

# Characterization of the Promoter of *Artemisia annua* Amorpha-4,11-diene Synthase (ADS) Gene Using Homologous and Heterologous Expression as well as Deletion Analysis

Mengmeng Zhu · Fangyuan Zhang · Zongyou Lv · Qian Shen ·  
Ling Zhang · Xu Lu · Weimin Jiang · Xueqing Fu · Tingxiang Yan ·  
Lingxian Chen · Guofeng Wang · Kexuan Tang

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**Abstract** Amorpha-4,11-diene synthase (ADS) is the first key enzyme of artemisinin biosynthetic pathway in *Artemisia annua* L. In this study, the promoter region of the ADS gene has been cloned and used to demonstrate the expression of GUS reporter gene in both glandular trichomes of *A. annua* and non-glandular trichomes of *Arabidopsis thaliana* following homologous and heterologous expression of ADS promoter–GUS fusion. Subsequently, 5' sequential deletion analysis of the ADS promoter revealed that a short sequence, –350 upstream of the transcription start site, was sufficient for trichome-specific expression in *A. thaliana* and that the region from –350 to –300 contained essential elements for this observed specificity. However, frequencies of transgenic *A. thaliana* plants displaying trichome-specific expressions varied between different lines, and all the lines with deleted fragments of the ADS promoter showed lower frequencies than the line with full-length ADS promoter. Most lines with deleted ADS promoter–GUS fusions showed GUS expressions in the guard

cells of stomata as well, which was not observed in *A. thaliana* plants transformed with the full-length ADS promoter. GUS activities varied among different transgenic lines as well, both in transiently transformed *Nicotiana benthamiana* and stably transformed *A. thaliana*, with promoter–deletion lines exhibiting higher GUS activities than the full-length ADS promoter line.

**Keywords** ADS promoter · Trichome specific · *Artemisia annua* L. · *Arabidopsis thaliana* · Promoter deletion · GUS

## Introduction

Artemisinin, a sesquiterpene lactone endoperoxide isolated from *Artemisia annua* L. (*A. annua*) of Asteraceae family, is a widely used anti-malarial drug. Malaria is a pandemic that threatens people in most tropical and subtropical regions of the world for decades. According to World Malaria Report 2010 (WHO 2011), there were an estimated 225 million cases of malaria worldwide in 2009, and among them 781,000 people died, accounting for 2.23 % of deaths across the world. Since resistance to traditional anti-malarial drugs has developed (Wellems 2002), such as chloroquine, and artemisinin as well as its derivatives are highly effective against the most severe and multidrug-resistant *Plasmodium falciparum* strains (Liu et al. 2006a), they now serve as primary components of the standard treatment worldwide for malaria. Nowadays, artemisinin is in great demand on international markets; however, *A. annua* plants, the main source of artemisinin, are of low content of this vital compound (0.1–0.8 %) (Abdin et al. 2003), resulting in the unaffordable cost of artemisinin-based treatment.

M. Zhu · F. Zhang · Z. Lv · Q. Shen · L. Zhang · X. Lu · W. Jiang ·  
X. Fu · T. Yan · G. Wang · K. Tang (✉)  
Key Laboratory of Urban Agriculture (South), Ministry of  
Agriculture, Plant Biotechnology Research Center, School of  
Agriculture and Biology, Fudan-SJTU-Nottingham Plant  
Biotechnology R&D Center, Shanghai Jiao Tong University,  
Shanghai 200240, People's Republic of China  
e-mail: kxtang@sjtu.edu.cn

K. Tang  
e-mail: kxtang1@yahoo.com

K. Tang  
e-mail: kxtang1@163.com

L. Chen  
Laboratory of Plant Biotechnology, College of Life and Environment  
Sciences, Shanghai Normal University, Shanghai 200234,  
People's Republic of China

To enhance artemisinin yield, researchers have taken great efforts for the past two decades in its production through varied approaches, including total chemical synthesis (Xu et al. 1986; Avery et al. 1992), in vitro culture (Arsenault et al. 2008), microbial production (Ro et al. 2006; Zeng et al. 2008), and in vivo production in transgenic plants (Chen et al. 2000; Sa et al. 2001; Zhang et al. 2009; Westfall et al. 2012); however, to date, not a single sound industrial-scale production system has been established. To address such a problem, thorough insights into the mechanisms of artemisinin biosynthesis are required.

Artemisinin is specifically produced and stored in glandular secretory trichomes (Nguyen et al. 2011; Ferreira and Janick 1996), and quite a few studies indicate that trichomes are of great consequence to the production of artemisinin (Kapoor et al. 2007; Arsenault et al. 2010). Therefore, it is considerably attractive for researchers to find a way of enhancing artemisinin through regulation of trichomes or reactions occurring in them, such as employing trichome-specific promoters.

Amorpha-4,11-diene synthase (ADS) is the first key enzyme of artemisinin biosynthetic pathway in *A. annua*. It catalyzes farnesyl pyrophosphate (FPP) into amorpha-4,11-diene to provide the carbon skeleton for artemisinin (Nguyen et al. 2011). Kim et al. (2008) revealed that the promoter of the *ADS* gene specifically drove downstream gene expression in non-glandular trichomes of *Arabidopsis thaliana*. Later on, Wang et al. (2011) cloned a short version of *ADS* promoter (1,929 bp) and found that it was trichome-specific in *A. annua* L. From these studies, it seems that *ADS* promoter can be a promising tool for artemisinin phyto-genetic engineering. However, the expression of *ADS* gene in *A. annua* features considerable variations. It not only responds to diverse environmental signals (Nguyen et al. 2011), but also displays temporal expression profile, with remarkably decreased levels as *A. annua* plant matures (Olofsson et al. 2011). Therefore it is worthy of modifying the *ADS* promoter to a simplified one, which is stronger than the original one, much shorter in length to make it easily manipulated both in promoter reconstruction and genetic engineering, while retaining its trichome specificity as much as possible.

In this study, we cloned a 2935-bp DNA fragment upstream of the *ADS* coding sequence from *A. annua* L. We demonstrated that this *ADS* promoter is specific for gene expression in both glandular trichomes of *A. annua* and non-glandular trichomes of *A. thaliana*, and its truncated versions (no less than –350 upstream of the transcription start site) are capable of retaining trichome specificity in *A. thaliana*, while exhibiting stronger activities (around two to four times) than the full-length one. Therefore, compared to the full-length *ADS* promoter, the deleted ones are probably more promising for future genetic engineering use.

