**AaWIN1, an AP2/ERF protein, positively regulates glandular secretory trichome initiation in *Artemisia annua***

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**Highlights**

* AaWIN1 positively regulates glandular secretory trichome (GST) initiation in *Artemisia annua*.
* AaWIN1 activates the expression of *AaGSW2*.
* AaWIN1 interacts with AaMIXTA1.
* Ectopic expression of *AaWIN1* results in fewer trichomes in *Arabidopsis thaliana.*

**Abstract**

Exploring the genetic network of glandular trichomes and manipulating genes relevant to secondary metabolite biosynthesis are of great importance and value. Artemisinin, a key antimalarial drug ingredient, is synthesized and stored in glandular secretory trichomes (GSTs) in *Artemisia annua*. WIN/SHN proteins, a clade of AP2/ERF family, are known as regulators for cuticle biosynthesis. However, their function in glandular trichome development is less unknown. In this study, we identified a *WIN/SHN* gene from *A. annua* and named it as *AaWIN1*. *AaWIN1* was predominantly expressed in buds, flowers and trichomes, and encoded a nuclear-localized protein. Overexpressing *AaWIN1* in *A. annua* significantly increased the density of GST as well as the artemisinin content. Furthermore, AaGSW2 was reported to play an important role in promoting GST initiation, and the expression of *AaGSW2* was induced in *AaWIN1*-overexpression lines. AaMIXTA1, a MYB protein positively regulating trichome initiation and cuticle biosynthesis, was confirmed to interact with AaWIN1. In addition, the ectopic expression of *AaWIN1* resulted in slender and curled leaves, fewer trichomes, and rising expressions of cuticle biosynthesis genes in *Arabidopsis thaliana*. Taken together, based on phenotype observations, content measurements and gene expression detections, AaWIN1 was considered as a positive regulator for GST initiation.

**Key words**: WIN/SHN, MIXTA, glandular trichome, initiation, *Artemisia annua*

**1 Introduction**

Trichomes are outgrowths derived from epidermal cells and present on most terrestrial plants, especially on leaves, floral organs and stems[1]. They play important roles in preventing plants from biological and physical attack, as well as in improving abiotic stress tolerance of UV irradiation and extreme temperature[2, 3]. Trichomes can be divided into branched and non-branched, unicellular and multicellular, or glandular and non-glandular trichomes across species[4-6]. In particular, glandular trichomes offer both chemical protections and commercial values by secreting mixtures of secondary metabolite, such as terpenoids, flavonoids, phenylpropenes and acylsugars[7-10]. Most of these specialized metabolites serve as feedstocks for medical, nutraceutical and cosmetic industries[11]. Therefore, investigations into glandular trichomes are of great importance and value.

In general, trichome development process consists of identity determination, initiation, morphogenesis and maturation[12]. Significant progress has been made in characterizing genes responsible for non-glandular trichome development, particularly in *Arabidopsis*[13-19]. By contrast, our understanding of glandular trichome development is still very limited[20]. Recent studies have shown that glandular trichomes might form through a distinct pathway from non-glandular trichomes. Investigations into glandular trichome development are including but not limited to tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), mint (*Mentha spicata*) and *A. annua* [21-23].

Among eight types of trichome in the tomato family, type Ⅰ, Ⅵ, Ⅳ and Ⅶ are glandular trichomes, and most studies are focused on type Ⅰ and Ⅵ[24]. MYB family SlMIXTA1 positively regulates trichome initiation especially for type I[25]. *Hair* is essential for type I trichome formation, encoding a C2H2 zinc-finger protein which regulates expression of *SlCycB2* and interacts with a HD-ZIP Ⅳ protein Wolly[26, 27]. The bHLH protein MYC1 is related to type Ⅵ trichome formation[28]. Two types of trichome present in *A. annua*,one isglandular secretory trichome (GST) acting as the putative bio-factory of artemisinin, the another is T-shaped trichome (TST)[4]. Several transcription factors are reported to regulate GST initiation in *A. annua*, such as WRKY, MYB, HD-ZIP Ⅳ and SAP[29-34]. Recent studies suggest that glandular trichomes are regulated by some functional homologues in tomato and *A. annua*, such as HD-ZIP Ⅳ family SlCD2 and AaHD8, or MYB family SlMIXTA-like and AaMIXTA1[35-38].

