Evaluation of the role of *Medicago truncatula* Zn finger CCHC type protein after heterologous expression in *Arabidopsis thaliana*

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**ABSTRACT**

Zinc finger proteins bind nucleic acids or act in transcriptional or translational regulation. The present study aimed to explore the effect of heterologous expression of the *Medicago truncatula* gene (*Mt-Zn-CCHC*), which encodes a Zinc finger CCHC type protein, in *Arabidopsis thaliana*. The *Mt-Zn-CCHC* gene, which affects seed size in *M. truncatula*, was used for construction of transgenic *A. thaliana* transcriptional reporter plants expressing *pMt-Zn-CCHC::GUS::GFP*, as well as lines with modified expression – overexpressed (OE) and knockdown (RNAi). *In silico* analysis of the promoter cis-elements of *pAt-Zn-CCHC* and *pMt-Zn-CCHC* suggested regulation during meristem activity, seed development, as well as cold stress. The expression of *pMt-Zn-CCHC* was localized in shoot apical meristem and in the base of the siliques. In the RNAi lines, successfully repressed endogenous *At-Zn-CCHC* expression resulted in shortened stem and reduction in silique number, silique size, seed number per silique, and decreased expression of the meristem marker *AtSWP*. In the gain-of-function lines, overexpression of *Mt-Zn-CCHC* acted as a positive regulator in silique and seed parameters, as well as increased *AtSWP* expression. Cold treatment of WT plants demonstrated upregulation of the endogenous *At-Zn-CCHC* and the *RD29A* cold marker gene. In the OE line, *RD29A* transcription was induced by cold faster but in the RNAi line, slower. The overall data support the roles of the studied Zn-CCHC gene in the development of shoot meristem, seeds and cold response, which highlights this protein as a conserved regulator in plant reproduction and stress signal transduction.

**Introduction**

Regulation of the gene expression in living organisms is important for their growth, development and stress responses [1]. Posttranscriptional regulation of gene expression – known as RNA metabolism – includes RNA processing, pre-mRNA splicing, and RNA export and decay. RNA metabolism has a key role in many processes in eukaryotic cells [2]. The regulation of RNA metabolism is carried out by direct or indirect binding of RNA-binding proteins (RBPs) to target RNAs [1]. RBPs include some conserved motifs, like RNA-recognition motif (RRM), zinc (Zn) finger motif, K homology (KH) domain, glycine-rich region, etc. [3]. There is a wide range of RBPs in plants, indicating that their functions in plant growth, development and stress responses is also diverse [4, 5]. For example, some RBPs that are central to plant growth and stress responses harbor an RRM at the N-terminus and a glycine-rich region at the C-terminus, i.e. glycine-rich RBP (GRP), zinc finger-containing GRP, cold shock domain protein (CSDP) and RNA helicase (RH) [6–8]. RBPs could have various types of Zn finger motifs in their structure. There are nine different types of Zn finger motifs described [9]; one of them is the CCHC Zn knuckle domain. The proteins possessing the Zn finger domains are widely distributed in plants and their role is related to regulation of nucleic acids function or participation in transcriptional or translational regulation [10].

The gene coding for a Zn finger CCHC type protein (ABE91952.1) was discovered from a Tnt1 transposon mutant collection of *Medicago truncatula* [11] and studied by application of reverse genetic approach in *M. truncatula* overexpressing (OE) lines, lines with silenced transcription of the gene (RNAi), and promoter lines *pMt-Zn-CCHC* with reporters [12]. Expression and...
phenotypical observations demonstrated localization in anthers of OE lines and reduction of seed size in RNAi \textit{M. truncatula} lines. In this study, \textit{M. truncatula} gene harboring Zn finger motif CCHC type (MT2G005460) was investigated in a heterologous system of \textit{Arabidopsis thaliana}. \textit{In silico} approach was applied to asses the promoter sequences of Mt-Zn-CCHC (MT2G005460) and its ortolog in \textit{A. thaliana} At-Zn-CCHC (At2G01050). Vectors carrying the open reading frame of Mt-Zn-CCHC gene were introduced in \textit{A. thaliana} plants. Both type of transgenic lines overexpressing (OE) and knockdown (RNAi) were successfully developed. Additionaly, \textit{A. thaliana} transcriptional reporter lines harboring the construct of the promoter sequence of pMt-Zn-CCHC fused to reporter genes were generated. The phenotypes of lines with modified expression were evaluated during plant development and their reproduction, as well as in response to cold stress.

