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ORIGINAL ARTICLE

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Seed-specific transcription factors ABI3 and FUS3: molecular interaction with DNA

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Abstract In *Arabidopsis thaliana* (L.) Heynh. the seed-specific transcription factors ABI3 and FUS3 have key regulatory functions during the development of mature seeds. The highly conserved RY motif [DNA motif CATGCA(TG)], present in many seed-specific promoters, is an essential target of both regulators. Here we show that, in vitro, the full-length ABI3 protein, as well as FUS3 protein, is able to bind to RY-DNA and that the B3 domains of both transcription factors are necessary and sufficient for the specific interaction with the RY element. Flanking sequences of the RY motif modulate the binding, but the presence of an RY sequence alone allows the specific interaction of ABI3 and FUS3 with the target in vitro. Transcriptional activity of ABI3 and FUS3, measured by transient promoter activation, requires the B3 DNA-binding domain and an activation domain. In addition to the known N-terminal-located activation domain, a second transcription activation domain was found in the B1 region of ABI3.

Keywords ABI3 · *Arabidopsis* · FUS3 · Transcription factor · DNA binding · Transient expression

Abbreviations ABI3: Abscisic acid insensitive 3 · FUS3: FUSCA 3 · GUS: β-Glucuronidase · LEC1(2): Leafy cotyledon 1 (2) · RY: DNA motif CATGCA(TG)

Introduction

Seed development in plants is divided into two major phases: early embryogenesis and seed maturation. During early embryogenesis all embryonic structures are formed (Mayer et al. 1991). After a growth phase, cell division arrests (Goldberg et al. 1994) and the seed enters a maturation pathway. This includes the accumulation of reserve compounds like storage proteins, carbohydrates and lipids. Finally, the seeds become dormant and tolerant to desiccation, necessary properties for the survival of the embryo. The transition from early embryogenesis to maturation has been extensively studied by genetic analysis. Several loci have been detected that control the maturation of embryos and germination, e.g. the *VP1* gene in maize (McCarty et al. 1991; McCarty 1995) and the genes *ABI3* (abscisic acid insensitive 3), *FUS3* (FUSCA3), *LEC1* (leafy cotyledon 1) and *LEC2* in *Arabidopsis thaliana* (Müller 1963; Koorneef et al. 1994; Meinke et al. 1994; West et al. 1994). The relationships between *ABI3*, *LEC1* and *FUS3* have been studied in detail by investigating the phenotypes of double mutants (Bäumlein et al. 1994; Meinke et al. 1994; Parcy et al. 1997). The combination of *abi3* with *lec1* or *fus3* leads to highly pigmented and extremely viviparous embryos with a dramatically reduced protein content (Raz et al. 2001). These data suggest that LEC1, FUS3 and ABI3 act in concert to regulate pathways controlling late embryogenesis and seed maturation. All four regulatory genes from *Arabidopsis*, *FUS3*, *LEC1*, *ABI3* and *LEC2*, have been cloned and sequenced (Giraudat et al. 1992; Luerssen et al. 1998; Lotan and Harada 1998; Stone et al. 2001). Genes homologous to *ABI3* and *VP1*, which are the prototypes of that class of gene, have been identified in various plant species (summarized by Kurup et al. 2000; Lazarova 2002). The corresponding proteins are all conserved in the B1, B2 and especially in the B3 regions, suggesting a general requirement of these basic domains for the function of *ABI3*/*VP1*-like genes (Holdsworth et al. 1999). A B3 DNA-binding domain is present also in the two other seed-specific transcription factors FUS3 and LEC2 (Luerssen et al. 1998; Stone et al. 2001). Many seed-specific gene promoters contain a highly conserved motif, called RY, with the sequence CATGCA(TG) about 100 bp upstream of the transcription

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start (Dickinson et al. 1988). The RY motif represents the central core of the previously identified 28-bp-long legumin box (Bäumlein et al. 1986) and its deletion abolishes most of the seed-specific promoter activities. This was shown for the legumin (Bäumlein et al. 1992) and for the napin promoter (Ellerström et al. 1996; Stalberg et al. 1996). Transient-expression studies in protoplasts demonstrated the functional link between the RY motif and the B3 domain containing transcription factors such as PvALF, ABI3 and FUS3 (Bobb et al. 1997; Ezcurra et al. 2000; Reidt et al. 2000, 2001). Moreover, the isolated B3 domain of the VP1 factor was found to bind specifically to the Sph/RY element TCCATGCAT involved in the VP1-mediated induction of the C1 gene (Suzuki et al. 1997). However, up to now there has been no clear evidence that the complete VP1 protein or the related ABI3 is able to bind to DNA. Specific binding of FUS3 to the RY element of the legumin gene promoter has been directly demonstrated by band-shift assay (Reidt et al. 2000). Here, we studied the molecular interactions of recombinant FUS3 and ABI3 proteins with RY-containing seed-specific promoter fragments, using BIAcore and ELISA (enzyme-linked immunosorbent assay) techniques. The use of these two techniques as an alternative to the band-shift assay has been proven in detail (Fisher et al. 1994; Benotmane et al. 1997; McKay et al. 1998). In addition, we investigated by transactivation experiments how both factors effect transcriptional activity.

