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Comprehensive Analysis of GRAS Gene Family and Their Expression under GA3, Drought Stress and ABA Treatment in *Larix kaempferi*

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Abstract: The *GRAS* family transcription factors play important roles in regulating plant growth and responses to abiotic stress, which can be utilized to breed novel plants with improved abiotic stress resistance. However, the GRAS gene family has been largely unexplored for tree species, particularly for *Larix kaempferi*, which has high economic and ecological values, challenging practices for breeding abiotic stress-resistant *L. kaempferi*. In order to improve the stress resistance by regulating the transcription factors in *L. kaempferi*, we identified 11 *GRAS* genes in *L. kaempferi* and preliminarily characterized them through comprehensive analyses of phylogenetic relationships, conserved motifs, promoter *cis*-elements, and expression patterns, as well as protein interaction network prediction. The phylogenetic analysis showed that the *LkGRAS* family proteins were classified into four subfamilies, including DELLA, HAM, SCL, and PAT1, among which the SCL subfamily was the largest one. Conserved motif analysis revealed many putative motifs such as LHRI-VHIID-LHRII-PFYRE-SAW at C-terminals of the *LkGRAS* proteins; we discovered a unique motif of the *LkGRAS* genes. Promoter *cis*-acting element analysis exhibited several putative elements associated with abiotic stresses and phytohormones; the abscisic acid-responsive elements (ABRE) and G-box are the most enriched elements in the promoters. Through expression profiles of *LkGRAS* genes in different tissues and under drought-stress and phytohormones (GA_3 and ABA) treatments, it was demonstrated that *LkGRAS* genes are most active in the needles, and they rapidly respond to environmental cues such as drought-stress and phytohormone treatments within 24 h. Protein interaction network prediction analysis revealed that *LkGRAS* proteins interact with various proteins, among which examples are the typical GA, ABA, and drought-stress signaling factors. Taken together, our work identifies the novel *LkGRAS* gene family in *L. kaempferi* and provides preliminary information for further in-depth functional characterization studies and practices of breeding stress-resistant *L. kaempferi*.

Keywords: *Larix kaempferi*; GRAS family; genome-wide analysis; phytohormone; drought stress; qRT-PCR

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1. Introduction

The GRAS gene family encodes a large transcription factor (TF) family crucial for plant growth, development, and responses to environmental stresses. Its name "GRAS" was derived from three TFs including GAI (Gibberellic Acid Insensitive), RGA (Repressor of GAI), and SCR (Scarecrow) which are the typical members of GRAS TFs [1]. The GRAS domain is conserved throughout the GRAS TFs at the carboxyl (C)-terminus, which mainly includes the five motifs, namely, LHR I (Leucine Heptapide Repeat I), LHR II, VHIID, PFYRE, and SAW [2], while they have a high degree of variability at the amino (N)-terminus [3]. It is currently known that the GRAS gene family consists of seven to 16 subfamilies, and the number depends on the plant species; seven in *Arabidopsis thaliana* [4], eight in *Oryza sativa* [3], 11 in *Citrus sinensis* [5], 13 in *Ricinus communis* [6], and 16 in *Medicago truncatula* [7].

The *GRAS* genes play significant roles in plant growth, development, and defense responses to various biotic and abiotic stresses, as well as phytohormone signaling and symbiosis formation. Their expression has been observed in various plant organs and tissues, including needle, stem, root, fruit, coleoptile, radicle, anther, and silk [8], and vary according to developmental stages and environmental conditions [9,10], suggesting their roles in plant development and response to environmental cues. DELLA, DLT, HAM, PAT1, LAS, LISCL, SCR, SCL3, SHR, and SCL4/7 are typical subfamilies of GRAS proteins [11] that have been implicated in plant development as follows. In *A. thaliana*, DELLA is a central regulator that plays a major role in regulating GA signal [12], and HAM is involved in chlorophyll synthesis, the proliferation of meristem cells, and polar organization [4,13,14]. PAT1 is a putative component of the phytochrome A signaling pathway [4], while the LAS subfamily increases inflorescence number [15], shortens flowering time [6], and promotes flowering induction [16] and lateral bud growth [17,18]. LlSCL regulates the pre-meiotic phase of anthers and promotes microspore genesis [19], and SCL3 integrates the gibberellin acid (GA) pathway [12]. The SHR and SCR complex participates in controlling plant organ development [20,21]. In addition, several *GRAS* genes are known to be associated with plant responses to abiotic stresses. In tobacco, *GRAS1* was induced by various stresses, which then increasing the level of reactive oxygen species [15]. Overexpression of *PAT1* enhanced tolerance to abiotic stress in *Arabidopsis* [22]. The SCL4/7 subfamily members in rapeseed enhanced tolerance against drought and salt stresses [23]. *GRAS6*-silenced tomato plants showed increased sensitivity to drought stress [20]. By regulating the expression of the stress-related gene, *GRAS23* has been demonstrated to enhance resistance against drought and oxidative stress in rice by regulating several stress-related genes [24]. In tomato, the *GRAS40* gene is essential to regulate the activation of abiotic stress-inducible promoters and auxin and gibberellin signaling [25].

L. kaempferi is an important fast-growing native tree species in northern China that has high economic and ecological value. *L. kaempferi* belongs to a conifer species, generally called larch trees, with great value for wood production and ecological afforestation. Larch trees constitute forests in large areas of China, Eastern Europe, and Western North America. Among larch trees, *L. kaempferi* has several superiorities over others; it grows faster at the juvenile stage, has longer, fibrous, denser wood, and can adapt more easily to the environment than other larch trees. Thus, *L. kaempferi* is now recognized as an important tree species for various economical uses, such as timber and pulp production and papermaking, as well as afforestation and ornamental purposes. The problem is that recent climate-change-derived abiotic stresses such as drought are severely challenging afforestation practices of *L. kaempferi*, which calls for breeding novel *L. kaempferi* varieties with improved abiotic stress resistance. The *GRAS* gene family is a candidate gene family that can be utilized to breed novel *L. kaempferi* varieties with improved abiotic stress resistance. However, the *GRAS* gene family has not yet been largely explored in *L. kaempferi*, probably due to the unavailability of *L. kaempferi* genome information. The whole genome of *L. kaempferi* was recently sequenced [26] and it is, therefore, possible to perform genome-wide identification analysis for important TFs such as the GRAS TFs.

In this study, we, for the first time, identified the GRAS gene family in the *L. kaempferi* whole genome and then performed comprehensive analyses. In total, we identified 11 *GRAS* genes from the *L. kaempferi* whole genome and analyzed the evolutionary relationship, conserved motifs, and promoter *cis*-elements. We further analyzed the expression pattern of *LkGRAS* genes in different organs and tissues, including the root, stem, and needles in *L. kaempferi*. We also analyzed the expression of the *GRAS* genes under GA3, ABA, and drought treatments. Finally, we predicted the protein interaction network of *LkGRAS* proteins. This study provides a comprehensive overview of the *L. kaempferi* GRAS gene family as well as a preliminary basis for further in-depth research on the roles of *LkGRAS* factors in regulating *L. kaempferi* responses to phytohormone and abiotic stresses. More importantly, this study provides valuable information for further studies of *L. kaempferi* to improve stress resistance by regulating transcription factors.

2. Materials and Methods

2.1. Genome-Wide Identification and Phylogenetic Analysis of LkGRAS Genes

The genomic DNA, CDS, and protein sequences of *L. kaempferi* were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) (accessed on 11 September 2021). Whole GRAS family members were searched in *L. kaempferi* using profile hidden Markov models (HMM); the GRAS binding domain (PF03514) was queried in the Pfam database (http://pfam.xfam.org/) (accessed on 11 September 2021) and then used to search all putative *L. kaempferi* GRAS protein members with the HMMER3 package. Redundant sequences were manually detected and eliminated, and then the remaining sequences were examined to confirm whether the GRAS binding domain is conserved throughout the sequences using the online programs CDD (https://www.ncbi.nlm.nih.gov/cdd) (accessed on 11 September 2021), Pfam (http://pfam.xfam.org/) (accessed on 11 September 2021), and SMART (http://smart.embl-heidelberg.de/) (accessed on 11 September 2021). The *L. kaempferi* GRAS protein sequences were aligned and visualized using EMBL-EBI (https://www.ebi.ac.uk/Tools/services/web/tool/) (accessed on 23 December 2021) and Jalview. We set the basic options, including "annotations, format, and color". The physical and chemical properties of the *L. kaempferi* GRAS proteins were analyzed using the ExPASy proteomics server (http://web.expasy.org/protparam/) (accessed on 23 December 2021) to analyze the characteristics of the GRAS proteins.

