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Promoters of *AaGL2* and *AaMIXTA-Like1* genes of *Artemisia annua* direct reporter gene expression in glandular and non-glandular trichomes

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Keywords: *AaGL2* promoter, *AaMIXTA-Like1* promoter, *cis*-elements, glandular trichome, non-glandular trichome

Abbreviations: ABRE, Abscisic acid responsive element; GARE, gibberellin-responsive element; GFP, Green fluorescent protein; GUS, β-Glucuronidase; MeJa, Methyl jasmonate; MS, Murashige and Skoog medium.

Herein, we report cloning and analysis of promoters of *GLABRA2* (*AaGL2*) homolog and a *MIXTA-Like* (*AaMIXTA-Like1*) gene from *Artemisia annua*. The upstream regulatory regions of *AaGL2* and *AaMIXTA-Like1* showed the presence of several crucial *cis*-acting elements. Arabidopsis and *A. annua* seedlings were transiently transfected with the promoter-*GUS* constructs using a robust agro-infiltration method. Both *AaGL2* and *AaMIXTA-Like1* promoters showed GUS expression preferentially in Arabidopsis single-celled trichomes and glandular as well as T-shaped trichomes of *A. annua*. Transgenic Arabidopsis harboring constructs in which *AaGL2* or *AaMIXTA-Like1* promoters would control GFP expression, showed fluorescence emanating specifically from trichome cells. Our study provides a fast and efficient method to study trichome-specific expression, and 2 promoters that have potential for targeted metabolic engineering in plants.

Trichomes are specialized unicellular or multicellular structures derived from epidermal cells which play prominent physiological roles in plants for interacting with the environment for adaptation. Two types of trichomes i.e. glandular and non-glandular prevail in higher plants. Apart from providing protection against herbivores and combating various stresses, glandular trichomes present in the aerial parts of plants have capacity to produce, secrete or store secondary metabolic compounds which have industrial importance such as aromas, flavours and pharmaceuticals.[1-3] *Artemisia annua* L. (Family: Asteraceae) is an important medicinal plant that synthesizes artemisinin, a sesquiterpene lactone, which has a great demand as an anti-malarial agent. *A. annua* is covered with T-shaped filamentous trichomes and 10-celled artemisinin producing biseriate peltate glandular trichomes.[4,5] The key genes such as amorpha-4,11-diene synthase (*ADS*), amorpha-4,11-diene monooxygenase (*CYP71AV1*), aldehyde dehydrogenase 1 (*ALDH1*) and artemisinic aldehyde Δ11 (13) reductase (*DBR2*) which are responsible for the biosynthesis of artemisinin are preferentially expressed in the trichomes.[6-8] Most secondary metabolites sequestered in the trichome cells in medicinal and aromatic plants involve crucial pathway genes which are either exclusively or abundantly expressed in the specialized cells of glandular trichomes. Hence, next generation high-throughput sequencing technologies are being exploited to analyze the transcriptome(s) of trichomes for better exploration of secondary metabolic activities at the genetic level.[9,10] Much progress has been made to understand the trichome development in Arabidopsis and a number of genes involved in trichome cell fate determination have been identified. Interactions of a WD40 protein (TRANSPARENT TESTA GLABRA1), a MYB-related factor (GLABRA1) and basic helix-loop-helix (bHLH) transcription factors (GLABRA3/ENHANCER OF GLABRA3) form a transcriptional-activation complex for promoting trichome initiation. This complex activates *GLABRA2* (*GL2*) gene which encodes a homeodomain leucine-zipper protein which further triggers downstream trichome differentiation events.[11,12] The *MIXTA-Like* genes of R2R3-MYB family (subgroup 9) normally control the development of conical cells in flower petals.[13,14] Other similar transcriptional regulators such as *NOECK* (MIXTA-Like MYB transcription factor) regulate trichome formation and function as a repressor of cell outgrowth.[15,16] In contrast to the Arabidopsis trichome development, the regulatory mechanisms and