AP2/ERF transcription factors play important roles in plant growth and metabolic process regulation. The clade of WIN/SHN proteins belongs to Ⅴa (B-6) group of ERF subfamily, and contains an APETALA2 (AP2) domain, a CMV-1 motif and a CMV-2 motif[39]. *WIN/SHN* genes are initially identified from *Arabidopsis* and then are successively characterized in important food crops and horticultural plants[40-48]. WIN/SHN proteins are known as regulators for cuticle formation or trichome development. For example, AtWIN1 and SlSHINE3 are required for cuticle biosynthesis in *Arabidopsis* and tomato, respectively[41, 49, 50]. Another homologue AaTAR1 is reported to regulate GST morphogenesis and artemisinin biosynthesis[51]. To date, however, no WIN/SHN protein is found to regulate trichome initiation in *A. annua.*

Artemisinin is considered as the most effective compound against malaria. In order to meet the huge demand, remarkable efforts have been made to improve artemisinin productivity[52]. One approach to gain high production of artemisinin is to improve GST density in *A. annua*, and many genes relevant to GST initiation remain to be discovered[37]. In this study, we identified an AP2/ERF transcription factor AaWIN1 as a positive regulator for GST initiation, which further advances the understanding of glandular trichome development. In addition, the interaction relationship between AaWIN1 and AaMIXTA1 also provides an insight into genetic network of WIN/SHN and MIXTA proteins.

**2 Materials and methods**

**2.1 Plant materials and growth conditions**

*Arabidopsis* ecotype Columbia-0, *A. annua* cultivar Huhao 1, and *Nicotiana benthamiana* were used as plant materials[53]. *Arabidopsis* plants grew at 22°C in a 16 h/8 h light/dark photoperiod. *A. annua* and *N. benthamiana* plants grew at 25°C in a 16 h/8 h light/dark photoperiod.

**2.2 Bioinformation prediction and analysis**

AP2/ERF proteins containing the conserved AP2 domain were identified from our unpublished transcriptome database by HMMER 3.0[54]. The phylogenetic tree was analyzed by MEGA 7.0 using maximum likelihood (ML) method based on LG+G model and neighbor-joining method. The hierarchical cluster analysis was performed by TBtools. The amino acid sequence alignment of WIN/SHN proteins was performed by GeneDoc. The cis-elements and subcellular localization were predicted by PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and ProtComp 9.0 (<http://www.softberry.com/berry.phtml>), respectively.

**2.3 Subcellular Localization**

The full-length cDNA of *AaWIN1* was amplified from leaf cDNA and inserted into pHB-YFP vector. The construct pHB-AaWIN1-YFP was transformed into *Agrobacterium tumefaciens* strain GV3101 and then was used to infiltrate 4-week-old *N. benthamiana* leaves. The treated *N. benthamiana* plants grew in darkness for 24 h and in light for another 24h. YFP signals were observed by confocal laser microscopy Leica TCS SP5-II. All experiments were repeated three times. Primers are listed in Table S1.

**2.4 Isolation of glandular trichomes**

Flower buds (20g) were mixed with 0.5mm glass beads (50g) and extraction buffer (250 mL). The mixture was shaken thoroughly for 1 minute; repeat three times. The mixture was sequentially passed through 350 μm, 105 μm and 20 μm nylon sieves to isolate the glass beads and tissue fragments. Glandular trichomes were collected on the 20 μm nylon sieves and were washed by buffer at least eight times. Tissues and glandular trichomes were stored and disposed in ice. This method was modified based on the previously described protocol[55].

