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Characterization of a Novel GT-box Binding Protein from Arabidopsis.

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Abstract

We isolated a cDNA encoding a novel GT-box binding protein from *Arabidopsis*, designated GT-4. The predicted open reading frame encodes a protein of 372 amino acids and the predicted protein sequence revealed the presence of a putative DNA-binding domain with 80% homology to the trihelix region of previously described light-responsive element binding protein, GT-1. Reverse transcription-PCR analysis showed that GT-4 transcripts are present in all tissues tested in light-grown plants, but light-regulated expression of GT-4 mRNA was observed only in etiolated seedlings. Electro-mobility shift assay using recombinant protein revealed that the GT-box-binding specificity of GT-4 is almost identical to that of GT-1. Transient expression of GT-4::GFP fusion protein in onion epidermal cells revealed the presence of a nuclear localization signal within the GT-4 protein. These results suggest the possibility that GT-4 is involved in GT-box-mediated gene expression by recognizing target sequences closely related to GT-1 binding sites.

Keywords: *Arabidopsis*, GT-box binding proteins, transcription factor

Accession number: The nucleotide sequence reported in this paper has been submitted to DDBJ under accession number AB072370 (GT-4 cDNA).

Abbreviations

CAB, chlorophyll a/b binding protein; RBCS, ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit

Introduction

Plants have evolved a complex signal transduction system and may respond to various environmental signals, such as plant hormones, pathogen and light. Recent studies on light signal transduction and regulated gene expression revealed that plants have a set of photoreceptors including phytochrome and cryptochrome. On the other hand, a large number of *cis*-regulatory elements and *trans*-acting factors that are crucial for light-responsive gene expression have been identified and characterized (Chamovitz and Deng, 1996; Chory *et al.*, 1996; Mustilli and Bowler, 1997). One of the GT-box elements, GT1-box, was first identified in pea ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (*RBCS*) promoters, and was defined as a light-responsive cis-regulatory element (LRE) (Bedbrook *et al.*, 1980; Kuhlemeier *et al.*, 1987; Kuhlemeier *et al.*, 1988; Kuhlemeier *et al.*, 1989; Gilmartin *et al.*, 1990; Lam and Chua, 1990).

GT-box binding proteins have a trihelix motif(s) as DNA-binding domain(s) that does not show significant homology to previously described DNA-binding domains found in other transcription factors in animals and yeast. There are two major subfamilies of GT-box binding proteins, GT-1 type and GT-2 type; the former has one trihelix, while the latter has twin DNA-binding domains (Nagano, 2000). GT-1 cDNA was cloned from tobacco by southwestern screening using a GT1-box sequence as a probe (Perisic and Lam, 1992; Gilmartin *et al.*, 1992). Based on the sequence homology, the GT-1 orthologue was cloned from *Arabidopsis* (Hiratsuka *et al.*, 1994). Molecular studies have revealed that GT-1 can multimerize via its C-terminal region,

and localize to the nucleus when fused to *β-glucuronidase* (GUS) (Hiratsuka *et al.*, 1994; Lam, 1995). The transcriptional activity of GT-1 has been confirmed by *in vivo* studies and modulation of DNA-binding activity by phosphorylation has been suggested by *in vitro* assays (Le Gourrierec *et al.*, 1999; Marechal *et al.*, 1999). Since the GT1-box element is known to act as LRE, GT-1 may play an important role as a terminal component of molecular light switch. In addition, GT-1 type of DNA binding protein has been shown to be involved in regulated expression of a pathogenesis-related protein gene of tobacco (Buchel *et al.*, 1996; Buchel *et al.*, 1999).

GT-2 that constitutes another major subtype of GT-box binding protein, has been cloned from rice and *Arabidopsis*, and characterized extensively. The GT-2 protein binds to GT2-box and GT3-box elements found in the promoter region of photophobic genes such as *phytochrome* A (PHYA) (Dehesh *et al.*, 1990; Kuhn *et al.*, 1993). GT-2 acts as a transcriptional activator (Ni *et al.*, 1996), and its binding activity is stimulated through association with the AT-rich sequence binding protein, HMG-I/Y (Martinez-Garcia and Quail, 1999).

Because the binding specificity of GT-2 is different from that of GT-1 (**Table 1**), it is presumable that GT-1 and GT-2 play distinct roles in light-responsive transcriptional regulation. This hypothesis is supported by the recent studies on the pea dark-inducible cis-regulatory element DE1 that is bound by a GT-2-like DNA binding protein, DF1 (Inaba *et al.*, 2000; Nagano *et al.*, 2001).

