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Research Article

Overexpression of *PmC3H32 from Pinus massoniana* Promotes Flowering, Enhance Physiological Parameter and Lead to Leaf Senescence

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Abstract

Plants in their natural habitat often encounter unfavorable conditions such as lack of water, low temperatures, and freezing, which can negatively impact their growth and productivity. The CCCH zinc-finger protein is a type of C3H motif that is commonly found in various plants. It is essential in controlling plant growth, development, and stress reaction. This paper investigates the effects of overexpressing the *PmC3H32* gene, derived from Pinus massoniana, in Arabidopsis thaliana, with a specific focus on the response to drought stress conditions. Utilizing genetic modification techniques, we induced the overexpression of the *PmC3H32* gene in Arabidopsis thaliana and monitored their growth and development under drought stress. Our investigation revealed that this overexpression resulted in enhanced growth traits, suggesting a promising genetic strategy for bolstering drought resistance in plants. However, our study also unveiled a complex side effect of this overexpression. We observed an onset of early leaf senescence and a reduction in silique weight, attributable to an accelerated life cycle, characterized by premature flowering, maturation, and senescence processes.

Our findings underscore the critical role of CCCH zinc-finger proteins in the regulation of plant growth, development, and stress responses. These proteins, as evidenced by our study, play a pivotal role in the intricate balance of plant physiology. The implications of our research are far-reaching. While the overexpression of the *PmC3H32* gene presents a potential pathway for improving drought resistance, the associated early senescence and reduced silique weight highlight the need for a more nuanced understanding of the gene's role in plant development. Future research should aim to unravel the complex interplay between CCCH zinc-finger proteins and plant physiology. The ultimate goal is to harness this knowledge to develop genetically modified plants that exhibit not only improved stress tolerance but also optimal growth and development traits. This endeavor could have profound implications for agriculture, particularly in regions prone to drought conditions.

Keywords: CCCH zinc finger proteins; P massoniana; Drought stress

Introduction

Zinc finger proteins are characterized by a zinc finger domain that binds to zinc ions, forming a finger-like structure. This domain is crucial for eukaryotic transcription factors [1]. Zinc finger domains can be grouped into different categories based on their structural features [2]. The zinc finger domain of transcription factors is characterized by the arrangement of cysteine (C) and histidine (H) residues, with the most common types being C2H2-type or

CCCC-type zinc fingers [3]. Plant-specific CCCH zinc finger proteins contain the conserved CCCH motif, which can occur in varying numbers, ranging from one to six copies [4]. Ongoing research on CCCH zinc finger proteins in many plants has revealed a similar pattern in other model plants [5]. The same study also found that the CCCH domain of CCCH zinc finger proteins is crucial for plant resilience [6]. A protein is considered a TZF protein if it has a tandem CCCH zinc finger (TZF) motif [7]. For example, the TZF protein AtTZF1 from Arabidopsis has two distinct motifs separated by 16 amino acids: C-X7-C-X5-C-X3-H and C-X5-C-X4-C-X3-H[8]. In cotton, GhZFP1 contains two different patterns of C-X8-C-X5-C-X3-H and C-X5-C-X4-C-X3-H zinc fingers, which are separated by a distance of 16 amino acids [2]. The function of the RR-TZF family has been extensively studied in various model plants, including Arabidopsis and rice [9]. Many TZF proteins have a plant-specific arginine-rich (RR) region in front of the TZF motif, known as RR-TZF [10]. Additionally, zinc finger proteins without tandem motifs are referred to as non-TZF proteins [11].

The localization of proteins within a cell is important for their proper functioning, and understanding their cellular localization can provide insights into their functions [12]. The nucleus contains several CCCH zinc finger proteins, including *GhZFP1*[13], *AtTZF11* [14], *OsDOS* [2], *AtZFP1* [15], *KHZ1* and *KHZ2* [16], *SAW1* [17] and *OsC3H10* [18]. Oxidation-related zinc fingers 1 (AtOZF1) are located at the plasma membrane [19], while ZFP36L3[20], ZC3H12a[21], and AtTZF2/3[22] are found in the cytoplasm. Proteins such as AtTZF1, AtTZF4, AtTZF5, AtTZF6, AtTZF7, and OsLIC exhibit bidirectional movement between the nucleus and cytoplasm [23]. Many plant CCCH proteins contain shuttle signals, which are believed to have important roles in signal transmission and stress response [24]. The transition from vegetative growth to flowering is a critical developmental phase in flowering plants, playing a significant role in their adaptation to the environment and achieving high agricultural productivity.

The genetic pathways regulating the transition of A. thaliana, a model plant, are complex and involve various factors, including photoperiod, vernalization, gibberellic acid (GA), age, autonomous, and ambient temperature signaling pathways. These pathways respond to both endogenous (age, GA) and environmental (day length, temperature, ambient temperature, and stress) stimuli. Recent studies have shown that the gene MsZFN from alfalfa delays flowering in A. thaliana. CCCH-type zinc finger proteins also play a role in floral transition [25], and *AtC3H17* promotes flowering in A. thaliana [26]. In rice, Ehd4 is a critical regulator that promotes flowering in the photoperiod pathway [27].

Drought is considered one of the most severe natural stresses that can negatively impact plant yield and quality [28]. To cope with this stress, plants have developed various complex mechanisms to detect and respond to drought at multiple levels, including molecular, biochemical, physiological, and morphological levels[29]. Many stress-responsive genes are involved in recognizing and adapting to the effects of drought stress. Based on their functional annotations, these genes can be categorized into two sets. The first set includes genes involved in drought signal transduction, such as protein kinases (MAP kinase, CDB kinase) and transcription factors (MYB, NAC, MYC, and DREB). The second set comprises functional genes involved in drought tolerance, including late embryogenesis abundant (LEA) proteins, osmotic proteins, water channel proteins, stomatal movement proteins, sugar and proline transporters, oxidative enzymes, and various proteases [30]. CCCH-type zinc finger protein genes have been found to have functions in stress response, including OsC3H10 and OsTZF5 (Oryza sativa CCCH-tandem zinc finger protein 5), which enhance rice's drought tolerance [18,31]. The genes AtSZF1 and AtSZF2 from *Arabidopsis thaliana* and GhZFP1 from *Gossypium hirsutum* are involved in responding to salt stress. They are zinc finger transcription factors of the CCCH type[2,14]. Some CCCH-type zinc finger protein genes respond to stress and also regulate flowering. Overexpression of AtZFP1 delays the flowering transition but improves salt tolerance in A. thaliana[32]. Overexpression of *AtTZF1*, a protein with two zinc fingers found in Arabidopsis thaliana, delays flowering and increases resistance to cold and drought [33].

Proper subcellular localization is essential for proteins to function effectively. Therefore, the cellular localization of a protein can provide valuable information about its role. In a previous study, we identified a CCCH-type zinc finger protein member called *PmC3H32* in *Pinus massoniana*[34], suggesting its potential involvement in responding to abiotic environmental factors. This study aims to investigate how *PmC3H32* contributes to the identification of stress-responsive genes. Additionally, transgenic experiments will be conducted in Arabidopsis to explore the involvement of *PmC3H32* in promoting flowering, improving physiological parameters, and inducing early leaf senescence under drought stress.

Materials and Methods

Test materials

Plant Material

Pinus massoniana seedlings, cultured in our laboratory, were used for extracting total RNA and genomic DNA. These seedlings were grown for 60 days after seed germination. *Arabidopsis thaliana* of the Columbia-0 (Col-0) type, obtained from seeds preserved in our laboratory, was used for genetic transformation after sterilization.

Strains and vectors

The *Escherichia coli* competent cell Trelief 5α was purchased from Beijing Qingke Biotechnology Co., Ltd., and the Agrobacterium perceptual cells EHA105 were purchased from Shanghai Weidi Biotechnology Co., Ltd. The pBI121-NG plasmids were obtained from E. coli strains preserved in our laboratory.

Preparation of culture medium and solution

LB liquid medium: 10g NaCl + 5g yeast extract + 10g trypsin, dissolved in 1 L deionized water and autoclaved at 121℃ for 20 min

MS solid medium: MS + sucrose 25 g·L-1+ agar 6.5 g·L-1

1 / 2MS solid medium: 1 / 2MS + sucrose 25 g·L-1+ agar 6.5 g·L-1

Primer synthesis and sequencing

The primers used in this experiment were synthesized by Shanghai Jirui Biotechnology Co., Ltd. and Beijing Qingke Biotechnology Co., Ltd. The primer sequences are provided in Table 1. Sanger sequencing service was performed by Shanghai Jie Biotechnology Co., Ltd. and Beijing Qingke Biotechnology Co., Ltd.

Table 1: All primers in this experiment.

