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Molecular Cloning and Functional Characterization of the *DELLA* Gene Family in *Liriodendron* Hybrids

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Abstract: DELLA proteins are key components of the gibberellins signal transduction pathway that play negative roles on promoting cell elongation and plant stature. However, the mechanisms underlying DELLA mediated growth inhibition in woody plant species are poorly understood. Here, we identified two *LhDELLA* genes including *LhSLR-like* (*LhSLRL*) and *LhRGA* from *Liriodendron* hybrids (*Liriodendron chinense* (Hemsl.) Sarg. × *L. tulipifera* L.), which is a horticultural tree with high-quality timber. Sequence analysis showed that *LhSLRL* and *LhRGA* possessed all typical conserved domains of DELLA proteins. Phylogenetic analysis showed that *LhRGA* was classified as the basal branch of DELLAs from species belonging to dicots. Interestingly, *LhSLRL* was a sister clade of DELLAs from the most ancient plant species such as bryophytes and ferns. Gene expression analysis showed that *LhSLRL* and *LhRGA* genes had the highest expression level in the stem. In addition, overexpression of *LhSLRL* decreased plant height and root length, increased branching and delayed flowering in *Arabidopsis thaliana*, while *LhRGA* overexpression enlarged leaves and reduced the number and length of roots. Overexpressing *LhSLRL* in tobacco caused a decreased plant height and the amount of root. Taken together, these results reveal that *LhDELLA* genes may play an important role in plant growth and development, especially in vegetative organs. Our results provide new insights into the function of *DELLA* genes in a woody plant, and contribute to a potential application of manipulating DELLA genetics.

Keywords: *Liriodendron* hybrids; DELLA; RGA; SLRL; gibberellin

1. Introduction

The plant hormones gibberellins (GAs) were first identified from rice diseased with the “bakanae” fungus (“Bakanae” fungus secretes a large amount of gibberellin, which makes rice grow steep) in 1926 and were officially named gibberellins in 1938. It is well known that GA induces a wide range of vegetative and reproductive developmental processes in plants, such as seed germination, hypocotyl elongation, stem elongation, leaf expansion, flower regulation, and fruit development [1,2].

Through the study of mutants defective in GA biosynthesis or signal transduction in rice (*Oryza sativa* cv Nipponbare) and *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.), several GA signaling pathway components were identified [3–6]. For example GIBBERELLIN INSENSITIVE 1 (GID1), which

acts as a hormone-sensitive lipase, can interact with DELLA proteins to modulate GA signaling [5–7]. Other GA regulatory proteins, including the F-box proteins (SLEEPY1 [SLY1] and SNEEZY (SNE)/SLY2 in *Arabidopsis* [8], GIBBERELLIN INSENSITIVE 2 [GID2] in rice [9]), have also been identified via various genetic and biochemical approaches.

DELLA proteins play a negative regulatory role in GA signaling and are named for their N-terminal DELLA domain, which belongs to a larger transcription factor (TF) family—the GRAS TF families [10]. DELLA genes have been cloned from dozens of plant species, such as *Arabidopsis* [11], rice [3], wheat (*Triticum aestivum* L.) [12], rape (*Brassica napus* L.) [13], strawberry (*Fragaria vesca* L.) [14] etc., and their functions and regulatory mechanisms have been studied in depth, with more emphasis on *Arabidopsis*, rice, and other model plants. In the *A. thaliana* genome, five DELLA genes are encoded: RGA (Repressor of *ga1*–3) [15,16], GAI (GA-Insensitive) [4], RGL1 (RGA-like1) [11], RGL2 [17], and RGL3 [18].

The DELLA proteins are a set of plant-specific TFs, whose typical architecture consists of a conserved GA-response specific domain, and a highly conserved GRAS domain. The GA-response specific domain is composed of DELLA, a TVHYNP motif and a poly S/T/V region. DELLA, LEQLE, and TVHYNP motifs confer the GA signal-sensing and transcriptional coactivation capacity of this domain, which is functionally conserved across all land plants [19]; the poly S/T/V region is a regulatory motif containing a protein phosphorylation and glycosylation modification site. The GRAS domain consists of NLS, LXXLL (or LZ), VHIID, SH2, and SAW motifs. The NLS motif is a nuclear localization signal motif and plays an important role in the localization of DELLA proteins. LZ (Leucine Zipper) is a leucine motif that promotes dimer formation, transcription co-activation, and interaction with nuclear receptors. VHIID, SH2, and SAW are repressive functional motifs [20].

The DELLA protein N-terminus can sense GA signaling, which is the structural basis of DELLA protein degradation. In the *A. thaliana* mutants *gai-1* and *rga-17*, the function of one DELLA protein is lost, resulting in reduced GA sensitivity. In these two mutants, GA cannot cause the degradation of DELLA through the ubiquitin-proteasome pathway, leading to plant dwarfing. Meanwhile, deletion of five amino acids from the DELLA motif at the N-terminus of the *d8-1* mutant DELLA protein (*Zea mays* L.) or mutation of the DELLALG motif to DELLALE in the *Sln1d* mutant (*Hordeum vulgare* L.), leads to a similar dwarfing phenotype [12,16,21,22]. In wheat, either an insertion of 30 amino acids into the DELLA structural domain or terminating mutations near the DELLA motif (*rht-d1c* and *Rht-b1b/Rht-d1b* mutants, respectively) weaken the interaction of the *Rht3* DELLA gene with GID1, and therefore, cause semi-dwarf and semi-dominant mutant phenotypes. In addition, single base variation in the DELLA domain resulted in no GA response and plant dwarfing [4,12]. The GRAS region at the C-terminus of DELLA proteins is responsible for the interaction with hundreds of TFs [23,24]. This interaction either downregulates [25,26] or enhances [27,28] the activity of the TFs, thereby regulating thousands of genes in different contexts [29].

