A Stress-Responsive NAC Transcription Factor from Tiger Lily (LlNAC2) Interacts with LlDREB1 and LlZFHD4 and Enhances Various Abiotic Stress Tolerance in Arabidopsis

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Abstract: Our previous studies have indicated that a partial NAC domain protein gene is strongly up-regulated by cold stress (4 °C) in tiger lily (Lilium lancifolium). In this study, we cloned the full-length of this NAC gene, LlNAC2, to further investigate the function of LlNAC2 in response to various abiotic stresses and the possible involvement in stress tolerance of the tiger lily plant. LlNAC2 was noticeably induced by cold, drought, salt stresses, and abscisic acid (ABA) treatment. Promoter analysis showed that various stress-related cis-acting regulatory elements were located in the promoter of LlNAC2; and the promoter was sufficient to enhance activity of GUS protein under cold, salt stresses and ABA treatment. DREB1 (dehydration-responsive binding protein1) from tiger lily (LlDREB1) was proved to be able to bind to the promoter of LlNAC2 by yeast one-hybrid (Y1H) assay. LlNAC2 was shown to physically interact with LlDREB1 and zinc finger-homeodomain ZFHD4 from the tiger lily (LlZFHD4) by bimolecular fluorescence complementation (BiFC) assay. Overexpressing LlNAC2 in Arabidopsis thaliana showed ABA hypersensitivity and enhanced tolerance to cold, drought, and salt stresses. These findings indicated LlNAC2 is involved in both DREB/CFB-COR and ABA signaling pathways to regulate stress tolerance of the tiger lily.

Keywords: NAC; DREB1; ZFHD4; interaction; abiotic stresses; lily

1. Introduction

Plants have evolved a series of complex mechanisms to deal with environmental stressors, such as drought, high salinity, and extreme temperatures [1]. As crucial regulatory proteins, transcription factors (TFs) can specifically bind to cis-acting regulatory elements in the promoter of target genes to up or down-regulate their transcript levels [2,3]. Multiple types of TFs have shown to play important roles in plant responses to stresses, such as AP2/EREBP [4,5], MYB [6,7], bHLH [8], WRKY [9,10], zinc finger [11,12], and NAC [13,14] families. Thus, the identification and functional characterization of TFs contribute significantly to our understanding of the transcriptional regulatory networks in plants under abiotic stresses, which are indispensable parts in developing transgenic crops with enhanced tolerance to unfavorable growth conditions as well.

As one of the largest plant-specific TF groups, NAC (NAM, ATAF1,2 and CUC2) domain proteins have been reported to be essential regulators for plant response to abiotic stresses [15,16], which contain conserved N-terminal NAC DNA binding domains and variable C-terminal regions involved in transcriptional activation [17,18]. Most of the NAC genes were demonstrated to play positive roles in plant stress resistance [19]. For instance, overexpression of eight NAC genes (e.g., OsNAC9,
OsNAC10 enhanced the drought and salinity tolerance significantly in rice (Oryza sativa) [1]. In wheat (Triticum aestivum), overexpression of TaNAC2a, TaNAC67, and TaNAC47 enhanced multi-abiotic stress tolerances in transgenic plants [20,21]. In Arabidopsis thaliana, overexpression of ANAC019, ANAC055, and ANAC072 genes enhanced plant tolerance to drought [22]; and ATAF1 exhibited positive regulation of drought ABA, salt, and oxidative tolerance in the transgenic plants [23]. Also, overexpressing the TsNAC1 (Thellungiella halophila), pumpkin (Cucurbita moschata) CmNAC1 [24], cotton (Gossypium hirsutum) GhNAC2 [25], rose (Rosa rugosa) RhNAC3 [26] genes showed increased tolerance to diverse abiotic stresses in transgenic plants. In contrast, only a few NAC genes were found to function as negative regulators of tolerance regulation to abiotic stress, such as NAC016 [27] and NTL4 [28] in Arabidopsis, and ShNAC1 (Solanum habrochaites) [19] and SISRNI (Solanum lycopersicum) in tomato [29].

On the other hand, the identification of C-repeat binding factor (CBF) genes has been known as one of the most important milestones in the cold signaling network elucidation [30]. CBFs (CBF1/2/3), also called dehydration-responsive binding protein 1 (DREB1B/1C/1A), are from the APETALA2/ethylene responsive factor (AP2/ERF) family, which can bind to C-repeat/dehydration-responsive elements (CRT/DREs) in the promoters of certain cold-responsive genes (COR) and regulate their expressions and functions [31–33]. Many findings have implied that CBF can function as a master regulator of the cold signaling pathway. Interestingly, some studies reported that some NAC TFs were also involved in the DREB/CBF-COR pathway. For instance, in Pyrus betulifolia, PbeNAC1 induces the expression of some stress-associated genes by interacting with PbeDREB1 and PbeDREB2A [30]. GmNAC20 can bind to the promoter of GmDREB1A and suppress the expression of GmDREB1C to regulate COR genes and mediate cold tolerance in soybean (Glycine max) [33]. MaNAC1 can interact with MaCBF1 to mediate cold tolerance of banana (Musa acuminata) [34].

Lily is one of the most important flower crops cultivated worldwide. Most commercial cultivars of lily are sensitive to abiotic stresses, such as cold, drought, and salt. However, Lilium lancifolium, also called tiger lily, is one of the most widely distributed wild lilies in Asia which has strong abiotic stress resistance [35]. It suggests that tiger lily may have distinctive molecular mechanisms of abiotic stress resistance [36]. In our previous study, the unigene contig16924 coding for a partial NAC domain of lily are sensitive to abiotic stresses, such as cold, drought, and salt. However, Lilium lancifolium, also called tiger lily, is one of the most widely distributed wild lilies in Asia which has strong abiotic stress resistance [35]. It suggests that tiger lily may have distinctive molecular mechanisms of abiotic stress resistance [36]. In our previous study, the unigene contig16924 coding for a partial NAC domain was found to be strongly up-regulated by low temperature in the tiger lily [36,37]. Thus, we characterized this NAC gene, LNAC2 in this report. LNAC2 was induced not only by cold stress but also by drought, salt, and exogenous ABA treatments. We also found the activity of LNAC2 promoter can be induced by cold, salt, and exogenous ABA treatments. In addition, A DREB1 from tiger lily (LIDREB1) was proved to bind to LNAC2 promoter, while LNAC2 can physically interact with LIDREB1 and zinc finger-homeodomain ZFHD4 from tiger lily (LIZFHD4). Furthermore, the transgenic plants of LNAC2 showed good tolerance under cold, drought, and salt treatments, and activated the expression of many stress-responsive genes in Arabidopsis. Together, our results indicate that LNAC2 plays a positive role in plant stress tolerance and can be a candidate gene utilized in transgenic breeding to enhance the abiotic stress tolerance of other crops.