Primer name	Sequence of primers (5'→3')
PmC3H32-F	TCCACCACTGTCACCATCTGCGTCTC
PmC3H32-R	GCCTTGGGCTTGCCAACCCTCTT
QPmC3H32-F	ACAGTCTTCCATCGACTCCCACA
QPmC3H32-R	TTGACCAGCTCATTAACCCAACC
TUA-F	CAAACTTGGTCCCGTATCCTC
TUA-R	CACAGAAAGCTGCTCATGGTAA
pBI121-PmC3H32- NG-F	atggccatggaggccgaattcATGTCAAGCGTTTCTGCAGAACA
pBI121-PmC3H32-NG -R	ccgctgcaggtcgacggatccTTACTTAACAAGCTCGTTCACCCA
M13F	GTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGAC

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using the FastPure Plant Total RNA Isolation Kit (RC401, Vazyme Biotech, Nanjing, China) [35]. The concentration and purity of RNA were measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was evaluated by 1.2% agarose gel electrophoresis. First-strand cDNA synthesis was performed using the One-step gDNA Removal and cDNA Synthesis Kit (AT311, TransGen Biotech, Beijing, China). Primers for quantitative real-time reverse transcription PCR (qRT-PCR) were designed using Primer 5.0 (see Supplementary Materials Table S1). The SYBR Green reagents were used to detect the target sequence. Each PCR mixture (10 μL) contained 1 μL of diluted cDNA (20× dilution), 5 μL of SYBR Green Master Mix (11184ES03, Yeasen Biotech, Shanghai, China), 0.4 μL of each primer (10 μM), and 3.2 μL of ddH2O. The PCR program consisted of an initial preincubation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension.

The PCR quality was assessed using melting curves. The α-tubulin (TUA) gene was used as a reference gene. Three independent biological replicates, with three technical replicates for each biological replicate, were examined. Quantification was achieved using comparative cycle threshold (Ct) values, and gene expression levels were calculated as 2^(-ΔΔCt) [ΔCT = CT Target - CT TUA. ΔΔCt = ΔCt Target - ΔCt CK]. The significance between different columns was analyzed using a Duncan test in IBM SPSS Statistics (Version 25), with lowercase letters used for marking the results. The letters indicated significant differences, with lowercase letters starting from the largest average. The same lowercase letters between different columns indicated no significant difference, while completely different lowercase letters indicated a significant difference ($p < 0.05$). If a column was marked "ab," it meant there was no significant difference between the "ab" column and the "a/b" column.

Identification of PmC3H32 Gene in Pinus massoniana

We acquired the hidden Markov model (HMM) profile of the CCCH domain (PF00642) from the Pfam database (http://pfam.xfam.org/, accessed on 16 April 2022). The HMM profile was used to search the CCCH members from the following three transcriptomes. Transcriptome data for P. massoniana were derived from the previously determined CO2 stress transcriptome, drought stress transcriptome (PRJNA595650) and young shoots transcriptome (PRJNA655997). A BLASTP search was performed against three transcriptome data using the hidden Markov model (HMM) profile. A transcription factor prediction tool (http://planttfdb.gao-lab.org/prediction.php, accessed on 16 April 2022) was used to predict CCCH proteins. Then, we used Pfam and NCBI's Conserved Domain search (CD Search) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 16 April 2022) to check the predicted CCCH domain of initial CCCH TFs.

Finally, sequences with complete CCCH domains were selected after deleting sequences with more than 97% similarity. The molecular weights and isoelectric points of the identified PmC3H proteins were computed from the ExPASy (Swiss Institute of Bioinformatics) website (https://web.expasy.org/compute_pi/, accessed on 17 April 2022) [34].

Cloning of ORF Rregion of PmC3H32 Gene

The complete sequence of *PmC3H32* gene was obtained by splicing the middle segment, 5'RACE segment and 3'RACE segment with SeqMan software. Take advantage of the NCBI's Open Reading Frame Finder. The ORF region of PmC3H32 gene was predicted by on-line software, and specific primers were designed at both ends of the primers. The first strand of *Pinus massoniana* cDNA synthesized in 2.2 was used as a template to amplify the ORF-region of the *PmC3H32* gene. The PCR reaction system and program setup of ORF region of PmC3H32 gene refer to Table 2.

The electrophoretic detection, gel cutting recovery, target fragment linking transformation, positive detection and sequencing of ORF fragment amplification products refer to the below experimental process.

Table 2: PCR amplification reaction system and procedure.

Reagent	Volume	Reaction procedure
cDNA	2.5 μl	98°C; 3 min
2×Phanta® Master Mix	25 μl	98°C; 15 sec
PmC3H32-F	2.5 μl	60°C; 15 sec 35 cycles
PmC3H32-R	2.5 μl	72°C; 1 min
ddH_2O	17 μl	72°C; 5 min
Total Volume	50 μl	4°C; ∞

Electrophoretic Detection and Gum Cutting Recovery

I. Electrophoretic detection

Electrophoretic detection: The 5μl PCR product was mixed with 1μl 6 × Loading Buffer and then placed into the prepared 1.2% agarose gel (containing Gel Stain) pores. Electrophoresis was performed at 200 V in 1 × TAE buffer solution for 20 min. After gel running, the size of the bands was observed with a gel imaging instrument, and pictures were taken.

II. Rubber cutting recovery

Mix the remaining PCR product with 6 × Loading Buffer and run the gel. The correct fragment gel was cut quickly under ultraviolet lamp irradiation, and the fragment was recovered by an agarose gel recovery kit. The procedure should be carried out strictly in accordance with the kit instructions.

Intermediate fragment join transform

I. Connection

Take 4 μl of the recovered product in a 1.5 ml centrifugal tube. Add 1 μl pEASY-Blunt, gently mix with a pipette gun, react at room temperature for 15 minutes, and place the centrifuge tube on ice.

II. Transformation

Add 50 μl Trelief to the joining product of 5 alpha competent cells, flick and mix well, followed by an ice bath for 30 min. Heat shock for 45 sec at 42 °C and immediately place on ice for 2 min. Then add 500 μl LB medium at 200 rpm and resuscitate at 37 °C for 1 h. Centrifuge at 5000rpm for 1 min, discard 450 μl of supernatant, blow and suck, and then coat on the blue-white screen plate containing Kan. The plate should be inverted and incubated overnight at 37 °C.

III. Positive detection and sequencing of bacterial fluid

Single white colonies were picked from the LB plate in 10 μl of ddH2O water. After blowing and sucking, 3 μl was taken for positive detection. The remaining bacterial liquid was added to 1 ml of LB liquid medium containing Kan and cultured at 200 rpm and 37 °C. At the end of the bacterial test, the correct bacterial solution was selected and sent to the company for sequencing. Positive test reaction system and procedures are listed in Table 3.

Table 3: PCR reaction system and procedure.

Reagent	Volume	Reaction procedure
2×Rapid Taq Master Mix	10 μl	95°C; 3 min
M13-F	1 μl	95°C; 15 sec
M13-R	1 μl	55°C; 15 sec 35 cycles
Bacteria solution	3 μl	72°C; 1 min
ddH_2O	5 μl	72°C; 5 min
Total	20 μl	4°C; ∞

Construction of genetic transformation vector

The plasmid vector used in this experiment was the pBI121-NG vector (Figure S1). The pBI121-NG vector contained the protein gene, which could be expressed as a marker gene along with the target gene link. Recombinant primers were designed based on the pBI121-NG vector sequence and the ORF sequence of the *PmC3H32* gene (Table S1). The pBI121-NG plasmid was extracted according to the instructions of the fast plasmid extraction kit. The extracted pBI121-NG plasmid (1 μg) was subjected to double enzyme digestion, with the reaction conditions shown in Table 4.

Table 4: Enzyme digestion of pBI121-NG plasmid.

Reagent	Volume	Temperature
pBI121-NG	1 μg	
10×QuickCut Buffer	5 μl	37°C; 30 min
Xba I	1 μl	80°C; 20min
BamH I	1 μl	4°C; ∞
ddH_2O	up to 50 μl	

The vector was constructed through homologous recombination. The reaction system was formulated on ice, including the linearized vector, insertion fragment, 5×CE II Buffer, Exnase II, and ddH2O. After a 30-minute reaction at 37°C, the mixture was immediately placed on ice. The transformation of the recombinant product into Escherichia coli and the positive detection method were conducted. The sequencing of the plasmid with correct sequencing results was completed by Beijing Qingke Biotechnology Co., Ltd., and the plasmid was stored at -20°C for backup.

Transformation of arabidopsis Thaliana by inflorescence leaching

Sowing and culture of *arabidopsis thaliana*

1) Disinfection of A. thaliana seeds: Proper amounts of *A. thaliana* seeds were placed in 1.5 ml centrifuge tubes and treated with 1 ml of 75% ethanol for 45 seconds, followed by the addition of 1 ml of 20% sodium hypochlorite for 5 minutes. The seeds were then rinsed repeatedly with sterile water.

2) Sowing: The sterilized A. thaliana seeds were cultured on 1/2MS medium using a liquid transfer gun.

3) *A. thaliana* culture: The culture medium containing A. thaliana seeds was sealed and cultured at 4°C for 2 days. Subsequently, the seeds were transferred to an artificial climate incubator for germination and growth. After approximately one week, healthy *A. thaliana* seedlings were transferred to nutrient-rich soil (black soil: vermiculite: perlite = 6:2:1) and covered with a fresh-keeping film. The film was removed on the third day.