**2.5 RNA extraction and QRT-PCR analysis**

Tissues (buds, flowers, young leaves, old leaves, roots, shoots, stems and trichomes) and leaves (leaf 0, leaf 1, leaf 2, leaf 3, leaf 4, leaf 5, leaf 6) of 5-month-old wild type *A. annua* plants were collected for *AaWIN1* expression pattern analysis. Leaf 0 (meristem) of 5-month-old transgenic *A. annua* plants, rosette leaves of 2-week-old wild type *Arabidopsis* plants and rosette leaves of 2-week-old transgenic *Arabidopsis* plants were collected for transcription level analysis. RNA of all samples was extracted using the RNA prep Pure Plant Kit (Tiangen, China) and were reverse transcribed into cDNA using the PrimeScript II RT Master Mix (Takara, China). QRT-PCR was performed using SuperReal PreMix Plus SYBR-Green (Tiangen, China) and Roche LightCycler 96 real-time PCR machine (Roche, Switzerland). All experiments were repeated three times. Primers are listed in Table S1.

**2.6 Transformation of *Arabidopsis***

The overexpression construct pHB-AaWIN1-YFP was transformed into *A. tumefaciens* strain GV3101 and then used to transform *Arabidopsis* by floral dip method, as previously described[56].

**2.7 Transformation of** ***A. annua***

The overexpression construct pHB-AaWIN1-YFP was transformed into *A. tumefaciens* strain EHA105, and then was used to transform *A. annua*, as previously described[57].

**2.8 GST density counting**

The fifth leaf below the meristem of wild type and transgenic *A. annua* plants (T0) were observed using the fluorescence microscopy (Olympus, Japan), and the excitation spectra was at 450–480 nm. The leaf area and GST number were measured by ImageJ program, as previously described[58]. All experiments were repeated three times.

**2.9 Artemisinin content measurement**

5-month-old leaves of wild type and transgenic *A. annua* plants (T0) were collected and dried at 50℃ for 3 days, then were ground to power. 0.1g powder was extracted with 1mL methanol under sonication at 55 Hz for 30 min, and then was centrifuged at 12000 rpm for 10 min. 2 mL supernatant in total was extracted and collected twice from the same sample, then was filtered through 0.22 μM nitrocellulose filter, and was measured by HPLC, as previously described[59]. All experiments were repeated three times.

**2.10 Yeast-two-hybrid (Y2H) assay**

The full-length coding sequence of *AaWIN1* and *AaMIXTA1*, and truncated sequences of *AaWIN1△C* and *AaWIN1△N* were inserted into pGADT7 vector and pGKBT7 vector. Various combinations of pGADT7, pGADT7-AaWIN1, pGADT7-AaWIN1△C, pGADT7-AaWIN1△N, pGKBT7 and pGKBT7-AaMIXTA1 were co-transformed into yeast strain AH109 and were cultivated on DDO (SD/-Leu/-Trp) plates for 3 days, as previously described[60]. Positive clones were transferred to TDO (SD/-Leu/-Trp/-His) and QDO (SD/-Leu/-Trp/-His/-Ade) plates, respectively. The growth condition of yeast cells was continuously observed for 4 days. All experiments were repeated three times. Primers are listed in Table S1.

**2.11 Bimolecular fluorescence complementation (BiFC) assay**

The full-length coding sequence of *AaWIN1* and *AaMIXTA1* were inserted into pXY104 vector (C terminal of YFP) and pXY106 vector (N terminal of YFP), respectively. All constructs were transformed into *A. tumefaciens* strain GV3101 and then were used to co-infiltrate 4-week-old *N. benthamiana* leaves. The treated *N. benthamiana* plants grew in darkness for 24 h and in light for another 24h. YFP signals were observed by confocal laser microscopy Leica TCS SP5-II. All experiments were repeated three times. Primers are listed in Table S1.

**2.12 Accession numbers**

Nucleotide and amino sequences were downloaded from National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) as follows: AaMIXTA1 (KP195023), AaMYB16 (KX465136), AaHD1 (KU744599), AaHD8 (KX465134), AaGSW2 (KX465129), proAaGSW2 (KX465141), AaTAR1 (EZ159016), AaORA (JQ797708), AaERF1 (JN162091), AaERF2 (JN162092), AaERF3 (JN695782), AaERF4 (JQ797709), AaERF5 (JQ797710), AaERF6 (JQ797711), AaERF7 (JQ797712), AaERF8 (JQ797713), AtWIN1 (NM\_101405), AtSHN2 (NM\_121157), AtSHN3 (NM\_122448.4), HaSHN1 (XP\_021989137), HaSHN2 (XP\_021985834), MeWIN1 (XP\_021602398), LsWIN1 (XP\_023763420), SlSHN1 (XP\_004235965).