The amino acid sequences of GRAS proteins in *A. thaliana* and *O. sativa* were downloaded from Phytozome (Phytozome v12.1: Home) and then aligned using Clustal X (version 2.0) and Bioedit (version 7.2.5) with a gap opening penalty and gap extension penalty of 10 and 0.1, respectively. Molecular features and phylogenetic relationships between the *GRAS* genes of *L. kaempferi*, *A. thaliana*, and *O. sativa* were analyzed using MEGA software (v7.0) with the maximum likelihood method parameters as the Poisson model, partial deletion (95%), and 500 bootstrap replications [27].

2.2. Conserved Motif and Promoter Cis-Element Analysis of LkGRAS Genes

Conserved motifs in the *LkGRAS* genes were investigated using MEME (Multiple Em for Motif Elicitation program 5.1.1; http://meme-suite.org/tools/meme) (accessed on 23 December 2021) with the following parameters: the maximum number of motifs was set to 15, and the optimum motif width was set to 6 to 50 residues [28]. The Pfam and SMART tools were used to perform each structural motif annotation.

The sequences of *LkGRAS* genes were downloaded from the *L. kaempferi* genome database in NCBI, and their promoters, 2000 bp upstream of the translation start site, were identified. Then, putative *cis*-elements were searched throughout the promoters using the online database PlantCARE [29].

2.3. Plant Materials and Treatments

Mature seeds of *L. kaempferi* were collected from 60-year-old trees in Qing Shan national Larch seed orchard in Heilongjiang province (the geographical coordinates are 133°53′28″–133°58′05″ E and 46°38′56″–46°44′20″ N) and stored at −20 °C. The seeds were sown in plastic pots (11 × 11 cm) containing a grit/soil mixture (1:3 ratio), and 30 days later, seedlings were transferred to 15 cm pots (one plant per pot) containing a grit/soil mixture (1:1 ratio). The seedlings were cultured for five months under a 16 h/8 h light/dark photoperiod, 150 μmol m^{-2} s^{-1} light intensity, 70% relative humidity [30], and the soil water content was kept at ≥70% field capacity [31].

We sampled roots, stem, and needles, respectively, before treatments to determine the tissue-specific expression pattern. For the ABA and GA treatment, the solution containing 100 μM ABA or 100 μM GA3 was prepared and sprayed on needles of the *L. kaempferi* seedlings [32]. The needles were then collected at 0, 6, 12, and 24 h after treatment [32] for further RNA extraction. In addition, for drought-stress treatment, watering was stopped, and soil moisture contents were temporally measured by the gravimetric method [26]. The degree of drought stress was determined by the soil moisture contents as follows: 70%–80%

(CK, non-drought), 50%–60% (mild drought, MD), and 20%–35% (severe drought, SD) of the maximum field water capacity [33]. Temporal change in soil water contents is shown in Figure S1 and the needles were sampled at 6, 9, and 12 d for further RNA extraction. The plants at 0 days were used as control. All the treatments were sampled with three biological repeats for each seedling. The needles were carefully sampled and frozen immediately in liquid nitrogen, stored at −80 °C until RNA extraction.

2.4. RNA Extraction and Gene Expression Analysis by qRT-PCR

Total RNA was extracted from the needles using the CTAB (cetyltrimethylammonium bromide) method [34] and then reverse-transcribed to cDNA by Hi Script® II Q Select RT Super Mix for qRT-PCR. The genome DNA was eliminated by gDNA Wiper Mix. The primers were designed and checked for *LkGRAS* genes using the NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools) (accessed on 23 December 2021). The qRT-PCR was performed to determine transcript levels of *LkGRAS* genes using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) according to the manufacturer's instructions. The whole-genome sequencing of *GRAS* genes in the *L. kaempferi* gene (Whole Genome Shotgun (WGS): INSDC: WOXR00000000.2) was used as target genes. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control gene [30]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression levels. All *LkGRAS* gene-specific primers used for qRT-PCR are listed in Table S1.

The qRT-PCR data were tabulated and loaded by HEML to generate a heat-map. We set "canvas" and "space" to resize the heat map. We also determined the position of the X and Y axes, meanwhile selecting "column and row" to generate the branch network. We set "note" to adjust the basic setting of the font, including size and color. In the end, we set "logarithmic 2" in the option of "statistics" and exported the image.

2.5. Protein Interaction Network Analysis

The STRING (version 11.0; https://string-db.org/cgi/input.pl) (accessed on 23 December 2021) database was employed to predict the protein interaction network of *LkGRAS* proteins; prediction was performed using amino acid sequence of *LkGRAS* proteins as query and *Arabidopsis thaliana* as the "organism". The basic settings included "evidence" and "text-mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence". The minimum required interaction score was set as medium confidence of 0.4.

2.6. Statistical Analysis of Data

The experimental data were analyzed by one-way analysis of variance (ANOVA) method using SPSS software (version 20, IBM, Chicago, IL, USA) to evaluate significant differences between the control and each treatment. Significant differences were defined as * $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1. Identification of GRAS Genes Family in L. kaempferi

To determine the information of the *GRAS* family member in *L. kaempferi*, we identified 11 GRAS genes in *L. kaempferi* genome using HMM profile of the GRAS binding domain (PF03514) as a query and then analyzed their basic information as follows. Domain search analysis using SMART and Pfam databases demonstrated that all encoded *LkGRAS* proteins possess GRAS domains. We named these genes from *LkGRAS1* to *LkGRAS11* (Table 1). The number of protein lengths, molecular weight, grand average of hydrophilicity (GRAVY), and isoelectric points are shown in Table 1. The length of GRAS proteins in *L. kaempferi* is between 223 and 730 amino acids, and the molecular weights are from 25.25 kDa to 86.22 kDa. The predicted theoretical point (pI) value varies from 5.12 to 7.07. GRAVY values of all *LkGRAS* proteins are below zero, ranging from −0.533 to −0.075, suggesting that *LkGRAS* proteins belong to the hydrophilic protein group. The instability index for most *LkGRAS* proteins is greater than 40, indicating that most *LkGRAS* proteins are unstable. Only

three *LkGRAS* proteins have a stable index from 37.84 to 39.67. The aliphatic index of all *LkGRAS* proteins ranged from 71.97 to 91.82. The research showed that the aliphatic index usually shows the domination of aliphatic side chains to indicate thermal stability [35].

Table 1. Basic information of *L. kaempferi GRAS* family members.

Name	Gene ID	Length	Molecular Weight (kDa)	Theoretical pI	GRAVY Value
LkGRAS1	Lk_f2p60_2509	619	68.86	5.12	−0.336
LkGRAS2	Lk_f2p57_2714	721	80.35	5.16	−0.533
LkGRAS3	Lk_f2p39_2015	594	64.40	5.65	−0.075
LkGRAS4	Lk_f4p60_3081	696	77.89	6.31	−0.423
LkGRAS5	Lk_f2p60_2987	730	82.16	5.67	−0.459
LkGRAS6	Lk_f2p49_1552	447	50.46	6.10	−0.331
LkGRAS7	Lk_f2p39_2775	781	86.22	5.19	−0.358
LkGRAS8	Lk_f2p16_2684	634	71.62	5.58	−0.291
LkGRAS9	Lk_f2p7_2221	476	51.89	7.07	−0.233
LkGRAS10	Lk_f2p60_2999	228	25.77	6.23	−0.258
LkGRAS11	Lk_f2p49_1141	223	25.25	5.66	−0.238

Figure 1 shows the multiple sequence alignments of the GRAS gene family members of *L. kaempferi*. In the multiple sequence alignments outcome, the blue color and its intensity represent conserved domains and their homology degrees; darker color means a higher homology level. There are four conserved domains, including LHR (C1), PFYRE (C2), VHIID (C3), and SAW (C4).

