Copy number variation of TdDof controls solid-stemmed architecture in wheat

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Stem solidity is an important agronomic trait of durum (Triticum turgidum L. var. durum) and bread (Triticum aestivum L.) wheat that provides resistance to the wheat stem sawfly. This dominant trait is conferred by the SS11 locus on chromosome 3B. However, the molecular identity and mechanisms underpinning stem solidity have not been identified. Here, we demonstrate that copy number variation of TdDof, a gene encoding a putative DNA binding finger protein, controls the stem solidity trait in wheat. Using map-based cloning, we localized TdDof to within a physical interval of 2.1 Mb inside the SS11 locus. Molecular analysis revealed that hollow-stemmed wheat cultivars such as Kronos carry a single copy of TdDof, whereas solid-stemmed cultivars such as CDC Fortitude carry multiple identical copies of the gene. Deletion of all TdDof copies from CDC Fortitude resulted in the loss of stem solidity, whereas the transgenic overexpression of TdDof restored stem solidity in the TdDof deletion mutant pitheless1 and conferred stem solidity in Kronos. In solid-stemmed cultivars, increased TdDof expression was correlated with the down-regulation of genes whose orthologs have been implicated in programmed cell death (PCD) in other species. Anatomical and histochemical analyses revealed that hollow-stemmed lines had stronger PCD-associated signals in the pith cells compared to solid-stemmed lines, which suggests copy number-dependent expression of TdDof could be directly or indirectly involved in the negative regulation of PCD. These findings provide opportunities to manipulate stem development in wheat and other monocots for agricultural or industrial purposes.

The wheat stem supports the leaves and grain-yielding inflorescence (spike), transporting water and minerals from the soil to the aboveground portions of the plant via the interconnected cells that form the xylem (2). Photoassimilates from source tissues are translocated through the stem phloem to sink tissues. Water-soluble carbohydrates (WSC), and to a lesser degree, starch, are stored in the stem and are later remobilized during grain filling (3). Fructan, the primary carbon storage molecule in the wheat stem, accumulates during periods of slow growth or when photosynthetic rates exceed sink tissue demands (4). The remobilization of water and WSC from the stem to the developing grain not only increases grain yield but also provides protection under moisture deficit (5, 6).

The wheat stem is divided into nodes at which the leaf sheaths are attached, and the regions between nodes are known as internodes. Between four and seven internodes elongate from a single internode primordium, depending on genetic and environmental factors (2). Stem elongation is a developmentally programmed stem | wheat | copy number variation (CNV) | DoF transcription factor | programmed cell death (PCD)

Wheat is the world’s most widely grown crop, with annual global production of ~750 million metric tons (1). The two most commonly grown types are bread wheat (Triticum aestivum L.) used for bread making and biscuits, and durum wheat (Triticum turgidum L. var. durum) used to produce pasta and couscous. Wheat consumption accounts for ~20% of the protein and calories consumed by humans (1). By 2050, the demand for wheat is predicted to increase by 50% to sustain a global population projected to surpass 9 billion people (1). Wheat production is threatened by the changing climate, which is expected to produce more severe and prolonged periods of extreme weather events such as heat, drought, and cold. Continuously evolving pathogen and insect populations also threaten wheat production on a global scale. A better understanding of the key genes involved in the physiological traits of wheat, including genes controlling stem development, will help breeders develop resilient cultivars equipped for these challenges to secure sustainable global food production.

Significance

Solid-stemmed wheat cultivars are resistant to the wheat stem sawfly, an important agricultural pest. Here, we identify TdDof as the causal gene that controls stem solidity in wheat. We show that copy number gain of TdDof correlates with its increased expression and the solid-stem phenotype. Our results suggest TdDof could function as a key regulator of genes involved in programmed cell death of the pith parenchyma cells. This research provides the framework to manipulate stem architecture in wheat and other monocots, which can be applied toward downstream agricultural and industrial applications. These include enhancing wheat stem sawfly resistance, modifying carbon partitioning and water-soluble carbohydrate remobilization in plants under drought and temperature stress, and bioenergy production.

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regulated process that begins from one of the lowermost nodes and progresses in succession upward toward the peduncle, which elongates last to support the spike. The intercalary meristem near the base of each internode gives rise to ground tissue comprising parenchyma cells, which differentiate to form the pith, and vascular strands that subsequently differentiate into vascular tissues surrounded by the cortex. In mature wheat stems, vascular bundles are separated by a layer of interfascicular parenchyma cells arranged in a ring surrounding a central cavity (internodal lacuna), which is formed by the collapse and breakdown of parenchyma cells that form the pith and causes the stems to become hollow (2). Pith is composed of undifferentiated parenchyma cells, which play important roles in water and WSC (i.e., sucrose, glucose, fructose, and fructan) storage (7). In moisture-limiting environments, the water-holding capacity of the pith parenchyma is proposed to be an important driver of drought and heat tolerance (8).