2. Results

2.1. Cloning and Sequence Analysis of LNAC2 Gene

LNAC2 gene contained a complete open reading frame (ORF) of 906 bp, with 74 bp 5’ UTR, 231 bp 3’ UTR. It encodes a polypeptide protein of 302 amino acids with a calculated molecular mass of 81.11 kDa and an isoelectric point of 5.03. Amino acid analysis revealed that the LNAC2 contained a diverse activation domain in C-terminal, and a conserved NAC domain in the N-terminal region which can be divided into five subdomains, namely A to E (Figure 1a). A phylogenetic tree based on the sequences of LNAC2 and some other known stress-responsive NACs was constructed, which revealed that LNAC2 was clustered closely to rice ONAC048 and Arabidopsis ATAF1, with 65% and 63% of identity (Figure 1b). We also BLAST the DNA sequence of LNAC2 to the whole-genome sequence of Arabidopsis thaliana using The Arabidopsis Information Resource (TAIR). The BLAST result showed
that the highest score (bits) significant alignment of LINAC2 was AT1G01720.1 (ATAF1), which was located in the No. 1 chromosome (Supplementary Figure S1).

Figure 1. Characterization of LINAC2. (a) Alignment of LINAC2 with Ziziphus jujuba ZjNAC2, Morus notabilis MnNAC2, Elaeis guineensis EgNAC48, Arabidopsis ANAC002, and Glycine max GmNAC2. Black arrowed lines indicate the locations of the five highly conserved subdomains A–E. The conserved NAC domain is boxed and identical amino acids are shaded in black. (b) Phylogenetic tree analysis of LINAC2 with other known stress-responsive NAC proteins. Protein sequences used in multiple sequence alignments and phylogenetic tree analysis are shown in Supplementary Table S2.
2.2. Subcellular Localization and Transactivation Assay of LlNAC2

The GFP-LlNAC2 fusion construct and the GFP control in pBI121-GFP vector driven by CaMV35S promoter were transiently expressed in tobacco epidermal cells and visualized under a laser scanning confocal microscope to determine the subcellular localization of LlNAC2. Results showed the fluorescence signal from GFP alone was widely distributed throughout the cells, whereas the GFP-LlNAC2 fusion protein fluorescence signal was mainly detected in the nucleus (Figure 2a), which demonstrated that LlNAC2 is a nuclear protein.

The entire coding region, N-terminal, and C-terminal domain coding sequence were inserted into the pGBDKT7 vector to investigate the transcriptional activity of LlNAC2 protein. The transactivation results showed that all transformed yeast cells grew well on SD/-Trp medium (Figure 2b). The yeast strain containing the full-length LlNAC2 (LlNAC2-A) and the C-terminus of LlNAC2 (LlNAC2-C) could grow well on the selection medium SD/-Trp/-His/-Ade, while the cells with the N-terminus of LlNAC2 (LlNAC2-N) and pGBDKT7 empty vector could not grow normally (Figure 2b). Furthermore, the yeast cells that grew well on the SD/-Trp/-His-x-α-gal medium appeared blue in the presence of α-galactosidase (Figure 2b). These results indicated that LlNAC2 is a transcriptional activator, and its transactivation domain locates in the C-terminal region.

Figure 2. Nuclear localization and transactivation assay of LlNAC2. (a) 35S-GFP and 35S-LlNAC2-GFP fusion proteins were transiently expressed in tobacco leaves and observed under a laser scanning confocal microscope. GFP, chlorophyll, bright field, and merged images were taken (Scale bar, 50 μm). (b) Full-length protein (LlNAC2-A), N-terminal fragment (LlNAC2-N) and C-terminal fragment (LlNAC2-C) were inserted to the pGBKT7 vector and expressed in yeast strain Y2HGold. Transformed yeasts were dripped on the SD/-Trp, SD/-Trp-His-x-gal and SD/-Trp-His-Ade plates after being cultured for 3 days under 30 °C. The pGBDKT7 vector was used as a negative control.
2.3. LINAC2 is Induced by Multiple Stresses and ABA

The qRT-PCR analysis revealed that the LINAC2 gene has relative high expression level in bulb, while its expression level was low in leaf and stem (Figure 3a). Since LINAC2 was identified from cold-treated RNA-seq data [36], we also analyzed its expression levels under cold stress in stems, roots, and bulbs (Figure 3b). The results showed that LINAC2 was up-regulated in root and stem after 2 h at 4 °C treatment. However, at the same time, the expression of LINAC2 was strongly down-regulated and then up-regulated in bulb after 16 h at 4 °C treatment. Thus, we suppose that LINAC2 might play a special role in cold resistance of bulb in the tiger lily. Under ABA treatment, the expression of LINAC2 was significantly and rapidly up-regulated within 2 h, leading to a three-fold to four-fold increase, and then it was induced specifically again at 24 h (Figure 3c). However, treatment of tiger lily plants with cold and drought stress conditions induced the expression of LINAC2 until 24 h, leading to a five-fold to six-fold increase (Figure 3c,f). Similarly, salt treatment also induced the expression of LINAC2 slowly, showing a three-fold to five-fold increase during 12-24 h after treatment (Figure 3e). These results showed that LINAC2 is a stress-responsive NAC gene in the tiger lily.

The 1527 bp upstream of ATG start codon LINAC2 promoter sequence was also analyzed. Sequences of various putative stress-related and hormone-responsive cis-acting regulatory elements were identified in the LINAC2 promoter, including DRE/CRT (dehydration responsive element/C-repeat element) motif, LTRE (low-temperature-responsive element), MYCRS (MYC recognition site), MYBRS (MYB recognition site), ABRE (ABA responsive element), ARF (auxin response factor binding site), TGA-element and CGTCA-motif (Figure 3g, Supplementary Table S3). These cis-acting regulatory elements may be responsible for the induce expression of LINAC2 under stresses.
Figure 3. Expression patterns of LlNAC2 in tiger lily seedlings under different stress treatments and the distribution of stress-related cis-elements in the LlNAC2 promoter. Expression patterns of LlNAC2 in leaves, bulbs, roots, and stems (a), and expression patterns of LlNAC2 after cold treatments in leaves (c), bulbs, roots, and stems (b), and after ABA (d), NaCl (e), drought (f) treatments by qRT-PCR analysis. Transcript levels were normalized to LlTIP1. Three biological replications were performed. The bars show the SD. Asterisks indicate a significant difference ** \( p < 0.01 \) compared with the corresponding controls. (g) Distribution of major stress-related cis-elements in the promoter region of LlNAC2 gene.

2.4. Promoter Activity of LlNAC2 Is Induced by Cold, Salt and ABA Treatments

To further clarify the regulatory mechanism underlying the stress-inducible expression of LlNAC2, the full-length LlNAC2 promoter (proLlNAC2) was cloned and used to drive the GUS expression in Arabidopsis. The results showed that the untreated and drought-treated proLlNAC2 transformant (proLlNAC2-trans) Arabidopsis showed apparent lighter blue than that of the CaMV35S
transformant (CaMV35S-trans) Arabidopsis plants (Figure 4a). However, the cold, salt, and ABA-treated proLINAC2-trans showed darker blue than the untreated proLINAC2-trans and the CaMV35S-trans Arabidopsis (Figure 4a), suggesting the expression of the GUS gene was increased after cold, salt stresses and ABA treatment, and the GUS transcript level driven by LINAC2 promoter is higher than that driven by CaMV35S. To further confirm these findings, GUS expression from the LINAC2 promoter was detected by qRT-PCR and fluorometric GUS assay. The expression of GUS gene increased significantly after 6 h or 12 h cold, salt, or ABA treatments in proLINAC2-trans Arabidopsis plants (Figure 4b). From the GUS enzyme activity assay, we also found the same phenomenon as that shown in GUS histochemical staining results. Thus, the LINAC2 promoter mediated GUS activity increased under cold, salt, and ABA treatments.