Transformation of arabidopsis thaliana

The main results were as follows:

1) Agrobacterium tumefaciens solution containing the PBI121-PmC3H32-NG recombinant plasmid was cultured on an LB plate containing 50 mg/L Kan and 25 mg/L Rif in an ultra-clean worktable [36].

2) A single colony with good growth was selected and cultured overnight at 28°C in 5 ml of LB liquid medium containing 50 mg/L Kan and 25 mg/L Rif.

3) The overnight cultured bacterial solution (1 ml) was inoculated into 50 ml of LB liquid medium containing 50 mg/L Kan and 25 mg/L Rif, and the culture was shaken until the optical density at 600 nm (OD600) reached approximately 0.8.

4) The bacterial solution was transferred to a sterile centrifuge tube, and the bacteria were collected by centrifugation at 5000 rpm for 10 minutes. The bacterial pellet was resuspended in 50 ml of pre-prepared osmotic buffer.

5) Arabidopsis thaliana plants that had bolted for approximately 4 weeks were selected, and the open flower buds were removed. The inflorescences were soaked in the infection solution for 30 seconds.

6) Dark culture was performed for 18-20 hours, followed by rinsing with clean water. The plants were then continued to be cultured in an incubator.

7) After 7-10 days, steps 1-6 were repeated for reinfection.

8) The transgenic T0 generation seeds were collected after the pods of Arabidopsis thaliana plants turned yellow. To promote seed maturity, the watering frequency was controlled appropriately when the seeds were nearing maturity.

Resistance screening of transgenic *arabidopsis thaliana* plants

The main results were as follows:

1) The disinfection method was consistent with the methods mentioned above when collecting the appropriate amount of T0 seeds in 1.5 ml centrifuge tubes.

2) The seeds were uniformly placed on 1/2MS medium containing 30 mg/L Hyg using a liquid transfer gun. After dark culture at 4°C for 2 days, the plates were transferred to an artificial climate incubator for further culture.

3) After approximately 2 weeks of culture, two resistant seedlings with green true leaves and normal root growth were selected and transplanted into vegetative soil. The seedlings were covered with a fresh-keeping film, which was removed on the third day.

4) Once the seeds matured, the T1 generation seeds were collected separately. The screening of T2 generation seeds was carried out until the T2 generation seeds were collected for further experiments [37].

PCR and qRT-PCR analysis of the transgenics

Genomic DNA was extracted from fresh leaves of T2 generation *Arabidopsis thaliana* using the FastPure Plant DNA Isolation kit [38]. The concentration and OD260/280 ratio of the extracted genomic DNA were determined using an ultramicro spectrophotometer. PCR detection was performed using genomic DNA as a template, with M13-F and M13-R primers. The PCR reaction system and procedure are described in Table 5.

Table 5: PCR reaction system and procedure.

Reagent	Volume	Reaction procedure
gDNA	2 µl	98°C; 3 min
2×Phanta® Master Mix	25 µl	98°C; 15 sec
M13-F	2 µl	55°C; 15 sec 35 cycles
M13-R	2 µl	72°C; 1 min
ddH2O	19 µl	72°C; 5 min
Total Volume	50 µl	4°C; ∞

The RNA was extracted from the samples and reverse transcribed to obtain cDNA using the methods provided in the Vazyme FastPure Plant Total RNA Isolation Kit (Polysaccharides and Polyphenolics-rich) instructions. RNA extraction, cDNA first-strand synthesis, qRT-PCR system, and program setup followed the methods described in section 2.2.

Phenotypic evaluation of transgenic plants under non-stressed conditions

Seedlings that were four weeks old and had spent two weeks on Murashige and Skoog (MS) plates, followed by two weeks on soil, were used to evaluate their growth and development under typical conditions. The plants were carefully removed from the soil to measure the length of their tap roots. Additionally, the longest and widest leaves of each plant were observed for further comparison.

Evaluation of survival rate and phenotypic observation of transgenic arabidopsis thaliana under drought stress

The survival rate of *Arabidopsis thaliana* under drought stress was assessed using the same-tray technique, where both wild-type and transgenic plants were cultivated on identical trays. Two-week-old seedlings were transferred from MS plates to soil and allowed to grow under normal watering conditions for two weeks before subjecting them to non-irrigation for 15 days. After the drought treatment, water was re-applied for three days, and the percentage of plants that recovered per genotype was evaluated. Photographs were taken before and after the drought treatment, as well as after the three-day recovery period. The assay was conducted with three biological replicates per genotype [39].

Expression analyses of *PmC3H32* genes under drought stress

After being exposed to drought treatment, transgenic Arabidopsis and wild-type plants were left dehydrated on a bench for 5 and 8 hours. The entire treated plants were then collected and frozen in liquid nitrogen. Purified total RNA was extracted from these samples using the Vazyme FastPure Plant Total RNA Isolation Kit (Polysaccharides and Polyphenolics-rich) instructions. DNA was extracted using the FastPure Plant DNA Isolation kit [38]. RNA extraction, cDNA first-strand synthesis, qRT-PCR system, and program setup followed the methods described in section 2.2.

Soil moisture analysis

Soil samples were collected from transgenic and wild plants at four different time points (0, 5, 10, and 15 days) during the drought treatment of *Arabidopsis thaliana*. Each sample consisted of approximately 5g of fresh soil, with two replicates labeled as A and B. The soil was placed in aluminum specimen boxes and weighed (m1). The boxes were heated in an oven at 105°C for 6-8 hours, cooled in a desiccator, and then weighed again (m2). The mass of the box without the soil was determined (m0). These measurements were used to calculate the soil moisture content (%) [40].

$$\text{Soil moisture content (\%)} = m1 - m2 \times \frac{100}{m1 - m0}$$

Statistical analysis

The statistical analyses, including ANOVA on all data sets and assessment of linear correlation using the Pearson correlation coefficient, were performed using GraphPad Prism 9.2.0 software (GraphPad Software, 2023). The objective was to determine if there were any statistically significant differences, considering p-values less than 0.05 as indicators of significance.

Results

Cloning and analysis of PmC3H32 gene from Pinus massoniana

Extraction of total RNA from *Pinus massoniana*

The total RNA extracted from *Pinus massoniana* was detected by ultramicrospectrophotometer. Total RNA of *Pinus massoniana* was detected by 1% agarose gel electrophoresis.

Amplification of the ORF region of *PmC3H32*

The ORF region of PmC3H32 gene was predicted in the ORF Finder, and specific primers were designed at both ends of the gene for PCR amplification (Figure 1). The complete ORF region was obtained by sequencing, with a length of 1578 bp and a total of 527 amino acids.

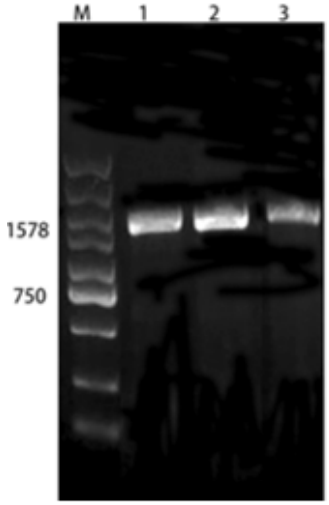

Figure 1: ORF amplification products of PmC3H32

Construction of the pBI121-PmC3H32-NG Recombinant Vector

The PBI121-PmC3H32-NG recombinant vector was successfully constructed by amplifying the target genes using primers with restriction sites (Figure 2A), followed by double digestion of the pBI121-NG plasmid and homologous recombination. The positive colonies were confirmed by PCR and sequencing (Figure 2B).

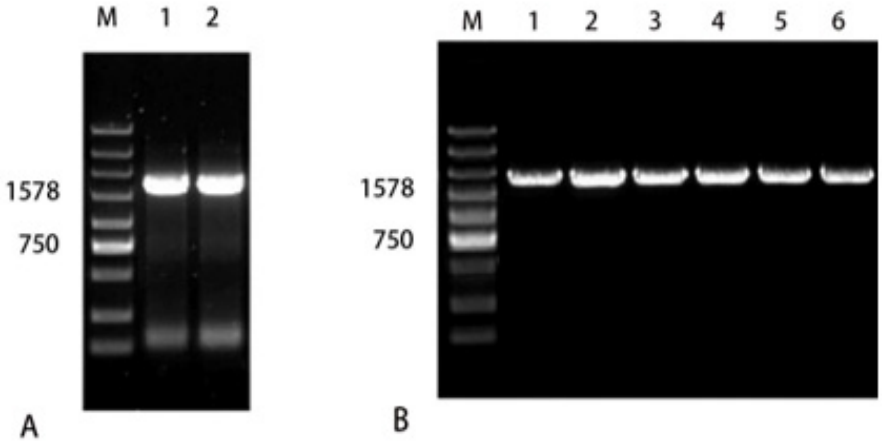

Figure 2: (A) *PmC3H32* insert fragment. (B) Electrophoresis of recombinant plasmid.

