Analysis of GT-3a identifies a distinct subgroup of trihelix DNA-binding transcription factors in *Arabidopsis*

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Abstract **Trihelix DNA-binding factors (or GT factors) bind to GT elements found in the promoters of many plant genes. Although the binding specificity and the transcriptional activity of some members (e.g. GT-1 and GT-2) have been studied, the regulatory function of this family of transcription factors remains largely unknown. In this work, we have characterised a new GT factor, namely GT-3a, and a closely related member, GT-3b. We show that (1) they can form either homo- or heterodimers but do not interact with GT-1; (2) they are predominantly expressed in floral buds and roots; (3) GT-3a cannot bind to the binding sites of GT-1 or GT-2, but binds to the *cab2* and *rbcS-1A* gene promoters via the 5′-GTTAC sequence, which has been previously shown to be the core of the Site 1 type of GT elements. These results suggest that GT-3a and GT-3b belong to a distinct subgroup of GT factors and that each subgroup of GT factors binds to a functionally distinct type of *cis*-acting GT elements.**

Key words: Transcription factor; GT element; Trihelix DNA-binding domain; GT-3a; *Arabidopsis*

1. Introduction

GT elements were initially identified in the pea *rbcS-3A* gene promoter as a light-responsive element called Box II [1] and later in many other promoters, some of them not regulated by light [2]. The regulatory function of GT elements in various promoters is generally not defined. However, in a few cases, GT elements have been found to play a distinct transcription regulatory role. For instance, the pea *rbcS-3A* Box II repeats could confer light responsiveness to otherwise light-unresponsive minimal or synthetic promoters [3,4], while those found in the rice *phyA* gene (called GT2-bx and GT3-bx) could activate transcription in the dark [5]. In addition, a GT element called Site 1, found in the ribosomal protein gene *rps1* promoter, has been shown to repress transcription in non-photosynthetic tissues or cells [6,7]. Box II-related elements found in a soybean *chs* gene promoter (called Box 1, Box 2 and Box 3) are likely to respond to elicitor treatments [8]. The sequences of these defined GT elements are different from the core sequence (5′-GGTTAA) of the pea *rbcS-3A* Box II, suggesting that there may exist distinct subclasses of GT elements that are recognised by different subgroups of transcription factors.

The GT-2 DNA-binding factor, with affinity to the GT elements of the rice *phyA* gene promoter, was first cloned from rice [5]. A Box II-binding protein, named GT-1a or B2F, was subsequently cloned from tobacco [9,10]. The cDNAs of *Arabidopsis* GT-1 and GT-2 were later isolated and characterised based on sequence homology [11,12]. These related proteins contain one or two plant-specific DNA-binding motifs known as trihelix (helix-loop-helix-loop-helix) [13]. GT-2 contains two trihelix motifs with the N-terminal one preferentially binding to GT3-bx and the C-terminal one to GT2-bx [11], whereas GT-1 contains one trihelix domain that binds to Box II with 100 times higher affinity than to GT2-bx or GT3-bx of *phyA* [12]. In a reverse experiment, GT-2 could not bind to the Box II sequences [11]. It has been shown that both GT-1 and GT-2 act as trans-activators in transient expression assays [14,15]. However, transgenic studies have shown that depending on the target promoter, GT-1 can be a positive or a negative transcription regulator [16].

The trihelix DNA-binding proteins are unique to plants, suggesting that they would be implicated in plant-specific gene regulation, as suggested for other plant lineage-specific factors [17]. However, little is known on the regulatory function of this class of transcription factors, which are encoded by a relatively small family of genes in the *Arabidopsis* genome [18]. In this work, we show that *Arabidopsis* GT factors can be divided into subgroups that bind to functionally distinct types of GT elements.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana Col-0 plants were used in this study. Wild-type plants were grown in soil in a greenhouse under a long-daylight cycle (16 h/8 h). Plant transformations using the *Agrobacterium* EHA105 strain were performed by the infiltration method [19]. For transgenic plant screening, *Arabidopsis* seeds were germinated in vitro on 0.5×MS salts supplemented with vitamins and 1% of sucrose in a growth chamber at 21°C either under the long-daylight cycle or in complete darkness.

2.2. Isolation of GT-3a genomic clone, GT-3a and GT-3b cDNAs

A *Sac*I genomic fragment was cloned by screening a genomic library, prepared from *A. thaliana* C24 plants, using a polymerase chain reaction (PCR)-generated DNA fragment corresponding to the conserved trihelix domain. This clone, corresponding to the *GT-3a* gene, was completely sequenced and deposited in databanks under the ac-

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cession number AF206715. The GT-3a and GT-3b cDNAs were subsequently isolated from total RNA of young seedlings by reverse transcription (RT) PCR using the primer sets: GT-3a upstream 5′-ATGGACCGACGTAACCCTTTCCAA-3′; GT-3a downstream 5′-CTTATGCTTAACACAAATAATGAG-3′; GT-3b upstream 5′-ATGGATGGACATCAGCATCATCACC-3′; GT-3b downstream 5′-TTAGAGGGAACCATCTCTAGTAAGC-3′. They were cloned into the pGEM-T Easy vector (Promega), sequenced and deposited in databanks under the accession numbers AY271677 and AY271678, respectively.