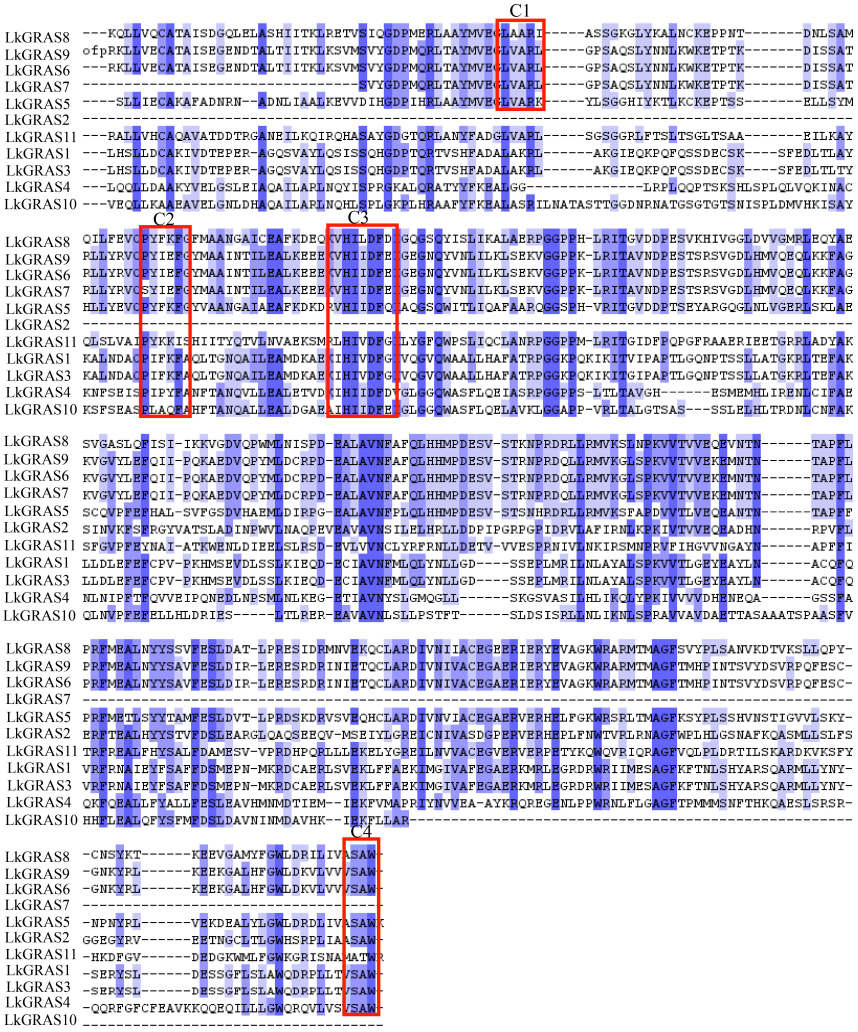

Figure 1. Multiple sequence alignments of the *L. kaempferi* GRAS gene family members. Blue shading marks identical residues, light blue shading marks conserved residues. Positions of the basic region of the GRAS domain and conserved domains (**C1–C4**) are demarcated by lines above sequences.

3.2. Phylogenetic Analysis of L. kaempferi GRAS Proteins

To investigate the evolutionary relationships and classification of the GRAS family in *L. kaempferi*, 37 *A. thaliana*, 63 *O. sativa*, and 11 *LkGRAS* proteins were used to construct a phylogenetic tree with the neighbor-joining (NJ) method in MEGA7.0 (Figure 2). According to the two clusterings and the relationship with *A. thaliana* and *O. sativa*, the GRAS proteins were classified into eight subfamilies (LISCL, RGL, PAT1, SCR, HAM, SCL3, SCL4/7, and DELLA). There are eight *LkGRAS* proteins belonging to the SCL (4) and PAT (4) subfamilies, while the other three proteins belong to the DELLA (1) and HAM (2). LkGRAS2, −5, −6, −7, −8, −9 proteins were clustered with the OsGRAS proteins, whereas LkGRAS1, −3, −4, −10, −11 were clustered with AtGRAS proteins. This indicates that the function of OsGRAS and AtGRAS proteins may provide a reliable reference to *LKGRAS* proteins.

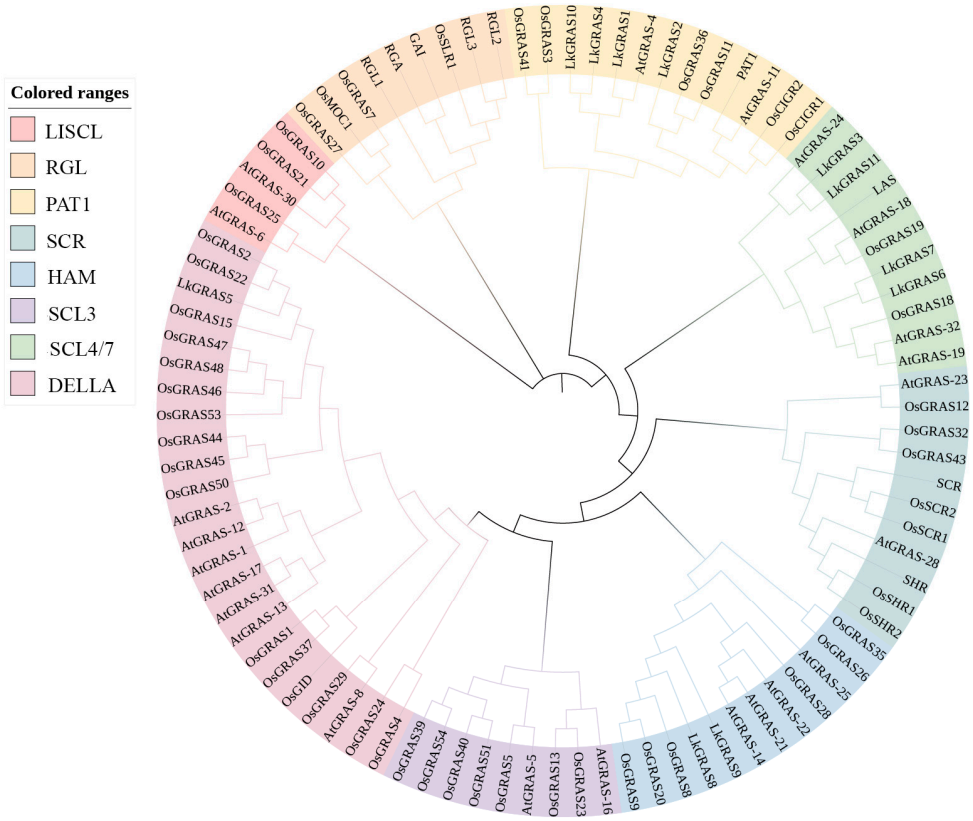

Figure 2. Phylogenetic analysis of the GRAS gene family members from *L. kaempferi*, *O. sativa*, and *A. thaliana*. Branches with less than 50% bootstrap support were collapsed. The phylogenetic tree was constructed using the maximum likelihood (ML) method of MEGA 7.0 with 500 bootstrap replicates.

3.3. Conserved Motifs of LkGRAS Proteins

The motifs analysis contributes to comprehensively understand the conserved characteristics of *LkGRAS* proteins and analyze structure in their conserved domain. We further confirmed the conserved motifs of *LkGRAS* proteins using MEME. In total, 15 distinct motifs were detected and named motif 1 to motif 15 (Figure 3). Since the structures and functions of the *LkGRAS* are not recognized completely, the motifs were defined based on sequence conservation. As per the previous research in GRAS domains characterization analysis, the LHRI-VHIID-LHRII-PFYRE-SAW structure domain determined the arrangements of motifs [1]. Motif 5 was highly conserved at the outermost part of C-terminal regions except for LkGRAS9 and LkGRAS10. The motifs were distributed mostly in the C-terminal. There were 10 motifs (motifs 1, 2, 3, 4, 5, 7, 8, 10, 12, and 14) in the C-terminal, while the remaining motifs (including motifs 6, 9, 11, 13, and 15) were at the N-terminal. Our results showed that conserved GRAS domains, including LHRI, VHIID, LHRII, PFYRE, and SAW domains

(previously discovered by Pysh et al., 1999), included motif 1 (in VHIID domain), motif 2 (in PRYRE and SAW domains), motif 4 (in LHRII domain), motifs 5 and 6 (in LHRI domain), motif 5 (in SAW domain), and motif 7 (in PRYRE domain) (Figure S2). The motif 3 and motif 8 to motif 15 were not found to form a structure in certain domains in *LkGRAS* proteins, but they were still an indispensable part of the conserved structure domain [36].

