Two lncRNAs of Chinese cabbage confer Arabidopsis with heat and drought tolerance

Yujie Wang[1], Shuqi Sun[1], Xuehuan Feng[2], Nan Li[1*] and Xiaoming Song[1*]

[1] *College of Life Sciences/Center for Genomics and Bio-computing, North China University of Science and Technology, Tangshan 063210, China*
[2] *Food Science and Technology Department, University of Nebraska-Lincoln, Lincoln, NE 68588, USA*
* Corresponding authors, E-mail: Limanxi1989@163.com; songxm@ncst.edu.cn

Abstract

Currently, frequent occurrences of abnormal weather events, including drought and extreme temperatures, are impacting plant growth and crop yields globally. Numerous lncRNAs in plants are known to be associated with stress responses, yet only a select few have been validated experimentally. Our prior research identified a set of lncRNAs in Chinese cabbage that were closely related to heat stress response through RNA-seq and bioinformatics analysis. In this study, two lncRNAs (*lnc000283* and *lnc012465*) were selected for genetic transformation in Arabidopsis to elucidate their functions. Arabidopsis plants over-expressing (OE) *lnc000283* and *lnc012465* accumulated higher levels of anthocyanins in the later growth stage when cultivated in a culture dish. When subjected to heat stress, both OE *lnc000283* and OE *lnc012465* plants exhibited enhanced thermotolerance, with *lnc012465* showing particularly pronounced effects. Moreover, the transgenic Arabidopsis plants demonstrated greater resistance to drought stress compared to the wild type. Under heat or drought treatments, the transgenic plants exhibited significantly higher fresh-to-dry weight ratios, POD activities, green leaf percentages, and survival rates compared to the wild type. Additionally, the transcript levels of *Hsp18.1-CI* were substantially elevated in the transgenic plants, both before and after heat treatment, and the expression levels of several drought-responsive genes were significantly higher than those in the wild type following drought stress. Altogether, the present results provide new ideas for the breeding of new multi-stress-tolerant varieties.

Introduction

Plants are continuously subjected to unpredictable environmental conditions and encounter a multitude of stressors throughout their growth and development, posing a significant challenge to global crop production and food security[1]. Heat and drought are undoubtedly the two most important stresses that have a huge impact on crops. Both elicit a wide array of biochemical, molecular, and physiological alterations and responses, impacting diverse cellular processes and ultimately influencing crop yield and quality[2].

A primary physiological consequence of both stresses is the diminished photosynthetic capacity, partially resulting from the degradation of chlorophyll due to leaf senescence under stress conditions. Chlorophyll accumulation was diminished in numerous plants subjected to drought or heat stress conditions[3,4]. Various environmental stresses prompt excessive generation of reactive oxygen species (ROS), initiating oxidative damage that compromises lipids, and proteins, and poses a serious threat to cellular functions[2]. To mitigate oxidative stress and minimize damage, plants have developed various protective mechanisms to neutralize ROS. Several antioxidant enzymes, such as SOD, POD, and CAT, are integral to cellular antioxidative defense mechanisms. Additionally, antioxidants such as anthocyanins and proline serve as crucial ROS scavengers[5,6]. The elevation in temperature typically induces the transient synthesis of heat shock proteins (Hsps), which function as molecular chaperones in protecting proteins from denaturation and

aggregation, with their activity primarily regulated at the transcriptional level by heat shock factors (Hsfs)[7]. The significance of Hsps and Hsfs in all organisms, including plants, has been assessed in various stress conditions that could disrupt cellular homeostasis and result in protein dysfunction[7]. Drought stress can also trigger the transcription of a suite of marker genes, including *RD29A, RD29B, NCED3, AREB1, Rab18,* etc., which assist plants in mitigating cellular damage during dehydration and bolstering their resilience to stress[8–10].

Previous research efforts focusing on the regulatory control of stress-related genes have largely centered around protein-coding genes. In recent years, non-protein-coding transcripts have emerged as important regulatory factors in gene expression. Among them, long non-coding RNAs (lncRNAs) lncRNAs have been identified as implicated in various abiotic stresses[11,12]. LncRNAs are a class of non-coding RNAs (ncRNAs) exceeding 200 nucleotides in length. They possess minimal or no protein-coding potential[13]. In plants, lncRNAs are specifically transcribed by RNA polymerases Pol IV, Pol V, Pol II, and Pol III[14,15]. LncRNAs exhibit low abundance and display strong tissue and cellular expression specificity relative to mRNAs. Moreover, sequence conservation of lncRNAs is was very poor across different plant species[13,16,17]. The widespread adoption of high-throughput RNA sequencing technology has revealed lncRNAs as potential regulators of plant development and environmental responses. In cucumber, RNA-seq analysis has predicted 2,085 lncRNAs to be heat-responsive, with some potentially acting as competitive endogenous RNAs (ceRNAs) to

execute their functions[18]. In radish, a strand-specific RNA-seq (ssRNA-seq) technique identified 169 lncRNAs that were differentially expressed following heat treatment[19]. In Arabidopsis, as*HSFB2a*, the natural antisense transcript of *HSFB2a* was massively induced upon heat stress and exhibited a counteracted expression trend relative to *HSFB2a*. Overexpression of as*HSFB2a* entirely suppressed the expression of *HSFB2a* and impacted the plant's response to heat stress[20]. For drought stress resistance, 244 lncRNAs were predicted in tomatoes to be drought responsive probably by interacting with miRNAs and mRNAs[21]. Under drought stress and rehydration, 477 and 706 lncRNAs were differentially expressed in drought-tolerant *Brassica napus* Q2 compared to drought-sensitive *B. napus*, respectively[22]. In foxtail millet and maize, 19 and 644 lncRNAs, respectively, were identified as drought-responsive[23,24]. Despite the identification of numerous lncRNAs by high-throughput sequencing, which suggests their potential involvement in various abiotic stress processes, only a minority have been experimentally validated for function.

In our previous study, we characterized 1,229 differentially expressed (DE) lncRNAs in Chinese cabbage as heat-responsive, and subsequent bioinformatics analysis reduced this number to 81, which are more likely associated with heat resistance[25]. *lnc000283* and *lnc012465* were selected from among them for further functional investigation. The findings indicated that both *lnc000283* and *lnc012465* could be promptly induced by heat shock (HS). Overexpression of either *lnc000283* or *lnc012465* in Arabidopsis plants enhanced their capacity to tolerate heat stress. Additionally, both *lnc000283* and *lnc012465* conferred drought tolerance to transgenic Arabidopsis.

Materials and methods

Plant materials, growth conditions, and stress treatment

The lncRNA sequences examined in this study were from Chiifu-401-42 Chinese cabbage and all Arabidopsis plants were of the Col-0 background. Transgenic plants expressing *lnc000283* and *lnc012465* were generated using the *Agrobacterium tumefaciens*-mediated floral dip method[26]. Single-copy and homozygous T3 plants were identified through genetic segregation on an agar medium supplemented with kanamycin. The T3 generation plants, or their homozygous progeny, were utilized in the experiments.

For phenotypic assessment, Arabidopsis seeds were initially sown on filter paper moistened with ddH$_2$O and placed in a 4 °C freezer for 2 d. Subsequently, they were evenly planted in nutrient-rich soil and transferred to a growth chamber operating a 16-h day/8-h night cycle, with day/night temperatures of 22 °C/18 °C and a light intensity of 250 μmol·m^{-2}·s^{-1}. After 10 d of growth, Arabidopsis plants with uniform growth were transferred to 50-hole plates. Arabidopsis plants grown in Petri dishes were firstly seed-sterilized and then sown on 1/2 MS medium supplemented with 10 g·L^{-1} sucrose. The seeds were then placed in a 4 °C refrigerator for 2 d in the dark before transferring them to a light incubator. The day/night duration was set to 16 h/8 h, the day/night temperature to 21 °C/18 °C, and the light intensity to 100 μmol·m^{-2}·s^{-1}.