Materials and methods

\textit{Gene cloning and plant transformation}

The promoter sequence of the Mt-Zn-CCHC gene (MT2G005460) was previously cloned into the vector pExK7SWFm14GW carrying β-glucuronidase (GuS) and green fluorescent protein (GFP) reporter genes [12]. Gene cloning procedures were described in detail by [12]. Briefly, the open reading frame of the Mt-Zn-CCHC gene was amplified and cloned in plant transformation vectors under the control of CaMV35S promoter [13]. RNA interference protocol [14] was applied for development of lines with silenced gene function. The successful gene silencing was \textit{in silico} predicted, and a 153-bp fragment located between 729 bp and 882 bp from the ORF of Mt-Zn-CCHC was used (Supplemental Figure S1). In the present work, the \textit{M. truncatula} constructs were introduced into \textit{Agrobacterium tumefaciens} strain C58C1, which was used for \textit{A. thaliana} transformation following the floral dip procedure [15]. Seeds from OE and RNAi lines were selected on Km media. T3 progeny positive plants were grown in greenhouse for seed production and phenotyping.

\textit{Plant material}

\textit{A. thaliana} (L.) Heynh. ecotype Columbia-0 (Col-0) was used in this work. Positive plants were selected on half-strength Murashige and Skooog (MS) medium containing 50mg L$^{-1}$ kanamycin selective agent and were used for analyses. The sterilized seeds were incubated in the dark at 4$^\circ$C for 48h and transferred to light. Plants were grown under controlled growth conditions (at 21$^\circ$C, continuous light, 100µmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation, 55% humidity) on vertical plates containing ½ MS medium supplemented with 0.8% plant tissue culture agar (LAB M Ltd, Heywood, UK) and 1% sucrose.

\textit{Bioinformatic analyses}

The alignment between At-Zn-CCHC (NP178215.1) and Mt-Zn-CCHC (ABE91952.1) proteins was performed by T-Coffee with M-Coffee [16] and visualized by Boxshade 3.21 - Pretty Printing and Shading of Multiple-Alignment files (created by Hofmann K and Baron MD, Swiss Institute of Bioinformatics http://www.ch.embnet.org/software/BOX_form.html) (Supplemental Figure S2). For phylogenetic tree construction, the At-Zn-CCHC and Mt-Zn-CCHC protein sequences were used for BLASTP search for proteins with a Zn-CCHC domain. The alignment of 9 amino acid sequences and subsequent construction of phylogenetic tree were performed using the MEGAX software by the Maximum Likelihood method and JTT matrix-based model [17–19]. The analysis for \textit{cis}-elements in At-Zn-CCHC (At2g01050) and Mt-Zn-CCHC promoters was performed by PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) [20].

\textit{Detection of GUS and GFP reporter genes}

Tissue specific localization of β-glucuronidase (GUS) activity was detected by the protocol of Jefferson et al. [21]. The procedure is described in detail in [12]. Binocular microscope (MZ16, Leika) equipped with a camera (Nikon) was used for image tracking. Fluorescence stereomicroscope SZX7 (460–490 nm excitation and 510–550 nm emission) with a DP73 digital camera (Olympus) for the images of plants expressing green fluorescent protein (GFP) were used.

\textit{Expression analysis}

Total RNA was extracted from 18-day-old seedlings. The protocol of RNeasy Plant Mini Kit (EurEx) was followed. Extracted RNA was quantified with a Nanodrop2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Copy DNA synthesis was performed with 1µg RNA according to the protocol of the iScript cDNA Synthesis Kit (BIO-RAD). The relative transcript level was monitored using 7300 Real-Time PCR System (Applied Biosystems). PCR reaction was performed with 5µL of 5x diluted first-strand cDNA in a 20-µL reaction including 0.5µmol/
μL gene-specific primers and iTaqUniversal SYBR green Super mix including ROX and fluorescein (BIO-RAD). The housekeeping gene *AtACTIN* was used as a reference gene for data normalization. All primers used in this study are listed in Supplemental Table S1. The relative expression levels were calculated by qBase 1.3.5 software.

