Characterization of GaWRKY1, a Cotton Transcription Factor That Regulates the Sesquiterpene Synthase Gene (+)-δ-Cadinene Synthase-A[1]

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The cotton (+)-δ-cadinene synthase (CAD1), a sesquiterpene cyclase, catalyzes a branch-point step leading to biosynthesis of sesquiterpene phytoalexins, including gossypol. *CAD1-A* is a member of *CAD1* gene family, and its promoter contains a W-box palindrome with two reversely oriented TGAC repeats, which are the proposed binding sites of WRKY transcription factors. We isolated several *WRKY* cDNAs from *Gossypium arboreum*. One of them, *GaWRKY1*, encodes a protein containing a single WRKY domain and a putative N-terminal Leu zipper. Similar to genes encoding enzymes of cotton sesquiterpene pathway, *GaWRKY1* was down-regulated in a glandless cotton cultivar that contained much less gossypol. *GaWRKY1* showed a temporal and spatial pattern of expression comparable to that of *CAD1-A* in various aerial organs examined, including sepal, stigma, anther, and developing seeds. In suspension cells, expression of both *GaWRKY1* and *CAD1-A* genes and biosynthesis of sesquiterpene aldehydes were strongly induced by a fungal elicitor preparation and methyl jasmonate. GaWRKY1 interacted with the 3× W-box derived from *CAD1-A* promoter in yeast (*Saccharomyces cerevisiae*) one-hybrid system and in vitro. Furthermore, in transgenic Arabidopsis plants, overexpression of *GaWRKY1* highly activated the *CAD1-A* promoter, and transient assay in tobacco (*Nicotiana tabacum*) leaves demonstrated that W-box was required for this activation. These results suggest that GaWRKY1 participates in regulation of sesquiterpene biosynthesis in cotton, and *CAD1-A* is a target gene of this transcription factor.

In cotton plants, biosynthesis of sesquiterpene phytoalexins could be induced by fungal and bacterial infection or other environmental stimuli (Bell and Stipanovic, 1977; Bell, 1986; Essenberg et al., 1990). Formation of gossypol and related sesquiterpene aldehydes is also developmentally regulated, and these secondary metabolites accumulate in pigmented glands of aerial tissues and in epidermal and subepidermal cells of roots (Bell and Stipanovic, 1977; Halloin and Bell, 1979; Meng et al., 1999). All cotton sesquiterpene phytoalexins identified so far have a cadinene-type skeleton, and biochemical and molecular evidence has suggested that (+)-δ-cadinene is the biosynthetic precursor (Chen et al., 1995; Davila-Huerta et al., 1995; Luo et al., 2001).

(+)-δ-Cadinene synthase (CAD1), a sesquiterpene cyclase, catalyzes the first committed step in the pathway of gossypol and related sesquiterpenes (Chen et al., 1995, 1996). In *Gossypium arboreum*, a diploid cotton species, *CAD1* is encoded by a gene family that can be divided into two subfamilies: *CAD1-A* with a single member and *CAD1-C* with several (Tan et al., 2000). Our previous reverse transcription (RT)-PCR analysis showed that *CAD1-C* genes were highly expressed in such aerial organs as sepal, petal, and developing seeds during early developmental stages, paralleling formation of pigment glands and accumulation of sesquiterpene aldehydes, whereas expression level of *CAD1-A* was low or undetectable when the same RT-PCR conditions were employed. In stem, *CAD1-A* transcripts were detectable only after elicitation, but those of *CAD1-C* were detected both before and after elicitation (Tan et al., 2000). In suspension cultured cells, both *CAD1-A* and *CAD1-C* genes were induced to express by elicitation (Chen et al., 1995, 1996). Thus, in cotton plants, genes *CAD1-A* and *CAD1-C* have different but overlapping expression patterns, and there appears to be a complex program modulating the expression of sesquiterpene cyclase genes.

WRKY proteins form a large transcription factor family with their occurrence limited to plants. There are estimated to be more than 70 WRKY proteins in Arabidopsis (Robatzek and Somssich, 2002; Singh et al., 2002; Dong et al., 2003; Kalde et al., 2003). WRKY proteins are featured by the WRKY domain, which is a 60-amino acid stretch containing a conserved amino acid sequence of WRKYGQK together with a zinc finger-like motif (Eulgem et al., 2000). There is either one or two WRKY domains in each protein; some members of the family have Leu zippers toward the N terminus (Eulgem et al., 1999; Cormack et al., 2002). Numerous in vitro and in vivo experiments

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have demonstrated that WRKY proteins specifically bind to the W-box (T)TGAC(C/T), a cis-acting DNA element found frequently in the promoter of defense-related genes (Rushton et al., 1996; Yang et al., 1999; Du and Chen, 2000; Yu et al., 2001).

In the past few years, there has been much progress in characterizing WRKY proteins involved in regulating plant defense responses. Many *WRKY* genes, such as *PcWRKY1*, *PcWRKY4*, *AtWRKY18*, and *AtWRKY70*, are involved in response to pathogen infection and elicitor treatment (Rushton et al., 1996; Chen and Chen, 2002; Cormack et al., 2002; Li et al., 2004). The parsley (*Petroselinum crispum*) PcWRKY1, for example, bound specifically to the W-box in *PR1-1* or *PR1-2* promoters, and its own expression was induced rapidly and transiently by the pathogen-derived elicitor pep25 (Rushton et al., 1996). Most of the WRKY proteins, such as AtWRKY6 and AtWRKY29, are transcription activators, whereas some WRKYs behave as repressors of transcription (Asai et al., 2002; Robatzek and Somssich, 2002). There is increasing evidence that WRKY proteins also play a diverse role in regulating other developmental and physiological processes of plants, such as trichome initiation (Johnson et al., 2002), senescence (Robatzek and Somssich, 2002; Hinderhofer and Zentgraf, 2001), and carbohydrate metabolism (Sun et al., 2003).