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genetic complements that control the formation of specialized glandular trichomes are poorly characterized. However, studies on the development of glandular trichomes have been carried out in relation to the secondary metabolites biosynthesis and phytohormonal regulation.[17,18] For improving the *in planta* artemisinin yield in *A. annua*, metabolic engineering of the pathway in glandular trichome-specific fashion is the most promising approach. For restricting the gene expression to specific cell type, tissue-specific promoters and understanding of associated *cis*-acting DNA elements are essential components. In the past few years, great progress has been done on the identification and evaluation of plant-derived tissue-specific and stress-inducible promoters.[19-21] But very few promoters conferring trichome-specific or preferential expression which include promoters of Arabidopsis *OASA1* and *AtTSG1*,[22,23] lipid transfer protein genes of cotton and tobacco,[24-26] and tobacco *CYP71D16*,[27] have been isolated and characterized in plants. Promoter of *A. annua ADS* gene was found to drive expression in the anthers and trichomes of Arabidopsis juvenile leaves,[28] while exclusively in stalk and secretory cells of *A. annua* glandular trichomes.[29] *A. annua CYP71AV1* gene promoter targets the reporter gene exclusively in glandular secretory trichomes of leaf primordia and top expanded leaves,[30] while on the other hand, linalool synthase (*LIS*) promoter was specifically active in T-shaped trichomes.[31] The availability of sequence and information of such genes and especially promoters provides material for in-depth studies of trichome-specific processes, and would facilitate precise genetic engineering of trichome-specific traits and secondary metabolism in plants. So far, a limited number of trichome-preferential promoters are known especially from *A. annua*, hence, the availability of information regarding diverse trichome-related promoters would be useful in making a choice of appropriate promoter as a biotechnological tool. In the present study, an attempt was made to identify *A. annua* sequences in the NCBI database which encode proteins similar to trichome development-related candidates, promoters of which are expected to exhibit a continued activity till trichome maturity. We cloned cDNAs and promoters of *GLABRA2* homolog and a *MIXTA-Like* gene from *A. annua*. Their promoter-reporter gene (*GUS* or *GFP*) constructs were prepared and tissue-specificity was studied. We further demonstrate that agro-infiltration method can be used as an efficient tool for analyzing gene expression in trichome cells.

