2017).

Several types of cell wall-related proteins function in cell wall remodeling, including glycoside hydrolases (GHs), glycosyltransferases, expansins, and peroxidase. GHs are broadly distributed enzymes that hydrolyze glycosidic bonds, leading to inversion and retention of the anomeric configuration of molecules (Ma et al. 2015; Wan et al. 2015). Plant GHs belong to 29 families, most of which were identified in Arabidopsis and rice. Endo-β-1,4-xylanases are major members of the plant GH family 10 (GH10). These enzymes play important roles in hydrolyzing xylan backbones into shorter, soluble xylo-oligo saccharides (Pollet et al. 2010). Two xylanases are strongly expressed during germination in barley (Hordeum vulgare), which primarily function in mobilizing nutrients from the aleurone layer and endosperm to the developing seed (Caspers et al. 2001). Maize (Zea mays) xylanase (Xyl) is thought to be involved in hydrolyzing the tapetum cell wall or stigma cell wall to facilitate pollen tube penetration into the silk (Li et al. 2012; Andriotis et al. 2016). AtXyn1, which is mainly expressed in Arabidopsis vascular bundles, is involved in rearranging or altering the orientation of hemicelluloses in the secondary cell wall (Suzuki et al. 2002). Members of GH10 are usually associated with carbohydrate binding domains (CMBs). Carbohydrate binding domains are structurally diverse noncatalytic domains that typically target enzymes to specific polysaccharide substrates and potentiate the activity of these enzymes against insoluble substrates such as cellulose, mannann, and xylan. Carbohydrate binding domains have been classified into 64 families (Cantarel et al. 2009), most of which have not been functionally characterized. Family 4 members display specificity for different polysaccharides, including amorphous cellulose, α-1,3-glucan, and xylan (Kognole and Payne 2015).

In maize, several wilty mutants—including the recessive mutant wilty1 and dominant mutants wilty2, wilty3 and wilty2445—have been previously described (Rock and Ng 1999). All of these mutants appear to be defective in vascular element development. None exhibited defects in the metabolism or signaling of abscisic acid (ABA), an important hormone for plant stomatal movement and drought tolerance. However, no wilty gene has been cloned to date, and the molecular mechanism underlying leaf wilting in maize is unknown. In the current study, we isolated and characterized the maize wilty5 (wi5) mutant, which exhibits marked dwarffing and chronic leaf wilting, even under normal growth conditions. WILTY5 encodes an active endo-1, 4-β-xylanase from the GH10 family. Our findings suggest that WILTY5 functions in the formation of the secondary cell wall and plays an essential role in increasing the thickness of the internode xylem required for efficient water transport, thereby helping plants develop normally under high transpiration and water-stress conditions.
RESULTS

Identification of the drought-sensitive mutant wi5 in maize

A wilty mutant, designated as wi5 (wilted5), was isolated from a maize Mutator (Mu) mutagenized M2 population. Under normal field conditions, wi5 plants displayed a drought-sensitive phenotype, with wilting and progressive leaf chlorosis after the 3-leaf stage; these symptoms were the most pronounced in young leaves (Figure 1A). This mutant phenotype was more severe and developed earlier under drought or high-temperature conditions. The wi5 mutant also exhibited marked dwarfing and reduced biomass (Figure 1B). Wilting occurred primarily at the leaf tips and spread around the leaf margins. Chlorosis developed after the leaves had fully expanded, followed by necrosis, which increased in severity during growth (Figure 1C). However, germination was unaffected in the mutant seeds, and no abnormal seedling phenotypes were observed.

We crossed wi5 plants with B73 plants. All F1 plants displayed a wild-type phenotype. Progeny derived from backcrossing F1 to wi5 plants showed an approximately 1:1 segregation ratio of wild-type : wi5 mutant phenotypes, suggesting that wi5 is a single-gene recessive nuclear mutant (Table S1).

Phenotypic and physiological characterization of wi5 plants

When plants encounter drought stress, an array of physiological and biochemical modifications become activated, including leaf wilting, reduced stomatal conductance, impaired photosynthesis and reduced growth rates. The chlorotic appearance of wi5 leaves suggests that this mutant has defects in photosynthetic pigment accumulation and photosynthesis. We therefore quantified photosynthetic pigment levels, photosynthesis rates, and other related physical indexes in wild-type and wi5 plants. We detected...
an approximately 40% and 20% reduction in chlorophyll (Chl) and carotenoid (Car) levels in w i 5 leaves compared to those of the wild-type, respectively (Figure 1D). The photosynthetic indicators of w i 5 plants were also strongly reduced relative to the wild-type (Table S2). The net photosynthetic rate (Pn) of w i 5 was strongly suppressed, and stomatal conductance (Cond), intercellular CO2 concentration (Ci), and transpiration rate (Tromml) were sharply reduced (by approximately 66%–90%) compared to the wild-type.