Here, we report that a palindrome of W-box elements is present in the *CAD1-A* promoter. We have isolated from *G. arboreum* a cDNA, *GaWRKY1*, coding for a Leu zipper-containing WRKY protein. GaWRKY1 interacted with the 3× W-box derived from *CAD1-A* promoter in yeast (*Saccharomyces cerevisiae*) one-hybrid system and in an electrophoretic mobility shift assay (EMSA). Expression of *GaWRKY1* activated *CAD1-A* promoter in both transgenic Arabidopsis plants and transiently transformed tobacco (*Nicotiana tabacum*) leaves, and disruption of the W-box abolished the activation. In the glandless cotton cultivar, expression of both *GaWRKY1* and *CAD1-A* genes was down-regulated, and in the glanded cultivar their temporal and spatial expression patterns were comparable. These data demonstrate that GaWRKY1 is likely a transcriptional activator of the CAD1 gene *CAD1-A*, participating in cotton sesquiterpene biosynthesis.

RESULTS

Isolation of *GaWRKY1*

To study the transcriptional regulation of the cotton CAD1, a key enzyme in the biosynthesis pathway of gossypol and related sesquiterpenes, promoters of *CAD1-A* and *CAD1-C3* were scanned with PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html). Both promoters were found to contain the typical W-box element (Fig. 1A). In *CAD1-A* promoter, two reversely oriented W-box cis-acting elements (AGTCAAAATTGACC) were located in the region between −340 and −323 bp, forming a palindrome

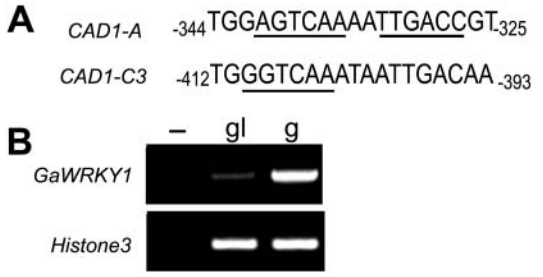

Figure 1. The W-box in the promoter region of *CAD1-A* or *CAD1-C3* (A) and RT-PCR detection of *GaWRKY1* transcripts in developing seeds of 25 DPA (B). The W-box is underlined. The seeds were collected from *G. hirsutum* L. cv Zhong-12, a glanded cultivar (g), and *G. hirsutum* cv GL-5, a glandless cultivar (gl). PCR was performed by 31 cycles of amplification. *Histone3* transcripts were amplified as an internal control.

(Fig. 1A). Deletion from 5′ end of the approximately 1.2-kb promoter of *CAD1-A* showed that the region containing the W-box was required for elicitor-induced transcription (our unpublished data). Since, as mentioned above, W-box elements are frequently found to be the binding sites of WRKY transcription factors, it is reasonable to assume that WRKY proteins are involved in transcriptional regulation of *CAD1* genes of cotton.

We then isolated *WRKY* cDNA fragments from *G. arboreum*. Expression of 10 *WRKY* genes in developing seeds collected at 25 d postanthesis (DPA) was examined by RT-PCR. One of them, *GaWRKY1*, showed a higher level of transcripts in seeds of *G. hirsutum* L. cv Zhong-12, a glanded cotton cultivar, than in *G. hirsutum* L. cv GL-5, a glandless cultivar (Fig. 1B). Expression of other nine *WRKY*s was either indistinguishable between the two cultivars or too low to be detected (data not shown). Previous investigations showed that aerial tissues of glandless cultivars were nearly gossypol free, and transcripts of *CAD1-A* and *CAD1-C* genes were undetectable (Liang et al., 2000; Tan et al., 2000). Therefore, GaWRKY1 was considered a candidate for transcription factors regulating the gossypol pathway.

The cDNA of *GaWRKY1* was isolated and found to encode a 313-amino acid protein with a predicted molecular mass of 34 kD. The deduced protein has a single WRKY domain with a zinc finger-like motif (C_2H_2) at its C terminus, categorizing it into Group II of the WRKY superfamily (Eulgem et al., 2000). In addition, GaWRKY1 has a putative Leu zipper in its N-terminal end (Fig. 2). GaWRKY1 exhibited high sequence identities (41%–45%) to Group IIa WRKY proteins, including PcWRKY4 of parsley (Cormack et al., 2002), AtWRKY18 of Arabidopsis (Chen and Chen, 2002), and Wizz of tobacco (Hara et al., 2000). Alignment of these protein sequences revealed that the WRKY domain, putative Leu zipper, nuclear localization site, and a C-terminal Ala-rich stretch were strictly conserved; outside these regions, however, there was little sequence conservation (Fig. 2).