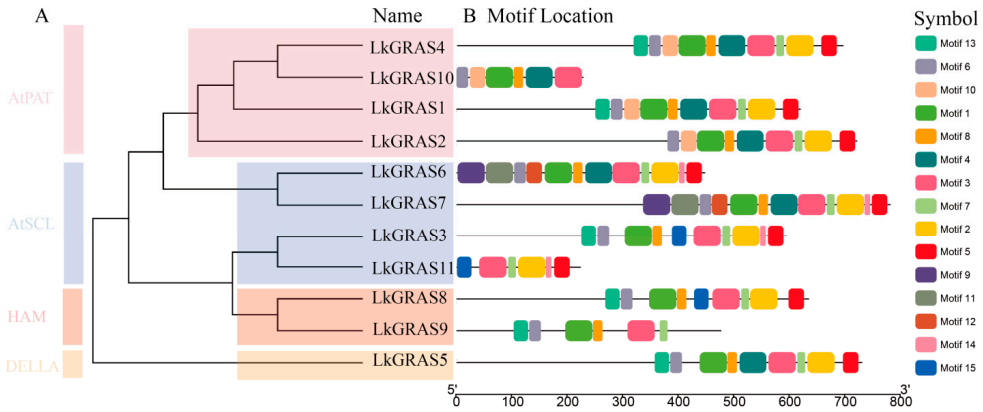

Figure 3. Phylogenetic relationships and conserved motifs of *LkGRAS* proteins. Phylogenetic tree (**A**) of *LkGRAS* proteins was constructed by using the neighbor-joining (NJ) method with 1000 bootstrap replicates in MEGA 7.0, and conserved motifs (**B**) were obtained using MEME.

3.4. Promoter Cis-Element Analysis

To understand possible regulation mechanisms of the *LkGRAS* genes, we analyzed the promoters of *LkGRAS* genes using PlantCARE and identified nine putative stress-related and phytohormone-related *cis*-elements (Figure S3). They include drought-inducibility elements (MBS) and low-temperature responsive elements (LTR), stress- and defense-responsive elements (TC-rich repeats elements), CGTCA/TGACG (MeJA-responsive elements), TCA-element (salicylic-acid-responsive elements), TGA-element (auxin-responsive elements), ABRE elements (abscisic-acid-responsive elements), and TA-rich repeats TC-box (gibberellin-responsive elements), as well as Box4 and G-box (light-responsive elements) (Table 2). The presence of these various stress- and phytohormone- responsive *cis*-elements suggested putative roles of *LkGRAS* genes in plant growth, development, and responses to abiotic stresses.

Table 2. *Cis*-element analysis of promoter regions of *LkGRAS* genes.

Name	MRE	MBS	LTR	ABRE	TGA	TCA	Box4	G-Box	CGTCA	TGACG
LkGRAS1	0	0	0	1	0	0	1	0	0	0
LkGRAS2	2	0	4	0	0	2	3	1	0	0
LkGRAS3	0	0	0	3	1	1	3	2	4	4
LkGRAS4	1	1	0	0	0	2	0	0	1	1
LkGRAS5	0	1	0	2	0	2	0	2	1	1
LkGRAS6	0	0	0	5	0	0	0	9	5	5
LkGRAS7	0	0	1	5	0	1	0	6	4	4
LkGRAS8	0	1	0	0	1	0	0	5	0	0
LkGRAS9	0	2	1	3	1	0	4	0	0	0
LkGRAS10	0	2	1	0	1	3	0	0	1	1
LkGRAS11	1	0	0	3	2	0	1	1	3	3

3.5. Tissue-Specific Expression Pattern of LkGRAS Genes

Tissue-specific expression profile for the genes belonging to a plant gene family reflects their tissue-specific functions. To determine tissue-specific expression profile of *LkGRAS* genes, we performed qPCR to analyze *LkGRAS* gene expression patterns in roots, stems, and needles at the same developmental stages and then generated a heat map (Figure 4) using

the qPCR data. Expression levels of 11 *LkGRAS* genes were different to each other in the same tissue. In addition, different tissues exhibited different expression levels of *LkGRAS* genes. Most *LkGRAS* genes were weakly expressed in root tissues except the *LkGRAS10*, while they showed much higher expression levels in needle and stem tissues. In addition, the *LkGRAS10* showed the highest expression level among the *LkGRAS* genes in roots and needles, as well as high expression level in stem tissue. Taken together, we demonstrated that *LkGRAS* genes are expressed in mostly needle, and among them, *LkGRAS10* showed relatively high expression levels in all kinds of tissues tested here.

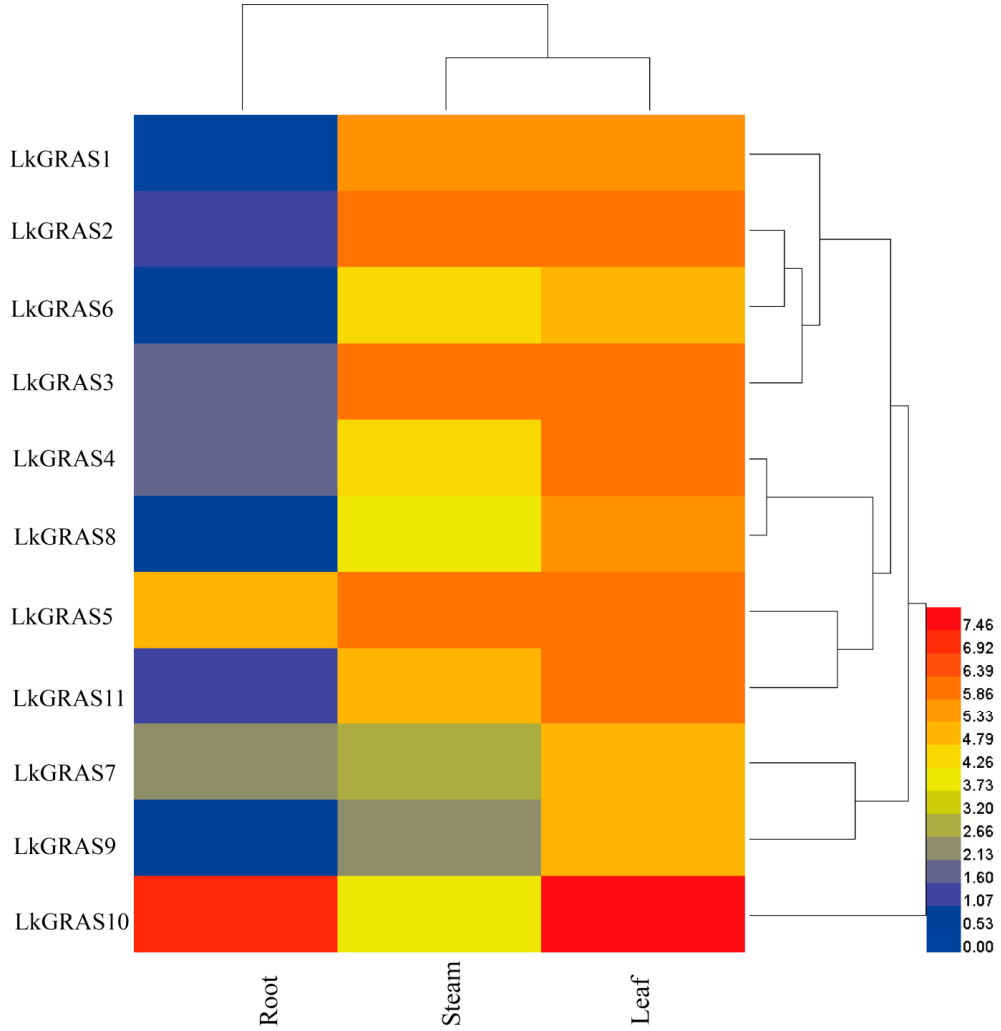

Figure 4. Tissue-specific expression pattern of *LkGRAS* genes. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Different colors represent different expression levels: blue, green, and red colors represent low, mild, and high expression levels, respectively.