Materials and methods

Production of recombinant proteins

The plasmid pcabi3-4F containing the ABI3 cDNA was kindly provided by J. Giraudat (Giraudat et al. 1992). The FUS3 cDNA was a gift from H. Luerssen (Luerssen et al. 1998) and the LEC1 cDNA a gift from J. Harada (Lotan and Harada 1998). cDNAs and fragments were subcloned into the expression vector pET23a (Novagen) via PCR amplification. The restriction sites *Mfe*I and *Sal*I, which are compatible with *Eco*RI and *Xho*I, were used for subcloning of the complete ABI3 cDNA, and *Eco*RI and *Sal*I for subcloning of the B3 domain of ABI3 (amino acids 559–677). The FUS3 cDNA and its subfragments were inserted into *Eco*RI and *Xho*I sites. The subfragments code for the amino acid sequences 74–312 (AN1), 131–312 (AN2) and 82–195 (B3). The same cloning strategy was used for LEC1. Primers were obtained from Metabion. Proteins were produced in the *Escherichia coli* strain BL21(DE3). Cells were grown at 35°C in LB-medium supplemented with 50 mg l^{-1} carbenicillin. Usually, 500 ml of fresh medium was inoculated with 10 ml of a log-phase pre-culture stored overnight at 4°C. Flasks were vigorously shaken. When the culture reached an optical density at 600 nm of 0.4–0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and cultivation was continued for 3 h. The weakly inducible ABI3 protein was produced from a 2-l culture. Cells were centrifuged, washed, resuspended in 15–20 ml binding buffer (see protein purification) per 500 ml culture and frozen at −70°C.

Primers used for PCR-mediated cloning were:

- For ABI3: abifor: GACCCAATTGAAAAGCTTGCATGTG GCG and abiback: CAGGTCGACTTTAACAGTTTGAGAA GTTGG; B3 of ABI3: abiBDfor: GACGAATTCCAGG GA TGGAAACCAGAA and abiBDback: CAGGTCGAC GCTC GGTTGTCTTACTTT.
- For FUS3: fusfor: GAGGTGAATTCGTTGATGAAAATG TGGAA and fusback: CAACCTCGAGGTAGAAGTCA TCGAGAGA; AN1: GAGGTGAATTCCCAACTCCTCTCC CCGCA and fusback; AN2: GAGGTGAATTCGAAGATTT GGACGGTTTT and fusback; B3 of FUS3: fusBDfor: GAG GTGAATTCATTGACCCAAGAAAGCTA and fusBDback: CAACCTCGAGTTCTTCCGATGCTTTTCT.
- For LEC1: lecfor: GAGGAATTCACCAGCTCAGTCATA GTA and lecback: CCACTCGAGCTTATACTGACCATA ATGGTA.

Protein purification

Cells were disrupted by ultrasonic treatment and centrifuged at 10,000 rpm at 4°C for 30 min. The supernatant was applied to a 1.5-ml Ni-NTA agarose (Qiagen) column to bind His-tagged proteins. Adsorption, washing and elution were performed as described by the manufacturer (Qiagen, Novagen). All buffers contained 20 mM Tris–HCl (pH 7.9), 300 mM NaCl and varying concentrations of imidazole: binding buffer 10 mM; washing buffer 20 mM for ABI3 protein and 30 mM for all other proteins, and elution buffer 100 mM and 250 mM, respectively. Glycerol was added to the eluted proteins to a final concentration of 50% to prevent precipitation. Proteins were stored at −20°C. The ABI3 protein was further purified by FPLC (Äkta Explorer; Amersham Pharmacia Biotech) with a Superdex 75 HR 10/30 column. 500 µl of solution containing 200 µg of protein was loaded onto the column and eluted with buffer containing 10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 (HBS-EP buffer; BIAcore).

Western blot analysis

Proteins were separated by SDS–PAGE and transferred to nitrocellulose. Commercially available anti-T7 tag (Novagen) and anti-His tag (Sigma) antibodies were used for the identification of the recombinant proteins. Immunological procedures were done as described by the manufacturer.

Oligonucleotides for binding studies

Pairs of base complementary 42-bp-long single-stranded oligonucleotides were obtained from Metabion and Operon. Each top strand was biotinylated at the 5′ end. Oligonucleotides were dissolved in sterile water at a concentration of 100 pmol μl^{-1}. To obtain double-stranded RY-containing and other DNA fragments, two complementary oligonucleotides (1 nmol each) were transferred to 500 µl TES buffer (10 mM Tris–HCl, 1 mM EDTA, 300 mM NaCl). The mixture was heated in a water bath to 95°C for 5 min and cooled slowly to room temperature for annealing.

BIAcore and ELISA

For BIAcore analysis, biotinylated double-stranded DNA fragments were immobilized on streptavidin-coated (SA) sensor chips. Sensor chips were pretreated as recommended by the manufacturer (BIAcore). DNA fragments were diluted to 0.1 pmol μl^{-1} in 10 mM HEPES (pH 7.4), 300 mM NaCl, 0.005% polysorbate 20 (HBS-P buffer; BIAcore), and a 40-µl aliquot was injected with a flow rate of 5 µl min^{-1}. The amount of immobilized oligonucleotide was about 1,000 resonance units (RU) corresponding to 1.2 ng DNA per mm^2 (Fisher et al. 1994). Proteins were diluted in HBS-P buffer to the extent that after injection of 30 µl the final elevation of RU was below 1,500. Flow conditions were 10 µl min^{-1}. For regeneration of the chip surface, 5 µl of 1 M NaCl followed by 5 µl of 0.05% SDS were injected after each binding experiment.