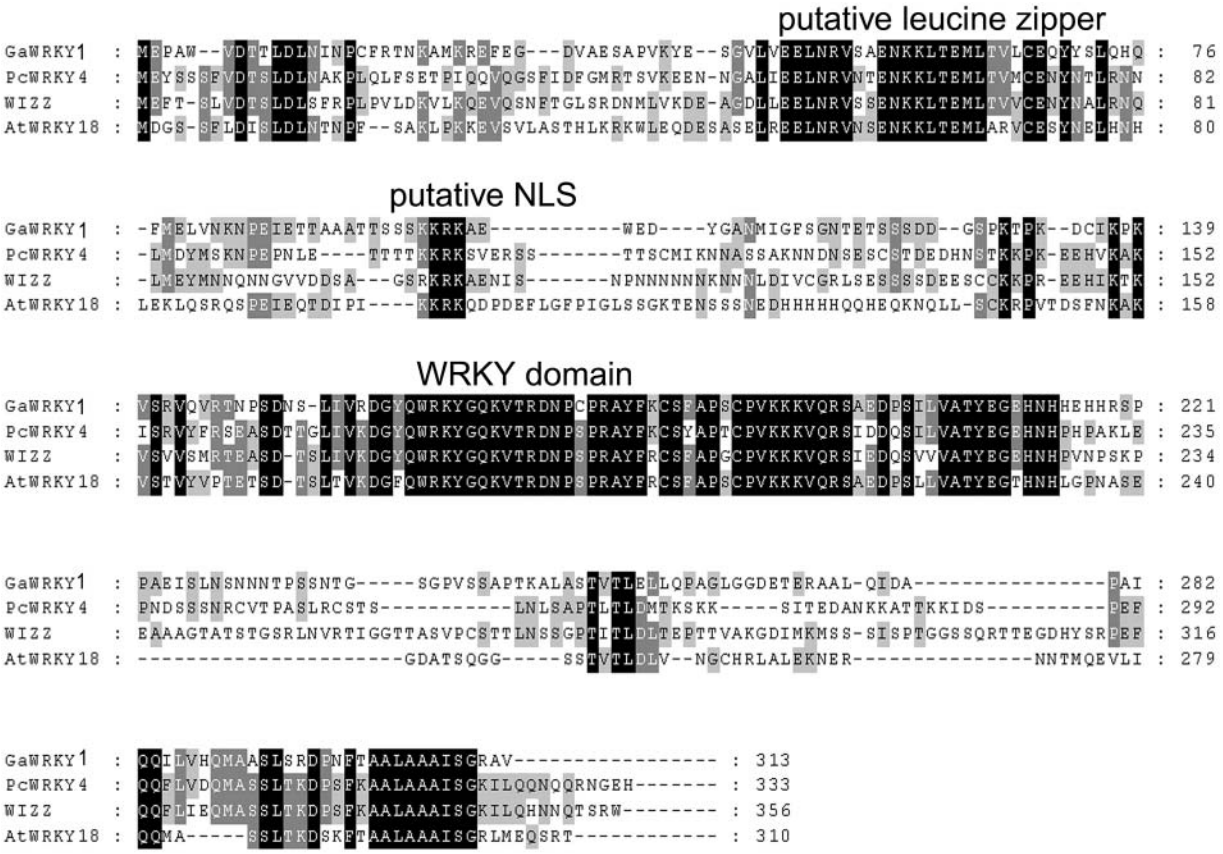

Figure 2. Alignment of GaWRKY1 with related WRKY proteins from other plant species. The deduced amino acid sequence of GaWRKY1 was aligned with PcWRKY4 (AAG35658), WIZZ (BAA87058), and AtWRKY18 (NP_567882) using ClustalW (Thompson et al., 1994) with default parameters through EMBnet (http://www.ch.embnet.org/software/ClustalW.html). Black and gray shadings, done with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html), indicate conserved amino acid residues. Predicted domains are indicated above the sequences.

Expression Patterns of *GaWRKY1* and *CAD1-A*

To see if *GaWRKY1* expression coincides with accumulation of sesquiterpene aldehydes in other tissues, transcription levels of *GaWRKY1* in the glanded and glandless cotton cultivars were further compared. RNA blot detected a significant level of *GaWRKY1* mRNA in cotyledon, stigma, and sepal of the glanded cultivar, and the level was low or undetectable in the glandless cultivar (Fig. 3A). The transcripts were also detected in root, peel, petal, stigma, and anther of the glanded cotton cultivar by RNA gel blot (data not show). In sepal, the *GaWRKY1* transcript level was higher at 0 DPA than at −3 DPA. Analysis of more samples by RT-PCR confirmed that the transcript abundance of *GaWRKY1* was increasing with the maturation of sepal (Fig. 3B).

Should GaWRKY1 target to *CAD1* genes, we would expect them to have similar or at least overlapping expression domains. In floral organs examined, the spatial and temporal pattern of *GaWRKY1* expression was highly similar to that of *CAD1-A*. In sepal and anther, both *GaWRK1* and *CAD1-A* genes had a low level of transcripts at −3 DPA, and both were up-regulated at the day of anthesis (Fig. 4A). In stigma, however, although *GaWRKY1* and *CAD1-A* showed the same temporal expression pattern, the dynamics of transcript accumulation were different: their mRNA levels were high at −3 DPA and decreased to an undetectable level at 0 DPA (Fig. 4A). Thus, toward flower maturation, the transcript abundance of *GaWRKY1* and *CAD1-A* was increasing in the sepal and anther but decreasing in stigma. In developing seeds, the *GaWRKY1* transcript level peaked around 25 DPA, then decreased; for *CAD1-A* the mRNA level also increased from 20 to 25 DPA, and the level continued to be high at 30 DPA (Fig. 4A).

As reported previously, *CAD1-C3* and probably other *CAD1-C* genes were highly expressed in the sepal of −3 DPA, and the transcript abundance decreased at 0 DPA (Tan et al., 2000). Further analysis by RT-PCR indicated that not only in sepal but also in anther, *CAD1-C3* showed opposite dynamics of mRNA accumulation to that of *GaWRKY1* and *CAD1-A*, i.e. during flower development, the transcript level of *CAD1-C3* in the sepal and anther was decreasing rather than increasing (Fig. 4B). Therefore, temporal

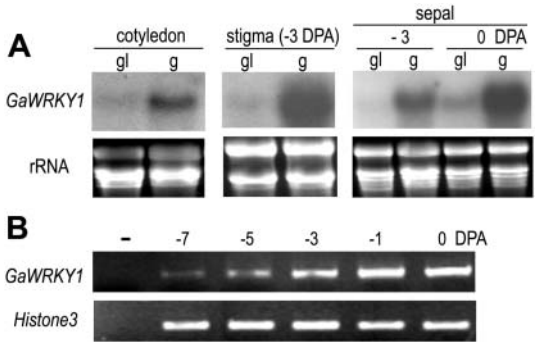

Figure 3. Expression of *GaWRKY1* in *G. hirsutum* L. cv Zhong-12 (g) and in *G. hirsutum* L. cv GL-5 (gl). A, Northern analysis of *GaWRKY1* transcripts in the cotyledon of 1-week-old seedlings and in stigma and sepal. B, RT-PCR analysis of *GaWRKY1* transcripts in developing sepals of *G. hirsutum* L. cv Zhong-12. For RNA gel blot, total RNA of 40 μg was loaded on each lane. PCR was performed by 31 cycles of amplification.

expression pattern of *CAD1-C3* was clearly different from that of *GaWRKY1* in these floral organs.

