



# The SPB-Box Transcription Factor *AaSPL2* Positively Regulates Artemisinin Biosynthesis in *Artemisia annua* L.

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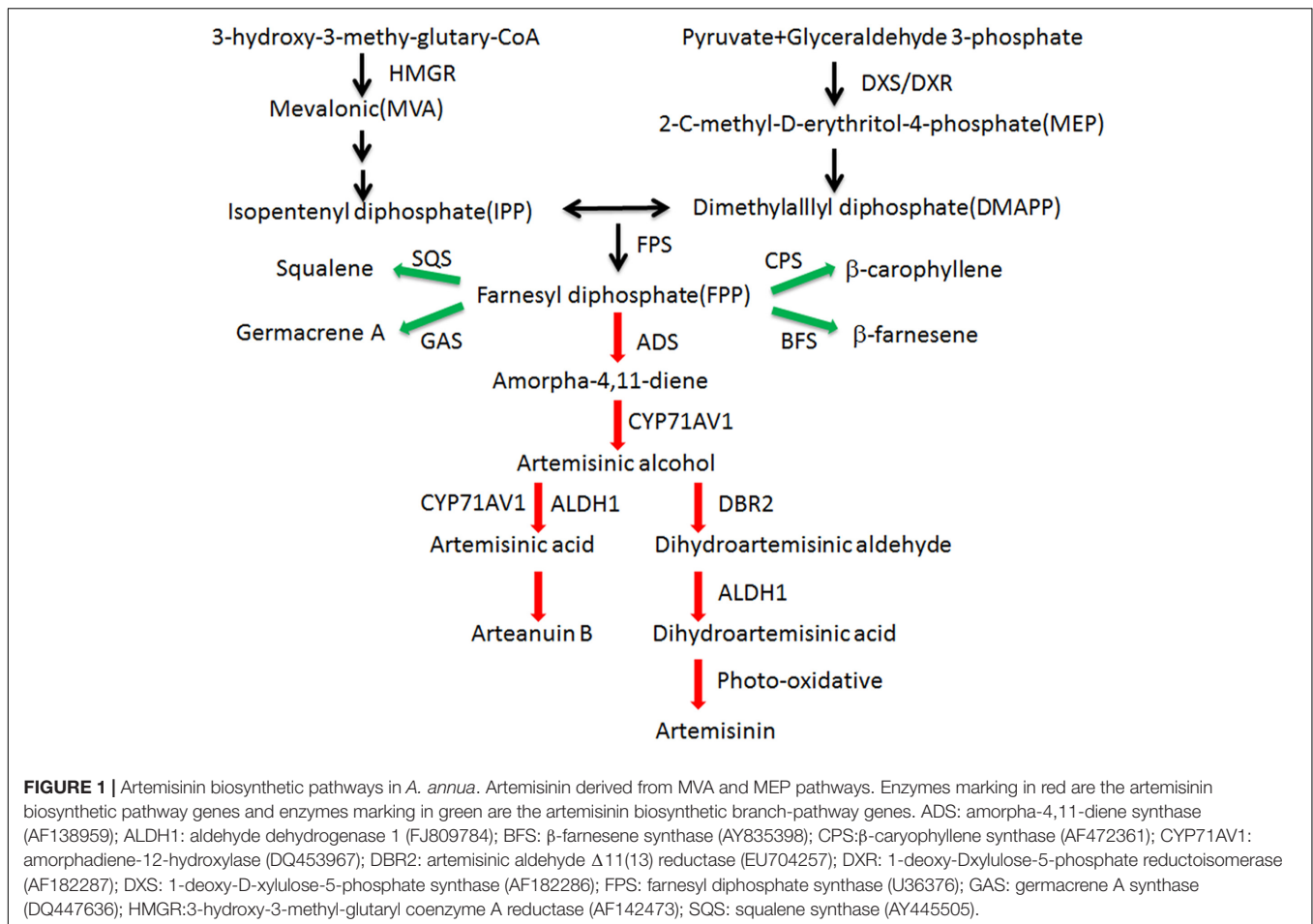
Artemisinin, an important compound produced by *Artemisia annua*, is the active ingredient in the treatment of malaria. Jasmonic acid, one of the phytohormones, is an important elicitor of artemisinin biosynthesis by enhancing transcription levels of transcription factors. SPL transcription factors are plant-specific transcription factors of plant growth, development, and secondary metabolism regulation. However, to date, the SPL transcription factors that regulate artemisinin biosynthesis is currently unclear. Here, we show that an SPL transcription factor can positively regulate artemisinin biosynthesis by binding to the promoter of artemisinin biosynthetic pathway genes. We screened *AaSPL2* by gene expression profiles analysis in 14 SPL transcription factors. We demonstrated that *AaSPL2* can activate the promoter of *DBR2* by dual-LUC assay. Moreover, in the *AaSPL2* overexpression plants, the artemisinin content was increased by 33–86%, and in the *AaSPL2* -RNAi transgenic plants, artemisinin content was decreased by 33–65%. These data suggest that *AaSPL2* and *DBR2* interact with a “GTAC” cis-element in the *DBR2* promoter, mediating the transcriptional activation of *DBR2* in response to JA and resulting in the improvement on artemisinin content.

**Keywords:** SPL transcription factor, jasmonic acid, transcript regulation, artemisinin, *Artemisia annua*

## INTRODUCTION

Malaria is a global infectious disease that 3.3 billion people are easy to infect it. Artemisinin combination therapy (ACT) has been the frontline treatment for malaria in recent years (World Health Organization, 2014). Artemisinin is produced in the glandular trichomes of *Artemisia annua* L (Duke and Paul, 1993). The artemisinin biosynthetic pathway is well studied (**Figure 1**).

**Abbreviations:** ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; CYP71AV1, amorpha-4,11-diene 12-hydroxylase; DBR2, artemisinic aldehyde  $\Delta^{11}(13)$  reductase; DHAA, dihydroartemisinic acid; YFP, yellow fluorescent protein.



In recent years, the semisynthesis of artemisinin following microbial production of artemisinic acid has already succeeded (Paddon et al., 2013). Because the price of artemisinin is fluctuating, the microbial deriving artemisinin may supplement the market but not replace agricultural production (Peplow, 2016). Our aim is to improve the content of artemisinin and reduce production costs.