3.6. Expression Analysis of LkGRAS Genes under GA_3, ABA Treatment, and Drought Stress

The presence of various stress- and phytohormone-responsive *cis*-elements suggested involvement of *LkGRAS* genes in plant growth, development, and responses to abiotic stresses. To examine whether the *LkGRAS* genes take part in the abiotic stress and phytohormone response, we performed qPCR to analyze the expression level of *LkGRAS* genes in needles of *L. kaempferi* plants subjected to GA_3 (100 μM), ABA (100 μM) treatment, and drought stress. Fold change > 2 was considered as significantly differentially expressed genes. Firstly, we analyzed the *LkGRAS* gene expression under GA_3 treatment. As shown in Figure 5, all *LkGRAS* genes showed responses to exogenous GA_3 treatment with diverse expression profiles; nine *LkGRAS* genes were upregulated, among which the expression

levels of *LkGRAS4*, *5*, and *7* shown were very significant. *LkGRAS6* and *10* did not show a significant response to GA_3 treatment (no more than twofold). Duration of GA_3 treatment also differentially influenced the expression pattern of the *LkGRAS* genes. *LkGRAS1*, *3*, and *8* were upregulated and reached a peak at 6 h, and *LkGRAS2*, *4*, *5*, and *7* at 12 h. The expression levels of *LkGRAS9* and *11* consistently increased for 24 h. *LkGRAS4*, *5*, and *7* had the highest expression levels among 11 *LkGRAS* genes in response to GA_3 treatment. Then, we analyzed the *LkGRAS* gene expression under drought stress. Except for *LkGRAS1*, *3*, *8*, and *9*, the other *LkGRAS* genes showed significant response to drought stress (Figure 6). *LkGRAS5*, *6*, and *10* were initially upregulated (at 6 d after drought treatment), and then declined gradually later. *LkGRAS2*, *4*, *7*, and *11* showed upregulation and reached a peak at 9 d after treatment. Finally, we analyzed the *LkGRAS* genes expression under ABA treatment. The *LkGRAS* genes were sensitive to ABA treatment except for *LkGRAS3*, *6*, *8*, and *9* (Figure 7). Though the *LkGRAS* genes showed different expression levels, they had a similar expression tendency under ABA treatment. Notably, the *LkGRAS* genes were significantly induced at various points in time under ABA treatment. The expression level was upregulated and reached a peak at 6 h, then downregulated later. Nearly all genes were in line with this trend, but the *LkGRAS3*, *6*, *8*, and *9* always showed dramatically downregulated expression levels. The expression level of *LkGRAS3*, *6*, and *8* were upregulated no more than twofold and showed lower expression levels together with *LkGRAS9*, while the expression levels of the other *LkGRAS* genes (*LkGRAS2*, *4*, *5*, *7*, *10*, and *11*) compared to them were significant, and the expression levels of *LkGRAS4*, *5*, *7*, and *11* were very significant.

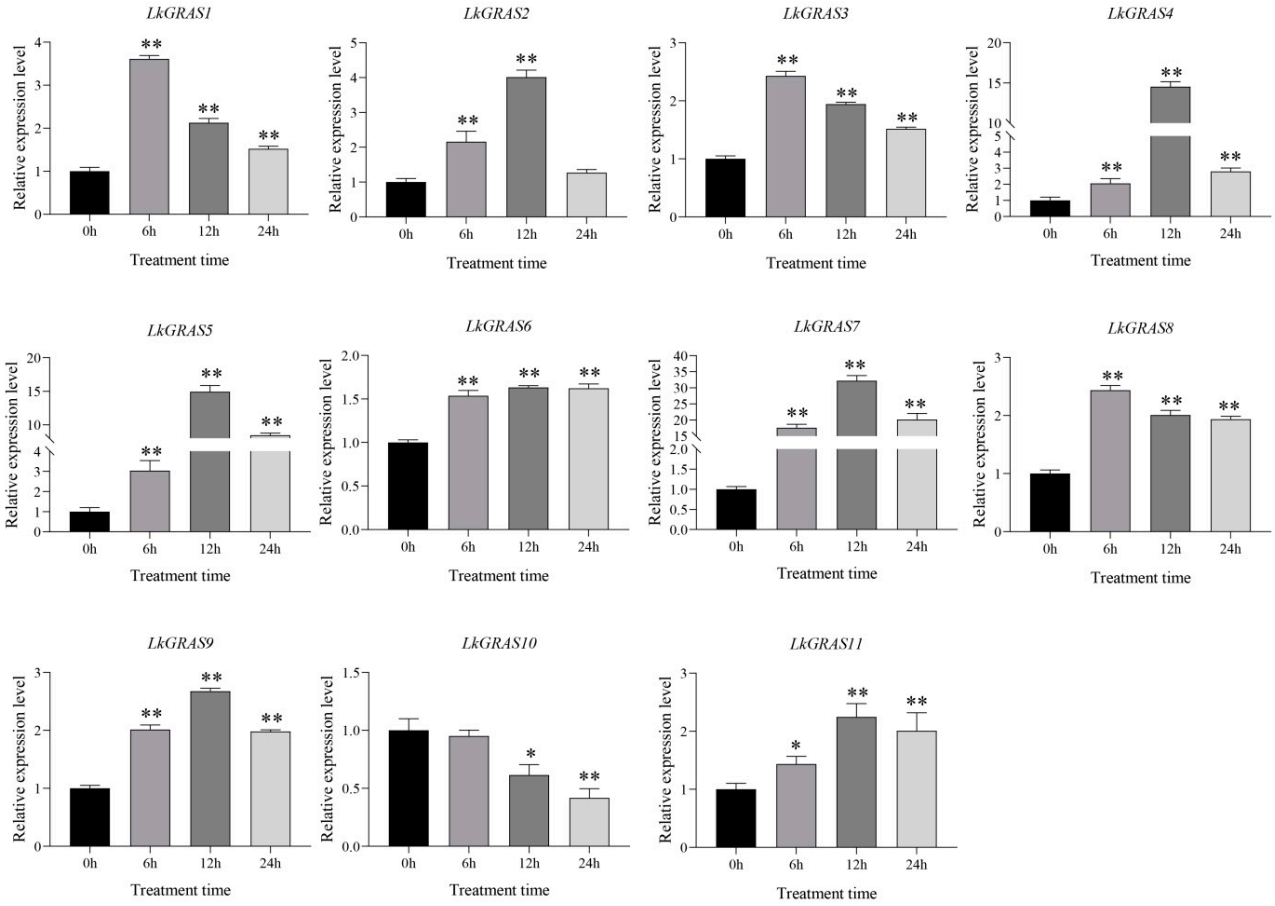

Figure 5. The relative expression level of the *LkGRAS* genes in needles under GA_3 treatment using qRT-PCR. Error bars represent the deviations from three biological replicates. The *x*-axis represents the time points after 100 μM GA3 treatment (* $p < 0.05$, ** $p < 0.01$).