The partial sequences of homologs of *GL2* (*AaGL2*) and *MIXTA-Like* (*AaMIXTA-Like1*), *in silico* identified in the EST/TSA dataset of *A. annua* in NCBI, were amplified and cloned. The cDNAs of *AaGL2* and *AaMIXTA-Like1* were completed to full-length by 5′ and 3′ RACE. In order to identify their upstream genomic region (promoter), *A. annua* genomic libraries were constructed by using Genome Walker™ Universal Kit (Clontech, USA) as per instruction manual. Adapter-specific nested forward primers (AP1 and AP2) and 2 gene-specific nested reverse primers for *AaGL2* (RP1: TGGAAGGATGAGTGTTATCGTTAGAAG /RP2: GATTAGTTTATGGTGATCGATAGATGC) and *AaMIXTA-Like1* (RP1: CCATGACCATGTTCTTCAATG TAAGCTA /RP2: CAATCCTACCTTATCGCAACAT GGA GAT) designed from the 5′ region of the cDNAs were used for primary and secondary PCR amplifications of genomic regions using Genome Walker libraries as per the conditions described in the instruction manual. Genomic fragments of 1613 bp and 1772 bp length could be amplified for *AaGL2* and *AaMIXTA-Like1*, respectively, in secondary PCR which were initially cloned in pGEM-T Easy vector (Promega, USA). The fragments were completely sequenced and their sequences were submitted in the GenBank (Accessions: KM892865 and KM892866). These cloned fragments are expected to contain most of the *cis*-acting elements and those needed for driving expression in trichome cells. The genomic sequences upstream to translation initiation site (ATG) of *AaGL2* and *AaMIXTA-Like1* were analyzed by using PlantCARE and PLACE databases, and the *cis*-regulatory elements that were identified are shown in **Figure 1**. In the *AaGL2* and *AaMIXTA-Like1* promoters (P_{AaGL2} and P_{AaMIX1}), a putative TATA box is identified at −31 and −30 positions, respectively. Several MYB transcription factor binding sites at positions −132 to −126, −499 to −494, −621 to −611, −892 to −887, −1304 to −1299 and −1312 to −1307 are detected in *AaGL2* promoter (**Fig. 1A**). The MYBs represent one of the largest transcription factor families regulating various cell fate determinations including trichome development.[12,32] MYB recognition sites were also observed in trichome-specific promoters of *AtTSG1*,[23] LTP3 and LTP6 genes from cotton,[24,25] and tobacco *CYP71D16*.[27] Two *cis*-regulatory elements responsible for light responsiveness (G-boxes) are recognized at positions −1187 to −1182 and −433 to −424 in *AaGL2* promoter. An E-box (CANNTG) could be detected at −1028 to −1023 position which is a binding site for most bHLH transcription factors and is also recognized by MYB and bZIP proteins. Other *cis*-acting elements present in *AaGL2* promoter include S-box (CACCTCCA at −50 to −57), heat stress responsive element (HSE at −786 to −777), proximal portion of B-box (CAAACACC at −420 to −413) and W-box (TGACT at −304 to −300 and −238 to −234). The S-box is conserved in many *rbcS* promoters and is responsive to sugar,[33] B-box is involved in abscisic acid-induced transcription,[34] and W-box is the consensus element required for the binding of WRKY transcription factors.[35] Since expression of *GL2* is controlled by *TRANSPARENT TESTA GLABRA2* (*TTG2*, a WRKY transcription factor) during trichome and root hair formation in Arabidopsis,[36] therefore, presence of W-box in *AaGL2* promoter is expected. A cell cycle-related MSA-like element (M phase-specific activity; TCTAACGGTCA) which resembles binding site of MYB factors (c-myb and v-myb) is also present in *AaGL2* promoter at position −87 to −77 and agrees with the consensus sequence (T/C)C(T/C)AACGG(T/C)(T/C)A reported earlier by Ito et al.[37] in *Catharanthus roseus* B-type cyclin gene *CYM*. Trichome size and branching is associated with cell cycle and endoreduplication events in the nucleus,[11] hence interaction of certain cell cycle proteins with the MSA-like element of *AaGL2* promoter cannot be ruled out. On the other hand, various *cis*-acting regulatory elements could also be identified in the *AaMIXTA-Like1* promoter (**Fig. 1B**). Apart from TATA box, other elements include regulatory element essential for the anaerobic induction (ARE at −1526 to −1521, −1190 to −1185, −1038 to −1033 and −939 to −934