In general, the average content of *A. annua* lies between 0.5–1.2%, while the purification yields are typically in the range of 50–80% of the crude extract (Kindermans et al., 2007). Because of the short supply and high costs of artemisinin, some strategies are employed to increase artemisinin production, such as blocking branch pathway genes (Zhang et al., 2009; Chen et al., 2011; Lv et al., 2016b), regulating transcription factors levels (Yu et al., 2015; Lv et al., 2016a) and modulating trichome numbers (Shi et al., 2017b; Yan et al., 2018). Semisynthesis of artemisinin by fermentation from engineered yeast is a feasible method of production of a precursor molecule, DHAA, which is easy to convert to artemisinin (Westfall et al., 2012).

Plant phytohormones increasing the secondary metabolites can be defined: the terpenoids, alkaloids and phenylpropanoids. In general, jasmonic acid (JA) is an important conserved elicitor of plant secondary metabolism. It triggers extensive transcriptional reprogramming and leads to an increase in the

expression level of the gene encoding the key enzyme for secondary metabolism biosynthetic pathway (De Geyter et al., 2012). JA can induce the mRNA levels of *ORCA3* in *Catharanthus roseus*, which is an important transcription factor for the regulation of terpenoid indole alkaloid biosynthesis (Zhang et al., 2011). *ERF189* can be induced by JA in tobacco and it regulates the nicotine biosynthesis by directly binding to the GCC-box in the promoter region of putrescine *N*-methyltransferase (PMT) (Shoji et al., 2010). JA is involved in the biosynthesis of artemisinin as well. Previously, JA increased the expression levels of transcription factor *AaWRKY1*, which enhanced artemisinin content by directly binding to the promoter of *ADS* (Ma et al., 2009). We have found that *AaNAC1* was induced by JA and played an important role in the modulation of artemisinin biosynthesis (Lv et al., 2016a). Many transcription factors can be induced by JA and many are involved in artemisinin biosyntheses, such as *AaERF1/AaERF2*, *AaORA*, and *AaMYC2*. The AP2/ERF transcription factors *AaERF1/AaERF2* and *AaORA* positively regulate the biosynthesis of artemisinin (Yu et al., 2012; Lu et al., 2013). The bHLH transcription factor *AaMYC2* is a positive regulator for artemisinin biosynthesis by binding to the G-box-like motifs within the promoters of the artemisinin biosynthetic pathway gene *CYP71AV1* and *DBR2* (Shen et al., 2016).

*SQUAMOSA Promoter-Binding Protein-Like (SPL)* genes are functionally diverse, including modulation of grain size, grain shape, branching, vegetative phase change, shoot regeneration and flowering time (Wang et al., 2009; Jiao et al., 2010; Zhang et al., 2015; Si et al., 2016). Recently, some studies indicate that *SPL* transcription factors can regulate secondary metabolism biosynthesis. AtSPL9 can regulate anthocyanin accumulation by inhibiting expression levels of anthocyanin biosynthetic pathway genes through destabilization of the Myb-bHLH-TTG1 complex (Gou et al., 2011). At the same time, AtSPL9 can regulate the content of (E)- $\beta$ -caryophyllene in *Arabidopsis thaliana* by binding to the promoter of *TPS21* (Yu et al., 2015). PpSPL1 negatively regulates the anthocyanin level in the peach flesh by inhibiting the transactivation activity of *NAC* transcription factors *BL* and *PpNAC1* through destabilization of the Myb-bHLH-TTG1 complex (Zhou et al., 2015).

Here, we also provide evidence that *SPL* transcription factors can regulate the artemisinin content by binding to the promoter of artemisinin biosynthesis gene *DBR2*. We provide evidence that overexpression of *AaSPL2* enhances artemisinin content, indicating that *AaSPL2* is a positive transcription factor of artemisinin biosynthesis in *A. annua*.

## MATERIALS AND METHODS

### Plant Material

Seeds of “Huhao 1” (Shen et al., 2016), which is a high artemisinin yielding *A. annua* strain, were first surface-sterilized in 75% ethanol for 2 min, followed by 10% (v/v) NaOCl (sodium hypochlorite) solution for 10 min, and rinsed three times with sterile water. Subsequently, the seeds were planted on MS medium (Murashige and Skoog, 1962) with 3.0% sucrose (m/v) and 0.8% agar (m/v) and cultured with a photoperiod of 16 h light/8 h dark and light at 8,000 Lux at 28°C. Three weeks later, the leaves of young seedlings of *A. annua* (about 5–7 cm in height) were cut and used as explants for transformation with the *Agrobacterium tumefaciens*-mediated leaf disk transformation method (Zhang et al., 2009).

Seeds of tobacco (*Nicotiana benthamiana*) were sown directly on soil (vermiculite: perlite: peat moss = 7: 0.5: 2), 1 week later, the tobacco seedlings were transferred into the new pots and cultured with the same conditions as *A. annua*. Five weeks later, the leaves of tobacco can be used for *A. tumefaciens* infiltration assay.

### Construction and Transformation of *A. annua*

The ORF of *AaSPL2* was introduced into the BamHI and SacI sites of pCAMBIA2300 (CAMBIA, Canberra, Australia) under the driven of CaMV35S promoter to generate CaMV35S::*AaSPL2* (overexpression vector). For RNAi interference assay, a 300 bp *AaSPL2* fragment was fused into PENTR vector via the BP reaction (Invitrogen, United States) and then introduce into the pHELLSGATE12 vector via the LR reaction (Invitrogen, United States) to generate the final RNAi- *AaSPL2* vector.

The overexpression constructs OX-*AaSPL2* and RNAi constructs *AaSPL2*-RNAi were introduced into *A. tumefaciens*

strain EHA105 by a conventional freezing-and-melting method (Zhang et al., 2009). The method of transformation of *A. annua* was according to the previous description (Lv et al., 2016b).

### Plant Hormone Treatments

MJ was purchased from Sigma-Aldrich (St Louis, MO, United States). For plant hormone treatment, 4-week-old *A. annua* seedlings were treated with 100  $\mu$ M MJ (Maes et al., 2011; Yu et al., 2012; Shen et al., 2016). Then the treated plants were sampled at time point 0, 1, 3, 6, 9, 12, and 24 h for RNA extracting and Q-PCR analysis.