2.3. Two-hybrid system

GT-3a, GT-3b and GT-1 (L36806) [15] were amplified by PCR, sequenced and cloned into the pGBT-9 (bait) and pGAD 424 (prey) vectors (Clontech). All the two-hybrid assay experiments were carried out according to the manufacturer's protocol.

2.4. Primer extension and RT-PCR analysis

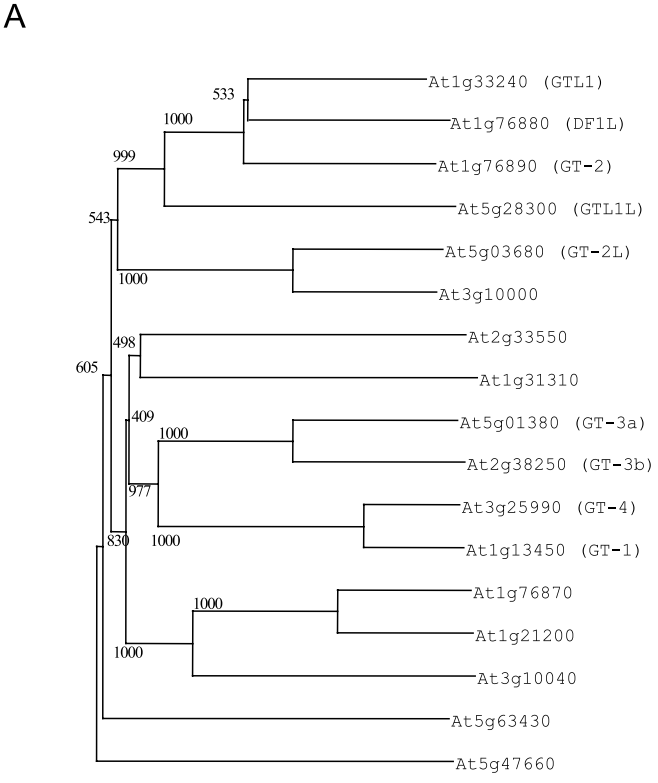
Total RNAs were extracted from plants grown on soil or on MS medium under a long-daylight cycle. Primer extension experiments were performed with total RNA from young seedlings using standard protocols. Reverse transcription reactions were carried out using RT Superscript II RNase H^- (Invitrogen) with Oligo$(dT)_{12-18}$ as primer (Invitrogen).

2.5. Promoter activity analysis and GUS staining

The *GT-3a* gene promoter region from −1709 to +10 was cut out from the genomic clone by *Taq*I and inserted into the pBI101 vector (Clontech). *A. thaliana* plants were transformed with this GT-3a::GUS construction and T2 generation plants were tested for GUS expression analysis using standard procedures [20]. Briefly, samples were prefixed in 90% acetone for 20 min at room temperature, rinsed in staining buffer without X-gluc and infiltrated with staining buffer (100 mM sodium phosphate buffer pH 7.2; 2 mM potassium ferrocyanide; 2 mM potassium ferricyanide; 2 mM X-gluc) under vacuum for 15 min and incubated at 37°C for about 14 h. After a progressive dehydration in ethanol, pictures were taken using a stereoscopic microscope SMZ-10A (Nikon) and a microscope Eclipse E800 (Nikon).

2.6. Nuclear localisation assays

The GT-3a coding region (without the codon stop) was amplified by PCR using the primers 5′-GGGGGATCCATGGACCGACGTAACCCT-3′ and 5′-GGGGGATCCCGAAACCTTGATTATGAT-3′ and inserted into the psmGFP vector [21] to produce the GT-3a/GFP fusion construct. Onion epidermal cells were transfected by biolistic bombardment using the PDS-1000/He system (Bio-Rad) according to the manufacturer's instructions and imaged with a confocal microscope (LSM 510, Zeiss).



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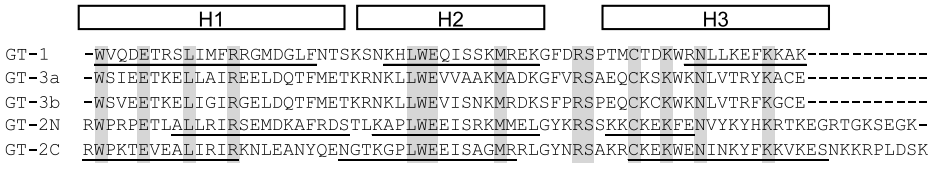


Fig. 1. A: Neighbour-joining tree of *Arabidopsis* GT factors constructed using the Clustal X multiple alignment package [22]. The bootstrap values for 1000 trials are indicated at each fork. B: Alignment of the trihelix domains from GT-1, GT-2, GT-3a and GT-3b. Amino acids conserved in all the listed domains are highlighted. Three helical regions (H1 to H3) are indicated. GT-2N: N-terminal DNA-binding domain; GT-2C: C-terminal DNA-binding domain.

