



Intragenic overexpression of *TONNEAU 1b* enhances grain length and weight in durum wheat

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ARTICLE INFO

Keywords:

Yield
Durum wheat
Grain size
Grain length
Cre-lox
Intragenesis

ABSTRACT

Grain yield is a complex trait determined by multiple genes, physiological and developmental processes, including those governed by cytoskeletal dynamics. In this study, we investigated the role of the *TONNEAU 1b* (*TON1b*) gene, a core component of the TON1-TRM-PP2A (TTP) complex involved in microtubule organization and cell division plane positioning, in the polyploid context of durum wheat (*Triticum turgidum* ssp. *durum*). To explore its function and agronomic potential, we developed intragenic Svevo lines overexpressing *TON1b* under the control of the endosperm-specific *1Dx5* promoter, using a CRE-LOX recombination system to produce marker-free genotypes. Molecular and phenotypic analyses of fixed intragenic lines revealed significant increases in grain length, grain weight, spikelet number and total starch content, without compromising grain number per spike. Transcriptomic profiling indicated consistent *TON1b* overexpression alongside downregulation of *CEN1* and *TON2*, and upregulation of *GW7*, suggesting a regulatory role in both cell division and elongation processes. These findings suggest a role of *TON1b* on cytoskeletal remodelling and grain development, offering a promising genetic lever for yield improvement in durum wheat.

1. Introduction

Wheat stands as a fundamental staple for 2.5 billion people worldwide. Specifically, durum wheat (*Triticum turgidum* ssp. *durum*) is a linchpin of Mediterranean and North African culinary traditions, providing essential ingredients for iconic dishes such as pasta, couscous, and various high-quality bread types. In the face of a rising global population and the escalating challenges posed by climate change, the imperative to enhance wheat yield is undeniable.

Grain yield in wheat is a complex quantitative trait governed by interactions between genetic factors and environmental conditions (Reynolds et al., 2009; Fischer et al., 2010). Yield is primarily determined by two direct components: grain number per unit area and individual grain weight, each resulting from the coordination of physiological processes during critical developmental stages, including spikelet initiation, floret fertility, and grain filling (Slafer et al., 2014; García et al., 2015). In addition to these core components, several ancillary traits such as total biomass, harvest index, plant architecture,

phenological adaptation, and stress resilience significantly contribute to yield potential (Araus et al., 2008; Reynolds et al., 2012), either by modulating source-sink dynamics or through mechanisms still under investigation at molecular and physiological levels (Shi et al., 2013; Tardieu et al., 2018). Among yield determinants, grain weight is strongly influenced by grain size, commonly defined by its length, width, and thickness (Bednarek et al., 2012).

Recent advances in cereal genomics have elucidated key molecular players underlying grain size and weight, especially in rice (*Oryza sativa*). Due to the high conservation of genomic structure among cereals, numerous orthologous genes regulating grain development have also been identified and functionally validated in wheat, enabling translational breeding strategies (Gasparis and Miłoszewski, 2023). G-protein signaling pathways include some of the most well-characterized regulators of grain size. In rice, *GS3* encodes a $G\gamma$ subunit that acts as a negative regulator of grain length, whereas *DEP1* positively affects both grain size and panicle architecture (Fan et al., 2006; Mao et al., 2010; Huang et al., 2009; Zhou et al., 2009). Their wheat orthologs, *TaGS3* and

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<https://doi.org/10.1016/j.plaphy.2025.110998>

Received 27 May 2025; Received in revised form 23 December 2025; Accepted 27 December 2025

Available online 28 December 2025

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TaDEP1, have been shown to retain these regulatory roles (Ren et al., 2021; Yang et al., 2019; Zhang et al., 2018). Similarly, the E3 ubiquitin ligase GW2 negatively regulates grain width in rice by targeting substrates that suppress cell proliferation (Song et al., 2007). Its wheat counterpart shows conserved function, and interacts additively with *TaDA1*, a ubiquitin receptor, to modulate thousand grain weight (TGW) (Liu et al., 2020; Sestili et al., 2019; Su et al., 2011).

Phytohormone signaling further contributes: *OsTGW6* acts as a negative regulator of grain length by limiting free auxin availability (Ishimaru et al., 2013). Its wheat ortholog (*TaTGW6*) conserve the same role in wheat (Hanif et al., 2016). *BG1* enhances grain size in both rice and wheat by promoting auxin-mediated cell expansion (Lo et al., 2020; Milner et al., 2021). Within the brassinosteroid pathway, *D11* and *BRI1* promote grain length via cell expansion whereas a Shaggy-like kinase GSK2 acts as a repressor by inactivating BR-responsive transcription factors (Morinaka et al., 2006; Zhu et al., 2015; H. Xu et al., 2022).

The mitogen-activated protein kinase (MAPK) cascade (e.g., SMG1, SMG2, and DSG1) promotes grain length in rice through enhanced cell proliferation in the spikelet hulls (Duan et al., 2014; Liu et al., 2015; R. Xu et al., 2018) and interacts with BR signaling via *OsWRKY53* (Tian et al., 2021). Conversely, in wheat, *TaMPK3* appears to negatively regulate grain width, indicating functional divergence within the MAPK module (Liu et al., 2022). In addition to hormone signaling and protein turnover, grain size and overall yield in plants are closely linked to the regulation of the cell cycle, which controls both cell division and expansion processes (Zhang et al., 2019).

Microtubule arrays (MTs) play a central role in these mechanisms by orchestrating cell growth and directing the axis of cell expansion. Moreover, these arrays display distinct spatial organization patterns that are critical for cell division and for the accurate positioning of the division plane (Drevensek et al., 2012). The preprophase band (PPB), a ring of MTs specific to plant cells, marks the division plane during mitosis. To date, only a limited number of genes have been identified as essential for the formation of the PPB. Among these, *TONNEAU1* (*TON1*) and *FASS/TONNEAU2* (*TON2*) are the only genes whose disruption results in the complete absence of PPB formation, highlighting their critical role in the spatial control of plant cell division (Spinner et al., 2013). In *Arabidopsis* *TON1* gene encodes a protein contains four main motifs: the TOF motif in the N-terminal region, the LisH motif, the PLL region, a phosphorylation site and a serine-rich region (Azimzadeh et al., 2008). This protein interacts with TRM (*TONNEAU1* Recruiting Motif) proteins, which mediate binding through their M2 and M3 domains: the M2 domain facilitates interaction with *TON1*, while the M3 domain enables association with *FASS/TON2*, the regulatory subunit of protein phosphatase 2A (PP2A) (Spinner et al., 2013; Kirik et al., 2012; Wu et al., 2024). These interactions lead to the assembly of the highly conserved TTP complex (*TON1*, TRM, PP2A), which plays a pivotal role in organizing the PPB during interphase by interacting with microtubule-associated proteins, (MAPs). Within this complex, PP2A acts as the central scaffold, interacting with both *TON1* and TRM, where *TON1* activates the complex. Additionally, *TON1* has been shown to interact with centrosomal proteins known as centrins (*CEN1*) (Wang et al. 2015, 2019). In *Arabidopsis*, functional characterization of *TON1* insertional mutants revealed the existence of two tandem gene isoforms, named *TON1a* and *TON1b*. These genes encode two 29 kDa polypeptides sharing an aminoacidic identity of 85 %. In rice, Wang et al. (2015) demonstrated that *OsGW7* (also known as *OsGL7*) encodes a rice homolog of the *Arabidopsis* LONGIFOLIA protein, a member of the TRM protein family (Lee et al., 2006). *OsGW7* interacts with *OsTON1b* and *OsTON2*, forming the highly conserved TTP protein complex, analogous to that identified in *Arabidopsis*. Wu et al. (2024) further revealed that *OsGW7* plays a crucial role in regulating cell elongation and organ morphology and exerts an epistatic effect on *DEP1* in modulating grain size and shape in rice. Their findings revealed that the truncated *depl* protein disrupting the interactions of *OsGW7* with *OsTON1b* and *OsTON2* significantly alters grain morphology, thereby impairing the