PCR and qRT-PCR Analysis of the Transgenics

Preliminary resistance screening on 1/2MS (Hyg) medium showed that the negative plants failed to grow normally, while the positive transgenic plants could grow normally. The normal Arabidopsis seedlings were transferred to vegetative soil for further cultivation. Genomic DNA and RNA were extracted from leaves, and the transgenic plants were identified at the gene and transcription levels. Firstly, the genomic DNA of wild type and transgenic Arabidopsis thaliana was amplified by PCR. The amplified bands of *PmC3H32* gene were the same size as the plasmid amplified products (Figure 3A). The transgenic lines that have been identified successfully at the gene level are then identified at the transcription level (Figure 3B). The results showed that the expression levels of different transgenic plants were different. Among the 10 transgenic Arabidopsis plants tested, the highest expression level was L10, followed by L4 and L6.

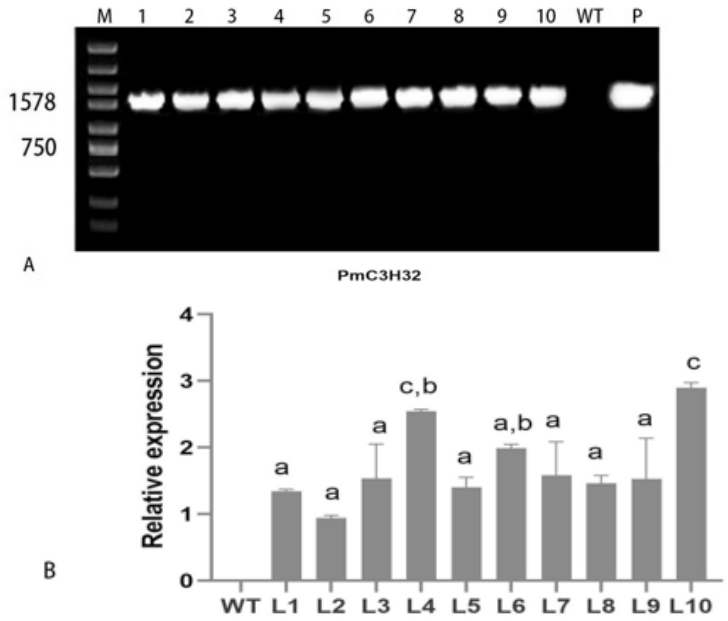

Figure 3: (A) PCR identification of transgenic A. thaliana. Note: M: 5000 bp Molecular weight mark-er; 1-10: transgenic Arabidopsis thaliana; WT: wild-type Arabidopsis thaliana; P: positive control (Plasmid of PBI121-PmC3H32-NG). (B) The PmC3H32 relative expression level detection in trans-genic plants.

Phenotypic evaluation of transgenic Plants under non-stressed conditions

The investigation employed three A. thaliana lines (L4, L6, L10) that overexpressed PmC3H32 for further examination (as illustrated in Figure 1). After being transplanted for two weeks, it was observed that the rosette leaves of the genetically modified plants were larger and wider, and that the root length was greater than those of the wild type (as depicted in Figure 4A-B). Furthermore, the genetically modified plants took an average of 21.26 days to bolt and 25.7 days from germination to flowering, whereas the WT plants required 25.82 days and 29.8 days respectively (Figure 4C-E). The research proposes that PmC3H32 may be accountable for the increased size of transgenic plant rosette leaves and long root length compared to WT. Its overexpression in these plants may affect flowering induction in *A. thaliana*.

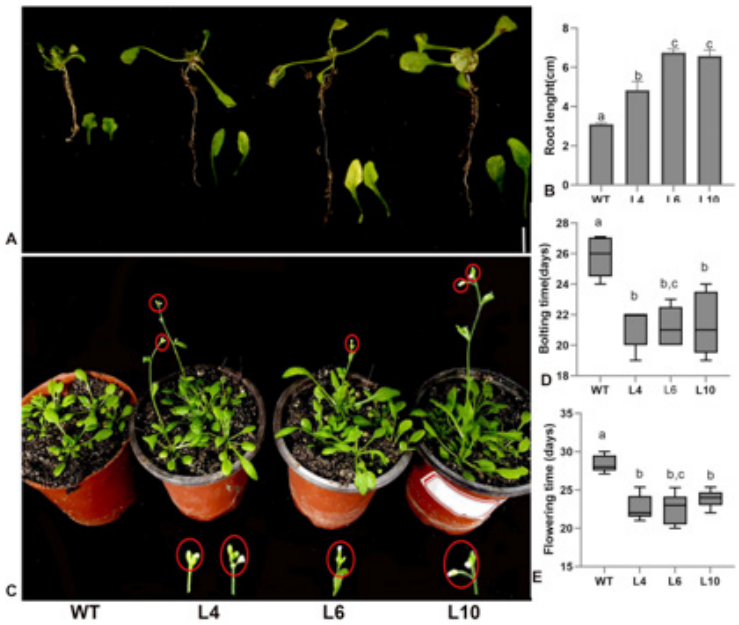

Figure 4: Phenotypic characteristics of three transgenic Arabidopsis lines (L4, L6, and L10) and wild-type (WT) plants carrying PBI121-PmC3H32-NG under normal growth conditions. (A) Obser-vation of rosette leaves and root length. (B) Measurement of root length. (C) Flower observation. (D) Time taken for bolting. (E) Time taken for flowering. Data in the figure are presented as the mean ± standard deviation (n=3). The letters above the bars indicate significant differences ($p < 0.05$). The presence of the same lowercase letter between two different columns indicates no significant differ-ence, while different lowercase letters among columns indicate a significant difference.

Overexpression of PmC3H32 in arabidopsis leads to an increase in physiological parameters and leaf senescence under drought stress

To investigate the function of *PmC3H32* in response to drought stress, we subjected transgenic A. thaliana (L4, L6, L10) and wild-type plants to a 15-day period without watering to simulate drought conditions. Before this, all plants had been growing normally with similar growth patterns. During the drought period, all plants showed some yellowing of leaves. After three days of normal watering (i.e., stress removal), the WT plant recovered significantly better than the transgenic plant due to the high number of green leaves observed compared to the transgenic plant with fewer green leaves (as shown in Figure 5A).

The average plant height of the three transgenic lines was 24.98 cm (L4: 25.16 cm; L6: 24.44 cm; L10: 25.33 cm), which was significantly higher than the wild-type height of 17.16 cm (Figure 5B). The average length of the silique was 12.79 mm (L4: 12.96 mm; L6: 12.32 mm; L10: 13.07 mm compared to 8.76 for the WT) (Figure 5C-D). The average number of siliques for each line also differed significantly between transgenic lines and wild-type plants (33, 32, and 35 for L4, L6, and L10 compared to only 9 for wild-type) (Figure 5E). The mean fresh weight of the siliques in the transgenic lines (L4, L6, and L10), as well as the wild type (WT), were 0.0209 g, 0.0232 g, 0.0235 g, and 0.03 g, respectively (Figure 5F). The average number of branches in the transgenic lines (L4, L6, L10) and WT were 17.66, 15, 19, and 9.66, respectively (Figure 5G).

The findings indicated that when plants undergo drought stress, their tissue functions can be enhanced by overexpressing the PmC3H32 genes. The transgenic lines (L4, L6, and L10) were found to have a lower weight of silique and less fresh leaf than the non-transgenic plants after the treatment, suggesting that the overexpression plants can initiate growth earlier and complete their life cycle before non-transgenic plants, even in varying environmental conditions like drought stress. The increased expression of this gene may be accountable for improving transgenic plants under drought stress leading to a fast leaf senescence.

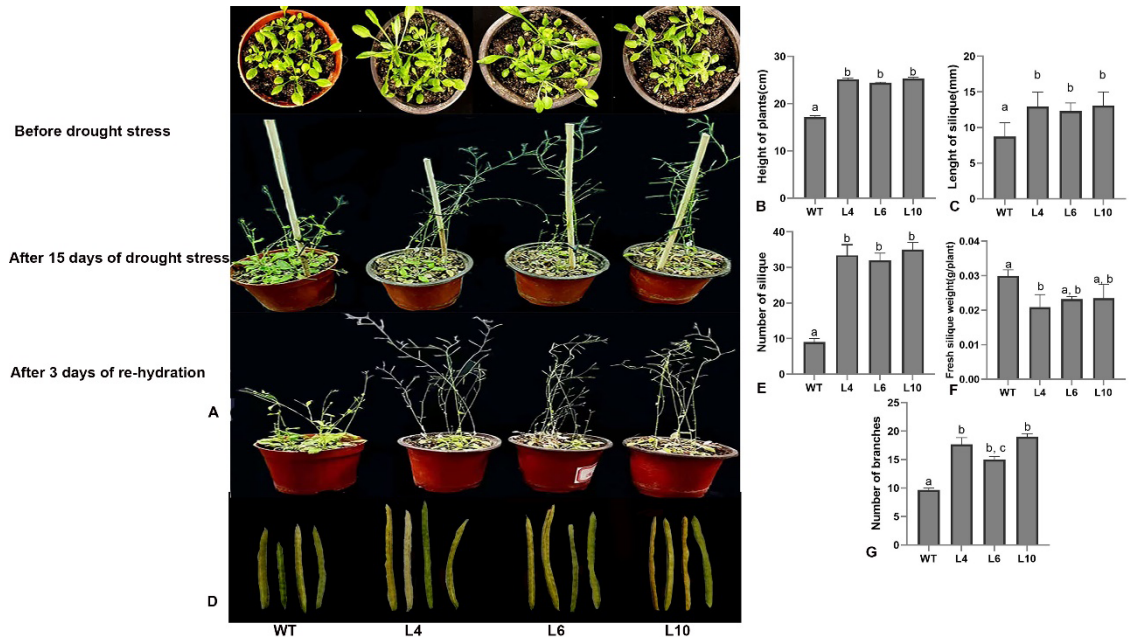

Figure 5: Evaluation of *PmC3H32*-transgenic Arabidopsis plants' resilience to drought conditions by assessing their survival rate and physiological characteristics. (A) Images of normal-type plants (WT) and three distinct transgenic lines (L4, L6, L10) before and after a 15-day period of soil drying fol-lowed by a 3-day re-watering phase. The plants used in the experiment were four weeks old. (B) Plant height. (C,D) Silique length. (E) Number of siliques. (F) Fresh weight of siliques. (G) Number of branches. Data in the figure are presented as mean ± standard deviation (n=3). The letters above the bars indicate significant differences ($p < 0.05$). The presence of the same lowercase letter between two different columns indicates no significant difference, while different lowercase letters among columns indicate a significant difference.