Figure 4. GUS activity mediated by LINAC2 promoter in transgenic Arabidopsis plants. (a) Beta-glucuronidase (GUS) expression in untreated (1), cold (3), NaCl (4), drought (5), and ABA-treated (6) proLINAC2-trans and CaMV35S-trans (2) Arabidopsis plants (Scale bar, 2 mm). (b) The GUS transcript levels and enzyme activity in the leaves of the transgenic Arabidopsis under cold (4 °C), salt, drought, and ABA treatments. The transgenic plants treated for 12 h under above treatments were used for fluorometric GUS assay. GUS activity from the CaMV35S-trans, untreated proLINAC2-trans (CK), and wild type (WT) served as controls. Twelve transgenic lines were acquired. Three biological replications were performed. The bars show the SD. Asterisks indicate a significant difference (**p < 0.01) compared with the corresponding controls.

2.5. LIDREB1 Can Bind to the Promoter of LINAC2

DREBs were known to play a vital role in plant response to abiotic stresses, and some NAC TFs were found to be involved in the DREB/CBF-COR pathway in many studies [30,33,34,38] which inspired us to further explore the NAC-DREB/CBF-COR signaling cascade. In our previous study, a novel DREB1 gene from the tiger lily (LIDREB1, NCBI accession No. KJ467618) induced by cold stress was identified [35]. Thus, we performed the Y1H assay to explore whether there is an interaction
between the LlDREB1 protein and LlNAC2 promoter. The minimal inhibitory concentration of Aureobasidin A (AbA) for bait yeast strains was found to be 150 ng/mL (Supplementary Figure S3). Yeast cells transformed with pGADT7-LlDREB1 and pAbAi-CRT/DREs grew well on SD/Leu plates with 150 ng/mL and 200 ng/mL AbA (Figure 5a). This suggested that LlDREB1 possessed the ability to bind CRT/DREs as general CBF/DREB1 TFs. The fragment (−487 to −312) of the LlNAC2 promoter containing three CRT/DREs was cloned and its interaction with LlDREB1 was detected (Figure 5b); the result showed LlDREB1 could bind to this fragment, indicating LlDREB1 might be involved in the regulatory pathway of LlNAC2.

Figure 5. Yeast one-hybrid analysis of LlDREB1 binding to LlNAC2 promoter. (a) Yeast one-hybrid analysis. Three tandem repeats of a CRT/DRE and the −487 to −312 region of the LlNAC2 promoter were inserted in front of the reporter gene AUR1-C. Yeast strain Y1HGold was co-transformed with bait (pAbAi-CRT/DREs or pAbAi-NAC-CRT/DREs) and a prey (pGADT7 or pGADT7-LlDREB1) construct. Interaction between bait and prey was determined by cell growth on SD medium lacking Leu in the presence of 200 ng mL⁻¹ AbA. (b) Schematic representation of CRT/DRE recognition elements in the LlNAC2 promoter.

2.6. LlNAC2 Interacts with LlDREB1 or LlZFHD4

We have already found LlNAC2 was highly co-expressed with another cold-responsive TF zinc finger homeodomain protein LlZFHD4 [35]. To further explore the interactions between LlNAC2, LlZFHD4, and LlDREB1 proteins, BiFC assay was performed with a yellow fluorescent protein (YFP). YFP was observed in tobacco epidermal cells, transformed with vectors containing LlNAC2-pSPYNE/LlZFHD4-pSPYCE and LlNAC2-pSPYNE/LlDREB1-pSPYCE localized to the nuclei; while no YFP was detected in negative control pSPYNE173/pSPYCE (M) and LlZFHD4-pSPYNE/LlDREB1-pSPYCE (Figure 6). The result showed that LlNAC2 could interact with LlDREB1 and LlZFHD4.
2.7. Overexpression of LlNAC2 in Arabidopsis Enhance Tolerance to Cold and Drought Stresses

Among 12 independent homozygous LlNAC2 transgenic lines, LlNAC2-5 (L5) and LlNAC2-6 (L6) with relatively high expression levels (Supplementary Figure S4) were selected for further analysis.

To study the effect of LlNAC2 overexpression on cold stress, LlNAC2 transgenic lines and wild-type (WT) plants were grown in equal amounts of potting soil for 3 weeks under normal conditions, and cold stress was applied by being exposed to various freezing temperatures for 12 h. The results showed that all plants grew well under −2 °C and −4 °C treatment as same as under normal temperature 22 °C (Figure 7a). When the temperature was decreased to −6 °C, most of the WT plants were dead with a survival rate at around 19%, but over half of two transgenic plants survived (Figure 7a,c). At −8 °C, all WT plants were dead whereas the survival rate of transgenic plants was observed at 30%–35% (Figure 7a,c). In a further experiment, 3-week-old plants were treated at 4 °C for 3 h, and the relative electrolyte leakage and soluble sugars were measured after treatment. As a result, the transgenic plants showed lower electrolyte leakage and higher levels of soluble sugars relative to WT plants (Figure 7d,e).
Figure 7. Overexpression of LlNAC2 in Arabidopsis enhances plant tolerance to cold and drought stresses. Performance of WT and LlNAC2 transgenic plants after freezing (a) and drought (b) treatments. (c) Survival rate of plants in (a) under freezing temperatures (blue region) and in (b) after drought treatment for 30 days (red region). Relative electrolyte leakage (d) and soluble sugar content (e) in WT and LlNAC2 transgenic lines after 4 °C and 16.1% PEG6000 (−0.5 MPa) treatment for 3 h. (f) Water loss rate of leaves from WT and LlNAC2 transgenic plants. Three biological replications were performed, each replication contains 30 plants. The bars show the SD. Asterisks indicate a significant difference 0.01 < *p < 0.05 and **p < 0.01 compared with the corresponding controls.

Similarly, to study drought stress tolerance, WT plants showed visible symptoms of drought-induced damage and even death after withholding water for 30 days, while some transgenic plants remained green with expanded leaves (Figure 7b). When plants were rehydrated, about 80% of the transgenic plants recovered well, whereas the WT plants failed and ultimately died (Figure 7b,c). Additionally, after being treated with 16.1% PEG6000 (−0.5 MPa) for 3 h, transgenic plants showed
lower electrolyte leakage and higher levels of soluble sugars compared to WT plants (Figure 7d,e). Also, the water-loss rates were found lower in transgenic plants after 3 h treatment (Figure 7f). These results suggest that the LINAC2 transgenic plants conferred tolerance to cold and drought stresses in some degree.