For ELISA measurements, streptavidin-coated microwell strips (Nunc) were used. The double-stranded biotinylated 42-bp-long DNA fragments were diluted to 0.1 pmol μl^{-1} in TBS-T [20 mM Tris–HCl (pH 7.4), 180 mM NaCl, 0.1% Tween-20]. 80 µl of this solution was added to each well and immobilized by incubation at 37°C for 1 h. Three washing steps followed, each extended to 5 min incubation as recommended by the manufacturer. Strips were blocked with 3% BSA in TBST for 1 h. Transcription factors and their fragments were diluted in HBS-P buffer or TBST containing 0.2% BSA. Binding to DNA, which occurs during incubation at room temperature for 1 h, was followed by washing three times with TBST. An antibody against T7 tag conjugated with alkaline phosphatase (Novagen) was used to detect bound transcription factors. Incubation was performed for 1 h, again followed by three washes with TBST. Phosphatase activity was determined with *p*-nitrophenyl phosphate (pNPP) dissolved in diethanolamine–HCl buffer (pH 9.8). After incubation at 37°C the optical density at 405 nm was measured with a plate reader (Dynatech MR7000).

Isolation of protoplasts from suspension cultures and transient-expression assay

Protoplasts were prepared from cells of of *Arabidopsis thaliana* (L.) Heynh. and transformed essentially as described for tobacco protoplasts by Reidt et al. (2000). Transformation was performed without heat shock. 20 µg DNA of the USP promoter–*β*-glucuronidase (GUS) reporter plasmid and 10 µg of the plasmid expressing the transcription factor or a fragment of it were used for transformation. The construction of the USP–GUS plasmid and the assay conditions have also been described previously (Reidt et al. 2000). For construction of the transfactor plasmids, cDNAs of ABI3 and FUS3, or derivatives of them, were transferred into the plasmid pRT103 via PCR-mediated cloning. Insertion sites used were *Bam*HI and *Xba*I for ABI3 and *Xho*I and *Xba*I for FUS3 constructs.

Primers used for PCR were:

- ABI3: RTabiN: CAGGGATCCATGAAAAGCTTGCATGTG and RTabiC: GACTCTAGATCATTTAACAGTTTGAG AAGT; ABI3/1: CAGGGATCCATGTCTTCCTTGGAACA GGAC and RTabiC; ABI3/2: CAGGGATCCATGG-ATC CAACAGGTTTC and RTabiC.
- FUS3: RTfusN: GGACTCGAGATGGTTGATGAAAAT GTG and RTfusC: CAGTCTAGACTAGTAGAAGTCA TCGAGAGA; FUS3/1: RTfusN and RTfusBDC: CAGTCT AGACTATTCTTCCGATGCTTTTCT; FUS3/2: RTfusBDN: GGACTCGAGATGATTGACCCAAGAAAG and RTfusC; FUS3/3: RTfusBDN and RTfusBDC.

Results

Interaction of ABI3 and FUS3 with DNA in vitro

Although the ABI3 protein contains a B3 DNA-binding domain, like FUS3, and requires the RY target motif for promoter transactivation (Ezcurra et al. 2000; Reidt et al. 2000), its binding to DNA is still obscure. We wanted to discern whether the full-length ABI3 protein can interact directly with DNA, like FUS3 (Reidt et al. 2000). In order to investigate the functional significance of the B3 domains for DNA binding, different fragments of both proteins were also checked for DNA binding. Protein–DNA interactions were studied using proteins as soluble binders to solid-phase fixed RY-DNA, both by surface plasmon resonance (BIAcore) and by ELISA-type tests. Soluble proteins were produced with the pET system in *E. coli* and purified by Ni-NTA technology. The recombinant proteins contain the T7 tag at the N-terminal end and the His tag at the C-terminus. In contrast to all other expressed proteins in this study, the ABI3 protein was poorly expressed, and purification and enrichment at the Ni-NTA matrix was less efficient. In spite of this, we could clearly demonstrate binding of the ABI3 protein contained in the Ni-NTA-purified fraction (IMAC) to RY-DNA, using BIAcore (Fig. 1a,d). To exclude the possibility that degraded fragments, rather than the full-length ABI3 protein, are the binding molecules, the protein was further purified on a Superdex 75 column by FPLC. Purification was monitored by SDS gel electrophoresis and tag-specific western blot analysis (Fig. 1a–c). DNA binding activity was found in the high-molecular-weight fractions (lanes 17–20, Fig. 1d), as would be expected for full-length ABI3. However, C-terminal His-tag-labelled degradation fragments are still visible within this preparation (Fig. 1c). No degradation products are detectable by labelling the N-terminus (T7 tag, Fig. 1b). Therefore, we chose ELISA as confirmation of binding of ABI3 to RY. With the help of an anti-T7-tag antibody the specific binding of full-length ABI3 to RY could be verified unambiguously (Figs. 2b, 4).