High expression of PmC3H32 maintained after drought stress in arabidopsis overexpressed plants

To explore the expression pattern of PmC3H32 after drought circumstance, the expression level of *PmC3H32* in Arabidopsis thaliana WT and Arabidopsis thaliana-overexpressing plants was determined after exposure to 15 days of drought stress and 3 days of re-hydration (Figure 6). The results showed that the expressions of PmC3H32 in L4, L6 and L10 were significantly higher than that of the WT seedlings.

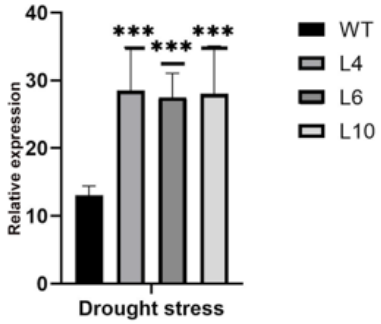

Figure 6: Expression levels of *PmC3H32* genes in wild-type (WT) and transgenic A. thaliana under drought stress. (Asterisks above columns indicate a significant difference compared to WT (*** $p \leq 0.001$).

Genes involved in the regulated Flowering and Drought Tolerance

HOMOLOG of BEE2 INTERACTING WITH IBH 1 (HBI1), which is known to play a role in floral transition and is predicted to be part of the photoperiod pathway, as well as *Isopentenyl transferase 3 (IPT3), Arabidopsis Thaliana Response Regulator 5 and 7 (ARR5, ARR7)*, which are predicted to be hormone-related proteins, were examined. Regarding drought tolerance, stomatal movement protein expansin1 (EXPA1) is a gene that encodes for a protein involved in cell wall expansion and growth [41](Table S2). The primers used for this experiment were listed on the Table S3.

Their expression patterns were analyzed using qRT-PCR and were found that HOMOLOG of BEE2 INTERACTING WITH IBH 1 (HBI1), Isopentenyl transferase 3 (IPT3), Arabidopsis Thaliana Response Regulator 5 and 7 (ARR5, ARR7) were upregulated in overexpression lines. At the same time, stomatal movement protein expansin1 (EXPA1) was downregulated in the transgenic plant under drought stress, but upregulated in the WT (Figure 7). However, the specific effects of this downregulating EXPA1 may depend on various environmental conditions such as drought stress and other genetic factors that might be responsible of the early leaf senescence of the transgenic plants. However, it's important to note that qPCR alone cannot directly demonstrate these physicals interaction between the two proteins encoded by these genes. For that, additional experimental techniques such as co-immunoprecipitation or yeast one-hybrid assays may be required.

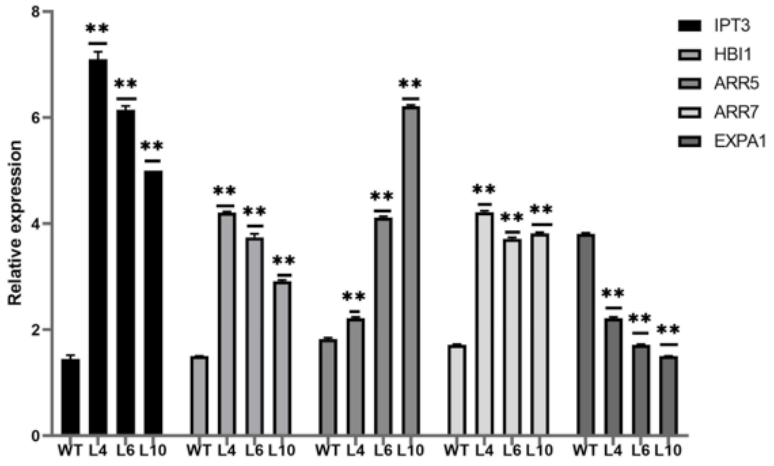

Figure 7: Expression validation of genes involved in floral transition or stress tolerance. The graph includes the wild-type (WT) and four overexpression lines (L4, L6, L10) of Arabidopsis thaliana. The error bars represent the standard deviation calculated from triplicate measurements. The asterisks (*) and double asterisks (**) indicate significant differences compared to WT at $p < 0.05$ and $p < 0.01$, respectively, as determined by the ANOVA test.

Soil moisture content

Upon further investigation of the soil moisture levels at 0, 5, 10, and 15 days of the transgenic lines (L4, L6, L10) and WT, it was observed that all plants experienced a similar decrease in moisture during the drought treatment (Figure 8). This suggests that the stress was evenly distributed among all tested plants.

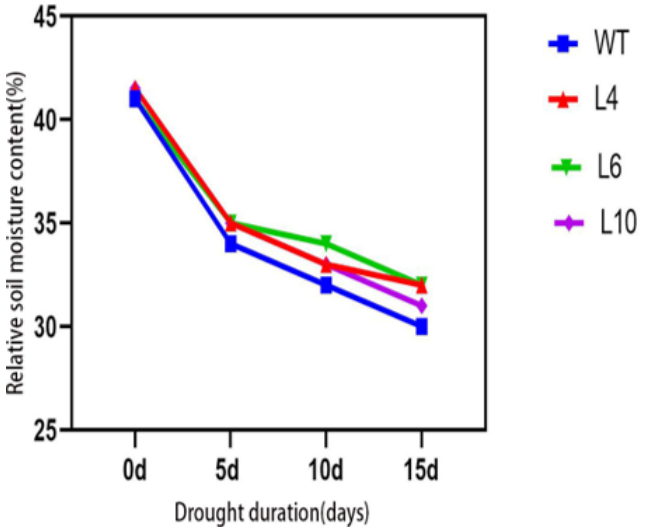

Figure 8: Average relative soil moisture content measured during the drought treatment. The data represents three replicates (n = 3).

Investigating relationships between different parameters of arabidopsis after drought stress with correlation analysis

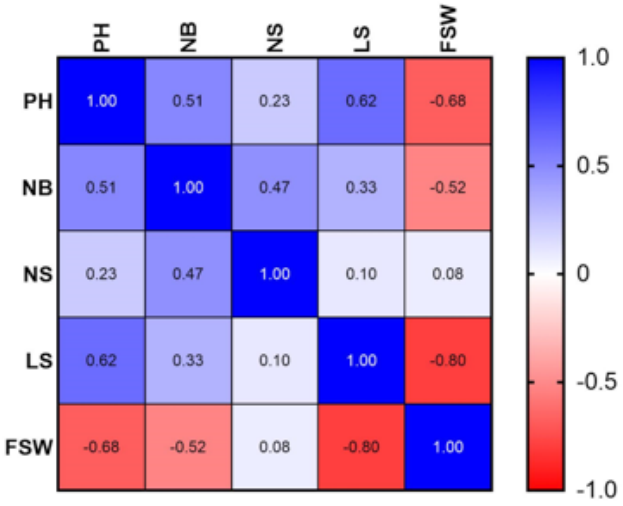

Figure 9: Pearson correlation matrix showing the relationships between different physiological parameters. The analysis was performed using GraphPad's Prism 9.3.0 software. The abbreviations used are as follows: PH (Plant Height), NB (Number of Branches), NS (Number of Silique), LS (Length of Silique), FSW (Fresh Silique Weight).

We investigated how plant height, number of branches, number of siliques, length of siliques, and fresh siliques weight are correlated. The correlation matrix shows the strength and direction of the relationship between different parameters. Based on the correlation matrix results, it can be observed that there are some strong positive correlations between plant height and the number of branches (0.51), length of siliques (0.62), and number of siliques (0.23). On the other hand, there is a strong negative correlation between plant height and fresh siliques weight (-0.68). Additionally, there are moderate positive correlations between the number of

branches and the number of siliques (0.47) and the length of siliques (0.33). However, there is a moderate negative correlation between the number of branches and the fresh siliques weight (-0.52) (Figure 9). The correlations between the other variables are weak or negligible. Overall, these results suggest that plant height has a significant impact on various aspects of plant growth and development, while fresh siliques weight is negatively affected by plant height. Additionally, the number of branches appears to have some influence on other variables, such as the number and length of siliques. However, further analysis is needed to determine the nature and strength of these relationships. In summary, our results suggest that some parameters have significant relationships while others have weaker or no relationships at all. Nevertheless, all parameters may positively impact physiological growth during drought stress conditions in transgenic lines (L4, L6, L10) and WT.