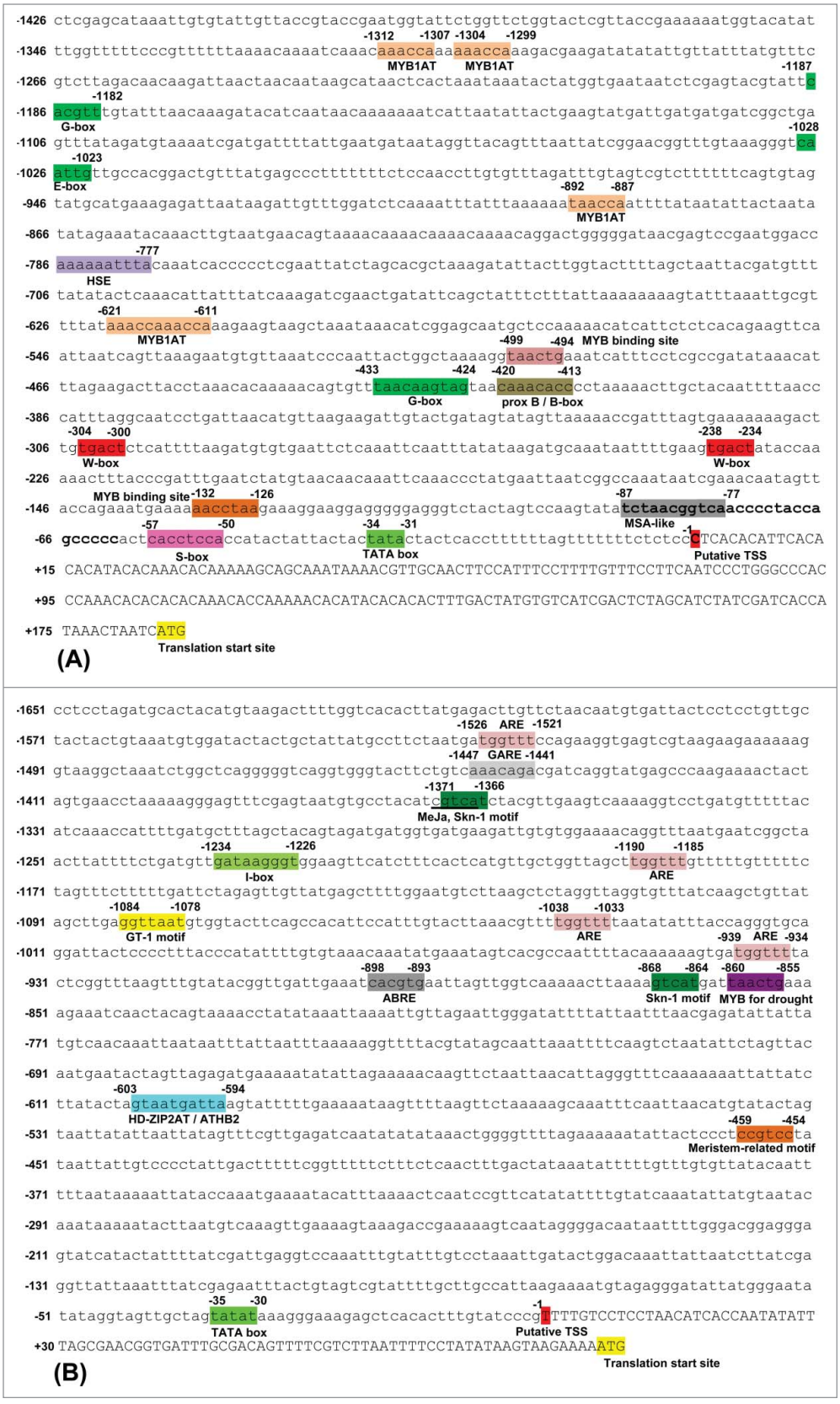

Figure 1. A schematic representation of the sequence of (**A**) *AaGL2* (P_{AaGL2}) and (**B**) *AaMIXTA-Like1* (P_{AaMIX1}) genomic fragments containing promoter region. The 1613 bp and 1740 bp (out of 1772 bp) genomic sequence upstream to ATG of *AaGL2* and *AaMIXTA-Like1*, respectively, were *in silico* analyzed by PLACE and PlantCARE. The positions of putative TSS, TATA box and various *cis*-acting elements identified are shown and marked in the sequence.