The modular structure of the ABI3 protein is shown schematically in Fig. 2a. In Fig. 2b the specific binding of the subcloned B3 domain of ABI3 is shown. The isolated B3 domain binds to DNA, as does the complete ABI3. The activation domain A and the basic regions B1 and B2 did not influence DNA binding. At concentrations higher than 2 $\mu g\ ml^{-1}$ for ABI3 and 0.5 $\mu g\ ml^{-1}$ for B3, both factors also showed progressive interactions with DNA in which RY was destroyed (RYdes) and with random nucleotide sequences. The seed-specific transcription factor LEC1 did not bind to RY-DNA or to RYdes-DNA and served as a negative control (Fig. 2b). To analyse the modular anatomy of binding of FUS3 to RY-DNA we constructed a series of truncated derivatives of FUS3 (Fig. 3a). Fragments of FUS3 containing the complete B3 domain (AN1, B3) bind to RY-DNA with the same efficiency as the complete protein. The fragment with a truncated B3 domain (AN2) lost the ability to recognize the RY motif (Fig. 3b). The binding behaviour of the complete FUS3 protein was identical to that of the isolated B3 domain in these tests. The specificity of DNA binding is demonstrated in Fig. 3c. Binding of FUS3 to RYdes was not observed at the highest concentration of 20 $\mu g\ ml^{-1}$, but the isolated B3 domain showed interaction with RYdes and a random sequence when used at concentrations higher than 0.5 $\mu g\ ml^{-1}$, comparable to B3 (ABI3). In summary, the B3 domains of ABI3 and of FUS3 are necessary and sufficient to bind the full-length proteins to the RY element.

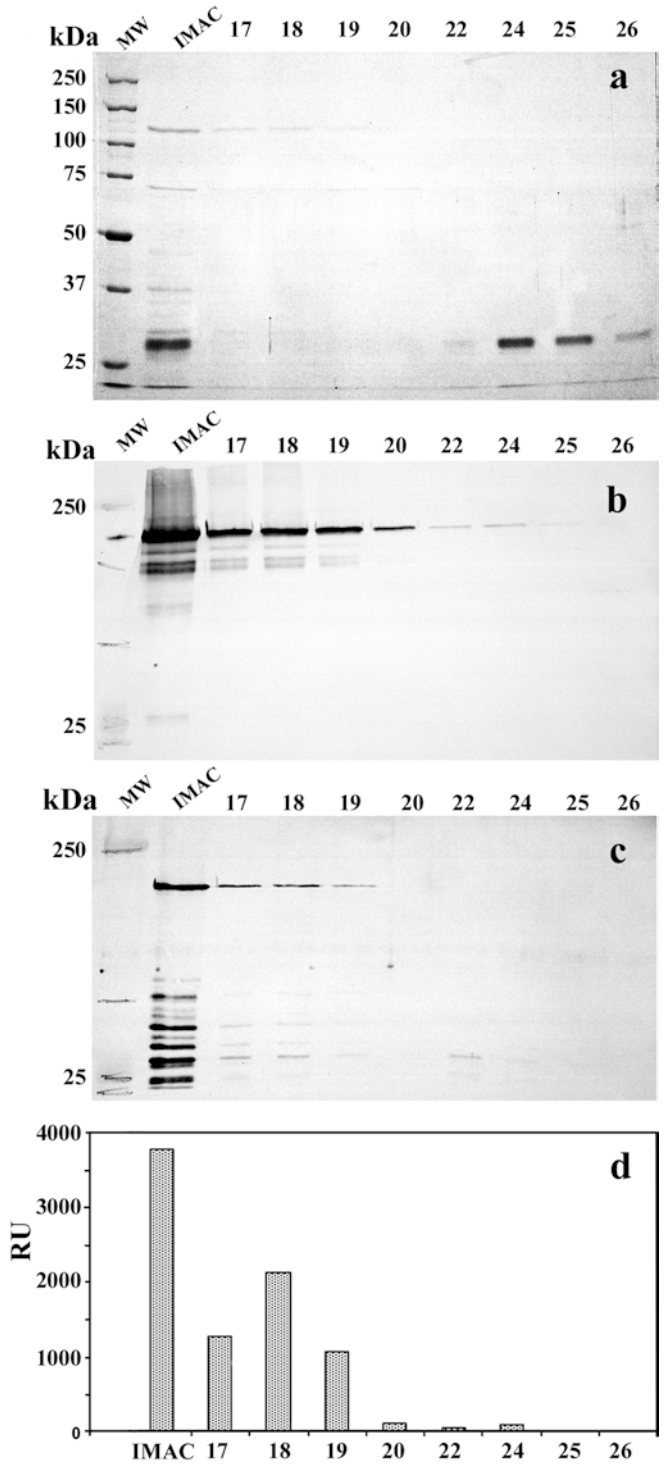

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Fig. 1a–d Purification of recombinant ABI3 protein and binding to RY-DNA. *Arabidopsis thaliana* ABI3 protein purified by Ni-NTA resin was further fractionated by FPLC on a Superdex G-75 column. Fractions were checked for purity by SDS–PAGE (**a–c**). Interaction with a 42-bp DNA fragment of the legumin box containing the RY motif was measured with BIAcore (**d**). **a** Coomassie-stained PAA-gel; **b,c** Identification of ABI3 protein by anti-T7 tag antibody (**b**) and by anti-His-tag antibody (**c**) in western blot analysis. **d** Binding activity measured with BIAcore is given in resonance units. *Lanes* : *MW* molecular weight standards; *IMAC (immobilised-metal affinity chromatography)* Ni-NTA-resin-purified ABI3 extract; *17–26* fractions of the FPLC separation of the IMAC-eluate. The apparent molecular mass of ABI3 as determined by SDS–PAGE (120 kDa) was higher than expected (80 kDa), as also observed by Parcy et al. (1994)