In some wheat cultivars, stem development has been reprogrammed to avoid the formation of a hollow stem. The main genetic factor conferring stem solidity resides within a major quantitative trait locus on chromosome 3B in durum (SSt1) and bread wheat (Qoss msu-3BL), hereafter referred to as SSt1 (9–11). The underlying gene, however, has not been identified. Several sources have been used to introduce stem solidity into elite breeding lines, including the commonly used Portuguese wheat landrace S-615 for bread wheat (12), and the German cultivar Biodor for durum wheat (13, 14). The trait in durum wheat is thought to have originated in the North African landrace Golden Ball. In all of these cultivars, the culm becomes partially or completely filled with pith (7).

The wheat stem sawfly (WSS) Cephus cinctus Norton (Hymenoptera: Cephidae) is a major pest in nearly the entire durum wheat-growing region of North America. Initially considered to be a pest of wild grasses, WSS developed a strong preference for wheat during the rapid expansion of this crop across the North American prairies beginning in the late 19th century (15). The ability of WSS to adapt to host preferences and thrive in new agroecological environments poses a risk to the geographical expansion of wheat that is expected to occur due to climate change. As sawfly populations also exist in Asia and Europe, they could pose a significant threat to wheat production if a similar shift in host preference were to occur (16). In North America, harvest losses attributed to WSS are estimated to exceed $350 million per year (15). Over the last century, the most effective way to minimize damage caused by WSS has been to grow solid-stemmed wheat cultivars (17). Solid stems provide resistance to WSS by deterring oviposition, and impeding larval development and growth inside the stem (18).

In the current study, we generate molecular and functional evidence that TdDof is the causative gene modulating stem solidity in wheat. Solid-stemmed cultivars carry multiple copies of TdDof, which correspond to its higher expression levels and the solid-stemmed phenotype. Our findings provide insight into putative TdDof-mediated regulatory functions associated with solid-stemmed architecture in wheat that may include regulation of key genes involved in programmed cell death (PCD). In addition, our results lay the foundation for developing genetic markers for screening and selecting desirable alleles for breeding wheat cultivars with the stem solidity trait.

**Results**

**Fine Mapping of the SSt1 Genomic Interval.** To narrow the genomic and genetic interval of SSt1, we performed fine mapping using an F2 population derived from a cross between durum (tetraploid) lines Kofa (hollow-stemmed) and W9262-260D3 (solid-stemmed) with markers designed to target the SSt1 region (SI Appendix, Table S1). Of 4,000 F2 lines examined, 24 critical recombinants were identified between flanking markers cks08_5169 and gwm247 (11). These lines were subsequently evaluated for stem solidity at maturity and used to position the SSt1 gene on the physical map (Fig. 1A). The closest flanking markers to SSt1 were usw306 and usw308, which were anchored to physical positions 828.5 and 830.6 Mb, respectively, on chromosome arm 3BL in the durum wheat reference genome assembly of the cultivar Svevo (19). This allowed us to further narrow the previously identified interval from 13 Mb (11) to 2.1 Mb (Fig. 1A). Within this interval, 42 protein-coding genes are annotated in the Svevo genome (SI Appendix, Table S2).

**Copy Number Variation of a Putative Dof Transcription Factor (TdDof) Is Associated with Stem Solidness in Wheat.** To identify candidate genes within the SSt1 fine-mapping interval, we generated an ethyl methanesulfonate mutant population derived from the solid-stemmed cultivar CDC Fortitude, which led to the selection of a hollow-stemmed mutant line “pithless1.” pithless1 was the only identified mutant exhibiting a complete loss of phenotypic expression of stem solidity. Within the SSt1 region in pithless1, a large deletion spanning 673.9 kb was detected via coverage analysis of short-read Chromium whole genome sequencing (10x Genomics). This sequencing platform provides long-range information through short reads by incorporating barcode oligos (GemCode) from the originating DNA segment, thereby providing increased power to detect structural variants. The deletion in pithless1 was validated by the presence of GemCode molecule associations between both ends of the deletion breakpoints (Svevo 3B positions 828,608,858 and 829,282,758 Mb) (Fig. 1B). The deletion contains 11 protein-coding genes, which are annotated to encode the following proteins: a Werner syndrome-like exonuclease, disease resistance protein RPM1, vacuolar protein sorting protein 25, two metallothioneins, plant invertase/pectin methyltransferase superfamily protein G, 30S ribosomal protein S17, 30S ribosomal protein S19, SANT domain-containing protein 2 G, very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase, and a Dof zinc finger protein (TdDof) (SI Appendix, Table S2). The hollow-stemmed phenotype of pithless1 and the deletion of these 11 genes suggest that the molecular determinant responsible for SSt1 is located within this region.