**3 Results**

**3.1 Identification of WIN/SHN from *A. annua***

To find out the WIN/SHN clade in *A. annua*, we screened the local trichome transcriptome database (unpublished), 216 proteins containing AP2 domain were grasped by HMMER program (Fig. S1). Phylogenetic tree confirmed that two candidate proteins, Aannua03223S367610 and Aannua07018S574920, were homologous to WIN/SHN proteins (Fig. 1A). Amino acid sequence alignment showed that Aannua03223S367610 and Aannua07018S574920 contained an AP2 domain in the N-terminal, a CMV-1 motif and a CMV-2 motif in the C-terminal (Fig. 1C). Moreover, Aannua03223S367610 and Aannua07018S574920 proteins shared 63.98% and 56.85% sequence identity with AtWIN1/SHN1, respectively. Hierarchical cluster analysis revealed that *Aannua03223S367610* showed a higher expression in trichomes, which was similar with several transcription factors related to trichome development in *A. annua* (Fig. 1B). Taken together, *Aannua03223S367610* was chosen for further investigation and was named as *AaWIN1*.



Fig. 1. Identification of WIN1/SHN in *A. Annua*. (A) Phylogenetic tree based on neighbor-joining method, the number of bootstrap replications was 1000. Three AP2/ERF proteins from *A. annua* (AaORA, AaERF2 and AaERF5) were defined as an outgroup. Aannua03223S367610 (AaWIN1) and Aannua07018S574920 were homologous to WIN/SHN proteins. (B) Hierarchical cluster analysis of *WIN/SHN* and genes encoding positive regulators for trichome development in *A. annua*. Aannua03223S367610, Aannua07018S574920 and AaTAR1 are WIN/SHN clade of AP2/ERF family; AaSAP1 is stress associated protein 1; AaMIXTA1 and AaMYB16 are MYB family proteins; AaHD1 and AaHD8 are HD-ZIP Ⅳ family proteins; AaGSW2 is a WRKY family protein. (C) Amino acid sequence alignment of WIN/SHN proteins in *Arabidopsis* (AtWIN1/SHN1, AtSHN2 and AtSHN3), *Solanum lycopersicum* (SlSHN1), *Helianthus annuus* (HaSHN1)and *A. annua* (AaTAR1, Aannua03223S367610 and Aannua07018S574920). AP2 domain was highlighted in green, CMV-1 and CMV-2 motifs were highlighted in yellow.

**3.2 Gene isolation and sequence analysis of *AaWIN1***

The transcript of *AaWIIN1* contained an open reading frame (ORF) of 624 bp with an intron of 158 bp, a 5’UTR of 133 bp, and a 3’UTR of 230 bp, based on *A. annua* genome database and unpublished full-length transcriptome database[61]. Correspondingly, *AaWIIN1* encoded a protein of 207 amino acids. In addition, 2161 bp nucleotide sequence upstream from transcription start site (TSS) of *AaWIN1* was selected and uploaded to PlantCARE to analyze. Apart from several ABA, Me-JA, abiotic stress and light responsiveness cis-elements, transcription factor binding sites MRE motif and W-box were predicted as well (Fig. S2).

**3.3 Expression pattern of *AaWIN1***

To better understand molecule function of *AaWIN1*, the spatial and temporal expression pattern was determined. QRT-PCR results showed that *AaWIN1* expressed highly in flowers and trichomes, moderately in buds, while weakly in roots, stems, shoots and leaves (Fig. 2A), and there was no distinction of expression level from leaf 0 to leaf 6 (Fig. 2B). Furthermore, AaWIN1 protein was predicted to locate on nuclei with 7.5 points by using ProtComp program. YFP fluorescence in subcellular localization experiment further indicated that AaWIN1 was a nuclear-localized protein, which correlated well with the function of transcription factors (Fig. 2C).