We stained leaves of w i 5 and wild-type plants with Trypan blue, a histochemical indicator of irreversible membrane damage or cell death. Deep blue staining was observed in w i 5 leaves, whereas no obvious staining was detected in wild-type leaves (Figure 1E). These results suggest that w i 5 plants exhibit severe cellular damage and an impaired photosynthetic system. Furthermore, w i 5 plants displayed stunted growth, with a height approximately 60% that of wild-type plants at the mature stage (Figure 1F). Finally, we compared the elongation patterns of internodes in w i 5 and wild-type plants. Mutant and wild-type plants had the same number of internodes, but all w i 5 internodes were shorter than those of the wild-type (Figure 1G). The wilting and chlorotic leaves, impaired photosynthetic system, and stunted growth of w i 5 suggest that these plants suffer water stress, even under normal growth conditions.

**w i 5 plants are hypersensitive to drought, high temperature, and NaCl at the seedling stage**

At the seedling stage, w i 5 plants were indistinguishable from wild-type plants under greenhouse conditions. However, w i 5 plants were more sensitive than wild-type plants to drought, high temperature (42°C), or NaCl stress, exhibiting severely suppressed growth and significantly reduced survival rates under these conditions. Figure 2A shows wild-type and w i 5 plants grown for 3 weeks in the same pot and treated with drought stress by withholding water. After 10 d of drought treatment, wild-type leaves remained turgid, whereas w i 5 leaves showed obvious wilting. After an additional 10 d, the wilting of w i 5 was more severe than that of the wild-type. When water was withheld from the soil for 30 d, both wild-type and w i 5 plants wilted. After watering was resumed, 80% of wild-type plants survived, whereas all w i 5 plants died.

The w i 5 plants were also more sensitive than wild-type plants to high-temperature stress (Figure 2B). When treated with 42°C for 12 h, w i 5 plants showed earlier leaf rolling and wilting than the wild type. After 48 h of treatment, the blades of w i 5 leaves curled sharply downward, whereas those of wild-type plants remained erect.

Since crosstalk occurs between drought and salt tolerance (Liu et al. 2010; Chien et al. 2014; Guo et al. 2016), we also examined the responses of w i 5 and wild-type plants to a 250 mmol/L NaCl treatment (Figure 2C). Similar to the results of drought treatment, after 6 d of watering with 250 mmol/L NaCl, the growth of w i 5 plants was completely suppressed, and the plants were more wilted and chlorotic than wild-type plants.

**w i 5 mutants exhibit a hypersensitive response to water stress, but impaired water transport system**

To determine why w i 5 plants are hypersensitive to drought stress and have an impaired water transport capacity, we analyzed the ABA contents, reactive oxygen species accumulation, water loss rate of isolated leaves, and injury flow of the seedlings. Drought treatment induced an increase in ABA content—both w i 5 and wild-type plants accumulated up to four times more ABA under drought conditions than well-watered conditions—and there was no significant difference in ABA contents between the mutant and wild-type leaves (Figure 3A).

H2O2 accumulation was monitored by 3,3′-diaminobenzidine (DAB) staining. Before osmotic stress treatment, little staining was observed in w i 5 and wild-type plants (left side of Figure 3B). After 24 h of treatment with 20% polyethylene glycol (PEG) and 30 µmol/L methyl viologen (MV), H2O2 accumulation was observed in both the mutant and wild-type (right side of Figure 3B). In the detached leaves of 3-week-old seedlings, water loss was a little slower for w i 5 than the wild-type (Figure 3C); the w i 5 mutant also displayed lower stomatal conductance than the wild-type (Table S2). These results imply that w i 5 plants can prevent water loss by closing their stomata and therefore have a normal physiological response to drought stress.

Furthermore, w i 5 seedlings had a significantly lower injury flow rate than the wild-type (Figure 3D),
indicating that the wilty phenotype of wi5 is the result of an impaired water transport system.