proper organization of microtubules. In bread wheat, CRISPR-Cas9-mediated editing of *TaGW7*, the ortholog of *OsGW7/OsGL7*, significantly alters grain shape and weight in a dosage-dependent manner (Wang et al., 2019). The *TaGW7*-centered gene co-expression analysis revealed an enrichment of genes involved in microtubule-associated processes and cell division, suggesting the existence of a conserved regulatory mechanism across species. To date, in rice and wheat *GW7* has undergone comprehensive functional characterization (Ma et al., 2017; S. Wang et al., 2015; Wang et al., 2019; Wu et al., 2024) whereas the biological role and mechanistic contribution of *TON1* remain largely unexplored.

To elucidate the functional role of the *TON1* gene in a polyploid cereal context and assess its potential to modulate agronomic traits, we overexpressed *TON1b*, by a CRE-LOX system (Mészáros et al., 2015), in *Triticum turgidum* ssp. *durum* cv. Svevo. This approach aimed not only to investigate the involvement of *TON1b* in cytoskeletal organization and seed development but also to explore its utility as a genetic lever to enhance grain yield.

2. Materials and methods

2.1. Isolation and phylogenesis of *TON1* sequences

The identification of *TdTON1* gene sequences in durum wheat was performed using the orthologous gene *OsTON1b* (Os11g0102600) from *Oryza sativa* as a query. A BLAST search was performed using the Ensembl Plants database to identify homologous sequences within the genome assembly Svevo.v1 of *Triticum turgidum* ssp. *durum*.

Protein sequences of *TON1* orthologs from major cereal species, as well as from *Arabidopsis thaliana*, were retrieved from the Ensembl database (<https://plants.ensembl.org/index.html>). Phylogenetic analysis was then conducted using MEGA11 software. A Maximum Likelihood (ML) tree was generated using default parameters, and node reliability was evaluated through 500 bootstrap replicates. The phylogenetic analysis included the actin protein sequence from *Triticum turgidum* ssp. *durum* (TdACT) as an outgroup. Multiple sequence alignments were performed using ClustalW and T-Coffee server (<https://tcoffee.crg.eu/>).

2.2. Vector assembly

The coding sequence of *TON1b* gene, homeoallele B (TRITD5Bv1G096040) was amplified by RT-PCR using total RNA extracted from immature seeds of durum wheat cv. Svevo at 18 days post-anthesis. First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. PCR amplification was performed in a final volume of 50 µl containing 1 µl of cDNA, 2.5 U of the FastStart High Fidelity PCR System (Roche Diagnostics), 1 × Taq PCR buffer, 50 ng of each primer, and 100 µM of each dNTP. The cDNA sequence was amplified by PCR using primers with 5' extensions harboring *Sall* and *XbaI* restriction sites (*Sall*-*TON1b*-F/*XbaI*-*TON1b*-R) (Table S1). The thermocycling program consisted of initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The resulting amplicon was digested with *Sall* and *XbaI* (<https://www.thermofisher.com>) at 37 °C for 1 h, then ligated into the pRDPT vector (Tosi et al., 2004) using T4 DNA ligase (<https://www.thermofisher.com>) at 20 °C for 10 min; the pRDPT carries the endosperm specific promoter and terminator sequences of the *1Dx5* high-molecular-weight glutenin subunit gene of *Triticum aestivum*. The entire cassette (*TaDX5p*:*TON1b*-cDNA::*TaDX5t*) was amplified by PCR using a primer pair with 5' extension harbouring *AvrII* restriction site (*AvrII*_PrDx5_F and *AvrII*_TerDx5_R). *TaDX5p*:*TON1b*-cDNA::*TaDX5t* cassette was digested with *AvrII* restriction enzyme (<https://www.thermofisher.com/>) at 37 °C for 2 h and ligated into the pGEM- T-easy--CreLox using T4 DNA ligase, as previously described. pGEM-

T-easy-CreLox plasmid carries *Cre* gene under the control of *WCS120* promoter (Ouellet et al., 1998), which is inducible by cold. Upon activation, *Cre* nuclease excises the transgenic DNA, leaving only the TaDX5p:*TON1b*-cDNA::TaDx5t sequence integrated into the plant genome, thereby producing marker-free intragenic plants (Rommens et al., 2007; Mészáros et al., 2015).

To prevent self-ligation of the vector prior to cassette insertion, the CRE-LOX plasmid was dephosphorylated using Shrimp Alkaline Phosphatase (SAP). The region containing both the cassette and the CRE-LOX system was excised from the assembled construct with the *NotI* restriction enzyme and the resulting linear DNA vector containing TaDX5p:*TON1b*-cDNA::TaDx5t + *WCS120p*-CreLox was used for biolistic transformation (Fig. S1).

2.3. Biolistic transformation and regeneration of durum wheat embryos

Immature embryos of *Triticum turgidum* ssp. *durum* cv. Svevo were isolated from surface-sterilized caryopses at 14–16 days post-anthesis and placed on induction medium (Sparks and Jones, 2014) with the scutella facing upwards. After 1–2 days of dark incubation at 22–23 °C to induce callus formation, a total of 1062 embryos were transformed via biolistic delivery of linear TaDX5p:*TON1b*-cDNA::TaDx5t + *WCS120p*-CreLox vector and coated onto 0.6 µm gold particles (Bio-Rad Laboratories Ltd., UK) using a Bio-Rad PDS1000/He particle bombardment system with a rupture pressure of 650 psi and vacuum of 28" Hg. The linear vector also contains *Bar* gene, the product of which confers resistance to the herbicide bialaphos, thereby providing a selectable marker for recognizing intragenic regenerants.

Following transformation, embryos were maintained on induction medium for 3–4 weeks to promote the development of embryogenic calli. Calli with somatic embryos were then transferred to regeneration medium (Sparks and Jones, 2014) and incubated under a 12-h photoperiod at 22–23 °C for an additional 3–4 weeks. Calli showing a positive response to selection were moved to regeneration medium without hormones in GA-7 Magenta vessels (Merck-Sigma Group, Darmstadt, Germany) and maintained under the same photoperiod and temperature conditions for 3–4 more weeks to promote shoot and root formation.

