Cloning and characterization of *DELLA* genes in *Artemisia annua*

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ABSTRACT. Gibberellins (GA) are some of the most important phytohormones involved in plant development. DELLA proteins are negative regulators of GA signaling in many plants. In this study, the full-length cDNA sequences of three *DELLA* genes were cloned from *Artemisia annua*. Phylogenetic analysis revealed that *AaDELLA1* and *AaDELLA2* were located in the same cluster, but *AaDELLA3* was not. Subcellular localization analysis suggested that AaDELLAs can be targeted to the nucleus and/or cytoplasm. Real-time PCR indicated that all three *AaDELLA* genes exhibited the highest expression in seeds. Expression of all *AaDELLA* genes was enhanced by exogenous MeJA treatment but inhibited by GA₃ treatment. Yeast two-hybrid assay showed that AaDELLAs could interact with basic helix-loop-helix transcription factor AaMYC2, suggesting that GA and JA signaling may be involved in cross-talk via DELLA and MYC2 interaction in *A. annua*.

Key words: DELLA; Gibberellin; *Artemisia annua*

INTRODUCTION

Artemisia annua, in the Asteraceae family, is known as sweet wormwood and occurs mostly in the northern hemisphere. *A. annua* is important to the pharmaceutical industry as a source of artemisinin (a sesquiterpene lactone) and its derivatives. Artemisinin is considered an effective anti-malarial drug and recommended by the World Health Organization. It is produced and stored in the glandular secretory trichomes of *A. annua* (Covello et al. 2007; Olsson et al. 2009). The commercialization of artemisinin, however, is greatly limited due to the low artemisinin content of *A. annua* (0.01-0.8% of dry weight). Research groups all over the world are using different strategies to enhance artemisinin content, such as spraying with exogenous phytohormones (Aftab et al., 2011; Caretto et al., 2011) and overexpressing key artemisinin biosynthetic pathway enzyme genes (Shen et al., 2012).

Gibberellins (GA) are tetracyclic diterpenoid plant growth regulators that affect many aspects of plant development, such as seed germination, stem elongation, and flowering (Davies, 2004). The GA signaling pathway is negatively regulated by DELLA proteins, which contain characteristic DELLA and VHYNP motifs at the N-terminus and the GRAS domain at the C-terminus (Willige et al., 2007). DELLA repression of GA signaling can be relieved through ubiquitin-mediated proteolysis or non-proteolytic GA signaling (Griffiths et al., 2006; Ariizumi et al., 2008; Murase et al., 2008). DELLA genes have been identified in various species, including *AtRGA*, *AtGAI*, *AtRGL1*, *AtRGL2*, *AtRGL3* in *Arabidopsis* (Lee et al., 2002; Wen and Chang, 2002; Piskurewicz and Lopez-Molina, 2009); *SLR1* in rice (Ikeda et al., 2001); and *GhRGL*, *GhSLR1b*, *GhGAI3a*, *GhGAI3b*, *GhGAI4a*, *GhGAI4b* in cotton (Aleman et al., 2008; Liao et al., 2009; Wen et al., 2012).

Exogenous GA treatment can increase artemisinin production in *A. annua* (Weathers et al., 2005; Zhang et al., 2005; Aftab et al., 2010; Banyai et al., 2011). However, the molecular mechanism of GA-modulated artemisinin production remains elusive. Research on hormone crosstalk has shown that *Arabidopsis* DELLA proteins can modulate jasmonate signaling through interaction with MYC2 and JAZ proteins (Hou et al., 2010; Hong et al., 2012; Wild et al., 2012; Wild and Achard, 2013), and MYC2-DELLA interaction is involved in regulation of the sesquiterpene synthase genes *AtTPS21* and *AtTPS11* (Hong et al., 2012). Since artemisinin is also a sesquiterpene, it is possible that GA treatment enhances artemisinin biosynthesis in *A. annua* through AaDELLA proteins, which regulate terpene synthase genes. Here, we have successfully cloned and analyzed three DELLA genes from *A. annua*: *AaDELLA1*, *AaDELLA2*, and *AaDELLA3*. Expression profiles, the subcellular location of *AaDELLA* genes, and the induction kinetics of MeJA and GA₃ treatment were analyzed. A yeast two-hybrid assay was used to investigate AaDELLA interaction with AaMYC2 and AaJAZs.