**Phenotypic characterization**

OE and RNAi transgenic lines of *A. thaliana* selected after qRT-PCR data were grown in a greenhouse and monitored phenotypically. The growth dynamics expressed by the length of the main stem was measured at two time points: 20 and 35-day-old plants. The characteristics related to the flower and seeds development, such as flowering time, number and size of siliques, as well as the seed number per siliques were assessed, and compared with the control plants of *A. thaliana*.

**Cold treatment**

Two-week-old plants from the T3 generation of *A. thaliana* OE and RNAi lines were grown in square Petri dishes 120/120 mm on MS medium at 22 °C under constant illumination. The plants were subjected to cold treatment at 4 °C for 0 h, 24 h and 48 h in a cold room. Total RNA from all tested plants was extracted at each time point and used for the following investigations. The relative expression under cold treatment was compared with the expression level of the gene *AtRD29A*, which was used as a positive control.

**Statistical analysis**

All experiments were performed in triplicate. Results are expressed as means values with standard error of the means (±SEM). Differences were assessed by Student’s *t*-test. Differences were considered statistically significant at the *p* < 0.05 level.

**Results and discussion**

**Phylogenetic analysis of Mt-Zn-CCHC**

In a previous report, the Mt-Zn-CCHC was shown to have a Zn-binding domain and to control seed size in *M. truncatula* [12]. To explore whether there are closely related proteins to Mt-Zn-CCHC in *A. thaliana*, BLASTP search was performed. Proteins with an identified Zn-CCHC domain were found and used for construction of a phylogenetic tree (Figure 1). In one cluster, the Mt-Zn-CCHC protein was grouped together with *Trifolium pratense* and *Glycine max*, as well as another member of the Fabaceae family, *Senna tora*. The *A. thaliana* ortholog, designated as At-Zn-CCHC, was grouped in a second cluster together with the Zn-CCHC representatives of the *Brassicaceae* family members *Capsella rubella* and *Brassica napus*. Alignment between the amino acid sequences of Mt-Zn-CCHC and At-Zn-CCHC showed 34% homology and confirmed the presence of common protein domains: a DUF domain of unknown function and a Zn-binding domain (Supplemental Figure S2).

**Regulatory cis-elements in the promoters of Mt-Zn-CCHC and At-Zn-CCHC**

The promoter assay of *Mt-Zn-CCHC* and *At-Zn-CCHC* could identify putative signaling pathways that are common for both plant species including seed development as reported by [12]. In confirmation of the functional study in *M. truncatula*, *Mt-Zn-CCHC* and *At-Zn-CCHC* were found to be enriched in regulatory
cis-elements related to seed development (Supplemental Figure S3; Tables 1 and 2). Strikingly, both promoters included motifs linked to cold stress. Some cis-motifs were annotated to be related to seed development and cold stress response together (Table 1). The approximate density of the cis-elements per promoter length was compared (Table 2). The results demonstrated that both genes have equal capacity to regulate seed development, and in respect to cold, the At-Zn-CCHC promoter seems to be more responsive (Table 2). Interestingly, there were meristem-related motifs in the At-Zn-CCHC promoter, whereas such were not observed in the Mt-Zn-CCHC sequence. In a study describing the Zn-CCHC gene family in *Triticum* sp., the authors discussed the existence of lots of cis-acting elements in the promoter regions of *TaCCHC-ZF* genes associated with environmental stress and phytohormone responsiveness, which might take part in multiple signalling pathways [22].