Recent advances in *Arabidopsis* genome research revealed the presence of many DNA sequences encoding putative GT-box binding proteins. cDNA sequence of a novel GT-box binding protein, GT-3a, has been reported recently. GT-3a has a single trihelix motif and shows 30-40% homology to the trihelix region of GT-1 and GT-2 types of GT-box binding proteins, suggesting that GT-3a composes a novel subtype of GT-box binding proteins. However, no further information other than its cDNA sequence has been published (Ayadi and Zhou,1999).

In an attempt to elucidate the mechanisms involved in GT-box-mediated transcriptional regulation, we have isolated and characterized a novel GT-box-binding factor, named GT-4, that shares striking homology to GT-1 within its putative DNA-binding domain. Here we describe the results of studies on cloning, expression, DNA-binding and intracellular localization of GT-4.

Table 1 Comparison of DNA-binding profiles of GT-4 with GT-1 and GT-2 protein. GT-1 binds to the GT1-box element most extensively, and binds to GT2-box and GT3-box with reduced affinity. Our data indicate that GT-4 also binds to the GT1-box element most tightly, and showed a DNA-binding property almost identical to that of GT-1. On the contrary, the GT-2 protein preferably bound to GT3-box and GT2-box.

	Profiles of the DNA-binding activity
AtGT-4	GT1-box > GT2-box > GT3-box
AtGT-1	GT1-box ≫ GT2-box > GT3-box
AtGT-2	GT2-box > GT3-box ≫ GT1-box

Materials and Methods

Plant materials

Arabidopsis thaliana ecotype Columbia was grown in a growth chamber at 23℃ and 70% humidity under long-day conditions (16 h of light/8 h of darkness) except as indicated. Young flowers, flower buds, stems, leaves, roots and siliques from *Arabidopsis* were harvested for RNA preparation. Three-day-old dark-adapted seedlings were transferred to continuous white light, and harvested after 3, 6, and 12 h.

Cloning of full-length GT-4 cDNA

Genomic database (Genome Survey Sequence; GSS) of *Arabidopsis* was searched for predicted amino acid sequence of the trihelix region of GT-1. A full-length cDNA clone of GT-4 was obtained by 5'- and 3'-RACE (Rapid Amplification of cDNA Ends) using the SMART RACE cDNA amplification kit according to the manufacturer's protocol (Clontech). Total RNA was isolated from flower buds of three-week-old *Arabidopsis* plant using RNeasy Plant (Qiagen). For the 5'-RACE procedure, first strand cDNA was synthesized with oligo (dT) primer, and GT-4 cDNA was amplified by nested-PCR using the SMART II oligonucleotide, Nested Universal Primer (NUP), and gene specific primers (60976C1.1 5'-GGAATCAACTTTAGCAGATGG-3', 60976C1.2 5'-CGTCTTGTGCCCATGTCTCTGC-3').

Expression of GT-4 recombinant protein

GT-4 cDNA fragment, amplified by PCR using gene-specific primers (GT-4P1 5'-CAGCCATGTTTGTTTCCGATAACAACAATCCTT-3', GT

-4P2 5'-CGGGATCC-TCAAACTCTCTCAGACATGTCC-3') was cloned as a *Nco*I-*Xho*I fragment into the expression vector pET30b (Novagen). GT-1 cDNA has been cloned into expression vector pET15b as described previously (Marechal *et al.*, 1999). These cDNAs were transcribed with T7 promoter *in vitro* using a Riboprobe *in vitro* transcription kit (Promega). RNA was translated with a Flexi rabbit reticulocyte lysate translation kit (Promega) in the presence of ^{35}S-methionine, according to the manufacturer's protocol. Protein production of each reaction was verified by SDS-PAGE followed by autoradiography.

Electro-mobility shift assays

Oligonucleotide probes consisting of tetrameric GT1-box (5'-TGTGT**GGTTAA**TATG-3'), GT2-box (5'-TGGC**GGTAATT**AAC-3'), GT3-box (5'-TCGA**GGTAAA**TCCG-3'), and GT1m-box (5'-TGTGT**CCTTAA**TATG-3') were prepared as described previously; the core sequences are in bold face. (Green *et al.*, 1988; Hiratsuka *et al.*, 1994). Electro-mobility shift reactions were performed in a volume of 16 μl containing 0.2 ng radiolabeled probe, 1 μg poly (dI-dC), 1 g poly (dA-dT), and 3 μl of rabbit reticulocyte lysate expressing recombinant proteins. For competition reactions, 25 ng of unlabeled competitor DNA was added to the reaction mixture.

Subcellular localization of GT-4

A pBI221 based vector, GFP (GA) 5II was digested with *Sal*I, blunted and digested with *Xho*I. Full length GT-4 coding sequence was digested with *Nco*I, blunted and digested with *Xho*I. Onion epidermal cells were bombarded with 1.6-μm gold particles coated by plasmid, carrying GFP::GT-4, or GFP, using PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). Subcellular localization of fusion proteins was observed 6 h after bombardment using a fluorescent microscope, BH-2 (Olympus).