## Materials and Methods

### Extraction of Genomic DNA of *A. annua*

The genomic DNA was extracted from fresh young leaves of *A. annua* using CTAB method. Around 0.1 g fresh young leaves were collected and ground in liquid nitrogen; 700  $\mu$ l preheated CTAB buffer containing both 2 %  $\beta$ -mercaptoethanol ( $v/v$ ) and 1 % PVP ( $w/v$ ) was then added to the ground leaves and then incubated in water bath at 65 °C for 1 h; 700  $\mu$ l chloroform–isopentanol mixture ( $v/v=24:1$ ) was then added to the CTAB–leaves mixture and shaken for 20 min (60 rev/min). The resulting mixture was then centrifuged at 10,000 rpm for 10 min, and after that the supernatant was separated with 600  $\mu$ l chloroform–isopentanol mixture ( $v/v=24:1$ ) added into it and shaken for 20 min (60 rev/min). The resulting mixture was then centrifuged at 10,000 rpm for 10 min, and then the supernatant was separated; 300  $\mu$ l cold isopropanol was subsequently added to the supernatant, and the resulting mixture was then incubated at 4 °C for 1 h. After that, the mixture was centrifuged at 10,000 rpm for 10 min, and the resulting supernatant was discarded, with the deposit kept and washed with 70 % EtOH twice. The washed deposit was then dried and dissolved with 100  $\mu$ l TE buffer, making the extracted *A. annua* genomic DNA solution.

### Cloning of the Promoter Region of the *ADS* Gene in *A. annua*

According to the published *ADS* gene promoter sequence (GenBank No.: AY528931.1), a pair of specific primers was designed and the digestion sites of two restriction enzymes *Bam*HI and *Nco*I (Fermentas FastDigest) were incorporated into these two primers as well. Using this pair of primers (*pADS-Bam*HI-F and *pADS-Nco*I-R; see details in Table 1), high-fidelity PCR (KOD Plus, TOYOBO) was conducted as follows: 94 °C for 3 min; 30 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 3 min; 72 °C for 10 min. The PCR products were then examined in gel electrophoresis and target fragments (~3,000 bp) were retrieved using JETQUICK Gel Extraction Spin Kit (GENOMED). The retrieved fragments were cloned into *pMD18-T* (Takara) and then transformed into competent cells of *Escherichia coli* strain DH5 $\alpha$  through heat shock (42 °C for 90 s). Positive clones were picked out on LB/amp, and their plasmids were extracted using JETQUICK Plasmid Miniprep Spin Kit (GENOMED) for sequencing of the target DNA fragment in BGI.

### Construction of the Expression Vector *ADSpro::GUS* and *ADS* Promoter Deletion Vectors

Both recombinant *pMD18-T* and another vector *pCambia1305.1* (Cambia) were double-digested with restriction enzymes *Bam*HI and *Nco*I, and the target DNA fragment

**Table 1** Primers used in this study

Primer name	Sequence
p <i>ADS-Bam</i> HI-F	CGCGGATCCGGCATAAGAACATACA AAGCA
p <i>ADS-800-Bam</i> HI-F	CGGGATCCAATAATTCTCGTAGCCAA ATTTAATTT
p <i>ADS-600-Bam</i> HI-F	CGGGATCCGGCGCCACATATGGGCCTT
p <i>ADS-550-Bam</i> HI-F	CGGGATCCATACAATGTCCATAGCCCCG
p <i>ADS-500-Bam</i> HI-F	CGGGATCCATACAATGTCCATAGCCCCGA
p <i>ADS-450-Bam</i> HI-F	CGGGATCCTAGAAGGTTCTTAATACA
p <i>ADS-400-Bam</i> HI-F	CGGGATCCATATTGTGCTTGAGTCAT ATTG
p <i>ADS-350-Bam</i> HI-F	CGGGATCCCTTAAATTGTAGTTAGAA
p <i>ADS-300-Bam</i> HI-F	CGGGATCCAGATATAGGACAACATGTAG
p <i>ADS-Nco</i> I-R	CATGCCATGGGGATTTTCAAAACTTT GAAT

Italicized bases indicate restriction enzyme sites

F forward, R reverse

and the resulting linear vector pCAMBIA1305.1 were ligated with T<sub>4</sub> DNA ligase (Thermo Scientific) (3 µl of the target DNA fragment, 1 µl of the linear vector, 1 µl of T<sub>4</sub> DNA ligase buffer, 4.5 µl of ddH<sub>2</sub>O) under the following condition: 22 °C for 30 min. This recombinant expression vector was designated ADSpro::GUS.

To construct promoter deletion vectors, several primers were designed according to the *ADS* promoter sequence (see details in Table 1). With these specific primers, deleted fragments of the *ADS* promoter were isolated from the cloning vector ADSpro-MD18-T. All these fragments and pCAMBIA1391z (Cambia) were double-digested by two restriction enzymes, *Bam*HI and *Nco*I, and the resulting digested *ADS* promoter fragments were constructed into pCAMBIA1391z, respectively, with T<sub>4</sub> DNA ligase, making recombinant expression vectors designated as Δ-800, Δ-600, Δ-550, Δ-500, Δ-450, Δ-400, Δ-350, and Δ-300, respectively.

All of those expression vectors stated earlier as well as an intact pCAMBIA1305.1 vector (designated as 35Spro::GUS) were introduced into the competent cells of *Agrobacterium tumefaciens* strain EHA105 using freeze–thaw method (frozen in liquid nitrogen for 5 min and then thawed at 37 °C for 3 min). Positive clones were selected out on LB (with kanamycin, rifampicin, and streptomycin) and then further examined for target DNA fragments via PCR amplification. These transgenic *A. tumefaciens* were prepared for further plant transformation.

#### *A. annua* and *A. thaliana* Stable Transformation

Plant materials for transformations are *A. annua* wild type Youqing (collected in Youyang County, Chongqing City of China) and *A. thaliana* wild type Columbia, respectively.

Following the method of Zhang et al. (2009), *A. annua* was transformed with both of the two types of recombinant *A. tumefaciens* containing ADSpro::GUS or 35Spro::GUS fusions and selected with hygromycin resistance.

*A. thaliana* transformation was conducted following the floral dip protocol of Zhang et al. (2006) with several recombinant vectors, respectively: ADSpro::GUS, Δ-800, Δ-600, Δ-550, Δ-500, Δ-450, Δ-400, Δ-350, and Δ-300. Transgenic seeds were screened on MS (with hygromycin and cefalexin) medium, and the obtained seedlings were grown in soil under 16-h days and 8-h nights at 22 °C.

#### Transient Gene Expression in *Nicotiana benthamiana*

Transient expression in *N. benthamiana* was achieved through agroinfiltration as described (Kapila et al. 1997) with modifications. *N. benthamiana* of around 40 days after sowing were used for transformation. *A. tumefaciens* EHA105 strains transformed with ADSpro::GUS, 35Spro::GUS, Δ-800, Δ-600, Δ-550, Δ-500, Δ-450, Δ-400, Δ-350, or Δ-300 were prepared in LB (with kanamycin, rifampicin, and streptomycin). These bacteria liquid cultures were centrifuged, and the obtained deposit cells were re-suspended in transformation buffer (10 mM MES, 10 mM MgCl<sub>2</sub>, and 100 µM acetosyringone) with a final OD<sub>600</sub> of around 1.0 and incubated for 3 h. The re-suspended cultures were then injected into the abaxial side of *N. benthamiana* leaves via a 1-ml syringe (needle removed). For each construct, at least three leaves were infiltrated to make sure of three replicates. For the purpose of quantitative expression comparisons between the full-length *ADS* promoter and other constructs, each leaf was treated with the left half injected with ADSpro::GUS *A. tumefaciens* stain while the right half was injected with stain of another construct. Infiltrated *N. benthamiana* plants were grown under 16-h day/8-h night at room temperature for 2–3 days before GUS assay.