### Isolation of Genomic DNA and PCR Analysis

The cetyltrimethylammonium bromide (CTAB) method was employed to obtain the genomic DNA of the transgenic plants (Allen et al., 2006). The forward primers of *AaTGA6* were used to detect the transgenic plants. A 20 bp sequence of the vector was designed as reverse primers. The 20 ml PCR with 10 pmol forward primer, 10 pmol reverse primer, 50 ng of plant genomic DNA, 7 ml of sterilized water and 10 ml of rTaq DNA polymerase mix (TaKaRa, Japan) was performed. The amplification procedure consisted of a 5-min incubation at 95°C, followed by 30 cycles of 95°C for 30 s, and 60 s at 55°C for primer annealing and 72°C for 600 s elongation.

### RNA Isolation and Real-Time PCR Analysis

Young leaves of *A. annua* plants were used for RNA isolation by plant RNA Kit (Tiangen) according to the manufacturer's instructions. The isolated total RNA was reverse-transcribed into cDNA using the TaKaRa cDNA synthesis kit. qRT-PCR assays using SYBR Green PCR Master Mix kit (Tiangen) as a detector were performed in Roch LightCycler<sup>®</sup>96 system according to the manufacturer's instructions. The comparative cycle threshold (CT) method was estimated relative transcript levels of samples and means of triplicate independent PCRs. The  $\beta$ -*actin* gene of *A. annua* was used to estimate relative mRNA levels. The qRT-PCR primers are listed in **Supplementary Table S1**. The process for amplification was 5 min at 95°C, followed by 40 cycles of 95°C for 10 s, and 40 s at 60°C for primer annealing and elongation. Then a dissociation stage for 20 s at 95°C, 40 s at 60°C, and 20 s at 95°C.

The  $2^{-\Delta\Delta CT}$  method was used for estimating the relative gene expression level according to the following formula (Livak and Schmittgen, 2001):

$$\Delta\Delta CT = (C_{T,Target} - C_{T,actin})_{Time\ x} - (C_{T,Target} - C_{T,actin})_{Time\ 0}$$

### Yeast One-Hybrid Assay

To analyze the specific binding of *AaSPL2* to GTAC-box of the *DBR2* promoters, the full length of *AaSPL2* was fused into the *EcoRI*-*XhoI* sites of the activation domain of the vector pB42AD (Clontech). Equal volume of the GTAC-box forward primer (10  $\mu$ M) and reverse primer (10  $\mu$ M) (**Table 1**) were mixed and incubated at these conditions: 95°C for 1 min,

**TABLE 1** | The character of SPLs in *A. annua*.

Gene name	Accession number	Gene length	PI	MW	miR156 target site
AaSPL1	MH090670	2484	4.9	206.73	
AaSPL2	MH090671	954	5.09	79.08	
AaSPL3	MH090672	2379	4.92	195.68	
AaSPL4	MH090673	879	5.1	72.71	✓
AaSPL5	MH090674	423	5.3	33.52	✓
AaSPL6	MH090675	2865	4.89	234.37	
AaSPL7	MH090676	1131	5.09	93.74	✓
AaSPL8	MH090677	1167	5.04	97.21	✓
AaSPL9	MH090678	906	5.1	74.37	✓
AaSPL10	MH090679	1284	5.02	107.01	✓
AaSPL11	MH090680	447	5.07	35.32	
AaSPL12	MH090681	516	5.27	40.57	✓
AaSPL13	MH090682	1197	5.03	99.08	✓
AaSPL14	MH090683	555	5.23	44.87	✓

85°C for 1 min, 75°C for 1 min, 65°C for 1 min, 55°C for 1 min, 45°C for 1 min, 35°C for 1 min, 25°C for 1 min. The reaction primer products were inserted into P178 vector (gifted by Dr. Yang Hongquan of Shanghai Jiao Tong University, Shanghai, China) with a single restriction site XhoI to form the reporter vector. Vectors containing the transcription factor gene and 3 × GTAC-box were introduced into the yeast strain EGY48 by LiAc mediated transformation method (Gietz and Schiestl, 2007). Yeast cells were selected on the synthetic dropout (SD) base medium with deficient in Trp and Ura

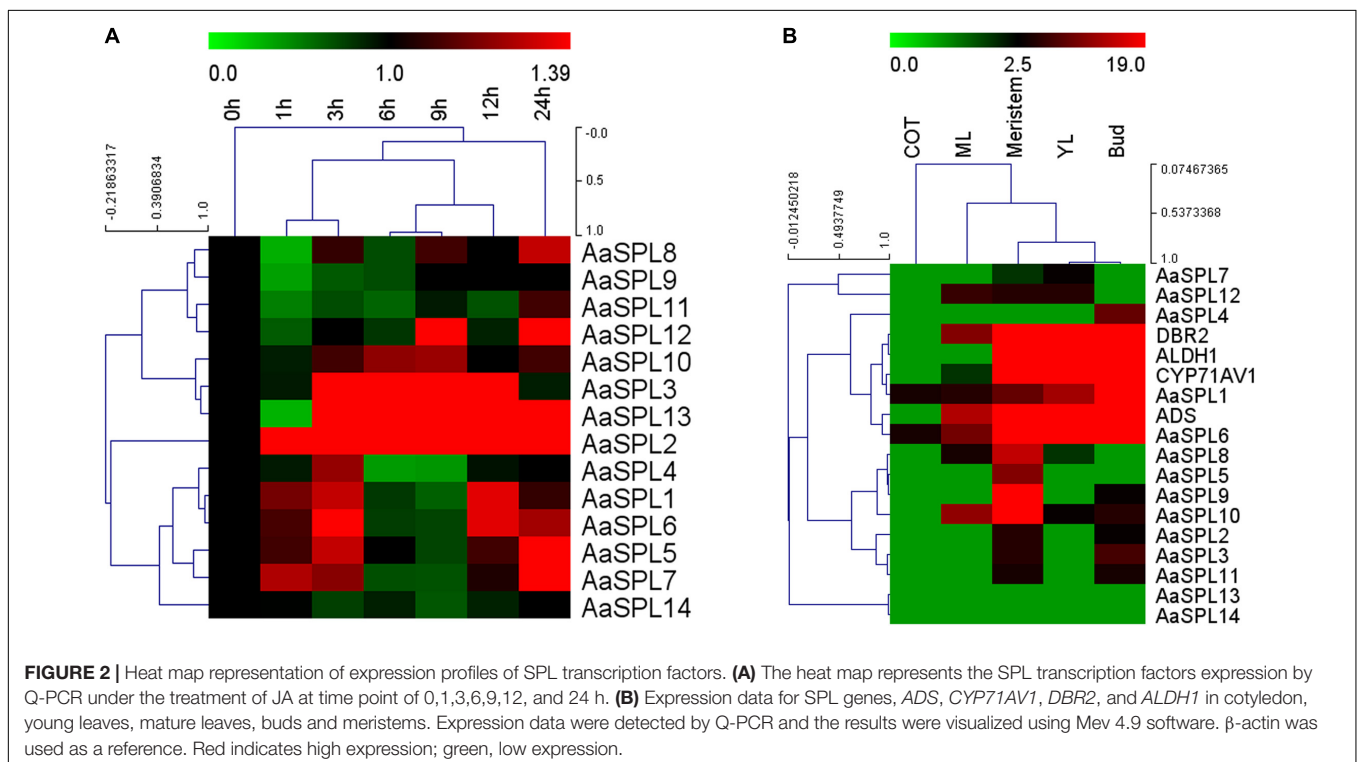
for 3 days at 30°C. Then yeast cells were resuspended with sterile water and dropped on an 5-bromo-4-chloro-3-indolyl-beta-Dgalactopyrano-side (X-gal) medium plate (Zhang et al., 2015). The sequences of the 3 × GTAC-box fragment are listed in **Supplementary Table S1**.