2.8. Overexpression of LINAC2 in Arabidopsis Increases Seed Sensitivity to ABA and Tolerance to NaCl

Salt tolerance and ABA sensitivity of LINAC2 transgenic plants were also assessed. WT and two LINAC2 transgenic lines were placed on MS agar plates supplemented with 50 mM or 100 mM NaCl and 2 μM ABA. There were no differences in plant morphology between WT and LINAC2 transgenic Arabidopsis on MS agar plates (Figure 8a,b). However, LINAC2 transgenic Arabidopsis displayed significantly higher germination ratios than WT plants following NaCl treatment according to both cotyledon greening and radicle protrusion (Figure 8a,c,d). In contrast, the germination ratio of transgenic plants seeds was remarkably lower than that of WT seeds in the MS medium containing 2 μM ABA according to cotyledon greening (Figure 8b,c). Therefore, we suggested that LINAC2 transgenic plants are more tolerant to salt stresses and more hypersensitive to ABA than WT plants.

Figure 8. LINAC2 transgenic lines showed ABA hypersensitivity and enhanced salt tolerance. Germination of WT of Col-0 and 35S-LINAC2 seeds on MS supplemented with (a) 50 mM or 100 mM NaCl and (b) 2 μM ABA assayed by both (c) cotyledon greening and (d) radicle protrusion. For (a) and (b), images were acquired after 7 days of incubation at 22 °C. Three biological replications were performed. The bars show the SD. Asterisks indicate a significant difference **p < 0.01 compared with the corresponding controls.

2.9. Altered Expression of Stress-Related Genes in LINAC2 Transgenic Arabidopsis Plants

LINAC2 transgenic Arabidopsis plants showed enhanced tolerance to cold, drought, and salt stresses. Thus, we detected the transcript levels of some stress-responsive genes from Arabidopsis in the transgenic plants, including AtRD20, AtCOR47, AtRD29A, AtRD29B, AtLEA14, AtGolS1, AtAPX2, and AtGSTF6 genes (NCBI accession numbers are shown in Supplementary Table S1). The qRT-PCR results showed higher transcripts of these genes accumulated in LINAC2 transgenic plants compared
to WT plants (Figure 9). We suppose the activated expression of these stress-responsive genes may contribute to the stronger stress tolerance in LINAC2 transgenic plants.

![Figure 9](image_url)

**Figure 9.** Transcript levels of the stress-related genes under normal condition in WT and LINAC2 transgenic Arabidopsis plants. Three biological replications were performed. The bars show the SD. Asterisks indicate a significant difference (**p < 0.01**) compared with the corresponding controls.

### 3. Discussion

In this study, we identified a novel stress-related NAC TF gene, *LINAC2*, from tiger lily. Sequence analysis shows that *LINAC2* has a highly conserved sequence in the N-terminal region and has high sequence identity with ATAF1 from Arabidopsis. Like most transcription factors, LINAC2 protein is localized in the nucleus with transactivation activity in the C-terminal region. Our previous transcriptome data analysis identified a unigene contig16924 coding for partial LINAC2, which showed significant changes in expression in the tiger lily under cold stress [35,36]. Here, we further confirmed that *LINAC2* not only participates in cold stress response, but also responds to drought and salt stress, and its expression is sensitive to ABA signaling molecules as well. Also, the *LINAC2* promoter is shown to be a stress-inducible promoter, and the GUS activity driven by the *LINAC2* promoter is significantly higher than that driven by the CaMV35S promoter. Given that using a stress-inducible promoter to drive transgene expression can not only result in remarkable gains in stress tolerance, but also avoid impacting important traits in crops negatively [39], the *LINAC2* promoter can be a candidate stress-inducible promoter utilized in transgenic breeding to enhance the stress tolerance of crops.

Many NAC domain proteins from different species were shown to function positively in regulation of plant stress tolerance [1,21,40,41]. We also found tolerance to cold, drought, and salt stresses were likely conferred by overexpressing *LINAC2* in Arabidopsis. At the morphological level, compared to WT plants, *LINAC2* transgenic lines showed a higher seed germination ratio under salt stress condition, and performed better growth performance and higher survival rate under drought and cold stress conditions. At the physiological level, the lower electrolyte leakage amount and higher soluble sugar level were observed in transgenic plants after dehydration and chilling treatment. Furthermore, the leaf water-loss ratios of the *LINAC2* transgenic plants were lower in transgenic plants than in WT plants. These physiological indices changes likely resulted in enhancing tolerance to stresses at the physiological level, which were consistent with previous reports about NAC TFs in maize, rice,
and rose [21,26,42]. At the gene transcription level, the LINAC2 gene may improve stress tolerance by regulating downstream stress-responsive genes. The results showed that higher transcript levels of 8 picked stress-responsive genes accumulated in LINAC2 transgenic plants. More importantly, putative NAC binding cis-elements were also reported to localize in the promoter sequences of these genes [27,43,44], indicating these genes might be transcriptionally regulated directly by LINAC2.

On the other hand, we found overexpression of LINAC2 resulted in improved ABA sensitivity, which was also observed on many NAC TFs from other species, such as GmNAC20, MINAC5, AtATAF1, ONAC022, and TaNAC47 [1,21,23,42,45]. Promoter analysis showed that there are two ABREs (YACGTGGC, Y = C/T) and eight core DPBF (Dc3 promoter-binding factor) (ACACNNG) cis-acting elements in LINAC2 promoter. ABREs can be recognized by ABRE-binding factors (ABRE/ABFs), which are involved in ABRE-dependent ABA signaling pathway in response to drought stress [46]; the core DPBF (Dc3 Promoter-Binding Factor) (ACACNNG) elements were also known to participate in ABA responsiveness [47,48]. The presence of these cis-elements might explain why LINAC2 was induced more quickly in response to ABA treatment compared with other stresses treatment in the tiger lily. Given that most of the picked genes above were also reported to be ABA-responsive genes, these results suggest that the LINAC2 may go through the more efficient ABA signaling pathway to enhance cold, drought, and salt stress tolerance in transgenic plants.

Except for the ABA signaling pathway, NAC domain proteins have received much attention as regulators in various stress signaling pathways [49], in which some studies showed some NAC TFs were also involved in the DREB/CBF-COR pathway [1,30,33,34]. CBF/DREB1 is known to activate stress-responsive genes under abiotic stress conditions by binding to specific cis-elements such as DRE/CRTs present in their promoters [4,50]. In the promoter region of LINAC2, we found there are one LTRE (CCGAC) and three CRT/DREs (GC/TCGAC) elements, which are known to be necessary and sufficient for gene transcription under cold stress [51–54]. Furthermore, a novel DREB1 gene from the tiger lily (LIDREB1) induced by cold stress was identified in our earlier study [35]. We thus further analyzed the interactions between LIDREB1 and LINAC2. Y1H assay confirmed that LIDREB1 could bind to the LINAC2 promoter. It provides a direct evidence for LINAC2 as a novel target of LIDREB1. Interestingly, BiFC assay showed that the LINAC2 protein can interact with the LIDREB1 protein in tobacco epidermal cells. These findings indicate that the LINAC2 gene might be regulated by LIDREB1 TF under abiotic stress conditions and LINAC2 TF can in turn interact with LIDREB1 to more efficiently regulate the expression of downstream stress-related genes. It may also explain why LINAC2 was induced more slowly but more strongly in response to cold compared with salt, drought, and ABA treatments in the tiger lily. However, in soybean, GmNAC20 was found to be an upstream protein of GmDREB1A which can directly bind to the promoter of GmDREB1A [1]. Thus, we suppose that there are various ways for NAC TFs participating in the DREB/CBF-COR pathway, and LINAC2 is involved in DREB/CBF-COR pathway, to mediate stress tolerance of the tiger lily.