Expression analysis of GT-4

Total RNA was extracted from flower, flower bud, leaf, stem, root and silique of *Arabidopsis* using total RNA extraction kit, RNeasy Plant (Qiagen). Three-day-old etiolated seedlings were treated with white light for 6 h. The seedlings were then dark adapted for 6 h. mRNA accumulation of GT-1, GT-4, CAB1, and EF-1a was analyzed by RT-PCR using gene specific *oligonucleotides* described as follows; GT-1dN68 5'-GGTCTAGAGACCATGGGAGAAAGCAGTGGAGAAGAT-3', JM001 5'-AGACGCCTTTCAAGGTTTAAGG-3', 60976N1.1 5'-CCTCGGAGAAAGCAGTG-3', GT-4P2 5'-CGGGATCCTCAAACTCTCTCAGACATGTCC-3', AtCAB1F 5'-ATTCTCTGGCGAATCACC-3', AtCAB1R 5'-GAGAACATAGCCAATCTTCC-3', F-EF1a 5'-TCGAGACCACCAAGTACTACTGC-3', R-EF1a 5'-AT-

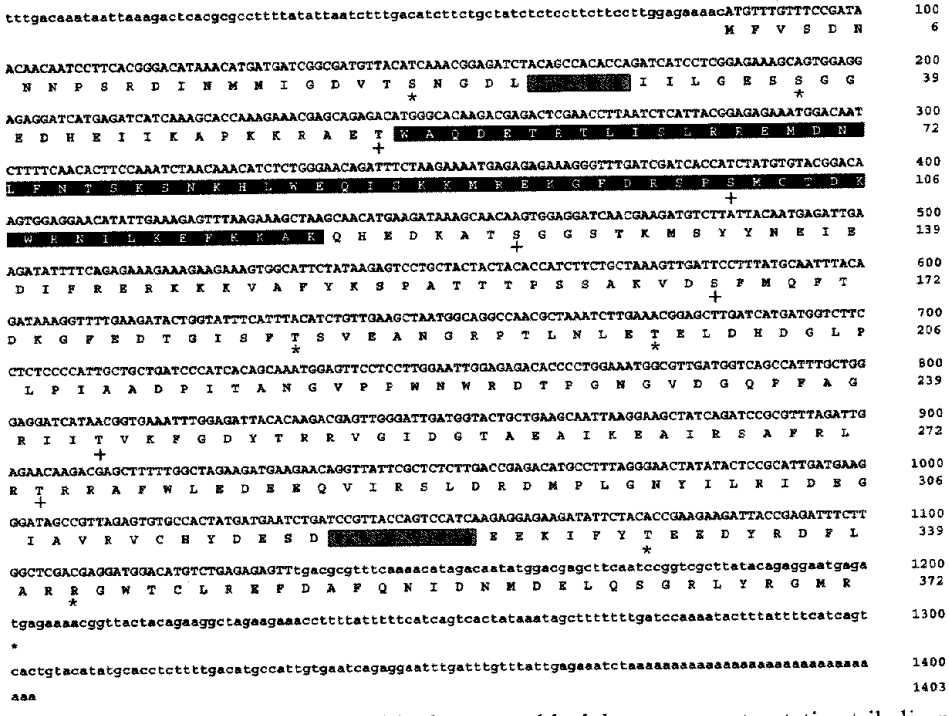

Fig. 1 cDNA sequence of GT-4. White letters on black box represent putative trihelix motifs, which are responsible for DNA binding. P/Q rich regions are boxed in shaded area. Putative phosphorylation sites for CKII (S/T-X-X-E/D) and CaMKII (K/R-X-X-T/S) are indicated by (*) and (+) respectively.

CATACCAGTCTCAACACGTCC-3'. PCR reactions were performed for 27 cycles at 94 ℃ for 30 s, at 56 ℃ for 30 s, and 72 ℃ for 40 s. Amplified cDNA fragments were then blotted onto Hybond-N+ (Amersham), and the gene specific probes were labeled using the Alk Phos Direct Labelling system (Amersham) according to the manufacturer's protocol.