**Map-based cloning and complementation of the wi5 mutant**

We performed map-based cloning to isolate the WI5 gene using a BC1F1 segregating population generated from a cross between the wi5 mutant and wild-type cultivar B73. The WI5 locus was initially mapped to the short arm of chromosome 5 in a 19-cM region between the two simple sequence repeat (SSR) markers bnlg1660 and umc2167 (Figure 4A). To determine the precise position of WI5, we used a larger BC1F1 mapping population consisting of 3,800 plants for fine mapping. One InDel marker (IDP), two SSR markers, and two cleaved-amplified polymorphic sequence (CAPS) markers were developed (Table S3) between markers umc2115 and umc2293. The WI5 locus was mapped to a 50-kb DNA region between markers 0821DP and Caps2 on a single bacterial artificial chromosome (BAC), AC208082. Within this region, three open reading frames (ORFs) were predicted using the program FGENESH (www.softberry.com), but only one expressed gene was identified. Reverse-transcription polymerase chain reaction (RT-PCR) analysis revealed that the candidate gene is 2,247 bp long, with three exons and two introns and encoding a 748 amino acid protein known as endo-1,4-β-xylanase. The wi5 allele contains a 20-bp deletion in the first exon that results in a frame-shift mutation, an early stop codon, and a truncated protein with 178 amino acids (Figure 4B). Thus, the mutation might severely reduce or completely disrupt the activity of the protein. A Basic Local Alignment Search Tool search revealed only one copy of WI5 in the maize genome.
We carried out a complementation test to confirm the mapping results. As wi5 is not a suitable transgene acceptor, we transferred a 4.632-kb wild-type DNA fragment containing the WI5 promoter region and the entire ORF into a wild-type acceptor in the X178 background. We crossed positive T0 plants with wi5 plants and determined that half of the F1 progeny harbored the transferred DNA. The positive F1 plants were self-pollinated to obtain an F2 population. Approximately 1/16 of the F2 plants exhibited the wilting and stunted growth phenotypes characteristic of wi5 (Table S4). We isolated an unsegregated F3 line and determined (by droplet digital PCR (ddPCR)) that it was a single copy event (Figure S1). These results demonstrate that WI5 is indeed responsible for the phenotypic changes observed in the wi5 mutant.

**Tissue-specific expression profiles of WI5**

To obtain further insight into the wi5 phenotype, we investigated the tissue-specific expression patterns of WI5 by quantitative RT-PCR. Although WI5 was expressed in all of the organs examined, it was expressed at the highest level in the developing stem and at very low levels in the ear, seed, embryo, and endosperm (Figure 5A). We then examined the transcript profiles of WI5 in various internodes in the stems of maize plants at the flowering stage. WI5 messenger RNA levels varied along the length of the stem, with higher expression in the middle internodes...
(eighth and ninth internodes, counted from the base of the plant) and lower expression in the basal and upper internodes (Figure 5B). The middle internodes were nonelongating internodes that had recently stopped elongating and were undergoing secondary cell wall assembly. These results suggest that WI5 contributes to secondary cell wall modification in maize.

To investigate the expression pattern of WI5 in more detail, we produced transgenic plants carrying the β-glucuronidase (GUS) reporter gene driven by the 1.5-kb promoter region upstream of WI5. GUS activity was predominantly localized to the vascular and epidermal tissues of the stem, a site of secondary cell wall deposition (Figure 5F, G). Some activity was also evident in the vascular tissue of the leaf sheaths (Figure 5D, E), but it was completely absent in younger leaves and roots (Figure 5C, H).

Since the wi5 mutant exhibited wilting leaves and short internodes, we were interested in determining whether the transcript levels of genes were altered in these tissues. We analyzed RNA-seq data for the 13th leaves and eighth internodes of wild-type and wi5 plants. Principal component analysis (PCA) revealed that the transcripts in internodes were significantly different between the wild-type and mutant, while those in leaves were only slightly different (Figure S2A). More differentially expressed genes (DEGs) were identified in the internodes than in the leaves based on PCA (Figure S2B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of internode data revealed many genes involved in synthesis and metabolism pathways that were down-regulated in wi5 internodes, in alignment with the repressed cell growth. Furthermore, genes that
function in DNA replication and repair were upregulated in these mutant internodes, likely to compensate for their metabolic deficiency (Figure S2C). These results suggest that WI5 is primarily expressed in internodes, functions in metabolic pathways, and mediates water transport and normal growth in the middle internodes.

**WILTED5 encodes a GH10 family member**

The GH10 family consists of endo-1,4-\(\beta\)-xylanases (EC3.2.1.8), endo-1,3-\(\beta\)-xylanases (EC 3.2.1.32), and cellobiohydrolases (EC 3.2.1.91). The major enzymes of this family are endo-1,4-\(\beta\)-xylanases, which are widely distributed among phyla, including numerous bacteria, fungi, and plant species. Endo-1,4-\(\beta\)-xylanases comprise...
both catalytic and noncatalytic modules held together by flexible linker regions. The PFAM (protein families database of alignments and hidden Markov models) prediction program indicated that WI5 contains two carbohydrate binding domains (CBM-4-9 and pfam02018) arranged in tandem at its N-terminus and one GH10 catalytic domain (pfam00331) at its C-terminus (Figure 6A).

Analysis of the complete genome sequence of maize revealed ten GH10 family members of WI5, which were designated as ZmxyI1 to ZmxyI10. Bioinformatics analysis revealed that these genes are located on different maize chromosomes, sharing 20%–50% identity with WI5 (Table S5). To determine the evolutionary relationships of maize GH10 family genes and xylanase genes of other species, we built a rooted phylogenetic tree using the neighbor-joining method (Figure 6B). Phylogenetic analysis revealed that these genes are divided into two subfamilies. Genes from different clades might have different functions. WILTED5 and ZmxyI1, 2, 3, and 4 belong to one clade and WI5 and Atxyn1 from Arabidopsis form a monophyletic clade with 88% bootstrap support. As Atxyn1 functions in secondary cell wall metabolism in vascular bundle cells (Suzuki et al. 2002), we propose that WI5 might have a similar function in maize.