The “Green Revolution” of the 1950s was made possible by beneficial mutations of GA20ox2 and DELLA proteins in rice and wheat, respectively, to achieve semi-dwarf breeding, leading to a strongly increased production of food [12]. Now, in a variety of crops, the DELLA protein expression level can be regulated using transgenics to create dwarf plants, increasing stress resistance and yield [30]. In addition, DELLA proteins play an important role in releasing seed dormancy, promoting early flowering, prolonging the flowering period, improving fruit quality, delaying plant senescence, and regulating the synthesis of secondary metabolites [17,31,32]. Therefore, the study of DELLA proteins has broad application prospects in regulating plant growth and development, and stress resistance [32,33].

The function of DELLA proteins is highly conserved among different plant species, but the number of DELLA genes and their mode of action differs across different species. In this study, we identified two *LhDELLA* genes, *LhSLRL* and *LhRGA*. We isolated and characterized these genes to speculate their role in *Liriodendron* hybrids growth and development.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Organs/tissues including root, stem, bud, leaf, flower, petal, stamen, and pistil from 25-year-old *Liriodendron* hybrids planted in the Xiashu Forest Farm of Nanjing Forestry University (32°7'48" N, 119°13'12" E), were collected and frozen in liquid nitrogen, then stored at −80 °C for further analysis.

The *Arabidopsis* ecotype used in this study is Col-0, while the tobacco used is wild-type tobacco (*Nicotiana tabacum* L.). *Arabidopsis* seedlings were grown on 1/2 MS Culture Medium, tobacco seedlings were grown on MS medium. Adult wild-type and transgenic plants of both species were grown in a greenhouse (21 to 25 °C, 16-h-light/8-h-dark cycle with supplemental light of $\sim 300 \mu\text{E m}^{-2} \text{s}^{-1}$, T5 28W 6400 K three-band linear fluorescent lamps and 60 to 80% humidity). Soil was composed of nutrient soil, vermiculite and perlite in a 5:1:1 ratio at identical dry weight per pot and was watered daily to maintain a water content of about 0.75 g of water per g of dry soil.

2.2. Identification of DELLA Genes in *Liriodendron* Hybrids

Protein sequences of the rice and *Arabidopsis* DELLA family were downloaded from NCBI (<http://blast.ncbi.nlm.nih.gov/>), and the windows native comparison software BioEdit v.7.1 (NCSU, Raleigh, NC, USA) was used to compare the BLASTP transcript histone sequence of *Liriodendron* hybrids to the protein sequences of rice and *Arabidopsis* DELLA to obtain candidate protein sequences. In order to further verify the reliability of the candidate protein, the sequence of the candidate protein was extracted from the transcript histone file, compared to the Pfam database (<https://www.ebi.ac.uk/Tools/pfa/pfamscan/>), and the genes with DELLA (Pfam 12041) and GRAS domain (Pfam 03514) were retained.

2.3. Analysis of Gene Structure and Motif

DNAMAN v.9.0 (Lynnon Corporation, San Ramon, CA, USA) was used to align the DELLA protein sequences of *Arabidopsis* and the *Liriodendron* hybrids. Conserved motifs in the protein sequences were verified by MEME (<http://meme-suite.org/tools/meme>), which provided information on their position in the protein sequence as well. TBtools v.1.0 (SCAU, Guangzhou, Guangdong, China) [34] was used to visualize amino acids conservation within motifs, as well as their distribution within each protein sequence.

2.4. Sequence Alignment and Phylogenetic Analysis

DELLA protein amino acid sequences were downloaded from the TAIR and NCBI databases, using *Arabidopsis thaliana* DELLA protein sequences as a query. Multiple sequence alignments were performed with Clustal W. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [35]. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Phylogenetic trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic analysis involved 35 amino acid sequences (Table S1). Evolutionary analyses were conducted in MEGA X v.10.1 (Temple, Philadelphia, PA, USA) [36].

2.5. Cloning of LhDELLAs

Total RNA from *Liriodendron* hybrids leaves was extracted using plant Trizol reagent (Tiangen, Beijing, China), and cDNA was synthesized using a HisScript[®] III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). According to the *Liriodendron* hybrids transcriptome, specific primers

were designed (Table S2) to carry out PCR amplification. PCR reaction conditions were: 98 °C pre-denaturation 2 min; 98 °C 30 s, 57 °C 30 s, 72 °C 1 min 40 s, 35 cycles; extension at 72 °C for 7 min. After the PCR product was recovered, it was ligated into the pMD19-T vector (Takara Japan) to construct a recombinant plasmid to transform *E. coli* DH5 α competent cells (Tiangen, Beijing, China), and positive clones were screened for sequencing.

2.6. Plasmid Construction and Transformation

The pMD-18 T vectors containing *LhSLRL* or *LhRGA* genes were double digested using BamHI and SacI (NEB, MA, USA), after which the inserts were ligated into the pBI121 vector to obtain *35S:LhSLRL* and *35S:LhRGA* constructs. Both PCR and double enzyme digestion were used to confirm the constructed plasmid, and the correct recombinant plasmid was introduced into GV3101 and EHA105 competent cells using the freeze-thaw method. As previously reported [37,38], the flower dip method was used for *Arabidopsis* (Col-0) transformation, and the leaf-disc method was used for tobacco transformation. The transgenic plants have been tested by PCR and RT-PCR (Figures S4–S7). The primers are in Table S3.

2.7. Quantitative RT-PCR

Total RNA was extracted from different organs/tissues of the adult *Liriodendron* hybrids (Figure S3): root, stem, bud, leaf, flower, petal, stamen, and pistil, using a General plant total RNA extraction kit (BioTeke, Beijing, China). Genomic DNA was removed from total RNA using DNase. cDNA was synthesized using a HiScriptII1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). qRT-PCR was performed on a LightCycler[®]480 (Roche, Switzerland), using the SYBR Green PCR master mix (Takara, Osaka, Japan). The *Liriodendron* hybrids *18S rRNA* gene was used as the reference gene for internal standard [39]. The primers used for qRT-PCR are listed in Table S3 (Figure S8). The relative expression value was calculated using 2(-Delta Delta C(T)) Method [40]. Three biological and three technical replicates were carried out for each gene.