Green plantlets with expanded leaves and well-developed root systems were transplanted into jiffy pots filled with soil and subjected to cold treatment (10–15 °C during the day and 6–9 °C at night, under a 12-h photoperiod) to induce CRE-LOX recombination. After vernalization, plants were transferred to growth chambers for tillering (18–20 °C day/14–16 °C night; 12–14 h photoperiod), followed by a maturation phase at 25 °C and a 12–14 h photoperiod, under consistent irrigation and fertilization. The same growing conditions were followed for T₁, T₂ and T₃ generations.

2.4. Selection of intragenic plants

The initial screening was conducted on the T₀ generation, followed by successive screenings on the T₁, T₂, T₃ and T₄ progenies to monitor segregation patterns and select the intragenic lines with the Dx5p:*TON1b*-cDNA::Dx5t insertion.

Genomic DNA was extracted according to the protocol described by De et al. (1990). Genomic DNA was used as template in PCR (Polymerase Chain Reaction) to detect the presence of the intragenically over-expressed *TdTON1b* gene (*TON1b*), using primer pairs spanning the promoter, *TON1b* sequence and terminator. PCR was performed using GoTaq® Hot Start Polymerase (Promega) following the manufacturer's protocol. 100 ng of genomic DNA template and 0.5 µM of each primer were used in a final volume of 10 µl for each sample. Amplification products were visualized by electrophoresis on 1.5 % agarose gel. Primers sequences and amplification conditions are described in Tables S1 and S2.

To determine the number of construct copies integrated into the genome of transformed plants, quantitative Real-Time PCR (qRT-PCR)

was performed using genomic DNA as template. The *SSIIa* gene, which is present in two copies in the durum wheat genome (TRIT-D7Av1G061960, TRITD7Bv1G038900), was used as the reference gene. qRT-PCR was conducted on the CFX96 Touch Real-Time PCR Detection System. Each reaction contained 5 µl of SsoAdvanced Universal SYBR Green Supermix (2 ×), 1 µl of genomic DNA, 0.5 µM of each primer, and H₂O up to the final volume of 10 µl. Primers sequences used for qRT-PCR are listed in Table S1. Amplification was performed with the following thermal cycling conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. A melt curve analysis was carried out from 55 °C to 95 °C, with a 0.5 °C increment every 5 s.

Relative quantification of gene copy number was performed using the ΔC_t method (Livak and Schmittgen, 2001), where the C_t value of the target gene (*TON1b*) was normalized to *SSIIa*. The ΔC_t was calculated as: $\Delta C_t = C_{tTON1} - C_{tSSIIa}$

Assuming the reference sample carries two copies of *SSIIa*, a ΔC_t of zero corresponds to two copies of *TON1b*. A lower C_t value in the test sample indicates a higher copy number, and vice versa. The relative copy number was calculated using the formula: Relative copy number = $2^{-\frac{\Delta C_t}{\Delta C_{tSSIIa}}}$.

2.5. Phenotypic characterization of intragenic plants

T₃ intragenic plants along with null-segregant genotypes were phenotypically characterized at full grain maturity. The following morphological traits were evaluated: plant height, number of spikes per plant, spike length, and number of spikelets per spike. Grain traits, including thousand kernel weight (TKW), number of seeds per plant, and seed dimensions (length, width, and area), were measured using the MARViTECH MARViN ProLine II system (<https://www.marvitech.de/en/products-for-seed-analysis>). For each intragenic line and its corresponding null-segregant line, seeds from all spikes of ten individual plants were analysed. The values obtained were assessed using the MARViN software.

2.6. Total starch determination

Total starch content was determined using the Total Starch Assay Kit (AA/AMG; Megazyme, Wicklow, Ireland), following the manufacturer's instructions. For sample preparation, 25 kernels per genotype were pooled and ground into whole-grain flour using a Cyclotec 1093 sample mill (Foss Tecator, Höganäs, Sweden). The assay was conducted on 100 mg of flour per sample.

The analysis included three independent intragenic lines as well as the corresponding null-segregant line. For each genotype, three biological replicates (independent pools of 25 kernels) were analysed, with three technical replicates per biological replicate to ensure reproducibility.

2.7. Total RNA extraction from immature caryopses and retro transcription reaction

Total RNA was extracted from immature caryopses at 21 days post-anthesis (DPA) using the Spectrum™ Plant Total RNA Kit (Merck-Sigma Group, Darmstadt, Germany), following the manufacturer's protocol. RNA concentration and purity were assessed using the Thermo Scientific™ µDrop Plate. Approximately 1 µg of total RNA was treated to remove genomic DNA and reverse transcribed into cDNA using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories), according to the manufacturer's instructions.

2.8. Quantitative real time PCR (qRT-PCR) to evaluate the expression of *TON1b* and yield associated genes

qRT-PCR was performed using the CFX96 Touch Real-Time PCR

Detection System (Bio-Rad Laboratories). For each reaction, 5 µl of SsoAdvanced Universal SYBRTM Green Supermix (2x), 1 µl of cDNA, 0.5 µM of each forward and reverse primer, and nuclease-free water were combined to a final volume of 10 µl. Primer sequences are provided in Table S1. PCR amplification conditions followed the protocol described by Garcia Molina et al. (2021).

Relative gene expression analysis was conducted using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Three biological replicates, each analysed with three technical replicas, were included for each genotype.

2.9. Statistical analysis

Statistical differences between intragenic lines and the null-segregant control were evaluated using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test, conducted via the Astatsa online platform (https://astatsa.com/OneWay_Anova_with_TukeyHSD/).

3. Results

3.1. Phylogenetic analysis of TON1b in Triticaceae and related species

The OsTON1b reference sequence (Os11g0102600), located on chromosome 11, was used to identify the orthologous sequences in durum wheat by using EnsemblPlants algorithm. This analysis led to the identification of TON1b homeoalleles in the A and B genomes of durum wheat, corresponding to TRITD5Av1G116910 and TRITD5Bv1G096040, respectively.

The phylogenetic analysis also confirmed the orthology between durum wheat TON1b and two TON1-related genes in *Arabidopsis thaliana*, namely AtTON1a and AtTON1b, which are located in tandem on chromosome 3 as a result of a gene duplication event. Protein sequence alignment revealed that the durum wheat TON1b proteins share 97.1 % identity with the rice OsTON1b protein, and 69.1 % and 71.3 % identity with *Arabidopsis* AtTON1a and AtTON1b, respectively. This level of sequence conservation suggests a potential functional conservation of TON1b across monocot and dicot species. In *Oryza sativa* and *Zea mays*, two paralogs were identified, located on chromosomes 11 and 12, and chromosomes 2 and 3, respectively. In contrast, a single copy of TON1b was identified in diploid species of the Triticaceae family, including *Hordeum vulgare* and *Aegilops tauschii*.