In Arabidopsis, an interaction between stress-inducible zinc finger homeodomain ZFHD1 and NAC TFs was detected in the report of Lam-Son Phan Tran [55]. It showed co-overexpression of ZFHD1 and NAC genes, enhanced expression of ERD1 in both Arabidopsis T87 protoplasts, and transgenic Arabidopsis plants. Also, in our previous study, LINAC2 was highly co-expressed with another cold-responsive TF, the zinc finger homeodomain protein 4 LIZFHD4 [36]; and here we confirmed that the LINAC2 protein can physically interact with the LIZFHD4 protein by BiFC assay. However, further analysis is needed to confirm whether this interaction functions the same as AtZFHD1 and AtNAC TFs in Arabidopsis.

In conclusion, LINAC2 is a nucleus-localized transcriptional activator which is regulated by cold, drought, and salt stresses and sensitive to ABA. The LINAC2 promoter is a stress-inducible promoter which showed higher promoter activity than CaMV35S under cold, salt, and ABA conditions. Overexpressing LINAC2 in Arabidopsis showed ABA hypersensitivity and enhancing tolerance of transgenic plants to freezing, dehydration, and salt conditions. We demonstrated that the LINAC2 gene is not only a novel direct target of LIDREB1, but the LINAC2 protein can also interact with the LIDREB1
protein. These data indicate LINAC2 is involved in both DREB/CFB-COR and ABA-dependent pathways to mediate stress tolerance of the tiger lily (Figure 10). Moreover, the protein interaction of the co-expressed LINAC2 and LIZFHD4 gene was confirmed, providing another pathway involving regulator LINAC2. Therefore, our future efforts will be focused on investigating the role of these interactive proteins in regulating expression and modulating the function of LINAC2 under various stress conditions.

![Figure 10. LINAC2 is involved in both DREB/CFB-COR and ABA-dependent pathways to mediate stress tolerance of the tiger lily.](image)

### 4. Materials and Methods

#### 4.1. Plant Materials and Abiotic Stress Treatments

The wild tiger lily (*Lilium lancifolium*) was used as experimental material in this study. The seedlings preparation method was described in our previous study [36]. The bulbs of the tiger lily were cleaned, disinfected, and then stored at 4 °C; in March, the bulbs were box-cultivated in a greenhouse (116.3° E, 40.0° N) under controlled conditions. The model plant *Arabidopsis thaliana* Columbia-0 (Col-0) was selected for the transgenic study of LINAC2. Arabidopsis plants were grown in 8 cm × 8 cm plastic pots containing a 1:1 mixture of sterile peat soil and vermiculite under controlled conditions (22/16 °C, 16 h light/8 h dark, 65% relative humidity, and 1000 lx light intensity). Seeds of *Nicotiana benthamiana* were planted and cultured under the same conditions.

For the expression analysis of LINAC2 in response to abiotic stress and ABA treatment, 8-week-old tiger lily seedlings were treated with 4 °C, 16.1% PEG6000 (~0.5 MPa), 100 mM NaCl and 100 μM exogenous ABA for 0, 1, 3, 6, 12, and 24 h, respectively. Leaf samples were collected and immediately frozen with liquid nitrogen and stored at −80 °C for RNA isolation.

#### 4.2. Full-Length cDNA Cloning and Sequence Analysis of LINAC2

The 3′-complete sequence cDNA of LINAC2 gene was obtained from the transcriptome data of cold-treated tiger lily leaves in our laboratory. Using the SMARTer RACE 5′/3′ Kit (Clontech, United States), we performed a 5′-rapid amplification of cDNA ends (5′-RACE) of LINAC2 according to the manufacturer’s protocol. Two reverse primer pairs (Supplementary Table S1) were designed to amplify...
the 5′-complete sequence cDNA of LINAC2. The 3′- and 5′-sequences were assembled by DNAMAN (version 7), resulting in the full-length cDNA of LINAC2 gene. The coding sequence of LINAC2 was amplified and cloned into pEASYT1-Blunt vector (TransGen Biotech, Beijing, China). Plasmid pEASYT1-LINAC2 was used as a template for all experiments after sequencing. Multiple sequence alignments were performed using DNAMAN (version 7). Phylogenetic tree analysis was performed using the neighbor-joining method in MEGA5 software with 1000 replications. The NCBI accession numbers of genes used in multiple sequence alignments and phylogenetic tree analysis are shown in Supplementary Table S2. The theoretical molecular weight and isoelectric point were calculated using ExPASy (http://expasy.org/tools/protparam.html). The NAC domain region was identified with SCANPROSITE (http://www.expasy.ch/tools/scanprosite).

4.3. RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was isolated using an RNAisomate RNA Easyspin isolation system (Aidlab Biotech, Beijing, China). First-strand cDNA synthesis was performed using Prime Script II 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan) according to the manufacturer’s instructions. The qRT-PCR was performed using a Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA) with SYBR® qPCR mix (Takara, Shiga, Japan) according to the manufacturer’s protocol. Relative mRNA content was calculated using the $2^{-\Delta\Delta C_t}$ method against the internal reference gene encoding tiger lily tonoplast intrinsic protein 1 (LlTIP1) [35] and Arabidopsis Actin gene (NCBI accession No. NM_112764). The primers used in this study were designed with Primer Premier 5 and are listed in Supplementary Table S1. All reactions were performed in three biological replicates. Student’s t-test was performed for all statistical analysis in this study.

4.4. Promoter Cloning and Sequence Analysis

LINAC2 promoter sequence was cloned from tiger lily genomic DNA using Genome Walker Universal Kits (Clontech, United States). The cis-acting motifs present in the LINAC2 promoter were predicted using the online search tool PLACE (http://www.dna.aaffrc.go.jp/PLACE/) database.

4.5. Subcellular Localization of LINAC2

By using ClonExpressII One Step Cloning Kits (Vazyme, Piscataway, NJ, United States), full-length LINAC2 was inserted into vector pBI121-GFP at Xhol and SalI sites. The pBI121-LINAC2-GFP plasmid and the pBI121-GFP empty vector were transformed into Agrobacterium tumefaciens GV3101 and infiltrated separately into N. benthamiana leaves. After infiltration, the plants were grown in a growth room under controlled conditions (22/16 °C, 16 h light/8 h dark, 65% relative humidity, and 1000 lx light intensity) for 32 h. GFP fluorescence signals were excited at 488 nm and detected under Leica TCS SP8 Confocal Laser Scanning Platform (Leica SP8, Leica, America) using a 500–530 nm emission filter.

4.6. Transcription Activation Assay of LINAC2 in Yeast

To investigate the transcriptional activity of the LINAC2 protein, the full-length coding sequence of LINAC2 and the sequence encoding the N-terminus (1–453 bp) and C-terminus (454–906 bp) were inserted into the EcoRI and BamHI sites of the pGBK7 vector, resulting in plasmid pGBK7-LINAC2-A, pGBK7-LINAC2-N (1–151 aa), and pGBK7-LINAC2-C (152–302 aa). These plasmids were transformed into the yeast strain Y2HGold separately by using Quick Easy Yeast Transformation Mix (Clontech, United States). The transformed yeast cells were incubated on SD/-Trp, SD/-Trp/-His-Ade and SD/-Trp-His-x-α-gal plates [24].