The overall structure of the catalytic domain of GH10 xylanases is a cylindrical eightfold α/β barrel resembling a salad bowl, with two active-site glutamates, one of which is the nucleophile and the other the acid-base (Juturu and Wu 2012). Site-directed mutagenesis studies identified Glu-128 and Glu-236 as essential residues in the active site of xylanase A from Streptomyces lividans. Asp-124 also contributes to the catalytic mechanism (Ding and Cai 2013). The active site of GH10 proteins predicted in PROSITE is [GTA]-X-X-[LIVN]-x-[IVMF]-[ST]-E-[LIY]-[DN]-[LIVMF].

We compared the reported xylanase catalytic domains of different organisms (Figure 6C), including various plant species such as Hordeum vulgare (barley X-I, X-II), Arabidopsis thaliana (Atxyn1), ZEA mays (xyl), Streptomyces lividans (Xylanase A), and Penicillium simplicissimum (xynA). WILTED5 and the other xylanases share a high level of sequence similarity over regions surrounding the enzymatic active site within the GH10 domain. Two nucleophilic residues (glutamic acid and asparagine) and one proton-donor residue (arginine) were identified. The sequence GLPLWFTELDV, located between amino acid residues 619 and 629 of WI5, is a perfect match for the PROSITE protein profile PS00591 for the GH10 active site.

**Mutation in wi5 alters secondary cell wall structure and xylan deposition**

WILTED5 encodes endo-1,4-β-xylanase and is mainly expressed in internodes undergoing secondary cell wall deposition. We reasoned that the mutation in wi5 might alter the cell wall structure of the mutant, leading to stunted growth. Therefore, we compared the cell wall morphology of wi5 and wild-type plants. Transverse sections derived from the eighth internode (Figure 7A) of field-grown mutant and wild-type plants at the elongating stage were used for these comparisons. Scanning electron microscopy revealed that wild-type sclerenchyma cell walls underlying the epidermis and surrounding the vascular bundles were much thicker than those of wi5 plants (Figure 7B–C). These results indicate that the wi5 mutant is primarily deficient in secondary cell wall formation.

To confirm that WI5 primarily functions in xylan modification, we labeled the eighth internode sections with monoclonal antibodies (Figure 8) LM10 and LM11, which recognize different epitopes on xylan polymers. LM10 is thought to be specific to unsubstituted or lower-substituted xylans, whereas LM11 binds to both lower and higher substituted xylans (Kim and Daniel 2012). These two antibodies predominantly labeled sclerenchyma cells and vascular bundles, which is consistent with the notion that xylan is an abundant polymer in maize secondary cell walls. However, the labeling intensity for both LM10 and LM11 was significantly reduced in wi5; this reduction was more dramatic for LM10 compared to LM11. These observations further confirm that the decreased secondary wall thickening in wi5 is due to a reduction in xylan deposition. Therefore, WI5 is specifically required for the modification of maize secondary cell walls in sclerenchyma cells and vascular bundles.

To better understand the changes in cell wall components in wi5, we analyzed the cellulose, xylan, and lignin contents of the eighth internodes of wild-type and wi5 plants. No significant difference in cellulose content was detected in the mutant, whereas xylan and lignin contents were reduced (Figure S3). Therefore, WI5 is required for xylan synthesis and lignin deposition.
Figure 6. Continued
To determine whether Wi5 has xylanase activity, we expressed wild-type and mutant versions of Wi5 in Escherichia coli (TransGen Biotech CD601-01) using a pET30a protein fusion system and purified the proteins using Ni-NTA Agarose (Qiagen 30210). We examined xylanase activity as described by (Bailey et al. 1992). Wild-type proteins had an activity of 11 U/mg under our experimental conditions, whereas the wi5 protein had a lower activity.

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Figure 6. Sequence analysis of WILTY5 (Wi5) encoded protein
(A) Protein structure of Wi5. WILTY5 contains two carbohydrate-binding domains (CBM4-9) at its C-terminus and one glycosyl hydrolase family 10 (GH10) catalytic domain at its N-terminus. (B) Phylogenetic tree of the maize GH10 genes compared to those of Arabidopsis thaliana, Hordeum vulgare, Streptomyces lividans, and Penicillium simplicissimum. The numbers at each node represent the bootstrap support (percentage). The scale bar is an indicator of genetic distance based on branch length. (C) Multiple sequence alignment of the GH10 catalytic domains of different organisms. The degree of conservation (percent identity) among equivalent residues is indicated as follows: black (100%), dark gray (75%), and light gray (50%). The catalytic residues E-128 and E-236 (corresponding to E-525 and E-626 of the protein sequence of Wi5) in Streptomyces lividans Xylanase A are indicated by an asterisk. The consensus sequences of the GH10 catalytic domains are boxed, corresponding to acidic residues 619–629 of Wi5. For members of Zea mays, please see Table S5.