To gain further insight into the relation of expression patterns between *CAD1-A* and *GaWRKY1*, promoters of both genes were fused to the β-glucuronidase (*GUS*) reporter gene and were transferred into tobacco plants, respectively. Histochemical staining revealed that both promoters directed *GUS* expression in stigma, sepal, and anther of the transgenic plants. And for the sepal, the GUS staining was stronger at 0 DPA than at -3 DPA (Fig. 4C), which was consistent with the northern results obtained from the cotton sepal (Fig. 4A). For both promoters, the expression in leaves was weak.

Induction of *GaWRKY1* and *CAD1-A* by Elicitation

Many *WRKY* genes that are involved in regulating defense-related genes are themselves elicitor responsive (Rushton et al., 1996; Yang et al., 1999; Yu et al., 2001; Yoda et al., 2002; Dong et al., 2003). Because *CAD1-A* transcription could be activated by fungal elicitation (Chen et al., 1996; Tan et al., 2000), we would expect an inducible expression of *GaWRKY1*, should it be a regulator of *CAD1-A*. We treated the *G. arboreum* suspension cells with an elicitor preparation of *Verticillium dahliae* (Vde) and several signaling molecules, including salicylic acid (SA), methyl jasmonate (MJ), and H_2O_2. Examination of transcript abundance by RT-PCR revealed that Vde and MJ significantly induced the transcription of *GaWRKY1* and *CAD1-A*, whereas SA and H_2O_2 exerted little effect (Fig. 5A). Coordinately, production of gossypol and related sesquiterpene aldehydes in *G. arboreum* cells also increased upon elicitation by Vde or MJ but not by SA neither H_2O_2 (Fig. 5B). Induced expression of chitinase genes in cotton plants treated with SA and the Vde elicitor has been reported (Hudspeth et al., 1996). When the chitinase gene *Chi2;1* was included in RT-PCR analysis, it was induced not only by Vde but also by the three signaling molecules (Fig. 5A), indicating that the *G. arboreum* cells responded to all these elicitors.

Furthermore, in Vde-treated cells the transcript level of *GaWRKY1* increased rapidly and peaked within 30 min, then declined to the original level. On the other hand, *CAD1-A* showed a comparatively slower rate of induction, and the transcript level was not significantly elevated until about 2 h postelicitation (Fig. 5C). Similar kinetics of transcript accumulation were also observed in MJ-treated cells (data not shown). Thus, the transcript level of *GaWRKY1* increased rapidly and transiently upon elicitation, preceding the *CAD1-A* induction. The concomitant responses of *GaWRKY1* and *CAD1-A* genes to different elicitor molecules and the subsequent gossypol accumulation support the assumption that GaWRKY1 participates in regulating the gossypol pathway.

Nuclear Localization and DNA-Binding Activity of GaWRKY1

To determine subcellular localization of GaWRKY1, the open reading frame (ORF) of *GaWRKY1* was in-frame fused to the green fluorescent protein (*GFP*) reporter gene. After introducing the construct into tobacco BY2 cells, GFP fluorescence was located exclusively in the nucleus (Fig. 6A). By contrast, BY2 cells transformed with *35S::GFP* showed GFP signal throughout the cell. This indicates that GaWRKY1 is a nuclear-localized protein.

To determine if GaWRKY1 binds to the *CAD1-A* promoter, yeast one-hybrid assay was performed. A

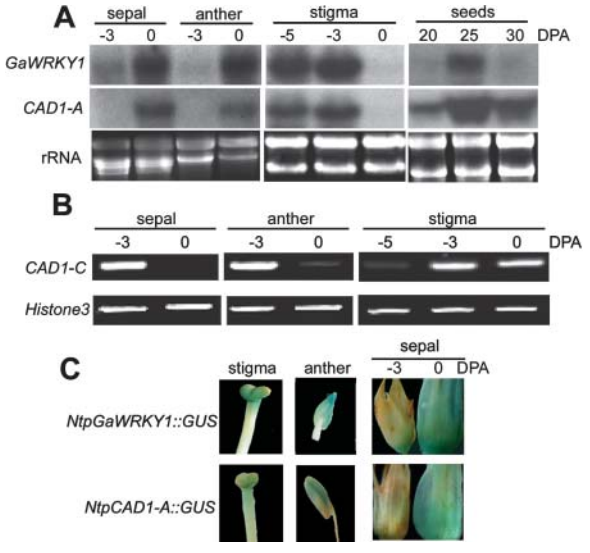

Figure 4. Comparison of expression patterns of *GaWRKY1* and *CAD1-A*. A, RNA gel blot analysis of stigma, sepal, anther, and seeds of *G. hirsutum* L. cv Zhong-12. B, RT-PCR analysis of *CAD1-C* (*CAD1-C3*) transcripts in developing stigma, sepal, and anther of *G. hirsutum* L. cv Zhong-12. PCR was performed by 23 cycles of amplification. C, Histochemical staining of GUS activities in stigma (-3 DPA), anther (0 DPA), and sepal of transgenic tobacco plants of *NtpGaWRKY1::GUS* and *NtpCAD1-A::GUS*. The tissues were stained for 24 h using X-Gluc as substrate.