2.8. GA₃ Treatment of T3 *Arabidopsis thaliana* Overexpressing *LhRGA*

The seeds of wild-type *Arabidopsis thaliana* and the T3 homozygous plants with the *LhRGA* overexpression were sterilized with 1% NaClO, which were sown on solid 1/2MS medium for germination. Seedlings one week post germination were transferred on a new solid 1/2MS medium containing different GA₃ concentrations. The GA₃ concentration used in the medium was 0, 0.1, 0.5, 1, and 5 μ mol/L, respectively. Three homozygous transgenic lines were selected and 10 plants from each line were treated under each GA₃ concentration, three experimental replicates for each testing, with wild-type *Arabidopsis* as a control. After 10 days, the root growth of transgenic plants and wild-type *Arabidopsis thaliana* was observed and analyzed.

3. Results

3.1. Identification of DELLA Genes in *Liriodendron* Hybrids

DELLAs mediate plant development by interacting with and regulating the activity of DNA-binding TFs to regulate the expression of downstream target genes [29]. First, to analyze the function of *DELLA* genes from *Liriodendron* hybrids (*Liriodendron chinense* (Hemsl.) Sarg. \times *L. tulipifera* L.) [41], we used the rice and *Arabidopsis* *DELLA* protein sequences downloaded from NCBI to perform a BLASTP search against the *Liriodendron* hybrids transcriptome [42]. Then, we further verified whether the non-redundant *DELLA* candidate sequence had both the *DELLA* and *GRAS* domains using the Pfam database, resulting in a total of two *DELLA* members from the *Liriodendron* hybrids transcriptome. Predicted characteristics of putative proteins encoded by *LhDELLA* genes are listed (Table 1). Their length and molecular weight are 524 and 540 aa, 57.5 and 59.6 kDa, respectively. It is noteworthy that all *LhDELLAs* encode weakly acidic proteins as supported by the predicted isoelectric points (Table 1). According to predictions, *LhDELLAs* presumably localize to the nucleus (Table 1), like all *DELLAs* in *A. thaliana*.

Table 1. List of putative DELLA gene family members of *Liriodendron* hybrids.

Protein Name ^a	Length (aa)	pI	MW (kDa)	Putative Proteins ^b			Protein Location ^c
				Instability Index	Aliphatic Index	GRAVY	
LhSLRL	524	5.66	57.53	50.46	87.21	−0.159	Nuclear
LhRGA	540	5.18	59.61	39.03	85.13	−0.23	Nuclear

^a Name refers to systematic designation to *Liriodendron* hybrids DELLAs in this work. ^b Molecular weight(kilodaltons), isoelectric point (pI), Instability index, Aliphatic index and Grand average of hydropathicity (GRAVY) of the deduced polypeptides were calculated using ProtParam (<https://web.expasy.org/protparam/>). ^c Protein location of the putative proteins was calculated using ProtComp v.9.0 (Softberry, Mount Kisco, NY, USA) (<http://linux1.softberry.com/>).

3.2. Phylogenetic Analysis of LhDELLA Proteins

To explore phylogenetic position of LhDELLAs, a phylogenetic tree was constructed with the protein sequences of LhSLRL, LhRGA, and 33 orthologues DELLAs from other species (Figure 1). The first clade comprised the DELLAs from species belonging to dicots. LhRGA was classified as the basal branch in the first clade, which is similar to the phylogenetic position of *Liriodendron* based on the genome sequence [42]. The second clade contained DELLAs from plants belonging to monocotyledons. Interestingly, LhSLRL together with *Physcomitrella patens* DELLAa, *P. patens* DELLAb, *Selaginella kraussiana* DELLA, *Amborella trichopoda* RHT1, *Aquilegia coerulea* DELLA3, *Nelumbo nucifera* GAI-like, and *N. nucifera* SLR1-like formed a clade, here named outgroup [19]. In the sequence alignment analysis, the DELLA motifs in LhSLRL in outgroup differed from those in the first clade (Figure 2). According to the most recent classification of Hernandez-Garcia et al., LhRGA may be “DELLA1” or “DELLA2” (hard to differentiate with this tree) and LhSLRL is a “DELLA3”, which is the only one found in early diverging angiosperms, but also some basal eudicots have it (like *Aquilegia coerulea* var. pinetorum and *Nelumbo nucifera* Gaertn. Fruct. et Semin.) [19].

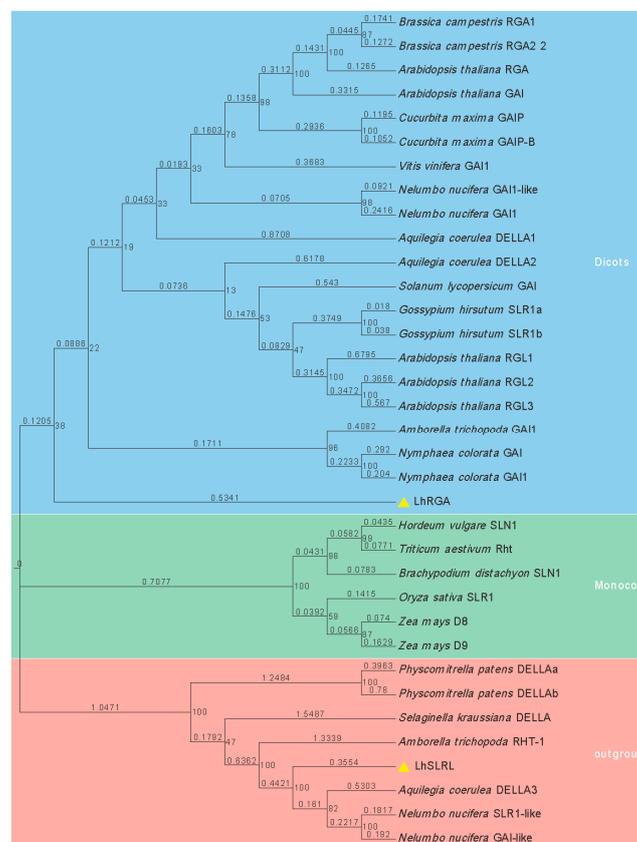


Figure 1. Phylogenetic tree of DELLA proteins from different plant species. Phylogenetic tree showing the predicted relationships between LhDELLAs and DELLA proteins of other species. Bootstrap support values are given at the nodes. This analysis involved 35 amino acid sequences. Evolutionary analyses were conducted in MEGA X.