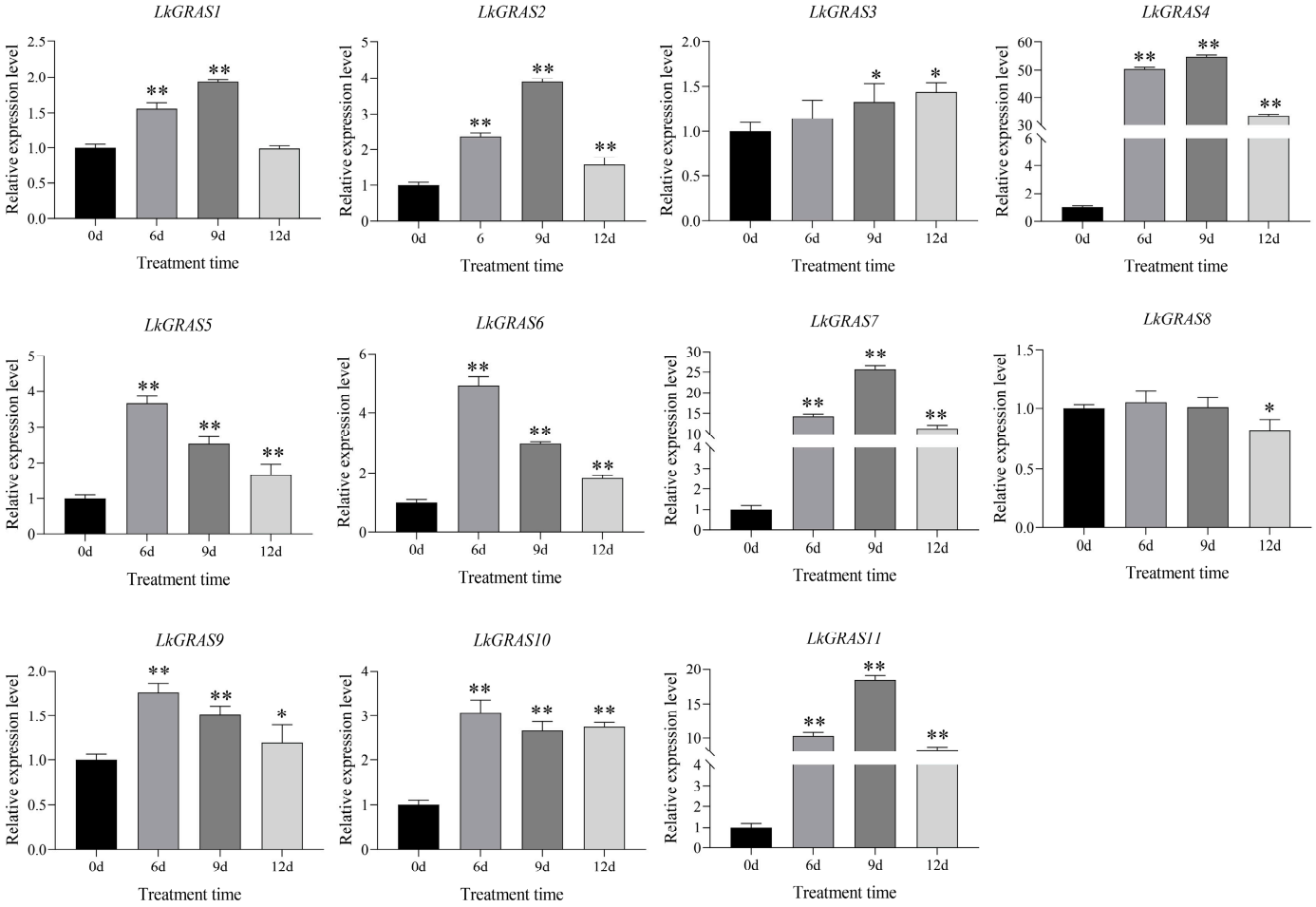

Figure 6. The relative expression levels of the *LkGRAS* genes in needles under drought stress using qRT-PCR. Error bars represent the deviations from three biological replicates. The *x*-axis represents the time points after drought stress (* $p < 0.05$, ** $p < 0.01$).

Collectively, the results showed that these *LkGRAS* genes responded to at least one kind of treatment. For instance, there were nine *LkGRAS* genes upregulated in the GA_3 treatment (*LkGRAS1*, *2*, *3*, *4*, *5*, *7*, *8*, *9*, *10*, and *11*) in which the *LkGRAS1*, *2*, *4*, *5*, *7*, *10* and *11* were upregulated in the ABA treatment. Apart from these *LkGRAS* genes, the *LkGRAS3* and *9* also showed opposite expression results. Moreover, among the six drought-inducible genes (*LkGRAS4*, *5*, *6*, *7*, *10*, and *11*), five were all upregulated by ABA (*LkGRAS4*, *5*, *7*, *10*, and *11*), and three by GA_3 (*LkGRAS4*, *5*, and *7*). Meanwhile, the expression levels of *LkGRAS4*, *5*, *7*, *10*, and *11* in ABA were consistent with those in drought, and *LkGRAS4* and *7* exhibited significantly positive responses to all three kinds of treatments.

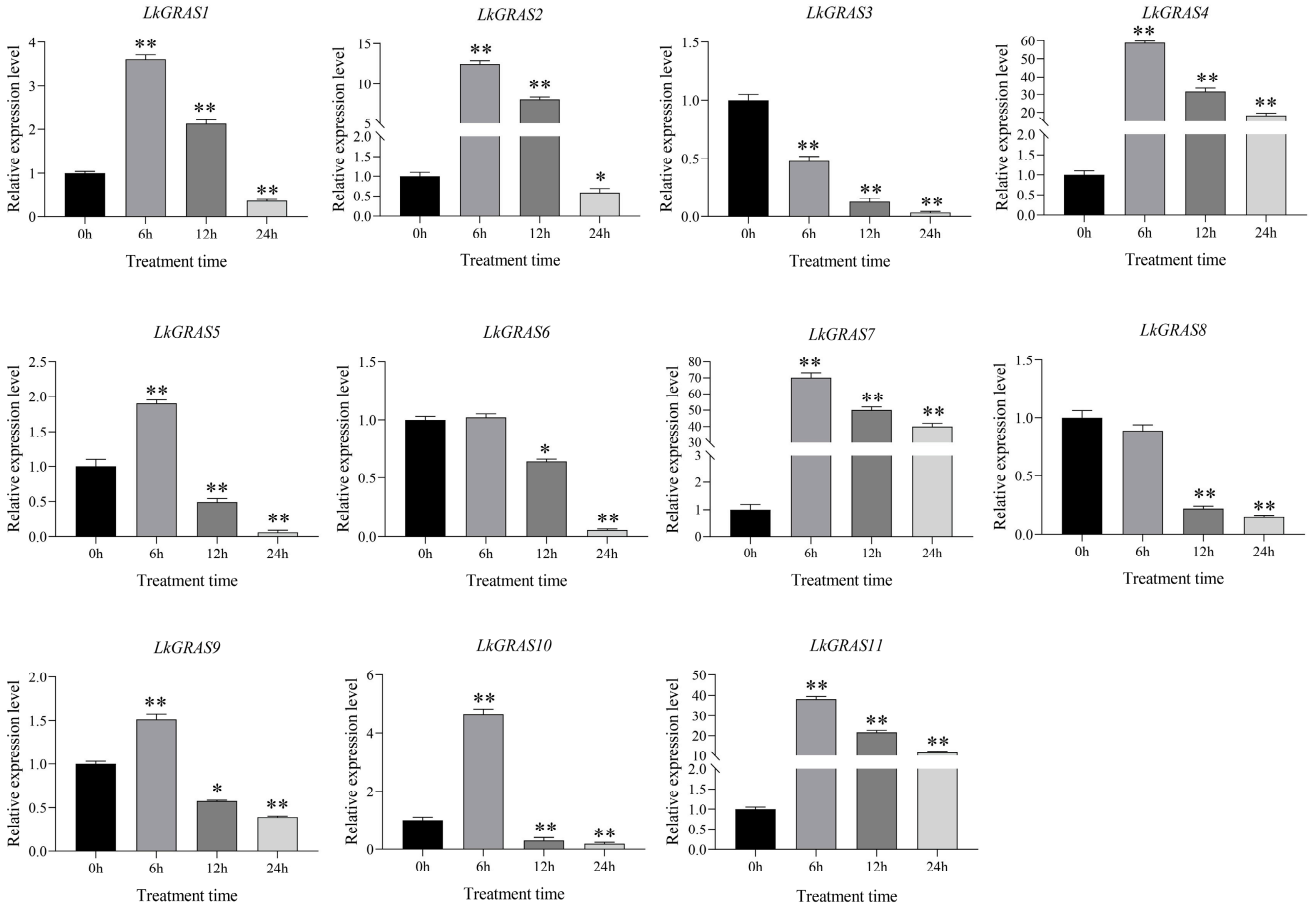

Figure 7. The relative expression levels of the *LkGRAS* genes in needles under ABA treatment using qRT-PCR. Error bars represent the deviations from three biological replicates. The *x*-axis represents the time points after 100 μM ABA treatment (* $p < 0.05$, ** $p < 0.01$).