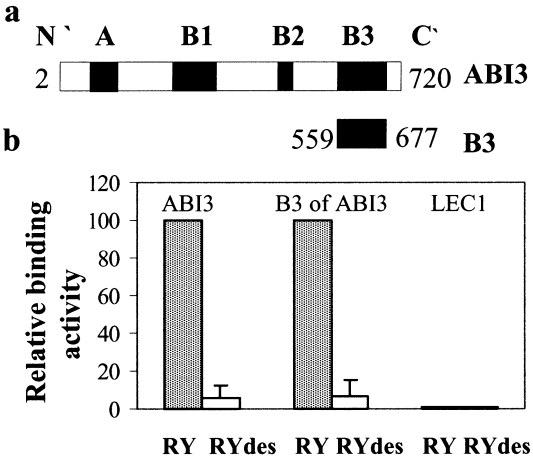

Fig. 2a,b Specific binding of *A. thaliana* ABI3 protein and isolated B3 domain of ABI3 to RY-DNA. **a** Diagram of the ABI3 protein showing conserved regions (*black boxes*): activation domain A and the three basic regions B1, B2 and B3. The isolated B3 domain is also shown. **b** Binding of ABI3 and the B3 domain of ABI3 to the legumin box DNA fragment containing an intact (*RY*) or destroyed (*RYdes*) RY motif. Binding was measured by ELISA. Transcription factor LEC1 served as a negative control. Data of three independent experiments are given as relative values. Sequences of the used oligonucleotides: RY: AATTCGGACTCCATAGC**CATGCATG**CTGAAGAATGTCACACA (Bäumlein et al. 1986; Reidt et al. 2000); RYdes: AATTCGGACTCCATAGC**TGACCA GC**CTGAAGAATGTCACACA

Influence of RY-element flanking sequences on DNA binding of FUS3 and ABI3

Up to now, only sequences from seed-specific promoters containing the RY element have been used to investigate their interactions with the seed-specific transcription factors FUS3, ABI3 and VP1. It has not been proven if RY motifs contained in other promoters can serve as targets for binding of FUS3 or ABI3. Little is known about the influence of RY flanking sequences on the binding characteristics of seed-specific transcription factors to the RY element (Bobb et al. 1997; Suzuki et al. 1997). Flanking regions may determine specificity; therefore, we studied the binding of FUS3 and ABI3 to 15 different natural RY-containing promoter sequences, each of 42 bp in length (Table 1). These were extracted from the *Arabidopsis* Genome Database at MIPS (http://mips.gsf.de/) by using the following parameters. First, a TATA box had to exist 40–100 bp downstream of the RY motif, a distance that is found in known seed-specific promoters. Second, a gene had to be annotated within 500 bp downstream of the TATA box. A series of about 200 sequences matched these conditions. 13 sequences from seed-specific and other promoters were

selected for binding experiments with FUS3 and ABI3 (Table 1). Additionally, we included the RY-containing sequences from the well-studied seed-specific USP and LeB4 promoters from *Vicia faba* (Bäumlein et al. 1991a, 1991b; Table 1), and an arbitrary sequence without the RY motif. All sequences, except the arbitrary one, contain the RY core motif CATGCA, as shown by the nucleotide-frequency analysis in Table 2. The binding of FUS3 and ABI3 to the selected RY-containing sequences was measured by ELISA-type experiments with biotinylated 42-bp-long DNA fragments fixed to the solid phase. FUS3 and ABI3 binding activities to the legumin box in the LeB4 promoter fragment were defined to be 100% (Fig. 4). The binding to the other DNA fragments is given relative to the binding activity of either ABI3 or FUS3 to the LeB4 sequence. FUS3 and ABI3 bound to all selected DNA fragments, which contained an RY motif. The relative binding activities differed within a range of ±50% and were similar in tendency for FUS3 and ABI3. The differences in binding to the targets were more pronounced for ABI3 than for FUS3. Remarkably, the highest relative binding activity was measured for the target containing two RY elements, the promoter of the 2S albumin gene (Fig. 4, column 13). The results show that the RY element itself mainly determines the interaction with FUS3 and ABI3.