In polyploid species, including *Triticum turgidum* ssp. *durum*, *Triticum turgidum* ssp. *dicoccoides*, and *Triticum aestivum*, TON1b homeoalleles were present across all subgenomes, but no additional paralogs were identified.

Deduced protein sequences were aligned using the ClustalW algorithm. The resulting multiple sequence alignment was subsequently used to construct a phylogenetic tree of TON1b using MEGA11 software (Fig. 1).

The resulting phylogenetic tree revealed two major clades of TON1b across the selected species. The first clade includes *Triticum turgidum* ssp. *durum* (TdTON1b), *Triticum turgidum* ssp. *dicoccoides* (TdicTON1b), *Triticum aestivum* (TaTON1b), *Aegilops tauschii* (AetTON1b), and *Hordeum vulgare* (HvTON1b), suggesting a close evolutionary relationship within the Triticaceae tribe. The second clade includes monocot species such as *Oryza sativa* (OsTON1b) and *Zea mays* (ZmTON1b), as well as the dicot species *Arabidopsis thaliana*.

The phylogenetic analysis clearly demonstrates that the evolution of TON1b is strongly conserved within the Triticaceae family.

In *Arabidopsis thaliana*, specific structural motifs have been identified in the TON1a and TON1b proteins and shown in Fig. S2 (Azimzadeh et al., 2008). To assess the conservation of these motifs across major cereal species, a multiple sequence alignment was performed using T-Coffee with the following protein sequences: TON1b-A1 and TON1b-B1 from durum wheat (*Triticum turgidum* ssp. *durum*), OsTON1b from rice (*Oryza sativa*), HvTON1b from barley (*Hordeum vulgare*), and

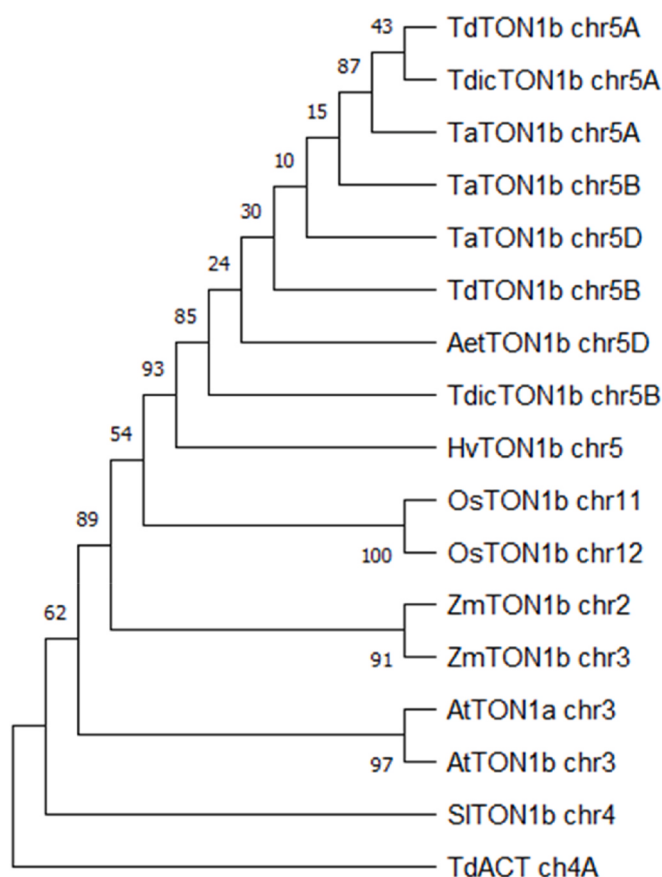


Fig. 1. Phylogenetic tree of TON1b protein sequences across diverse plant species.

The tree was constructed using the Maximum Likelihood (ML) method with 500 bootstrap replicates in MEGA 11. Protein sequences are annotated with species abbreviations and corresponding Ensembl Protein IDs: *Triticum turgidum* (TdTON1b: TRITD5Av1G116910, TRITD5Bv1G096040), *T. dicoccoides* (TdicTON1b: TRIDC5AG026020, TRIDC5BG027160), *T. aestivum* (TaTON1b: TraesCS5A02G156800, TraesCS5B02G155000, TraesCS5D02G162200), *Aegilops tauschii* (AetTON1b: AET5Gv20398800), and *Hordeum vulgare* (HvTON1b: HORVU.MOREX.r3.5HG0466870), *Oryza sativa* (OsTON1b: Os11t0102600, Os12t0102200), *Zea mays* (ZmTON1b: Zm00001eb094350_T001, Zm00001eb163130_T001) and dicot species *Arabidopsis thaliana* (AtTON1a: AT3G55000; AtTON1b: AT3G55005) and *Solanum lycopersicum* (SITON1b: Solyc04g049710). The actin gene from *T. turgidum* (TdACT: AT5G09810) was used as an outgroup to root the tree. Bootstrap values are indicated at each node.

ZmTON1b from maize (*Zea mays*) (Fig. S1). The analysis revealed a high degree of conservation for both the TOF motif and the LisH dimerization domain between cereal proteins and their *Arabidopsis* counterparts. Specifically, the TOF motif located in the N-terminal region is fully conserved across the analysed species, with the exception of the first amino acid residue, which is substituted by glutamic acid (E) in cereals, whereas *Arabidopsis* carries an aspartic acid (D) at the same position. The LisH motif is also widely conserved, although some sequence variations are observed between cereal TON1b proteins and the *Arabidopsis* paralog TON1a, potentially reflecting functional or structural divergence between the paralogs. In contrast, the C-terminal region of cereal TON1b proteins lacks both the serine-rich domain and the adjacent phosphorylation site found in *Arabidopsis*, suggesting a possible divergence in post-translational regulation or function.

3.2. Generation of durum wheat intragenic lines expressing TON1b gene

A total of 1062 immature embryos (15 DPA) from *Triticum durum* cv.

Svevo were transformed using the linear TaDX5p:*TON1b*-cDNA::TaDx5t + WCS120p-CreLox vector (Fig. S1). Out of 138 T₀ regenerated plants, 63 were positive to at least one primer pair used for the PCR screening (Table S1). Among these 63 plants, 25 plants produced seeds and 8 of which (T4C3, T4C6, T4C10B, T4C23B, T10C1B, T10C9, T10C14, and T10C23) were positive to both primer pairs spanning the entire introgressed sequence (Dx5p:*TON1b*-cDNA::Dx5t). No significant morphological or developmental differences were observed between the regenerated transformants and untransformed controls, all of which responded efficiently to the *in vitro* regeneration stages (Fig. S3).

Seeds from eight *TON1b*-positive T₀ plants (hereafter referred to as lines) were sown and advanced to obtain the T₁ generation, alongside null-segregants. The PCR results are summarized in Table S3.