3.7. Protein Interaction Network of LkGRAS Proteins

Proteins hardly implement their functions independently, but interact with other proteins to regulate cellular biological processes and prediction of the knowledge of protein–protein interactions (PPIs); therefore, they can untangle the cellular behaviors and functionality of the proteins. To identify the relationship of *LkGRAS* proteins with other proteins, we predicted the protein interaction network for *LkGRAS* proteins using STRING. Each *LkGRAS* protein sequence could obtain more than one network, and only the networks with the highest scores are shown in Figure 8. The networks revealed that *LkGRAS* proteins within a subfamily interact with the same proteins. For example, LkGRAS1 and LkGRAS2 of the PAT1 subfamily interact with SCL28, while LkGRAS1 and LkGRAS10 of the same subfamily interact with WAK. LkGRAS6 and LkGRAS7 of the SCL subfamily interact with MYB87, whereas LkGRAS3 and LkGRAS11 of the same subfamily interact with GID1. LkGRAS8 and LkGRAS9 of the HAM subfamily interact with WOX4. It seems that the proteins in a subfamily have highly similar motif alignments and therefore share the same protein targets to interact with each other. In addition, there are several GA, ABA, and drought-stress-related proteins, including SCL28/30, JAZ1, GID1, SLY1, GA3Ox1, PIF3, XBAT35, WDR55, and AT5G67411, among the interacting proteins, implying the interactions between them and *LkGRAS* proteins under GA, ABA, and drought-stress treatment.

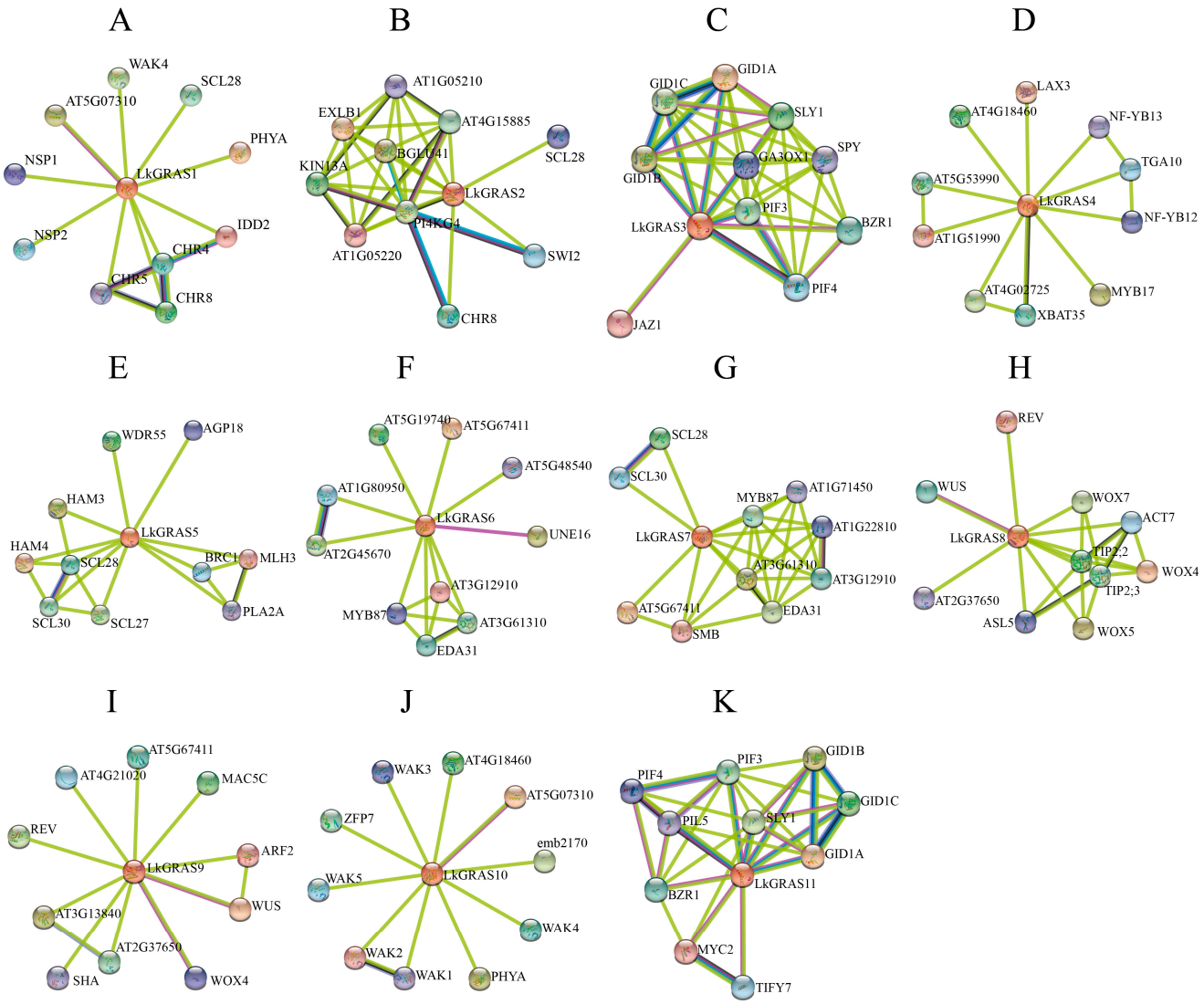

Figure 8. The predicted protein interaction network of *LkGRAS* proteins. (**A**–**K**) The potential protein interaction networks of each protein were predicted by the STRING database. Different colored lines represent different evidence of an interaction.

4. Discussion

The GRAS gene family encodes plant-specific TFs, which play essential roles in various biological processes. To date, the *GRAS* gene family has been extensively reported in various plant species including *A. thaliana* [37], *Brassica campestris* [38], *Brassica juncea* [16], *C. sinensis* [5], *Glycine max* [39], *Gossypium hirsutum* L. [10], *Ipomoea trifida* [40], *Juglans regia* L. [41], *Malus domestica* [42], *Manihot esculenta* [43], *M. truncatula* [7], *Nelumbo nucifera* [44], *O. sativa* [3], *Panax ginseng* [45], *Populus* L. [46], *R. communis* [6], *Solanum lycopersicum* [47], *Triticum aestivum* [48], and *Zea mays* L. [49]. Notably, the GRAS gene family has been largely unexplored in tree species; only reported in cassava [43] and poplar [46]. In our work, we identified the GRAS gene family in *L. kaempferi*, which is an economically and ecologically important tree species in northeastern China, for the first time. Then, we performed comprehensive analyses including phylogenetic analysis, conserved motif, and promoter *cis*-element analyses, tissue-specific and phytohormone and abiotic stress-triggered expression profile analysis, as well as protein interaction network prediction analysis for the *L. kaempferi* GRAS gene family.

Genome-wide identification and phylogenetic analysis revealed that the *LkGRAS* gene family (abbreviation of *LkGRAS* gene family) includes 11 *GRAS* genes which are further

classified into four main subfamilies: DELLA, HAM, SCL, and PAT1. Other subfamilies, such as DLT, LAS, LISCL, SCR, and SHR, are not found in the *LkGRAS* gene family; this would probably be due to incompleteness of *L. kaempferi* genome database or unique feature of the *L. kaempferi* species. The structure of *LkGRAS* genes further showed that they have highly conserved motifs at C-terminal regions; conserved motifs were arranged as LHRI-VHIID-LHRII-PFYRE-SAW at C-terminals, while their N-terminal regions showed high variability that may be associated with functional divergence among the *LkGRAS* proteins. All *LkGRAS* proteins except LkGRAS9 and 10 have the SAW motif in the C-terminal region, consistent with previous findings [4] that reported the presence of the SAW motif in the C-terminal region in the *A. thaliana* GRAS family. We also found that the *LkGRAS* proteins in the same subfamily have a similar motif arrangement in the C-terminal region. For example, the *LkGRAS* proteins of the PAT1 subfamily all have a motif5 and a motif7 arranged at the C-terminal region. In addition, the motif2 domain is present in both PAT1 and SCL subfamilies. It postulates that these *LkGRAS* genes might have similar functions in biological processes. In addition, promoter *cis*-element analysis indicated that the promoters of *LkGRAS* genes contain many *cis*-acting elements such as drought-inducibility elements (MBS) and low-temperature responsive elements (LTR), stress- and defense-responsive elements (TC-rich repeats elements), CGTCA/TGACG (MeJA-responsive elements), TCA-element (salicylic-acid-responsive elements), TGA-element (auxin-responsive elements), ABRE elements (abscisic-acid-responsive elements), and TA-rich repeats TC-box (gibberellin-responsive elements), as well as Box4 and G-box (light-responsive elements), suggesting the roles of GRAS TFs in the *L. kaempferi* response to environmental cues (drought, low temperature) and phytohormones (auxin, ABA, gibberellin, MeJA, and salicylic acid).