Discussion

The domain of genetically engineered plants with enhanced growth and stress resilience is a well-established research field, offering potential solutions for adapting to unpredictable environmental conditions [42]. Prior research has demonstrated the feasibility of introducing exogenous genes to develop transgenic plants with increased biomass and improved resistance to abiotic stress [43]. In this context, the CCCH type of zinc finger proteins, including PmC3H32, plays a pivotal role in plant growth, stress responses, and the transition from vegetative growth to flowering [9,41].

Contrasting our findings with extant research, we observe that while previous studies have illuminated the signal transduction and molecular mechanisms of CCCH zinc finger proteins in plant stress resistance [34], the functional studies of the CCCH gene family in Masson pine remain largely unexplored. Therefore, our study contributes to the selection of candidate genes for further functional investigation in this species.

To engineer stress-resistant and high-yielding Arabidopsis plants via genetic modification, efficient transgenic technology, a well-developed transformation material, and superior genes are essential factors. Arabidopsis was chosen as the transformation material due to its adaptability, efficiency, and fully decoded genome, enabling a comprehensive analysis of the effects of PmC3H32 overexpression [43].

Our successful production of transgenic Arabidopsis lines expressing *PmC3H32* with high transformation efficiency (100%) and significant transcription levels in L4, L6, and L10 underscores the importance of CCCH proteins in regulating growth, development, and stress responses [44,45]. The increased expression of PmC3H32 in Arabidopsis plants resulted in several notable phenotypic changes, including larger rosette leaves, longer root length, and early flowering. These effects suggest that the upregulation of *PmC3H32* might stimulate shoot apical meristem growth and various hormone pathways, such as auxins, cytokinins, gibberellins, abscisic acid, ethylene, and florigen, which collectively contribute to leaf tissue development, root growth, and promotion of plant flowering [18,46].

Under stressful conditions, such as drought stress, the transgenic Arabidopsis plants exhibited taller height, longer siliques, higher siliques number, and a greater number of branches compared to the wild type. These phenotypic enhancements are attributed to the augmented function of meristematic tissue at the root and shoot tips, as well as the dermal tissue acting as a protective barrier against water loss, pathogens, and physical damage [47,48]. However, it is important to consider potential negative consequences associated with the overexpression of *PmC3H32*.

One potential negative consequence is the observed early senescence of leaves and siliques under drought stress in the transgenic lines (L4, L6, and L10). This suggests a trade-off between enhanced growth and accelerated senescence [49,50]. Additionally, the lower weight of fresh siliques in the transgenic lines compared to the wild type under various environmental factors, including drought stress, indicates that overexpression of PmC3H32 may expedite growth and maturation, resulting in reduced silique weight. It is crucial to further investigate the long-term effects and potential trade-offs associated with *PmC3H32* overexpression to comprehensively understand its impact on plant fitness and overall physiology.

In terms of drought stress tolerance, our findings align with the effects of overexpressing the CpC3H3 gene from Chimonanthus praecox, which demonstrated improved drought tolerance and early flowering in Arabidopsis thaliana [41]. The extensive presence and diverse functions of zinc finger proteins in plant growth and development further support their significance in enhancing stress tolerance [41]. For instance, a computational analysis identified 36 CCCH zinc-finger family genes in Aegilops tauschii Coss, with AetTZF1 exhibiting the highest expression level under drought stress conditions [51].

To gain insights into the regulatory mechanisms underlying the effects of *PmC3H32* on flowering and drought stress, we examined the transcriptome profiles of wild-type and transgenic plants. The overexpression of *PmC3H32* resulted in the upregulation of genes such as *IPT3, HBI1, ARR5, and ARR7*, which are associated with flowering regulation mechanisms [34]. This supports the hypothesis that *PmC3H32* may promote flowering through photoperiod and hormone signaling pathways. However, further confirmation and additional verification are necessary to fully understand these regulatory mechanisms.

It is worth noting that the regulation of stomatal movement, mediated by AtEXPA1, is crucial for drought stress tolerance as it facilitates a balance between water conservation and gas exchange [45]. By controlling the stomatal aperture, plants can minimize water loss while ensuring adequate carbon dioxide uptake for photosynthesis, thereby maintaining metabolic processes even under conditions of limited water availability. The *EXPA1* gene in *Arabidopsis thaliana* is instrumental in regulating cell expansion and growth during the early stages of plant development. As plants mature and transition into the senescence phase, the need for cell expansion and growth diminishes, which accounts for the downregulation of *EXPA1* during this stage. Early leaf senescence observed under drought stress conditions has been associated with this al-

tered regulation of *EXPA1* [52]. However, it's important to note that while this association has been observed, definitive causation has not been established. To confirm that the early leaf senescence is indeed due to the downregulation of EXPA1 under drought stress, additional tests, such as Yeast One-Hybrid (Y1H) assays, would be necessary. Y1H assays can help identify and validate the interactions between EXPA1 and other proteins or genes that may be involved in the process of leaf senescence under drought stress. This further investigation would provide more concrete evidence of the role of *EXPA1* in this process.

In the context of drought stress, our findings are consistent with a study on the gene *OSCA1* in Arabidopsis [53], which found that overexpression of this gene led to earlier flowering under drought stress conditions, implying its sensitivity to drought stress and shorter survival compared to the wild-type under the same conditions. This suggests that the overexpression of certain genes, such as *PmC3H32* and *OSCA1*, can alter the plant's response to drought stress, potentially leading to earlier flowering and shorter survival under drought conditions.

Regarding the soil moisture content, both the transgenic lines and the wild type exhibited a similar decrease during the drought treatment, indicating a comparable response to water scarcity. This observation suggests that the enhanced stress tolerance of the transgenic lines is not primarily attributed to differences in soil moisture absorption but rather to other physiological and molecular mechanisms involved in stress responses. This aligns with findings that the overexpression of specific genes such as *GmWRKY16, NRGA1, VlWRKY3, and GmST1*, which are known to regulate molecular and physiological stress responses, are the primary contributors to the enhanced drought tolerance observed in our transgenic *Arabidopsis thaliana lines* [54-57].

The analysis of the correlation matrix between physiological plant parameters provides valuable insights into the interrelationships among different traits and their impact on plant growth and development [58]. In our study, we focused on plant height, number of branches, number of siliques, length of siliques, and fresh silique weight. These parameters were found to have significant correlations, which align with the findings of previous studies [58,59].

For instance, a positive correlation was observed between plant height and the number of branches, suggesting that taller plants tend to have more branches. This is in line with the findings of a study by Granier et al. [60] who used the PHENOPSIS phenotyping platform to measure *Arabidopsis thaliana* plant traits. This finding is significant as it indicates that plant height can serve as an indicator of branching patterns and overall plant architecture.

Moreover, plant height exhibited a positive correlation with the length of siliques, implying that taller plants tend to produce longer siliques. This is supported by Wang et al. [61] who highlighted the significant role of silique wall photosynthesis in seed-related traits. Understanding this correlation is important as it suggests that plant height can influence the reproductive structures and ultimately affect seed production.

Interestingly, a negative correlation was found between plant height and fresh silique weight, suggesting that as plants grow taller, their individual silique weights decrease. This could be due to factors such as reduced nutrient uptake or increased resource competition among plant parts, as indicated by a high-throughput non-destructive phenotyping study. Exploring this correlation further helps us understand the trade-offs between plant growth and reproductive allocation [62].

Additionally, a positive correlation was noted between the number of branches and the number of siliques, indicating that plants with more branches tend to have a higher number of siliques. However, a negative correlation was found between the number of branches and fresh silique weight, suggesting that plants with more branches tend to have smaller individual siliques. This aligns with a study that showed adaptive diversification of growth allometry in *Arabidopsis thaliana.*

Interestingly, there was only a weak positive correlation between the number of siliques and the length of siliques, and no significant correlation between the number of siliques and fresh silique weight. These results suggest that factors other than the quantity of siliques on the plant influence the size and weight of individual silique. Notably, a strong negative correlation was observed between the length of siliques and fresh silique weight, indicating that longer siliques tend to have lower weights compared to shorter ones. This relationship underscores the importance of considering both length and weight when evaluating silique characteristics.

Conclusion

In conclusion, our research led to the successful creation of genetically modified *A. thaliana* plants expressing the *PmC3H32* gene from *P. massoniana*. These transgenic plants exhibited enhanced growth characteristics, including increased plant height, higher silique count, and more branches, particularly under drought stress conditions. However, the overexpression of *PmC3H32* also led to early leaf senescence and reduced silique weight, likely due to accelerated flowering, maturation, and senescence processes.