2.7. Recombinant protein production and gel shift assays

The GT-3a coding region was cloned into the pQE30 expression vector (Qiagen). The His-tagged GT-3a protein production and gel shift assays were performed as previously described [15]. DNA fragments, corresponding to the promoter region of *cab1* (J04098), *cab2* (X15221), *rbcS-1A* (X13611) and *chs* (M20308) used as probes, were obtained by PCR using the primer sets: *cab1* −52 to −228 (5′-GGGAATCTGCGAAGTGCGAGCC-3′ and 5′-TTTCTAGAGCCAAGAAATGGGTGG-3′); *cab2* −37 to −201 (5′-GGGAATTCTGGTGGACTAGAGATTG-3′ and 5′-TTTCTAGACTTGTGAGTGAGAGTG-3′); *cab2* −51 to −463 (5′-GGTAGTGATGATGAACTAATTACC-3′ and 5′-GTGATTAAAACTGGTTCGATAGTG-3′); *rbcS-1A* −20 to −371 (5′-ATTCCCCATAAGGAAAGGGCCAAG-3′ and 5′-TGTGACTGAGGTTTGGTCTAGTGC-3′); *chs* −73 to −513 (5′-AGAACTGGGAAGTGAAACCTCCTG-3′ and 5′-GGAAATATACCGGAGAGTGAGAGC-3′). Box II and Site 1 DNA fragments as well as their mutant versions were obtained as described [7,15]. Oligonucleotides were synthesised to obtain dimers of GT2-bx and GT3-bx as well as cab2w and cab2m: GT2-bx 5′-GGCGGTAATTAAC-3′; GT3-bx 5′-CGAGGTAAATCCG-3′; cab2w 5′-ATATAACAAACGTTACAATATCCCTATATA-3′; cab2m 5′-ATATAACAAACCTCATAATATCCCTATATA-3′.

3. Results

3.1. Two closely related small GT factors in Arabidopsis

A neighbour-joining tree of 17 *Arabidopsis* GT factors was constructed using the Clustal X multiple alignment package [22] (Fig. 1A). This tree revealed two closely joined members (At5g01380, At2g38250), which had 53% amino acid sequence identity throughout the proteins (not shown). The highest conservation is within the trihelix domain with 78% amino acid sequence identity. There is no significant conservation with the other characterised GT factors, except the trihelix domains (Fig. 1B). With 323 or 289 amino acids in length, these putative proteins were much smaller than previously characterised GT-1 (At1g13450) and GT-2 (At1g76890), and were named GT-3a and GT-3b, respectively.