## Dual-LUC Assay

For the dual-LUC assay, the promoter of *DBR2* was fused to firefly luciferase gene on the plasmid pGreenII 0800-LUC. The sequenced plasmid was introduced into *A. tumefaciens* strain EHA105. Then incubated *Agrobacterium* cells were harvested by centrifugation and resuspended in MS medium (containing 10 mM MES and 150 mM acetosyringone) to an OD600 nm of 0.6. After 3 h incubation at room temperature, bacteria (reporter strain DBR2pro::LUC mixed with effector strain 35Spro::AaSPL2 1:1) were injected into tobacco leaves. Forty-eight hours' later, the injected tobacco leaves were collected for dual-LUC assay. The dual-Luciferase Reporter Assay system of Promega was used for dual-Luc assays according to the manufacturer's instructions.

## Quantification of Artemisinin Using HPLC-ELSD (HPLC-Evaporative Light Scattering Detection)

The upper parts leaves of *A. annua* were collected at pre-flowering stage and dried at 50°C for 24 h. then all the leaves were ground into powder. A sample of 0.1 g powder was suspended in ethanol (2 mL) for ultrasonication. Sampling was carried out in triplicate. Thirty min later, Samples were centrifuged for 15 min, 4°C, at 12 000 g. The supernatant was filtered through a 0.22 mm pore size filter and then analyzed by the Waters Alliance 2695





HPLC system. Artemisinin standard (98% pure) was purchased from Sigma. The approach for detecting artemisinin using HPLC-ELSD has already been described (Zhang et al., 2009).

## RESULTS

### Characterization of AaSPLs in *A. annua*

The SQUAMOSA promoter-binding protein (SBP) genes contain a conserved domain, which encodes 76 amino acid residues in length for the interaction with DNA (Cardon et al., 1999). To identify the SPLs in *A. annua*, the putative conserved SBP domain was used for searching the transcriptome database (Graham et al., 2010; Hao et al., 2017) using the HMMER3.0 search program<sup>1</sup> (Finn et al., 2011). Through molecular cloning, we identified a total of 14 SPLs (*AaSPL1*~*AaSPL14*) in *A. annua* with the GenBank accession number (Table 1). The open reading frame (ORF) of the SPL is varied in length from 447bp to 2865bp. All the SPLs encode protein products 149 to 955 amino acids with the calculated isoelectric point<sup>2</sup> of 4.89 to 5.3. The details of the gene character of SPLs are shown in the Table 1.

MiR156 plays important role in modulation of transcription levels of targeting SPLs for translational repression or cleavage (Wang et al., 2009; Wu et al., 2009). So the miR156 in *A. annua* has been studied recently (Pani et al., 2011). To confirm the targets of miR156, we screened all of the SPLs in *A. annua*. Nine of SPLs have target site of miR156 and they are predicted to be the potential targets of miR156 (Table 1).

### Expression Patterns of SPLs in *A. annua*

JA plays a crucial role in inducing the artemisinin accumulation (Jing et al., 2009; Maes et al., 2011; Yu et al., 2012). We hypothesized that JA may be used for screening the SPL which takes part in modulating artemisinin biosynthesis in *A. annua*. First, we checked mRNA levels of *AaSPL1*~*AaSPL14* at different time points (0, 1, 3, 6, 9, 12, and 24 h) after treatment with JA (100  $\mu$ M). To this end, the expression intensity and hierarchical clustering (HCL) of SPL genes were formatted as a heatmap by Mev4.9.0 software.<sup>3</sup> Exogenous application of JA to *A. annua* seedlings resulted in strong activation of both *AaSPL2* and *AaSPL13* (Figure 2A), indicating that both of them may play a role in the regulation of artemisinin.