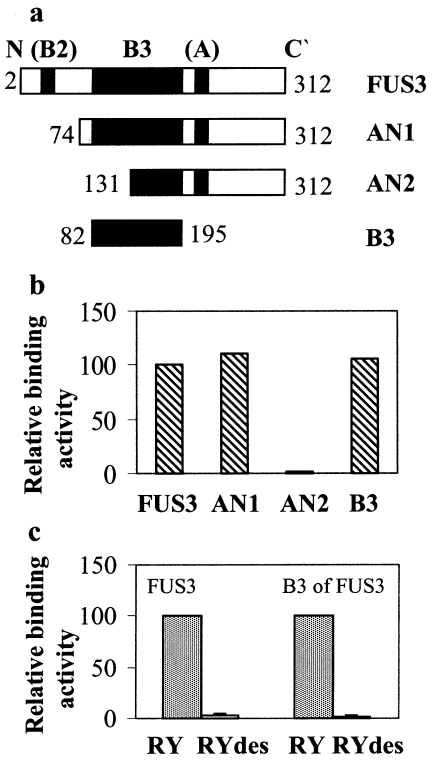

Fig. 3a–c Binding of *A. thaliana* FUS3 and FUS3 fragments to RY-DNA. **a** FUS3 protein and the FUS3 fragments AN1, AN2, and B3. Putative protein domains B2, B3 and A are indicated (*black boxes*). **b** Binding of complete FUS3 and FUS3-derived fragments to the RY-containing DNA fragment, measured by ELISA. **c** Specificity of binding of FUS3 and the B3 domain of FUS3 to intact RY. Data of three independent experiments are given as relative values. Sequences of the oligonucleotides RY and RYdes are given in the legend of Fig. 2

Transcriptional activity of ABI3 and FUS3

Binding to DNA is one precondition for the action of the factors in the activation of transcription. A protoplast-based transient-expression system was used to study the functions of other domains in transcription initiation in vivo, as described by Reidt et al. (2000). Protoplasts of *Arabidopsis thaliana* were transformed with a plasmid carrying the *GUS* gene under control of the USP promoter as a target construct and a second plasmid coding for the transfactor/fragments under control of the 35S cauliflower mosaic virus (CaMV) promoter. Cells transformed with the USP–GUS

Table 1 Selected RY-containing DNA sequences for binding studies. Genes known to be expressed specifically in seeds are labelled with an *asterisk* (*). The RY element is indicated in *bold* letters

Genes downstream of the selected RY regions from *Arabidopsis thaliana* and *Vicia faba* (1 and 2)	Sequence of the RY-containing DNA fragments
1 Legumin (LeB4)*	AATTCTGACTCCATAGC**CATGCATG**CTGAAGAATGTCACACA
2 USP (unknown seed protein)*	ACACAAGTTTTGAGGTG**CATGCATG**GATGCCCCTGTGCCACT
3 Myo-inositol-1-phosphate-synthase	GAAGCATCACTAGCTCA**CATGCAT**TGGACAAATAGGGACCAT
4 Peroxidase ATP24a	ATTGTAGATGAAAATAT**CATGCA**CTAATACGAGTTTCCTTAA
5 Oxidoreductase, putative	TGGAGATGTGTGAACCA**CATGCATG**TGGAATTTATGATTAAT
6 Beta-glucosidase	ACACAACGGTGCGAGGT**CATGCA**ATGAATCATCCTATATATA
7 12S cruciferin, seed storage protein*	GCTTAGACTGAAGTCCC**CATGCA**AACCTAATCCTACGTGGCT
8 Lipase, putative	TGAAAATGATTTATACG**CATGCAT**CATGAGCATTATTGTATT
9 Peroxidase, putative	CCACTTTCTTAATTATG**CATGCAT**CTCCGAGGCGCACCCTTG
10 Gibberellin-regulated protein GASA2	CAAAGAAAAACTACGGA**CATGCATG**CACCCAATTATATACAT
11 Putative trypsin inhibitor*	TAGTATAAAAACTTTCT**CATGCA**ATTATATAGAAATGTATAT
12 Germin-like protein	CGTACACCCAAAGTTCA**CATGCATG**CAATGCTAAAAAAATAA
13 2S albumin precursor, napin*	CTCTCTCCACACACACT**CATGCATGCATGCA**TTCTTACACGT
14 Globulin-like protein*	CCACTAATACCGATATG**CATGCAT**ATCTCAATATCGCATCGA
15 Cell division-controlling protein kinase, putative	GGCTTTACATCAAAGAT**CATGCAT**AGATTCATTCAACTAAAC
16 Arbitrary sequence	GTACTCATACTGTCGCAATGAGTCATCTGTCACAGAGTTCAG

Table 2 Nucleotide frequency analysis of the 15 potential promoter sequences of Table 1. The numbers 1–42 in the top row are the base-pair numbers of the 42-bp oligonucleotides of Table 1. The values in the table show the distribution of the single nucleotides within the 15 sequences. Sequence 16 is not included since it is not a potential promoter

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
A	4	4	7	4	5	9	5	4	7	3	6	6	9	4	5	2	4	0	15	0	0	0	15	3	3	2	8	3	6	6	7	5	4	4	6	4	4	5	5	6	7	5
C	5	5	2	4	4	0	3	6	2	3	4	4	0	3	2	7	2	15	0	0	0	15	0	1	2	5	3	2	3	6	3	2	4	3	2	1	5	4	4	4	3	1
G	3	4	2	2	2	1	3	3	1	3	1	3	4	1	4	3	4	0	0	0	15	0	0	0	6	4	2	3	3	2	3	2	1	1	3	3	4	1	1	1	2	1
T	3	2	4	5	4	5	4	2	5	6	4	2	2	7	4	3	5	0	0	15	0	0	0	11	4	4	2	7	3	1	2	6	6	7	4	7	2	5	5	4	3	8