For heat treatment, 3-week-old seedlings were subjected to 38 °C for 4 d within a light incubator, subsequently transferred to their original growth conditions under the same light/dark cycles. For drought treatment, 3-week-old Arabidopsis seedlings were deprived of water for 10 d, followed by rehydration to facilitate a 2-d recovery period. Plants were photographed and surveyed both before and after treatment.

Vector construction

The lncRNA sequences (*lnc000283* and *lnc012465*) were chemically synthesized based on RNA-seq data, with restriction sites for *BamH1* and *Kpn1* engineered upstream and downstream. The resultant lncRNA constructs were subcloned into the pCambia2301 binary vector, incorporating a cauliflower mosaic virus (CaMV) 35S promoter. The recombinant vectors were transformed into *Escherichia coli* TOP10 competent cells (Clontech), incubated at 37 °C overnight, after which single clones were selected for PCR verification, and the confirmed positive colonies were submitted for sequencing. Following verification, the correct plasmids were introduced into *A. tumefaciens* strain GV3101 using the freeze-thaw method and subsequently transformed into Arabidopsis wild-type (Col) plants.

Chlorophyll and anthocyanin content determination

To quantify the chlorophyll content, the aerial portions of wild-type and transgenic Arabidopsis plants, grown in Petri dishes were weighed, minced, and then subjected to boiling in 95% ethanol until fully decolorized. Aliquots of 200 μL from the extract were transferred to a 96-well plate and the absorbance at 663 nm and 645 nm was measured *via* spectrophotometry by a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). Three biological replicates were analyzed for WT and each transgenic line. Chlorophyll content was determined according to the formula of the Arnon method[27]: Chlorophyll a = (12.72A$_{663}$ − 2.59A$_{645}$) v/w, Chlorophyll b = (22.88A$_{645}$ − 4.67A$_{663}$) v/w, Total chlorophyll = (20.29A$_{645}$ + 8.05A$_{663}$) v/w.

The quantification of anthocyanin was performed as follows: aerial parts of wild-type and transgenic Arabidopsis plants, cultivated in Petri dishes, were weighed and ground to powder in liquid nitrogen. Subsequently, the samples were incubated in 600 μL of acidified methanol (containing 1% HCl) at 70 °C for 1 h. Following this, 1 mL of chloroform was added, and the mixture was vigorously shaken to remove chlorophyll. The mixture was then centrifuged at 12,000 rpm for 5 min, after which the absorbance of the aqueous phase was determined at 535 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). Three biological replicates were analyzed for WT and each transgenic line. The relative anthocyanin content was calculated according to anthocyanin concentration and extraction solution volume. One anthocyanin unit is defined as an absorption unit at a wavelength of 535 nm in 1 mL of extract solution. In the end, the quantity was normalized to the fresh weight of each sample.

Physiological analysis

Three-week-old transgenic and WT *A. thaliana* plants, subjected to normal conditions or varying durations of heat or drought stress, were utilized for subsequent physiological assessments. All assays were performed in accordance with the method described by Chen & Zhang[28]. In brief, 0.1 g of fresh leaf tissue was homogenized in 500 μL of 100 mM PBS (pH 7.8) while chilled on ice. The homogenate was then centrifuged at 4 °C, and the resultant supernatant was employed for further analysis. For the determination of MDA content, 100 μL of the

supernatant was combined with 500 μL of a 0.25% thiobarbituric acid (TBA) solution (which was prepared by dissolving 0.125 g of TBA in 5 mL of 1 mol·L⁻¹ NaOH before being added to 45 mL of 10% TCA) and boiled for 15 min. Following a 5 min cooling period on ice, the absorbance was measured at 532 nm and 600 nm. The activity of POD was determined as follows: initially, 28 μL of 0.2% guaiacol and 19 μL of 30% H_2O_2 were sequentially added to 50 mL of 10mM PBS (pH 7.0), after thorough heating and mixing, 1 mL was transferred into a cuvette, then 50 μL of the supernatant was added to the cuvette and the absorbance at 470 nm was monitored every 15 s for 1 min. To determine the proline content, a reaction solution was prepared by mixing 3% sulfosalicylic acid, acetic acid, and 2.5% acidic ninhydrin in a ratio of 1:1:2, then 50 μL of the supernatant was added to 1 mL of the reaction solution, which was then subjected to a boiling water bath for 15 min (the solution turned red after the boiling water bath). Following cooling on ice, the absorbance at 520 nm was recorded. For the quantification of proline, an L-proline standard curve was prepared by dissolving 0, 5, 10, 15, 20, 25, and 30 μg of L-proline in 0.5 mL of ddH_2O, followed by the addition of 1 mL of the reaction solution and measuring the absorbance at 520 nm. The proline content in the samples was then determined based on the L-proline standard curve.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from the aerial parts of Arabidopsis using the TaKaRa MiniBEST Plant RNA Extraction Kit, followed by purification and reverse transcription using the PrimeScript RT reagent Kit with gDNA Eraser (Takara). The cDNA product was diluted 10 times and real-time PCR was conducted in triplicate for each biological replicate using SYBR PCR Master Mix (Applied Biosystems) on the ABI 7500 system under the following conditions: 98 °C for 3 min, followed by 40 cycles of 98 °C for 2 s and 60 °C for 30 s. The relative expression levels of each gene were normalized against the transcript abundance of the endogenous control UBC30 (At5g56150) and calculated using the $2^{-\Delta C_T}$ method. The specific primers employed for qRT-PCR are detailed in Supplemental Table S1.

Results

Screening and characterization of lncRNAs

In our prior investigation, dozens of lncRNAs associated with the heat stress response in Chinese cabbage were identified through informatics analysis. Two lncRNAs (lnc000283 and lnc012465) were chosen for genetic transformation in Arabidopsis to elucidate their functions comprehensively. Transcriptome data analysis indicated that the expression of lnc000283 and lnc012465 in Chinese cabbage were both induced by HS. To verify the accuracy, the expression patterns of lnc000283 and lnc012465 were confirmed through quantitative real-time PCR (qRT-PCR), and the results from qRT-PCR were consistent with those obtained from RNA-seq (Fig. 1a). The corresponding homologous genes in Arabidopsis were identified as CNT2088434 and CNT2088742, exhibiting sequence similarities of 88% and 87%, respectively (Supplemental Fig. S1). Subcellular localization predictions using the Inclocator database (www.csbio.sjtu.edu.cn/bioinf/lncLocator) suggested that both lncRNAs are localized within the nucleus (Supplemental Table S2). Bioinformatics analysis was conducted using the CPC tool (http://cpc.cbi.pku.edu.cn/) indicated that lnc000283 and lnc012465 are

noncoding sequences, with coding probabilities of 0.0466805 and 0.0432148, respectively comparable to the well-characterized lncRNAs COLDAIR and Xist, but significantly lower than those of the protein-coding genes UBC10 and ACT2 (Fig. 1b).