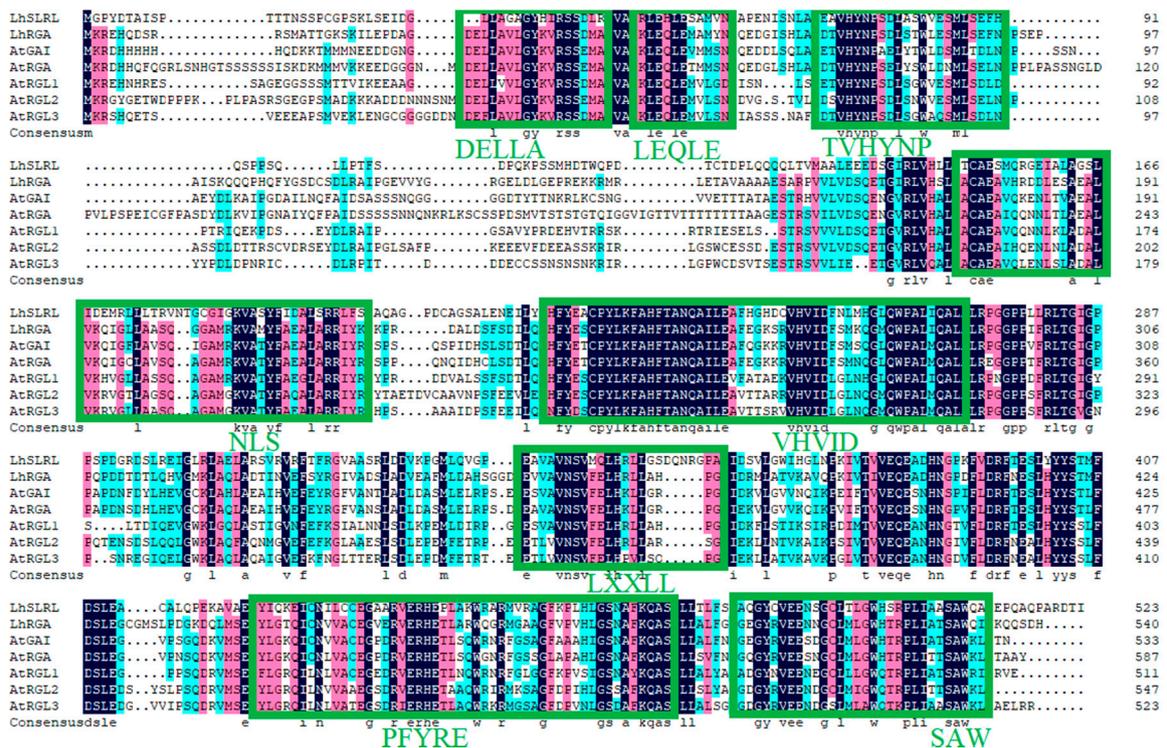


Figure 2. Sequence alignments of LhDELLAs to AtDELLAs. Amino acid sequences were aligned using DNAMAN 8. The conserved domains of the DELLA protein are framed by green lines. Identical and similar amino acid residues were shaded with black, pink, and blue, respectively.

3.3. Structural Analysis of LhDELLA Genes

To comprehend the conserved characteristics of *Liriodendron* hybrids DELLA proteins and analyze their sequence differences, we performed a multiple sequence alignment between LhDELLAs and their homologs. The results showed that LhSLRL and LhRGA proteins have 35.83% to 40.07% and 50% to 55.54% similarity with AtGAI, AtRGA, AtRGL1, AtRGL2, and AtRGL3 from *Arabidopsis*, respectively (Table S4). In order to better understand the sequence conservation of LhDELLAs and hypothesize about their functionality, we identified all typical and conserved amino acid motifs present across 16 DELLA proteins from nine different plant species. All selected proteins, with length varying from 511aa to 651 aa, contain more than ten motifs with a very similar arrangement (Figure 3a,b). Additionally, proteins located on the same branch of the phylogenetic tree have a similar number and arrangement of motifs. *A. trichopoda* GAI1, *Nymphaea colorata* GAIa, and *N. colorata* GAIb contain 17 motifs, whereas proteins from monocotyledonous plants contain motif19, which is not present in other DELLAs (Figure 3a,b). We furthermore confirmed the presence of two different motifs (DELLA, LEQLE, and TVHYNP) described previously [43] to be present in DELLA domains of *Liriodendron* hybrids and five different motifs (LHRI, LHRII, VHID, PFYRE, and SAW) described previously [43] to be present in GRAS domains of *Liriodendron* hybrids (Figures 2 and 3b). Differences between LhRGA and AtDELLA sequences mainly lie outside the DELLA and GRAS domains, whereas the divergence of the DELLA domain sequence in LhSLRL is more substantial (Figure 2). Compared with AtDELLA sequences, LhRGA possesses motif21 but no motif14, while LhSLRL lacks motif13, motif17, motif14, motif15, motif12, and motif16 (Figure 3a). The DELLA domain of AtRGA was “DELLAVLGYKV”, same as the one in LhRGA; however, the DELLA domain of LhSLRL was “DGLLAGAGYHI” (Figure 2). Together, these data indicate the divergence of extra motifs might cause changes in the characteristics of LhRGA, whereas divergence of extra motifs and variations in the DELLA domain might cause changes in the characteristics of LhSLRL.