MATERIAL AND METHODS

Plant materials

A. annua seeds were obtained from Southwest University, Chongqing, China. The seeds were first surface-sterilized in 70% ethanol for 1 min, followed by 10% sodium hypochlorite solution for 15 min, and then rinsed three times with sterile water. The seeds were plated on Murashige and Skoog (MS) medium (Sigma-Aldrich, USA) with 88 mM sucrose and 0.7% agar (pH 5.8).

Tissue samples (roots, stems, and leaves) were gathered from five individual five-month-old plants grown in a greenhouse. Young alabastrums and mature alabastrums were collected 7 and 14 days after budding, respectively, and seed samples were previously collected and kept by our lab.

Cloning of *AaDELLA* genes from *A. annua*

A search of GenBank found a single candidate sequence encoding an *A. annua* DELLA protein (accession number GQ468552). Based on this sequence, we searched an *A. annua* cDNA library previously constructed by our laboratory (Lu et al., 2013). From this, another two putative full-length *DELLA* sequences were obtained.

Then, we designed *DELLA*-specific primers (Table 1) to amplify the coding regions of these genes. PCR was performed with ExTaq DNA polymerase (Takara, Dalian, China). The PCR mixtures were heated to 94°C for 3 min, then subjected to 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and to a final extension at 72°C for 10 min. The resulting PCR products were subcloned into pMD18-T vector (Takara) and confirmed by sequencing.

Table 1. Primers used in this study.

Primer	Sequence (5'-3')	Purpose
RT-DELLA1F	GATGCTGGAGTTGATGAG	qPCR
RT-DELLA1R	GTGCGCTGGAATGGACTG	qPCR
RT-DELLA2F	ATGGTCAAAGGCTGGTC	qPCR
RT-DELLA2R	TACCCAGAACCACCGCCAC	qPCR
RT-DELLA3F	GGTGGTGAATGTTCTCG	qPCR
RT-DELLA3R	GAGAATCAACCAGCACAA	qPCR
RT-ACTINF	CCAGGCTGTTCACTCTGTAT	qPCR
RT-ACTINR	CGCTCGGTAAGGATCTTCATCA	qPCR
DELLA1F	ATGAAACGACATTACCCAAACATG	Clone
DELLA1R	TCAGTCGATACACCGAAGCTTCC	Clone
DELLA2F	ATGAAAAAACACACACTTGGTTGC	Clone
DELLA2R	TTACTTTAGTTTCCAAGCTGAGG	Clone
DELLA3F	ATGAAACGAGATCGTGAGCG	Clone
DELLA3R	TCATGACTCGATACCGCTAGG	Clone
TOPO-DELLA1F	CACCATGAAACGACATTACCC	Clone
TOPO-DELLA1R	GTCGATACACCGAAGCTTCCACG	Clone
TOPO-DELLA2F	CACCATGAAAAAACACACACTTGG	Clone
TOPO-DELLA2R	CTTTAGTTTCCAAGCTGAGGTCG	Clone
TOPO-DELLA3F	CACCATGAAACGAGATCGTGAGCG	Clone
TOPO-DELLA3R	TGACTCGATACCGTAGGTTTCC	Clone

Bioinformatic analyses

Comparative and bioinformatic analyses of *AaDELLA* genes were performed using online tools (<http://www.ncbi.nlm.nih.gov>). Sequence analyses were carried out using BLAST (<http://www.ncbi.nlm.nih.gov/Blast.cgi>) and the Vector NTI software (Invitrogen, USA). Phylogenetic analysis of *DELLA* genes from various species was performed with CLUSTAL X, using default parameters. A maximum likelihood phylogenetic tree with 1000 bootstrap replicates was constructed using MEGA 5.0 (<http://www.megasoftware.net>). Tertiary structure prediction was performed using SWISS-MODEL online tools (<http://swissmodel.expasy.org/>; Schwede et al., 2003; Arnold et al., 2006).