The increased drought tolerance observed in the transgenic *A. thaliana* plants can be attributed to the overexpression of *PmC3H32*, despite the accelerated senescence process. The upregulation of genes such as *IPT3, HBI1, ARR5, and ARR7*, which are involved in flowering regulation through photoperiod and hormone signaling pathways, was also noted. Concurrently, the downregulation of *EXPA1*, a gene crucial for cell expansion and growth, may contribute to the observed early senescence under drought stress conditions.

Interestingly, both the transgenic and wild-type plants demonstrated a similar rate of decrease in soil moisture content, suggesting that the enhanced stress tolerance in the transgenic lines is not primarily due to differential soil moisture absorption. The strong correlations observed between various physiological parameters provide valuable insights for potential improvements in the physiological resilience of transgenic plants under drought stress.

These findings contribute to our understanding of the role of CCCH-type zinc finger proteins in plant growth and stress responses, offering a valuable genetic resource for enhancing flowering control and drought resistance in transgenic plants.

Future research will expand on these findings by exploring the molecular mechanisms of drought stress response at the proteome level in *P. massoniana*. Further assays, including those involving SAG12 and SAG13, which are known to be induced during developmentally related senescence, will be conducted to confirm the role of the senescence program in the early leaf senescence observed in *PmC3H32*-overexpressing plants under drought stress. Additionally, further investigation is needed to verify the expression levels of EXPA1 during early plant development stages and under drought stress conditions in Arabidopsis thaliana. This comprehensive approach will further elucidate the responses of *PmC3H32*-overexpressing plants to water deficiencies.

Author Contributions

Conceptualization, RHA and DW; methodology, RHA and DW; software, RHA; validation, JZ; formal analysis, MZ; investigation, RHA and SY; resources, DW; writing-original draft preparation, RHA; writing-review and editing, RHA, SMM., DW and KJ All authors have read and agreed to the published version of the manuscript.

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None.

Conflicts of Interest

No conflict of interest.

References

1. Yan A, Wu M, Zhao Y, Zhang A, Liu B, et al. (2014) Involvement of C2H2 Zinc Finger Proteins in the Regulation of Epidermal Cell Fate Determination in Arabidopsis. J Integr Plant Biol 56(12): 1112-1117.
2. Guo YH, Yu YP, Wang D, Wu CA, Yang GD, et al. (2009) GhZFP1, a Novel CCCH-Type Zinc Finger Protein from Cotton, Enhances Salt Stress Tolerance and Fungal Disease Resistance in Transgenic Tobacco by Interacting with GZIRD21A and GZIPR5. New Phytol 183(1): 62-75.
3. Matthews JM, Sunde M (2002) Zinc Fingers -Folds for Many Occasions. IUBMB Life 54: 351-355.
4. Blackshear PJ (2002) MRNA Degradation: An Important Process in Controlling Gene Expression. Tritetraprolin and Other CCCH Tandem Zinc-Finger Proteins in the Regulation of MRNA Turnove. Biochem Soc Trans 30: 945-952.
5. Pi B, He X, Ruan Y, Jang JC, Huang Y (2018) Genome-Wide Analysis and Stress-Responsive Expression of CCCH Zinc Finger Family Genes in Brassica Rapa. BMC Plant Biol 18(1): 373.
6. Zhuang Y, Wang C, Zhang Y, Chen S, Wang D, et al. (2020) Overexpression of PdC3H17 Confers Tolerance to Drought Stress Depending on Its CCCH Domain in Populus. Front. Plant Sci 10: 1-10.
7. Bogamuwa SP, Jang JC (2014) Tandem CCCH Zinc Finger Proteins in Plant Growth, Development and Stress Response. Plant Cell Physiol 55(8): 1367-1375.
8. Pomeranz MC, Hah C, Lin PC, Kang SG, Finer JJ (2010) The Arabidopsis Tandem Zinc Finger Protein AtTZF1 Traffics between the Nucleus and Cytoplasmic Foci and Binds Both DNA and RNA. Plant Physiol 152(1): 151-165.
9. Han G, Qiao Z, Li Y, Wang C, Wang B (2021)The Roles of Ccch Zinc-Finger Proteins in Plant Abiotic Stress Tolerance. Int J Mol Sci 22(15): 8327.
10. D'Orso F, De Leonardis AM, Salvi S, Gadaleta A, Ruberti I, et al. (2015) Conservation of AtTZF1, AtTZF2, and AtTZF3 Homolog Gene Regulation by Salt Stress in Evolutionarily Distant Plant Species. Front Plant Sci 6: 394.
11. Seok HY, Nguyen LV, Park HY, Tarte VN, Ha J, et al. (2018) Arabidopsis Non-TZF Gene AtC3H17 Functions as a Positive Regulator in Salt Stress Response. Biochem. Biophys. Res Commun 498: 954-959.
12. Wang W, Wei X, Jiao G, Chen W, Wu Y, et al. (2020) GBSS-BINDING PROTEIN, Encoding a CBM48 Domain-Containing Protein, Affects Rice Quality and Yield. J Integr Plant Biol 62: 948-966.
13. Kong Z, Li M, Yang W, Xu W, Xue Y (2006) A Novel Nuclear-Localized CCCH-Type Zinc Finger Protein, OsDOS, Is Involved in Delaying Leaf Senescence in Rice. Plant Physiol 141: 1376-1388.
14. Sun J, Jiang H, Xu Y, Li H, Wu X, et al. (2007) The CCCH-Type Zinc Finger Proteins AtSZF1 and AtSZF2 Regulate Salt Stress Responses in Arabidopsis. Plant Cell Physiol 48: 1148-1158.
15. Han G, Wang M, Yuan F, Sui N, Song J, et al. (2014) The CCCH Zinc Finger Protein Gene AtZFP1 Improves Salt Resistance in Arabidopsis Thaliana. Plant Mol Biol 86: 237-253.
16. Yan Z, Jia J, Yan X, Shi H, Han Y (2017) Arabidopsis KHZ1 and KHZ2, Two Novel Non-Tandem CCCH Zinc-Finger and K-Homolog Domain Proteins, Have Redundant Roles in the Regulation of Flowering and Senescence. Plant Mol Biol 95: 549-565.
17. Wang B, Fang R, Chen F, Han J, Liu YG, et al. (2020) A Novel CCCH-Type Zinc Finger Protein SAW1 Activates OsGA20ox3 to Regulate Gibberellin Homeostasis and Anther Development in Rice. J Integr Plant Biol 62(10): 1594-1606.
18. Seong SY, Shim JS, Bang SW, Kim JK (2020) Overexpression of Osc3h10, a Ccch-Zinc Finger, Improves Drought Tolerance in Rice by Regulating Stress-Related Genes. Plants 9: 1298.
19. Huang P, J HW, Min JH, Zhang X, Chung JS, et al. (2012) Molecular and Physiological Characterization of the Arabidopsis Thaliana Oxidation-Related Zinc Finger 2, a Plasma Membrane Protein Involved in ABA and Salt Stress Response through the ABI2-Mediated Signaling Pathway. Plant Cell Physiol 53(1): 193-203.
20. Frederick ED, Ramos SBV, Blackshear PJ (2008) A Unique C-Terminal Repeat Domain Maintains the Cytosolic Localization of the Placenta-Specific Tristetraprolin Family Member ZFP36L3. J Biol Chem 283(21): 14792-14800.
21. Matsushita K, Takeuchi O, Standley DM, Kumagai Y, Kawagoe T, et al. (2009) Zc3h12a Is an RNase Essential for Controlling Immune Responses by Regulating MRNA Decay. Nature 458: 1185-1190.
22. Lee SJ, Jung HJ, Kang H, Kim SY (2012) Arabidopsis Zinc Finger Proteins AtC3H49/AtTZF3 and AtC3H20/AtTZF2 Are Involved in ABA and JA Responses. Plant Cell Physiol 53: 673-686.
23. Bogamuwa S, Jang JC (2013) The Arabidopsis Tandem CCCH Zinc Finger Proteins AtTZF4, 5 and 6 Are Involved in Light-, Abscisic Acid- and Gibberellic Acid-Mediated Regulation of Seed Germination. Plant Cell Environ 36(8): 1507-1519.
24. Chai G, Hu R, Zhang D, Qi G, Zuo R, et al. (2012) Comprehensive Analysis of CCCH Zinc Finger Family in Poplar Populus Trichocarpa. BMC Genomics 13: 253.
25. Chao Y, Zhang T, Yang Q, Kang J, Sun Y, et al. (2014) Expression of the Alfalfa CCCH-Type Zinc Finger Protein Gene MsZFN Delays Flowering Time in Transgenic Arabidopsis Thaliana. Plant Sci 215-216: 92-99.

26. Seok HY, Woo DH, Park HY, Lee SY, Tran HT, et al. (2016) AtC3H17, a Non-Tandem CCCH Zinc Finger Protein, Functions as a Nuclear Transcriptional Activator and Has Pleiotropic Effects on Vegetative Development, Flowering and Seed Development in Arabidopsis. Plant Cell Physiol 57(3): 603-615.

