

# 14-3-3 Proteins are components of the transcription complex of the *ATEM1* promoter in *Arabidopsis*

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**Abstract** The *AtEm1* and *AtEm6* gene products accumulate exclusively in embryos during *Arabidopsis* seed maturation. The transcription factor ABI3 and the phytohormone abscisic acid are required for normal expression of both genes. However, the expression of these genes occurs in extremely small embryos limiting the availability of tissue to directly study DNA–protein interactions. We generated callus lines derived from embryos to determine if the regulation of *Em* expression was similar to wild type embryos. Expression of *AtEm1* and *AtEm6* was strongly induced by abscisic acid in callus derived from wild type embryos, but not in embryo callus derived from ABI3 mutant embryos (*abi3-6*). Epitopes to 14-3-3 proteins were found in complexes with the *AtEm1* promoter in mobility shift experiments using nuclear extracts derived from both wild type and *abi3-6* calli. Using phosphorylated peptides that bind to 14-3-3 proteins, we show that 14-3-3 proteins are required for the maintenance of the transcriptional complex generated in nuclear extracts. Chromatin immunoprecipitation experiments using a 14-3-3 antibody display the expected

241-bp band from the *AtEm1* promoter. Hence, 14-3-3 proteins are physically present in the *AtEm1* transcriptional complex in vivo and are required for the maintenance of the transcriptional complex in vitro.

**Keywords** 14-3-3 Proteins · ABI3 · Abscisic acid · Em · Embryo calli

## Abbreviations

ABA Abscisic acid  
ChIP Chromatin immunoprecipitation  
DAP Days after pollination  
EMSA Electrophoretic mobility shift assay

## Introduction

During plant seed development, regulatory programs control the expression of distinct gene sets. During later stages of seed maturation, the major classes of induced genes include those encoding storage proteins (such as 2S albumins and 12S globulins) and late embryogenesis abundant (LEA) proteins that are involved in the acquisition of desiccation tolerance among other traits (Thomas 1993; Wise 2003). The identification of *cis*-acting promoter elements and transcription regulators has assisted in understanding the molecular mechanisms that control the seed-specific expression of these gene sets. The phytohormone abscisic acid (ABA) plays an important role during seed maturation and seed germination. ABA levels start to increase in the middle stage of embryogenesis and reach its highest levels during late embryogenesis (Rock and Quatrano 1995). ABA levels decline after the accumulation of reserves and the onset of desiccation. ABA also prevents precocious germination. Several studies have shown that ABA deficient

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mutants (*aba*) generally germinate precociously and have reduced dormancy (Léon-Kloosterziel et al. 1996). Moreover, ABA insensitive mutants (*abi1* to *abi5*) have altered expression of several ABA inducible maturation proteins (Koorneef et al. 1984; Nambara et al. 1992, 1994; Ooms et al. 1993; Finkelstein 1994; Parcy et al. 1994; Söderman et al. 2000; Carles et al. 2002; Kroj et al. 2003). One of these loci, *abi3*, is orthologous to the maize *viviparous1* (*vp1*) gene and encodes an essential transcriptional regulator mainly involved in late stages of seed development (Giraudat et al. 1992; Nambara et al. 1995). In addition, it has been shown that ABI3 is expressed in arrested apical meristems of dark-grown *Arabidopsis* seedlings (Rohde et al. 1999) and also involved in determining plastid identity in the shoot apex (Rohde et al. 2000). Mutations in this locus result in seeds displaying precocious germination, desiccation intolerance, poor storage protein accumulation and seeds that remain green that are insensitive to ABA (Giraudat et al. 1992; Parcy et al. 1994). Recently, it was demonstrated that ABI3 is part of a hierarchical network of key regulators of seed maturation, together with FUS3 and LEC1 (Kagaya et al. 2005; To et al. 2006). Several mutant alleles of the ABI3 locus have been identified and examined (Koorneef et al. 1984; Nambara et al. 1992, 1994; Ooms et al. 1993; Parcy et al. 1997). All *abi3* mutants are able to germinate with relatively high concentrations of ABA. In some mutant alleles, the embryos are normal and can be rescued by germinating immature seeds, giving rise to phenotypically normal plants (Nambara et al. 1992, 1994). In particular, *abi3-6* mutant seeds are highly non-dormant and germinate within 2 days if they are imbibed before desiccation. In this mutation, about one-third of the coding region is deleted, causing complete loss of ABI3 protein function (Nambara et al. 1994). Furthermore, *abi3* mutants exhibit reduction in the expression of maturation and *LEA* mRNAs associated with the establishment of embryo dormancy and desiccation (Parcy et al. 1994; Nambara et al. 1995).