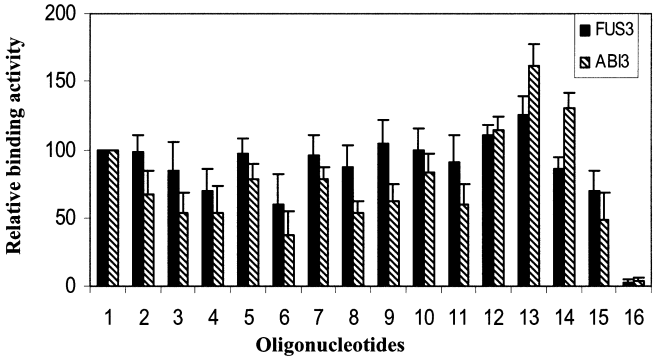

Fig. 4 Binding of *A. thaliana* FUS3 and ABI3 to 42-bp DNA fragments, which differ in flanking sequences of the RY element CATGCA. Sequences of the DNA fragments and the corresponding genes are listed in Table 1. *Sequences*: *1, 2* promoter elements from *Vicia faba*; *3–15* virtual extracted promoters from the genome of *Arabidopsis thaliana*; *16* an arbitrary sequence without the RY element, as a control. Columns represent relative binding values. Binding to the legumin promoter-derived fragment (former RY) was defined as 100%. Each value represents the mean and SD of three independent ELISA experiments

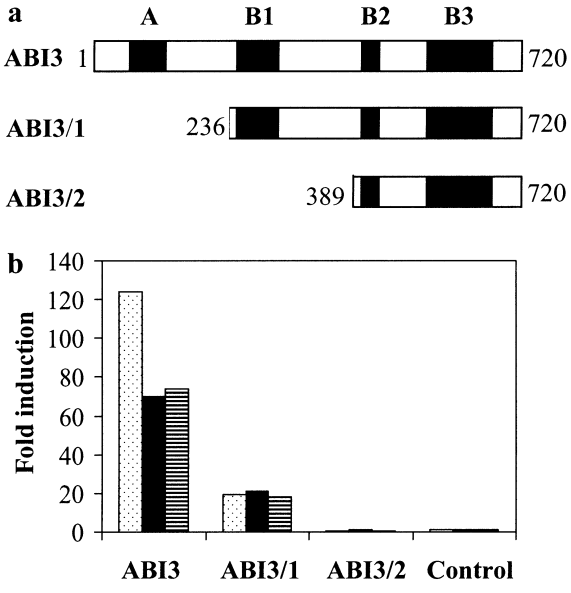

Fig. 5a,b Transcriptional activities of the *A. thaliana* ABI3 and ABI3 fragments. **a** Scheme showing the fragments used. **b** Transient-expression assay of the USP promoter activity. Three independent experiments are shown. The effects of ABI3 and derived fragments on USP promoter activity are given as *n*-fold induction compared to the promoter construct alone

construct alone showed low GUS-basis expression. The effect of the ABI3 protein and protein fragments, and FUS3 protein and protein fragments, on the stimulation of the USP promoter was determined by the *n*-fold increase in GUS activity related to the base activity. The protein fragments used and the binding results achieved are shown in Figs. 5 and 6. In the presence of ABI3 protein the USP promoter is stimulated 60–120-fold. Deletion of the N-terminally located activation domain reduced the stimulation, but not completely. The promoter was still stimulated about 20-fold. Deletion from the N-terminus to the basic B2 domain depleted stimulation completely, and in two of three experiments the activity was below the background activity (Fig. 5). FUS3 protein stimulated USP activity 15–40-fold (Fig. 6). When the N-terminal part upstream to the B3 domain was deleted the stimulation of the USP promoter was even increased compared to the whole FUS3 protein. In contrast, deletion of the C-terminal part caused the loss of any stimulation. Obviously, in that case the activation domain had been deleted. An activation domain localized downstream of the B3 domain was also found by Reidt (2002) using the yeast two-hybrid system. The B3 domain alone did not show any effect on USP promoter activity (Fig. 6).

Discussion

Previous genetic data suggest that the transcription factors FUS3 and ABI3 act in concert to regulate key pathways during late embryogenesis and seed maturation (Bäumlein et al. 1994; Parcy et al. 1997; Raz et al. 2001). Transactivation and binding studies demonstrated that the FUS3 factor physically interacts with the RY element of seed-specific gene promoters (Reidt et al. 2000). In transient-expression assays based on tobacco protoplasts, RY-containing seed-specific promoters can be strongly induced both by FUS3 and ABI3, either separately or in combination (Reidt et al. 2000, 2001). In similar transactivation studies the B3 domain of ABI3 was shown to be essential for RY-mediated induction of the napin promoter (Ezcurra et al. 2000). Although these data together suggested the physical interaction of ABI3 with RY elements, this could not be demonstrated. The only previous indication was the interaction of the isolated glutathione *S*-transferase (GST)-tagged B3 domain of the structurally and functionally related VP1 protein