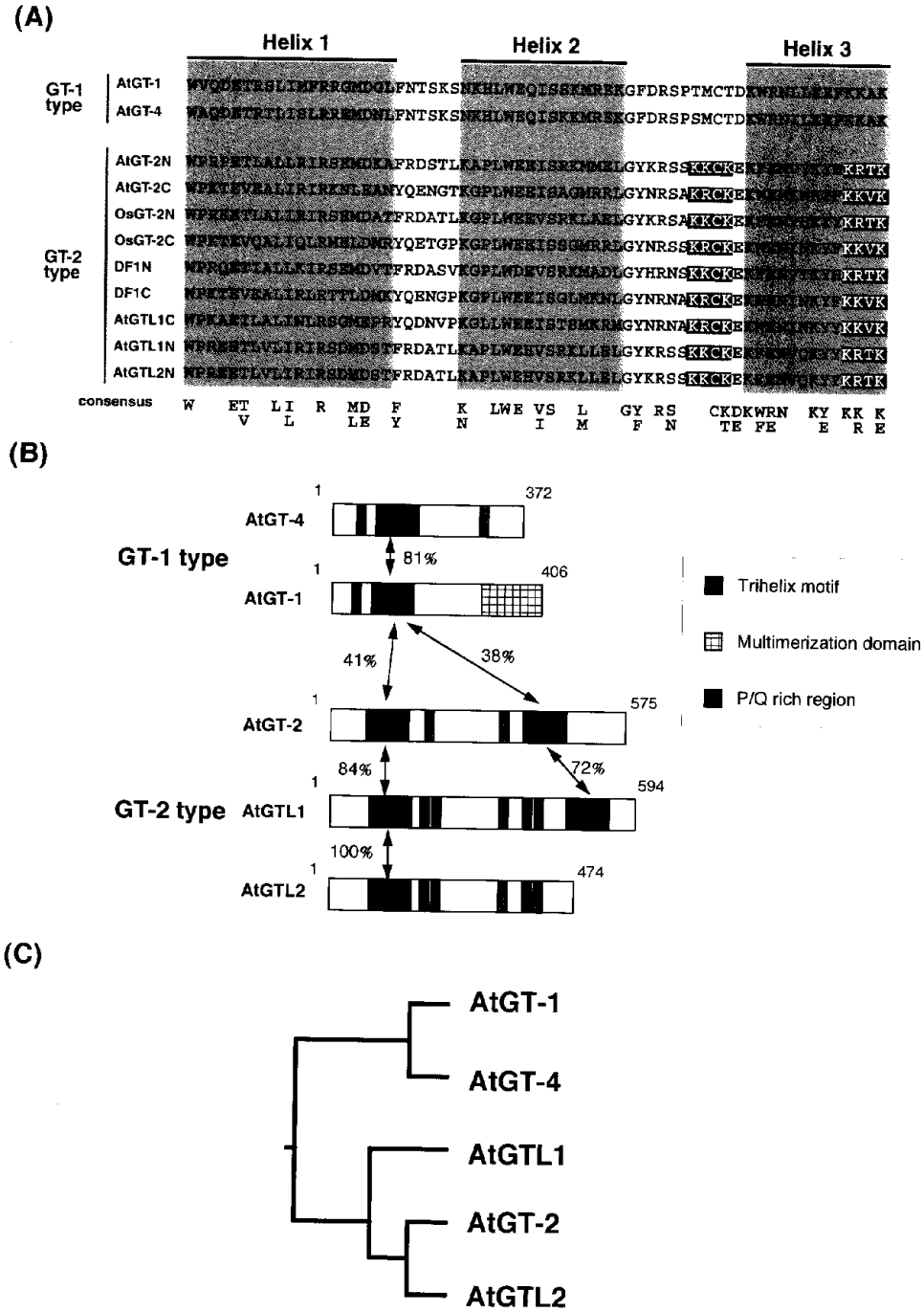

Fig. 2 *Arabidopsis* GT-box binding proteins. GT-box binding proteins have plant specific trihelix motif(s) in DNA-binding domain(s). (A) Sequence alignment of GT-box binding proteins among their trihelix region. GT-1 and GT-4 have only one DNA-binding domain and GT-4 shows striking homology to GT-1. There are some residues, as indicated below, which are conserved among all GT-box binding factors from *Arabidopsis*. Nuclear localization signals (NLSs) are indicated by filled squares. (B) Schematic structure of GT-box binding factors. Homology between trihelices is indicated in percentage. (C) Phylogenetic tree of GT-box binding proteins in their DNA-binding domain, trihelices. The tree is drawn with WebPHYLIP Version 2.0 program (http://sdmc.krdl.org.sg:8080/~lxzhang/phylip/). The protein sequences used for the alignment were as follows: GT-1; (AAA66473). GT-2; (CAA51289). GTL1; (CAA05995). GTL2; (CAA05996). GT-4; (AB072370).

Results

Cloning of GT-1-like genes from Arabidopsis

In order to isolate genes encoding a novel GT-box binding protein, we searched the *Arabidopsis* database for GT-1 related genes with the cDNA sequence corresponding to the GT-1 DNA binding domain. By using the BLAST program (Altschul *et al.*, 1990), we found one genomic fragment, B60976, from the *Arabidopsis* GSS database. Because the B60976 fragment showed high homology to GT-1, we cloned the cDNA of the putative GT-box binding protein encoded by the genomic sequence. After confirming the mRNA expression by RT-PCR, we isolated a putative full-length cDNA by 5'-RACE and 3'-RACE methods (**Fig. 1**). Presence of an in-frame stop codon in 75-bp upstream of putative first methionine codon suggests that we have obtained full-length cDNA encoding the corresponding DNA binding protein, designated GT-4.