#### Histochemical GUS Staining

After around 20 days following regeneration, two to three fresh young leaves of non-transgenic *A. annua* plants and transgenic *A. annua* plants with ADSpro::GUS and with 35Spro::GUS were sampled respectively for histochemical GUS staining using the method of Jefferson (1987).

For *A. thaliana* transgenic lines, lotus leaves of each regenerated plant of around 2–3 weeks old were sampled for GUS staining. The specificity percentage was thus calculated based on the staining results.

For *A. annua* and *A. thaliana* leaves bearing good GUS staining effects, optical microscope was employed to picture their staining patterns.

## Quantitative Analysis of *GUS* Gene Expression

The fluorogenic assay was performed as described (Jefferson 1987).

For each agroinfiltrated *N. benthamiana* leaf, the left half and the right half were sampled separately in that they were transformed with different constructs (as stated earlier). The GUS activity of the right half of the leaf was then normalized to that of the left half so that results would be in relative forms (relative activity of a certain construct to that of ADSpro::GUS) and simultaneously the error between different leaves could be minimized.

For each *Arabidopsis* transgenic line (ADSpro::GUS,  $\Delta$ -800,  $\Delta$ -600,  $\Delta$ -550,  $\Delta$ -500,  $\Delta$ -450,  $\Delta$ -400, and  $\Delta$ -350) of around 2–3 weeks old, no less than three lotus leaves were sampled and assayed separately to constitute at least three biological replicates. The detected GUS activities of all deleted *ADS* promoter constructs were then normalized to that of ADSpro::GUS, resulting in relative GUS activities.

Statistical analysis was carried out with the methods of one-way ANOVA and paired-samples *t*-test using SPSS Version 20 software (IBM Corp.).

## Results

### Isolation and Bioinformatic Analysis of the *ADS* Gene Promoter

We cloned a 2,935-bp DNA fragment upstream of the translation initial codon (ATG) of the *ADS* gene from the genomic DNA of *A. annua* L. through high-fidelity PCR amplification and followed by sequencing.

To achieve an in-depth understanding of this sequence, in silico analysis was conducted via several online tools. As TSSP (<http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter>) predicted, three promoter/enhancer(s) are located at 2868 LDF-, 1247 LDF-, and 774 LDF-, respectively; the 2868 LDF- site is then considered as the transcription start site (+1). Through PlantCARE (Rombauts et al. 1999; Lescot et al. 2002) (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) assay of this sequence, quite a number of putative *cis*-acting elements are present as shown in Fig. 1. For general promoter features, a well-conserved TATA-box (TATAAA) (a core promoter element) was found –12 bp upstream of the transcription start site (TSS); furthermore, several putative CAAT boxes, a common type of *cis*-acting elements in promoter and enhancer regions, are present at around –50 to –200 bp upstream of TSS.

Previous studies showed that expression of the *ADS* gene is regulated by abiotic stress, biotic stress, and multiple hormone

treatments. Our in silico analysis also reveals multiple binding sites of different transcription factor families.

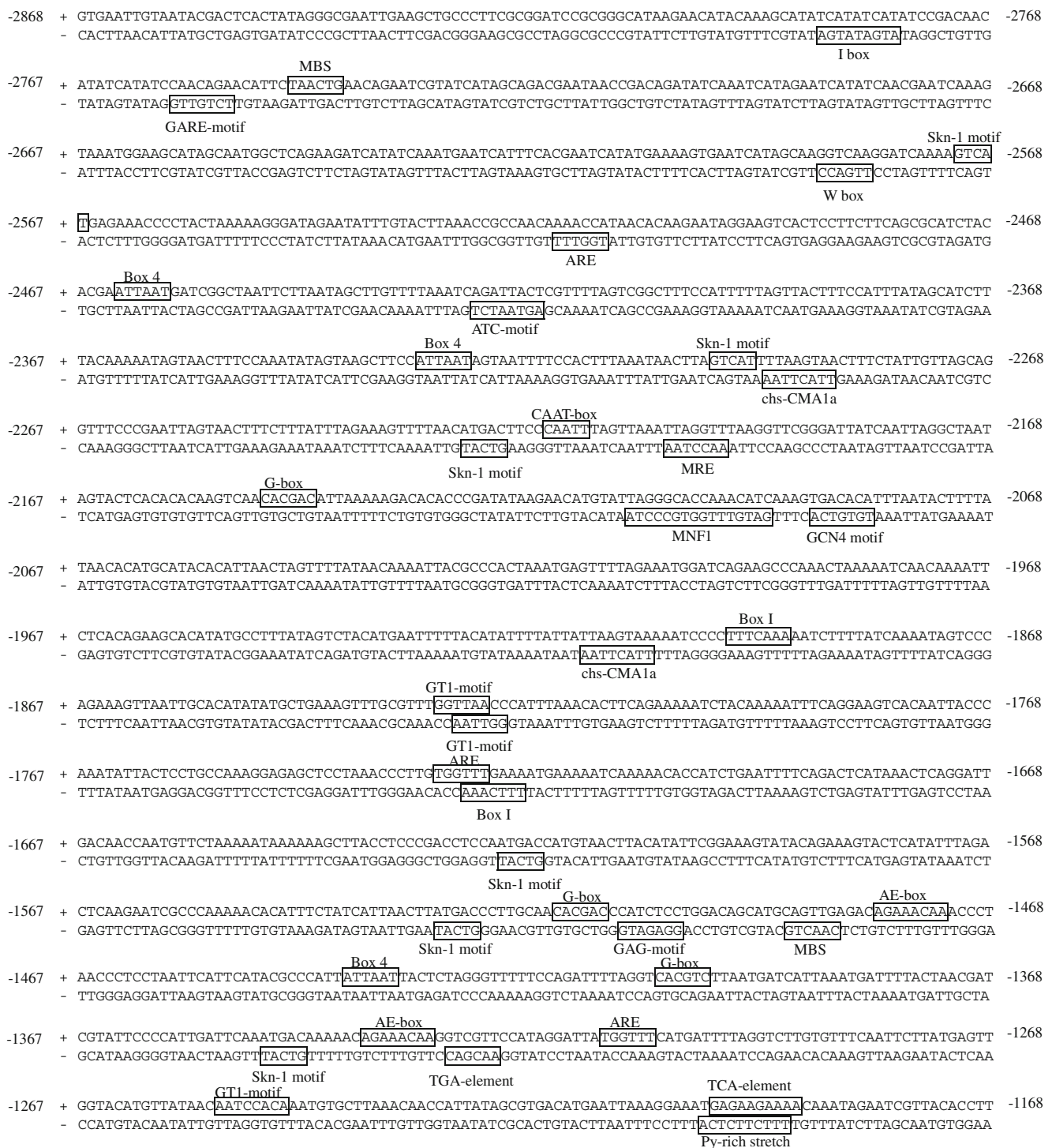
First of all, three W boxes (TTGACC), known as the binding site of WRKY transcription factors, are predicted, and this consists well with the previous discovery that *Aa*WRKY1 protein binds to W box in *ADS* promoter (Ma et al. 2009). In addition, two putative MBSs (CAACTG, TAACTG), the MYB binding site involved in drought inducibility (Yamaguchi-Shinozaki and Shinozaki 1993), are localized at –1485(+) and –2744(–) bp respectively. These are probably associated with the discovery (Yang et al. 2010) that expression of the *ADS* gene in shoots rises when *A. annua* roots were dried for 6 h. Moreover, six elements related to anaerobic induction (ARE, TGGTTT) and one involved in defense and stress responsiveness (TC-rich repeats, ATTTCTTCA) are found as well, indicating that the *ADS* gene expression may be controlled by stress through those elements.