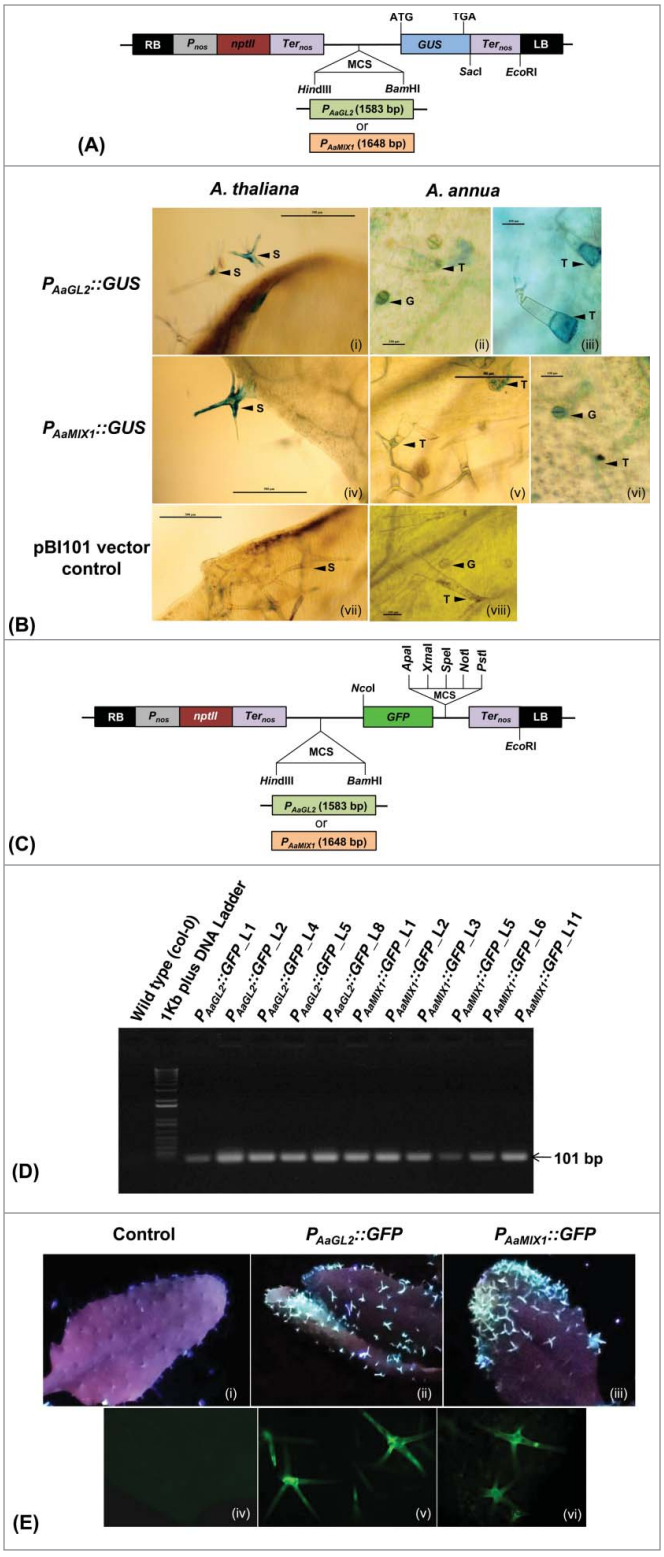

Figure 2. Tissue-specific expression analysis of *AaGL2* (P_{AaGL2}) and *AaMIXTA-Like1* (P_{AaMIX1}) promoters. (**A**) Scheme of the constructs of P_{AaGL2}::*GUS* and P_{AaMIX1}::GUS in pBI101 binary vector used for transient transformation of Arabidopsis and *A. annua*. (**B**) Localization of GUS in the single-celled trichomes of Arabidopsis (i, iv), and glandular as well as non-glandular trichomes of *A. annua* (ii, iii, v, vi). No GUS staining was observed in the trichomes of Arabidopsis (vii) and *A. annua* (viii) transiently transformed with mock vector (pBI101) control. S, Single-celled trichome; G, Glandular trichome; T, T-shaped non-glandular trichome. Scale bar; 500 μm (i, iv, vii), 100 μm (ii, iii, vi, viii), 300 μm (v). (**C**) Schematic diagram of the P_{AaGL2}::*GFP* and P_{AaMIX1}::*GFP* constructs in pBIGFP (modified pBI121) binary vector used for stable transformation of Arabidopsis. (**D**) Gel image showing the amplification of GFP-specific fragment (101 bp) by PCR in representative Arabidopsis transgenic lines harboring P_{AaGL2}::*GFP* or P_{AaMIX1}::*GFP* constructs (**E**) GFP fluorescence in the leaf trichomes of T3 lines of transgenic Arabidopsis harboring P_{AaGL2}::*GFP* (ii, v) and P_{AaMIX1}::*GFP* (iii, vi) constructs. Leaf of un-transformed control plants (i, iv) did not show any green fluorescence. Magnified fluorescent images of trichomes (iv, v, vi) were taken under 10X objective.