Generation of lncRNA overexpression lines

To elucidate the role of lnc000283 and lnc012465 in response to abiotic stress, overexpression vectors were constructed for these lncRNAs, driven by the CaMV 35S promoter, and they were introduced into Arabidopsis thaliana (Col-0 ecotype). Through PCR identification and generational antibiotic screening, two homozygous positive lines for lnc012465 and lnc000283 were obtained. The relative expression levels of these lncRNAs were assessed using qRT-PCR (Fig. 2a). When plants were grown in 1/2 MS medium, with the consumption of nutrients, and reduction of water, the leaves of WT began to turn yellow, but the lnc000283 and lnc012465 overexpression lines developed a deep purple color of leaf veins (Fig. 2b).
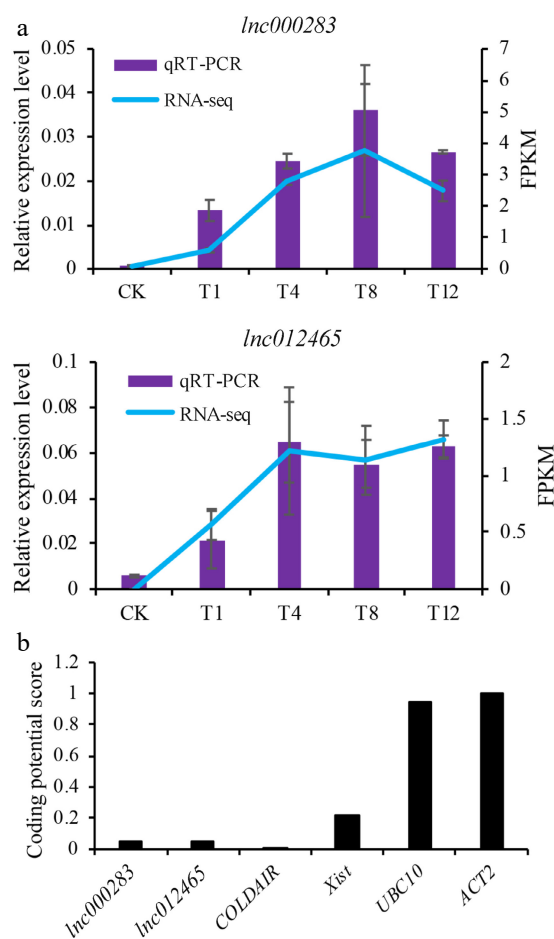

Fig. 1 Characteristics of lnc000283 and lnc012465. (a) Expression level of lnc000283 and lnc012465 in Chinese cabbage leaves treated at 38 °C at different time points, as determined by qRT-PCR and RNA-seq. CK is a representative plant before heating, and T1, T4, T8, and T12 denote plants that were subjected to 38 °C for 1, 4, 8, and 12 h, respectively. The expression levels were normalized to the expression level of Actin. (b) Analysis of coding potential for lnc000283 and lnc012465. The coding potential scores were calculated using the CPC program. UBC10 (At5g53300) and ACT2 (At3g18780) are positive controls that encode proteins. COLDAIR (HG975388) and Xist (L04961) serve as negative controls, exhibiting minimal protein-coding potential.

Wang et al. Vegetable Research 2024, 4: e029

Page 3 of 11

Examination of chlorophyll and anthocyanin contents in the plants revealed that both overexpression lines had higher levels of chlorophyll and anthocyanin compared to the WT, suggesting that the transgenic plants might possess enhanced resistance to nutritional or water stress (Fig. 2c, d).

Effect of lncRNA overexpression on heat tolerance in Arabidopsis

Given that *lnc000283* and *lnc012465* were highly induced by heat, the thermotolerance of the overexpressing (OE) plants

were compared to that of the wild type. Arabidopsis plants were initially exposed to a an HS treatment at 38 °C for 4 d, followed by recovery at room temperature. The death caused by HS was processive. Post-severe HS challenge for 4 d, OE plants initially appeared similar to WT, but upon recovery, their leaves started to fold or curl, followed by a transition to yellow, white, and eventually drying out (Fig. 3a). OE *lnc000283* and OE *lnc012465* plants exhibited enhanced thermotolerance compared to WT, with *lnc012465* showing particularly strong
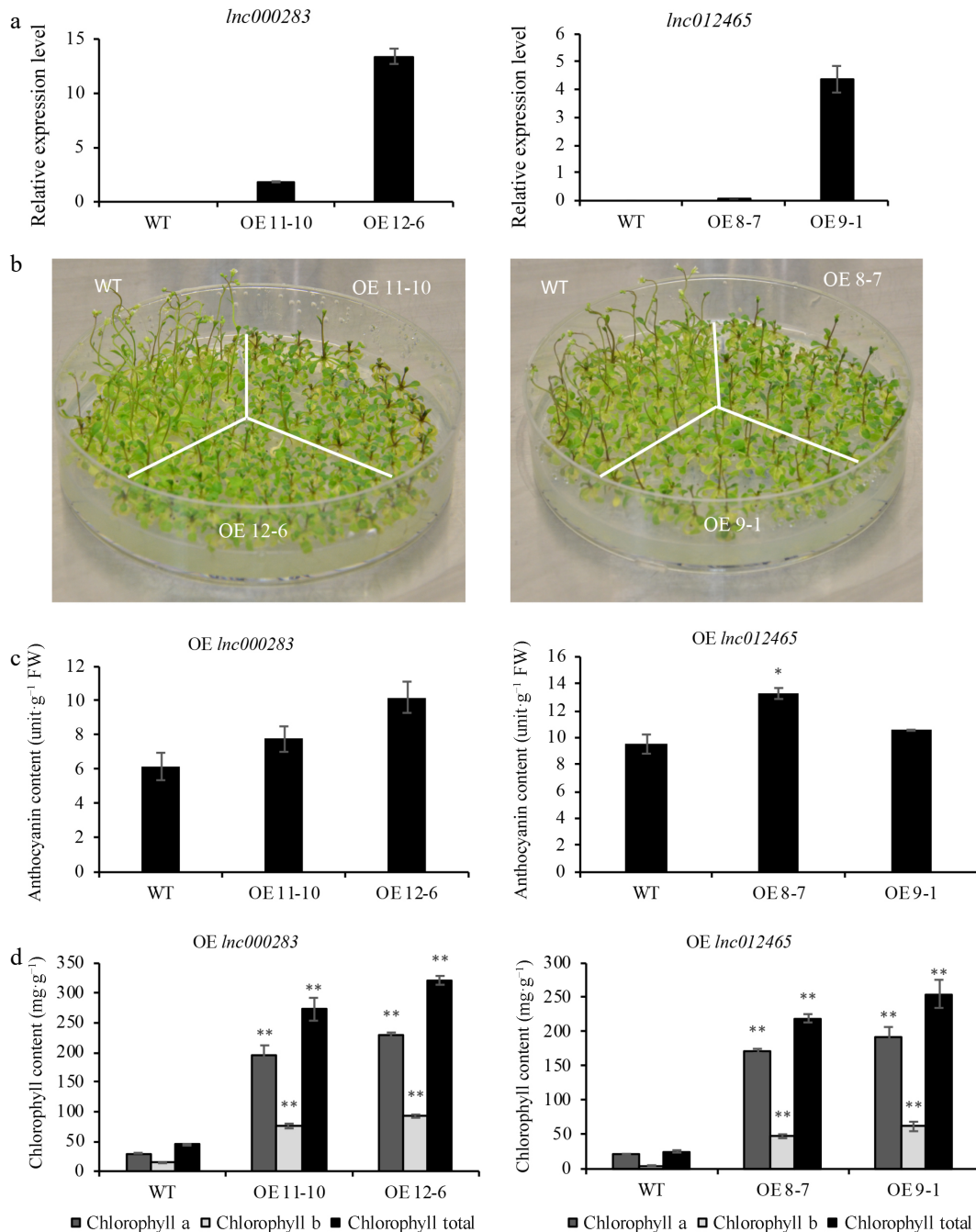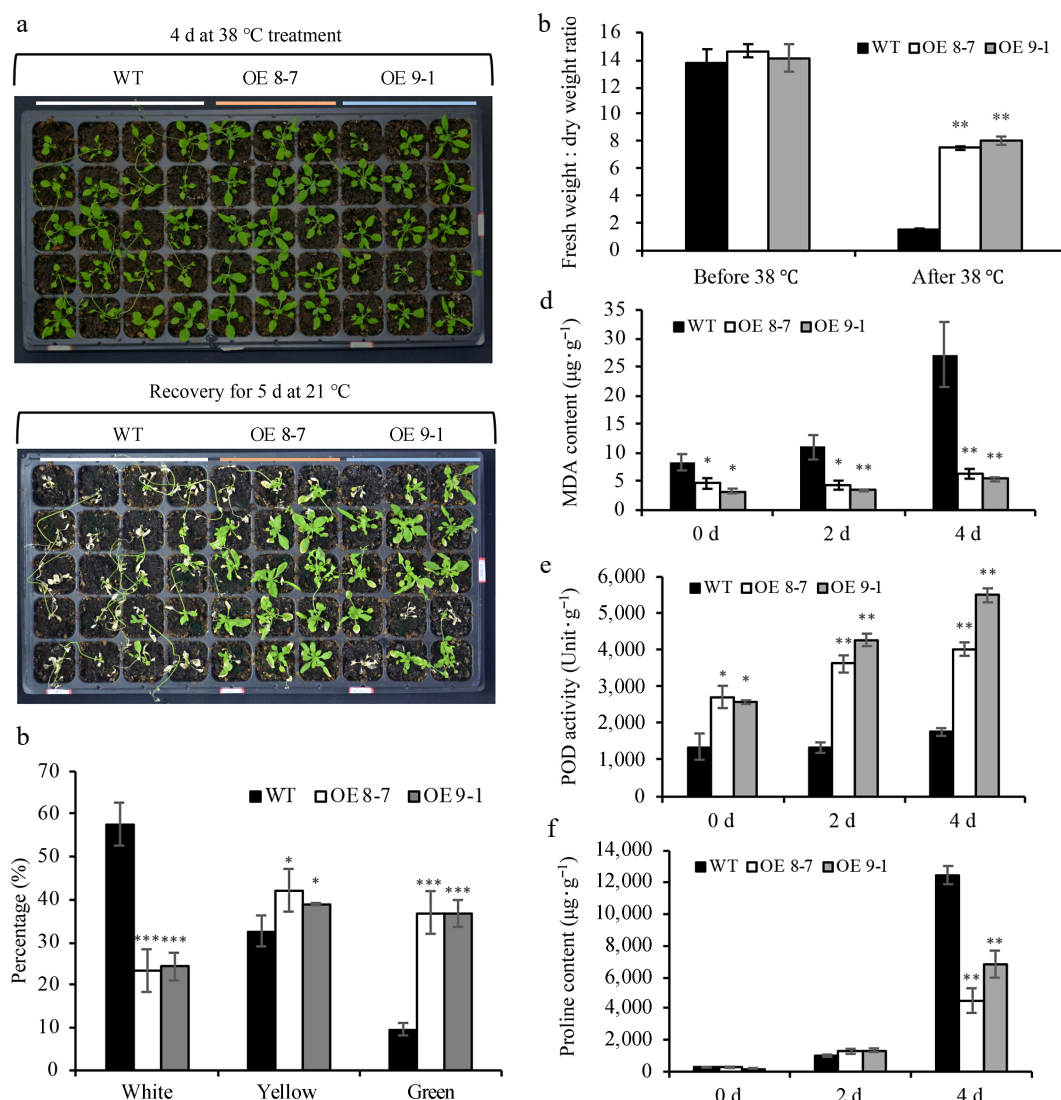