3.2. Dimerisation between GT-3a and GT-3b

To determine whether there was any interaction between

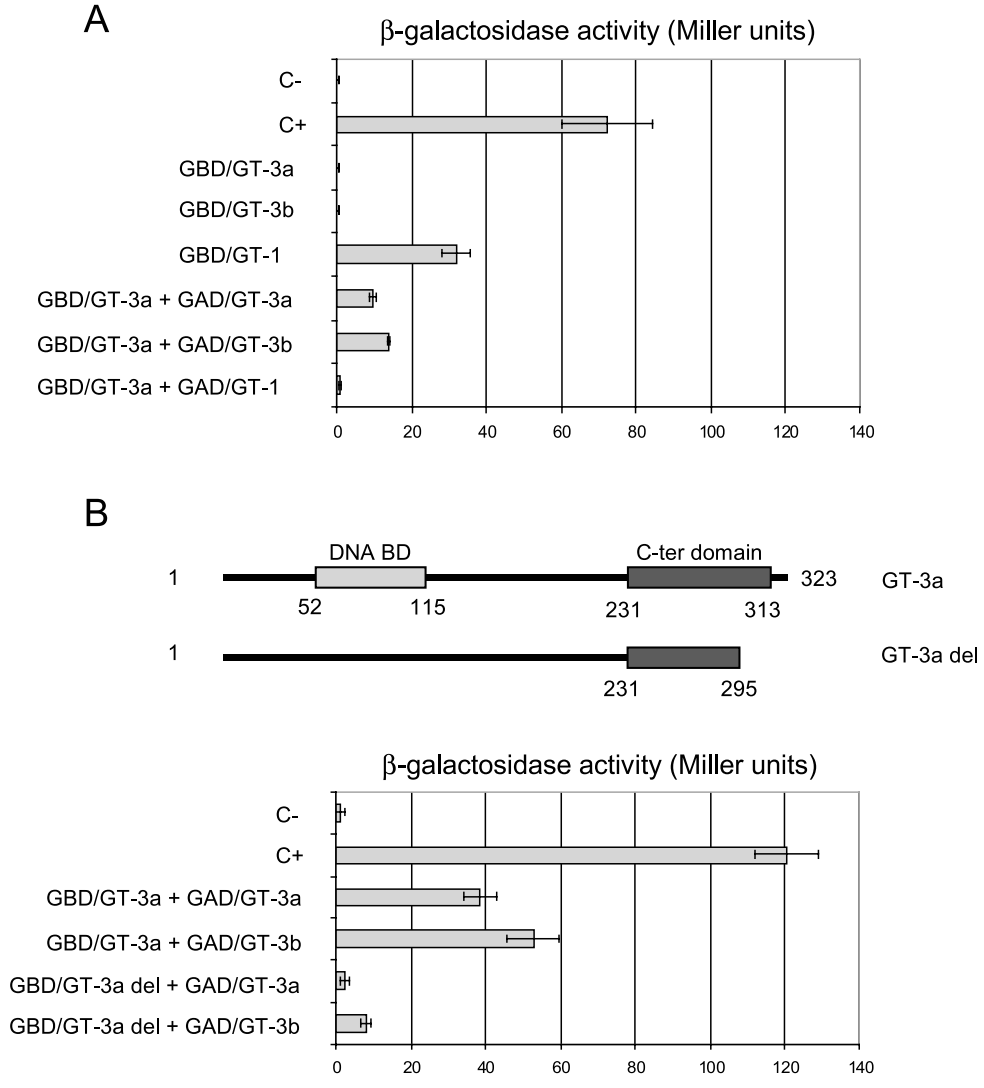


Fig. 2. Two-hybrid analysis of protein interaction between GT-3a and other GT factors in yeast. A: Average values of β-galactosidase activities measured from three independent transformations with combinations of bait (GBD/protein X) and prey (GAD/protein Y) constructs. B: Two-hybrid assays achieved with the GT-3a truncated protein (GT-3a del). C−: negative control (GAL 4 DNA-binding domain); C+: positive control (GAL 4 protein).

GT-3a, GT-3b and other GT family members, we carried out protein interaction assays in a yeast two-hybrid system. The results presented in Fig. 2A showed that both GT-3a and GT-3b could form either homo- or heterodimers, but no interaction between each of the two proteins and GT-1 was detected. The dimerisation domain seemed to be located at the C-terminus, since deletion of the last 28 amino acids of the protein in GT-3a sufficed to abolish the dimerisation activity of the protein (Fig. 2B).

3.3. Expression profile of GT-3a

Primer extension experiments with total RNA from young seedlings revealed two transcription start sites, one located at −19 bp and the other at −93 bp relative to the ATG start codon of GT-3a (not shown). This is consistent with the presence of two putative TATA boxes found in the promoter region. In order to study the GT-3a expression pattern, the GT-3a promoter fragment spanning from −1709 to +10 (relative to the first transcription start site) was used to control the expression of the GUS (*uidA*) reporter gene in transgenic plants. GUS activity was detected in germinating seeds (Fig. 3A), in the vascular system of leaves, stems and roots (Fig. 3B,C,E,F) and in flower buds (Fig. 3F,G). The GUS activity decreased during flower development and disappeared in ma-