Hormone treatments

A. annua plants were grown on MS medium at $25 \pm 1^\circ\text{C}$, with a 16/8 h light/dark cycle, for two weeks. They were then transferred onto Petri dishes lined with moist filter paper and sprayed with either 100 μM GA₃ or 100 μM MeJA (Wang et al., 2008; Lu et al., 2012). For gene expression analysis, plants were selected randomly at different time points before treatment (0 h) and after treatment (1, 3, 6, 12, and 24 h).

RNA extraction and qRT-PCR analysis

Expression profiles of *AaDELLA* genes in various tissues and in response to hormone treatments were analyzed via quantitative real-time PCR. Tissues were collected for total RNA extraction using RNeasy Pure Plant kit (Qiagen Biotech, Beijing, China), following the manufacturer protocol. Reverse transcription was conducted using a PrimeScript RT reagent kit (Takara), according to the manufacturer protocol. Real-time PCR was conducted on a Peltier Thermal Cycler PTC200 (Bio-Rad, Hercules, CA, USA) using the gene-specific RT primer pairs listed in Table 1. SYBR Premix qPCR Kit (Tiangen) was used to perform PCR, with the following parameters: 3 min at 95°C ; 40 cycles of 20 s at 95°C , 20 s at 54°C , 20 s at 72°C ; and 72°C for an additional 5 min. Melting curves were assessed to test product specificity by gradually increasing the temperature of PCR products from 65 to 95°C in 0.5°C increments. Quantification of *AaDELLA* gene expression was analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method (Schmittgen and Livak, 2008).

Subcellular localization of AaDELLA-YFP fusion proteins

AaDELLA cDNA was amplified and cloned into the Gateway entry vector pENTR/SD/D-TOPO. Primers were designed according to the Invitrogen protocol; these are shown in Table 1. The full-length open reading frame was fused upstream of the yellow fluorescent protein (YFP) gene and placed under the control of the CaMV 35S promoter in the pEarleyGate104 vector, to construct a 35S::*AaDELLA*-YFP fusion protein, using LR Clonase Reaction (Invitrogen) for each entry clone. The resulting recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain EHA105. Transient expression in *Nicotiana tabacum* epidermal cells using agroinfiltration has been described previously (Voinnet et al., 2003). Three days after infiltration, YFP fluorescence in tobacco epidermal cells was observed using TCS SP5 laser scanning confocal imaging (Leica Microsystems, Buffalo Grove, IL, USA), and the pEarleyGate104 vector alone was used as positive control. Fluorescence was detected at 530–600 nm for YFP.

Yeast two-hybrid assay

To examine protein-protein interactions between *AaDELLA* and jasmonate signaling proteins (*AaMYC2* and *AaJAZs*), we performed a yeast two-hybrid assay using ProQuest Two-Hybrid System (Invitrogen). To create prey and bait vectors, the cDNA of *AaMYC2*, *AaJAZ1* (KJ651999), *AaJAZ2* (KJ652000), *AaJAZ3* (KJ652001), *AaJAZ4* (KJ652002), and each *AaDELLA* gene was first cloned into Gateway entry vector pENTR/SD/D-TOPO. Then, the gene of interest for the bait plasmid was cloned into pDEST32, and the gene of interest for the

prey plasmid was cloned into pDEST22, using LR clonase (Invitrogen). pDEST32 and pDEST22 were Gateway-adapted destination vectors. In this experiment, the *AaDELLA* coding region was ligated into the prey vector while *AaMYC2* and *AaJAZs* were cloned into the bait vectors. The prey and bait plasmids were cotransformed into yeast strain AH109 following the manufacturer protocol, and were subsequently plated on SD-Leu-Trp medium for initial selection. Eight transformants were randomly chosen and plated on SD-Leu-Trp-Ade and SD-Leu-Trp-His-Ade medium for protein-protein interaction testing.

RESULTS

Cloning of full-length cDNA encoding *A. annua* DELLA proteins

Three DELLA protein genes were obtained from *A. annua*; these were named *AaDELLA1* (GenBank accession No. KJ651996), *AaDELLA2* (GenBank accession No. KJ651997), and *AaDELLA3* (GenBank accession No. KJ651998). They have lengths of 1593, 1794, and 1662 bp, and encode proteins of 530, 597, and 553 amino acid residues, respectively. N-terminal DELLA and VHYNP motifs, as well as the C-terminal GRAS domain, were found in all three *AaDELLA* amino acid sequences (Figure 1). BLAST results from NCBI indicated that the amino acid sequences shared high similarity with *A. thaliana* DELLA proteins, *Lactuca sativa* DELLA1 protein, and *Solanum tuberosum* GAI protein.