In contrast to our observations in pithless1, we identified an increase in read coverage within the SSt1 interval spanning a 34.8-kb interval (Svevo 3B positions: 829,118,858 and 829,153,686 Mb) in the solid-stemmed lines CDC Fortitude and W9262-260D3, indicating that additional copies of this segment are present in each of these lines (Fig. 1B). Importantly, this region of copy number variation (CNV) contains only one gene (TRTID3Bv1G280530) in Svevo that encodes a putative Dof zinc finger protein (TdDof) and is one of the 11 deleted genes in the pithless1 mutant (SI Appendix, Table S2). Analysis of the GemCode molecule associations spanning this region in CDC Fortitude and W9262-260D3 indicated that all copies of TdDof are arranged in tandem (Fig. 1B and C). In contrast, visualization of the read data for the hollow-stemmed lines Svevo and Kofa indicated that a single copy of the region containing TdDof is present in these lines (Fig. 1B). The overlap between the deleted region in pithless1 and the CNV in solid-stemmed lines supports TdDof as a strong candidate for SSt1.

**Solid-Stemmed Cultivars Carry Three Identical Copies of TdDof.** To resolve the TdDof CNV region in solid-stemmed lines, we performed targeted sequencing and assembly of the region in CDC Fortitude following a modified CRISPR-Cas9 protocol from Oxford Nanopore Technologies. The reason we chose this approach was twofold; first, to fully resolve the tandem copies of TdDof, ultralong reads that span the CNV breakpoints are required, and, second, because of the size of the wheat genome, targeted sequencing of the TdDof region was a more efficient
Fig. 1. CNV of TdDof is associated with stem solidity in wheat. (A) Fine-map interval of SSt1 in the Kofa/W9262-260D3 F2 population. The region between closest flanking markers usw306 and usw308 is shown in blue. (B) Chromium read coverage plots within the SSt1 interval on chromosome 3B of five durum lines. The y axis of each coverage plot denotes the minimum and maximum read coverage for each line within the interval. GemCode molecule associations spanning structural variants are indicated by the green bars (CDC Fortitude, W9262-260D3) and red bar (pithless1). Positions are in megabases. (C) Diagram showing the organization of the TdDof CNV region in CDC Fortitude (solid-stemmed) and Kofa (hollow-stemmed) with CNV breakpoints indicated by the black bars. Three copies of TdDof (TdDof1-3) are present in CDC Fortitude vs. a single copy in Kofa. At the CNV breakpoint between TdDof1-2 and TdDof2-3, a unique 25-bp insertion is present that is only found in solid-stemmed cultivars. (D) High-throughput KASP (kompetitive allele-specific PCR) marker usw275 accurately distinguishes stems that are hollow (red) and solid (blue) in both bread and durum wheat.
strategy. We generated an assembly of 230,823 base pairs (bp) that contained three copies of **TdDof** (**TdDof**<sub>1</sub>; **SI Appendix**, Fig. S1A), confirming the presence of two additional copies of **TdDof** in CDC Fortitude arranged in tandem orientation, each with an identical upstream putative regulatory region (Fig. 1C and **SI Appendix**, Fig. S1 A and B). A unique 25-bp insertion sequence (AGATGCTCTAGAAACGTCTTAAAG) was identified at the insertion breakpoint between **TdDof**<sub>1</sub> and **TdDof**<sub>2</sub> and between **TdDof**<sub>2</sub> and **TdDof**<sub>3</sub> that was not present in any of the hollow-stemmed lines examined (Fig. 1C and **SI Appendix**, Fig. S1B). We used Droplet Digital PCR (ddPCR), which provides absolute quantification of target DNA or RNA molecules, to confirm the presence of three copies of **TdDof** and two copies of the unique 25-bp insertion in CDC Fortitude compared to the single copy of **TdDof** in Kofa and no copies of the insertion sequence (**SI Appendix**, Fig. S1C). We also developed a dominant PCR marker to detect the CNV region and validated it in three lines derived from common representative sources of stem solidness: Golden Ball, Biodur, and S-615 (**SI Appendix**, Fig. S1D). These results suggest that increased copy number of **TdDof** is associated with the **SSt1** solid-stem phenotype.

**TdDof** is located between positions 829,151,607 and 829,152,953 on chromosome 3B in the Svevo reference genome (**SI Appendix**, Table S2). The gene contains two exons and one intron (**SI Appendix**, Fig. S2), and the coding sequence (CDS) of 1,134 nucleotides encodes a predicted protein of 377 amino acids (**SI Appendix**, Fig. S2). The predicted protein sequence encoded by **TdDof** is identical between hollow- and solid-stemmed lines. In solid-stemmed lines, each **TdDof** copy (**TdDof**<sub>2</sub>-**TdDof**<sub>3</sub>) encodes an identical protein. The predicted protein contains a zinc finger DoF domain (IPR003851) between amino acid residues 110 and 164 (**SI Appendix**, Fig. S2). The DoF domain is a highly conserved DNA-binding domain that is specific to a family of transcription factors observed in vascular plant species.