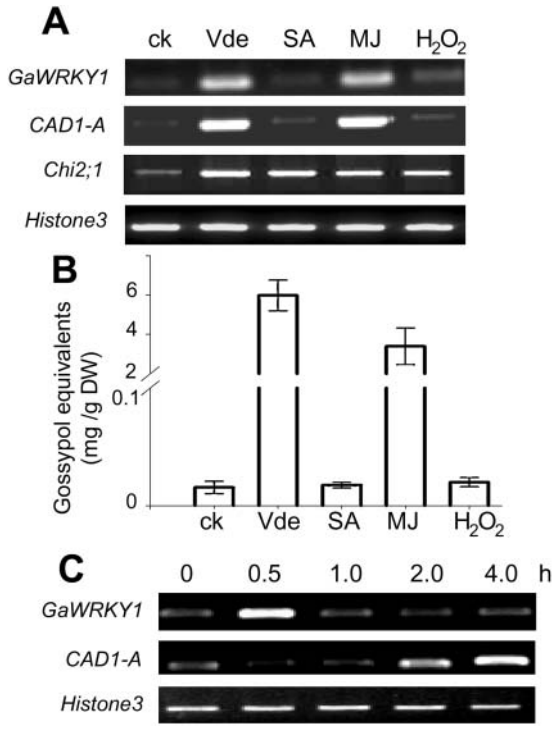

Figure 5. Induced gene expression of *GaWRKY1* and *CAD1-A* and sesquiterpene aldehyde accumulation in *G. arboreum* suspension cultured cells. A, RT-PCR analysis of gene expression in *G. arboreum* cells treated with Vde, SA (5 mM), MJ (45 μM), and H_2O_2 (100 μM) for 30 min (*GaWRKY1*) or 4 h (*CAD1-A* and *Chi2;1*). B, Accumulation of sesquiterpene aldehydes (gossypol equivalents) in *G. arboreum* cells collected at 24 h postelicitation. C, Transcript levels of *GaWRKY1* and *CAD1-A* in Vde-treated suspension cells for the time period postelicitation as indicated. PCR was performed by 30 cycles of amplification for *GaWRKY1* and 29 cycles for *CAD1-A*.

DNA fragment of 3× W-box (triple tandem repeats of the W-box palindrome of *CAD1-A*) was introduced into yeast cells, forming HWLW. The plasmid of pGAD-GaWRKY1, encoding the fusion protein of GAL4 binding and GaWRKY1, and the plasmid of pGAD424 were introduced into HWLW cells, respectively. We found that only the yeast clones harboring pGAD-GAWRKY1 grew on the −His−Ura−Leu synthetic dextrose (SD) base containing 5 mM 3-amino-1,2,4-triazole (Fig. 6B), indicating that GaWRKY1 bound to the 3× W-box and activated transcription in yeast. Furthermore, deletion of the GaWRKY1 showed that the zinc finger-like motif (C_2H_2) at the C terminus was the binding domain, and the putative Leu zipper at the N terminus significantly increased the binding affinity (data not shown).

In EMSA, the recombinant protein of glutathione *S*-transferase (GST)-GaWRKY1 showed binding affinities to the 3× W-box. The DNA-binding specificity was confirmed in a competition experiment with 50-fold excess of the unlabeled probe of 3× W-box (Fig. 6C). Thus, in vitro GaWRKY1 was able to specifically recognize and interact with the W-box palindrome of *CAD1-A*.

Activation of *CAD1-A* Promoter in Plants by GaWRKY1

To provide evidence that GaWRKY1 protein regulates *CAD1-A* gene transcription, we used transgenic plants of Arabidopsis to assay their interactions. An approximately 1.2-kb promoter of *CAD1-A* fused with either the *GUS* reporter gene (*pCAD1-A::GUS*) or the *35S::GaWRKY1* recombinant gene was transferred into Arabidopsis plants, followed by crossing the plants' T2 lines. The *GaWRKY1* transcripts were clearly detectable in a T2 line of *35S::GaWRKY1* (36-2; Fig. 7A). Plants transformed with *35S::GaWRKY1* did not show clear phenotypic changes. In the T2 plants of *pCAD1-A::GUS* (5-7), the GUS activity in leaves was low or too weak to be stained. However, the GUS activities became significantly higher in F_1 plants (*35S::GaWRKY1* × *pCAD1-A::GUS*) that contained a *35S::GaWRKY1* gene (Fig. 7, A and B). Analysis by

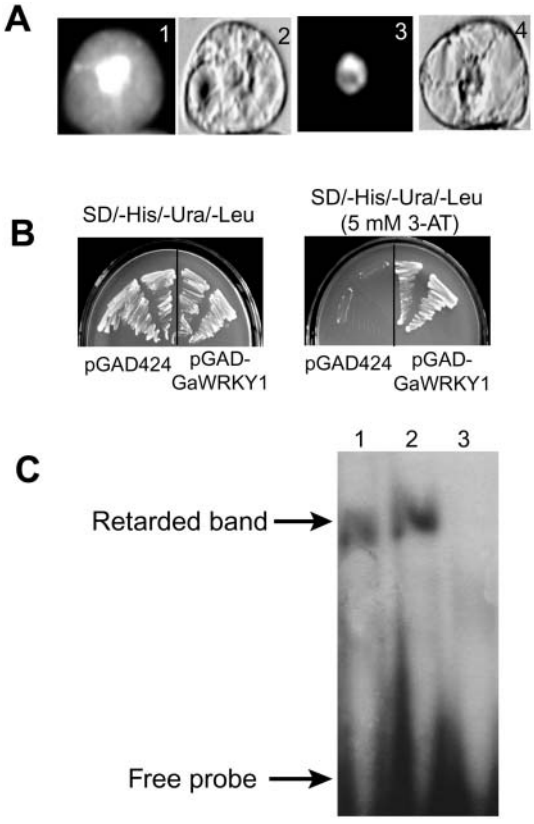

Figure 6. Nuclear localization and DNA-binding activity of GaWRKY1. A, Subcellular localization of GFP (1 and 2) and GFP-GaWRKY1 fusion protein (3 and 4) in BY2 cells. B, Yeast one-hybrid assay using the 3× W-box as bait. Yeast cells carrying pGAD424 or pGAD-GaWRKY1 were grown for 3 d at 30°C in SD/−His/−Ura/−Leu or SD/−His/−Ura/−Leu plus 5 mM 3-amino-1,2,4-triazole (3-AT), respectively. C, EMSA performed by incubation of the purified GST-GaWRKY1 protein with ^{32}P-labeled DNA fragment of 3× W-box. The three lanes stand for ^{32}P-labeled 3× W-box (1), DNA fragment of the pBSK vector without the binding site as a nonspecific competitor (2), and 50-fold excess of the unlabeled 3× W-box as a competitor (3). The 3× W-box contained triple tandem copies of the W-box palindrome of *CAD1-A* promoter.