**Generation of transgenic A. thaliana lines**

In order to investigate the Mt-Zn-CCHC function in a heterologous system, stable transgenic *A. thaliana* plants with modified expression of the Mt-Zn-CCHC (overexpressed-OE) and knockdown of At-Zn-CCHC gene (RNAi) were developed. Three overexpressing lines (OE 2, 4 and 5) with a higher transcript level of the exogenous Mt-Zn-CCHC and three RNAi *A. thaliana* lines with silenced expression of the endogenous gene At-Zn-CCHC (RNAi 2, 3 and 4) were selected after analysis of transcript levels (Figure 2).

**Tissue localization of pMt-Zn-CCHC in A. thaliana**

The produced *A. thaliana* transcriptional reporter lines (pMt-Zn-CCHC::GUS::GFP) were analyzed for tissue localization of the marker genes expression. A strong GFP signal was detected in the base of the shoot apical meristem (SAM) (Figure 3A). GUS monitoring during SAM development showed that the signal was localized specifically in this point (Figure 3B and C). Later in the development, the GUS signal was detected in the base of siliques (Figure 3D). The data demonstrated the specific localization of the Mt-Zn-CCHC gene in tissues with high dividing cell activity. It has been reported that *A. thaliana* RNA-binding protein AtGRP2 was specifically detected in SAM of 6-day-old seedlings [23]. In another study [24], the tissue localization of AtCSP2 was in shoot and root apical regions, as well as in the reproductive organs. Specific accumulation in tissue with high meristematic activity was described.

![Figure 2](image)

*Figure 2.* *A. thaliana* OE and RNAi transgenic lines. Expression levels of Mt-Zn-CCHC on OE lines (A) and At-Zn-CCHC in RNAi lines (B) in T3 generation. Note: The expression levels were calculated and normalized according to the housekeeping gene *AtACTIN*. Data are mean values ± SEM. Asterisks indicate statistically significant differences compared to the control: p < 0.05*; p < 0.01**; p < 0.001***.

![Figure 3](image)

*Figure 3.* Tissue-specific pMt-Zn-CCHC expression in *A. thaliana*. GFP signal localization in shoot apical meristem (A); GUS signal in the base of shoot apical meristem at 13 (B) and 27 days-old seedlings (C), and in the base of the siliques (D).
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these type of proteins are RBPs that harbour Zn-CCHC domains.

Phenotypic characteristics of A. thaliana OE and RNAi lines

The selected T3 OE and RNAi A. thaliana lines were grown under greenhouse conditions and monitored phenotypically. The dynamics of the central stem development was measured in 20-day-old plants and 15 days later. The results demonstrated that 20-day-old plants from both OE and RNAi lines grew slowly in comparison to the wild-type (WT) control, and the length of the central stem was shortened (data not shown). At this time point it was observed that the plants from OE lines started flowering similar to the WT control plants (data not shown). In contrast, the RNAi lines were characterized with delayed flowering.

Table 1. Regulatory cis-elements in the promoter regions of prMt-Zn-CCHC and prAt-Zn-CCHC.