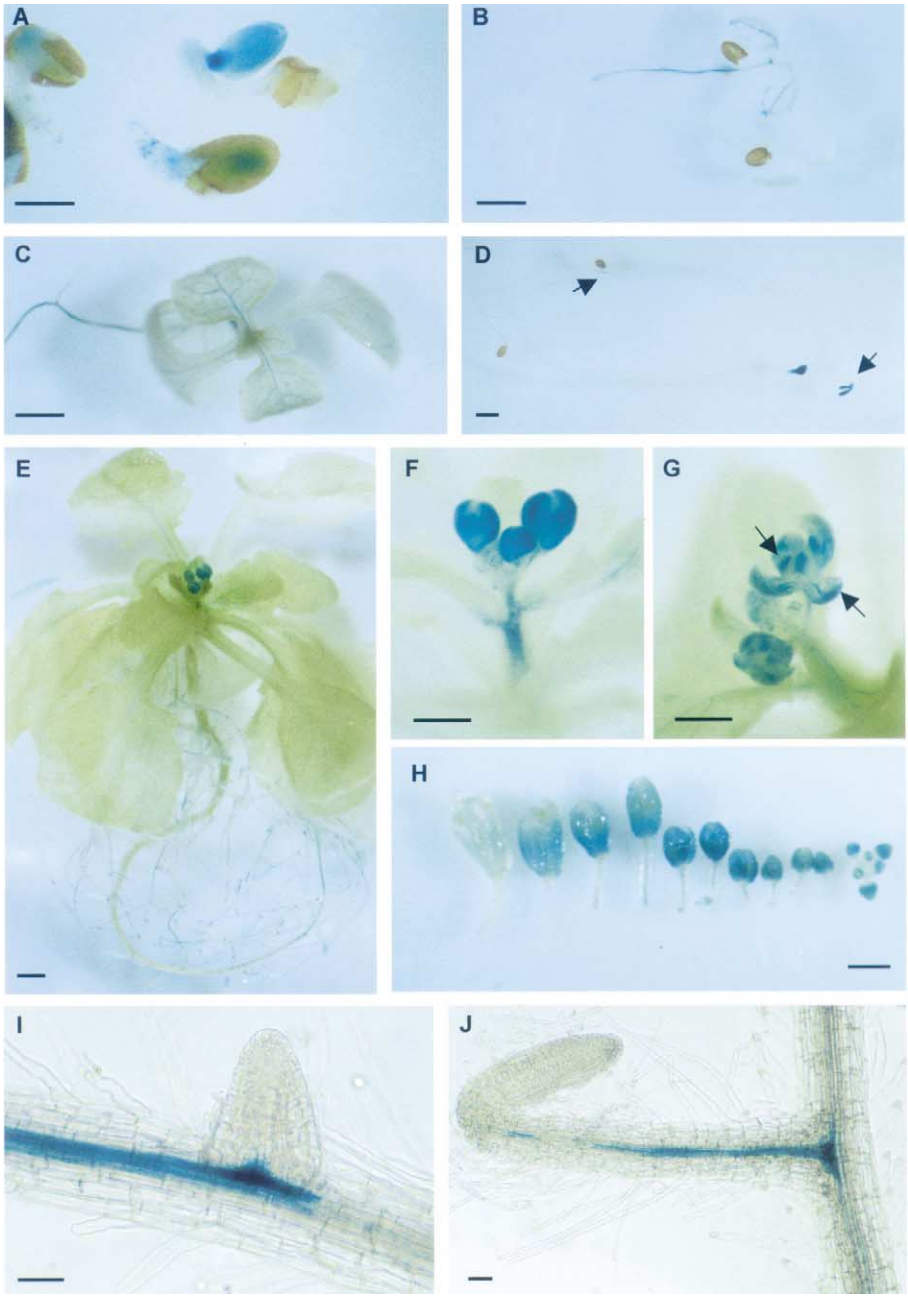


Fig. 3. GUS staining of *Arabidopsis* plants transformed by GT-3a::GUS. A: Germinating seeds. B: Three-day-old seedlings. C: A light-grown 8-day-old plant. D: Dark-grown 8-day-old plants, arrow indicates GUS staining in etiolated cotyledons. E: A plant at flowering stage. F: Enlarged view of the inflorescence shown in E. G: An opened flower showing staining in sepals and stamen filament. H: Flowers of an inflorescence, showing that the staining decreases in more developed flowers. I,J: GUS staining in lateral roots. Bars in A, F and G: 0.5 mm; bars in B–E and H: 1 mm; bars in I and J: 0.05 mm.

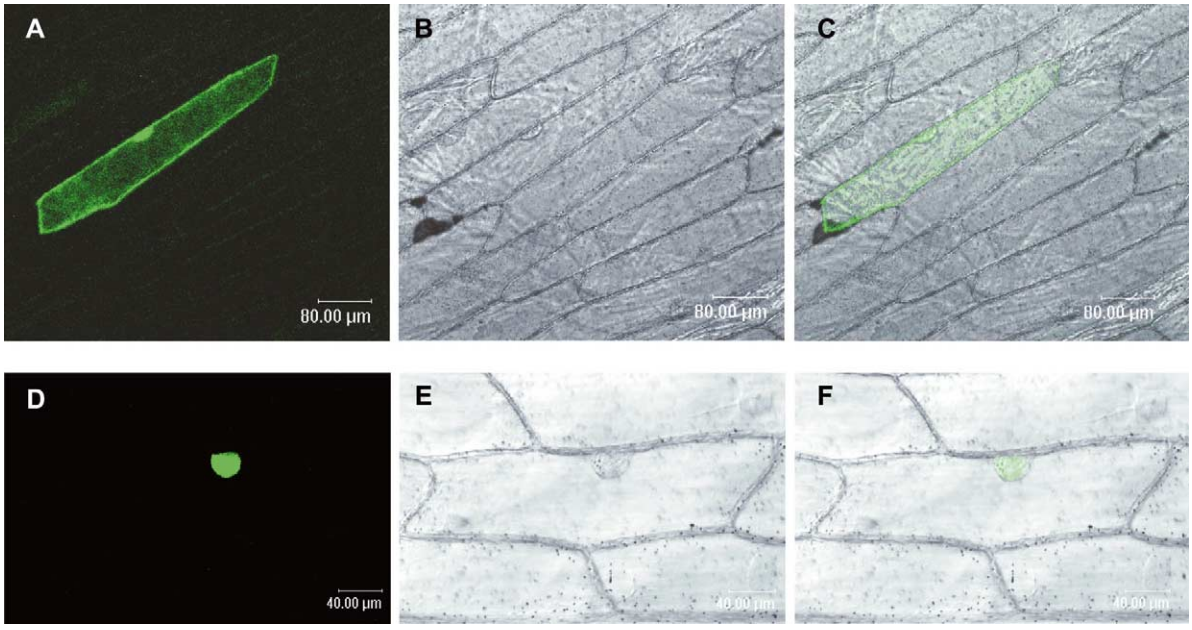


Fig. 4. Nuclear localisation of GT-3a in onion epidermal cells. Onion epidermal cells were transfected by 35S::GFP (A–C) or 35S::GT-3a/GFP (D–F) and photographed under a confocal microscope at 488 nm after 48 h (A,D). B and E are respectively the transmission image of A and D. C and F are merged images of A and B or D and E respectively.

ture flowers (Fig. 3H). High level of GUS activity was observed at the onset of secondary root development (Fig. 3I,J). Although no significant GUS activity was detected in cotyledons and leaves of 8-day-old light-grown plants (Fig. 3C), strong GUS activity was observed in cotyledons of 8-day-old etiolated plants (Fig. 3D). GT-3a seemed to be predominantly expressed in floral buds and roots, which was confirmed by semi-quantitative RT-PCR experiments that also revealed a similar expression profile for GT-3b (not shown).

3.4. *GT-3a targeted to the nucleus*

Sequence analysis showed the existence of a putative nuclear localisation signal in both GT-3a and GT-3b (not shown). To test whether GT-3a is targeted to the nucleus, a GT-3a/GFP fusion construct under the control of the CaMV 35S promoter was introduced into onion epidermal cells. The expressed fusion protein was localised exclusively in the nucleus, while the GFP alone was located throughout the cell (Fig. 4), indicating that GT-3a is targeted to the nucleus.