Fig. 2   Arabidopsis plants overexpressing *lnc000283* and *lnc012465* had higher anthocyanins and chlorophyll content. (a) The relative expression level of *lnc000283* and *lnc012465* in WT and different transgenic lines. *UBC10* (*At5g53300*) was used as an internal control. Each value is mean ± sd (n = 3). (b) The phenotype of WT and Arabidopsis overexpressing *lnc000283* or *lnc012465* grown on 1/2 MS medium 50 d after sowing. The (c) anthocyanin and (d) chlorophyll content of WT and transgenic Arabidopsis overexpressing *lnc000283* or *lnc012465*. The asterisks above the bars indicate statistical significance using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

tolerance (Fig. 3a; Supplemental Fig. S2a). After 5 d of recovery, leaf coloration indicated that transgenic plants maintained a significantly higher percentage of green leaves and a lower percentage of bleached leaves compared to WT (Fig. 3b; Supplemental Fig. S2b). Under non-heat-stress conditions, WT and OE plants possessed comparable water content. However, following heat stress, the fresh-to-dry weight ratio of OE *lnc000283* and *lnc012465* lines was significantly greater than that of WT (Fig. 3c; Supplemental Fig. S2c). Abiotic stresses frequently trigger the production of excessive reactive oxygen species (ROS), which are believed to cause lipid peroxidation of membrane lipids, leading to damage to macromolecules. Leaf MDA content is commonly used as an indicator of lipid peroxidation under stress conditions; therefore, the MDA content in both transgenic and WT plants was assessed. Figure 3d shows that the MDA content in WT plants progressively increased after heat treatment, whereas in the two lines

overexpressing *lnc012465*, the MDA content increased only slightly and remained significantly lower than that in WT at all time points. In plants overexpressing *lnc000283*, the MDA content did not significantly differ from that of WT before heat stress. However, after 4 d of heat treatment, the MDA content was significantly lower compared to WT (Supplemental Fig. S2d). The results suggested that the expression of both *lnc012465* and *lnc000283* can mitigate injury caused by membrane lipid peroxidation under heat-stress conditions. Peroxidase (POD) is a crucial antioxidant enzyme involved in ROS scavenging. Figure 3e and Supplemental Fig. S2e demonstrate that POD activity increased in both transgenic and WT plants after heat treatment. However, the increase in WT plants was modest, whereas OE *lnc000283* and OE *lnc012465* plants exhibited consistently higher POD activity. As anticipated, proline levels were induced in response to stress in all studied plants (Fig. 3f; Supplemental Fig. S2f). However, under normal

Fig. 3 Overexpressing *lnc012465* lines are more tolerant to heat stress. (a) Phenotypes of WT and OE *lnc012465* plants were assessed before and after exposure to heat stress. The heat treatment was applied to 25-day-old Arabidopsis plants. (b) The percentage of leaves with different colors in Arabidopsis after heat treatment and recovery for 5 d. (c) The fresh-to-dry weight ratio of Arabidopsis leaves was measured before and after 38 °C heat treatment. (d)–(f) depict the MDA content, POD activity, and proline content in Arabidopsis leaves at varying durations of heat stress. The asterisks above the bars indicate statistical significance using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

conditions and 2 d post-heat stress treatment, the proline content in OE *lnc000283* and OE *lnc012465* plants did not exhibit significant changes compared to WT (Fig. 3f; Supplemental Fig. S2f). Moreover, after 4 d of heat stress, the proline content in OE *lnc012465* lines was significantly lower than in WT, and the OE *lnc000283* transgenic line 12-6 also showed a marked decrease in proline content compared to WT (Fig. 3f; Supplemental Fig. S2f). The results indicated that the thermotolerance of plants overexpressing either *lnc000283* or *lnc012465* was independent of proline accumulation.

Effect of lncRNA overexpression on the expression quantity of genes related to high-temperature response in Arabidopsis

To elucidate the molecular mechanisms by which lncRNAs enhance thermotolerance in Arabidopsis, the expression of the *Hsf* gene *HsfA7a* and three *Hsps* (*Hsp25.3*, *Hsa32*, and *Hsp18.1-CI*) in OE *lnc000283*, OE *lnc012465*, and WT Arabidopsis plants were investigated at various time points following heat treatment. As shown in Fig. 4 and Supplemental Fig. S3, both *Hsf* and *Hsps* exhibited a rapid response to heat stress with strong induction. Notably, the transcripts of *HsfA7a* and *Hsp25.3* were significantly upregulated at 1 h after heat exposure, then experienced a sharp decrease. *Hsa32* and *Hsp18.1-CI* were highly induced at 1 h and, unlike the other proteins, sustained high expression levels at 3 h (Fig. 4; Supplemental Fig. S3). At 1 h post-heat treatment, the transcript levels of *Hsa32* and *HsfA7a* in OE *lnc000283* did not significantly differ from those in WT. However, by 3 h, *Hsa32* expression was roughly 50% of the WT level, while *HsfA7a* expression was approximately double that of WT (Supplemental Fig. S3). The overexpression of *lnc000283* did not significantly affect the transcript level of *Hsp25.3* at any of the tested time points. Notably, *Hsp18.1-CI* expression in both lines overexpressing *lnc000283* was significantly induced at all three detection points post-heat treatment, reaching approximately 4-9-fold higher levels than in the WT (Supplemental Fig. S3). In Arabidopsis plants with elevated expression of *lnc012465*, the expression patterns of all *Hsp* and *Hsf* genes were similar to those in plants overexpressing *lnc000283*, with the notable exception of *Hsa32*. Unlike the WT, *Hsa32* did not show a trend of down-regulation at 3 h post-heat treatment (Fig. 4). The findings suggest that the substantial induction of *Hsp18.1-CI* may play a role in enhancing the thermotolerance of Arabidopsis plants overexpressing *lnc000283* and *lnc012465*.