**Overexpression of **TdDof** Induces Stem Solidness in Kronos and **pithless1**. To investigate the phenotypic effects of increased **TdDof** gene expression, we generated a series of independent transgenic lines in the hollow-stemmed wheat lines **pithless1** and Kronos that overexpress the **TdDof** CDS under control of the rice **ACTIN** promoter. All 79 lines were analyzed for **nptII** copy number to confirm the presence of the **TdDof** transgenic construct. Both the **pithless1** and Kronos constructs-regenerated from calli carried 0 transgene copies, whereas the transgenic lines carried between 1 and 4+ copies of the **TdDof** construct (**SI Appendix**, Table S3). For the **pithless1**-derived transgenic plants, 9 out of 10 **T<sub>0</sub>** lines had the maximum level of stem solidness, in contrast to the **pithless1** control which had hollow stems (**SI Appendix**, Table S3). For the Kronos-derived transgenic plants, 27 out of 30 **T<sub>0</sub>** lines had stems that were solid compared to the Kronos control, which had hollow stems (**SI Appendix**, Table S3). To further validate these observations, we advanced the **T<sub>0</sub>** plants in both backgrounds to the **T<sub>1</sub>** generation. Homozygous **T<sub>1</sub>** plants were phenotypically similar to the controls, except for the accumulation of pith. Some variation in the color of pith was observed between transgenic lines, which ranged between green and white. Importantly, the **T<sub>1</sub>** progeny maintained the stem solidness phenotype that was observed in the **T<sub>0</sub>** generation (Fig. 2 A–H). The increased copy number of the **TdDof** constructs in the transgenic lines was associated with increased **TdDof** transcript levels (Fig. 2 I–L). Therefore, overexpressing **TdDof** in two independent genetic backgrounds (Kronos and the **pithless1** mutant) resulted in stem solidness, providing strong evidence that **TdDof** is the **SSt1** locus is a causal genetic factor of stem solidness.

**Gene Expression Signatures Identify Candidate Pathways Involved in **TdDof**-Mediated Stem Solidness.** We performed gene expression profiling of six durum wheat lines with hollow- and solid-stemmed types to identify differentially expressed genes (DEGs) during early internode elongation (Zadoks stage 32). This is typically when the first sign of pith breakdown begins to appear in hollow-stemmed cultivars. The experiment was performed in three replications for each line. Within the **SSt1** interval, 21 of the 42 genes were expressed (raw mean read count >15), including a cluster of eight genes (including **TdDof**) that were differentially expressed between **pithless1** and CDC Fortitude (Fig. 3A). All eight genes are contained within the **pithless1** deletion, and thus were not expressed in the mutant. Of the eight genes, only **TdDof** was consistently differentially expressed (up-regulated in solid-stemmed lines) across all pairwise comparisons (Fig. 3A). These results indicate that **TdDof** expression is associated with stem solidness, and its gene expression pattern is positively associated with the genomic copy number of **TdDof** (Fig. 3 B and C).

To investigate genome-wide regulatory pathways involved in stem solidness, we performed weighted gene coexpression network analysis (Fig. 3D and Table 1). In a pairwise comparison between **pithless1** and CDC Fortitude, only 195 DEGs were identified, which were distributed across all chromosomes (adjusted *P* < 0.01) (**Dataset S1**). Of these, we identified 12 “primary” genes that were codifferentially expressed in all hollow- by solid-stemmed pairwise comparisons (Table 1). These genes have been annotated to encode the following proteins: peroxidase (**TRITD1Bv1G046830**), two NAM/ATAF1/2/CUC2 (NAC) domain proteins (**TRITD2Av1G226090** and **TRITD2Bv1G188540**), three cysteine proteases (**CIP**; **TRITD3Av1G213290**), **TRITD3Bv1G195680** and **TRITD3Bv1G196700**, a Dof zinc finger protein, **TdDof** (**TRITD3Bv1G280530**), two metal transporters (**TRITD4Av1G000570** and **TRITD4Bv1G174140**), a pectin lyase-like superfamily protein (**TRITD6Av1G021640**), and two **HTK** transmembrane (**TRITD7Av1G226240** and **TRITD7Bv1G172380**). The expression of **TdDof** in the solid-stemmed lines was negatively correlated with the expression of all 11 other primary genes (Table 1). Notably, this list includes three **CIP** genes, and two NAC transcription factor genes whose orthologs have been implicated in PCD in other species (Table 1), and a pectin lyase-like superfamily protein putatively involved in cell wall degradation.

Next, we used the list of “primary” genes to extract “secondary” genes from the weighted gene coexpression network based on their correlated expression patterns (Table 1 and **Dataset S1**). Using this approach, we constructed a subnetwork consisting of 32 genes from seven different coexpression modules (Fig. 3D). Secondary genes were annotated as being putatively involved in cell wall modification/degradation, transcriptional regulation, central metabolism, and signal transduction (Table 1). Taken together, our studies indicate that higher levels of **TdDof** expression in solid-stemmed cultivars is correlated with the suppression of genes involved in several processes including cell wall modification/degradation, and the regulation and execution of PCD.