4.7. Yeast One-Hybrid (Y1H) Assays

Y1H assay was carried out using the Matchmaker™ Gold Yeast One-Hybrid System (Clontech, United States). Three tandem copies of CRT/DRE were generated by oligonucleotide synthesis
and cloned into the pAbAi (bait) vector (Clontech, United States). A fragment (−312 to −487) of the LlNAC2 promoter was amplified by PCR, and also cloned into the pAbAi (bait) vector to generate pAbAi-NAC-CRT/DREs plasmid (shown in Figure 4B). Full-length LlDREB1 (NCBI accession No.KJ467618) was amplified and inserted into pGADT7 (prey) vector (Clontech, United States) yielding plasmid pGADT7-LlDREB1. The bait plasmids were linearized and transformed into the yeast strain Y1HGold. Positive yeast cells were then transformed with pGADT7-LlDREB1 plasmid. The DNA–protein interaction was determined based on the growth ability of the co-transformants on SD/-Leu medium with Aureobasidin A (AbA).

4.8. BiFC Assay

Full-length LlNAC2 and zinc finger homeodomain protein LlZFHD4 (sequence shown in Supplementary Figure S2) were cloned into the pSPYNE173 vector, LlDREB1 and LlZFHD4 were cloned into the pSPYCE(M) vector [56], respectively. Co-expression was executed on N. benthamiana leaves as described in subcellular localization assessments. After infiltration, the plants were grown under 24 h dark and then 16 h light/8 h dark for 32 h. YFPs were excited at 514 nm. Images were generated through Leica TCS SP8 Confocal Laser Scanning Platform using a 500–530 nm emission filter.

4.9. Generation of LlNAC2 Transgenic Arabidopsis

The LlNAC2 open read frame (ORF) was cloned into the pBI121 vector under control of a CaMV35S promoter; the LlNAC2 promoter region was inserted into the CaMV35S-GUS vector by replacing the CaMV35S promoter. The recombinant vectors and empty GUS vector were transformed into 5-week-old Arabidopsis ecotype Col-0 plants by the floral-dip method [57]. Transformed seeds were selected on MS medium containing 50 mg/L kanamycin. All transgenic lines were identified by RT-PCR; T3-generation homozygous lines were selected for gene functional analysis.

4.10. Histochemical Staining and Fluorometric GUS Assay

Histochemical staining and fluorometric GUS assay analysis for GUS activity was carried out as described before [58]. Transgenic LlNAC2 Arabidopsis plants were treated with 4 °C, 16.1% PEG6000 (−0.5 MPa), 100 mM NaCl and 100 μM exogenous ABA for different durations before sampling. The leaves of stress-treated transgenic LlNAC2 Arabidopsis were incubated in GUS reaction buffer (Huayueyang Biotech, Beijing, China). Photos of those stained samples were obtained by a Leica TL3000 Ergo microscope under white light. Leaves of stress-treated transgenic Arabidopsis were also used to examine the GUS gene expression level by qRT-PCR, and determine GUS enzyme activity.

4.11. Abiotic Stress Tolerance Assays and ABA Sensitivity Analysis

For cold and drought treatments, 3-week-old seedlings were kept at 4 °C for 3 h, then at −2, −4, −6 or 8 °C, respectively, for 12 h. After that, the plants were kept at 4 °C for 3 h before transferring to a normal condition at 22 °C [34]. For the drought treatment, the water intake of 3-week-old seedlings in water-saturated substrate was withheld for 30 days, followed by rehydrating the seedlings for 7 days [24].

For determining the salt tolerance and ABA sensitivity in transgenic plants, Arabidopsis seeds were cultivated on MS medium supplemented with 0 and 2 μM ABA or 50 and 100 mM NaCl, respectively, under continuous light at 22 °C in a growth chamber. The germination rate was scored on the 7th day after planting on the plates.

4.12. Measurements of Relative Electrolyte Leakage, Soluble Sugar, and Water Loss Rate

The relative electrolyte leakage, soluble sugar content, and water loss rate were evaluated following the method described previously [21,59]. The relative electrolyte leakage was evaluated by determining the relative conductivity of fresh leaves (100 mg) in solution using a conductivity detector.
The anthrone-sulfuric acid colorimetry was used for determining the soluble sugar. The water loss rate was calculated related to the initial fresh weight of the leaf samples; the samples were placed on the lab bench (20–22 °C, humidity 45–60%) and weighed at designated time points. All the measurements were performed with ten plants in triplicate.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/20/13/3225/s1.

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Abbreviations

ABA abscisic acid
TF transcription factor
Y1H yeast one-hybrid
BiFC bimolecular fluorescence complementation
GFP green fluorescent protein
AbA aureobasidin A
GUS β-glucuronidase

References

1. Hong, Y.; Zhang, H.; Huang, L.; Li, D.; Song, F. Overexpression of a Stress-Responsive NAC Transcription Factor Gene ONAC022 Improves Drought and Salt Tolerance in Rice. *Front. Plant Sci.* 2016, 7, 4. [CrossRef] [PubMed]
2. Chung, P.J.; Jung, H.; Choi, Y.D.; Kim, J.K. Genome-wide analyses of direct target genes of four rice NAC-domain transcription factors involved in drought tolerance. *BMC Genom.* 2018, 19, 40. [CrossRef] [PubMed]
3. Liu, C.; Wang, B.; Li, Z.; Peng, Z.; Zhang, J. TsNAC1 Is a Key Transcription Factor in Abiotic Stress Resistance and Growth. *Plant Physiol.* 2018, 176, 742–756. [CrossRef] [PubMed]
4. Mizoi, J.; Shinozaki, K.; Yamaguchi-Shinozaki, K. AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta* 2012, 1819, 86–96. [CrossRef] [PubMed]
5. Du, C.; Hu, K.; Xian, S.; Liu, C.; Fan, J.; Tu, J.; Fu, T. Dynamic transcriptome analysis reveals AP2/ERF transcription factors responsible for cold stress in rapeseed (*Brassica napus* L.). *Mol. Genet. Genom.* 2016, 291, 1053–1067. [CrossRef] [PubMed]
6. Xiong, H.; Li, J.; Liu, P.; Duan, J.; Zhao, Y.; Guo, X.; Li, Y.; Zhang, H.; Ali, J.; Li, Z. Overexpression of *OsMYB48-1*, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. *PLoS ONE* 2014, 9, e92913. [CrossRef] [PubMed]
7. Wei, Q.; Luo, Q.; Wang, R.; Zhang, F.; He, Y.; Zhang, Y.; Qiu, D.; Li, K.; Chang, J.; Yang, G.; et al. A Wheat R2R3-type MYB Transcription Factor TaODORANT1 Positively Regulates Drought and Salt Stress Responses in Transgenic Tobacco Plants. *Front. Plant Sci.* 2017, 8, 1374. [CrossRef] [PubMed]
8. Castilhos, G.; Lazzarotto, F.; Spagnolo-Fonini, L.; Bodanese-Zanettini, M.H.; Margis-Pinheiro, M. Possible roles of basic helix-loop-helix transcription factors in adaptation to drought. *Plant Sci.* 2014, 223, 1–7. [CrossRef]
9. Rushton, D.L.; Tripathi, P.; Rabara, R.C.; Lin, J.; Ringler, P.; Boken, A.K.; Langum, T.J.; Smidt, L.; Boomsma, D.D.; Emme, N.J.; et al. WRKY transcription factors: Key components in abscisic acid signalling. *Plant Biotechnol. J.* 2012, 10, 2–11. [CrossRef]
10. Chen, L.; Song, Y.; Li, S.; Zhang, L.; Zou, C.; Yu, D. The role of WRKY transcription factors in plant abiotic stresses. *Biochim. Biophys. Acta* 2012, 1819, 120–128. [CrossRef]
11. Huang, J.; Sun, S.J.; Xu, D.Q.; Yang, X.; Bao, Y.M.; Wang, Z.F.; Tang, H.J.; Zhang, H. Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. *Biochem. Biophys. Res. Commun.* 2009, 389, 556–561. [CrossRef] [PubMed]