Specifically, eleven T₁ plants derived from T₀ lines tested positive for the Dx5p:*TON1b*-cDNA::Dx5t construct, while twelve carried the *Cre* gene, indicating that excision of the selectable marker cassette had not occurred. This outcome is consistent with earlier reports showing that the WCS120 promoter-driven *Cre* recombinase sometimes fails to remove the lox-flanked cassette efficiently, either due to not low enough temperature for promoter induction or too low temperature thereafter for the *Cre* enzyme to function (Éva et al., 2018; Mészáros et al., 2015), although excision may still occur in subsequent generations.

Interestingly, three T₁ plants derived from the regenerated lines T4C10B, T4C23B, and T10C23 showed partial integration: the *Cre* gene was present, but the Dx5p:*TON1b*-cDNA::Dx5t sequence was absent (Table S3) and for this reason were discarded. Such partial insertions are

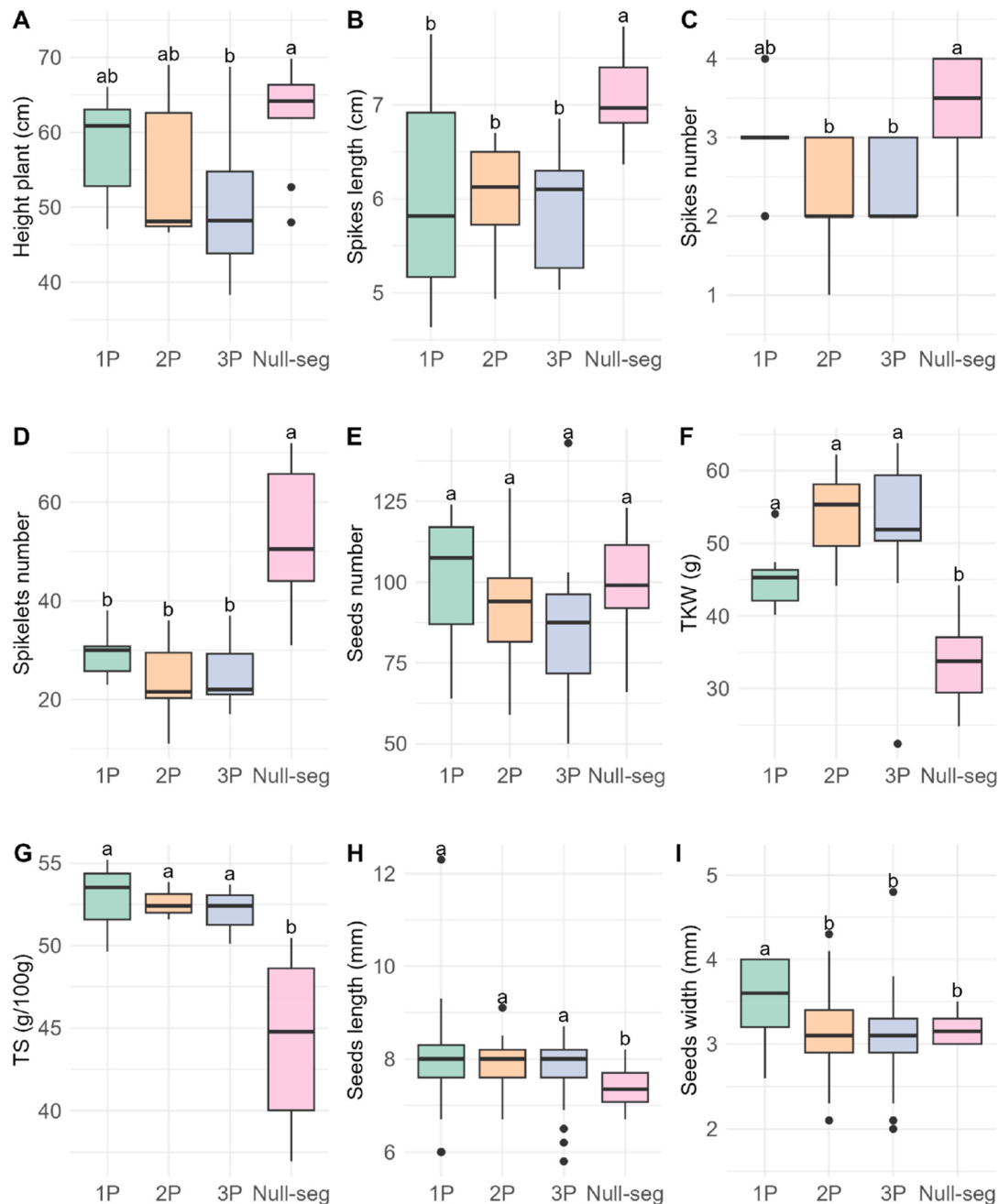


Fig. 2. Phenotypic and seed-related traits in intragenic and null segregant lines. (A) Height of plants; (B) Spikes length; (C) Spikes number; (D) Spikelets number; (E) Seeds number per plant; (F) 1000-kernels weight; (G) Total starch content (g/100g); (H) Seed length; (I) Seed width. Seed traits were measured using Marvin Tech image analysis software. Bars indicate standard errors. Different letters indicate statistically significant differences ($p < 0.05$) based on Tukey's post hoc comparisons.

common in biolistic transformation due to DNA fragmentation or incomplete integration (Šramková et al., 2009). The *TON1b*-positive lines were selected and advanced to obtain T₂ and T₃ progenies, which were screened for the presence of the intragenically over-expressed *TON1b* gene and the excision of the Cre cassette. By the T₃ generation, the stable integration of *TON1b* construct and the complete excision of Cre-lox system were confirmed, leading to the identification of three fixed intragenic lines (IP1, IP2, and IP3) derived from the T₀ lines T4C3, T4C6, and T10C9, respectively. These lines were used in subsequent phenotypic and gene expression analyses.

To estimate *TON1b* copy number in IP1, IP2, and IP3, relative quantification was performed by qRT-PCR using the 2^{-ΔCt} method, with *SSIIa* (present in two copies in durum wheat genome) as reference. The ΔCt values were -3.4 (IP1), -2.8 (IP2), and -3.0 (IP3), corresponding to estimated transgene copy numbers of approximately 5, 4 and 4.5, respectively (Fig. S3).

3.3. Phenotypic characterization and yield performance of *TON1b* intragenic lines

Morphological trait analysis revealed a significant decrease in spike length and number of spikes per plant, in the intragenic lines compare to the null-segregant lines (Fig. 2B and C).

With regard to yield components, a significant increase was observed in the 1000-kernel weight, while the number of spikelets per spike significantly decreased. The total number of seeds per spike remained unchanged (Fig. 2D, 2E, 2F).

A detailed seed morphometric analysis conducted using Marvitech software showed a significant increase in seed length ($p < 0.01$) across all intragenic lines compared to the null segregant (Figs. 2H and 3). Notably, line IP2 exhibited a larger seed width and a significantly greater seed area ($p < 0.01$) (Fig. 2I). Total starch content was significantly higher in the intragenic lines compared to the null segregant control, increasing from 44 % in the null segregant to 53 % in line IP1 (Fig. 2G).