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Fig. 2. Expression pattern of *AaWIN1*. (A) Relative expression level of *AaWIN1* in different tissues, in which root was taken as 1. *AaActin* was used as housekeeping gene. Values were given as means ± SD from three biological replicates. T-test was used to analyze significance (P-value<0.01, extremely significant, \*\*; P-value<0.05, significant, \*). (B) Relative expression level of *AaWIN1* in different leaves, in which leaf 0 (meristem) was taken as 1. *AaActin* was used as housekeeping gene. Values were given as means ± SD from three biological replicates. (C) Subcellular localization of AaWIN1. Colocalization of AaWIN1 and nuclei was determined by DAPI staining. Bars were 20 μm.

**3.4 Overexpressing *AaWIN1* increased GST density in *A. annua***

To explore function of *AaWIN1*, transgenic *A.annua* plants were generated. After transgene test, four *AaWIN1* overexpression lines from eight lines were randomly selected for phenotype analysis, namely OE-AaWIN1-4, OE-AaWIN1-5, OE-AaWIN1-8 and OE-AaWIN1-12. Relative expression level of *AaWIN1* was detected in each line (Fig.3B). Strikingly, the density of GST on adaxial side of leaves increased at least 50% in *AaWIN1*-overexpression lines (Fig. 3C). It was reported that artemisinin synthesized and stored in GSTs[59]. Correspondingly, the artemisinin content was 50%-80% higher in transgenic lines than in wild type (Fig. 3D). Furthermore, AaMIXTA1, AaHD1 and AaGSW2 were reported to positively regulate trichome initiation[34, 37, 62]. In particular, AaGSW2 functioned as a crucial GST-specific transcription factor. QRT-PCR results showed that expression of *AaGSW2* was induced significantly in transgenic lines, while no obvious change was detected in either *AaMIXTA1* or *AaHD1* (Fig. 3E-3G). These results indicated that overexpressing *AaWIN1* promoted GST initiation in *A. annua*.



Fig. 3. Overexpressing *AaWIN1* in *A.annua*. (A) GST on the adaxial side of mature leaves. The *A. annua* leaf was observed under fluorescence microscopy. The yellow spots are autofluorescence of glandular secretory trichome (GST), and the red background is chlorophyll. Bars were 1mm. (B) Relative expression level of *AaWIN1* in *AaWIN1*-overexpression *A.annua*. *AaActin* was used as housekeeping gene. Values were given as means ± SD from three biological replicates. T-test was used to analyze significance (P-value<0.01, extremely significant, \*\*; P-value<0.05, significant, \*). (C) GST density on the adaxial side of mature leaves. Values were given as means ± SD from three biological replicates. T-test was used to analyze significance (P-value<0.01, extremely significant, \*\*; P-value<0.05, significant, \*). (D) Artemisinin content measured by HPLC. Values were given as means ± SD from three biological replicates. T-test was used to analyze significance (P-value<0.01, extremely significant, \*\*; P-value<0.05, significant, \*). (E-G) Relative expression level of three positive regulators for GST initiation, namely *AaMIXTA1*, *AaHD1* and *AaGSW2*, in *AaWIN1*-overexpression *A.annua*. *AaActin* was used as housekeeping gene. Values were given as means ± SD from three biological replicates. T-test was used to analyze significance (P-value<0.01, extremely significant, \*\*; P-value<0.05, significant, \*).

**3.5 AaWIN1 interacted with AaMIXTA1**

Previous studies have reported that the MIXTA-like MYB transcription factors MYB106 and MYB16 regulate cuticle development coordinately with WIN1/SHN1 in *Arabidopsis* and *Torenia fournieri*, and SlSHINE3 positively regulates *SlMIXTA-like* in tomato[41, 63]. Based on the complementary DNA (cDNA) library of *A.annua,* we performed a yeast-two-hybrid (Y2H) screen for putative proteins interacting with AaWIN1, and the MIXTA protein AaMIXTA1 was identified[37]. Hierarchical cluster analysis also showed that *AaWIN1* shared a similar expression pattern with *AaMIXTA1* (Fig. 1B). To further verify the relationship between AaWIN1 and AaMIXTA1, the Y2H assay was generated, and AaWIN1 was confirmed to interact with AaMIXTA1 in yeast cells. Additionally, the domain deletion analysis revealed that AaWIN1△N alone, which simultaneously contained CMV-1 and CMV-2 motifs, was able to interact with AaMIXTA1, while AaWIN1△C alone could not (Fig. 4A). As an alternative approach, the reconstituted YFP fluorescence signal in bimolecular fluorescence complementation (BiFC) assay confirmed the interaction between AaWIN1 and AaMIXTA1 in *N. benthamiana* (Fig. 4B).