| Role | Factor or site name | cis-elements | Short description | Frequency | PLACE # |
|------|---------------------|--------------|-------------------|-----------|---------|
| seed | CAATBOX1 | CAAT | CAAT; legA; seed | 12 | S000028 |
|      | SEF4MOTIFGFM75 | RTTTTTR | soybean; seed; storage protein; 7S; globulin; beta-conglycinin; KW SEF | 2 | S000103 |
| -300ELEMENT | EBOXBNAPAPA | TGHAARAK | −300 element; hordein; gliadin; glutenin; seed | 2 | S000122 |
|      | MYREPEATBNAPAPA | CATGCA | napA; storage protein; ABRE; E-box; seed | 18 | S000144 |
|      | MYCCONSNSUSAT | CANTNG | MYC; rd22BP1; ABA; leaf; seed; stress; CBF3; cold; CBF; DREB1; CBF; CBF | 18 | S000407 |
|      | MYB1AT | WAACCA | MYB; rd22BP1; ABA; leaf; seed; stress | 9 | S000408 |
| cold | LTRECOREATCOR15 | CCGAC | low temperature; cold; LTRE; drought; ABA; cor15a; BN115; leaf; KW shoot; phytochrome | 1 | S000153 |
|      | DRECRTCOREAT | RCGAC | DRE; CRT; drought; high-light; cold; DREB; DREB1; DREB2; CBF | 1 | S000418 |
|      | CBFHV | RYGCAC | CBF; AP2 domain; CRT; DRE; low temperature | 1 | S000497 |
| prAt-Zn-CCHC | seed | AMYBOX1 | TACARARA | amyrase box | 1 | S000020 |
|      | CAATBOX1 | CAAT | CAAT; legA; seed | 11 | S000028 |
|      | MYBPNAPA | CATGCA |RY repeat; legumin box; seed; storage protein | 1 | S000100 |
|      | -300ELEMENT | EBOXBNAPAPA | TGHAARAK | −300 element; hordein; gliadin; glutenin; seed | 2 | S000122 |
|      | CANBINAPA | CNAACAC | napA; storage protein; ABRE; E-box; seed | 4 | S000144 |
|      | MYBP2M | CCWACC | P; P gene; P; gene; MYB; myb; seed | 2 | S000179 |
|      | MYBGAHV | TAAACAA | myb; Myb; GAmyb; GB; gibberellin; GARC; alph-amyrase; amyrase; KW aleurone; GARE; seed | 1 | S000181 |
|      | CGAGCGOSAMY13 | CGAG | amyrase | 1 | S000205 |
|      | MYB2CONSNSUSAT | YAACKG | MYB; rd22BP1; ABA; leaf; stress | 3 | S000409 |
|      | GAREAT | TAACAR | GARE; GA | 1 | S000439 |
| cold | LTRECOREATCOR15 | CCGAC | low temperature; cold; LTRE; drought; ABA; cor15a; BN115; leaf; KW shoot; phytochrome | 2 | S000153 |
|      | CRTDREHCRCBF2 | GTCGAC | cold; AP2; CRT; DRE; CBF | 4 | S000411 |
|      | DRECRTCOREAT | RCGAC | DRE; CRT; drought; high-light; cold; DREB; DREB1; DREB2; CBF | 1 | S000418 |
|      | CBFHV | RYGCAC | CBF; AP2 domain; CRT; DRE; low temperature | 8 | S000497 |

The PLACE software was used for search of promoter motifs in the respective 1500 bp and 1549 bp upstream non-coding regions. Boldface indicates common sites for both plant species. Different colours indicate motifs associated with seed development (dark green), cold response (turquoise) or both (light green). The approximate frequency of occurrence (i.e. number of sites) is indicated, and the identification number of each site in PLACE is included.

*Motifs are shown in the promoter sequences in Supplemental Figure S3.

Table 2. Density of cis elements in the promoter regions of prMt-Zn-CCHC and prAt-Zn-CCHC.

| Role | Seed cis-motifs | Seed/cold cis-motifs | Cold cis-motifs |
|------|-----------------|---------------------|----------------|
| prMt-Zn-CCHC | 9.16% | 6.87% | 0.76% |
| prAt-Zn-CCHC | 10.51% | 1.54% | 3.85% |

The number of specific cis motifs related to ‘seed’ only, ‘seed and cold’ and ‘cold’ only which were identified by the PLACE software is divided to the total number of motifs found in the studied promoter regions.

for OsCSP1 and OsCSP2 genes in rice [25]. These type of proteins are RBPs that harbour Zn-CCHC domains.