Figure 7. Scanning electron microscopy of the sclerenchyma of an internode
(A) Wilty (wi5) and wild-type plants used for observation. The above plant is wi5, and below is wild-type. White arrows show the sampling position of wi5 and wild-type. (B–G) Scanning electron micrographs of the sclerenchyma cell of wi5 and wild-type stems. (B) Low-magnification views of wi5 epidermis and vascular bundles. (C–D) Detailed views of wi5 epidermal sclerenchyma cells shown in red boxes and vascular bundles sclerenchyma cells in white boxes in (B). (E) Low-magnification views of wild-type epidermis and vascular bundles. (F–G) Detailed views of wild-type epidermal sclerenchyma cells shown in red boxes and vascular bundles sclerenchyma cells in white boxes in (E). White arrows show the different between wi5 and wild-type.
no significant xylanase activity (Figure S4). Thus, the mutant-impaired xylanase activity of WI5 affects the xylan content of the cell wall.

DISCUSSION

WILTED5 is a member of the GH10 family and is highly active in internodes undergoing secondary cell wall thickening

PFAM-based annotation and sequence analysis revealed that WI5 belongs to the GH10 family, with a domain containing two glutamic acid residues essential for catalysis (E525 and E626) and an active site located between amino acid residues 619 and 629. Indeed, we demonstrated the xylanase activity of WI5 in vitro (Figure S4).

The regulatory mechanisms of large gene families are more complex than those of smaller gene families, with many members playing partially redundant roles. We identified ten additional GH10 genes in the maize genome. This gene family does not contain highly conserved sequences. The genes are widespread in the genome, and no recent duplication events were detected. Phylogenetic analysis identified two distinct GH10 subfamilies. WILTED5 and Zmxyl1–4 belong to the same clade and share higher sequence identity with WI5 (33%–53%) than Zmxyl5–10, which belongs to another clade, pointing to the functional specialization of different isoforms. Since tissue-specific gene expression patterns might explain specific phenotypes in some cases, we examined the gene expression profiles of WI5. WILTED5 was mainly expressed in maize stems, especially in nonelongated internodes undergoing secondary cell wall thickening, suggesting that WI5 activity is restricted to wall deposition in specialized cells after elongation. In addition, due to the strong mutant phenotype, the possibility of functional redundancy could be eliminated.

Altered cell wall structure and composition lead to stunted growth in wi5

The wi5 mutant displayed stunted growth, with short internodes and a plant height approximately 60% that of wild-type plants. These traits indicate that WI5 is involved in normal growth and development in maize. Our analysis of the cell wall structure and cell wall components of wi5 internodes showed that mutations in WI5 affect the structure and composition of secondary cell walls. The strong differences between
wild-type and wi5 plants were mainly detected in mechanical tissues, such as sclerenchyma cells and vascular bundles. The sclerenchyma cell wall of the wi5 mutant was thinner than that of wild-type plants, especially for cells under the epidermal layer. Immunochemical analysis revealed reduced xylan deposition in the secondary cell wall.

These observations indicate that the wi5 mutation results in developmental changes to the structure and composition of the maize secondary cell wall. A deficiency in the formation of secondary cell walls in Arabidopsis usually leads to thinner cell walls (He et al. 2018), collapsed xylem, and globally altered morphology. By contrast, the cell shape and xylem structure did not differ substantially between wi5 and wild-type plants. Perhaps changes in the secondary cell wall structure have different effects on the overall structure of the xylem in monocot and dicot plants.

Possible role of WI5 in regulating plant growth and transporting water

The plant cell wall is a dynamic composite structure that provides mechanical support to the plant cell and participates in biotic and abiotic stress responses by remodeling matrix polysaccharides and regulating the cell wall biosynthetic machinery (Wolf et al. 2012). The disruption of the plant cell wall and cell wall enzymes often has obvious consequences on plant growth and development or responses to environmental stimuli (Hamann et al. 2009). The role of the cell wall in resistance to plant diseases and pathogen attack has been extensively studied (Bacete et al. 2018). The pivotal role of the cell wall in mediating abiotic stress responses has also been reported. However, only a few studies have focused on this topic. In the Arabidopsis lew2 mutant, which has a defect in the cellulose synthase gene AtCesA8, xylem cells are collapsed and water transport is impeded, resulting in wilting. The altered cell structure appears to be involved in a feedback response with the accumulation of sugar, proline, and ABA, which enhances drought tolerance (Chen et al. 2005). LEW3 encodes an α-1,2-mannosyltransferase. The mutation in lew3 leads to reduced protein glycosylation, impairs cellulose synthesis, and results in xylem collapse, stunted growth, and leaf wilting (Zhang et al. 2009a).