Due to the presence of putative stress and phytohormone-related *cis*-acting elements in the promoters of *LkGRAS* gene family members, the expression profiles of *LkGRAS* genes were investigated under drought, GA_3, and ABA treatments. Before this, expression of the *LkGRAS* genes was examined in different tissues and it was demonstrated that the *LkGRAS* genes were highly expressed in needles. Then, expression of the *LkGRAS* genes in needles was further investigated under drought, GA_3, and ABA treatments. Upon GA_3 treatment, *LkGRAS4*, *5*, and *7* showed relatively high expression compared to others. *LkGRAS5* belongs to the *DELLA* family gene. The DELLA proteins are known as repressors of gibberellin response in plants [50]; DELLA proteins are essential components in the intracellular GA_3 degradation system, negatively regulating GA_3 signaling in *Arabidopsis*. Many previous studies reported that the GA-DELLA module is conserved and plays a central role in GA signaling in plants [51–53]. Upregulation of *LkGRAS5* (*DELLA* subfamily) upon GA_3 treatment in our work was consistent with the findings in the above previous studies. These findings also verified our result indirectly, that when we apply exogenous GA to *L. kaempferi*, the GA oxidases genes of the *LkGRAS* family will show high expression levels of degraded gibberellin. In addition, under ABA treatment, *LkGRAS* that belong to *PAT1* and *SCL* subfamilies exhibited high expression, indicating that *PAT1* and *SCL* subfamilies are associated with the ABA pathway. *LkGRAS2*, *4*, *5*, *7*, and *11* showed relatively high expression levels compared to the other genes, and among them, *LkGRAS5*, *7*, and *11* were expressed at the highest levels. The presence of ABRE elements in the promoters of *LkGRAS5*, *7*, and *11* would be one of the putative reasons why they showed strong upregulation under ABA treatment. Moreover, the *LkGRAS* genes showed different expression patterns under GA_3 and ABA. This might be due to the antagonistic roles of GA and ABA in plant growth and development [54]. In our work, *LkGRAS2*, *4*, *5*, *7*, *10*, and *11* showed higher expression levels under ABA treatment than under GA_3 treatment (except for *LkGRAS2* and *11*), and *LkGRAS3*, *9*, and *10* showed contrasting patterns of expression under GA_3 and ABA treatments. These results implied that *LkGRAS* genes might be involved in the antagonistic effects of GA and ABA on plant growth and development. In addition to GA and ABA, plant response to drought stress is also known to be related to *GRAS* genes. Previous works revealed that the *AtPAT1* subfamily of the *GRAS* family gene in *Arabidopsis* could increase the tolerance of the plant to abiotic stress, such as cold,

drought, and salt [23,55]. The *SCL* subfamily was also demonstrated to participate in drought-stress response [24]. Consistently, our work also manifested that most of the *LkGRAS* genes responded to drought stress; among which *LkGRAS4* belongs to the *PAT1* subfamily and *LkGRAS7* and *11* belong to the *SCL* subfamily. It can be inferred that the *PAT1* and *SCL* subfamilies of *GRAS* genes in *L. kaempferi* are involved in drought-stress response. In addition, drought-stress-related *cis*-acting elements are present in promoters of the differentially expressed *LkGRAS* genes under drought stress. Among the drought-inducible genes, *LkGRAS4*, *7*, *10*, and *11* showed high expression levels in drought-stress and ABA treatment. In plants, signaling pathways of ABA and drought-stress response are interrelated with each other. It, therefore, appeared that drought and ABA treatments both induced the expression of *LkGRAS4*, *7*, *10*, and *11*. *LkGRAS4* and *7* also belong to the *PAT1* subfamily, which showed a high expression level under GA and ABA treatments. Overall, expression profiles of the *LkGRAS* genes showed consistency with the prediction from the *cis*-acting elements in promoters of the *LkGRAS* genes. The *LkGRAS* genes with drought-inducibility elements showed high expression levels under drought stress. The highly induced *LkGRAS* genes under GA_3 or ABA treatments also possess GA- or ABA-related *cis*-acting elements in their promoters. Each of the *LkGRAS* genes contains at least two *cis*-elements related to phytohormone or abiotic stress responsiveness.

Moreover, the protein interaction network of *LkGRAS* proteins was predicted using the STRING database, which could provide a supplementary understanding of orthologous proteins' roles in biological processes [56]. We found that the *LkGRAS* proteins within the same subfamily revealed similar protein interaction networks. Among the interacting proteins, we found that several factors, such as SCL28, JAZ1, GID1, SLY1, GA3Ox1, PIF3, XBAT35, WDR55, and AT5G67411, have previously been known to be associated with GA, ABA, and drought-stress responses. SCL28, which is a GRAS type TF in *A. thaliana* [57] and is known to be involved in ABA-mediated stress responses [58], interacts with LkGRTAS1, 2, 5, and 7 proteins. JAZ1, WDR55, and XBAT35 are also the ABA response factors [59–61], which are predicted to interact with LkGRAS3, 4, and 5 proteins, respectively, in our study. In addition, JAZ1 and WDR55, which can regulate drought-stress responses through ABA pathways, interact with LkGRAS3 and 5, respectively. PIF3, which is previously known to enhance resistance to drought stress [62], interacts with LkGRAS3 and 11. In addition to ABA and drought-related factors, there are GA response factors such as GA3Ox1, GID1, and SLY1 among the total interacting factors. GA3Ox1, which is the enzyme for GA biosynthesis, interacts with LkGRAS3 and GID1, which is a gibberellin receptor protein [63,64] and interacts with LkGRAS11. SLY1, which is known to positively regulate GA signaling [65], interacts with both LkGRAS3 and 11.

5. Conclusions

In conclusion, we identified 11 *GRAS* family genes in *L. kaempferi* and analyzed their phylogenetic tree, conserved motifs, and promoter *cis*-elements. The 11 *LkGRAS* genes are classified into four subfamilies, including *DELLA*, *HAM*, *SCL*, and *PAT1*. The *LkGRAS* proteins all have conserved LHRI-VHIID-LHRII-PFYRE-SAW motifs at C-terminals and their promoters contain many *cis*-acting elements associated with abiotic stresses and phytohormones. In addition, we evaluated the expression patterns of *LkGRAS* genes in different tissues and under GA_3, ABA, and drought-stress treatments using qRT-PCR. *LkGRAS* genes were mainly expressed in needles and were significantly induced upon exogenous treatment by phytohormones (GA_3 and ABA) and drought stress. We also predicted the protein interaction network of *LkGRAS* proteins. Preliminary results of our work on the *LkGRAS* gene families provided knowledge that would be the basic information for further in-depth functional characterization of *LkGRAS* family genes in *L. kaempferi*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13091424/s1, Figure S1: Temporal change in the soil water contents under non-watered condition; Figure S2: Conserved GRAS domains (LHRI, VHIID, LHRII,

PFYRE, and SAW domains); Figure S3: The nine putative stress-related and phytohormone-related cis-elements; Table S1: Primers for quantitative qRT-PCR.

Author Contributions: Conceptualization, M.M. and C.L.; experimental design: C.L.; material collection and performing the experiments: M.M., X.W., L.L. and C.Z.; data analysis: M.M.; software, M.M.; writing—original draft: M.M.; writing—review and editing: M.M., S.P. and C.L. All authors approved the final draft. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data is available on request from the corresponding author.

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