Effect of lncRNA overexpression on drought tolerance in *Arabidopsis*

Prior research has implicated a significant proportion of genes in conferring resistance to various abiotic stresses. To elucidate the functions of *lnc000283* and *lnc012465* more thoroughly, WT and transgenic plants were subjected to drought stress by depriving them of water for 9 d. It was noted that the majority of leaves in WT plants withered and dried, whereas the OE *lnc000283* and OE *lnc012465* plants exhibited reduced withering, with only a minority displaying dryness (Fig. 5a; Supplemental Fig. S4a). Eight days post-rewatering, a negligible fraction of WT seedlings exhibited recovery, whereas the overwhelming majority of transgenic plants regained vigorous growth (Fig. 5a; Supplemental Fig. S4a). The transgenic plants demonstrated a significantly higher survival rate compared to the WT plants. Following 9 d of water deficit treatment, less than 40% of the WT plants survived, whereas the OE 012465 lines 8-7 and 9-1 exhibited survival rates of 100% and 95%, respectively, and the OE 000283 lines 11-10 and 12-6 had survival rates of 87% each. (Fig. 5b; Supplemental Fig. S4b). Water loss serves as a critical metric for assessing plant drought tolerance, hence the fresh-to-dry weight ratio of excised leaves was assessed *via* desiccation analysis. Following 4 d of drought treatment, the fresh-to-dry weight ratio for WT plants was reduced to 43%, whereas for OE *lnc000283* lines 11-10 and 12-6, it was reduced to 73% and 75%, respectively. For OE 012465 lines 8-7 and 9-1, the ratios were reduced to 67% and 62%, respectively (Fig. 5c; Supplemental Fig. S4c). The findings indicated that *lnc000283* and *lnc012465* endow the transgenic plants with drought tolerance.
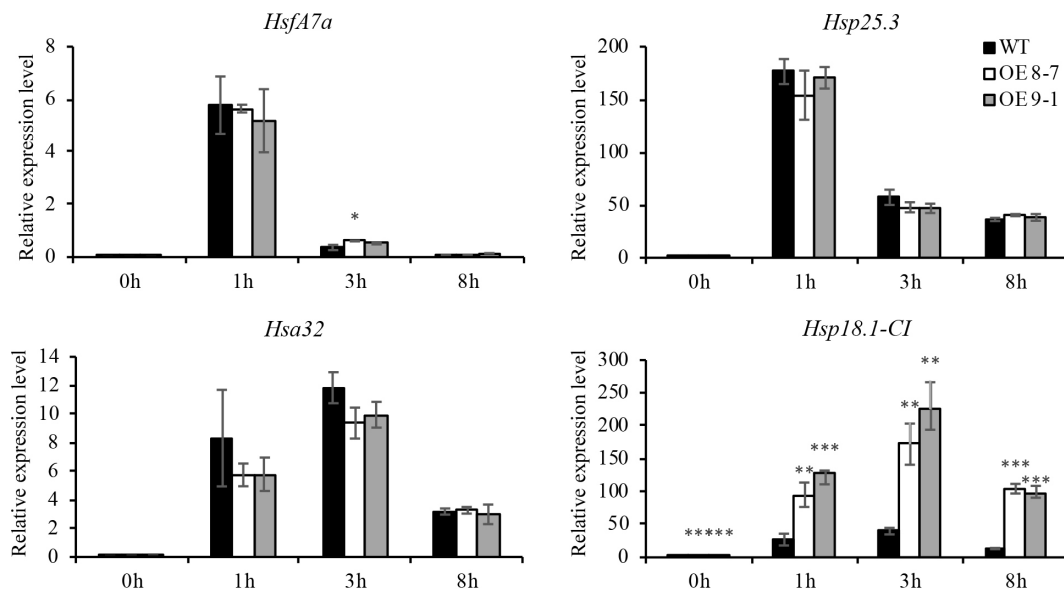
Fig. 4 The expression of *HSF* and *HSP* genes in *lnc012465* overexpressing lines before and after different heat treatment times. Gene expression levels were quantified using RT-qPCR and normalized to *UBC10* (*At5g53300*). Each value represents the mean ± standard deviation (n = 3). The asterisks above the bars indicate statistical significance using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).
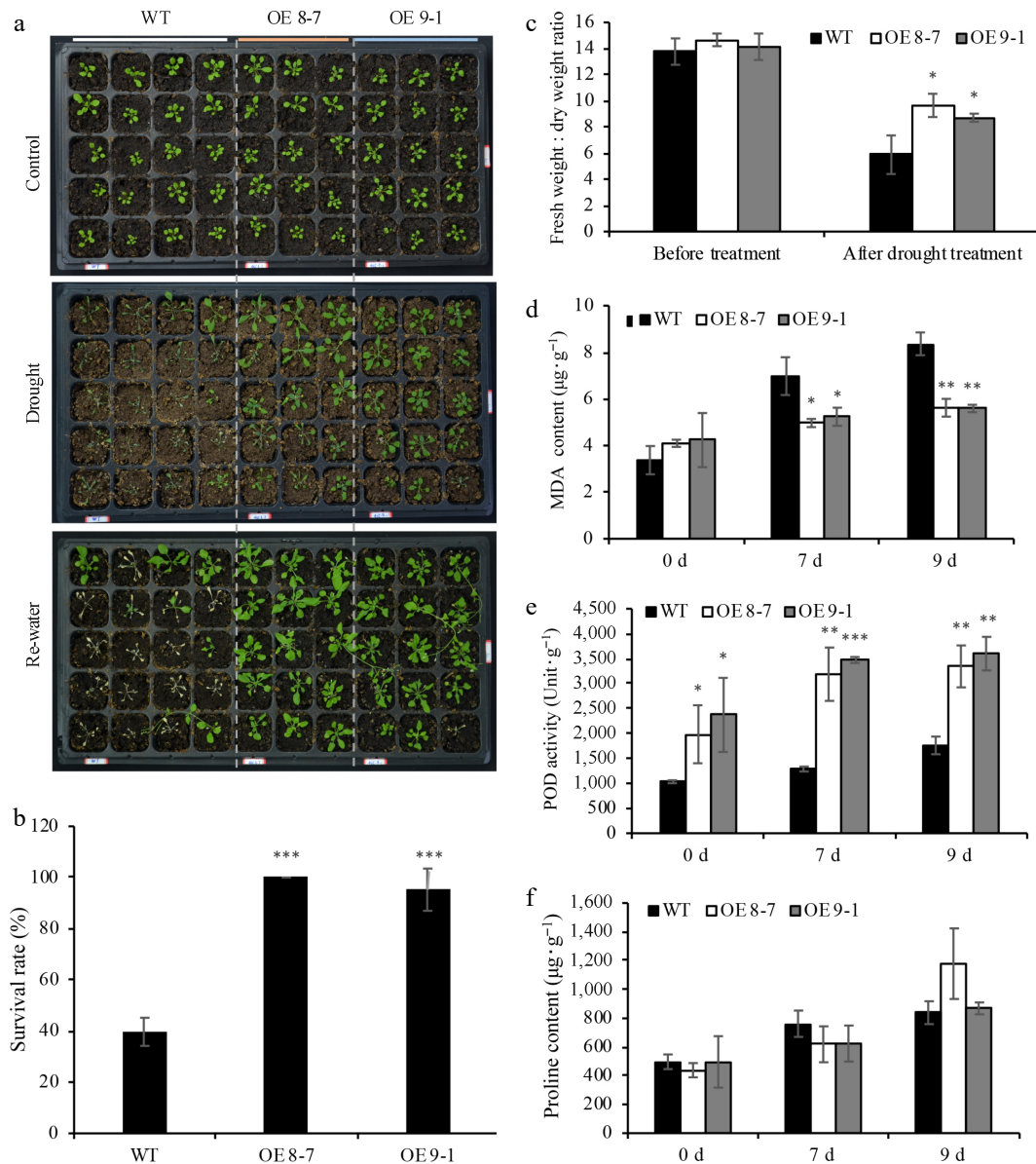
Fig. 5 Overexpressing *lnc012465* lines are more tolerant to drought stress. (a) Phenotype of WT and OE *lnc012465* plants before and after subjecting to drought stress. Drought treatment was carried out on 20-day-old Arabidopsis plants. (b) The percentage of leaves with different colors in Arabidopsis after heat treatment and recovery for 5 d. (c) The fresh weight to dry weight ratio of Arabidopsis leaves before and after undergoing 38 °C heat treatment. (d)−(f) MDA content, POD activity, and proline content in Arabidopsis leaves under different times of heat stress. The asterisks above the bars indicate statistical significance using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