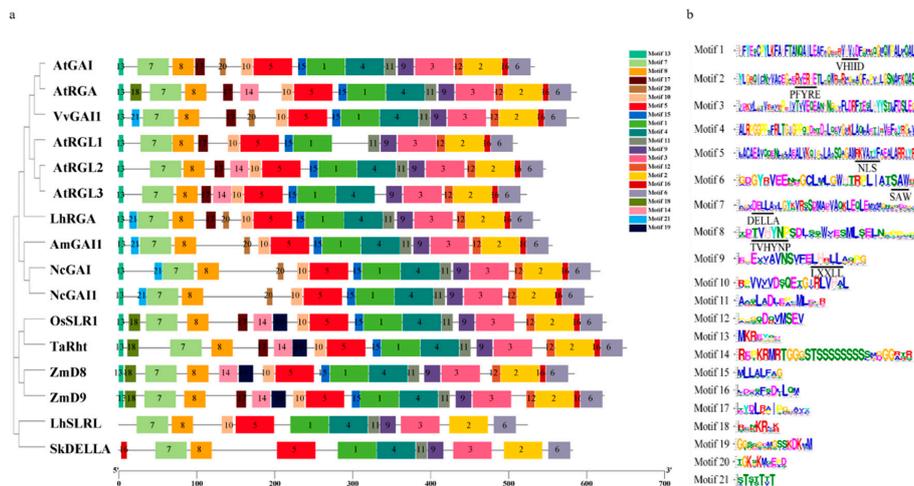


Figure 3. Structural analysis of LhDELLAs proteins. (a,b) Motifs identified (a) and their positions (b) in 16 DELLA proteins from nine different plant species. Different colors correspond to different motifs. The total height of each stack indicates the sequence conservation at that position, and the height of the symbol in each stack reflects the relative frequency of the corresponding amino acid (MEME motif search tool).

3.4. Expression Patterns of LhDELLAs in Liriodendron Hybrids

To further analyze *LhDELLA* function, we cloned the putative *LhDELLA* gene coding sequence using *Liriodendron* hybrids cDNA. We obtained a more accurate spatiotemporal distribution of *LhDELLA* transcripts by using quantitative real-time PCR (qRT-PCR) analysis across vegetative and reproductive organs. This analysis indicated that *LhDELLAs* are expressed across almost all organs/tissues, including root, stem, bud, leaf, flower, petal, stamen, and pistil, yet with largely varying expression levels. In vegetative organs, the *LhSLRL* and *LhRGA* have the highest expression level in stem, and the lowest level in root (Figure 4). However, in reproductive organs, the expression levels of *LhSLRL* and *LhRGA* genes are the highest level in petal, and the lowest in stamen. qPCR analysis revealed their unique spatial expression patterns in vegetative and reproductive organs of *Liriodendron* hybrids, indicating their potential functional diversification.

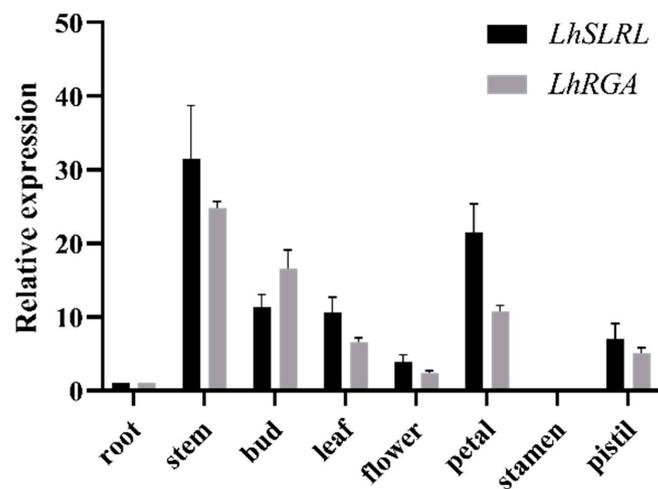


Figure 4. Expression profile of *LhDELLAs* in *Liriodendron* hybrids. Quantitative real time PCR analysis of *LhDELLAs* in the following organs/tissues from *Liriodendron* hybrids: root, stem, bud, leaf, flower, petal, stamen, and pistil. The *Liriodendron* hybrids 18S gene was used as a reference gene. Error bars represent ± SD from three biological repeats.

3.5. *LhDELLA* Overexpression Alters *Arabidopsis* Development

To further investigate *Liriodendron* hybrids *DELLA*s functionality, we generated *Arabidopsis* transgenic plants overexpressing *LhDELLA*s under the control of the CaMV 35S promoter. In *Arabidopsis* *LhSLRL*-overexpressing lines, the flowering date changed significantly, with transgenic plants flowering three–six days later than wild-type plants (Figure 5a,b). Flowering time showed no significant difference among four *LhSLRL*-overexpressing lines (Figure 5b). Furthermore, *LhSLRL* overexpression promoted stem development and caused branching and dwarf-like growth in transgenic plants (Figure 5c–e). Likewise, in *Arabidopsis*, the root length of *LhSLRL* overexpressing plants was shortened compared to wild-type plants (Figure 5f,g). We also observed these similar phenotypes in tobacco overexpressing *LhSLRL* (Figure S1).