12. Zang, D.; Wang, L.; Zhang, Y.; Zhao, H.; Wang, Y. ThDoF1.4 and ThZFP1 constitute a transcriptional regulatory cascade involved in salt or osmotic stress in *Tamarix hispida*. *Plant Mol. Biol.* 2017, 94, 495–507. [CrossRef] [PubMed]

13. Mao, H.; Yu, L.; Han, R.; Li, Z.; Liu, H. ZmNAC55, a maize stress-responsive NAC transcription factor, confers drought resistance in transgenic Arabidopsis. *Plant Physiol. Biochem.* 2016, 105, 55–66. [CrossRef] [PubMed]

14. Mao, X.; Chen, S.; Li, A.; Zhai, C.; Jing, R. Novel NAC transcription factor TaNAC67 confers enhanced multi-abiotic stress tolerances in Arabidopsis. *PLoS ONE* 2014, 9, e84359. [CrossRef]

15. Olsen, A.N.; Ernst, H.A.; Leggio, L.L.; Skriver, K. NAC transcription factors: Structurally distinct, functionally diverse. *Trends Plant Sci.* 2005, 10, 79–87. [CrossRef] [PubMed]

16. Zhang, L.; Zhang, L.; Xia, C.; Zhao, G.; Jia, J.; Kong, X. The Novel Wheat Transcription Factor TaNAC47 Regulates Abiotic Stress Response. *Plant Physiol. Biochem.* 2018, 124, 55–66. [CrossRef]

17. Tran, L.S.; Nakashima, K.; Sakuma, Y.; Simpson, S.D.; Fujita, Y.; Maruyama, K.; Fujita, M.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors. *Plant Cell* 2010, 22, 1249–1263. [CrossRef] [PubMed]

18. Liu, H.; Zhou, Y.; Li, H.; Wang, T.; Zhang, H.; Ouyang, B.; Ye, Z. Molecular and functional characterization of ShNAC1, an NAC transcription factor from Solanum habrochaites. *Plant Sci.* 2018, 271, 9–19. [CrossRef] [PubMed]

19. Liu, X.; Wang, T.; Bartholomew, E.; Black, K.; Dong, M.; Zhang, Y.; Yang, S.; Cai, Y.; Xue, S.; Weng, Y.; et al. Comprehensive analysis of NAC transcription factors and their expression during fruit spine development in cucumber (*Cucumis sativus* L.). *Hortic. Res.* 2018, 5, 31. [CrossRef] [PubMed]

20. Olsen, A.N.; Ernst, H.A.; Leggio, L.L.; Skriver, K. NAC transcription factors: Structurally distinct, functionally diverse. *Trends Plant Sci.* 2005, 10, 79–87. [CrossRef] [PubMed]

21. Wu, Y.; Deng, Z.; Lai, J.; Zhang, Y.; Yang, C.; Yin, B.; Zhao, Q.; Zhang, L.; Li, Y.; Yang, C.; et al. Dual function of VND-INTERACTING2, a NAC domain transcription factor, negatively regulates xylem vessel formation in Arabidopsis. *Plant Cell* 2010, 22, 1249–1263. [CrossRef] [PubMed]

22. Tran, L.S.; Nakashima, K.; Sakuma, Y.; Simpson, S.D.; Fujita, Y.; Maruyama, K.; Fujita, M.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. *Plant Cell* 2004, 16, 2481–2498. [CrossRef] [PubMed]

23. Wu, Y.; Deng, Z.; Lai, J.; Zhang, Y.; Yang, C.; Yin, B.; Zhao, Q.; Zhang, L.; Li, Y.; Yang, C.; et al. Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. *Cell Res* 2009, 19, 1279–1290. [CrossRef] [PubMed]

24. Cao, H.; Wang, L.; Nawaz, M.A.; Niu, M.; Sun, J.; Xie, J.; Kong, Q.; Huang, Y.; Cheng, F.; Bie, Z. Ectopic Expression of Pumpkin NAC Transcription Factor CmNAC1 Improves Multiple Abiotic Stress Tolerance in Arabidopsis. *Front. Plant Sci.* 2017, 8, 2052. [CrossRef] [PubMed]

25. Gunapati, S.; Naresh, R.; Ranjan, S.; Nigam, D.; Ouyang, B.; Hans, A.; Verma, P.C.; Gadre, R.; Pathre, U.V.; Sane, A.P.; Sane, N.A. Expression of *GhNAC2* from *G. herbaceum*, improves root growth and imparts tolerance to drought in transgenic cotton and Arabidopsis. *Sci. Rep.* 2014, 4, 7734. [CrossRef] [PubMed]

26. Shi, P.; Ouyang, Z.; Zhang, Y.; Li, X.; Hong, Y.; Huang, L.; Liu, S.; Zhang, H.; Li, D.; Song, F. Tomato NAC transcription factor SISR11 positively regulates defense response against biotic stress but negatively regulates abiotic stress response. *PLoS ONE* 2014, 9, e102067. [CrossRef]
30. Jin, C.; Li, K.Q.; Xu, X.Y.; Zhang, H.P.; Chen, H.X.; Chen, Y.H.; Hao, J.; Wang, Y.; Huang, X.S.; Zhang, S.L. A Novel NAC transcription factor, PbeNAC1, of Pyrus betulifolia confers cold and drought tolerance via interacting with PbeDREBs and activating the expression of stress-responsive genes. *Front. Plant Sci.* **2017**, *8*, 1049. [CrossRef] [PubMed]

31. Wang, K.; Yin, X.R.; Zhang, B.; Grierson, D.; Xu, C.J.; Chen, K.S. Transcriptomic and metabolic analyses provide new insights into chilling injury in peach fruit. *Plant Cell Environ.* **2017**, *40*, 1531–1551. [CrossRef] [PubMed]

32. Zhu, J.; Dong, C.H.; Zhu, J.K. Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr. Opin. Plant Biol.* **2007**, *10*, 290–295. [CrossRef] [PubMed]

33. Shan, W.; Kuang, J.-F.; Lu, W.J.; Chen, J.Y. Banana fruit NAC transcription factor MaNAC1 is a direct target of MaICE1 and involved in cold stress through interacting with MaCBF1. *Plant Cell Environ.* **2014**, *37*, [CrossRef] [PubMed]