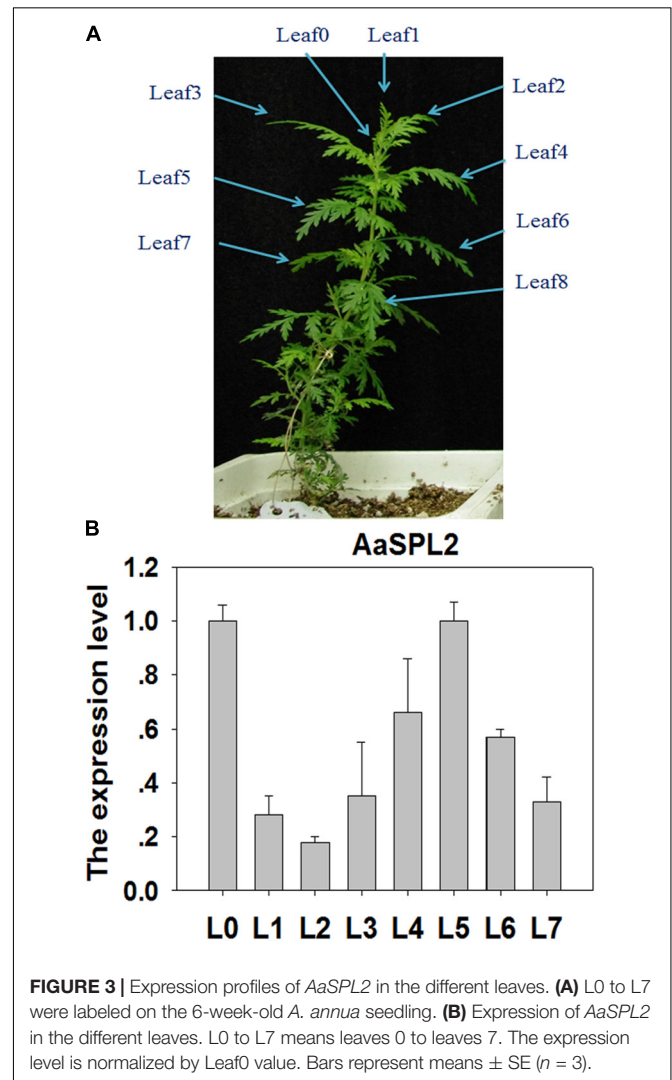
In the process of artemisinin biosynthesis, the artemisinin biosynthetic pathway genes *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* mRNA levels were accumulated in the young leave tissues (YL) and buds, confirming previous studies that artemisinin was produced in the glandular trichomes (Maes et al., 2011). To confirm the expression files of SPLs of *A. annua*, we tested SPL expression levels in the RNA-sequence of different tissues (Graham et al., 2010). As shown in Figure 2B, heatmap results indicated that the mRNA levels of *AaSPL2* are higher in bud compared with *AaSPL13*, therefore, we focus our attention on *AaSPL2* according to the results of the expression profiles of SPLs.

### Characterization of AaSPL2

SPL transcription factors are known to regulate the biosynthesis of secondary metabolites, such as sesquiterpene, flavonoid (Gou et al., 2011; Yu et al., 2015). We hypothesized that SPL may modulate the biosynthesis of sesquiterpene lactone artemisinin by directly or indirectly regulation of artemisinin biosynthetic pathway genes. To investigate the gene function and control mechanism, a 954-bp segment of the *AaSPL2* ORF region was isolated from cDNA. The full-length cDNA of *AaSPL2* encodes a polypeptide of 318 amino acid residues with a calculated mass of 79 kDa and a pI of 5.09 (Table 1). We also detected the gene expression level of *AaSPL2* in leaf0~leaf7 (L0~L7) (Figure 3A). The Q-PCR results indicated that *AaSPL2* expressed at a high level in the L0, L4, L5, and L6 (Figure 3B).

### Phylogenetic Analysis of AaSPL2 Proteins

The analysis on full-length nucleotide sequence showed that *AaSPL2* contains a conserved SBP domain in the 289–471