MDA content in leaves is a standard biomarker for assessing the extent of drought stress-induced damage. Prior to drought stress exposure, MDA levels in WT and transgenic plants were comparable. However, following 7 and 9 d of water deficit, the MDA content in the transgenic plants was markedly reduced compared to the WT, suggesting a less severe degree of membrane lipid peroxidation in the transgenic plants (Fig. 5d; Supplemental Fig. S4d). Oxidative stress frequently coincides with drought stress, hence the activity of POD was assessed to evaluate the ROS scavenging ability. The findings indicated that as the duration of drought treatment increased, POD activity progressively rose. Before drought exposure, the POD activity in lines 11-10 and 12-6 of OE 000283 was 2.4-fold and 2.2-fold higher than that of the WT, respectively (refer to Supplemental

Fig. S4e). Following drought treatment, the POD activity in the transgenic lines remained significantly elevated compared to the wild type, although the enhancement was less pronounced than before the treatment (Supplemental Fig. S4e). In the OE 012465 plants, the POD activity in lines 8-7 and 9-1 significantly surpassed that of the wild type, with the discrepancy being more pronounced during drought stress (Fig. 5e). The proline content in WT and OE 000283 plants exhibited no significant differences before and after 7 d of treatment. However, after 9 d of drought, the proline content in OE 000283 plants was significantly lower compared to that in the WT (Supplemental Fig. S4f). OE 000465 plants showed no significant difference from the wild type before and after drought treatment (Fig. 5f). The findings were consistent with those

under heat stress, indicating that the enhanced stress resistance due to the overexpression of *lnc000283* and *lnc012465* in Arabidopsis is not reliant on proline accumulation.

Following drought stress treatment, the expression levels of drought-related genes such as *RD29A*, *RD29B*, *NCED3*, *AREB1*, and *Rab18* were significantly elevated in plants overexpressing *lnc000283* and *lnc012465* compared to WT plants. These findings suggest that *lnc000283* and *lnc012465* modulate Arabidopsis drought tolerance by regulating the expression of genes associated with the drought stress response (Fig. 6; Supplemental Fig. S5).

Discussion

The integrity of global food security is under threat due to the confluence of rapid population expansion and profound climatic shifts[29]. Amidst the shifting climatic landscape, heat and drought stress have emerged as primary limitations to crop yield and global food security. Understanding how plants detect stress cues and acclimate to challenging conditions is a pivotal biological inquiry. Moreover, enhancing plant resilience to stress is essential for maintaining agricultural productivity and fostering environmental sustainability[2]. Concurrently, the advancement of next-generation sequencing (NGS) technology has led to the identification of a substantial number of lncRNAs that participate in diverse stress responses, with functional analyses having been conducted on several of these molecules.[30] For instance, in the case of potatoes, the lncRNA *StFLORE* has been identified to modulate water loss through its

interaction with the homologous gene *StCDF1*[31]. LncRNA *TCONS_00021861* can activate the IAA biosynthetic pathway, thereby endowing rice with resistance to drought stress[32]. In wheat, the expression of *TalnRNA27* and *TalnRNA5* was upregulated in response to heat stress[33]. Our prior investigation identified a total of 81 lncRNAs in Chinese cabbage that engage in intricate interactions with their respective mRNA targets across various phases of heat treatment[25]. Two lncRNAs, *lnc000283* and *lnc012465*, were chosen for subsequent functional analysis. Findings confirmed that these lncRNAs endow transgenic Arabidopsis plants with enhanced tolerance to both heat and drought, thereby offering novel resources for enhancing stress resistance through genetic engineering.

Abiotic stresses frequently trigger the synthesis of anthocyanins, serving as natural antioxidants that mitigate oxidative damage by neutralizing surplus reactive oxygen species (ROS), thereby protecting plants from growth inhibition and cell death, allowing plants to adapt to abiotic stresses[34,35]. For instance, during chilling stress, the accumulation of anthocyanins within leaves can mitigate oxidative damage, thereby enhancing the photosynthetic rate[36]. Consequently, the level of abiotic stress tolerance can be inferred from the concentration of anthocyanins. The reduction of photosynthetic ability is one of the key physiological phenomena of stresses, which is partly due to the degradation of chlorophyll caused by leaf senescence during stress. The reduced accumulation of chlorophyll in the plants was seen in many plants when exposed to drought or heat stress conditions. The current investigation revealed that lncRNA-overexpressing plants cultivated in Petri
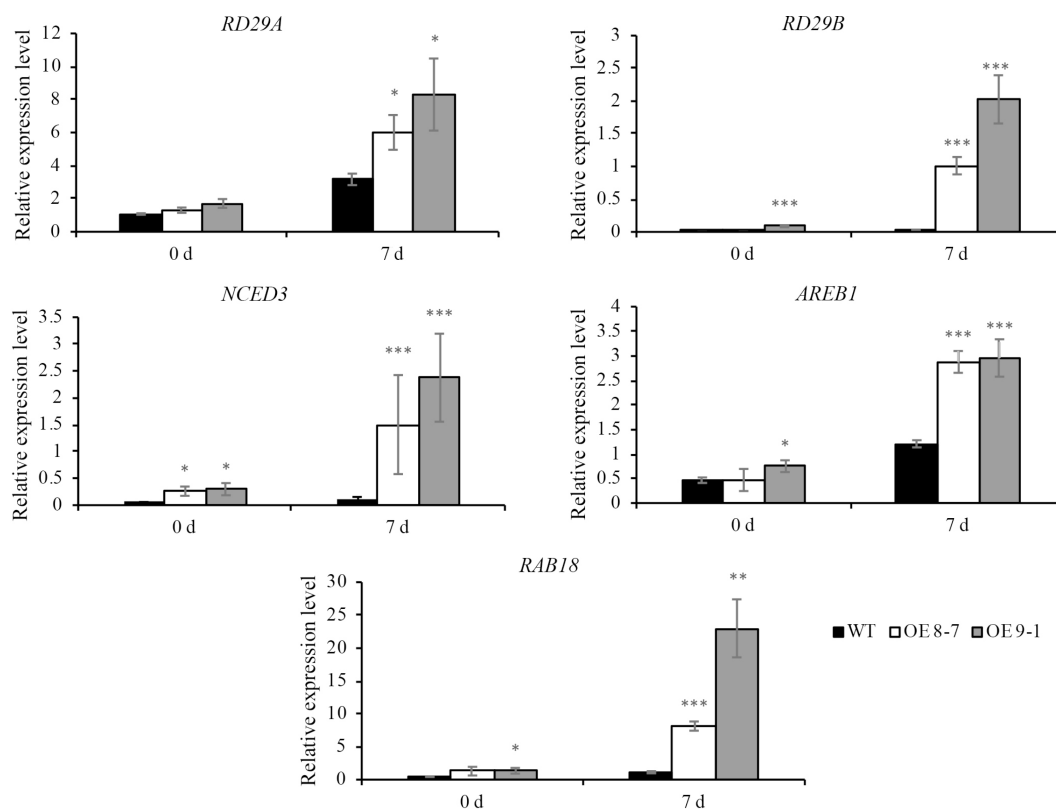
Fig. 6 The expression of drought-responsive genes in *lnc012465* overexpressing lines before and after different drought treatment time. Gene expression levels were determined by qRT-PCR normalized against *UBC10* (*At5g53300*). Each value is mean ± sd (n = 3). The asterisks above the bars indicate statistical significance using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

dishes exhibited increased accumulation of both chlorophyll and anthocyanins in advanced growth phases, indicating that these transgenic plants, overexpressing *lnc000283* and *lnc012465*, demonstrated enhanced stress tolerance and superior growth performance relative to WT (Fig. 2c, d).