**Developmental and Metabolite Analyses Identify Structural and Metabolomic Consequences of **TdDof**-Mediated Stem Solidness.** To address the developmental pathways involved in the distinct stem solidness phenotypes in wheat, we performed a detailed comparison of stem pith anatomy between CDC Fortitude and **pithless1** using stem cross-sections sampled at Zadoks stage 32 (**SI Appendix**, Fig. S3). Light and electron microscopy revealed clear differences in the central pith parenchyma cells of **pithless1** compared to CDC Fortitude, including irregular, broken, and collapsed parenchyma cells and cell wall buckling. These anomalies in pith parenchyma cell size and shape prompted us to analyze the spatial expression pattern of **TdDof** in the stem for any potential associations with these morphological changes. We performed in situ PCR using stem cross-sections
Fig. 2. Transgenic expression of TaDof induces stem solidness in hollow-stemmed lines *pithless1* and Kronos. Cross-sections of the second internodes of nontransgenic (Left, hollow stem) and TaDof overexpression T1 transgenic lines (Right, solid stem) derived from *pithless1* (A) and Kronos (B) at Zadoks stage 34. Scanning electron microscopy images of stem cross-sections showing the detailed stem anatomy in *pithless1* Control_6 (nontransgenic line) (C and D) compared to TaDof overexpression T1 transgenic line *pithless1* 4_3_3 (F and G). Light microscopy images of stem cross-sections (E and H). In the high-magnification view of the central regions of these sections (D, E, G, and H), parenchyma cells in and surrounding the central pith region are intact and more uniform in size in the TaDof overexpression T1 transgenic lines (G and H), whereas, in nontransgenic control lines in the comparable pith region, the cells are irregular or broken and collapsed (D and E). (Scale bars: A–C and F = 2 mm; D, E, G, and H = 500 μm.) Molecular analysis via ddPCR of TaDof transgene copy number and gene expression in independent T1 transgenic lines of *pithless1* (I and J) and Kronos (K and L). Error bars denote SD across replicates (I–L). The same lines imaged in A and B were selected for molecular studies.
collected at Zadoks stages 32 and 34, from *pithless1*, Kofa, and CDC Fortitude (Fig. 4). *TdDof* transcripts were predominantly expressed in pith parenchyma cells of Kofa and CDC Fortitude during second internode formation (Fig. 4). During stem elongation, hollow pith gradually formed in Kofa (Zadoks stage 34), while *TdDof* was only expressed in intact parenchyma cells (Fig. 4). By contrast, the expression pattern of *TdDof* in CDC Fortitude at Zadoks stage 34 remained constant and was similar to Zadoks stage 32, which was associated with reduced cell death and pith degeneration (Fig. 4). As expected, no signals above the background level were detected in *pithless1*, which is consistent with the deletion of *TdDof* (Fig. 4).

To investigate the potential cause of cell collapse in the hollow stems of *pithless1*, we compared cell viability in the pith parenchyma cells at Zadoks stage 32 in the stem internodes of CDC Fortitude and *pithless1* using two complementary cell viability...
stains, fluorescein diacetate (FDA) and propidium iodide (PI), followed by confocal laser scanning microscopy (Fig. 5 A–H). PI stains only nuclei from dead cells with ruptured cell membranes, making them appear fluorescent, whereas nuclei from living cells are not stained by this dye. FDA stains viable, living cells producing nuclear and nucleocytoplasmic signals around a dark vacuole where stain is quenched. In our analysis, the absence of nuclear staining by PI alone was not used to determine the

Table 1. List of genes identified in coexpression network analysis

| Svevo gene      | Mod     | Annotation                              | Chr | Position | P vs. F | K vs. F | L vs. F | K vs. LG | K vs. W | L vs. LG | L vs. W |
|-----------------|---------|-----------------------------------------|-----|----------|---------|---------|---------|---------|---------|---------|---------|
| TRITD1AvG0046830 | 5       | Peroxidase                              | chr1B | 128,533,413 | -1.0    | -2.7    | -2.0    | -3.2    | -3.5    | -2.0    | -2.6    |
| TRITD2AvG226090 | 10      | NAC domain–containing protein           | chr2A | 619,702,812 | -2.1    | -2.8    | -2.6    | -2.2    | -2.0    | -1.8    | -1.8    |
| TRITD2AvG11850540 | 10      | NAC domain protein,                     | chr2B | 558,687,739 | -1.2    | -1.9    | -1.6    | -2.7    | -2.2    | -1.5    | -1.7    |
| TRITD3AvG213290 | 5       | Cysteine proteinase                     | chr3A | 587,481,650 | -1.4    | -3.9    | -2.9    | -5.1    | -3.5    | -3.0    | -2.6    |
| TRITD3AvG195680 | 4       | Cysteine proteinase                     | chr3B | 597,195,875 | -1.3    | -4.6    | -3.8    | -5.1    | -2.9    | -3.5    | -2.2    |
| TRITD3AvG196170 | 2       | Cysteine proteinase                     | chr3B | 598,187,812 | -1.4    | -2.9    | -2.5    | -5.1    | -2.5    | -3.9    | -2.1    |
| TRITD3AvG280530 | 3       | Dof zinc finger protein                 | chr3B | 829,151,607 | 4.7     | 2.1     | 1.7     | 2.5     | 2.5     | 1.9     | 2.0     |
| TRITD4AvG0000570 | 5       | Metal transportor, putative             | chr4A | 1,055,669  | -1.3    | -3.4    | -2.3    | -3.8    | -4.2    | -2.4    | -2.8    |
| TRITD4AvG174140 | 17      | Metal transportor, putative             | chr4B | 591,093,455 | -0.9    | -2.7    | -1.8    | -2.4    | -2.6    | -1.3    | -1.6    |
| TRITD6AvG021640 | 2       | Pectin lyase–like superfamily protein   | chr6A | 51,804,402  | -1.2    | -2.7    | -2.0    | -2.8    | -2.7    | -1.8    | -1.9    |
| TRITD7AvG226240 | 5       | HKT23 transporter                      | chr7A | 606,221,441 | -2.0    | -3.9    | -3.7    | -2.2    | -3.6    | -1.8    | -3.1    |
| TRITD7AvG172380 | 5       | HKT23 transporter                      | chr7B | 544,995,054 | -1.1    | -1.8    | -1.8    | -1.7    | -2.2    | -1.5    | -2.0    |