Structural feature of GT-4

The GT-4 cDNA encodes a protein of 372 amino acids with a relative molecular mass of 40.9 kDa. Analysis of the predicted secondary structure has revealed that the GT-4 cDNA contains a trihelix motif showing 80% homology to that of GT-1 (**Fig. 1, 2B**). Moreover, other characteristic features among known GT-box binding proteins are well conserved. Conservation of proline-rich and glutamine-rich regions and putative phosphorylation sites for calcium/calmodulin kinase II (CaMKII) and casein kinase II (CKII) indicates that the GT-4 is closely related to previously described GT-box binding factors (**Fig. 2B**). Unlike the GT-2 type of GT-box binding proteins, however, GT-4 does not contain the typical bipartite nuclear localization signal (NLS) within its trihelix motif (**Fig. 2A**). Phylogenetically GT-4 is categorized into a GT-1 type of GT-box binding protein (**Fig. 2C**).

DNA-binding specificity of GT-4

To investigate the DNA-binding specificity of GT-4, we performed electro-mobility shift assays (EMSA) using recombinant GT-4 protein expressed in a rabbit reticulocyte lysate system. The mobility of the *in vitro* translated protein in SDS-PAGE is consistent with the size of the protein calculated from the deduced amino acid sequence (40.9 kDa), assuring that the GT-4 cDNA we obtained encodes the predicted open reading frame in its cDNA (**Fig. 3A**). GT-4 protein binds to a probe consisting of four copies of the GT1-box, but not to the GT1m-box competitor, with two guanines of the core sequence substituted by cytosines (**Fig. 3B**). This observation suggests that GT-4 binds to GT1-box in a sequence-specific manner, and the first two guanines within the GT1-box core sequence are crucial for GT-4 binding as in GT-1. Two different bound complexes, a faster and a slower migrating band, were detected under the experimental condition exploited in this study. This might be due to the presence of multiple binding sites in four copies of GT1-box probe sequence.

GT-box elements are found in the promoter region of chlorophyll a/b binding protein (*CAB*), *RBCS*, and other light-responsive genes such as rice phytochrome A (Dehesh *et al.*, 1992). They are categorized into three types: GT1-box, GT2-box, and GT3-box. Their core sequences -important for recognition by GT-box binding proteins- are slightly different as described previously (Dehesh *et al.*, 1992; Hiratsuka *et al.*, 1994). To investigate the binding specificity of GT-4 to those three types of GT-boxes, we carried out cross competition analysis using GT-box oligonucleotide as competitors. As shown in **Fig. 4**, GT-4 recombinant protein binds to the GT1-box most tightly, and to the other GT-box elements with weaker affinity. These observations indicated that the DNA-binding property of GT-4 is almost identical to that of GT-1 (**Fig. 4A, 4B**). Comparison of the DNA-binding profile

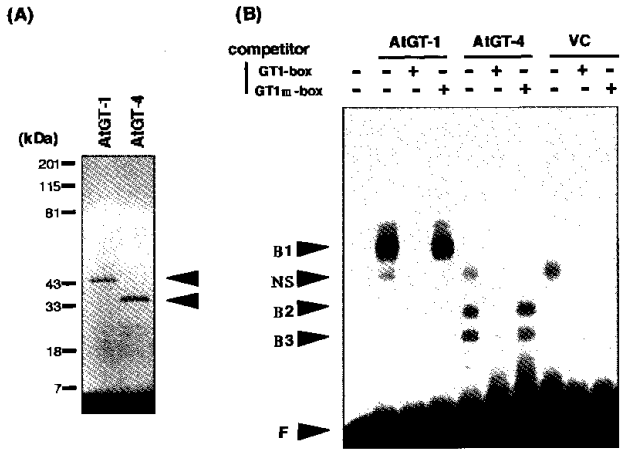

Fig. 3 Recombinant GT-4 protein binds to GT1-box element specifically. (A) SDS-PAGE analysis of *in vitro* translation products synthesized using rabbit reticulocyte lysate system. (B) Electro mobility shift assay using recombinant GT-1 and GT-4 protein. 10 ng of GT1-box and GT1m-box are used as competitor fragments. Each reaction mixture contains 0.2 ng DNA probe. probe: 4xGT1-box, VC: vector control, B1: GT-1-DNA complex, B2, 3: GT-4-DNA complex, F: free probe, NS: non-specific complex.