In addition to the stress (abiotic and biotic)-responsive elements, several *cis*-acting elements linked to phytohormone or signal molecule responsiveness were found as well, including one GARE motif (TCTGTTG, GA responsive), one TCA element (GAGAAGAAAA, involved in salicylic acid responsiveness), and one TGA element (AACGAC, auxin-responsive). Such prediction results are closely related to previous studies: An abrupt rise of *ADS* expression occurred at 6 h post GA<sub>3</sub> application (Banyai et al. 2011); SA application gave rise to a temporary peak in *ADS* gene expression at 24 h after application (Pu et al. 2009).

Moreover, a 5' UTR Py-rich stretch (TTTCTTCTCT), an element responsible for high transcription levels, has been predicted at –1190(–) bp, implying that *ADS* promoters are capable to drive the expression of downstream genes actively.

Moreover, various putative elements in relation to light response are abundant in *ADS* promoter sequence, signifying that this promoter is probably subject to light regulation. These elements include two AE boxes (AGAAACAA), one ATC motif (AGTAATCT), one ATCT motif (AATCTAATCT), four Box 4 sites (ATTAAT), four Box I sites (TTTCAA), two chs-CMA1a sites (TACTTAA), five G boxes (CACGAC, CACGTC, CACGTT, CACATGG), one GA motif (ATAG ATAA), one GAG motif (GGAGATG), one Gap box (AAATGGAGA), three GT1 motifs (GGTTAA or AATCC ACA), one I box (ATGATATGA), one MNF1 (GTGCCC), one MRE site (AACCTAA), and one TCT motif (TCTTAC).

Interestingly, two types of *cis*-acting elements required for endosperm expression are found in the *ADS* promoter region: nine Skn-1 motifs (GTCAT) and three GCN4 motifs (TGT GTCA, TCAGTCA), suggesting that this promoter may also have activity during seed development. This may explain that, although *A. annua* seeds are trichome-absent, several artemisinin precursors are still found in them (Brown et al. 2003).



**Fig. 1** Nucleotide sequence and putative *cis*-acting elements of the cloned *ADS* promoter. A brief introduction of *cis*-acting elements present in this figure is as follows: *ARE*, a *cis*-element related to anaerobic induction; *CAAT box*, a common type of *cis*-acting elements in promoter and enhancer regions; *GARE motif*, GA-responsive element; *GCN4 motif*, a *cis*-acting element required for endosperm expression; *MBS*, MYB binding site involved in drought inducibility; *Py-rich stretch*, a *cis*-element responsible for high transcription levels; *Skn-1 motif*, a *cis*-acting

element required for endosperm expression; *TATA box*, a core promoter element; *TCA element*, a *cis*-element involved in salicylic acid responsiveness; *TC-rich repeats*, a *cis*-element involved in defense and stress responsiveness; *TGA element*, an auxin-responsive element; *W box*, the binding site of WRKY transcription factors; elements in relation to light response: *AE box*, *ATCT motif*, *Box 4 site*, *Box I site*, *chS-CMA1a site*, *GAG motif*, *GA motif*, *Gap box*, *G box*, *GT1 motifs*, *I box*, *MNFI*, *MRE site*, *TCT motif*

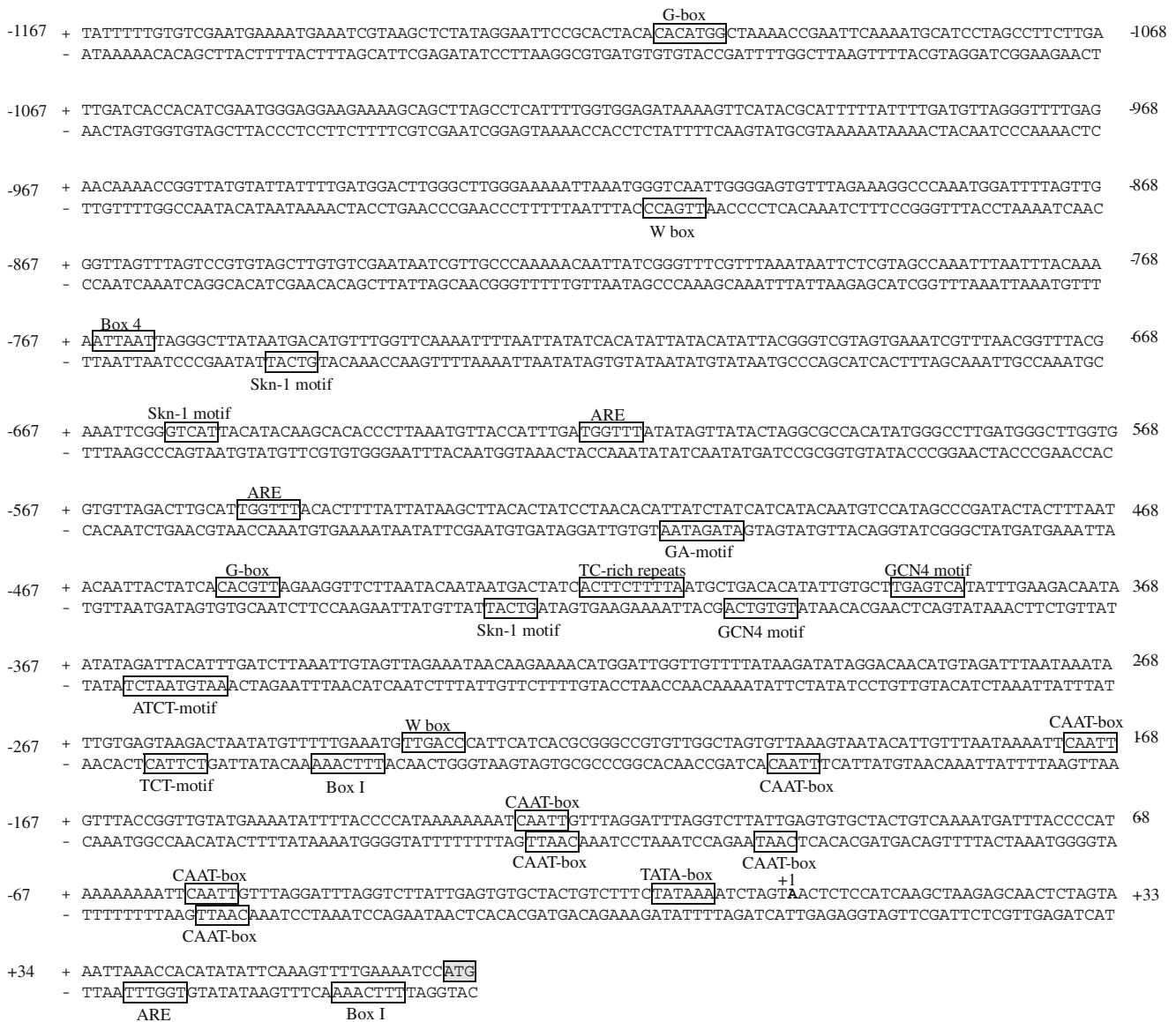


Fig. 1 (continued)

Those in silico analysis results reveal that the *ADS* promoter consists of multiple regulatory elements, which is comported well with previous study that *ADS* is under complex control.