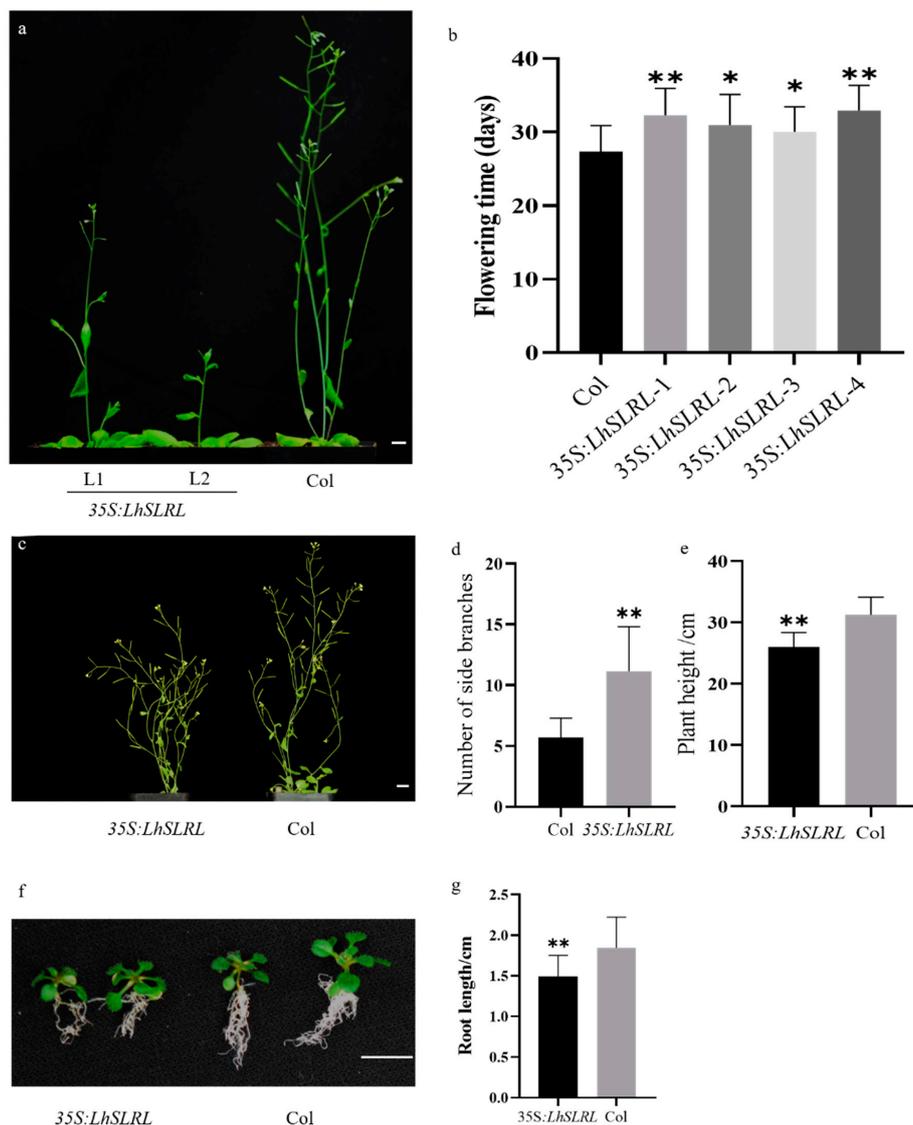


Figure 5. Morphological phenotypes of *Arabidopsis* overexpressing *LhSLRL*. (a) Phenotypes of 40-day-old wild-type and transgenic plants. (b) Measurement of flowering time in the wild-type and transgenic lines (a). (c) Phenotypes of 55-day-old wild-type and transgenic plants. (d) Number of side branches in the wild-type and transgenic lines (c). (e) Measurement of plant height branches in the wild-type and transgenic lines (c). (f) Phenotypes of 15-day-old wild-type and transgenic plants. (g) Measurement of root length in the wild-type and transgenic lines (f). Error bars: \pm SD. * $p < 0.05$; ** $p < 0.01$. Scale bars: 1 cm (a,c,f).

Secondly, overexpression of *LhRGA* in *Arabidopsis* caused changes in plant height, flowering time, and number of side branches, but no significant difference was observed compared with the wild-type (Figure 6). However, rosette leaf length significantly increased in transgenic plants, ranging from an increase of 0.22 to 0.94 cm among different transgenic lines (Figure 7a,b). In addition, we found that 27% of all adult transgenic plants started to show strongly darkened green leaves (Figure S2).