Phenotypic characteristics of A. thaliana OE and RNAi lines

The selected T3 OE and RNAi A. thaliana lines were grown under greenhouse conditions and monitored phenotypically. The dynamics of the central stem development was measured in 20-day-old plants and 15 days later. The results demonstrated that 20-day-old plants from both OE and RNAi lines grew slowly in comparison to the wild-type (WT) control, and the length of the central stem was shortened (data not shown). At this time point it was observed that the plants from OE lines started flowering similar to the WT control plants (data not shown). In contrast, the RNAi lines were characterized with delayed flowering.
On day 35 the plants from OE lines were able to compensate the delay of the central stem development in comparison to the control, but RNAi lines continued to grow slowly (Figure 4A). The delayed flowering described for RNAi lines was accompanied with deviation in the silique development: reduced silique number and size (Figure 4B and C). The siliques formed on the OE lines were a bit larger than those of the control plants, while the siliques on RNAi lines were dramatically smaller ($p < 0.001$, Figure 4C). Additionally, there was deviation from the normal development in respect to the reduced number of siliques formed by RNAi Arabidopsis lines (Figure 4B). This observation was accompanied with a low number of seeds per siliques (Figure 4D). The phenotypic data demonstrated that the seeds production of Arabidopsis RNAi lines was significantly disturbed due to the knockdown of the endogenous gene. A similar phenotypic defect developed in Arabidopsis mutant (11-0561-1, mutation after transposon insertion – photo is available in TAIR for AT2G01050).

In the research of Clay and Nelson [26], the STRUWELPETER (SWP) gene known to affect cell number and shoot meristem development was studied in Arabidopsis. Additionally, SWP interacts with SMP1 and SMP2 encoding CCHC zinc finger proteins with similarities to step II splicing factors. The authors demonstrated similar to the phenotypic data reported here in the mutant swp line: shorter organs, fewer siliques with reduced seed number [26]. In the present study, the transcript level of AtSWP gene was monitored in two OE and two RNAi Arabidopsis lines to check for a possible role of the studied Mt-Zn-CCHC gene and its ortholog At-Zn-CCHC in cell proliferation (Figure 5). The obtained data demonstrated a significant expression level of the AtSWP gene in OE lines and reduction in the gene expression in RNAi lines (Figure 5).

The results presented here support previous evidence that Mt-Zn-CCHC gene can influence the plant development and seeds size of M. truncatula [12]. A recent report identified genes with direct or indirect influence on plant reproduction in water yam (Dioscorea alata L.), among which was the Mt-Zn-CCHC gene [27]. Additionally, high-density SNP-based association mapping of seed treat in Fenugreek identified

![Figure 4](image-url). Phenotyping of OE and RNAi Arabidopsis lines. Length of central stem in 35-days old plants (A); siliques number per line (B); siliques size (C) and seed number per siliques (D). Note: Data are mean values ± SEM from analyses of two independent transgenic lines. Asterisks indicate statistically significant differences compared to the control: $p < 0.05^*$; $p < 0.01^{**}$; $p < 0.001^{***}$. 
dDocent_Contig_466_145, which indicates an association between the Znf-C2HC domain (corresponding to the domain in Mt-Zn-CCHC) and its role in gene transcription and effect on seed size [28]. Fusaro et al. [23] demonstrated that AtCSP2 knockdown plants had a reduced number of stamens and high rates of abnormal development of seeds/embryos. The gene AtCSP2 possesses Zn-CCHC knuckle domains. The relation between the function of CSPs (cold shock proteins) and their role in flowering time and reproductive tissue development was discussed [29, 30].

Mt-Zn-CCHC gene and its ortholog At-Zn-CCHC play a role in the plant cold response

Based on the promoter data analysis, next, we tested the sensitivity to cold of the endogenous At-Zn-CCHC and the heterologous Mt-Zn-CCHC. First, WT A. thaliana plants were treated for 0, 24 and 48 h at 4 °C in a cold chamber. The relative expression of At-Zn-CCHC was monitored at each time point and compared to the expression of the AtRD29A gene, which is a cold-inducible marker used as a positive control [31]. This gene was reported to be strongly up-regulated in transgenic A. thaliana with chilling tolerance [32]. The expression analysis revealed strong induction of both genes at 24 h, corresponding to 8% upregulation for At-Zn-CCHC and 40% for AtRD29A. The induction continued and after 48 h of cold treatment up-regulation of almost 60% for At-Zn-CCHC and 85% for AtRD29A was detected (Figure 6 A).

In support of the transcriptional regulation of the endogenous At-Zn-CCHC by cold, the expression profile of the gene transcript in WT and RNAi lines showed a similar trend with significant induction after cold treatment (Supplemental Figure S4A). Notably, at 48 h of the treatment, the signal obtained from RNAi lines was lower than that in WT. In the OE lines, it was confirmed lack of Mt-Zn-CCHC induction under cold after 24 and 48 h of treatment – which is logical because the heterologous gene Mt-Zn-CCHC is under the 35S promoter (Supplemental Figure S4B).