3.5. *GT-3a binding to the* cab2 *and* rbcS-1A *promoters*

In order to study whether GT-3a binds to GT element-containing promoters, we produced recombined His-tagged GT-3a protein in *Escherichia coli* to test its binding activity in gel shift assays. Promoter fragments from four *Arabidopsis* light-responsive genes were selected for the analysis: *cab1* (lhcb1.3, accession number J04098), *cab2* (lhcb1.1, X15221), *rbcS-1A* (X13611) and *chs* (M20308) for the tests. Analysis using the PLACE program [23] revealed the presence of sequences related to previously characterised GT elements such as Box II, GT2-bx and GT3-bx (not shown). DNA fragments corresponding to the promoter regions −228 to −52 of *cab1*, −463 to −51 of *cab2*, −371 to −20 of *rbcS-1A* and −513 to −73 of *chs* (all relative to the translational start codons) were used as probes in gel shift assays. These experiments revealed three shifted bands with the *cab2* fragment and one with the *rbcS-1A* fragment, but none with the *cab1* or the *chs* fragment (Fig. 5). The shifts resisted competition by an unrelated DNA fragment in 200 molar excess, but not that by unlabelled (cold) probes in 100 molar excess. This suggests that the binding of GT-3a to the *cab2* and *rbcS-1A* promoters was specific.

3.6. *Localisation of a GT-3a-binding site within the* cab2 *promoter*

Gel shift assays with the *cab2* promoter region from −201 to −37 showed a single shifted band (not shown). To find out

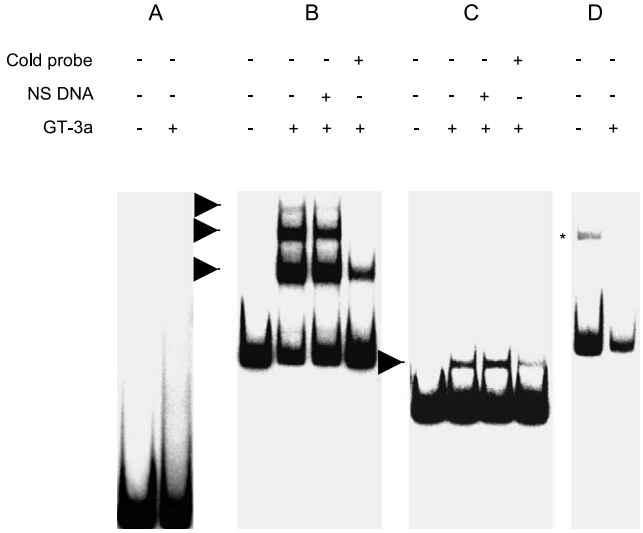


Fig. 5. DNA-binding assays of recombined GT-3a to a few putative target promoters. Promoter fragments from *cab1* (−52 to −228) (A), *cab2* (−51 to −463) (B), *rbcS-1A* (−20 to −371) (C) and *chs* (−73 to −513) (D) were used as probes and incubated with recombined GT-3a produced from *E. coli* as indicated (all the indicated positions are relative to the initiating ATG codon). Non-specific (NS DNA) or specific (Cold probe) competitors were included where indicated. Asterisk indicates a non-specific band.