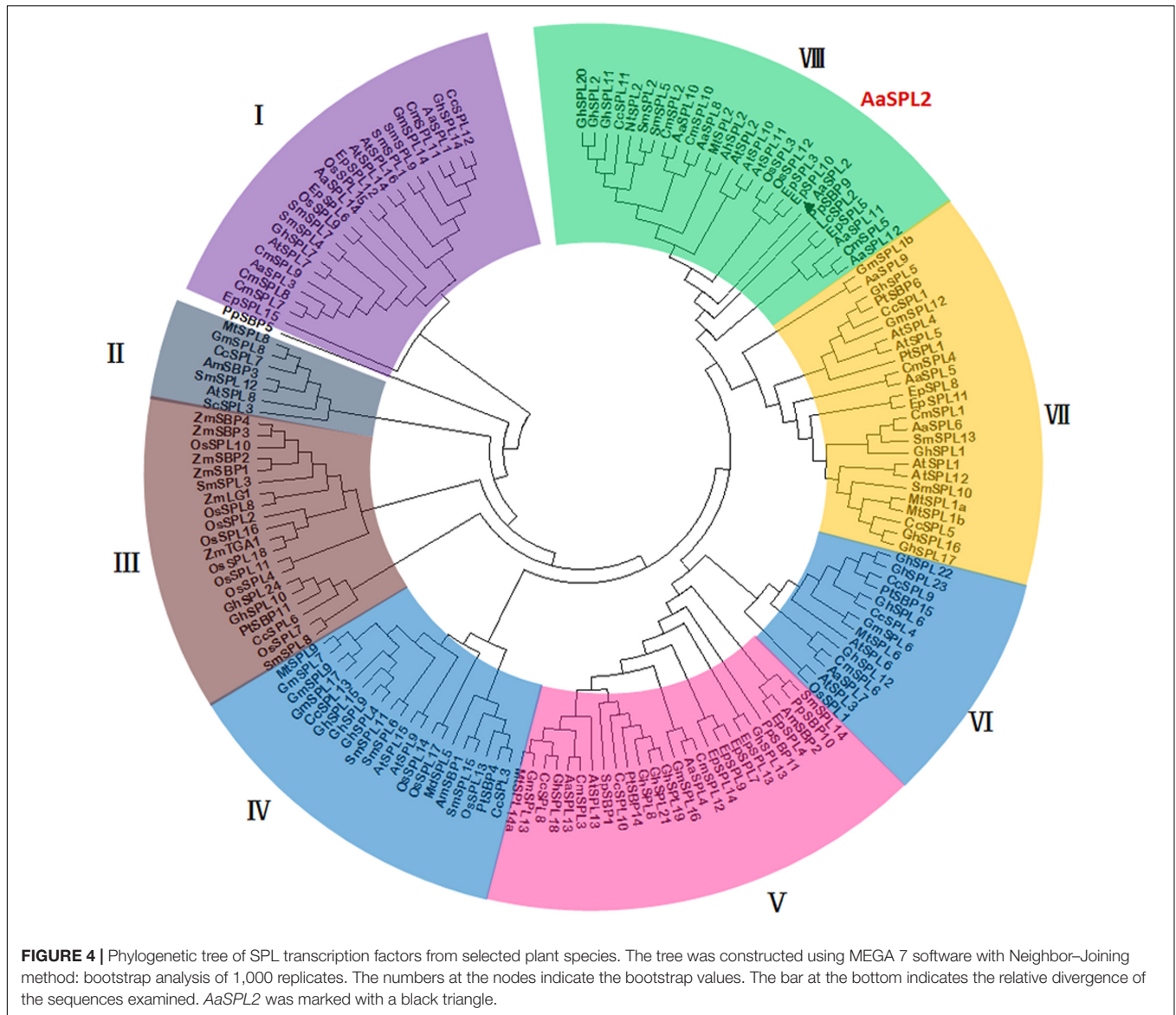


**FIGURE 3 |** Expression profiles of *AaSPL2* in the different leaves. **(A)** L0 to L7 were labeled on the 6-week-old *A. annua* seedling. **(B)** Expression of *AaSPL2* in the different leaves. L0 to L7 means leaves 0 to leaves 7. The expression level is normalized by Leaf0 value. Bars represent means  $\pm$  SE ( $n = 3$ ).

<sup>1</sup><http://hmmer.janelia.org>

<sup>2</sup>[https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/)

<sup>3</sup><http://www.mybiosoftware.com/mev-4-6-2-multiple-experiment-viewer.html>



**FIGURE 4 |** Phylogenetic tree of SPL transcription factors from selected plant species. The tree was constructed using MEGA 7 software with Neighbor-Joining method; bootstrap analysis of 1,000 replicates. The numbers at the nodes indicate the bootstrap values. The bar at the bottom indicates the relative divergence of the sequences examined. AaSPL2 was marked with a black triangle.

regions. A phylogenetic tree by using nucleic acid sequences was built to investigate the phylogenetic relationships among the SPL proteins in *A. annua*, Arabidopsis and rice other species. The results indicated that AaSPL2 belongs to the subfamily of AtSPL2/10/11 transcription factors (Figure 4). AtSPL2/10/11 has been reported to modulate vegetative phase change and trichome development (Shikata et al., 2009), suggesting that AaSPL2 has a potential role in the regulation of phase transition or trichome initiation in *A. annua*.

### Subcellular Localization of AaSPL2

To investigate the subcellular localization of the AaSPL2 protein, a YFP was fused to the C-terminus of AaSPL2 and the AaSPL2-YFP construct was expressed under the control of the double 35S Cauliflower mosaic virus (CaMV) promoter. Agroinfiltration method was performed to infiltrate *N. tabacum* leaves (Zhang et al., 2015). Controls showed

GFP expressed throughout the cytoplasm (Figure 5), whereas the AaSPL2-YFP fusion was targeted to the nucleus (Figure 5).

### AaSPL2 Activates the Transcription of *DBR2* *in vivo*

The SQUAMOSA promoter binding protein transcription factors can activate the transcription of MADS transcription factors by binding to “GTAC” *cis*-element in the promoter (Franco-Zorrilla et al., 2014). There are 9 “GTAC” *cis*-elements in the promoter of *DBR2* (Jiang et al., 2014). Thus, we hypothesized that AaSPL2 may potentially induce the transcription of *DBR2*. First, a dual luciferase assay (dual-LUC) was employed to detect if the expression of *DBR2* can be regulated by the product of the transcription factor AaSPL2 (Figure 6A). The results of dual-LUC

indicated that *AaSPL2* activates the transcription of *DBR2* (Figure 6B).

To further substantiate this finding, we tested whether *AaSPL2* is able to bind to the “GTAC” box in the *DBR2* promoters. Therefore, the ninth “GTAC” elements were cloned into the reporter vector p178 (Figure 6C). The results of the Y1H indicated that *AaSPL2* can bind to the “GTAC” of the *DBR2* promoters directly (Figure 6D).

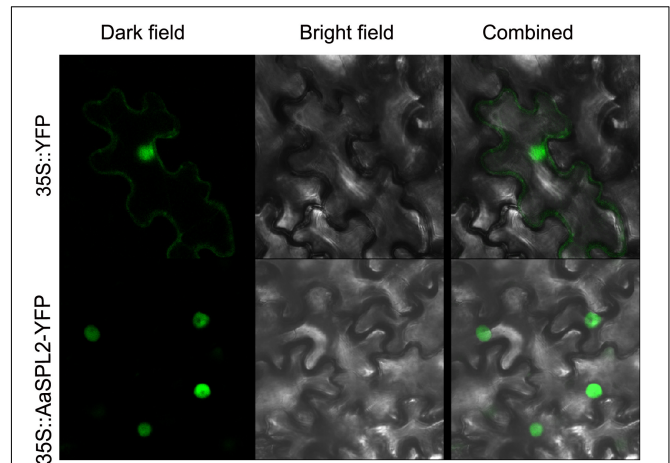
## Overexpression of *AaSPL2* in *A. annua* Increases Artemisinin Biosynthesis

The function of SPL transcription factors is diverse. To investigate the functional role of *AaSPL2*, we overexpressed *AaSPL2* (abbreviated as *AaSPL2*-OX) in *A. annua*. The transgenic plants were detected by PCR. Overexpression of *AaSPL2* in *A. annua* resulted in enhanced *AaSPL2* expression upon wild-type (Figure 7). The expression levels of artemisinin biosynthetic pathway genes *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* were also detected. Similar results were obtained with the expression levels of *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* (Figure 7). Therefore, to investigate whether *AaSPL2* is a positive regulator for artemisinin biosynthesis, the artemisinin content was detected. In the *AaSPL2* overexpression plants, the artemisinin and DHAA content was increased to 33–86% and 26–159%, respectively (Figure 8A and Supplementary Figure S1A). In the *AaSPL2*-RNAi transgenic plants, the mRNA levels of *AaSPL2* and artemisinin biosynthetic pathway genes all decreased (Figure 7). The artemisinin and DHAA content was decreased by 33–65% and 3–16%, respectively (Figure 8B and Supplementary Figure S1B). Thus, *AaSPL2* is a positive regulator for artemisinin biosynthesis. The transgenic plants displaying normal phenotype overexpressed in *Arabidopsis* (Supplementary Figure S2) and in *A. annua* (Supplementary Figure S3).