27. Gao H, Zheng XM, Fei G, Chen J, Jin M, et al. (2013) Ehd4 Encodes a Novel and Oryza-Genus-Specific Regulator of Photoperiodic Flowering in Rice. PLoS Genet 9(2): e1003281.

28. Yang HW, Akagi T, Kawakatsu T, Tao R (2019) Gene Networks Orchestrated by MeGI: A Single-Factor Mechanism Underlying Sex Determination in Persimmon. Plant J 98(1): 97-111.

29. Aamir M, Karmakar P, Singh VK, Kashyap SP, Pandey S, et al. (2021) Novel Insight into Transcriptional and Epigenetic Regulation Underlying Sex Expression and Flower Development in Melon (Cucumis Melo L.). Physiol Plant 173(4): 1729-1764.

30. Zhang X, Wei X, Wang M, Zhu X, Zhao Y, et al. (2019) Overexpression of NtabDOG1L Promotes Plant Growth and Enhances Drought Tolerance in Nicotiana Tabacum. Plant Sci 287: 110186.

31. Selvaraj MG, Jan A, Ishizaki T, Valencia M, Dedicova B, et al. (2020) Expression of the CCCH-Tandem Zinc Finger Protein Gene OsTZF5 under a Stress-Inducible Promoter Mitigates the Effect of Drought Stress on Rice Grain Yield under Field Conditions. Plant Biotechnol J 18(8): 1711-1721.

32. Wang W, Zheng H, Wang Y, Han G, Sui N (2018) Overexpression of CCCH Zinc Finger Protein Gene Delays Flowering Time and Enhances Salt Tolerance in Arabidopsis by Increasing Fatty Acid Unsaturation. Acta Physiol Plant 40: 1-13.

33. Lin PC, Pomeranz MC, Jikumaru Y, Kang SG, Hah C, Fujioka S, et al. (2011) The Arabidopsis Tandem Zinc Finger Protein AtTZF1 Affects ABA- and GA-Mediated Growth, Stress and Gene Expression Responses. Plant J 65(2): 253-268.

34. Wang D, Yao S, Agassin RH, Zhang M, Lou X, et al. (2022) Transcriptome-Wide Identification of CCCH-Type Zinc Finger Proteins Family in Pinus Massoniana and RR-TZF Proteins in Stress Response. Genes (Basel) 13(9): 1639.

35. FastPure Universal Plant Total RNA Isolation Kit Instruction for Use.

36. Zhang X, Henriques R, Lin SS, Niu QW, Chua NH (2006) Agrobacterium-Mediated Transformation of Arabidopsis Thaliana Using the Floral Dip Method. Nat Protoc 1(2): 641-646.

37. Ee SF, Khairunnisa MB, Zeti-Azura MH, Noor Azmi S, Zamri Z (2014) Effective Hygromycin Concentration for Selection of Agrobacterium-Mediated Transgenic Arabidopsis Thaliana. Malaysian Appl Biol 43: 119-123.

38. Plant F Isolation, DNA FastPure Plant DNA Isolation Mini Kit. Vol. 3-5.

39. Liang X, Luo G, Li W, Yao A, Liu W, et al. (2022) Overexpression of a Malus Baccata CBF Transcription Factor Gene, MbCBF1, Increases Cold and Salinity Tolerance in Arabidopsis Thaliana. Plant Physiol Biochem 192: 230-242.

40. Hoang XLT, Nguyen NC, Nguyen YNH, Watanabe Y, Tran LSP, et al. (2019) The Soybean GmNAC019 Transcription Factor Mediates Drought Tolerance in Arabidopsis in an Abscisic Acid-Dependent Manner. Int J Mol Sci 21(1):286.

41. Liu H, Xiao S, Sui S, Huang R, Wang X, et al. (2022) Tandem CCCH Type Zinc Finger Protein Gene CpC3H3 from Chimonanthus Praecox Promotes Flowering and Enhances Drought Tolerance in Arabidopsis. BMC Plant Biol 22(1): 506.

42. Suprasanna P (2020) Plant Abiotic Stress Tolerance: Insights into Resilience Build-Up. J Biosci 45: 120.

43. Esmaeili N, Shen G, Zhang H (2022) Genetic Manipulation for Abiotic Stress Resistance Traits in Crops. Front Plant Sci 13: 1011985.

44. Cai C, Wang W, Ye S, Zhang Z, Ding W, et al. (2020) Overexpression of a Novel Arabidopsis Gene SUPA Leads to Various Morphological and Abiotic Stress Tolerance Alternations in Arabidopsis and Poplar. Front Plant Sci 11: 560985.

45. Zhang Q, Zhang J, Wei F, Fu X, Wei H, et al. (2023) The CCCH-Type Zinc-Finger Protein GhC3H20 Enhances Salt Stress Tolerance in Arabidopsis Thaliana and Cotton through ABA Signal Transduction Pathway. Int J Mol Sci 24(5): 5057.

46. Murray JAH, Jones A, Godin C, Traas J (2012) Systems Analysis of Shoot Apical Meristem Growth and Development: Integrating Hormonal and Mechanical Signaling. Plant Cell 24: 3907-3919.

47. Li Z, Liang F, Zhang T, Fu N, Pei X, et al. (2021) Enhanced Tolerance to Drought Stress Resulting from Caragana Korshinskii CkWRKY33 in Transgenic Arabidopsis Thaliana. BMC Genomic Data 22(1): 11.

48. Gupta S, Misra S, Kumar M, Mishra SK, Tiwari S, et al. (2022) Enhancement of Drought Tolerance in Transgenic Arabidopsis Thaliana Plants Overexpressing Chickpea Ca14-3-3 Gene. J Plant Growth Regul 42: 1544-1557.

49. Guo Y, Ren G, Zhang K, Li Z, Miao Y, et al. (2021) Leaf Senescence: Progression, Regulation, and Application. Mol Hortic 1: 5.

50. Zhou Y, Zhang X, Chen J, Guo X, Wang H, et al. (2022) Overexpression of AHL9 Accelerates Leaf Senescence in Arabidopsis Thaliana. BMC Plant Biol 22(1): 248.

51. Jiang AL, Xu ZS, Zhao GY, Cui XY, Chen M, et al. (2014) Genome-Wide Analysis of the C3H Zinc Finger Transcription Factor Family and Drought Responses of Members in Aegilops Tauschii. Plant Mol Biol Report 32: 1241-1256.

52. Neilson B, Rossiter N (2005) Scholar (16) From Precarity to Precariousness Back Again Labour, Life Unstable Networks pp. 5.

53. Yao J, Sun D, Cen H, Xu H, Weng H, et al. (2018) Phenotyping of Arabidopsis Drought Stress Response Using Kinetic Chlorophyll Fluorescence and Multicolor Fluorescence Imaging. Front Plant Sci 9: 603.

54. Ren S, Lyle C, Jiang GL, Penumala A (2016) Soybean Salt Tolerance 1 (GmST1) Reduces ROS Production, Enhances ABA Sensitivity, and Abiotic Stress Tolerance in Arabidopsis Thaliana. Front Plant Sci 7:445.

55. Li CL, Wang M, Ma XY, Zhang W(2014) NRGA1, a Putative Mitochondrial Pyruvate Carrier, Mediates ABA Regulation of Guard Cell Ion Channels and Drought Stress Responses in Arabidopsis. Mol Plant 7(10): 1508-1521.

56. Guo R, Qiao H, Zhao J, Wang X, Tu M, et al. (2018) The Grape VLWRKY3 Gene Promotes Abiotic and Biotic Stress Tolerance in Transgenic Arabidopsis Thaliana. Front Plant Sci 9: 545.

57. Ma Q, Xia Z, Cai Z, Li L, Cheng Y, Liu Jet al. (2019) GmWRKY16 Enhances Drought and Salt Tolerance through an ABA-Mediated Pathway in Arabidopsis Thaliana. Front Plant Sci 9: 1979.

58. Ahmad S, Belwal V (2020) Study of Correlation and Path Analysis for Yield and Yield Attributing Traits in Mungbean. Int J Chem Stud 8: 2140-2143.

59. Sinyangwe C, Graham K, Nicholas S, King R, Mukupa S, et al. (2016) Assessing the Quality of Care for Pneumonia in Integrated Community Case Management: A Cross-Sectional Mixed Methods Study. PLoS One 11: e0152204.

60. Granier C, Aguirrezabal L, Chenu K, Cookson SJ, Dauzat M, et al. (2006) PHENOPSIS, an Automated Platform for Reproducible Phenotyping of Plant Responses to Soil Water Deficit in Arabidopsis Thaliana Permitted the Identification of an Accession with Low Sensitivity to Soil Water Deficit. New Phytol 169(3): 623-635.

61. Wang L, Czedik-Eysenberg A, Mertz RA, Si Y, Tohge T, et al. (2014) Comparative Analyses of C4 and C3 Photosynthesis in Developing Leaves of Maize and Rice. Nat Biotechnol 32(11): 1158-1165.

62. Awlia M, Nigro A, Fajkus J, Schmoeckel SM, Negrão S, et al. (2016) High-Throughput Non-Destructive Phenotyping of Traits That Contribute to Salinity Tolerance in Arabidopsis Thaliana. Front Plant Sci 7: 1414.