Figure 1. Amino acid sequence alignment of *Artemisia annua* DELLA proteins (*AaDELLA1*, *AaDELLA2*, *AaDELLA3*) and those of other plants. Conserved DELLA, VHYNP, and GRAS domains are boxed. LsDELLA1: *Lactuca sativa* DELLA1 (AB370239); AtGAI: *Arabidopsis thaliana* GAI (Y1593); AtRGA1: *Arabidopsis thaliana* RGA1 (Y11336); StGAI: *Solanum tuberosum* GAI (JF834157); AtRGL1: *Arabidopsis thaliana* RGL1 (NM_105306); AtRGL2: *Arabidopsis thaliana* RGL2 (NM_111216); AtRGL3: *Arabidopsis thaliana* RGL3 (NM_121755); GhGAI3a: *Gossypium hirsutum* GAI3a (HM034760); OsSLR1: *Oryza sativa* SLR1 (AB262980).

Molecular evolution analyses of *AaDELLAs*

Using BLAST for protein sequences, we found that *AaDELLAs* shared the characteristic DELLA/VHYNP motif and GRAS domain found in other DELLA proteins. A phylogenetic tree of DELLA proteins from various organisms was constructed using MEGA 5.0. This revealed that DELLA proteins originated from a common ancestor and diverged into two groups: *AaDELLA1*, *AaDELLA2*, and *LsDELLA1* form a closely related subgroup, while *AaDELLA3* shares some similarity with *StGAI*, *SIGAI-like*, and *LeGAI-like* (Figure 2).

Figure 2. Maximum likelihood phylogenetic tree of plant DELLA proteins. Numbers on the branches represent bootstrap support for 1000 replicates. *Artemisia annua* DELLA proteins are boxed in red. Also included: *Arabidopsis thaliana* RGA1 (Y11336), *Arabidopsis thaliana* GAI (Y15193), *Phaseolus vulgaris* GAI1 (AB304457), *Pyrus bretschneideri* DELLA (JF304103), *Artemisia annua* DELLA2(KJ651997), *Lactuca sativa* DELLA1 (AB370239), *Artemisia annua* DELLA1(KJ651996), *Vitis vinifera* GAI-like (AF389125), *Arabidopsis thaliana* RGL2 (NM_111216), *Arabidopsis thaliana* RGL3 (NM_121755), *Arabidopsis thaliana* RGL1 (NM_105306), *Artemisia annua* DELLA3(KJ651998), *Solanum tuberosum* GAI (JF834157), *Solanum lycopersicum* GAI-like (NM_001247436), *Lycopersicon esculentum* GAI-like (AY269087), *Oryza sativa* SLR1(AB262980), *Triticum aestivum* Rht-B1 (KC434135), *Hordeum vulgare* SLN1 (AF460219), *Triticum aestivum* Rht-D1a (AJ242531), *Oryza sativa* GAI (AY464568), *Malus domestica* DELLA (DQ007888), *Gossypium hirsutum* GAI3a (HM034760).

Three-dimensional structure of AaDELLA proteins

Analysis of the protein tertiary structure conducted using SWISS-MODEL indicated that the three AaDELLA proteins are highly similar to the *Arabidopsis thaliana* DELLA protein AtRGA1 (Figure 3). From the 3-D structures, we observed two obvious candidates for the DELLA and GRAS domains, and the links between the domains constituted the differences among the four proteins. Therefore, we speculate that AaDELLAs may function similarly to AtRGA1, which functions as a repressor of GA signaling and regulates JA signaling via interaction with MYC2 proteins (Wild et al., 2012).

Figure 3. Predicted tertiary structure of *Artemisia annua* and *Arabidopsis* DELLA proteins. (A) AaDELLA1, (B) AaDELLA2, (C) AaDELLA3, (D) *Arabidopsis thaliana* RGL1.