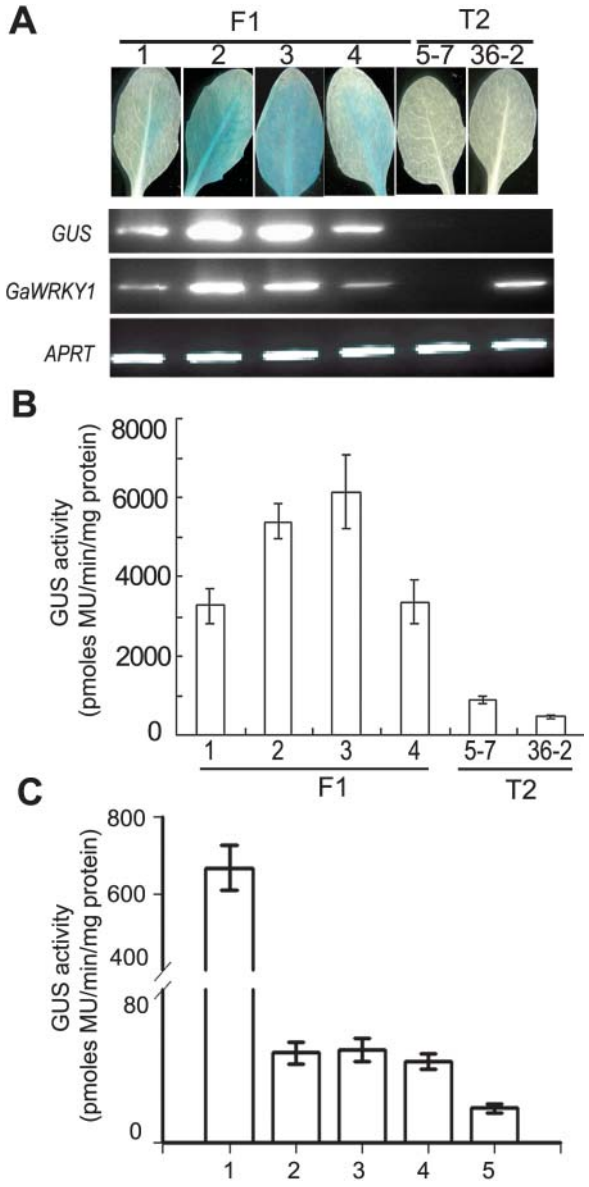

Figure 7. Activation of *CAD1-A* promoter by constitutive or transient expression of *GaWRKY1* in plants. A, GUS staining and RT-RCR analysis of the *GUS* and *GaWRKY1* transcripts in mature leaves of T2 plants of 5-7 (*pCAD1-A::GUS*) and 36-2 (*35S::GaWRKY1*) lines and their F_1 plants containing both constructs. B, Quantitative determination of GUS activities in mature leaves of the plants as indicated. PCR was performed by 28 cycles of amplification, and Arabidopsis Ade phosphoribosyl transferase (*APRT*) transcripts were amplified as an internal control. C, Transient assay of GUS activities in tobacco leaves 2 d after infiltration with Agrobacterium cells harboring *pCAD1-A::GUS/35S::GaWRKY1* (1), *pCAD1-A::GUS* (2), *mpCAD1-A::GUS/35S::GaWRKY1* (3), *mpCAD1-A::GUS* (4), and *35S::GaWRKY1* (5), respectively.

RT-PCR proved that in plants transformed with *pCAD1-A::GUS* only, the *GUS* transcripts were undetectable, whereas in F_1 plants, significant levels of both *GaWRKY1* and *GUS* transcripts were present. Moreover, there was a good correlation between the transcript levels of *GaWRKY1* and *GUS* in leaves of F_1 plants (Fig. 7A). Clearly, in transgenic Arabidopsis plants, constitutive expression of *GaWRKY1* strongly activated the *CAD1-A* promoter.

To investigate whether the W-box in the *CAD1-A* promoter was indispensable for the recognition by GaWRKY1, we generated a mutant form of *pCAD1-A* (*mpCAD1-A*), in which the W-box palindrome of AGTCAAAATTGACC was mutated to ATTCAAA-ATTGAAC. We then tested the interactions of GaWRKY1 with the wild-type and mutant promoters by Agrobacterium-mediated transient expression of a *GUS* reporter gene in tobacco leaves. The GUS activity was low in leaves expressing either *pCAD1-A::GUS* or *mpCAD1-A::GUS* alone. Coexpression of *pCAD1-A::GUS* with *35S::GaWRKY1* increased the GUS activity by approximately 20-fold. By contrast, *35S::GaWRKY1* did not activate *mpCAD1-A* (Fig. 7C). These results indicate that activation of the *CAD1-A* promoter by GaWRKY1 requires the W-box palindrome.

DISCUSSION

Terpenoids, the largest group of plant secondary metabolites, play a diverse role in plant-microbe, plant-herbivore, plant-plant, and plant-environment interactions (Chappell, 1995; Mahmoud and Croteau, 2002; Pichersky and Gershenzon, 2002; Aharoni et al., 2003). In cotton, gossypol and other sesquiterpene phytoalexins not only have fungistatic properties and insecticidal activities but also are toxic to mammals (Bell and Stipanovic, 1977; Davila-Huerta et al., 1995; Puckhaber et al., 2002). In previous studies, we have cloned three enzymes that catalyze consecutive steps from isopentenyl diphosphate and dimethylallyl diphosphate to 8-hydroxy-(+)-δ-cadinene of the gossypol pathway: farnesyl diphosphate synthase (Liu et al., 1999), CAD1 (Chen et al., 1995, 1996; Tan et al., 2000), and (+)-δ-cadinene-8-hydroxylase (CYP706B1; Luo et al., 2001). Genes encoding enzymes of the gossypol pathway characterized so far are all down-regulated in the glandless cultivar of *G. hirsutum*, as is the WRKY protein gene *GaWRKY1* reported herein. As shown in Figure 1A, both *CAD1-A* and *CAD1-C3* promoters contain W-box elements. In fact, W-boxes are also present in the promoter of *CYP706B1* gene (our unpublished data). These data strongly suggest the involvement of WRKY proteins in regulation of cotton sesquiterpene biosynthesis.