Further, the relative expression of AtRD29A was evaluated under cold treatment in two OE and two RNAi A. thaliana lines. The data demonstrated a significant induction of the transcript level at 24 h in OE lines in comparison to WT corresponding to 45% (Figure 6 B). It was interesting to observe that induction was also obtained in the RNAi lines, and the transcript level in the RNAi profile was higher – 70%, in comparison with WT and OE. At 48 h cold treatment, both transgenic genotypes possessed different response, and in the OE profile the signal was reduced, whereas in RNAi lines the transcript level continued to grow (Figure 6 B). These results suggest less sensitivity to cold in OE lines. In OE lines the accumulation of endogenous At-Zn-CCHC and overexpressed Mt-Zn-CCHC transcripts probably lead to abundance of the Zn-CCHC protein so plants become less sensitive to the cold stress. On the other hand, RNAi lines demonstrated hypersensitivity to cold probably related with deficiency of At-Zn-CCHC protein. It can be hypothesized that Mt-Zn-CCHC protein level correlates with cold tolerance, however, additional experiments are required to clarify the precise role of Mt-Zn-CCHC in the cold response.

The overall data obtained by promoter analysis and functional studies demonstrated the sensitivity of both genes Mt-Zn-CCHC and its ortholog At-Zn-CCHC to cold. Both genes have similar conservative motifs in their protein structure: DUF4283 domain and zinc knuckle - zinc binding motif (Supplemental Figure S2). There is scarce information about the function of these conservative domains. The DUF4283 domain is described as a binding/guiding region. The zinc binding motif was described as an important motif, largely found in a variety of regulatory proteins, which can specifically bind DNA or RNA sequences, and take part in protein interactions [33].

The results presented support the observation for cold induction regulated by the promoter of the endogenous gene At-Zn-CCHC. In our study, the gene AtRD29A used as a positive control is known to encode the low-temperature responsive protein 78 (LTI78) taking part in the cold stress signaling pathway [34] by
overexpressing CBF/DREB1. It is clear that the behavior of Mt-Zn-CCHC and At-Zn-CCHC genes followed the dynamics of AtRD29A gene transcription level under cold treatment. These results indicate that both genes, Mt-Zn-CCHC and its ortholog At-Zn-CCHC, are sensitive to cold treatment and participated in the plant response to this abiotic stress factor.

In another study [25], two cold shock proteins, OsCSP1 and OsCSP2, demonstrated the stronger influence of both genes on flower and seed development, and their transient upregulation in response to low temperature in rice cultivars. A report about AtCSP2 gene in A. thaliana confirmed that the gene is regulated by both cold and developmental signals [24]. Additional experiments will broaden the knowledge about the functional role of both genes, Mt-Zn-CCHC and its ortholog At-Zn-CCHC, in plant development and plant response to cold.

**Conclusions**

In this study the function of Mt-Zn-CCHC gene was evaluated in the heterologous background of A. thaliana ecotype Columbia. The results demonstrated the role of this gene in plant development and reproduction, which was hardly repressed by RNAi interference of the endogenous gene At-Zn-CCHC. Additionally, the results showed that Mt-Zn-CCHC and its ortholog gene At-Zn-CCHC are sensitive to cold treatment based on the presence of cis-regulatory elements in the promoter sequences. The functional study outlines a direction to think that Mt-Zn-CCHC and At-Zn-CCHC play a role in the response to cold stress. A new family of proteins harboring a Zn binding domain was discovered by a reverse genetic approach and two members of this family had a role in seed development and cold response.
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Disclosure statement

No potential conflict of interest was reported by the authors.

Consent to participate

All the authors have approved their participation in the final manuscript.

Consent for publication

All the authors have read and approved the final manuscript and its submission for publication.

Availability of data and material (data transparency)

All data that support the findings reported in this study are available from the corresponding author upon reasonable request.

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