## DISCUSSION

### MJ Induced Artemisinin Biosynthesis

Since JA and its cyclic precursors and derivatives play important roles in inducing the genes encoding enzymes involving in the biosynthesis of secondary metabolites in the plant (Van der Fits and Memelink, 2000; De Boer et al., 2011). So JA becomes the hotspot as the secondary metabolites inducer. Previously, it was shown that overexpression of artemisinin biosynthetic pathway genes (*ADS*, *CYP71AV1*, *DBR2*, *ALDH1*, *CPR*) can redirect carbon flux to the artemisinin pathway and increase artemisinin contents for several folds (Chen et al., 2013; Shi et al., 2017a). JA enhances artemisinin biosynthetic pathway genes mRNA levels and plays the same roles as overexpression artemisinin biosynthetic pathway genes (Maes et al., 2011). JA improved artemisinin content by increasing mRNA levels of transcription factors as well, which regulate gene expression level through specific binding to *cis*-acting elements in the target genes promoter. Many



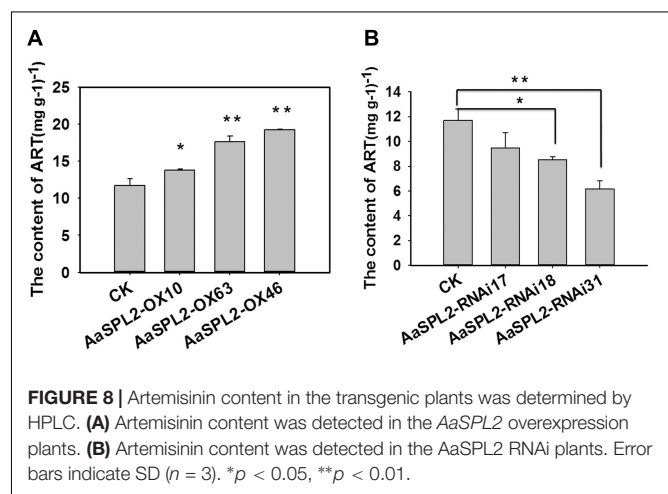
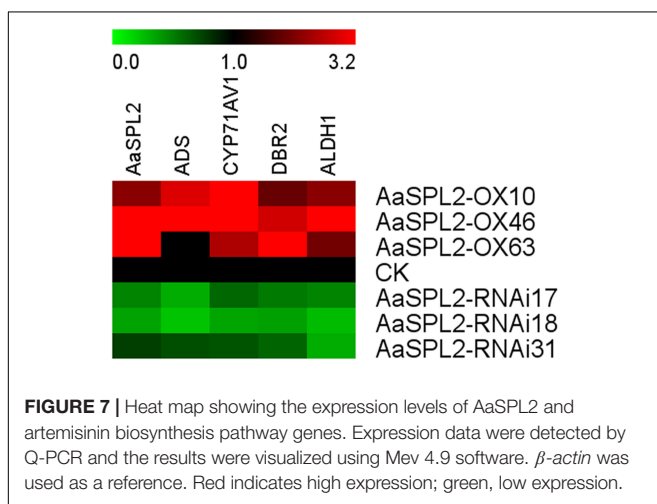
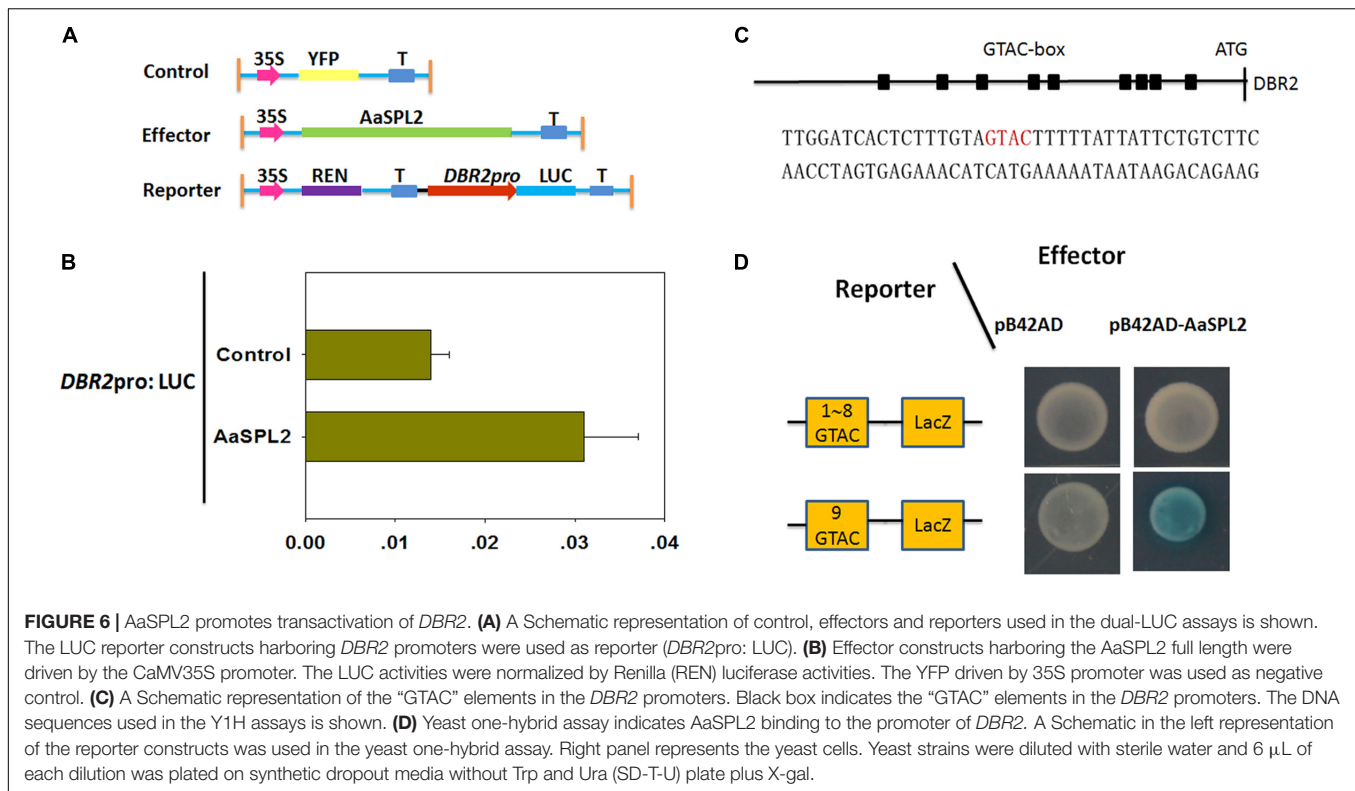
**FIGURE 5 |** Subcellular localizations of *AaSPL2* in *N. benthamiana* leaves. Constructs 35S::AaSPL2-YFP were introduced into *Agrobacterium* and transferred into *N. benthamiana* by infiltration. Constructs 35S::YFP were used as control.