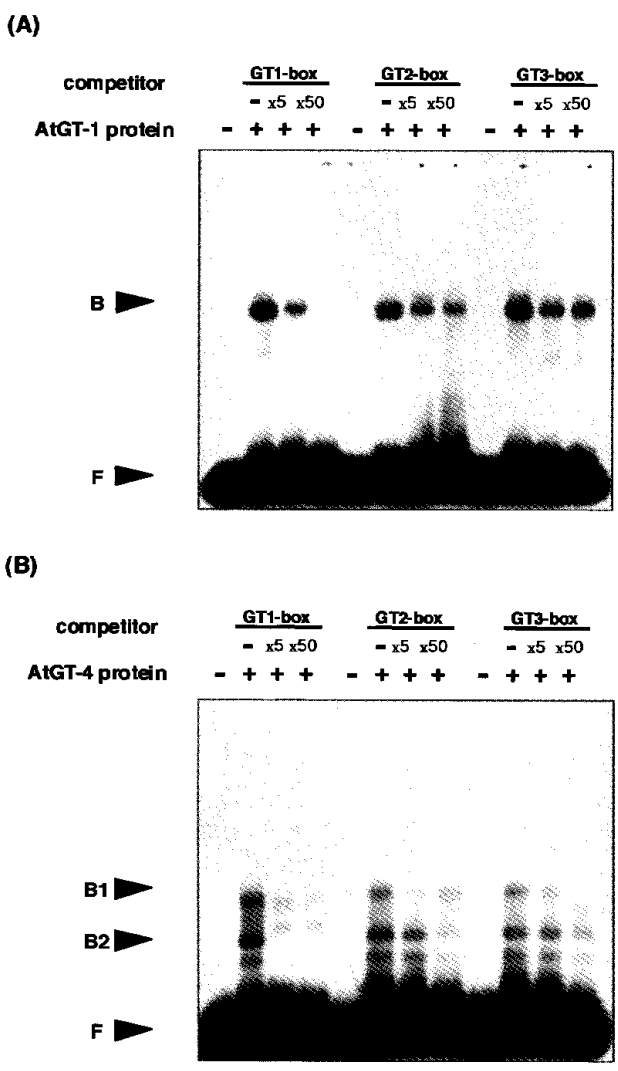

Fig. 4 (A) Cross competition assays using recombinant GT-1 protein. Each reaction mixture contains 0.2 ng DNA probe, *in vitro* translation products, and competitor. B: GT-1-DNA complex, F: free probe. (B) Cross competition assay using GT-4 recombinant protein. Each reaction mixture contains 0.2 ng DNA probe. B1, B2: GT-4-DNA complex, F: free probe.

of GT-1, GT-2 and GT-4 for various GT-box *cis*-regulatory elements is summarized in **Table 1**.

Subcellular localization of GT-4

In order to identify the subcellular localization of GT-4, a chimeric gene encoding the GT-4 protein fused to Green Fluorescent Protein (GFP) was introduced into onion epidermal cells using a particle bombardment system and transiently expressed by the cauliflower mosaic virus 35S promoter. GFP fluorescence was investigated under fluorescent microscopy six hours after bombardment. **Fig. 5** shows examples of the microscopic image of onion epidermal cells bombarded with GFP::GT-4 and

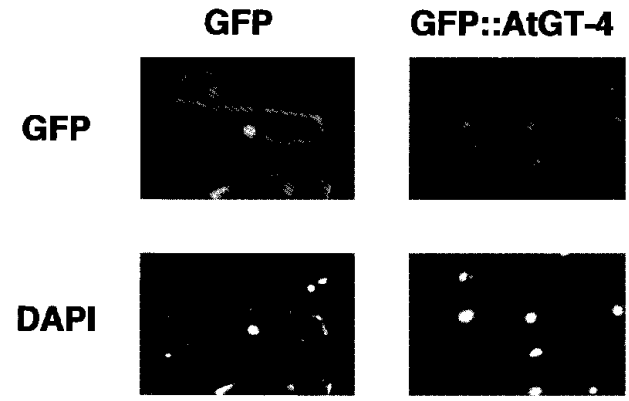

Fig. 5 GT-4::GFP fusion protein localizes to nucleus. Onion epidermal cells were transformed transiently with 1.6-μm gold particles coated with plasmid carrying GFP::GT-4, or GFP using a model PDS-1000/He particle delivery system (Bio-Rad). GFP fluorescence was observed 6 h after bombardment under fluorescent microscopy.

GFP constructs, respectively. The green fluorescence derived from the fusion protein was detected mainly in the nucleus. By contrast, the fluorescence signal derived from the GFP was detected in the cytoplasm. These results suggest the presence of nuclear localization signal(s) within the GT-4 molecule.