In cotton suspension cultured cells, both *GaWRKY1* and *CAD1-A* expression and sesquiterpene aldehyde biosynthesis responded to the Vde elicitor and MJ but not to SA or H_2O_2. The coordinated responses suggest that GaWRKY1 and CAD1-A are components of the same pathway of cotton defense reaction, which involves induced biosynthesis of sesquiterpene phytoalexins. Searching of databases revealed the presence of W-box elements in the promoter region of

sesquiterpene cyclase genes of other plants, such as 5-epi-aristolochene synthase gene *EAS4* of tobacco (Yin et al., 1997), putative sesquiterpene cyclase genes of Arabidopsis (At1g31950), and rice (*Oryza sativa*; BAC99543.1). Therefore, WRKY-W-box interactions are likely operating also in other plants in regulation of sesquiterpene biosynthesis.

Among the WRKY proteins reported so far, only a subset of members, such as ABF2, WIZZ, PcWRKY4, and PcWRKY5, have putative Leu zippers (Rushton et al., 1995; Eulgem et al., 2000; Hara et al., 2000; Cormack et al., 2002). Function of the Leu zipper has been proposed to mediate dimerization and increase the DNA-binding affinity of WRKY proteins (Cormack et al., 2002). Our results from yeast-one hybrid assay support this assumption, since removing the N-terminal Leu zipper from GaWRKY1 decreased its binding affinity to the W-box of *CAD1-A* promoter.

In elicitor-treated suspension cells of *G. arboreum*, different kinetics of induction of transcript accumulation were observed for genes encoding the transcription factor GaWRKY1, enzymes of the gossypol pathway, and a PR-10 protein. The response of *GaWRKY1* to elicitation is quick and instant (Fig. 5C), whereas the induction rate of the sesquiterpene cyclase genes *CAD1-A* and *CAD1-C3* and the cytochrome P450 gene *CYP706B1* was moderate, and mRNA levels of these enzymes peaked in several hours postelicitation (Chen et al., 1995, 1996; Luo et al., 2001). For *GaPR-10*, which encodes a pathogenesis-related protein with in vitro ribonuclease activities, induction of gene expression was slow, and the peak level of mRNA did not appear until 12 h postelicitation (Zhou et al., 2002). Many defense-related *WRKY* genes, such as *PcWRKY1*, *PcWRKY4*, and *AtWRKY*18, also show fast and transient induction that precedes the induced expression of downstream defense genes (Rushton et al., 1996; Chen and Chen, 2002; Cormack et al., 2002). However, function of GaWRKY1 is unlikely limited to concerting defense responses, since it shows developmentally mediated expression in floral tissues. Accumulation of both *GaWRKY1* and *CAD1-A* transcripts was increasing in the sepal and anther along with maturation, and the highest level appeared at the day of anthesis (Fig. 4, A and C). Certain senescence-associated WRKY genes, such as *AtWRKY6*, are expressed in floral tissues (Quirino et al., 1999), and maturation of the floral organ is also considered a senescence process (Bleecker and Patterson, 1997). Although the temporal expression pattern of *GaWRKY1* in sepal and anther correlates with maturation of both organs, evidence that GaWRKY1 is involved in floral senescence is still lacking. In fact, expression of *GaWRKY1* (and of *CAD1-A* as well) in mature stigma (0 DPA) is down- rather than up-regulated. The biological significance of differential regulation of *GaWRKY1* and *CAD1-A* genes in different floral organs needs further investigation.

Although *GaWRKY1* and *CAD1-A* genes showed a complex expression pattern in floral organs, their coordinated expression in various aerial organs and in suspension cultured cells indicates that regulation of *CAD1-A* by GaWRKY1 is spatially and temporally possible. In combination with results from DNA-binding assay and transgenic plant analyses, our data strongly suggest that *CAD1-A* is a target gene of GaWRKY1. The discrepancy of temporal expression patterns between *CAD1-C* (such as *CAD1-C3*) and *GaWRKY1* in floral organs suggests that other WRKY proteins or bZIP-type proteins may be involved in regulation of *CAD1-C* genes. However, this does not exclude the possibility that GaWRKY1 may also regulate *CAD1-C* genes in other organs. In developing seeds, for example, genes of *CAD1-A* and *CAD1-C* showed similar temporal patterns of expression (Meng et al., 1999). Alternatively, GaWRKY1 may function as a repressor of *CAD1-C* genes in flower. Besides W-box palindrome, other cis-acting elements, such as those of MYB and MYC, are also present in the *CAD1-A* promoter. It is clear that expression of cotton *CAD1* genes and biosynthesis of sesquiterpene aldehydes are mediated by both developmental and defense-related programs. Isolation of more transcription factors regulating the gossypol pathway and further elucidation of regulatory machinery of *CAD1* genes will be a great help in manipulating cotton secondary metabolism and disease resistance.

MATERIALS AND METHODS

Materials

Plants of cotton (*Gossypium arboreum* L. cv Qingyangxiaozhi, *G. hirsutum* L. cv Zhong-12, and a glandless cultivar *G. hirsutum* cv GL-5) and tobacco (*Nicotiana tabacum*) were grown in a greenhouse. Fresh materials were collected as described previously (Tan et al., 2000; Luo et al., 2001). Plants of Arabidopsis (ecotype Columbia) were grown in a growth chamber at 22°C with a photoperiod of 16 h of light and 8 h of dark. *G. arboreum* suspension cells were cultured in liquid Murashige and Skoog medium. Elicitation was performed by treating the cells with SA (5 mM), MJ (45 μM), H_2O_2 (100 μM), or a crude preparation of fungal elicitor (Vde), which was obtained by precipitation of the culture fluid of *Verticillium dahliae* and applied at a final concentration of 1 μg of Suc equivalent per milliliter of culture, as described previously (Liu et al., 1999). Sesquiterpene aldehydes were extracted and quantitated by the phloroglucinol/HCl method (Meng et al., 1999).