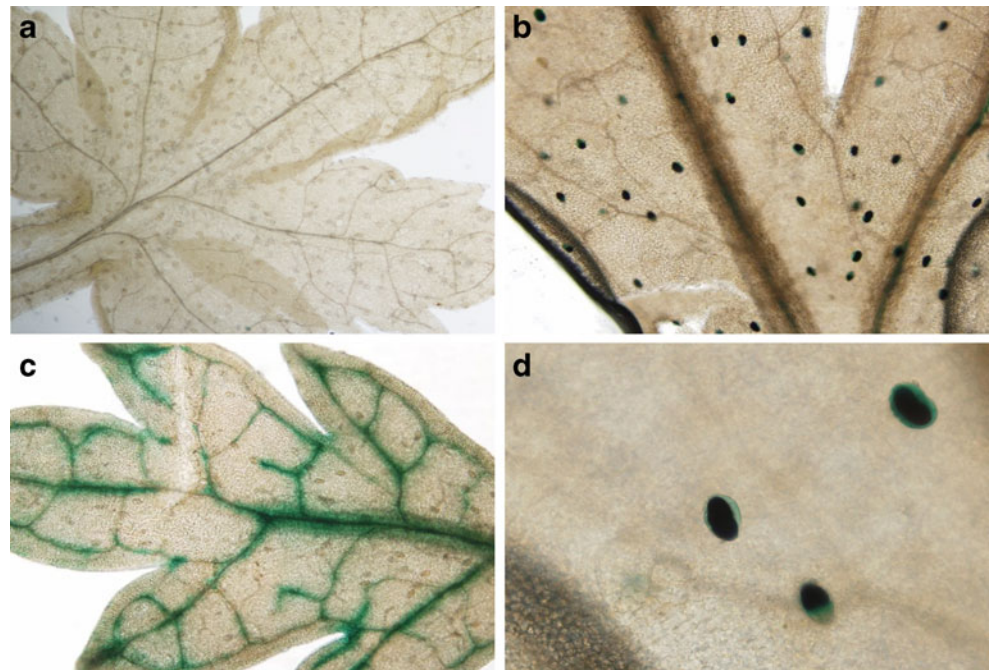
The *ADS* Promoter is Specific for Gene Expression in Both Glandular Trichomes of *A. annua* L. and Non-glandular Trichomes of *A. thaliana*

To explore the expression pattern of the *ADS* promoter, we constructed the ADSpro::GUS fusion vector (along with 35Spro::GUS as a positive control) and transformed wild-type *A. annua* with them. As shown in Fig. 2, while most leaf veins but not trichomes of the transformants with 35Spro::GUS were stained blue (Fig. 2c), in contrast blue signals of the leaf transformed with ADSpro::GUS fusion were well confined to

glandular trichomes (T-shaped trichomes not included) (Fig. 2b, d). No blue traces occurred in the non-transgenic *A. annua* leaf (Fig. 2a), which serves as a negative control for GUS assay. These results indicate that the *ADS* promoter specifically drives downstream gene expression in *A. annua* trichomes.

*A. thaliana* trichomes have long been utilized as a model for plant trichome development researches. Kim et al. (2008) have previously isolated an *ADS* promoter sequence (2,574 bp, Genbank accession number DQ448294), created its corresponding transgenic *A. thaliana* plants, and demonstrated that sequence is specifically expressed in *Arabidopsis* non-glandular trichomes. The *ADS* promoter sequence we obtained is around 400 bp longer than DQ448294 at the 5' end and features several base differentiations as well (data not shown).

**Fig. 2** Histochemical GUS staining of transgenic *A. annua* plants. **a** Juvenile leaf of non-transgenic plants (*control*). **b** Juvenile leaf of plants stably transformed with ADSpro::GUS fusion. **c** Juvenile leaf of plants stably transformed with 35Spro::GUS fusion. **d** Trichomes on juvenile leaf epidermis of plants stably transformed with ADSpro::GUS fusion

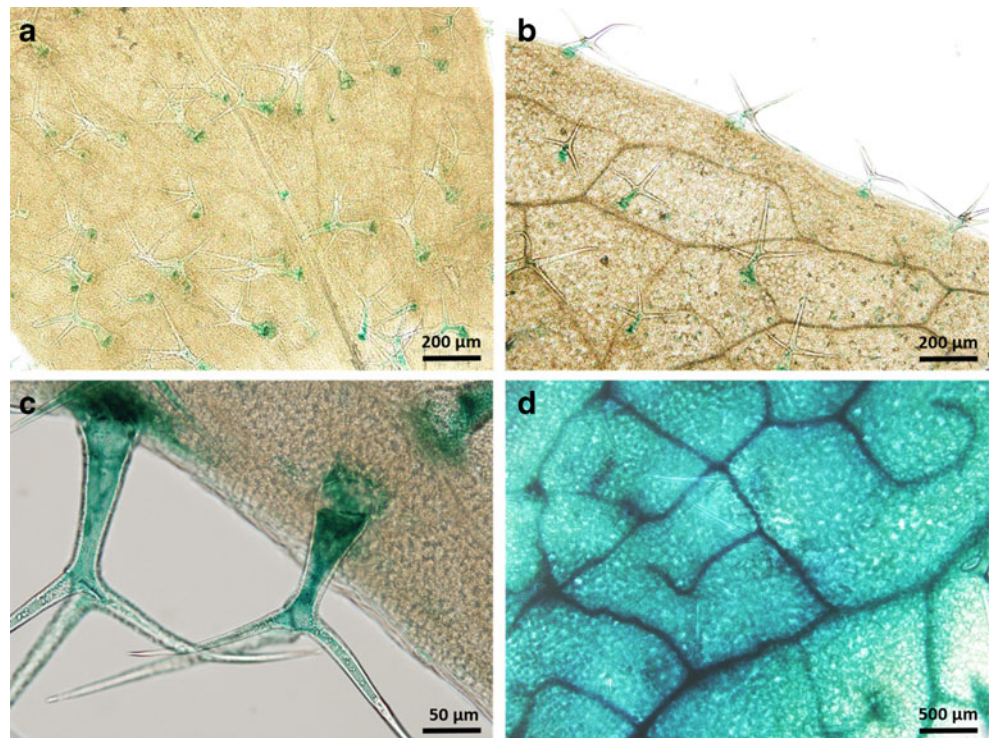


Therefore, similarly, we introduced our ADSpro::GUS fusion into wild-type *A. thaliana* Columbia, and GUS staining assay revealed that blue staining occurred specifically in *Arabidopsis* trichomes as well despite the existence of discrepancies between the two sequences, as is shown in Fig. 3a. Such results reveal that the trichome specificity of the *ADS* promoter in *A. thaliana* is not determined by those discrepancies.