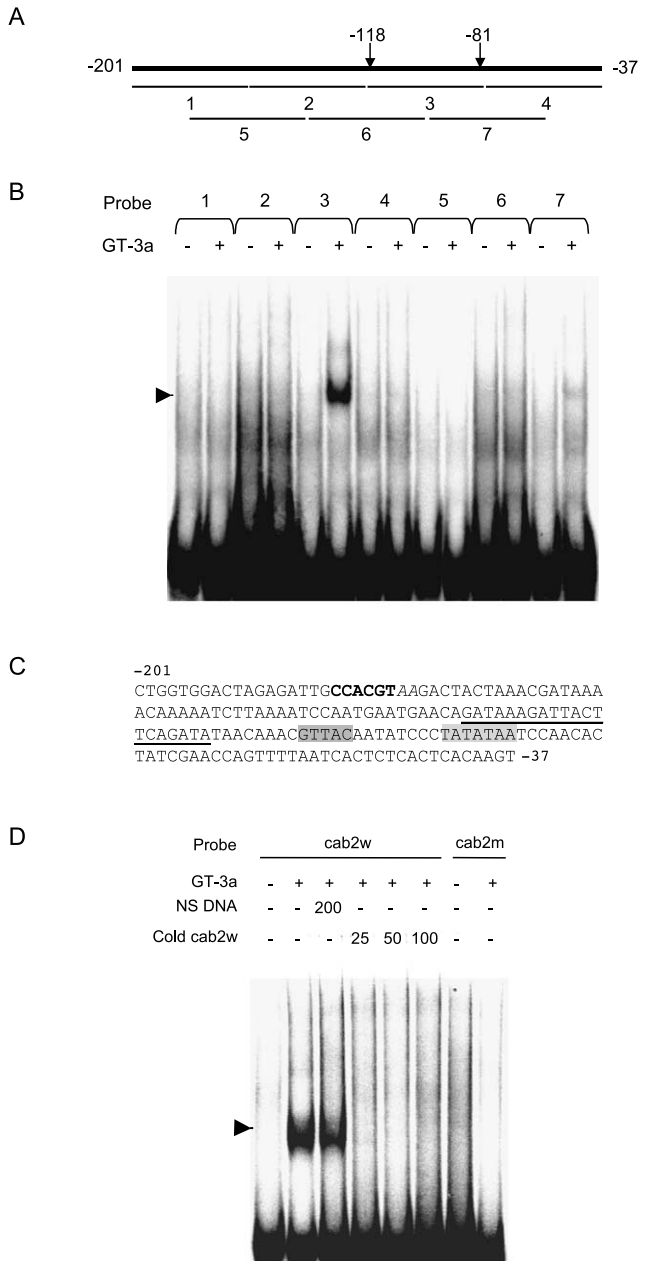
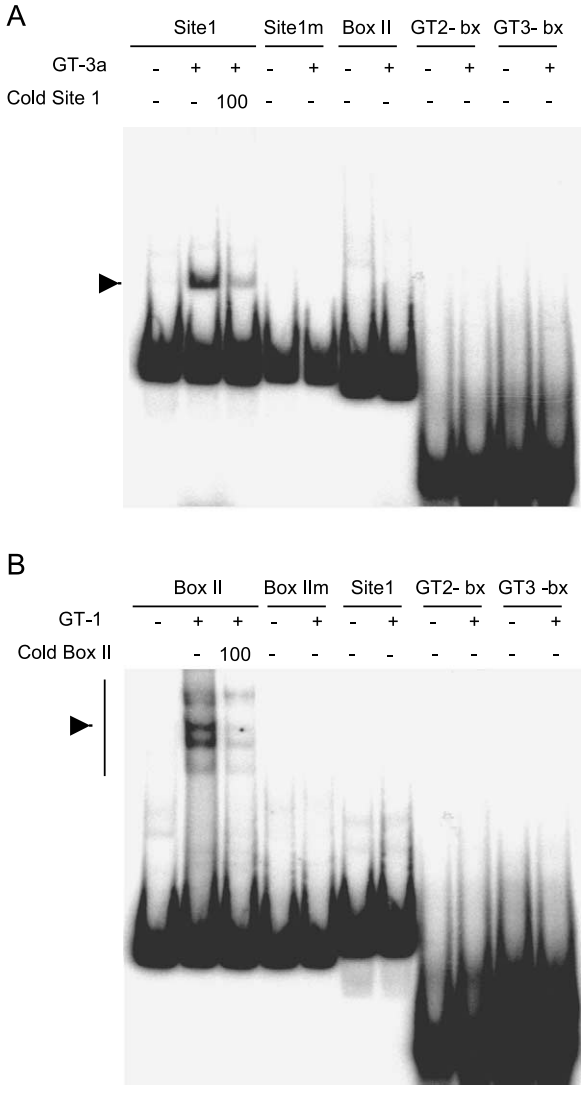


Fig. 6. Binding site localisation of GT-3a within the *cab2* −37 to −201 promoter region. A: Schematic representation of the oligonucleotide pairs used as probes. B: Gel shift assays with the GT-3a recombinant protein and the seven oligonucleotide pairs. C: DNA sequence of the promoter region from −37 to −201. Previously characterised *cis* elements on this promoter are highlighted by bold-faced letters (G-Box) or underlined (GATA motifs). The putative TATA box is shaded by light grey and the putative GT-3a-binding site is shaded by dark grey. D: Gel shift assay with dimers of either the wild-type (cab2w) or the mutant version (cab2m) of the *cab2* promoter sequence from −81 to −110 used as probes (see Section 2.7). Chevrons indicate retarded bands.

the binding sequence of GT-3a within the *cab2* region from −201 to −37, seven pairs of oligonucleotides of 40 bases were synthesised and used as probes in gel shift assays. Pairs 1, 2, 3 and 4 covered head to tail the promoter region and pairs 5, 6 and 7 overlapped with pairs 1 and 2, 2 and 3 and 3 and 4, respectively (Fig. 6A). A clear shift was observed with pairs 3 and 7, but a much stronger signal was produced with pair 3 (Fig. 6B). Competition experiments showed that the binding was specific (not shown). No clear binding was observed with pair 6 that contains sequences corresponding to the 5′ half of pair 3, suggesting that the DNA bases necessary for the binding were located somewhere from the middle to the 3′ half of pair 3. Comparison of this region to previously characterised GT elements showed the core sequence 5′-GTTAC of Site 1



C

	TGT**GGTTAA**TA	BoxII	I
	TGT*CC***TTAA**TA	BoxIIm	
	GGC**GGTAAT**TA	GT2-bx	II
	CGA**GGTAAA**TC	GT3-bx	
	ATT**GTTAC**CAT	Site1	III
	ATT*C***T***CGA*CAT	Site1m	
-102	AAC**GTTAC**AAT -91	cab 2	
-271	ACG**GTTAC**ACT -281	cab 2	
-397	AAT**GTTAC**TTT -387	cab 2	
-194	GCC**GTTAC**CAA -184	rbcS-1A	

defined from the *rps1* promoter [6] matched exactly the sequence situated between −99 and −95 of the *cab2* promoter (Fig. 6C). Two additional pairs of oligonucleotides were synthesised, one corresponding to the wild-type sequence of the *cab2* region from −110 to −81 (cab2w), the other carrying the same sequence except that the GTTAC bases were replaced by CTCAT (cab2m) (see Fig. 6D). These oligonucleotides were tested by gel shift assays with GT-3a. This mutation completely abolished the binding, suggesting that this sequence was required for the binding of GT-3a.