The table presents 12 primary genes that met the criteria of differential expression across all hollow–vs. solid-stemmed comparisons. This list was used to identify secondary genes that were correlated in their expression across samples with at least one of the primary DEGs. The table lists the gene accession from the Svevo annotation, coexpression module (Mod), Svevo gene description, chromosome (Chr), and position. Values on the right of the table are log2 expression fold-change values for each hollow by solid comparison. Positive values indicate expression was greater in the solid-stemmed line denoted by blue shading, and negative values indicate expression was greater in the hollow-stemmed line denoted by red shading. Genotypes used in pairwise comparisons are labeled as follows: P, pithless1; F, CDC Fortitude; L, Langdon; LG, Langdon-GB-3B; K, Kofa; W, W9262-2603.
viability status of a cell, as select nuclei may be out of the optical plane or excluded by specimen preparation. Thus, putatively viable cells were defined as FDA stain positive, while dead or dying cells were defined by a PI-stained nucleus. The occasional plane or excluded by specimen preparation. Thus, putatively vi-

Four. In situ PCR demonstrating enrichment of TdDof messenger RNA in internode parenchyma cells in pithless1, Kofa, and CDC Fortitude. Purple staining (labeled by red arrows) indicates the presence of in situ PCR amplified TdDof transcripts. Representative micrographs of internode cross-sections from Zadoks stages 32 and 34 show similar high expression levels of TdDof in parenchyma cells in which the negative controls were generated under the same conditions but with the reverse transcription steps omitted. Three panels from Left to Right represent internode tissues from pithless1, Kofa, and CDC Fortitude. (Scale bars = 200 μm.)

Developing a DNA Marker for Breeding Solid-Stemmed Cultivars. A high-throughput universal marker for the solid-stemmed trait that can be used in both durum and bread wheat germplasm has remained elusive. We previously described the marker umw204, which is currently used in some breeding programs (20); however, this marker scores hexaploid and tetraploid cultivars inversely (SI Appendix, Fig. S7A). To overcome this limitation, we mined the Chromium sequence datasets to call single-nucleotide polymorphisms and designed additional markers. We identified a region spanning position 828.9 Mb to 829.6 Mb on chromosome 3B of the Svevo assembly in which the allelic states of solid-stemmed hexaploid and tetraploid cultivars were identical (SI Appendix, Fig. S7B). This interval is consistent with the fine-mapped interval in durum wheat that contains the SSt1 gene TdDof. We successfully developed a marker (umw275) tightly linked (<4.3 kb) to TdDof that produces accurate results in both bread and durum wheat and, most importantly, is ame-