3.4. Impact of *TON1b* overexpression on genes related to *TON1* and involved in kernel size and starch synthesis

The abundance of *TON1b* transcripts was quantified by qRT-PCR on total RNA extracted from T₃ immature kernels, 21 DPA, using specific primers for *TON1b* (Table S1). Actin gene expression was used as an internal control (housekeeping). Overexpression of *TON1b* was observed in all intragenic lines (IP1, IP2, and IP3), with transcript levels showing a 2.1-fold increase in IP1, 2.0-fold in IP2, and 3.8-fold in IP3 compared to the null segregant control line (Fig. 4).

To investigate the effect of overexpression of *TON1b* on the other components of TTP complex (GW7 and TON2), as well as its downstream impact of genes associated with grain size including *CEN1*, *GW2*, *TPP7*, *TPP3*, and *TPS7*, qRT-PCR were performed.

Notable changes in gene expression were observed across the intragenic lines. Specifically, *CEN1*, *TON2* and *TPS7* were consistently downregulated in all intragenic lines, with transcript levels reduced to approximately 30–50 % of the control levels. In contrast, *GW7* expression was significantly upregulated in IP1, showing a 2.5-fold increase compared to the control (Fig. 4).

In addition, qRT-PCR analyses of key starch biosynthesis genes

(granule bound starch synthases, *GBSSI*, *GBSII*; ADP-glucose pyrophosphorylase, *AGPase*; starch synthases, *SSI*, *SSII*; starch branching enzymes, *SBEIIa*, *SBEIIb*) revealed changes in transcript levels in the *TON1b*-overexpressing lines compared to the control (Fig. 4). Specifically, *GBSSI*, *AGPase* and *SBEIIa* were strongly upregulated in IP1 and IP3, while IP2 showed moderate increases. *SSII* expression increased by approximately 2-fold in IP1, whereas IP2 and IP3 displayed smaller increases (17–20 %).

No significant differences were detected in the expression of *GW2*, *TPP3*, *TPP7*, *SSI*, *SBEIIb* and *GBSII*, suggesting that their expression remains stable regardless of *TON1b* overexpression.

4. Discussion

The focus of agricultural research has undergone a significant transformation over the decades. During the Green Revolution, the primary goal was to maximize crop yields through the widespread use of high-yielding varieties, chemical fertilizers, and irrigation. This approach was instrumental in addressing the food security challenges of the time. However, contemporary agricultural research has shifted toward a more holistic and sustainable paradigm. Today, the emphasis is on improving the nutritional quality of crops, reducing reliance on chemical inputs and enhancing resilience to a broad range of abiotic and biotic stresses that are becoming increasingly prevalent due to climate change. Overall, the productivity has become a primary necessity driven by the geopolitical instability and the continued growth of the global population.

In this study, we aim to enhance seed size in durum wheat by inducing the overexpression of the *TON1b* gene and to deepen our understanding of its molecular function in the context of a polyploid crop. While *TON1b* has been characterized in *Arabidopsis thaliana*, its role in complex genomes such as *Triticum turgidum* ssp. *durum* (durum wheat) remains largely unexplored. In *Arabidopsis*, two closely related genes, *TON1a* and *TON1b*, are in tandem and share high sequence similarity (Azimzadeh et al., 2008). In *Arabidopsis* *TON1a* is part of a conserved protein complex with GW7/TRM1 and FASS/TON2, and together with *TON1b* plays a key role in regulating cell division in complex tissues (Drevensek et al., 2012; Hashimoto, 2015). This complex is essential for organizing cortical microtubules, particularly during mitosis (Drevensek et al., 2012). Notably, *TON1* has been shown to interact with centrin, a core component of microtubule organizing centers, suggesting a conserved role in the spatial arrangement of microtubule arrays, analogous to centrosomal organization in animal cells (Azimzadeh et al., 2008). Mutations in *TON1* or *FASS/TON2* impair the formation of the preprophase band (PPB), a microtubule structure critical for defining the future division plane. The evolutionary conservation of this mechanism across kingdoms, from plants to animals, highlights the fundamental and ancient role of *TON1*-related proteins in cytoskeletal organization and cell division. In our study, orthology and phylogenetic analyses revealed notable evolutionary divergence among the species examined. In *Arabidopsis thaliana*, as well as in monocots such as *Oryza sativa* (rice) and *Zea mays* (maize), two isoforms of the *TON1* gene were identified, suggesting either a gene duplication event or the retention of a paralog. In contrast, related cereals belonging to the Triticeae tribe, including *Triticum dicoccoides*, *Aegilops tauschii*, *Triticum aestivum* (bread wheat), *Triticum turgidum* ssp. *durum* (durum wheat), and *Hordeum vulgare* (barley), appear to possess only a single copy of the *TON1b* gene, with no



Fig. 3. Variation with respect to grain length between IP1 line (bottom) and null-segregant line (top).