It was reported that AaMIXTA1 regulated genes related to cuticle biosynthesis in *A.annua*[37]. Given the interaction between AaWIN1 and AaMIXTA1, we detected expressions of these genes in transgenic lines. QRT-PCR results showed that expressions of *AaCYP77A1*, *AaKCS5* and *AaABCG12* were significantly induced in *AaWIN1*-overexpression lines (Fig. 4C). These results suggested that AaWIN1 might function cooperatively with AaMIXTA1.



Fig. 4. Interaction between AaWIN1 and AaMIXTA1. (A) Yeast-two-hybrid (Y2H) assay. Three types of screening medium, DDO (SD/-Leu/-Trp), TDO (SD/-Leu/-Trp/-His) and QDO (SD/-Leu/-Trp/-His/-Ade). AD-AaWIN1 and BD-AaMIXTA1 were full-length ORF; AD-AaWIN1△C was N-terminal containing AP2 domain; AD-AaWIN1△N was C terminal containing CMV-1 and CMV-2 motifs. Empty vector AD and BD were negative controls. (B) Bimolecular fluorescence complementation (BiFC) assay, the recombinant YFP fluorescence was observed in *N. benthamiana* inwhich co-expressed *AaWIN1-cYFP* and *AaMIXTA1-nYFP*. AaWIN1-cYFP represented that AaWIN1 was fused with the C-terminal fragment of YFP, AaMIXTA1-nYFP represented that AaMIXTA1 was fused with the N-terminal fragment of YFP. Colocalization of AaWIN1 and nuclei was determined by DAPI staining. Bars were 20 μm. (C) Relative expression level of three genes related to cuticle biosynthesis in *AaWIN1*-overexpression *A. annua* lines. *AaActin* was used as housekeeping gene. Values were given as means ± SD from three biological replicates. T-test was used to analyze significance (P-value<0.01, extremely significant, \*\*; P-value<0.05, significant, \*).

**3.6 Ectopic expression of *AaWIN1* resulted in fewer trichomes in *Arabidopsis***

To further investigate its function, *AaWIN1* was ectopically expressed in *Arabidopsis*. After transgene test, three *AaWIN1*-overexpression lines were randomly selected for phenotype analysis, namely OE-AaWIN1-7, OE-AaWIN1-8, OE-AaWIN1-12. Relative expression level of *AaWIN1* and *AtWIN1* were detected in each line (Fig. 5F, Fig. S4).Comparing with wild type, most transgenic plants showed obvious phenotypes with small plants, slender rosette leaves and curled cauline leaves (Fig. 5A, 5B, 5E). Moreover, fewer trichomes were observed on rosette leaves, and most of them located on leaf blade margins (Fig. 5C-5D). These phenotypes were consistent with *shn* mutant or *AtWIN1*-overexpression *Arabidopsis* plants[49, 50, 64].

It was reported that overexpressing *AtWIN1* upregulated expressions of genes related to cuticle biosynthesis in *Arabidopsis*, such as *AtCER1*, *AtKCS1*, *AtCYP86A4,* *AtCYP86A7* and *AtLACS2*. We assumed that *AaWIN1* regulated these genes as well. QRT-PCR results showed that expressions of *AtCYP86A4* and *AtCYP86A7* were dramatically increased in transgenic *Arabidopsis* lines, and expressions of *AtCER1*, *AtKCS1* and *AtLACS2* were induced as well (Fig. 5F).