3.7. GT-3a binding to the Site 1-type GT elements

To test whether GT-3a could bind to Site 1 and other previously characterised GT elements including Box II, GT2-bx and GT3-bx, these elements and a few mutant versions were used as probes for gel shift assays. As shown in Fig. 7, GT-3a was only able to bind to wild-type Site 1 in the gel shift assays (Fig. 7A), whereas recombined GT-1 bound only to Box II (Fig. 7B). These data indicate that GT-3a binds specifically to the 5′-GTTAC core-containing Site 1 type of GT elements. As shown earlier, the *cab2* promoter may have at least three GT-3a-binding sites. Indeed, two additional 5′-GTTAC sequences were found in the position between −278 and −274 (in reverse orientation) and −394 to −390 of the promoter (Fig. 7C). Consistent with the gel shift results, only one site was found in *rbcS-1A* at −191 to −187 relative to the ATG initiation codon (Fig. 7C). No 5′-GTTAC was found in the *cab1* or the *chs* promoter fragment.

4. Discussion

Plant transcription factors belong to many large families that may have been produced as a result of extensive gene duplication [18]. The amplification of these transcription factors in plants implies their functional redundancy and/or regulatory diversity obtained through differential expression and combinatory interaction. GT factors are among the first transcription factors identified in plants. However, their regulatory function remains largely unknown. No developmental and physiological function identified through mutant screens has been assigned to GT factors so far, which may be in part due to functional redundancy of this family of transcription factors.

High conservation and protein dimerisation between GT-3a and GT-3b as well as their similar expression profiles suggest that they may have similar evolutionary origin and function. The DNA regions containing the GT-3a (in chromosome 5) and GT-3b (in chromosome 2) genes indeed belong to a large segmental duplication of the *Arabidopsis* genome: between the segments from At5g01030 to At5g01870 and At2g37930 to At2g38530 (http://wolfe.gen.tcd.ie/athal/dup). GT-3a and GT-3b are likely to have been generated after a duplication event.

Unlike GT-1 and GT-2 that are expressed constitutively [11,12], the expression of GT-3a and GT-3b showed some tissue or organ specificity (Fig. 3, not shown). High expression levels of GT-3a in flower buds and in roots suggest that it may be involved in the control of expression of target genes in those organs.

Our data (Fig. 2) and previous results [12,24] suggest that single-trihelix-domain GT factors may function in forms of dimers. The truncation of the C-terminus of GT-1 that is responsible for the dimerisation abolishes the binding activity of GT-1 [12,24]. There exists a GT-1 homologous factor gene (At3g25990) named GT-4 [25]. This homologous factor could bind specifically to Box II and form dimers with GT-1 (unpublished results), suggesting that GT-1 and this closely related neighbour may function as heterodimers to regulate gene expression. The dimerisation domain of GT-3a also seems to be located at the C-terminal end of the protein (Fig. 2). The coils2 program predicts that the C-terminus of both GT-3a and GT-3b has a coiled-coil protein dimerisation domain, which is not observed in GT-1 or GT-2. This may explain why no cross-interaction between GT-1 and GT-3a or GT-3b was observed and suggests that both subgroups of factors may function independently.

Like GT-1, GT-3a and GT-3b contain one single trihelix domain, while GT-2 and related factors contain two trihelix domains and bind to two GT elements at once [11,12]. The binding specificity of GT-1-type factors is distinguished from that of the GT-2-type factors [11,12], although the core sequences of the binding sites are quite similar (compare Box II to GT2-bx and GT3-bx, Fig. 7C). Our experiments could not detect binding activity of GT-3a to either Box II, GT2-bx or GT3-bx (Fig. 7A). Conversely, recombined GT-1 could not bind to the GT-3a site (i.e. Site 1) either (Fig. 7B). Comparison between the sequences of Site 1 of *rps1* and the GT-3a-binding sites found in *cab2* and *rbcS-1A* showed that only the 5′-GTTAC core sequence was conserved. The immediate flanking nucleotides were divergent (Fig. 7C), suggesting that they may be not essential for the binding. These data indicate that GT-3a is specific to the Site 1 type of GT elements, and that GT factors bind to DNA in a strikingly high sequence-specific manner.

Taken together, the data presented in this report suggest that GT-3a and GT-3b belong to a third subgroup of GT factors. Each subgroup recognises a specific type of GT elements previously shown to have distinct regulatory function [3,5,6].

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Fig. 7. GT-3a binds specifically to Site 1-type GT element via the 5′-GTTAC core. Gel shift assays with the GT-3a (A) and GT-1 (B) recombinant proteins. GT elements previously characterised were used as probes as dimers, except Box II and Box IIm that were in tetramer. For sequences of these elements, see C. Where indicated, cold probes and non-specific (NS) DNA in different molar excess were included in the binding assays. Chevrons indicate shifted bands. Site 1m: mutant version of Site 1; Box IIm: mutant version of Box II. C: DNA sequences of three types of GT elements (I, II, III) and the mutant versions (Box IIm, Site 1m) used in the gel shift assays. The GT-3a-binding sites found in the *cab2* and *rbcS-1A* gene promoters are classified into type III. The consensus sequences are in boldfaced letters, substitutions in the mutant versions are in italic letters.

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