In the current study, we determined that WI5 encodes an endo-1,4-β-xylanase that remodels the plant cell wall by hydrolyzing cell wall xylan. The disruption of WI5 affected secondary cell wall structure and composition. The sclerenchyma cell walls were thinner and the xylan and lignin contents were severely reduced in wi5 compared to wild-type plants. These deficiencies likely result in a lack of structural strength in the xylem, ultimately resulting in defects in water transport capacity. As a result of reduced water uptake from the roots, wi5 plants were hypersensitive to drought, high temperature, and NaCl, with early wilting leaves, severely inhibited growth, and reduced survival. In addition, wi5 was highly sensitive to the oxidative stress reagent MV.

Further examination of the effect of the wi5 mutation on H2O2 levels by DAB staining revealed that wi5 seedling leaves accumulated more H2O2 under normal conditions and that wi5 plants accumulated much higher amounts of H2O2 under PEG and MV treatment compared to the wild-type (Figure 3). The wi5 plants exhibited a clear leaf wilting phenotype under normal growth conditions after the 3-leaf stage. Wilting was initially observed in the leaf margins of wi5, and the leaves became quite necrotic and withered under severe conditions. This may be because impaired water transport in the stem led to an abnormal thickening of the cell wall.

The xylan is synthesized in Golgi apparatus and then secreted by vesicles outside the cell. According to the activity of xylan hydrolytic enzyme measured in this experiment, it is speculated that the function of WI5 may modify the synthesis of xylan through its glycoside hydrolytic activity. The specific process is speculated as follows: mature xylan chain which takes part in hemicellulose formation has a particular mature structure, and the xylan precursor produced in the process of xylan synthesis may include some abnormal structures (possibly long beta-1,4 xylose branched chain and too long xylose poly backbone chain). WILTED5 included in a downstream modification pathway, is necessary to process the precursor into the correct structure before it can be secreted out of the cell to participate in the secondary cell wall. Due to the lack of WI5 protein, the xylan precursor could not be processed into mature xylan and could not be secreted out of the cell. The inhibition of the processing of xylan precursor results in the inhibition of the upstream xylan chain synthesis process, which ultimately leads to the reduction of the
accumulation of total xylan content, the failure of the formation of secondary cell walls and the decline of water transport capacity due to the lack of secondary cell walls in xylem, which result in the wilted phenotype of wi5 mutants.

The inevitable problem is how xylan can be modified rather than hydrolyzed by Wi5. Maybe the xylan included in the xylem is glucuronoroarabinoxylan which is enriched in side chains, while Wi5 plays an hydrolyze active with xylan with fewer side chains. The evidence for this conjecture comes from reports: hydrolysis of glycolytic enzymes from other families that hydrolyze gluconic acids and Arabian side chains (Rogowski et al. 2015). Further, in our previous preliminary tests to determine xylan activity (data not shown), Wi5 showed no hydrolytic activity for xylan (glucuronoraroabinosylxylan) from corn cob, but showed significant activity for xylan from beechwood which lacks side chains.

In summary, we identified an endo-1,4-xylanase gene that plays an essential role in secondary cell wall synthesis in maize, as demonstrated by both morphological and molecular analyses.

MATERIALS AND METHODS

Plant materials and growth conditions
The wi5 mutant was isolated from M2 population of W22 background Mutator transposon mutants (Mu9) crossed with inbred line Zong31. The plants were grown in the Shang Zhuang Experimental Station of the China Agricultural University for phenotypic observation and measurement of physiological indicators. For fine mapping, wi5 was backcrossed into the B73 genetic background to generate a BC1F1 backcross population which included more than 4,000 individual plants. wilted5 was backcrossed into the B73 genetic background for four generations and selfing once to generate a BC4F2 population. The homozygotes (wi5/wi5) and heterozygotes (wi5/+ ) from the BC4F2 population were used as the mutant and wild-type, respectively.

To observe the phenotypes of wi5 under different stress treatments, wi5 and wild-type plants were planted side-by-side in the same pots in the greenhouse. Water was continuously supplied until the plants reached the 5-leaf stage. The plants were subsequently treated with drought, high temperature (42°C), 250 mmol/L NaCl, or 50 µmol/L MV. The plants were photographed at different time points before and after treatment.

Measurement of water loss of detached leaves
Leaves of the same position were detached from 3-week-old wild-type and wi5 seedlings and their fresh weights were measured (at t = 0). Then the weight was measured every 30 min for 6 h in a ventilated dry room. Wild-type and wi5 plants were each measured three times. Water loss was calculated as: water loss rate = (Ws – Wt)/Ws (Ws = fresh weight of leaf at time = 0; Wt = weight of detached leaf at time t).