#### Sequentially Deleted Fragments of the *ADS* Promoter Resulted in Unexpected Expression Patterns in *A. thaliana*

To thoroughly investigate the *ADS* promoter, especially what it is that endows it with trichome specificity, we conducted 5' sequential deletion, obtained eight 5' deleted fragments of the *ADS* promoter, used each of them into the expression vector

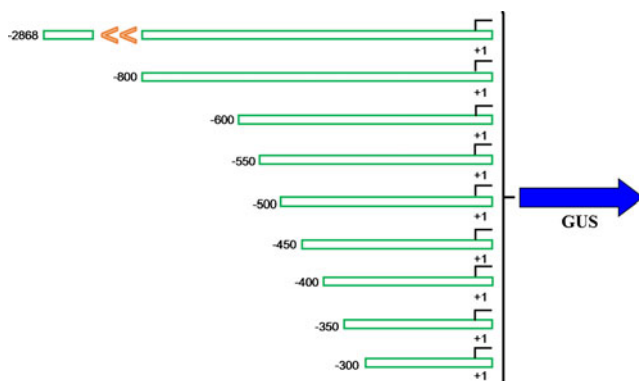
**Fig. 3** Histochemical GUS staining of transgenic *A. thaliana* leaves. **a** *A. thaliana* plants transformed with full-length-ADSpro::GUS fusion. **b** *A. thaliana* plants transformed with  $\Delta$ -800/ $\Delta$ -600/ $\Delta$ -550/ $\Delta$ -500/ $\Delta$ -450/ $\Delta$ -400/ $\Delta$ -350. **c** Closer view of *A. thaliana* trichomes with GUS staining. **d** *A. thaliana* plants transformed with  $\Delta$ -300



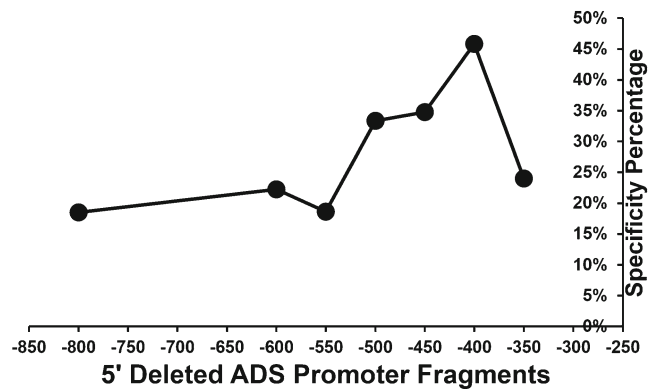
pCAMBIA1391z containing *GUS* as the downstream gene of those promoter fragments, and numbered them as  $\Delta$ -800,  $\Delta$ -600,  $\Delta$ -550,  $\Delta$ -500,  $\Delta$ -450,  $\Delta$ -400,  $\Delta$ -350, and  $\Delta$ -300, respectively, as is shown in Fig. 4. Stably transformed *A. thaliana* wild type Columbia with those fusion vectors exhibited differentiated expression patterns after being stained with X-gluc, as Fig. 3b, d shows. Through 5' sequential deletion, we found that, as short as merely -350 upstream the transcription start site (TSS), the promoter fragment still confers *GUS* expression specificity in *A. thaliana* trichomes, and when it was shortened to -300, we did not obtain any transformed lines displaying trichome-specific *GUS* staining pattern.

However, not all transgenic lines containing the deleted fragments of the *ADS* promoter equal to or longer than -350 exhibit trichome specificity; matter-of-factly, quite a small portion of them do so, and the rest feature constitutive *GUS* expression pattern, with blue staining observed in trichomes as well as in other parts of transformed *Arabidopsis* leaves. Figure 5 reveals the specificity percentages of all transgenic lines, from which we found that transgenic lines such as  $\Delta$ -800 and  $\Delta$ -550 featured specificity percentages as low as around one fifth, and from  $\Delta$ -550 on, the percentage increased until the highest point of 45.83 %, which lay in  $\Delta$ -400, and then plummeted to below 25 % when deleted to -350. However, the specificity percentage of the full-length *ADS* promoter transgenic line was around 70 %.

*Arabidopsis* trichomes are unicellular, protuberating from leaf or stem epidermis with a short stem and generally three branches (Folkers et al. 1997). Regarding the detailed trichome-specific *GUS* staining feature of both the full-length and all deleted *ADS* promoter fragments, blue staining was basically found in the lower region of the trichome stem close to the epidermis and could also be observed in branches



**Fig. 4** Schematic representation of the *ADS* promoter deletion constructs for assaying *GUS* expression in transgenic *A. thaliana*. Sequentially 5' deleted fragments of the *ADS* promoter were fused to the *GUS* gene to make corresponding constructs ( $\Delta$ -800,  $\Delta$ -600,  $\Delta$ -550,  $\Delta$ -500,  $\Delta$ -450,  $\Delta$ -400,  $\Delta$ -350, and  $\Delta$ -300)



**Fig. 5** Specificity percentages of *A. thaliana* lines transformed with constructs of the deleted fragments of the *ADS* promoter that are able to confer specific *GUS* expression in *A. thaliana* trichomes

and basal cells in some transgenic plants with relatively high *GUS* expressions. Generally, trichomes located at the leaf margin exhibited higher *GUS* expressions than those close to the central leaf vein, with the highest expression occurring in the ones at the very tip of the leaf.

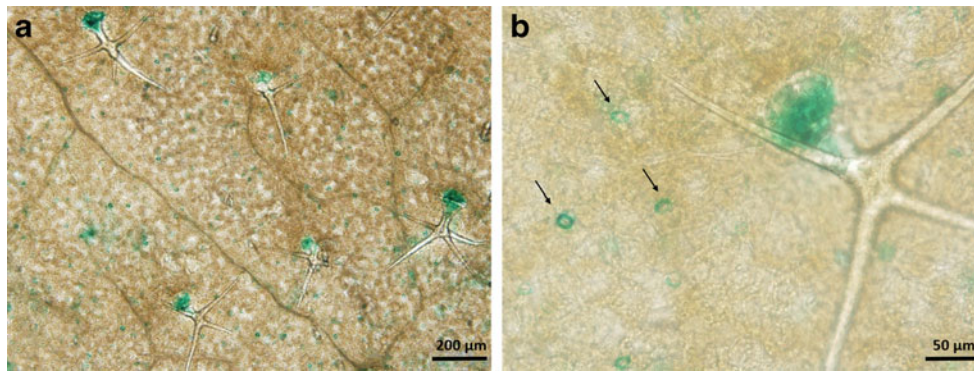
Besides the trichome specificity of all deleted fragments of the *ADS* promoter, which is similar to that of the full-length *ADS* promoter, we have observed an unusual expression pattern: Apart from transgenic *Arabidopsis* lines with the full-length *ADS* promoter, in all the other lines with deleted fragments (except  $\Delta$ -500), blue staining was commonly found in the guard cells of stomata, as shown in Fig. 6.