Isolation of *GaWRKY1* cDNA

A degenerate primer [5′-TGG(A/C) IAA(A/G) TA(C/T) GGICA(A/G) AA(A/G)-3′], corresponding to the conserved peptide sequence of WRKYGQK (Eulgem et al., 2000), together with a vector-specific T7 primer, was used in amplification of *WRKY* fragments from a λ-Unizap cDNA library, which was constructed from elicitor-treated *G. arboreum* cells (Chen et al., 1995). The PCR products were cloned into pGEM-T vector (Promega, Madison, WI) and sequenced. The cDNA of *GaWRKY1* was isolated by a PCR-mediated 96-well method, with the primers GaWRKY1P0 (5′-GTCACAAGAGATAACCCCTG-3′) and GaWRKY1M0 (5′-TGATACTC-TTCTCCCATTGAACCTAC-3′).

RNA Isolation and Analysis

Total RNA preparation, RT-PCR, and RNA gel blot were performed as described previously (Tan et al., 2000; Luo et al., 2001), except that in RT-PCR 1 μL of reverse transcription products was used as template without dilution. The primers used were GaWRKY1P1 (5′-ATCTTGGATCTCAACATCA-ACCC-3′) and GaWRKY1M1 (5′-CTATTATCAGAAGGATTGTGCGG-3′) for

GaWRKY1, Histone3P (5′-GAAGCCTCATCGATACCGTC-3′) and Histone3M (5′-CTACCACTACCATCATGG-3′) for cotton *Histone3* (AF024716), and Chi2P (5′-ACTCCACAGTCACCGAAACC-3′) and Chi2M (5′-ATCTTATTCCATCTCCACGG-3′) for the cotton chitinase gene *Chi2;1* (Z68152). For *CAD1-A*, *CAD1-C3* and Arabidopsis Ade phosphoribosyl transferase (*APRT*; NM_179383), primers were the same as reported (Payne et al., 2000; Tan et al., 2000). DNA probes of *GaWRKY1* and *CAD1-A* were obtained by ^{32}P-labeling of the PCR products amplified from the corresponding cDNA clones, using the same primers as for RT-PCR.

Subcellular Localization of GFP-GaWRKY

The *GaWRKY1* ORF was amplified by GFPWP (5′-CGGGATCCACGTTCAGTAATG GAACCAG-3′) and GFPWM (5′-GCTCTAGACTAAATTACTTCGGCACAGAGT-3′), which contained a *Bam*HI and *Xba*I site (underlined), respectively. The PCR products were digested and inserted into pGFP-JW vector, harboring an ORF encoding the GFP driven by a *35S* promoter (our unpublished data), forming a chimerical gene encoding GFP-GaWRKY1. The plasmids of GFP-GaWRKY1 and pGFP-JW were then introduced into tobacco BY2 cells, respectively, via Agrobacterium-mediated transformation (Gu and Verma, 1997).

Binding Assay in Yeast One-Hybrid System

The binding assay utilized a yeast one-hybrid system (CLONTECH, Palo Alto, CA). The bait was the triple tandem copies of the *CAD1-A* W-box palindrome (3× W-box, 5′-TGGAGTCAAAATTGACCGTTGGAGTCAAAATTGACCGTTGGAGTCAAAATTGACCGT-3′). This fragment was inserted into pHISi and pLacZi vectors, respectively, and the two resultant plasmids were introduced into yeast cells, forming HWLW. The ORF of the *GaWRKY1* was in-frame fused with the GAL4 activation domain of the one-hybrid vector pGAD424, forming pGAD-GaWRKY1. HWLW cells were then transformed with the pGAD424 and pGAD-GaWRKY1, respectively.

Electrophoretic Mobility Shift Assay

The ORF of *GaWRKY1* was inserted into the expression vector pGEX-4T-1 (Amersham Pharmacia Biotech, Uppsala). GST and GST-GaWRKY1 proteins were then purified according to the instruction manual. Isolation of nuclear proteins from Vde-treated suspension cells of *G. arboreum* and EMSA were performed according to the published protocols (Rushton et al., 1995; Yang et al., 1999).

Plant Transformation and GUS Activity Assay

The promoters of *CAD1-A* (*pCAD1-A*) and *GaWRKY1* (*pGaWRKY1*) were inserted into the pBI121 vector (CLONTECH), respectively, replacing the 35*S* promoter. These two promoter-GUS plasmids were transferred into tobacco plants by a leaf-disc method, as described previously (Liang et al., 2000), and the resulting transgenic plants were named *NtpCAD1-A::GUS* and *NtpGaWRKY1::GUS*, respectively. Arabidopsis plants were transformed with the constructs of *35S::GaWRKY1* and *pCAD1-A::GUS* by a floral-dip method (Clough and Bent, 1998), and the transgenic plants were named *At35S::GaWRKY1* and *AtpCAD1-A::GUS*, respectively.

The W-box (AGTCAAAATTGACC) in *pCAD1-A* was mutated into ATTCAAATTGAAC by mega-primer method (Barik, 1993) with the primer mWboxP (5′-GGAAGTTGGATTCAAAATTGAA-3′); the resulting promoter was named *mpCAD1-A*. *pCAD1-A* and *mpCAD1-A* were inserted into the modified pBI121 vector harboring an intron-containing *GUS*, replacing the 35*S* promoter, respectively. For *35S::GaWRKY1*, the ORF of *GaWRKY1* was inserted into pBI121, replacing *GUS*. The two promoter-GUS plasmids and the *35S::GaWRKY1* were introduced into Agrobacterium strain GV3101, respectively. Transient assay using tobacco leaves was performed as described (Yang et al., 2000). The ratio of *35S::GaWRKY1* to *pCAD1-A::GUS* or to *mpCAD1-A*::*GUS* was adjusted 3:5 for coinfiltration.

A fluorimetric assay with 4-umbelliferyl-D-glucuronide as substrate was used to determine GUS activities, and the expression pattern was visualized with a histochemical staining using X-Gluc as substrate (Jefferson, 1987). Quantitative analysis was performed at least three times throughout this investigation.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY507929.

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