AaDELLA proteins localize in the nucleus and cytoplasm

To analyze the subcellular localization of AaDELLAs, we constructed the expression vector pEarleyGate104-*AaDELLA* and performed an *Agrobacterium*-mediated transient expression assay in tobacco epidermal cells. The results showed that AaDELLA1 was targeted to the nucleus while AaDELLA2 and AaDELLA3 localized in both the nucleus and the cytoplasm (Figure 4). We therefore speculate that AaDELLA2 and AaDELLA3 may act as nucleocytoplasmic shuttling proteins. It needs to be stressed that AaDELLA2 and AaDELLA3 exhibited stronger expression in the nucleus than in the cytoplasm, which indicates that their primary function is in the nucleus.

Expression profiles of *AaDELLAs* in *A. annua* tissues

To study the expression profiles of *AaDELLA* genes, total RNA from *A. annua* tissues were extracted. Real-time PCR results show *AaDELLA1* and *AaDELLA2* expression in all tissues, while *AaDELLA3* exhibited basal expression in roots and flowers (Figure 5). All three genes exhibited their highest expression levels in dormant seeds, and this result is consistent with *DELLA* expression patterns found in *Arabidopsis* (Tyler et al., 2004) and with the view that DELLA proteins are required in seed dormancy and germination (Penfield et al., 2006). The relatively lower expression levels of *AaDELLAs* in other tissues suggests that their suppression may be required for plant development.

Figure 4. Subcellular location of *Artemisia annua* DELLA proteins in tobacco epidermal cells. Cells were transiently transformed with constructs containing either control 35S::YFP or 35S::AaDELLAs::YFP under the control of the CaMV35S promoter. YFP fluorescence (left), bright-field (middle), and merged overlay (right).

Figure 5. Relative expression of *AaDELLA1*, *AaDELLA2*, *AaDELLA3* in different tissues of five-month-old *Artemisia annua* plant. From left to right, tissues are as follows: roots, stems, leaves, green young alabastrums (alabastrums1), mature alabastrums (alabastrums2), flowers, and seeds. β -Actin was used as an internal control. Data are reported as means $\pm$ SD of four repeated samples.

Expression profiles of *AaDELLAs* in response to hormone treatment

DELLA proteins are always involved in plant hormone regulation, and are well known in the GA and JA signaling pathway in *Arabidopsis* and *Oryza* (Ikeda et al., 2001; Wen and Chang, 2002). To find out whether *AaDELLA* proteins play a similar role in response to these hormones, *AaDELLA* expression patterns were analyzed by qRT-PCR after treatment with MeJA (100 μ M) and GA₃ (100 μ M). The results showed that all three *AaDELLAs* exhibited similar expression patterns in response to hormone treatments. As is indicated in Figure 6, in response to MeJA treatment, all three *AaDELLA* transcripts were induced and peaked 12 h after treatment. In contrast, in response to GA₃ treatment, *AaDELLA* mRNA levels were all suppressed and reached their lowest levels at 6 h after treatment. The suppression of *AaDELLA* gene expression by GA₃ treatment is similar to that observed in the previously reported DELLA gene *BnSLY1* in *Brassica napus* (Gao et al., 2012). In addition, we found that *AaDELLA3* expression was more sensitive to hormone treatment, showing both greater enhancement and suppression after MeJA and GA₃ treatments than the other two *AaDELLA* genes.

AaDELLAs interact with *AaMYC2* proteins *in vitro*

Based on the fact that DELLA can modulate jasmonate signaling through interaction with both MYC2 and JAZ proteins in *A. thaliana* (Hou et al., 2010; Hong et al., 2012; Wild et al., 2012; Wild and Achard, 2013), and that *AaDELLA* proteins have similar structures and expression profiles as their homologs in *Arabidopsis*, we speculated that a similar interaction pattern also exists in *A. annua* and performed a yeast two-hybrid assay to test this hypothesis. We found that all three *AaDELLAs* were able to interact with *AaMYC2*, while none could interact with *AaJAZ* proteins (Figure 7). This is a slightly different result from the DELLAs

in *A. thaliana*, which do interact with AtJAZ proteins (Lu et al., 2012). The fact that all three *AaDELLAs* displayed a similar interaction pattern with the JA signaling protein MYC2 indicates that the regulatory model of JA and GA crosstalk is largely conserved in plants.