#### Quantitative Analysis of *GUS* Gene Expression

To explore the driving capabilities of the full-length *ADS* promoter as well as all its deleted promoter fragments, we conducted *GUS* quantitative assays in both transiently and stably transformed lines of full-length/deleted *ADS* promoter (fragments).

We first injected transformed *A. tumefaciens* into *N. benthamiana* leaves to achieve transient expressions of all the deleted fragments of the *ADS* promoter and the full-length one along with CaMV 35S promoter as the positive control. Quantitative analysis of the transformed tobacco leaves revealed diversified *GUS* expression profiles. Compared with leaves carrying the full-length *ADS* promoter, those with 5' sequentially deleted fragments expressed higher *GUS* activities, ranging from around 1.5 to three times that of the full-length one. Of the 5' deleted fragments, from  $\Delta$ -800 to  $\Delta$ -300, *GUS* activities of the corresponding leaves fluctuated upward, with a minimum of 1.58 times ( $\Delta$ -800) that of the original *ADS* promoter and a maximum of 2.89 times ( $\Delta$ -300) (Fig. 7a). As expected, the positive control of 35S promoter led to considerably strong *GUS* expression, with 8.16 times of activity that of the original *ADS* promoter. Statistical analysis





**Fig. 6** GUS staining in the guard cells of stomata exhibited on the leaf epidermis of *A. thaliana* plants transformed with constructs of the deleted fragments of the *ADS* promoter ( $\Delta$ -800,  $\Delta$ -600,  $\Delta$ -500,  $\Delta$ -450,  $\Delta$ -400,

or  $\Delta$ -350). **a** Transgenic *A. thaliana* leaf surface with GUS staining in both trichomes and guard cells of stomata. **b** Closer view of GUS staining in guard cells of stomata (arrows)

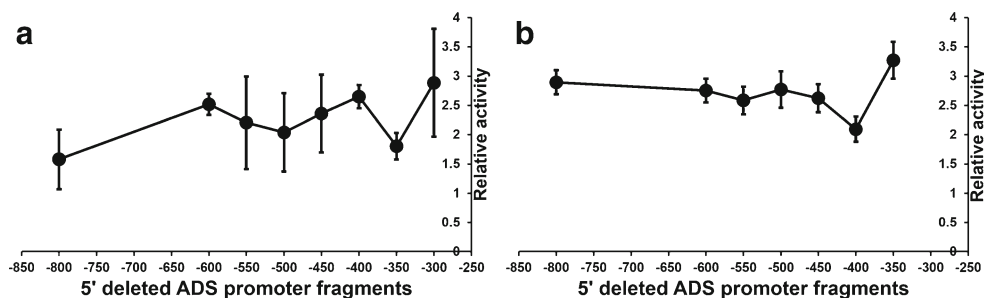
results showed that there were no significant differences between the activities of the eight deleted fragments; however, significant differences were found between the activity of 35S promoter and that of those deleted fragments. Paired-samples *t*-test results showed that activities of  $\Delta$ -600,  $\Delta$ -400,  $\Delta$ -350, and 35S promoter were significantly higher than that of the full-length *ADS* promoter.

In stably transformed *Arabidopsis* plants, as is mentioned earlier, all deleted *ADS* promoter fragments except  $\Delta$ -300 rendered trichome specificity. We therefore examined the GUS activities of both the full-length *ADS* promoter and all the other deleted fragments ( $\Delta$ -300 not included) in transgenic *A. thaliana*. As is displayed in Fig. 7b, all the examined 5' deleted fragments exhibited higher expression activities than the original *ADS* promoter, with a minimum of 2.09 times ( $\Delta$ -400) and a maximum of 3.27 times ( $\Delta$ -350). From  $\Delta$ -800 to  $\Delta$ -450, activities change fairly slightly, followed by a drop of  $\Delta$ -400 and a soaring rise of  $\Delta$ -350. Statistical analysis results showed that activities of the seven deleted fragments of the *ADS* promoter were all significantly higher than that of the full-length one, and the activity of  $\Delta$ -350 is significantly higher than that of  $\Delta$ -400.

## Discussion

### The Cross-Species Feature of Trichome Specificity of the *ADS* Promoter

Trichomes differentiated from plant epidermis are generally categorized into two types: non-glandular trichomes and glandular trichomes (Werker 2000). From *Arabidopsis* trichomes which serve as a research model for cell differentiation to cotton fibers which dictate huge economic values, non-glandular trichomes have long been the research focus for decades. Glandular trichomes are economically important as well. However, they are far more complex than their non-glandular counterparts (Tissier 2012a). From multicellular trichomes on most composite plants to unicellular ones on tobacco, glandular trichomes exhibit diverse forms that we are striving to attain deeper comprehension of. In this study, the *ADS* promoter derived from Asteraceae species *A. annua* is specifically expressed not only in *A. annua* multicellular glandular trichomes but in *A. thaliana* single-cell non-glandular trichomes as well. This result indicates the conservation of gene regulations between non-glandular and glandular trichomes.



**Fig. 7** GUS quantitative analysis of the *ADS* promoter. **a** Relative GUS activities of *N. benthamiana* leaves transiently transformed with the deleted fragments of the *ADS* promoter compared to that of the full-

length *ADS* promoter. **b** Relative GUS activities of *A. thaliana* leaves stably transformed with the deleted fragments of the *ADS* promoter compared to that of the full-length *ADS* promoter

## Complexity of the Trichome-Specific Gene Expression Directed by the *ADS* Promoter

Efforts have long been contributed to the elucidation of the mechanism that regulates trichome-specific expressions. For the past decade, sub-regions of trichome-specific promoters from various species (*Gossypium hirsutum*, *Nicotiana tabacum*, *A. thaliana*, *Nicotiana glauca*, etc.) that contribute to trichome specificity have been discovered, with lengths ranging from 50 to over 300 bp (Hsu et al. 1999; Wang et al. 2002; Gutierrez-Alcala et al. 2005; Liu et al. 2006b; Wu et al. 2007; Ni et al. 2008; Ennajdaoui et al. 2010). However, until now, few *cis*-acting elements that control such specificity are discovered. The only discovery lies in the promoter of a cotton fiber gene *RDL1* which contains an L1 box and a MYB binding motif that synergistically determine the promoter's trichome specificity in *A. thaliana* (Wang et al. 2004). Mutation of each *cis*-element alone did not deprive the promoter of trichome specificity, only reducing its activity greatly; double mutation of both elements completely inactivated the promoter. In this study, with the online tool PLACE (<http://www.dna.affrc.go.jp/PLACE/index.html>) (Prestridge 1991; Higo et al. 1999), no L1 boxes (TGCA TTTA) were found in the full-length *ADS* promoter. Concerning the MYB binding motif, seven MYBCORE elements (CNGTT[A/G]) were discovered across the *ADS* promoter (-2758(-), -2745(-), -2713(-), -1491(+), -960(+), -680(-), and -162(-)); however, none of these was located in the region between -350 and -300. These findings apparently illustrated that the mechanisms controlling the trichome specificity of the *ADS* promoter are far distinguished from the "L1 box + MYB binding motif" mechanism of the *RDL1* promoter.