Fig. 5. Phenotypes of *AaWIN1*-overexpression *Arabidopsis*. (A) *Arabidopsis* plants. (B) Leave shape of rosette leaves; adaxial side. (C-D) Trichome numbers and locations on rosette leaves; adaxial side. (E) Leave shape of cauline leaves; abaxial side. Bars in (A) and (B) were 1cm; bars in (C), (D) and (E) were 1mm. OE-AaWIN1 and WT *Arabidopsis* were on the left and right, respectively, in every group of pictures. (F) Relative expression level of genes related to cuticle biosynthesis in *AaWIN1*-overexpression *Arabidopsis* lines. *AtActin2* was used as housekeeping gene. Values were given as means ± SD from three biological replicates. T-test was used to analyze significance (P-value<0.01, extremely significant, \*\*; P-value<0.05, significant, \*).

**4 Discussion**

Over the last two decades, tremendous progress has been made in characterizing enzymes related to the artemisinin biosynthesis pathway[23]. However, the regulation of GST development remains poorly elucidated. Using well-characterized AP2 domain from *Arabidopsis* as an input, we identified 216 AP2/ERF proteins in *A.annua*. Among them, the reported WIN/SHN protein AaTAR1 was corresponded with Aannua00739S133030 in our database (Fig. S1). Besides, two potential homologues to the WIN/SHN clade from *Arabidopsis* were identified (Fig. S1). Of the two candidates, *Aannua03223S367610* showed a higher expression in trichomes, and thus, was chosen for further investigation and was named as *AaWIN1*. Interestingly, the phylogenetic analysis across species showed that AaWIN1 clustered with three WIN/SHN proteins from *Arabidopsis* (Fig. 1A), although AaTAR1 shared a higher amino acid identity (67%) with AtWIN1 than AaWIN1 (64%)[51].

Previous studies suggest that differences in expression pattern of *WIN/SHN* genes confer functional variations. For example, *AtWIN1* and *AtSHN2* were preferentially expressed in floral tissues and siliques, while *AtSHN3* was actively and broadly expressed in most organs[49]. Expression of *AtWIN1* was also detectable in stems and rosette leaves, which might be related to its functional role in cuticle biosynthesis[50].In our study,apart from the high expression in buds and trichomes, *AaTAR1* was moderately expressed in leaves and stems as well as *AaHD8*, suggesting their functional roles in cuticle formation[37, 38, 51]. By contrast, *AaWIN1* shared a similar expression pattern with *AaMIXTA1*, suggesting a cooperative function in trichome development (Fig. 1B). Specifically, *AaWIN1* was mainly expressed in buds, flowers and trichomes separated from floral tissues (Fig. 2A). A high-efficiency method has been reported to isolate GSTs from leaf epidermis by using laser capture microdissection recently[65]. With this new method, it will be particularly interesting to compare expression of *AaWIN1* within trichomes between floral tissues and leaves.

Previous studies have reported that the MIXTA-like MYB transcription factors MYB106 and MYB16, which regulate epidermal cell morphology, also regulate cuticle development coordinately with WIN1/SHN1 in *Arabidopsis* and *Torenia fournieri*; and SlSHINE3 positively regulates *SlMIXTA-like* in tomato[41, 63]. AaMIXTA1, a known MIXTA protein, was reported to prompt GST initiation and regulate cuticle biosynthesis in *A. annua*. However, AaMIXTA1 did not appear to regulate AaTAR1, a reported WIN/SHN transcription factor which affects trichome morphology rather than the density of the trichome[37]. It is thus interesting to investigate the relationship between AaMIXTA1 and other WIN/SHN transcription factors. In this study, we wondered whether AaWIN1 coordinately regulated GST initiation and/or cuticle formation with AaMIXTA1. Therefore, we conducted several experiments to confirm our conjecture. We firstly detected the expression of *AaMIXTA1* in *AaWIN1*-overexpression lines, and the QRT-PCR result suggested that *AaMIXTA1* was not influenced by AaWIN1 at transcriptional level (Fig. 3E). Furthermore, we screened the cDNA library of *A. annua* and found AaMIXTA1 interacting with AaWIN1, which demonstrated by Y2H and BiFC assays as well (Fig. 4A, 4B). In addition, previous studies suggested that trichome initiation was closely linked to cuticle biosynthesis. AaMIXTA1 was found to affect cuticle biosynthesis in *A.annua*. In particular, expressions of genes *AaCER1*, *AaKCS5*, *AaCYP77A1*, *AaCYP86A1* and *AaABCG12* were significantly reduced in *AaMIXTA1-*RNAi lines[37]. In our study, AaWIN1 was confirmed to interact with AaMIXTA1 in yeast and tobacco cells, and expressions of three genes *AaKCS5*, *AaCYP77A1* and *AaABCG12* were induced in *AaWIN1*-overexpression lines (Fig. 4C). These results suggest a potential role of *AaWIN1* in cuticle biosynthesis in *A.annua.*