Upon exposure to heat stress, there is a marked induction of transcription for numerous genes that encode molecular chaperones in plants, with the vast majority of these genes contributing to the prevention of protein denaturation-related damage and the augmentation of thermotolerance[37–39]. The present investigation identified multiple heat-inducible genes in plants overexpressing *lnc000283* and *lnc012465*, as well as in WT (Fig. 4; Supplemental Fig. S3). The findings indicated that of the four HSP or HSF genes examined, *Hsp18.1-CI* exhibited a significantly greater abundance in both OE *lnc000283* and OE *lnc012465* plants compared to the WT following heat treatment for several days. *Hsp18.1-CI,* formerly referred to as *Hsp18.2* has been the subject of investigation since 1989.[40] Following the fusion of the 5' region of *Hsp18.2* in frame with the *uidA* gene of *Escherichia coli*, the activity of *GUS*, serving as the driver gene was observed to increase upon exposure to HS[40]. The Arabidopsis *hsfA2* mutant exhibited diminished thermotolerance after heat acclimation, with the transcript levels of *Hsp18.1-CI* being substantially reduced compared to those in wild-type plants following a 4-h recovery period[41]. The findings revealed that the upregulation of *Hsp18.1-CI* protein is a critical mechanism by which plants achieve enhanced protection against heat stress in adverse environmental conditions, thereby bolstering their thermotolerance.

Plants cultivated in natural settings are often subjected to concurrent multiple abiotic stresses, which can exacerbate threats to their routine physiological functions, growth, and developmental processes[42,43]. Elucidating the molecular mechanisms underlying plant responses to abiotic stress is crucial for the development of new crop varieties with enhanced tolerance to multiple abiotic stresses. Previous research has indicated that the overexpression of certain protein-coding genes can endow plants with resistance to a variety of abiotic stresses. For instance, tomatoes with robust expression of *ShCML44* demonstrated significantly enhanced tolerance to drought, cold, and salinity stresses[44]. Overexpression of *PeCBF4a* in poplar plants confers enhanced tolerance to a range of abiotic stresses[45]. With respect to lncRNAs, transgenic Arabidopsis plants that overexpress lncRNA-*DRIR* displayed marked increased tolerance to salt and drought stresses compared to the wild-type[46]. In the present study, both overexpression lines of *lnc000283* and *lnc012465* exhibited resistance to heat and drought stresses, thereby contributing to the enhancement of plant resilience against multiple stresses (Figs 3, 5; Supplemental Figs S2, S4).

The number of genes implicated in plant drought resistance is regulated by both ABA-dependent and ABA-independent pathways[47,48]. It is well established that the expression of *RD29A* exhibits a high level of responsiveness to drought stress, operating through both ABA-dependent and ABA-independent mechanisms[49]. *RD29B*, *AREB1*, and *RAB18* are governed by an ABA-dependent regulatory pathway[10,49,50]. *NCED3* is involved in ABA biosynthesis[51]. In the present study, the transcript levels of *RD29A*, *RD29B*, *NCED3*, *AREB1*, and *RAB18* were significantly elevated in OE *lnc000283* and OE *lnc012465* plants compared to those in the WT plants (Fig. 6; Supplemental Fig. S5). The findings indicated that the drought tolerance imparted by

OE *lnc000283* and OE *lnc012465* plants is contingent upon an ABA-dependent mechanism.

Prior research has indicated that certain long non-coding RNAs (lncRNAs) can assume analogous roles across diverse biological contexts. For example, the lncRNA *bra-miR156HG* has been shown to modulate leaf morphology and flowering time in both *B. campestris* and Arabidopsis[52]. Heterogeneous expression of *MSL*-lncRNAs in Arabidopsis has been associated with the promotion of maleness, and similarly, it is implicated in the sexual lability observed in female poplars[53]. In the present study, *lnc000283* and *lnc012465* were induced by heat in Chinese cabbage, and their heterologous expression was found to confer heat tolerance in Arabidopsis. Additionally, sequences homologous to *lnc000283* and *lnc012465* were identified in Arabidopsis (Supplemental Fig. S1). The data suggest that these sequences may share a comparable function to that of heat-inducible sequences, potentially accounting for the conservation of *lnc000283* and *lnc012465*'os functionality across various species.

Conclusions

In conclusion, the functions of two heat-inducible lncRNAs, *lnc000283* and *lnc012465* have been elucidated. Transgenic Arabidopsis lines overexpressing these lncRNAs accumulated higher levels of anthocyanins and chlorophyll at a later stage of growth compared to the WT when grown on Petri dishes. Furthermore, under heat and drought stress conditions, these OE plants exhibited enhanced stress tolerance, with several genes related to the stress resistance pathway being significantly upregulated. Collectively, these findings offer novel insights for the development of new varieties with tolerance to multiple stresses.

Author contributions

The authors confirm contribution to the paper as follows: study conception and supervision: Li N, Song X; experiment performing: Wang Y, Sun S; manuscript preparation and revision: Wang Y, Feng X, Li N. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (https://www.maxapress.com/article/doi/10.48130/vegres-0024-0029)

Dates

References

1. Zhu JK. 2016. Abiotic stress signaling and responses in plants. *Cell* 167:313−24

2. Fahad S, Bajwa AA, Nazir U, Anjum SA, Farooq A, et al. 2017. Crop production under drought and heat stress: plant responses and management options. *Frontiers in Plant Science* 8:1147

3. Dutta S, Mohanty S, Tripathy BC. 2009. Role of temperature stress on chloroplast biogenesis and protein import in pea. *Plant Physiology* 150:1050−61

4. Abid M, Ali S, Qi LK, Zahoor R, Tian Z, et al. 2018. Physiological and biochemical changes during drought and recovery periods at tillering and jointing stages in wheat (*Triticum aestivum* L.). *Scientific Reports* 8:4615

5. Gill SS, Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48:909−30

6. Das K, Roychoudhury A. 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science* 2:53

7. Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M. 2013. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International Journal of Molecular Sciences* 14:9643−84

8. Ding Y, Fromm M, Avramova Z. 2012. Multiple exposures to drought 'train' transcriptional responses in *Arabidopsis*. *Nature Communications* 3:740

9. Kuromori T, Sugimoto E, Shinozaki K. 2011. Arabidopsis mutants of *AtABCG22*, an ABC transporter gene, increase water transpiration and drought susceptibility. *The Plant Journal* 67:885−94

10. Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, et al. 2005. AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *The Plant Cell* 17:3470−88

11. Jha UC, Nayyar H, Jha R, Khurshid M, Zhou M, et al. 2020. Long non-coding RNAs: emerging players regulating plant abiotic stress response and adaptation. *BMC Plant Biology* 20:466

12. Zhao Z, Zang S, Zou W, Pan YB, Yao W, et al. 2022. Long non-coding RNAs: new players in plants. *International Journal of Molecular Sciences* 23(16):9301

13. Mercer TR, Dinger ME, Mattick JS. 2009. Long non-coding RNAs: insights into functions. *Nature Reviews Genetics* 10:155−59

14. Wierzbicki AT, Haag JR, Pikaard CS. 2008. Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. *Cell* 135:635−48

15. Zhang YC, Chen YQ. 2013. Long noncoding RNAs: new regulators in plant development. *Biochemical and Biophysical Research Communications* 436:111−14