To investigate the mechanism of the trichome specificity of the *ADS* promoter, at first, we considered the model plant *A. thaliana* an efficient shortcut with which to pin down a unique sub-region (even element) of the *ADS* promoter that explains its trichome specificity since fortunately it is specifically expressed in *Arabidopsis* trichomes as well. However, our study reveals that it is not that simple as we previously envisaged. With the sequential deletion progressing, remaining fragments as short as barely -350 still confers trichome specificity, however, at the cost of specificity ratio, namely, the ratio between transgenic lines with trichome-specific GUS expression and the ones with constitutive GUS expression dropped compared with that of the full-length *ADS* promoter transgenic lines. Such a result indicates that regions upstream of -800 probably contribute to reflect partial trichome specificity, not qualitatively but quantitatively, through a mechanism we have not yet known. Within the progressive deleted region, the specificity percentages of transformed *Arabidopsis* lines with different deleted fragments varied considerably as well. The trend did not display as total decrease or total increase but rather

a peak shape with a long tail. It seems that within the deeply investigated region from -800 to -300, all the small sub-regions of 50–200 bp all contribute to trichome specificity. Some sub-regions do it negatively, such as the sub-region of -550 to -500, deletion of which increases trichome specificity, while others positively, such as the sub-region -400 to -350, deletion of which decreases trichome specificity sharply. Therefore, from this study, the trichome specificity of *ADS* promoter is more like a quantitative trait than a qualitative one. These unexpected results inevitably suggest that the mechanism that lies behind trichome-specific expression is far more complicated than we previously perceived it, at least in the case of the *ADS* promoter. Further studies based on alternative methods are probably needed to eventually elucidate the mechanism.

## Deleted Fragments of the *ADS* Promoter Yielded Unexpected *GUS* Gene Expression in SGuard Cells of Leaf Tissues

It is intriguing that besides GUS expression in *Arabidopsis* trichomes, guard cells of stomata of most transformed *A. thaliana* lines with deleted fragments of the *ADS* promoters were found to be with GUS staining as well. Apart from the full-length *ADS* promoter and  $\Delta$ -500, all the other deleted *ADS* promoters are able to confer guard cell expressions in their corresponding transgenic *Arabidopsis* plants. We therefore scrutinized the *ADS* promoter sequence for particular *cis*-acting elements that are putatively responsible for the specific expression in the guard cells of stomata and eventually found 37 Dof binding elements across the whole *ADS* promoter sequence with the online tool PLACE (<http://www.dna.affrc.go.jp/PLACE/index.html>) (Prestridge 1991; Higo et al. 1999). Dof proteins are transcription factors that constitute the Dof family, and they play diverse roles in plants, ranging from light and defense responses, seed development, and germination, etc. (Yanagisawa 2002). Recent studies reveal that they regulate guard cell-specific gene expression as well through binding a short *cis*-acting DNA sequence [A/T]AAAG, namely, Dof binding element (Plesch et al. 2001; Galbiati et al. 2008; Yang et al. 2008; Gardner et al. 2009). Among the 37 Dof binding elements found in *ADS* promoter sequence, six of them (16.2 %) are located in the region from -800 to the transcription start site, and the location sites are -543(-), -473(-), -415(-), 198(+), -17(-), and +52(+), respectively. However, the full-length *ADS* promoter containing a staggering 37 Dof binding elements fails to direct guard cell expression, while owners of no more than six Dof binding elements ( $\Delta$ -800,  $\Delta$ -600,  $\Delta$ -550,  $\Delta$ -450,  $\Delta$ -400,  $\Delta$ -350) succeed in it. Besides, among all of the deleted *ADS* promoters, only  $\Delta$ -500 lines did not display any staining in guard cells. From these findings, we may easily conclude that only Dof binding elements themselves are not sufficient for guard cell expressions. There ought to be hidden mechanisms that

regulate these expressions of *ADS* promoter in a more complicated yet unknown way.

#### Prospects for Artemisinin Genetic Engineering via Deleted Fragments of the *ADS* Promoters

Serving as the currently most effective anti-malarial compound, artemisinin dictates almost all economic values of *A. annua* L. With no other industrial-scale production systems available, land-produced *A. annua* plants serve as the paramount source for artemisinin-based anti-malarial medication. To improve artemisinin yield in *A. annua* is thus of great value.

CaMV 35S promoter is a constitutive promoter that is extensively used in both basic and applied research. Recognized bottlenecks occur in the application of constitutive promoters in that the resulting overexpression of target genes is non-economical, bringing about waste of energy, and over-expressed products in non-target areas can be harmful to the plant itself. Artemisinin is specifically biosynthesized and sequestered in *A. annua* glandular trichomes (Nguyen et al. 2011) partly due to its phytotoxic properties (Duke et al. 1987). From this study, an attractive phenomenon occurred in that CaMV 35S promoter did not guide the GUS expression in *A. annua* trichomes. Although the causes of the occurrence are to be further elucidated, together with those bottlenecks of constitutive promoters, it does not appear promising to employ constitutive promoters such as 35S promoter to genetically modify *A. annua* for high artemisinin yield when target genes are specially expressed in glandular trichomes.

Therefore, highly effective glandular trichome-specific promoters play a critical role in genetic engineering-based *A. annua* breeding. A fairly large number of trichome-specific promoters from various other species have been discovered (Tissier 2012b), but none of them have been demonstrated to be able to retain their trichome specificity and function effectively in gene engineering in *A. annua*. The *ADS* promoter is one of only a few trichome-specific promoters that are isolated from *A. annua*. Whether it is a good choice for engineering is still an open question in that the *ADS* gene is expressed in not only spatial but also highly temporal way—with extraordinarily high expression in juvenile stage of *A. annua* but extremely low level in the senescent stage (Olofsson et al. 2011). The *ADS* promoter corresponds well to its gene expression profile just as expected, with high GUS activity in *A. annua* young leaves while nearly not detectable in old leaves (data not shown). It is hence fairly worthwhile to demobilize *ADS* promoter, i.e., to achieve a compact trichome-specific core promoter through removing all the unnecessary negative-regulatory elements. Beyond the core promoter, enhancers can probably be added to create a “super promoter” that embraces both trichome-specific and high-level expression. In this

study, we shortened the *ADS* promoter to various extents and investigated the resulting promoter fragments’ capabilities of driving downstream gene expression. It is worth noting that all of the deleted fragments of the *ADS* promoter are more powerful in directing gene expression than the full-length one, among which  $\Delta$ -350 conferred the highest GUS expression (over three times that of the full-length promoter). Therefore, these deleted fragments can be candidates for future studies on “super promoters” and engineering application. For example, although the specificity percentage of the  $\Delta$ -400 transgenic line is lower (around 45 %) than that of the full-length *ADS* promoter,  $\Delta$ -400 can still probably serve as the backbone for the construction of a super promoter or at least be employed for the trial of artemisinin genetic engineering in that it is far easier to be manipulated (significantly shorter than the original *ADS* promoter), boasting trichome specificity and higher activity.

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