On the other hand, as a part of the regulatory network of GST development, AaGSW2 seems to act very downstream, which is regulated by a HD-ZIP-MYB complex, to positively regulate GST initiation[31]. We found that expression of AaGSW2 was also increased in *AaWIN1*-overexpression lines by QRT-PCR(Fig. 3G). GCC-box and RAV1AAT (RAA) motif are important binding sites for AP2/ERF transcription factors in *A.annua*[51, 66]*.* Consistently, a GCC-box and five RAA motifs were predicted in *AaGSW2* promoter by PlantCARE (Fig. S3). We speculated that *AaGSW2* was a potential target gene for AaWIN1 in GST initiation. However, whether AaWIN1 directly regulates AaGSW2 or through other regulators remains unclear, which needs further investigation.

Overexpressing *AaWIN1* resulted in an increase of GST density in *A.annua*, while resulted in fewer trichomes in *Arabidopsis* (Fig. 3C, 5C) [49, 50]. Notably, GST consists of 10 cells and a subcuticular space, by contrast, the trichome in *Arabidopsis* is unicellular and non-glandular[67, 68]. The structural difference of trichomes in cell number and secretory capacity might contribute to this distinction. Furthermore, previous studies suggest that AtWIN1 modulates cuticle formation in *Arabidopsis* by regulating genes related to cuticle biosynthesis, including *AtCER1*, *AtKCS1*, *AtCYP86A4,* *AtCYP86A7* and *AtLACS2*[50, 64]. Expressions of these genes were significantly induced when *AaWIN1* was ectopically expressed in *Arabidopsis* (Fig. 5F). Fewer trichomes on *Arabidopsis* rosetteleaves might be a secondary effect caused by the alteration in cuticle biosynthesis[41]. However, further investigation is needed to clarify the link between trichome development and cuticle biosynthesis.

As described above, the function of *AaWIN1* was mainly relevant to trichome initiation. Notably, gene repression lines were considered as ideal substantial for trichome morphogenesis investigation in *A.annua*. For example, silencing *AaTAR1* caused defects in GST cells with abnormal top and reduced cell numbers, and repressing *AaTAR2* resulted in shrivelled GSTs[32, 51]. In order to observe morphological phenotypes, we attempted to generate *AaWIN1*-RNAi transgenic lines but ultimately failed. *AaWIN1* significantly influenced trichome initiation, however, its functional role in trichome morphogenesis has not been examined until now. It is worth mentioning that CRISPR/Cas9 system has been gradually applied to *A.annua*, which is advantageous for further investigation[32, 34].

Taken together, based on phenotype observations, content measurements and gene expression detections, AaWIN1 is considered as a positive regulator for GST initiation. Based on current evidence, AaWIN1 might be implicated in two regulation mechanisms in *A. annua* (Fig. 6). For one thing, AaWIN1 functions in regulation of GST initiation through activating *AaGSW2* at transcriptional level. For another, AaWIN1 interacts with AaMIXTA1, suggesting its potential role in cuticle biosynthesis.



Fig. 6. A simplified regulation model of AaWIN1 in *A.annua*. AaWIN1 might be implicated in two regulation mechanisms in *A. annua*. For one thing, AaWIN1 functions in regulation of GST initiation through activating *AaGSW2* at transcriptional level. For another, AaWIN1 interacts with AaMIXTA1, suggesting its potential role in cuticle biosynthesis. AaGSW2, a WRKY transcription factor which positively regulates GST initiation. AaMIXTA1, a MIXTA transcription factor which prompts GST initiation and regulates cuticle biosynthesis. Black arrow, up-regulation; dotted line, lack of precise evidence for the direct activation.

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**6 Declaration of Competing Interest**

The authors declare no conflicts of interest.

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