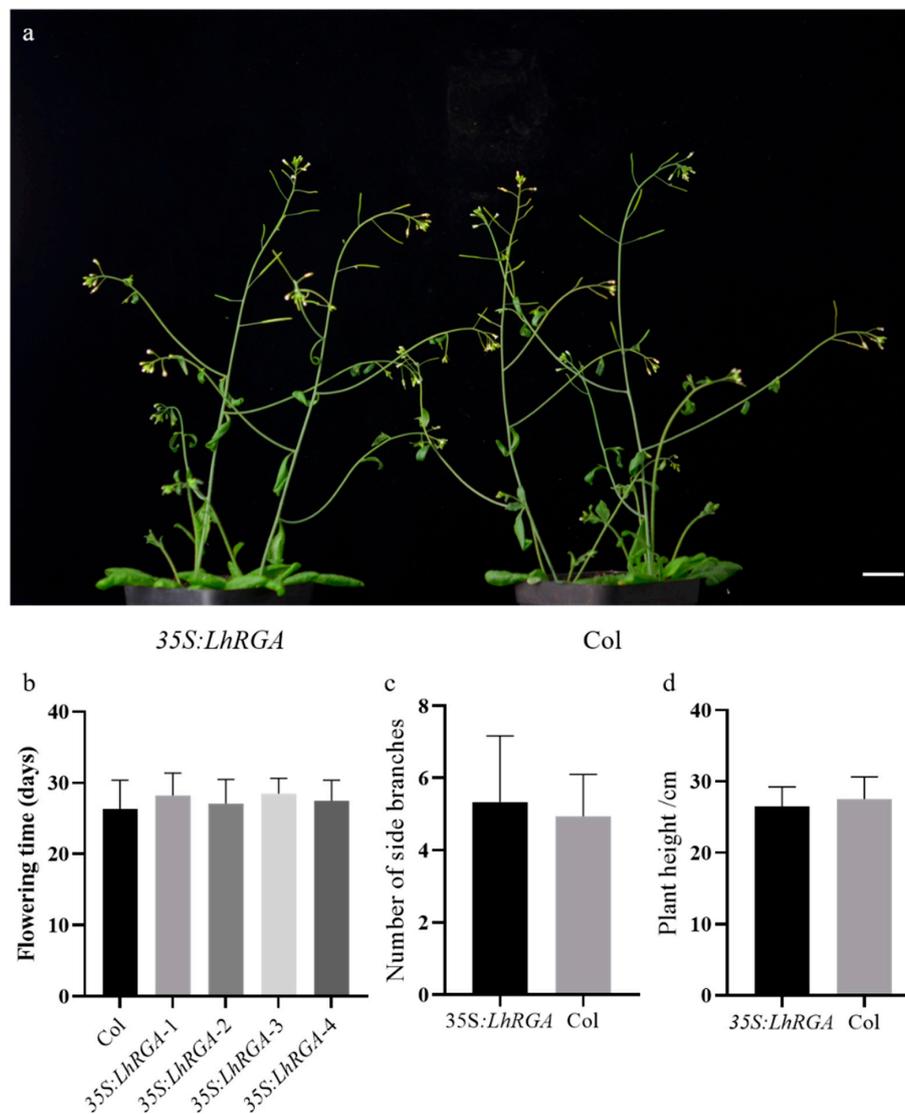


Figure 6. Morphological phenotypes of *Arabidopsis* overexpressing *LhRGA*. (a) Phenotypes of 30-day-old wild-type and transgenic plants. Scale bars = 2 cm (b) Measurement of flowering time in the wild-type and transgenic lines (a). Error bars: \pm SD. (c) Number of side branches in the wild-type and transgenic lines (a). Error bars: \pm SD. (d) Measurement of plant height branches in the wild-type and transgenic lines (a). Error bars: \pm SD.

As we had seen that *LhSLRL* may shorten plant roots in the *Arabidopsis* (Figure 5f,g), we wondered whether *LhRGA* might also affect root development. To study this, we designed an experiment using different concentrations of GA_3 to treat T3 *LhRGA* transgenic plants and wild-type plants. We found that low concentrations of GA_3 were able to promote root elongation of both wild-type and transgenic plants (Figure 8a–f). T-test analysis showed that the root length of wild-type was not significantly different from transgenic plants in the absence of GA_3 ; however, under treatment with different concentrations of GA_3 , the root length of transgenic plants was significantly shorter than

wild-type plants, with the strongest effect being observed at a GA₃ concentration of 0.5 μM (Figure 8f). Taken together, these results show that *LhRGA* overexpression also leads to shorter roots and regulates root development. However, its effect is only apparent upon GA₃ treatment.

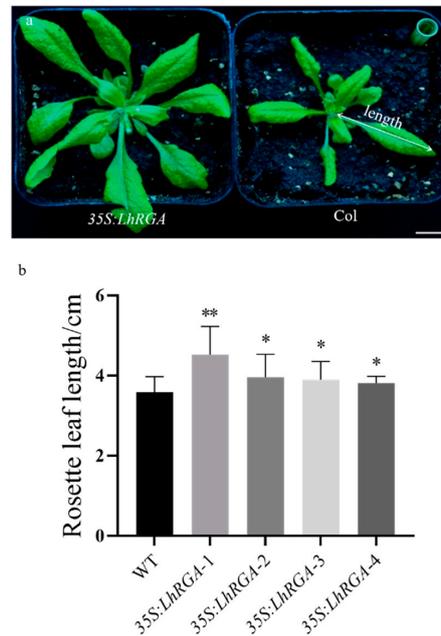


Figure 7. Morphological phenotypes of *Arabidopsis* overexpressing *LhRGA*. (a) Phenotypes of 28-day-old wild-type and transgenic plants. Scale bar = 1 cm. (b) Measurement of rosette leaf length in the wild-type and transgenic lines (a). Error bars: \pm SD. * $p < 0.05$; ** $p < 0.01$.

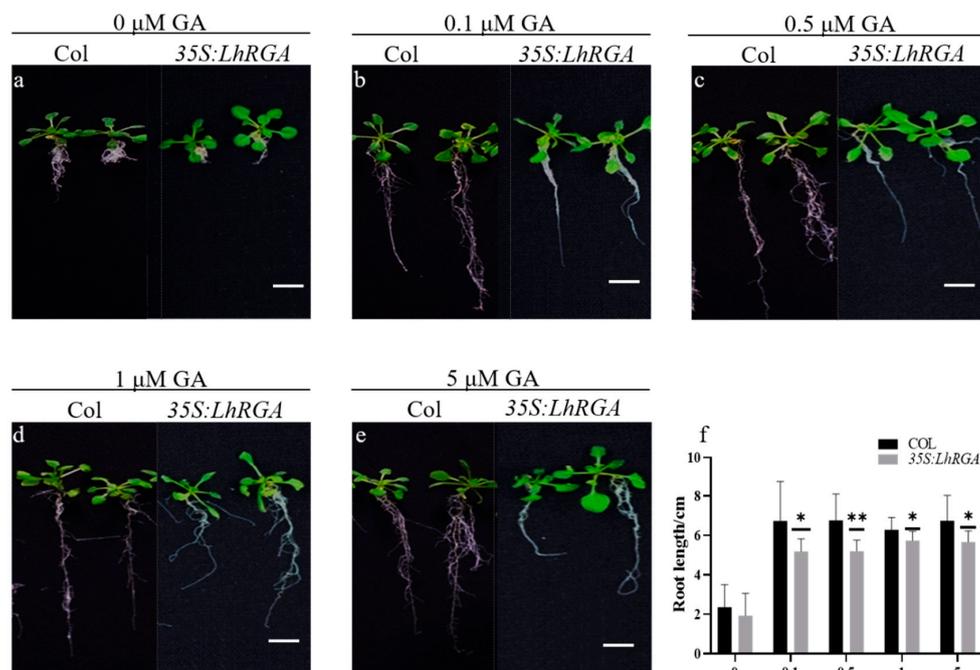


Figure 8. Morphological phenotypes of *Arabidopsis* overexpressing *LhRGA* after GA treatment. (a–e) Phenotypes of 15-day-old wild-type and transgenic plants treated with different concentrations of GA. Scale bar = 1 cm. (f) Measurement of root length in the wild-type and transgenic lines (a–e). Error bars: \pm SD. * $p < 0.05$; ** $p < 0.01$.