Measurement of leaf ABA content
Leaves of the same position were detached from wild-type and wi5 seedlings planted in the same pot and their ABA content was measured under the followed conditions: D0, 3-week-old seedlings watered normally; D20, 20 d drought treatment; D30, 30 d drought treatment; Re-watering for 10 d. Wild-type and wi5 plants were measured three times each. ABA content was measured as previously described (Zhang et al. 2009b).

Histochemical analysis
Hydrogen peroxide accumulation was measured by DAB staining (Sigma). Leaves were submerged in 1.0 mg/mL DAB solution and incubated in darkness at room temperature for 6–8 h. To visualize cell death, leaves collected from wi5 and wild-type plants at the 10-leaf stage were submerged in lactophenol Trypan blue solution (5 mL lactic acid, 10 mL of 50% glycerol, 1 mg Trypan blue (Sigma), and 5 mL phenol) at 30°C for 2–3 h. After each staining, the leaves were detached for 10 min in boiling 95% ethanol. Samples were observed under a dissecting microscope (Olympus, SZX16-DP72).

Genetic analysis and marker development
A fine mapping population including 4,000 BC1F1 segregation plants was constructed by crossing wi5 with B73. Genomic DNA was extracted and subjected to cosegregation analysis using available SSR markers listed in http://www.maizegdb.org. New SSR markers were identified using the SSR Hunter 1.3 Simple
Sequence Repeat Search tool and designed using Primer 5.0 based on B73 genome sequence information in http://ensembl.gramene.org/Zea_mays/Info/Index. CAPS markers were developed using SNP2CAPS software based on sequence comparisons between the wi5 mutant and B73 according to the B73 sequence in http://www.ncbi.nlm.nih.gov.

Copy number analysis and testing of a transgenic complementation event
A homozygous T3 generation line was isolated from a T2 individual. Genomic DNA was extracted from 14-d-old seedlings of the homozygous T3 line and wi5. The DNA was diluted to 100 ng/μL and digested with FastDigest HindIII (Thermo Scientific™ FD0504). The following primers were used for ddPCR: HMGA-F1, 5′-GAAATCCCTGAGGAGTGGTA-3′ and HMGA-R1, 5′-AGTAACAAACCTTGAAGCATC-3′ as the internal reference; and Wi5_DDPCR_F1, 5′-TGGGCAGGAGTCCTGGAG-3′ and Wi5_DDPCR_R1, 5′-GAGACCTGGTAGG TGCCGTAG-3′ for amplification of both endogenous and exogenous Wi5. Sample amplification and data analysis were performed using QX200 ddPCR EvaGreen Supermix (Bio-Rad 186-3022) and the QX200 Droplet Digital PCR System (Bio-Rad 186-4001).

Sequence and phylogenetic analyses
GH10 genes were identified by searching the whole genome sequence database of maize using DNA or protein sequences of the maize Wi5 gene as queries. Gene prediction was performed using FGENESH (Salamov and Solovyev 2000). Subcellular localization was predicted with ProtComp v. 9.0 (www.softberry.com). Multiple amino acid sequence alignments and phylogenetic analyses were performed using DNAMAN version 6.0 (LynnonBiosoft) and MEGA software version 4.0.

Xylanase activity assay
The complementary DNA (cDNA) fragments from wild-type and wi5 plants were amplified and inserted into expression plasmid pET30a, which was expressed in E. coli (TransGen Biotech CD601-01). The protein was purified using Ni-NTA Agarose (Qiagen 30210). Purified protein was diluted to 0.2 mg/mL. The xylanase substrate used in this experiment was xylan produced from a 10 mg/mL solution (Megazyme P-XYLNB-E10G) dissolved in sodium acetate buffer, pH 5.0 at 0.1 mol/L. A 200 μL aliquot of protein solution was mixed with 1.8 mL substrate, incubated at 50°C for 10 min and cooled in ice water. A solution containing sodium acetate buffer instead of protein served as the reagent blank. A standard curve was constructed using 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of 1 mg/mL xylose dissolved in 0.1 mol/L, pH 5.0 sodium acetate and diluted with sodium acetate buffer to 2 mL, combined with 3 mL 3,5-Dinitrosalicylic acid, incubated in a boiling water bath for 10 min, and diluted with water to 15 mL. The color produced at 540 nm was measured against the reagent blank and the released xylose was calculated based on the standard curve: 1 U represents the amount of enzyme that 1 μmol xylose releases per minute.