transcription factors are involved in JA signaling cascades and regulate the biosynthesis of artemisinin. Such as AP2/ERF transcription factors *AaERF1* and *AaERF2*, they bind to the promoter of *ADS* and *CYP71AV1* directly (Yu et al., 2012). Another AP2/ERF transcription factor *AaORA* (Lu et al., 2013), which increases artemisinin content by improving artemisinin biosynthetic pathway genes mRNA levels. *AaMYC2* and *AaNAC1* activate the expression level of artemisinin biosynthetic pathway genes *CYP71AV1*, *DBR2*, and *ADS*, respectively (Lv et al., 2016a; Shen et al., 2016). *AaSPL2* mRNA level was enhanced under the treatment of JA and the content of artemisinin was increased in the *AaSPL2* overexpression plants also (Figure 2). *AtSPL9* attended the JA pathway through interacting with known JA signaling factor (such as *JAZ3*) to regulate biosynthesis of secondary metabolism glucosinolate and  $\beta$ -caryophyllene (Yu et al., 2015; Mao et al., 2017). On the other hand, JA improved artemisinin content by increasing of trichome number (Maes et al., 2011). Artemisinin was specifically synthesized in the glandular trichome and was related to the trichome number. The increase of trichome number may decrease feedback inhibition of artemisinin content (Arsenault et al., 2010). In our study, trichome number changes not obviously (Supplementary Figure S3F), suggesting that *AaSPL2* may not take part in trichome regulation.

### SPL Transcription Factors Regulation of Terpenoid Products

Through genome-wide identification, a total of 14 SPLs have been found in *Salvia miltiorrhiza* (Zhang et al., 2014). There are 19 SPL genes in the rice and 16 SPLs in *Arabidopsis* (Birkenbihl et al., 2005; Manning et al., 2006). A systematical study reported that 59 SPLs were identified in the Genome-wide of *G. hirsutum*. SPLs play important roles in fiber initiation, branching, root growth, grain size and yield, suggesting that SPL transcription





factors play key roles in *G. hirsutum* growth and development. The reasons for so many SPLs in the *G. hirsutum* are the tandem duplication events or by assembly error (Cai et al., 2018). In this study, a total of 14 SPLs were cloned. AaSPL2 mainly expressed in the buds (Figure 2B) and induced by MJ (Figure 2A), play important role in artemisinin biosynthesis (Figure 8). However, AaSPL3 has the same expression patterns with AaSPL2 (Figures 2A,B), therefore, it is a potential gene for improving artemisinin content.

SPL transcription factors can regulate gene expression through binding to *cis*-regulatory specific core “GTAC”

consensus sequences in the promoters of their target genes. Previously, the core “GTAC” consensus sequence of *DFR* has been identified as the binding motif of SPL9 (Gou et al., 2011). Plant terpenoids have important physiological and ecological functions, such as serving as signals in biocommunications and interactions, and phytoalexins in defense against pests and pathogens (Sharma et al., 2017). Terpene synthase 21 (*TPS21*) is responsible for the vast majority of floral sesquiterpenes and it can catalyze the conversion of FPP into  $\beta$ -caryophyllene. Transcription factor SPL9 directly binds to *TPS21* promoters and activates its expression to regulate the biosynthesis of



$\beta$ -caryophyllene (Yu et al., 2015). Artemisinin is a sesquiterpene, hence, we postulated that SPL in *A. annua* may function similarly as an SPL in *Arabidopsis*. The phylogenetic tree indicated that AaSPL2 may homologous to the AtSPL3 in *Arabidopsis*.

The expression patterns indicated that AaSPL2 mainly expressed in the buds (Figure 2), which is partly same as artemisinin biosynthetic genes expression patterns, so AaSPL2 in *A. annua* may play the role in the regulation of sesquiterpene artemisinin. AaSPL2-OX and AaSPL2-RNAi results show that SPL2 can modulate artemisinin (Figure 8) and DHAA accumulation (Supplementary Figure S1). The fact that the overexpression of AaSPL2 partially increases the transcription levels of artemisinin biosynthetic gene *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1* supported the hypothesis that enhancing of AaSPL2 expression level may be a plausible mechanism for increase artemisinin content.

## AUTHOR CONTRIBUTIONS

ZL, KT, WC, and LZ conceived and designed the entire research plans. BP, YL, and ZL performed the most of the experiments. BP and ZL provided the technical assistance. ZL wrote the manuscript. ZL, YW, and LZ helped with the organization and editing.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2019.00409/full#supplementary-material>

**FIGURE S1** | DHAA content in the transgenic plants was determined by HPLC. (A) DHAA content was detected in the AaSPL2 overexpression plants. (B) DHAA content was detected in the AaSPL2 RNAi plants. Error bars indicate SD ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ .

**FIGURE S2** | The phenotype of AaSPL2 overexpressed in *Arabidopsis*.

**FIGURE S3** | The phenotype of AaSPL2 overexpressed in *A. annua*. (A) A schematic representation of overexpression vector and RNAi vector used in the *A. annua* transformation is shown. (B) the phenotype of overexpression and RNAi transgenic plants. (C) Glandular trichome of control. (D) Glandular trichome of overexpression plants. (E) Glandular trichome of RNAi plants. (F) Trichome number of *A. annua*. Scale bar represents 0.5 mm.

**TABLE S1** | Primers used in this study.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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