4. Discussion

In recent years, a large number of genes encoding DELLA proteins, a key regulator of the gibberellin signaling pathway, have been identified in poplar (*Populus delotides* × *P. euramericana* cv. ‘Nanlin895’) [44] and breadfruit (*Artocarpus altilis* cv. Cannonball) [45] and were subsequently isolated and cloned from other timber species. Although cloning of related genes in *Liriodendron chinense* (Hemsl.) Sarg. has been reported [46], the functionality of these genes has not yet been verified. Here, we successfully isolated two genes encoding DELLA proteins from *Liriodendron* hybrids. *Liriodendron* is a tertiary glacial relic plant, an ancient angiosperm with a history of more than 100 million years. However, the DELLA proteins of *Liriodendron* hybrids are strongly conserved, and have a similar number and arrangement of motifs as the DELLA proteins of other plants. This indicates that the *Liriodendron* hybrids DELLA proteins may have similar functions to those of other plants. However, compared with the DELLA protein of *A. thaliana*, the DELLA protein of *Liriodendron* hybrids lacks several motifs, suggesting that they may have different functions. More importantly, the DELLA domain of LhSLRL is significantly different from the same domain in DELLA proteins of other plants. The DELLA motif conserved in the DELLA domain of most plants is “DELLAALGYKV”, whereas the DELLA motif in LhSLRL is “DGLLAGAGYHI”. Single-base variation in the DELLA domain will cause plants to appear unresponsive to GA and show dwarfism. The *sln1d* dwarf mutant in barley is due to mutation of the conserved DELLALG motif in the DELLA domain to “DELLALE” [21]. In *Arabidopsis* overexpressing *LhSLRL*, there are significant differences in plant height, number of side branches, flowering time, and root length compared with the wild-type. However, compared with the wild-type, overexpressing *LhRGA* in *Arabidopsis*, only caused significant differences in rosette leaves, as well as the root length after GA₃ treatment.

For a long time, it has been found that GAs can control branching, as evidenced by all GA-deficient mutants showing increased branching compared to wild-type [47]. The number of stem collaterals of *Arabidopsis* overexpressing *LhSLRL* was significantly higher than that of the wild-type, which is similar to the phenotype of GA-deficient mutants. This may be because the plant endogenous GAs are unable to degrade the overexpressed *LhSLRL*. Another possibility is that LhSLRL causes this phenotype through interaction with the plant hormone strigolactone (SL). SL is a terpene lactone, which was found to inhibit the growth of axillary buds. Studies have found that rice DELLA SLR1 can interact with D14 in an SL-dependent manner and regulate downstream SL signaling [48], thereby controlling the growth of axillary buds.

Recent studies have shown that GA enhances the anisotropy of leaf swelling [49]. Excessive GA makes the leaves narrow and long, whereas the GA-deficient *dwarf3* mutant has short and wide leaves. The leaves of *Arabidopsis* overexpressing *LhRGA* are long, which is different from the results of existing studies. This may be caused by divergence of extra motifs in LhRGA, or it may depend on additional factors being at play. In addition to the GA-DELLA module, there are five other modules that are important for the determination of *Arabidopsis* leaf size: ubiquitin receptor DA1-ENHANCER OF DA1 (EOD1), GROWTH REGULATING FACTOR (GRF)-GRF-INTERACTING FACTOR (GIF), SWITCH/SUCROSE NON-FERMENTING (SWI/SNF), KLU, and PEAPOD (PPD). These six modules form a complex regulatory network that regulates the size of *Arabidopsis* leaves [50]. The phenotypic effects observed when individual members of different modules are incorrectly expressed may also be balanced at the leaf level [51–53]. This may be the reason why the leaves of *Arabidopsis* overexpressing *LhRGA* are long.

GAs can stimulate root elongation. Different concentrations of GA were applied to *Arabidopsis* overexpressing *LhRGA*, and it was found that the roots of *A. thaliana* had obvious elongation, but not as much as the wild-type. However, the effect of GAs on the formation of lateral and adventitious roots is still controversial. Early studies have shown that in some cases, exogenously administered GAs can inhibit this process [54], whereas in other cases it can promote this process [55]. In this study, both the lateral roots of *Arabidopsis* overexpressing *LhSLRL* and overexpressing *LhRGA* were significantly reduced, which was the opposite of the phenotype of the DELLA-deficient *Populus*

tree [56]. The results showed that in *Liriodendron* hybrids, *LhDELLA* may inhibit the formation of lateral roots and adventitious roots, while GA promotes the formation of lateral roots and adventitious roots.

5. Conclusions

Overexpression of *LhSLRL* and *LhRGA* in *Arabidopsis* shows that both genes have remarkable effects on the vegetative growth of transgenic plants, but their functions may be different. Our results provide a preliminary understanding of *LhDELLA* function, supplying an important basis for further understanding of the mechanism of GA signal transduction and its role in the growth and development of *Liriodendron* hybrids.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/12/1363/s1>, Figure S1: Morphological phenotypes of tobacco overexpressing *LhSLRL*, Figure S2: Morphological phenotypes of *Arabidopsis* overexpressing *LhRGA*, Figure S3: Sampling method, Figure S4: PCR testing for *Arabidopsis* overexpressing *LhSLRL*, Figure S5: PCR testing for *Arabidopsis* overexpressing *LhRGA*, Figure S6: RT-PCR analysis of wild types and *Arabidopsis*/tobacco overexpressing *LhDELLAs*, Figure S7: PCR testing for tobacco overexpressing *LhSLRL*, Figure S8: The thermal profile of qRT-PCR, Table S1: DELLA proteins of different species, Table S2: Primers used for gene cloning, Table S3: Primers used for qRT-PCR, RT-PCR, and PCR testing, Table S4: Identity matrix for *LhSLRL*/*LhRGA* and five DELLA proteins in *Arabidopsis*.

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