positions), gibberellin-responsive element (GARE motif at −1447 to −1441), skn-1 motifs for endosperm expression (GTCAT at −1371 to −1366 and −868 to −864), MYB binding site (TAACTG at −860 to −855) involved in drought-inducibility, light responsive elements such as I-box (GATAAGGGT at −1234 to −1226) and GT-1 motif (GGTTAAT at −1084 to −1078), abscisic acid responsive element ABRE (CACGTG at −898 to −893), HD-ZIP binding consensus sequence (GTAATGATTA at −603 to −594), meristem-specific activation element (CCGTCC at −459 to −454) and a MeJa-responsive element (CGTCA at −1370 to −1365) overlapping with one of the skn-1 motif. Gibberellin and jasmonic acid showed a synergistic effect on the trichome induction in Arabidopsis and led to significant increase in trichome number on leaf surface.[38] Presence of gibberellin- and MeJa-responsive elements in *AaMIXTA-Like1* promoter indicates toward its participation in gene regulatory roles in the trichomes.

We further analyzed tissue-specific activity of the 2 promoters (KM892865 and KM892866) cloned by us by transforming promoter-*GUS* construct (**Fig. 2A**) in Arabidopsis and *A. annua*. For directional cloning in pBI101 binary vector, forward and reverse primers harboring *Hin*dIII and *Bam*HI restriction sites, respectively, were synthesized for *AaGL2* (Forward: CGTAAGCTTCTCGAGCATAAAATTGTGTAT /Reverse: ACTGGATCCTAGAGTCGATGACACATAGTC) and *AaMIXTA-Like1* (Forward: GCGAAGCTTCCTAGATGCACTACATGTAAGAC /Reverse: ATAGGATCCCGGGATACAAAGTGTGAGCTCT). After PCR amplification using respective pGEM-T Easy clones as templates, finally 1583 bp and 1648 bp genomic fragment containing the promoter region of *AaGL2* and *AaMIXTA-Like1*, respectively, were inserted directionally which would drive *GUS* reporter gene in pBI101. The binary constructs (P_{AaGL2}::*GUS* or P_{AaMIX1}::*GUS*) were introduced into *Agrobacterium tumefaciens* strain AGL1 by freeze thaw method. The strains harboring P_{AaGL2}::*GUS* or P_{AaMIX1}::*GUS* were used for transient transformation of *A. thaliana* (Col-0) and *A. annua* seedling (2 to 3-week-old) with trichomes at the stage of initiation and development. Agro-infiltration method described by Ma et al.[39] with modifications was used for transient transformation. *Agrobacterium* culture was raised in 50 ml YEM medium containing 50 mg/l kanamycin, 100 mg/l rifampicin, 10 mM MES and 20 μM acetosyringone at 28°C/200 rpm until $OD_{600} = 0.5$. The cells were harvested by centrifugation at 3500 × g for 5 min and washed with re-suspension