34. Hao, Y.J.; Wei, W.; Song, Q.X.; Chen, H.W.; Zhang, Y.Q.; Wang, F.; Zou, H.F.; Lei, G.; Tian, A.G.; Zhang, W.K.; et al. Soybean NAC transcription factors promote abiotic stress tolerance and root formation in transgenic plants. *Plant J.* **2011**, *68*, 302–313. [CrossRef] [PubMed]

35. Wang, J.; Wang, Q.; Yang, Y.; Liu, X.; Gu, J.; Li, W.; Ma, S.; Lu, Y. De novo assembly and characterization of stress transcriptome and regulatory networks under temperature, salt and hormone stresses in *Lilium lancifolium*. *Mol. Biol. Rep.* **2014**, *41*, 8231–8245. [CrossRef] [PubMed]

36. Yong, Y.B.; Li, W.Q.; Wang, J.M.; Zhang, Y.; Lu, Y.M. Identification of gene co-expression networks involved in cold resistance of *Lilium lancifolium*. *Biol. Plant.* **2018**, *62*, 287–298. [CrossRef]

37. Wang, J.; Yang, Y.; Liu, X.; Huang, J.; Wang, Q.; Gu, J.; Lu, Y. Transcriptome profiling of the cold response and signaling pathways in *Lilium lancifolium*. *BMC Genom.* **2014**, *15*, 203. [CrossRef]

38. Yang, S.D.; Seo, P.J.; Yoon, H.K.; Park, C.M. The Arabidopsis NAC transcription factor VNI2 integrates abscisic acid signals into leaf senescence via the COR/RD Genes. *Plant Cell* **2011**, *23*, 2155–2168. [CrossRef]

39. Pino, M.T.; Skinner, J.S.; Park, E.J.; Jeknić, Z.; Hayes, P.M.; Thomashow, M.F.; Chen, T.H. Use of a stress inducible promoter to drive ectopic AtCBF expression improves potato freezing tolerance while minimizing negative effects on tuber yield. *Plant Biotechnol. J.* **2007**, *5*, 591–604. [CrossRef]

40. Huang, L.; Hong, Y.; Zhang, H.; Li, D.; Song, F. Rice NAC transcription factor ONAC095 plays opposite roles in drought and cold stress tolerance. *BMC Plant Biol.* **2016**, *16*, 203. [CrossRef]

41. Wang, L.; Li, Z.; Lu, M.; Wang, Y. ThNAC13, a NAC transcription factor from *Tamarix hispida*, confers salt and osmotic stress tolerance to transgenic Tamarix and Arabidopsis. *Front. Plant Sci.* **2017**, *8*, 635. [CrossRef] [PubMed]

42. Gao, F.; Xiong, A.; Peng, R.; Jin, X.; Xu, J.; Zhu, B.; Chen, J.; Yao, Q. OsNAC52, a rice NAC transcription factor, potentially responds to ABA and confers drought tolerance in transgenic plants. *Plant Cell Tissue Organ Cult.* **2010**, *100*, 255–262. [CrossRef]

43. Liu, X.; Hong, L.; Li, X.-Y.; Yao, Y.; Hu, B.; Li, L. Improved drought and salt tolerance in transgenic Arabidopsis overexpressing a NAC Transcriptional Factor from *Arachis hypogaea*. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 443–450. [CrossRef]

44. Jiang, G.; Jiang, X.; Lü, P.; Liu, J.; Gao, J.; Zhang, C. The Rose (*Rosa hybrida*) NAC transcription factor 3 Gene, *RhNAC3*, involved in ABA signaling pathway both in rose and Arabidopsis. *PLoS ONE* **2014**, *9*, e109415. [CrossRef] [PubMed]

45. Yang, X.; Wang, X.; Ji, L.; Yi, Z.; Fu, C.; Ran, J.; Hu, R.; Zhou, G. Overexpression of a *Miscanthus lutarioriparius* NAC gene *MlnAC5* confers enhanced drought and cold tolerance in Arabidopsis. *Plant Cell Rep.* **2015**, *34*, 943–958. [CrossRef] [PubMed]

46. Yoshida, T.; Mogami, J.; Yamaguchi-Shinozaki, K. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Curr. Opin. Plant Biol.* **2014**, *21*, 133–139. [CrossRef]

47. Kim, S.Y.; Chung, H.J.; Thomas, T.L. Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. *Plant J. Cell Mol. Biol.* **1997**, *11*, 1237–1251. [CrossRef]

48. Lopez-Molina, L.; Chua, N.H. A null mutation in a bZIP factor confers ABA-insensitivity in Arabidopsis thaliana. *Plant Cell Physiol.* **2000**, *41*, 541–547. [CrossRef]

49. Puranik, S.; Sahu, P.P.; Srivastava, P.S.; Prasad, M. NAC proteins: Regulation and role in stress tolerance. *Trends Plant Sci.* **2012**, *17*, 369–381. [CrossRef]
50. Nakashima, K.; Ito, Y.; Yamaguchi-Shinozaki, K. Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. *Plant Physiol.* 2009, 149, 88–95. [CrossRef]

51. Jiang, C.; Ju, B.; Singh, J. Requirement of a CCGAC cis-acting element for cold induction of the BN115 gene from winter *Brassica napus*. *Plant Mol. Biol.* 1996, 30, 679–684. [CrossRef][PubMed]

52. Xue, G. Characterisation of the DNA-binding profile of barley HrCBF1 using an enzymatic method for rapid, quantitative and high-throughput analysis of the DNA-binding activity. *Nucleic Acids Res.* 2002, 30, e77. [CrossRef][PubMed]

53. Agarwal, M.; Hao, Y.; Kapoor, A.; Dong, C.H.; Fujii, H.; Zheng, X.; Zhu, J.K. A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J. Biol. Chem* 2006, 281, 37636–37645. [CrossRef][PubMed]

54. Svensson, J.T.; Crosatti, C.; Campoli, C.; Bassi, R.; Stanca, A.M.; Close, T.J.; Cattivelli, L. Transcriptome analysis of cold acclimation in barley *albina* and *xantha* mutants. *Plant Physiol.* 2006, 141, 257–270. [CrossRef][PubMed]

55. Tran, L.S.; Nakashima, K.; Sakuma, Y.; Osakabe, Y.; Qin, F.; Simpson, S.D.; Maruyama, K.; Fujita, Y.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. *Plant J.* 2007, 49, 46–63. [CrossRef][PubMed]

56. Waadt, R.; Schmidt, L.K.; Lohse, M.; Hashimoto, K.; Bock, R.; Kudla, J. Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. *Plant J.* 2008, 56, 505–516. [CrossRef][PubMed]

57. Bent, A. Arabidopsis thaliana floral dip transformation method. *Methods Mol. Biol.* 2006, 343, 87–103. [CrossRef][PubMed]

58. Jefferson, R.A.; Kavanagh, T.A.; Bevan, M.W. GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 1987, 6, 3901–3907. [CrossRef][PubMed]

59. Cao, W.H.; Liu, J.; He, X.J.; Mu, R.L.; Zhou, H.L.; Chen, S.Y.; Zhang, J.S. Modulation of ethylene responses affects plant salt-stress responses. *Plant. Physiol.* 2007, 143, 707–719. [CrossRef]

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