Expression analysis of GT-4

The expression pattern of GT-4 mRNA was investigated by RT-PCR using total RNA obtained from the flower, flower bud, stem, leaf, root and silique (**Fig. 6A**). Because no apparent tissue-specific expression of GT-1 has been reported, mRNA expression of GT-1 was also investigated for comparison. The GT-4 mRNA was detected from all tissues tested, while the expression of GT-1 in silique was relatively low under the experimental condition exploited in this study.

Because mRNA levels of GT-1 are not affected by the light condition in mature plants, GT-1 protein is considered to be involved in the primary response of light-regulated gene expression. In order to survey the GT-4 expression in response to light, we investigated the accumulation pattern of GT-4 mRNA in three-day-old etiolated seedlings by RT-PCR. *CAB1* gene expression was monitored as a control for the light condition and light-induced transcription. Unlike GT-1, which expresses consititutively, the accumulation of GT-4 transcripts in etiolated seedlings was significantly low. GT-4 mRNA accumulation was observed after a one-hour exposure to white light and gradually increased up to 12 h under a continuous light

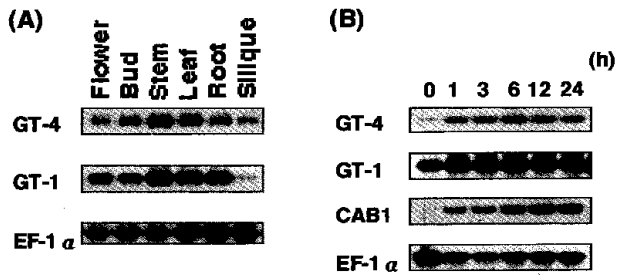

Fig. 6 mRNA expression of GT-4. (A) RT-PCR was performed using 1 μg of total RNA isolated from each plant tissue. *Elongation Factor-1a* (*EF-1a*: Gen Bank accession number X16430) was used as a control for equal amount of RNA sample, (B) mRNA expression of *GT-1* and *GT-4* in response to light condition. Three-day-old etiolated seedlings were exposed to white light, and sampled after 0, 1, 3, 6, 12 and 24 h. Total RNA was extracted from each sample and exploited as template for RT-PCR. Chlorophyll a/b binding protein (*CAB1*: Gen Bank accession number J04098) was used as positive control for light condition. Total RNA extracted from three-day-old, dark grown seedlings of *Arabidopsis* was subjected. RT-PCR was carried out using specific primers for each gene and blotted to Hybond-N+ (Amersham-Pharmacia Biotech), then hybridized with each probe.

condition. Compared with *CAB1* mRNA expression pattern, the GT-4 light response was slightly earlier (**Fig. 6B**).

Discussion

GT-1 that binds the GT1-box and related *cis*-regulatory elements is the only protein whose cDNA has been isolated and characterized in detail. In this study, we cloned a cDNA that encoded a novel GT1-box binding protein, named GT-4.

The GT-4 cDNA showed 80% homology to the trihelix region of GT-1 and the analysis of *Arabidopsis* genome database has revealed that GT-4 has the highest homology to GT-1 among the predicted *Arabidopsis* gene products (Murata *et al.*, unpublished data). These results suggest a close phylogenetic origin and the functional relationship between GT-4 and GT-1.

A previous study on the DNA-binding specificity of GT-1 indicated that the recombinant GT-1 protein can bind to the GATA-box-containing *cis*-regulatory element of *Arabidopsis CAB2* and the box-III element of pea *RBCS* gene (Hiratsuka *et al.*, 1994; Teakle and Kay, 1995). Recent studies have revealed that GT-1 can bind to various promoters including pathogenesis-related 1a (*PR-1a*), pollen-specific late anther tomato 52 (*LAT52*), defense-related terpenoid indole alkaloid (*TIA*) and tryptophan decarboxylase (*TDC*) (Eyal *et al.*, 1995; Buchel *et al.*, 1996; Ouwerkerk *et al.*, 1999; Pasquali *et al.*, 1999; Zhou, 1999). These promoters have GT-box element(s) and/or GT-box-related sequences in the TATA-box proximal region of their promoters.

Recently, a GT-2 type of GT-box binding protein, named DF1, was cloned from pea and the DNA-binding specificity of DF1 was characterized extensively (Nagano *et al.*, 2001). DF1 has dual DNA-binding specificity; DF1 binds to two distinct cis-regulatory elements, one of which is DE1 (Inaba *et al.*, 2000), that confers dark-inducible and light down-regulated gene expression of *pra2* gene, and the other is the GT2-box. Because the nucleotide sequences of these two *cis*-regulatory elements show low similarity, the GT-2 type of GT-box binding proteins, for example DF1, might also have a broad DNA-binding specificity like GT-1. Because GT-4 shows high homology to GT-1 within its DNA-binding domain, GT-4 protein was estimated to have DNA-binding specificity similar to that of GT-1. In fact, an electro-mobility shift assay using recombinant protein has revealed that the DNA-binding preference of GT-4 is similar to that of GT-1. Although these findings are obtained by binding assays *in vitro*, our results suggest that GT-4 might share the target sequences with GT-1 *in vivo*. To compare the sequence specificity of both proteins, however, it is necessary to test other GT-1 binding sequences. Further investigation of DNA-binding using non-GT-box containing elements such as GATA-boxes and PR-promoter elements may provide further insight into DNA-binding characteristics and target genes of the GT-4 protein.