Figure 6. Relative expression of *AaDELLA1*, *AaDELLA2* and *AaDELLA3* in response to methyl jasmonate (100 μ M) and GA₃ (100 μ M), over a course of 24 h. Fold changes in transcript levels are relative to levels at 0 h. Blue lines represent mock treatments.

Figure 7. Yeast two-hybrid assays reveal AaDELLA interactions with AaMYC2 and AaJAZ proteins. Empty vectors *pDEST22* and *pDEST32* were used as negative controls.

DISCUSSION

The phytohormone GA plays an essential role in many aspects of plant development, including dormancy breaking, stem elongation, and flowering (Davies, 2004). The DELLA family of transcription factors consists of negative regulators of GA signaling in many species, including *A. thaliana*, *Oryza sativa*, and *Gossypium hirsutum* (Ikeda et al., 2001; Lee et al., 2002; Wen and Chang, 2002; Aleman et al., 2008; Piskurewicz and Lopez-Molina, 2009; Liao et al., 2009; Wen et al., 2012). In this study, we found that DELLA expression in *A. annua* was suppressed in all tissues examined, except in seeds. This indicates that, in *A. annua*, DELLA expression is required for the inhibition of GA response in dormant seeds, while DELLA suppression is required for GA response and plant development. We also revealed *AaDELLA* expression to be suppressed in response to GA₃. It is very likely that AaDELLA proteins are also negative regulators of GA signaling in *A. annua*.

However, we found that AaDELLAs are slightly different from DELLAs in *A. thaliana*. It was previously reported that DELLAs in *A. thaliana* localize in the nucleus (Thomas and Sun, 2004). However, our results suggest that AaDELLA2 and AaDELLA3 localize in both the nucleus and the cytoplasm, suggesting that DELLA transcription factors in *A. annua* can act as nucleocytoplasmic shuttling proteins. This is not surprising, since an AP2/ERF transcription factor in *A. annua* was recently shown to be a nucleocytoplasmic shuttling protein (Lu et al., 2012). It is possible that AaDELLA2 and AaDELLA3 may have additional functions in the cytoplasm, in contrast to AaDELLA1. We also discovered that DELLAs do not interact with JAZ proteins in *A. annua*, in contrast to the DELLAs in *A. thaliana* (Hou et al., 2010). The question remains as to what evolutionary mechanism resulted in this arrangement and its significance for *A. annua* species.

Both JA and GA₃ are known to influence artemisinin production (Zhang et al., 2005; Maes et al., 2011). JA-induced MYC2 proteins are highly involved in the regulation of secondary metabolites such as terpenoid indole alkaloids in *Catharanthus roseus* (Zhang et al., 2011), nicotine in *N. tabacum* (Zhang et al., 2012), and indole glucosinolates and anthocyanins in *A. thaliana* (Dombrecht et al., 2007). Additionally, *Arabidopsis* MYC2 can

enhance the expression of sesquiterpene synthase genes (Hong et al., 2012). Recently, our laboratory found that overexpressing *AaMYC2* gene in *A. annua* increased artemisinin content. The strong interaction between AaDELLA and AaMYC2 proteins suggests that AaDELLAs may potentially affect artemisinin production through their interactions with AaMYC2. However, there are other potential mechanisms through which AaDELLAs may influence artemisinin yield. The suppression of *AaDELLA* expression may be required for flowering, which can induce the conversion of artemisinic acid to artemisinin (Zhang et al., 2005). It is also possible that AaDELLA protein degradation liberates GA-induced transcription factors, some of which may be involved in regulation of artemisinin production. For example, the GA-induced GLABROUS1 (GL-1) transcription factor is required for trichome formation in *Arabidopsis*, and the trichome is the site of artemisinin biosynthesis and storage in *A. annua* (Covello et al., 2007; Olsson et al., 2009). Therefore, suppression of *AaDELLA* or degradation of the protein may be able to relieve GL1-like transcription factors and induce trichome development in *A. annua*.

Conflicts of interest

The authors declare no conflict of interest.

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