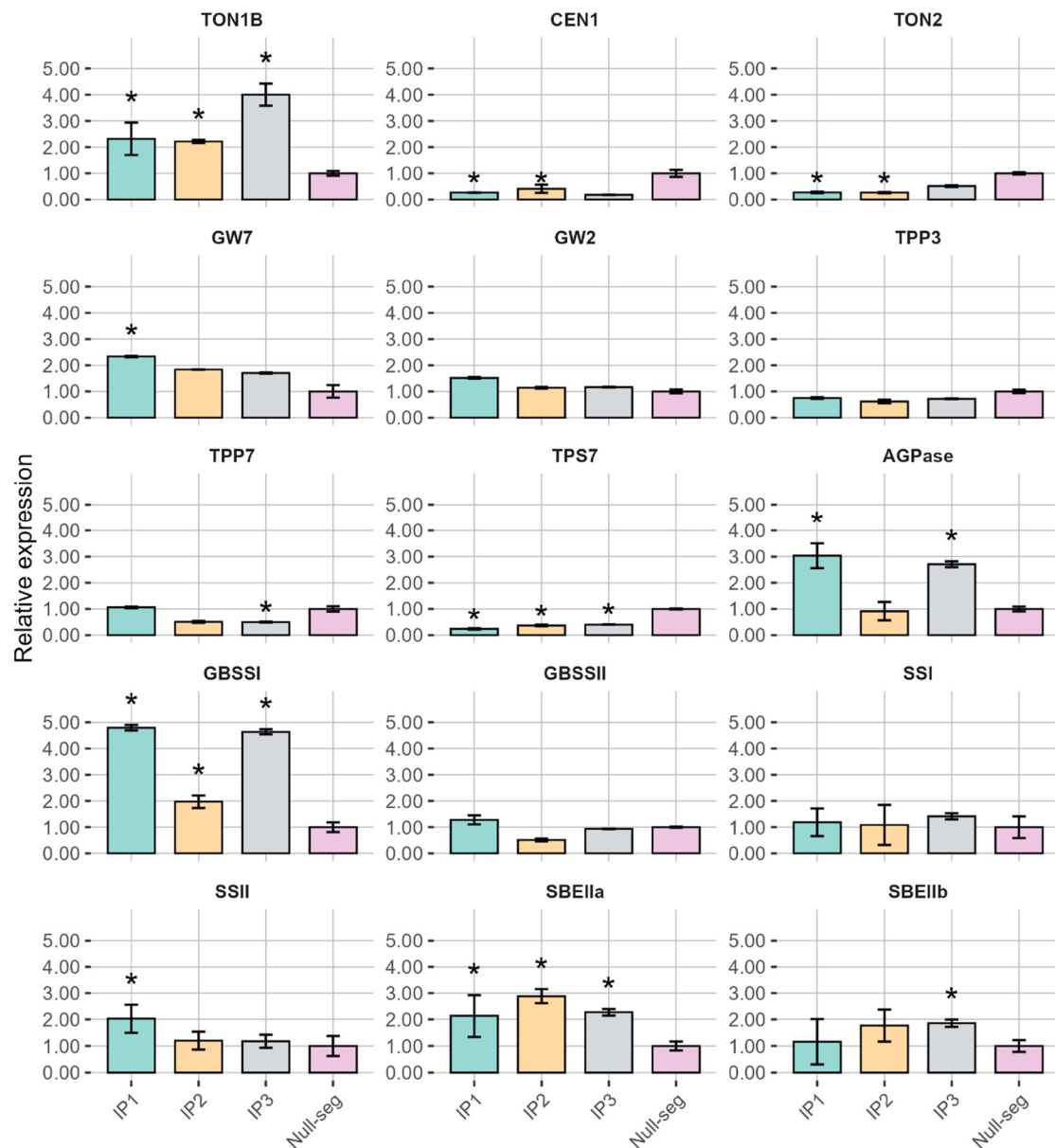


Fig. 4. Quantitative Real-Time PCR analysis of gene expression in T_3 immature seeds. Transcript levels of *TON1b*, *CEN1*, *GW7*, *GW2*, *TON2*, *TPP7*, *TPP3*, *TPS7*, *AGPase*, *GBSSI*, *GBSSII*, *SSI*, *SSII*, *SBEIIa* and *SBEIIb* were measured by qRT-PCR. The analysis was performed on three different intragenic lines (IP1, IP2, and IP3) and a null segregant line as the control. Data are presented as fold changes relative to the null segregant line using the $2^{-\Delta\Delta Ct}$ method (Rel. exp. = relative expression), with bars representing standard errors.

evidence of recent duplication.

The observed divergence in the *TON1* gene family among plant species can be explained by several evolutionary mechanisms. Tandem gene duplications are a common feature in nearly all sequenced plant genomes. After duplication, the resulting paralogs can follow different evolutionary outcomes: one copy may become nonfunctional due to mutations (*non-functionalization*), one may acquire a new function (*neo-functionalization*), or both may partition the original function (*sub-functionalization*) (Force et al., 1999; Lynch and Force, 2000; Gossmann and Schmid, 2011). Complete redundancy between duplicates is rarely maintained over long evolutionary periods, as mutational drift tends to inactivate one of the copies (Clark, 1994; Lynch and Vaishali, 2004; O'Hely, 2006). However, Azimzadeh and colleagues (2008) demonstrated that the functions of *TON1a* and *TON1b* are fully redundant in *Arabidopsis*.

The divergence in *TON1* gene copy number, two isoforms in *Arabidopsis*, rice, and maize versus a single copy in Triticeae lineages, can be

attributed to lineage-specific evolutionary pressures. These pressures shape gene duplication and retention across different plant lineages. In *Arabidopsis* and certain monocots, gene duplication events likely led to the retention of multiple *TON1* isoforms. These duplicates may have been preserved through subfunctionalization, offering adaptive advantages under specific developmental or environmental conditions (Gossmann and Schmid, 2011). Conversely, the retention of a single *TON1b* copy in Triticeae lineages suggests a different evolutionary trajectory. Studies in wheat have shown that duplicated genes often evolve faster and exhibit higher tissue specificity compared to non-duplicated genes, a pattern that may be influenced by the polyploid nature of these genomes (Cui et al., 2023). This suggests that selective pressures in Triticeae may promote the retention of single-copy genes for core cellular functions, while duplicated genes are either lost or repurposed for specialized roles.

The multiple sequence alignment (MSA) highlighted that several conserved motifs, previously identified in Azimzadeh et al. (2008) are

preserved across species, including the LisH, TOF, and PLL motifs. The LisH motif, located near the N-terminal region, is known to mediate protein–protein interactions and dimerization, suggesting a central role in TON1b function, particularly in anchoring microtubules to cortical sites. The conservation of this motif across species supports its essential role in cytoskeletal organization. Additionally, the alignment highlights a serine-rich region only present in the C-terminal portion of the *Arabidopsis* proteins, along with predicted serine phosphorylation sites. These may represent regulatory domains subjected to post-translational modification, potentially influencing the TON1 subcellular localization, interaction with partner proteins, or its stability. Overall, the conserved nature of the LisH, TOF, and PLL motifs, supports a model in which TON1b functions as a scaffold protein conserved across angiosperms.

In this study, to investigate the role of *TON1b* in durum wheat, an intragenic approach was employed using a Cre-lox system to over-express the gene. This system is a powerful tool for generating cisgenic and intragenic lines, as it enables the removal of transgenic sequences, thereby preventing the production of GM proteins in various crop species (Chen et al., 2017; Boszorádová et al., 2019; Mészáros et al., 2015). However, it also presents the drawback of frequently inserting multiple copies of the transgene. This can lead to the formation of inverted repeats or transgene rearrangements, which may result in gene silencing, abnormal expression patterns in subsequent generations, or even loss of the inserted gene (Ismagul et al., 2018). To assess the number of inserted copies in the intragenic lines, quantitative PCR (qPCR) analysis was performed, revealing between four and five copies of the construct. In order to avoid pleiotropic effects due to the *TON1b* overexpression a kernel-specific promoter was used (Sestili et al., 2019).

Molecular expression analyses confirmed the successful over-expression of *TON1b* in all intragenic lines. Interestingly, this was accompanied by a notable downregulation of *CEN1* and *TON2/FASS* transcripts. *CEN1*, which encodes a centrin protein, is well-known for its involvement in microtubule organization and cell division (Azimzadeh et al., 2008). The observed reduction in *CEN1* and *TON2/FASS* transcript levels following *TON1b* overexpression suggests the activation of a compensatory regulatory mechanism to maintain cellular homeostasis during cell division. It is plausible that this fine-tuned feedback loop is critical for ensuring the proper formation and organization of the pre-prophase band (PPB) during mitosis, a key step in seed development. These findings underscore a functional interplay between *TON1b*, *CEN1*, and *TON2/FASS*, supporting the hypothesis of their coordinated role within the TTP complex, as previously described in *Arabidopsis* (Azimzadeh et al., 2008; Wu et al., 2024). Moreover, line IP1 exhibited a significant upregulation of the *GW7* gene, which is implicated in cell division, particularly in relation to grain size regulation. Although *GW7* transcript levels also increased in lines IP2 and IP3, these changes were not statistically significant. The positive correlation observed between *TON1b* expression and *GW7* upregulation provides evidence of interaction of *TON1b* and *GW7* in the TPP complex during cell division cycle (Wang et al., 2015).