A root GT-box binding factor (RGTF), with GT-1 related, but different DNA-binding activity, has been found in tobacco root nuclear extract reported (Villain *et al.*, 1996). An electro-mobility shift assay revealed faster migrating complexes for this root-specific GT1-box binding activity compared with authentic GT-1 DNA-binding activity. Our data presented in this report suggest that GT-4 may be one of the candidates for RGTF, because the GT-4-DNA complex gives a faster migrating band than the GT-1-DNA complex.

Recent studies on transcription factors have revealed that post-translational regulation, in particular phosphorylation, plays an important role for regulating the DNA-binding activity. The DNA-binding activity of GT-1 has been shown to be regulated by CaMKII-mediated phosphorylation

(Marechal *et al.*, 1999). Phosphorylation of T133 in GT-1 was shown to be critical for its enhanced DNA-binding. Because the putative CaMKII phosphorylation sites of GT-1, for example T133, are well conserved in GT-4, the DNA-binding activity of GT-4 may also be modulated by CaMKII phosphorylation (**Fig. 1**). Detailed characterization of the effect of phosphorylation on DNA-binding activity of GT-4 remains to be investigated.

Transcription factors must localize to the nucleus when they function as components of transcriptional machinery. In this study, we have shown that the GT-4 protein is localized to the nucleus in onion epidermal cells when fused to GFP. This strongly suggests that GT-4 is a nuclear protein. As reported earlier, transient expression of the GT-1::β-glucuronidase fusion protein revealed the presence of NLS within the first 215 amino acids of GT-1. However, no NLS consensus sequence was found within the GT-1 amino acid sequence (Hiratsuka *et al.*, 1994). On the other hand, it has been shown that GT-2 type of GT-box binding proteins contain typical bipartite type of NLSs (Kalderon *et al.*, 1984; Dehesh *et al.*, 1995). Although it is possible that GT-4 also contains NLS, we could not identify any previously described NLS consensus sequences within the amino acid sequence of GT-4. The difference in the structures of NLS between GT-1 type and GT-2 type of GT-box binding proteins suggests the possibility that the GT-1 type and GT-2 type of GT-box binding proteins would bind to distinct NLS-binding proteins. Hence the machinery of subcellular translocation of these two types of proteins are possibly different.

Expression analysis of GT-4 mRNA revealed that GT-4 mRNA is expressed in all organs examined, with preferential expression in bud, stem and leaf. On the other hand, expression of GT-1 mRNA in silique was relatively low in our experimental condition. Hence, it is possible that GT-4, instead of GT-1, would function as a major transcriptional regulator of light-responsive genes in silique. There is another member of GT-box binding proteins, GTL1, shows precedent expression in silique (Smalle *et al.*, 1998). Although characterization of DNA-binding specificities of GTL1 and GTL2 have not been reported, they might bind to the GT2-box element because GTL1 and GTL2 share high homology with the GT-2 type of GT-box binding protein within their DNA-binding domains. Consequently, GTL1 and GTL2 may not share binding sequences with GT-1 and GT-4. These findings are consistent with the idea that multiple GT-box binding proteins possibly regulate transcription in an organ-specific manner (Villain *et al.*, 1996).

Furthermore, unlike GT-1 whose expression is unaffected by light, the GT-4 mRNA in etiolated seedlings clearly responded to white light. This observation suggests that the expression of GT-4 may be under the control of primary light-regulated gene transcription. Therefore, GT-4 may act as a transcriptional activator that strengthens the activity of light-dependent transcription to obtain a maximal level of gene expression. It is also feasible that GT-4 is involved in a secondary step of light-responsive transcription. Taken together, available evidence suggests that GT-4 acts downstream of GT1-box mediated transcriptional regulation which is supposed to play a major role in a primary step of light-regulated transcription.

Although the number of GT-box binding proteins cloned is growing larger, the *in vivo* function of GT-box binding proteins still remains to be uncovered. GT-4 belongs to the GT-1 type of GT-box binding proteins, shares the target sequence with GT-1, and possibly acts as a transcription factor like GT-1. Therefore, it is possible that GT-4 is involved in various types of gene regulation mediated by GT1-box and related cis-regulatory elements. Further study with transgenic plants or mutants lacking the functional GT-4 gene should provide information on the in vivo function of the GT-4 protein.

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