RT-PCR and real-time PCR
Total RNA was extracted from various organisms and subjected to reverse-transcription. The RNA was digested with DNasel for 40 min at 37°C. The reaction was stopped by adding stop solution and was then incubated at 65°C for 10 min, and the RNA was reverse-transcribed into cDNA with oligos and avian myeloblastosis virus reverse transcriptase. For RT-PCR, tubulin primers were used as a control (5′-CTGTCCTGTCACCCACTCTCT-3′ and 5′-GGAACCTGTCATCAACGGTTC-3′). Expression analysis of Wi5 was carried out using specific primers (5′-CGGTAACACTGGTCTTCAACAA-3′ and 5′-TGTTGTGATAAAGCCGCCAT-3′). Each 30 μL reaction included 0.5 μM of each primer and 1× SYBR Green PCR Master Mix (Takara). The reactions were carried out in a Real-Time PCR System as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 34 s, and 72°C for 15 s.

Transcriptome analysis
The eighth internodes and 13th leaves of wi5 and wild-type plants at the 13-leaf stage were sampled for RNA-seq library construction. An average of 1.98 G clean data was aligned to the B73 V3 reference genome. PCA was performed based on gene expression level. DEGs were identified using DESeq2, and KEGG (https://www.kegg.jp/) was used for pathway enrichment analysis of the DEGs.

Xylan immunolocalization
Stem sections were incubated with LM10 and LM11 antibodies (Plant Probes; 1/20 dilution) for 1 h (Kim and Daniel 2012). The samples were washed in

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phosphate-buffered saline and incubated for 1 h with fluorescein isothiocyanate-conjugated anti-rat secondary antibody (1/100 dilution; Jackson ImmunoResearch). The samples were visualized using a Leica fluorescence microscope.

**Cell wall component analysis**

The eighth internodes of wi5 and wild-type plants at the 13–15-leaf stage were dried and ball-milled into a powder. The cell wall residues were treated with pullulanase M1 (Megazyme) and α-amylase (Sigma) in 0.1 mol/L sodium acetate buffer (pH 5.0) overnight to remove starch. To analyze neutral sugar and cellulose contents, the alcohol-insoluble residues (AIRs) were hydrolyzed with trifluoroacetic acid. The supernatants were reduced and analyzed by gas chromatography – mass spectrometry (Agilent) to determine the monosaccharide content as previously described. The remaining material was treated with Updegraff reagent and hydrolyzed with 72% sulfuric acid to measure the cellulose content. The lignin content was measured using the acetyl bromide method (Foster et al. 2010).

**Injury flow test**

**WILTED5** and wild-type kernels were surface disinfected in 10% (v/v) H2O2 for 30 min, pre-germinated in saturated CaSO4 for 6 h, and washed three times in pure water. Kernels were then germinated in darkness between two layers of wet filter paper. The endosperm was removed from seedlings exhibiting the same growth trends and the embryos were grown in liquid Murashige and Skoog medium until they reached the 3-leaf stage. The embryos were then transferred into a 100% relative humidity environment and the middle of their hypocotyls were cut. Injury flow was collected for 4 h. Roots of detected seedlings were dried at 42°C and weighed. Injury flow rate was calculated as: injury flow (µL)/time (h)/weight of dehydrated root (g).

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**AUTHOR CONTRIBUTIONS**

X. H., H. Z., and Y. C. designed the experiments. X. H., Y. C., and X. L. performed most of the experiments and analyzed the data. The other authors assisted in the experiments and discussed the results. X. H., Y. C., and H. Z. wrote the manuscript. All authors read and approved the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12923/suppinfo

**Figure S1.** WI5 gene copy numbers of positive transgenic event and wi5
Gene copy numbers were calculated by the ratio of WI5 to reference. F3, homozygous plant by crossed positive T0 plants with wi5 plants and two-generation self-crossing.

**Figure S2.** Transcriptome analysis of leaves and internodes of wild-type and wi5
(A) Principal component analysis. (B) Venn diagram of Sample_WT_S vs Sampel_wi5_S and Sample_WT_L vs Sample_wi5_L. (C) Pathway enrichment of down-regulated genes (left) and up-regulated genes (right). Sample_WT_L and Sample_wi5_L: leaf data of wild-type and wi5 mutant; Sample_WT_S and Sampel_wi5_S: Internodes data of wild-type and wi5 mutant.

**Figure S3.** Comparison of cell wall component between wild-type and wi5 in eighth internode
Cellulose, xylan, and lignin contents of the eighth internodes of wild-type and wi5 plants. Error bars indicate ± SE (n = 3). Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001).

**Figure S4.** Xylanase activity of wild-type and wi5
1 U represents amount of enzyme that releases 1 μmol xylose per minute. CK represents blank tested additional three times for error calibration. Error bars indicate ± SE (n = 3). Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001).

**Table S1.** Genetic pattern of wi5

**Table S2.** The photosynthesis indexes of wild-type plant and wi5 mutant

**Table S3.** Markers used in the positional cloning of WI5 gene

**Table S4.** Statistical analysis of transgenic F2 generation segregation ratio

**Table S5.** Overview of the 11 members of the GH10 family identified in maize