Our findings provide new evidence that *TON1b* overexpression in durum wheat modulates the expression of key genes involved in cell division and expansion, reinforcing its central role in cytoskeletal organization and developmental regulation in wheat.

The improvements of some phenotypic traits observed in the intragenic wheat lines overexpressing *TON1b*, notably increased starch content, seed size, and specific yield components, suggest a multifaceted potential role of TON1b in modulating both cellular division and metabolic pathways. The expression analysis revealed an upregulation of *AGPase*, *GBSSI*, and *SBEIIa* in the intragenic lines. *AGPase* catalyzes the first committed step of reserve starch biosynthesis; *GBSSI* is involved in the amylose formation; and *SBEIIa* introduces branch points in the amylopectin polymer (James et al., 2003). The coordinated upregulation of these key genes is consistent with the significant increase in total starch content observed in the *TON1b*-overexpressing lines.

This enhanced transcriptional activity, together with the improved

endosperm cellular organization previously associated with TON1/Tonneau proteins, suggests that TON1b may create a more favorable cellular environment for carbon allocation and starch deposition. Such structural and metabolic reinforcement may also explain the observed increase in thousand kernel weight, as enhanced starch biosynthesis typically correlates with greater grain filling. Furthermore, the potential interactions between TON1 and GW7 could contribute to these phenotypes. Studies in rice have shown that GW7 influences grain shape and modulates the expression of starch biosynthetic genes (Wang et al., 2015), supporting the hypothesis that TON1b may participate in a broader regulatory network affecting both starch metabolism and grain physical traits.

To investigate the pleiotropic effects of *TON1b* overexpression on sucrose partitioning during grain filling, we analysed the expression of transcripts involved in trehalose metabolism. Notably, the observed downregulation of *TPS7* gene in *TON1b*-overexpressing lines suggests a potential reduction in Trehalose 6-phosphate (T6P) accumulation during the grain development. A decrease in T6P levels may mimic a low-energy status in the developing grains, triggering compensatory responses that enhance the translocation of sucrose from source tissues to the sink organs. This hypothesis aligns with previous findings indicating that reduced T6P levels relieve the feedback inhibition typically exerted on sucrose transport, thereby increasing the flux of photoassimilates toward the developing grains (O'Hara et al., 2013; Liu et al., 2023). This hypothesis is also supported by the increase in total starch content, grain size and weight observed in the intragenic lines. The reduced expression of *TPS7* in *TON1b*-overexpressing plants may therefore facilitate a more efficient remobilization and utilization of sucrose into the developing endosperm, ultimately resulting in improved grain filling and yield-related traits. Although the precise role of *TON1b* in this regulatory cascade remains unclear, it may modulate *TPS7* expression either directly, through transcriptional regulation, or indirectly, via structural or signalling changes that influence carbohydrate metabolism. Given the established role of TON1-related proteins in cytoskeletal organization and cell division, it is plausible that *TON1b* overexpression alters sink strength or cellular dynamics within developing grains, reshaping metabolic signalling pathways such as those governed by the T6P. Altogether, these results reveal a previously unrecognized link between *TON1b* activity and trehalose metabolism, offering a plausible mechanistic explanation for the enhanced grain traits observed in the transgenic lines. However, further studies, including metabolite profiling and functional characterization, are needed to elucidate the molecular interactions among TON1b, TPS7, and T6P-mediated signaling during grain development.

Notably, the significant increase in thousand kernel weight, together with the absence of changes in the total number of grains per spike, indicates that TON1b primarily enhances grain size rather than grain number. Although the intragenic lines displayed a reduced number of spikelets, the total grain number per spike remained unchanged. This can be explained by the fact that, in the *TON1b*-overexpressing lines, both basal and apical spikelets contained four fully developed grains, a condition rarely observed in durum wheat, where terminal florets typically undergo floral or early post-floral abortion. This enhanced fertility of the terminal spikelets suggests that TON1b has a positive effect on reproductive processes, consistent with previous studies in cereals where overexpression of genes such as *TaBG1-A* or *OsGW7* increased grain size through modifications in cell expansion or spikelet development (Milner et al., 2021; Wang et al., 2015). Considering that the *1Dx5* promoter is endosperm-specific and, according to *WheatExp* (*expVIP*) data, becomes active around eight days after anthesis, it is plausible that *TON1b* expression driven by *1Dx5* promoter enhances early grain development. In this time, increased TON1b activity may improve endosperm cellular organization and support the grain filling. As a consequence, post floral abortion in terminal spikelets is reduced, allowing the total grain number to remain unchanged despite the lower number of spikelets.

The detailed morphometric analysis revealing significant increases in seed length across all intragenic lines, and a notable increase in seed area in line IP3, underscores the role of *TON1b* in seed development. These enhancements are likely due to *TON1b* involvement in cytoskeletal organization, which is crucial for cell division and expansion during seed formation. Similar phenotypic outcomes have been reported with the overexpression of genes like *OsGW7*, *TaGW7* and *TaGL3.3-5B*, which are associated with larger and heavier grains in rice and wheat, respectively (Wang et al., 2015; W. Wang et al., 2019; C. Wang et al., 2022).

The phenotypic analysis carried out in this study highlighted a parallelism between *TON1b* and *GW7* overexpression in durum wheat, bread wheat and rice, further confirming previous studies that demonstrated the interaction of *OstON1b* and *OstON2* with *GW7* in rice (Wang et al., 2015). In rice *GW7* encodes a TONNEAU1-recruiting motif protein that influences grain shape by modulating cell division patterns (Wang et al., 2015). Overexpression of *GW7* has been shown to increase grain length and weight, similar to the effects observed with *TON1b* overexpression in wheat. These findings suggest that manipulating genes involved in cytoskeletal organization, such as *TON1b* and *GW7*, can be a viable strategy for improving grain size and yield in cereal crops.

5. Conclusion

Collectively, the overexpression of *TON1b* in durum wheat appears to orchestrate a complex interplay between cytoskeletal dynamics and metabolic pathways, culminating in enhanced starch accumulation, improved grain morphology, and favorable yield components. These findings position *TON1b* as a promising target for genetic interventions aimed at improving wheat productivity and grain quality.

CRedit authorship contribution statement

Chiara D'Attilia: Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Francesco Camerlengo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Samuela Palombieri:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Arianna Frittelli:** Writing – review & editing, Methodology, Formal analysis. **Csaba Éva:** Writing – review & editing, Resources. **Francesco Sestili:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Funding

The research was carried out within the framework of the Ministry for Education, University, and Research (MIUR) initiative “Department of Excellence” (Law 232/2016) DAFNE Project 2023–27 “Digital, Intelligent, Green and Sustainable (acronym: D.I.Ver.So)”.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2025.110998>.

Data availability

Data will be made available on request.

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