Discussion
Here, we identified TdDof (TRITD3Bv1G280530), which encodes a putative Dof (DNA binding with one finger) protein, as the causal gene conferring stem solidity corresponding to SSt1 on chromosome arm 3BL. We confirmed this via transgenic overexpression of TdDof in two independent hollow-stemmed durum wheat genotypes which yielded transformants with solid stems. Although all cultivars we examined carry the genetic sequence to encode identical functional TdDof proteins irrespective of stem type, our findings indicate the solid-stemmed phenotype is caused by increased expression of the TdDof gene.
Fig. 5. Cell viability in the pith parenchyma cells of CDC Fortitude and pithless1 stems. Stem cross-sections from the second internodes of CDC Fortitude (A–D and I) and mutant pithless1 (E–H and J) plants viewed as single frame images. Viability staining of pith cells viewed by confocal laser-scanning microscopy phase contrast image (A and E), PI signal (B and F), FDA signal (C and G), and PI and FDA merged view (D and H). Parenchyma cell walls stained with PI in CDC Fortitude (B) and pithless1 (F), with fluorescent nuclei indicating penetration of the dye into cells surrounding the hollow pith of pithless1 stem sections. Penetration and conversion of nonfluorescent FDA stain into green fluorescent fluorescein by CDC Fortitude (C) and pithless1 (G) pith parenchyma cells. The * symbol indicates a putatively viable cell, with bright FDA signal around a dark vacuole, and the Θ symbol indicates a cell considered to be dead, with PI-positive nucleus. The viability status of cells without labels is undetermined. TUNEL assay for DNA fragmentation in CDC Fortitude (I) and pithless1 (J) stems, as viewed by confocal laser scanning microscopy. Arrowheads indicate positive nuclear signals in pith cells. (K) Quantification of TUNEL assay results for three biological replicates of CDC Fortitude (Fort), pithless1 (P1), positive control using TACS nuclease treatment (+), and unlabeled experimental control (–), with the labeled nuclei per the total number of pith parenchyma cells plotted as the mean percentage ±SD. (Scale bars: A–H = 20 μm; I and J = 100 μm.)

Three identical copies of TdDof in solid-stemmed durum wheat are tandemly arranged within a 34.8-kb triplicated segment within the SSl1 locus. The expression of TdDof during early stem elongation is positively correlated with its genomic copy number, and higher expression likely induces the solid-stem phenotype. Given that the upstream putative regulatory region is present for each of the three TdDof copies, coupled with an approximate threefold increase in gene expression, it is likely that all three copies are expressed in solid-stemmed cultivars. However, we cannot rule out the possibility that one or more copies of TdDof contribute more to the observed expression levels.

Stem solidness is a trait that has been deployed in wheat to control devastating outbreaks of the WSS in North America. The conventional sources used to introgress stem solidness into wheat were the Portuguese landrace S-615 (hexaploid durum wheat), the South African landrace Golden Ball (tetraploid durum wheat), and the German cultivar Biodur (tetraploid durum wheat) (12–14). Despite their diverse global origins and differing ploidy levels, the unique TdDof CNV signature is found in all three of these sources of stem solidness. This finding, combined with an accurate DNA marker that predicts stem solidness within both wheat species, not only supports that TdDof is responsible for stem solidness in both durum and bread wheat but also indicates that stem solidness originated from a common genetic progenitor. However, the original source has not been identified, and it remains to be determined whether stem solidness in wheat originated pre- or postdomestication.

TdDof is part of a large family of plant-specific transcription factors that were first identified in maize (21). Since their discovery, Dof transcription factors have been shown to regulate a wide range of developmental and physiological processes, including carbon and nitrogen assimilation (22), hormone signaling (23), light responses (24), seed germination and development (25), flowering time (26–28), pollen development (29), shoot branching (30), interfascicular cambium/vascular tissue development (31), biotic stress tolerance (32), and drought and salt tolerance (28). There are 62 Dof genes in durum wheat and 116 in bread wheat. The recent release of the first genome sequences for durum and bread wheat (19, 20), along with high-quality gene models, has set the stage for unraveling the functions of this diverse gene family and their associated regulatory functions. Here, we demonstrated that TdDof controls stem architecture, and leads to reduced expression of genes putatively involved in PCD such as NAC (TRIDT2Av1G226080 and TRIDT2Bv1G188540) and CEP (TRIDT3Av1G213290, TRIDT3Bv1G195680, and TRIDT3Bv1G196170). Furthermore, TdDof may also impact response to oxidative stress (peroxidase: TRIDT1Bv1G046830), metal ion transport (TRIDT4Av1G000570 and TRIDT4Bv1G174140), cell wall modification (pectin lyase-like superfamily: TRIDT6Av1G021640) and cation transport (HKT2: TRIDT7Av1G226240 and TRIDT7Bv1G172380).