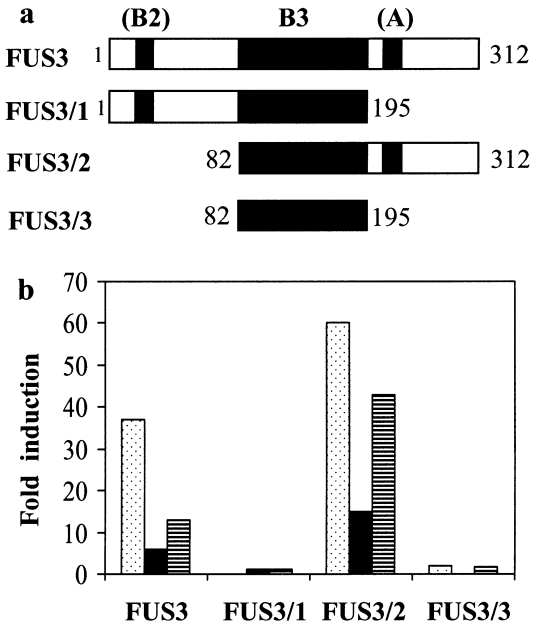

Fig. 6a,b Transcriptional activities of the *A. thaliana* FUS3 and FUS3 fragments. **a** Scheme showing the fragments used. **b** Transient-expression assay of the USP promoter activity. Three independent experiments are shown. The effects of FUS3 and derived fragments on the USP promoter activity are given as *n*-fold induction compared to the promoter construct alone

with RY elements (Suzuki et al. 1997). Since in this experiment binding could not be demonstrated for the complete VP1 protein, the authors suggested that protein regions adjacent to the B3 domain might prevent binding to DNA and that other auxiliary docking proteins are required for VP1/ABI3–DNA interaction. Here we demonstrate for the first time, that the full-length ABI3 protein does physically interact with RY-promoter elements. Thus, our observations solve a long-standing contradiction and consolidate previous genetic and transactivation data. The specific interaction of transcription factors and *cis*-elements is usually based on specific, structurally highly conserved DNA-binding domains. For the transcription factors FUS3 and ABI3 the protein–DNA interaction is thought to be mediated by the conserved B3 domain and the RY element of seed-specific gene promoters. To test this hypothesis in more detail, both FUS3 and ABI3 have been truncated to various extents. The binding behaviour of the truncated versions demonstrates that the B3 domains of both FUS3 and ABI3 are necessary and sufficient for the binding of the full-length proteins to the RY-containing legumin box. The specificity of this binding was shown by control experiments demonstrating that FUS3- and ABI3-derived B3 domains fail to bind to destroyed RY motifs. The results described here, as well as promoter binding and transactivation studies (Bobb et al. 1997; Ezcurra et al. 2000; Reidt et al. 2000), have shown that the minimal RY sequence CATGCA is necessary and sufficient for in vitro binding and in vivo function. This is not in contradiction to the data reported above showing that a duplication of the RY motif—as found in the napin gene promoter—results in stronger binding of both FUS3 and ABI3, and does not exclude enhancing effects of multiple RY elements in front of a CaMV 35S minimal promoter as reported by Bobb et al. (1997) and Ezcurra et al. (2000). Only a few results are available concerning the role of sequences that directly flank the RY core element in transcription-factor binding. Suzuki et al. (1997) reported that the isolated B3 domain of VP1 has a higher affinity to an RY element embedded in a 103-bp DNA fragment of the C1 gene promoter than to an isolated RY dimer. We checked binding of ABI3 and FUS3 to 15 totally different promoter sequences, identical only in the RY sequence. Our data demonstrate that FUS3 and ABI3 do bind in vitro to each RY element with the correct core sequence CATGCA. Promoter RY elements derived from genes known to be expressed specifically in seeds do not show preference in binding to FUS3 and ABI3. Obviously, the in vitro binding of ABI3 and FUS3 to RY-DNA is independent of the flanking sequences, at least in the 15 sequences tested here. Therefore, the question arises of how the seed promoter-specific RY elements are selected in vivo. The data suggest complex interactions of other parts of B3-domain-containing proteins with additional factors of the transcription machinery as a prerequisite for precise gene regulation. In BIAcore analysis we could not detect interactions between ABI3 and FUS3, ABI3 and LEC1 or FUS3 and LEC1 (data not shown). ABI5/TRAB1 and four general transcription factors were found to interact physically with ABI3/VP1 in yeast two-hybrid studies (Hobo et al. 1999; Kurup et al. 2000; Nakamura et al. 2001). Whether these interactors, however, direct the promoter target recognition of ABI3 remains an open question. The B3 domain directs the transcription factors ABI3 and FUS3 to the DNA. To initiate transcription the additional presence of an activation domain is not only required but also sufficient, at least in the transient-expression system used. According to the sequence similarity between ABI3 and VP1 the activation domain of ABI3 should be localized at the very N-terminal end. However, deletion of that protein region does not completely abolish the transcriptional activity. Therefore, we conclude that either this activation region is more extended or a second activation domain is present near to the B1 region. The transcriptional activity of a FUS3 fragment deficient in the N-terminal protein domain is even higher than that of the complete FUS3. We suggest that the N-terminal domain of the protein is active in case FUS3 acts as repressor. The activation domain in FUS3 is localized downstream from the B3 domain, as indicated by two-hybrid experiments (Reidt 2002). Obviously, FUS3 and ABI3/VP1 show a functional modular composition. In ABI3, the B1 region was mapped to be responsible for ABI5 binding (Nakamura et al. 2001). Single modules are responsible for DNA binding, transcription activation or repression, cellular communication and other unknown functions.

In conclusion, the presented data contribute to our understanding of the functional modular structure of the transcription factors ABI3 and FUS3, and focus on the problem of how these factors discriminate in vivo between seed promoter regulatory RY elements and those present in other promoters or elsewhere in the genome.

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