buffer (MS salts, 10 mM MES, 2% sucrose and 200 μM acetosyringone). Pellet was finally re-suspended in re-suspension buffer (50 ml) containing 0.005% Silwet L-77. The seedlings were dipped in the *Agrobacterium* suspension in a beaker placed in a desiccator, and a vacuum was applied at inHg strictly for 1.0 min. Subsequently, air was slowly released into the desiccator to avoid any tissue damage. Following agro-infiltration, the medium was removed and the seedlings were placed on a blotting paper in petridishes followed by co-cultivation in dark at 25°C for 48 h. The leaf, stem/hypocotyl and root of the seedlings were subjected to GUS histochemical staining for overnight (12 to 14 h), followed by gentle washing with 70% ethanol to leach out chlorophyll from green tissues for clear visualization of GUS stain. Each experiment was performed for at least 3 times using different batches of seedlings. Finally, the processed tissues were visualized under a light microscope (DMI3000 B, Leica, Germany). Both the gene promoters showed GUS expression exclusively in the trichome cells of Arabidopsis. GUS expression was also observed in the glandular secretory cells and T-shaped trichomes of *A. annua* (**Fig. 2B**). No significant staining was observed in the stem/hypocotyl and root of the seedlings. Our results suggest that these 2 promoter fragments carry *cis*-acting regulatory elements which are necessary for trichome expression. For further validation, promoter activity was also studied in the stable transgenic Arabidopsis expressing an alternative reporter gene, green fluorescent protein (GFP), driven by these promoters (P_{AaGL2} and P_{AaMIX1}). For preparing the promoter-*GFP* constructs, pBI121 binary vector was modified in which *GUS* cassette ($P_{CaMV35S}$:*GUS*:Ter_{nos} between *Hin*dIII and *Eco*RI restriction sites) was replaced with a *GFP* cassette ($P_{CaMV35S+omega}$:*GFP*:Ter_{nos}) taken from psGFPcs_1/pUC18 vector,[40] at the same site to finally obtain pBIGFP binary vector. The initially cloned genomic fragments of *AaGL2* and *AaMIXTA-Like1* genes (i.e., P_{AaGL2} and P_{AaMIX1} in pGEM-T Easy vector) were inserted directionally at *Hin*dIII/*Bam*HI sites replacing the *CaMV35S+omega* promoter. P_{AaGL2}::*GFP* and P_{AaMIX1}::*GFP* binary constructs were obtained (**Fig. 2C**) which were stably transformed in Arabidopsis by using floral dip method,[41] and transgenics were obtained. A total of 13 and 11 kanamycin resistant lines were generated for P_{AaGL2}::*GFP* and P_{AaMIX1}::*GFP*, respectively, which were further confirmed by PCR using GFP-specific primers (Forward: GGGCACAAGCTGGAGTACAACT /Reverse: ATGTTGTGGCGGATCTTGAAG). PCR amplification in 5 and 6 representative transgenic lines of P_{AaGL2}::*GFP* and P_{AaMIX1}::*GFP*, respectively, is shown in **Figure 2D**. The T3 lines of transgenic plants were analyzed for GFP expression in the tissues by illumination with UV torch (365 nm). Leaf surface was further visualized under a fluorescence microscope (DMI3000 B, Leica, Germany) at 365 nm excitation and photographed. The fluorescence of GFP was clearly and specifically observed in the trichomes of transgenic plants harboring either of the 2 constructs while un-transformed control plants did not show any GFP expression (**Fig. 2E**). Root, stem/hypocotyl and flower bud of the transgenic lines did not show GFP fluorescence above the background. The trichome-specific expression of reporter gene in stable transgenic lines of Arabidopsis further confirms the tissue-specific activity of these 2 promoters and existence of crucial *cis*-elements in their sequence. The ATP fragment of *OASA1* gene also conferred similar trichome-specific activity in non-glandular and glandular trichome of Arabidopsis and tobacco, respectively.[22] A *cis*-acting element to direct trichome-specific expression was also recognized in the promoter of *AtTSG1*.[23]

In this investigation, we could identify 2 promoters which have the capability to target gene of interest in glandular as well as non-glandular trichomes, and may have immense biotechnological applications especially for the improvement of *A. annua*. The use of agro-infiltration method to study gene expression in trichomes was demonstrated for the first time. Since the expression of foreign gene using constitutive promoters may have adverse effect on plant development and physiology, therefore, the availability of such trichome-specific promoters may help in genetic engineering of trichome-related traits and metabolic engineering of secondary metabolic pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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