Our findings suggest that TdDof may impact PCD pathways through direct or indirect regulation of NAC and CEP genes, as supported by histochemical evidence of PCD of the pith parenchyma in hollow-stemmed cultivars during the early stages of stem elongation. PCD is an important regulatory mechanism used by plants to eliminate unwanted cells, a process required for normal plant growth and development (33, 34). One example of
PCD is the hypoxia-driven occurrence of pith autolysis, a wide- spread phenomenon in many plant species that is necessary for eliminating pith parenchyma cells and forming aerenchyma to facilitate gas exchange within the stem (35). In the current study, we provided several key lines of evidence suggesting PCD is associated with stem hollowness in wheat. Gene coexpression analysis identified two NAC genes on chromosomes 2A and 2B and three CEP genes on chromosomes 3A and 3B that were strongly upregulated in hollow stems where TdDof expression was low. In Arabidopsis, the close ortholog to TRITD2Av1G226090, TRITD2Bv1G188540 is the gene KIRA1, whose expression was implicated as a key regulator of PCD-associated genes in the stigma (36). Recent studies identified the D gene (Sobic.006G147400) in sorghum (Sorghum bicolor L.), encoding an NAC transcription factor that functions as a transcriptional switch to regulate PCD in stems (37, 38). The D gene activates various cell death-related enzymes, including the direct activation of the cysteine protease CEPI (37, 38). The most closely related genes to the sorghum D gene in the durum wheat annotations share 86% protein sequence similarity with NAC protein TRITD2Bv1G188540 encoded on chromosome 2B and 66% sequence similarity with NAC protein TRITD2Av1G226090 encoded on chromosome 2A, indicating that these proteins are orthologous between sorghum and wheat. Cysteine protease genes, including CEPI that is regulated by the sorghum D gene, belong to a large family of enzymes that are ubiquitous across plants, animals, and microbes. These enzymes function downstream of transcription factors and are involved in protein degradation and PCD (39, 40). The Arabidopsis ortholog, CEPI, has been implicated in PCD during xylem development (41). Along with CEPI, several other known protease genes are often coexpressed in cells that are actively undergoing PCD (42–44). Given the strong negative correlation we observed between the expression of TdDof and PCD-related genes, this observation suggests that NAC and CEP genes could be target genes that are negatively regulated by TdDof. Nevertheless, the direct link between TdDof expression and the occurrence of PCD remains unclear and warrants further investigation.

We also obtained histochemical evidence suggesting the cells in pithless1 undergo PCD prior to collapsing into the culm lumen. However, unlike stems in sweet and juicy sorghum cultivars, whose pith remains largely intact, the complete collapse of the pith in most hollow-stemmed wheat occurs during the early stages of stem elongation. Despite this difference, our findings suggest that the physiological processes identified in sorghum (37, 38) are likely similar to the processes that occur in hollow-stemmed wheat cultivars. Targeting genes involved in pith PCD, including those identified in this study such as the NAC and CEP genes, might represent a new avenue for enhancing the expression of stem solidness in crop species. For example, because solid stems have higher mass per unit length than hollow stems, this could have positive implications for biomass partitioning and may be useful in the production of bioethanol. If PCD pathways are conserved within the stem between species, genetic engineering involving TdDof could be a strategy to increase accessible WSC content in other monocot crops such as switchgrass (Panicum virgatum L.), which is used in the biofuel industry for ethanol production (45). Increasing WSC content in straw could increase the extraction efficiency of ethanol while lowering the amount of enzymatic pretreatment required during processing (46).

Our findings lay the foundation for future research aimed at optimizing the expression of stem solidness, which could have additional downstream agricultural and industrial applications. An important breeding target should be to maximize pith production when female WSS are actively laying eggs, which would provide maximum levels of resistance to this insect. However, it may be beneficial to begin degrading the pith during grain filling to enhance water and WSC remobilization capacity to the developing grain, particularly during periods of abiotic stress. Drought poses the single largest constraint to crop production in many parts of the world and is expected to become more widespread due to the changing global climate. Increasing the capacity of the pith to store water and WSC for later remobilization under periods of abiotic stress has been suggested (8), but remains largely unexplored as a breeding target for enhancing drought stress tolerance. Indeed, in the current study, we observed the accumulation of higher levels of the fructan 1-ketose in the stems of CDC Fortitude relative to the hollow-stemmed controls. Consistent with this possibility, during periods of drought, hexose sugars from hydrolyzed fructans reduce leaf intracellular water potential to maintain turgor and to promote continued leaf expansion (4). Higher sugar concentrations also increase the chemical potential of water, which lowers the freezing point in nonfrozen tissues or promotes the melting of ice in frozen tissues (47), making it possible for plants with solid stems to have better cold stress tolerance than hollow-stemmed plants.

In summary, we determined that TdDof controls pith development in wheat. We propose that CNV of TdDof drives increased gene expression and the solid-stemmed phenotype. When we deleted all copies of TdDof in the solid-stemmed parent CDC Fortitude to generate the mutant pithless1, we observed a corresponding loss of the solid-stemmed phenotype associated with this deletion. Adding TdDof back into the pithless1 genetic background via exogenous overexpression restored the solid-stemmed dominant phenotype of this mutant. This gain of function was also observed in the unrelated genetic background of hollow-stemmed cultivar Kronos. Our newly developed molecular marker usw275 will be useful for tracking TdDof-mediated stem solidity to facilitate research and breeding efforts. Our results also provide insight into the downstream pathways that may be regulated by TdDof, particularly the repression of PCD as a plausible mechanism for stem solidness.

Materials and Methods

Details regarding sample preparation, experimental procedures, and data analysis, along with the associated references, are presented in SI Appendix, SI Materials and Methods. The 10x Genomics Chromium whole-genome sequencing data and Oxford Nanopore and RNA-seq (Illumina) data have been submitted to Sequence Read Archives at National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA630287 (48).

Data Availability. Sequencing data have been deposited in NCBI (PRJNA630287) (48). All study data are included in the article and SI Appendix.

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