16. Wang X, Ai G, Zhang C, Cui L, Wang J, et al. 2016. Expression and diversification analysis reveals transposable elements play important roles in the origin of Lycopersicon-specific lncRNAs in tomato. *New Phytologist* 209:1442−55

17. Liu J, Jung C, Xu J, Wang H, Deng S, et al. 2012. Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in *Arabidopsis*. *The Plant Cell* 24:4333−45

18. He X, Guo S, Wang Y, Wang L, Shu S, et al. 2020. Systematic identification and analysis of heat-stress-responsive lncRNAs, circRNAs and miRNAs with associated co-expression and ceRNA networks in cucumber (*Cucumis sativus* L.). *Physiologia Plantarum* 168:736−54

19. Yang Z, Li W, Su X, Ge P, Zhou Y, et al. 2019. Early response of radish to heat stress by strand-specific transcriptome and miRNA analysis. *International Journal of Molecular Sciences* 20(13):3321

20. Wunderlich M, Groß-Hardt, Schöffl F. 2014. Heat shock factor HSFB2a involved in gametophyte development of *Arabidopsis thaliana* and its expression is controlled by a heat-inducible long non-coding antisense RNA. *Plant Molecular Biology* 85:541−50

21. Eom SH, Lee HJ, Lee JH, Wi SH, Kim SK, et al. 2019. Identification and functional prediction of drought-responsive long non-coding RNA in tomato. *Agronomy* 9:629

22. Tan X, Li S, Hu L, Zhang C. 2020. Genome-wide analysis of long non-coding RNAs (lncRNAs) in two contrasting rapeseed (*Brassica napus* L.) genotypes subjected to drought stress and re-watering. *BMC Plant Biology* 20(1):81

23. Qi X, Xie S, Liu Y, Yi F, Yu J. 2013. Genome-wide annotation of genes and noncoding RNAs of foxtail millet in response to simulated drought stress by deep sequencing. *Plant Molecular Biology* 83:459−73

24. Zhang W, Han Z, Guo Q, Liu Y, Zheng Y, et al. 2014. Identification of maize long non-coding RNAs responsive to drought stress. *PLoS One* 9(6):e98958

25. Song X, Hu J, Wu T, Yang Q, Feng X, et al. 2021. Comparative analysis of long noncoding RNAs in angiosperms and characterization of long noncoding RNAs in response to heat stress in Chinese cabbage. *Horticulture Research* 8:48

26. Zhang X, Henriques R, Lin SS, Niu QW, Chua NH. 2006. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols* 1:641−46

27. Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* 24:1−15

28. Chen T, Zhang B. 2023. Correction notice: measurements of proline and malondialdehyde contents and antioxidant enzyme activities in leaves of drought stressed cotton. *Bio-protocol* 5(13):e4752

29. Lesk C, Rowhani P, Ramankutty N. 2016. Influence of extreme weather disasters on global crop production. *Nature* 529:84−87

30. Liu D, Mewalal R, Hu R, Tuskan GA, Yang X. 2017. New technologies accelerate the exploration of non-coding RNAs in horticultural plants. *Horticulture Research* 4:17031

31. Ramírez Gonzales L, Shi L, Bergonzi SB, Oortwijn M, Franco-Zorrilla JM, et al. 2021. Potato CYCLING DOF FACTOR 1 and its lncRNA counterpart *StFLORE* link tuber development and drought response. *The Plant Journal* 105:855−69

32. Chen J, Zhong Y, Qi X. 2021. LncRNA TCONS_00021861 is functionally associated with drought tolerance in rice (*Oryza sativa* L.) via competing endogenous RNA regulation. *BMC Plant Biology* 21(1):410

33. Xin M, Wang Y, Yao Y, Song N, Hu Z, et al. 2011. Identification and characterization of wheat long non-protein coding RNAs responsive to powdery mildew infection and heat stress by using microarray analysis and SBS sequencing. *BMC Plant Biology* 11:61

34. Naing AH, Kim CK. 2021. Abiotic stress-induced anthocyanins in plants: their role in tolerance to abiotic stresses. *Physiologia Plantarum* 172:1711−23

35. Zhou B, Zheng B, Wu W. 2024. The ncRNAs involved in the regulation of abiotic stress-induced anthocyanin biosynthesis in plants. *Antioxidants* 13(1):55

36. Meng X, Yin B, Feng HL, Zhang S, Liang XQ, et al. 2014. Overexpression of R2R3-MYB gene leads to accumulation of anthocyanin and enhanced resistance to chilling and oxidative stress. *Biologia Plantarum* 58:121−30

37. Shan Q, Ma F, Wei J, Li H, Ma H, Sun P. 2020. Physiological functions of heat shock proteins. *Current Protein & Peptide Science* 21:751−60

38. Jacob P, Hirt H, Bendahmane A. 2017. The heat-shock protein/chaperone network and multiple stress resistance. *Plant Biotechnology Journal* 15:405−14

39. Haider S, Raza A, Iqbal J, Shaukat M, Mahmood T. 2022. Analyzing the regulatory role of heat shock transcription factors in plant heat stress tolerance: a brief appraisal. *Molecular Biology Reports* 49:5771−85

40. Takahashi T, Komeda Y. 1989. Characterization of two genes encoding small heat-shock proteins in *Arabidopsis thaliana*. *Molecular and General Genetics MGG* 219:365−72

41. Charng YY, Liu HC, Liu NY, Chi WT, Wang CN, et al. 2007. A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. *Plant Physiology* 143:251−62

42. Zhang H, Zhu J, Gong Z, Zhu JK. 2022. Abiotic stress responses in plants. *Nature Reviews Genetics* 23:104−19

43. Zandalinas SI, Mittler R. 2022. Plant responses to multifactorial stress combination. *New Phytologist* 234:1161−67

44. Munir S, Liu H, Xing Y, Hussain S, Ouyang B, et al. 2016. Overexpression of calmodulin-like (*ShCML44*) stress-responsive gene from *Solanum habrochaites* enhances tolerance to multiple abiotic stresses. *Scientific Reports* 6:31772

45. Tian Q, Chen J, Wang D, Wang HL, Liu C, et al. 2017. Overexpression of a *Populus euphratica CBF4* gene in poplar confers tolerance to multiple stresses. *Plant Cell, Tissue and Organ Culture* 128:391−407

46. Qin T, Zhao H, Cui P, Albesher N, Xiong L. 2017. A nucleus-localized long non-coding RNA enhances drought and salt stress tolerance. *Plant Physiology* 175:1321−36

47. Soma F, Takahashi F, Yamaguchi-Shinozaki K, Shinozaki K. 2021. Cellular Phosphorylation signaling and gene expression in drought stress responses: ABA-dependent and ABA-independent regulatory systems. *Plants* 10:756

48. Liu S, Lv Z, Liu Y, Li L, Zhang L. 2018. Network analysis of ABA-dependent and ABA-independent drought responsive genes in *Arabidopsis thaliana*. *Genetics and Molecular Biology* 41:624−37

49. Msanne J, Lin J, Stone JM, Awada T. 2011. Characterization of abiotic stress-responsive *Arabidopsis thaliana RD29A* and *RD29B* genes and evaluation of transgenes. *Planta* 234:97−107

50. Hallouin M, Ghelis T, Brault M, Bardat F, Cornel D, et al. 2002. Plasmalemma abscisic acid perception leads to *RAB18* expression via phospholipase D activation in Arabidopsis suspension cells. *Plant Physiology* 130:265−72

51. Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, et al. 2001. Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxy-carotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *The Plant Journal* 27:325−33

52. Zhou D, Zhao S, Zhou H, Chen J, Huang L. 2023. A lncRNA *bra-miR156HG* regulates flowering time and leaf morphology as a precursor of miR156 in *Brassica campestris* and *Arabidopsis thaliana*. *Plant Science* 337:111889

53. Mao J, Wei S, Chen Y, Yang Y, Yin T. 2023. The proposed role of MSL-lncRNAs in causing sex lability of female poplars. *Horticulture Research* 10(5):uhad042