In 1979, the first *LEA* protein, Em, was identified in wheat (Cuming and Lane 1979). *LEA* mRNAs and proteins reach the maximum level in dry seeds and decline rapidly after germination (Bies et al. 1998). Their accumulation pattern suggests that these proteins are involved in desiccation tolerance. This role has also been supported by ectopic expression of *LEA* genes. When expressed in yeast, the wheat *Em* gene caused attenuation of the growth inhibition normally seen in high osmolarity media (Swire-Clark and Marcotte 1999). The barley *LEA* gene, *HVA1*, has been shown to confer tolerance to drought and salt stresses when expressed in transgenic rice (Xu et al. 1996). Recently, an anti-aggregation activity in synergism with trehalose was demonstrated for the wheat Em protein (Goyal et al. 2005). Several *LEA* genes can be precociously induced in immature seeds or vegetative tissues by applying ABA or by

water deficit or osmotic stress (Skriver and Mundy 1990; Jakobsen et al. 1994).

*Arabidopsis thaliana* contains two *Em* genes, *AtEm1* and *AtEm6* (Gaubier et al. 1993) that have been used as molecular markers of late embryogenesis. Although both are expressed at high levels in mature seeds, they are not expressed exactly in the same tissues: *AtEm1* is expressed in pre-vascular tissues and root tips, whereas *AtEm6* expression is ubiquitous (Vicent et al. 2000). Parcy et al. (1994) demonstrated that the transcription factor ABI3 is required for normal expression of both *Arabidopsis Em* genes since both are down-regulated in *abi3* mutants. Moreover, it was shown that both late embryogenesis markers are also down-regulated in INSOMNIAC (*nsm*) mutants, which lack the stratification dependency to break dormancy (Baumbusch et al. 2004). Expression of *LEA* genes, including *AtEm1* and *AtEm6*, is also up-regulated by ABA. Their transcription is controlled by ABA responsive elements (ABRE) (Marcotte et al. 1988) present in their promoters (Gaubier et al. 1993; Delseny et al. 2001), which are recognition sites for bZIP transcription factors (Guilinan et al. 1990). The bZIP factor ABI5 has been shown to interact with the *AtEm1* and *AtEm6* promoters in vitro (Carles et al. 2002) and with the *AtEm6* promoter in vivo (Lopez-Molina et al. 2002).

Previous work has identified 14-3-3 proteins interacting with the ABA–VP1 response complex in the wheat *Em* promoter (Schultz et al. 1998). In plants, these proteins were initially identified in association with G-box elements (like ABREs) in promoters that respond to different transduction pathways (de Vetten et al. 1992; Lu et al. 1992; Liu et al. 1995). 14-3-3 proteins are involved in a number of processes like signal transduction, regulation of transcription and enzymatic activity (Roberts 2003). They frequently act as dimers and usually bind phosphorylated motifs in their target proteins (Ferl 1996).

In this work, we examine whether 14-3-3 proteins are also in association with the *AtEm1* promoter, a characteristic transcriptional complex regulated by ABA and ABI3 in *Arabidopsis* embryos. Obtaining sufficient embryo tissue to carry out all biochemical and molecular studies is difficult and time-consuming. Thus, we generated callus lines from immature embryos that retained the same capacity of regulating *Em* genes as intact embryos. We demonstrated that 14-3-3 proteins are present within the *AtEm1* promoter–protein complex in vitro, as well as in vivo.

## Materials and methods

### Plant material

*Arabidopsis thaliana* ecotype Columbia wild type seeds were sterilized with ethanol for 10 min, dried for 1 h, and

plated on MS plates (MS salts, 2% sucrose and 6 g/l agar). Plates were incubated 3 days at 4°C and then grown at 25°C and a 16-h-light/8-h-dark photoperiod. Mutant *Arabidopsis abi3-6* seeds (Nambara et al. 1994) were taken out before the siliques dried out, and plated on MS plates as above at 25°C without incubation at 4°C.

#### Callus generation and culture

To generate embryogenic calli, plants were grown on soil at 25°C and a 16 h/8 h light/dark photoperiod. Staging of the developing siliques was performed by tagging individual flowers on the day of pollination, which was defined as the day when the stigma first extruded from the corolla. Seven to nine DAP siliques were excised and opened under a dissecting scope in sterile conditions. The embryos were taken one by one, placed in callus-inducing medium (CIM, MS salts, 2% sucrose, 0.5 g/l MES, 0.25 g/l BactoTryptone, 2 g/l casein hydrolysisate, 0.5 mg/l 2,4-D, 0.05 mg/l kinetin, 5 mg/l nicotinic acid, 10 mg/l pyridoxine, 2 mg/l glycine, 100 mg/l myo-inositol, 100 mg/l thiamine and 6 g/l of agar for solid media, pH was adjusted to 5.6–5.8) and incubated at 24–25°C and a 16-h-light/8-h-dark photoperiod. They were transferred to new plates after 15 days and then sub-cultured once a month.

#### ABA treatment

Callus liquid cultures were prepared by placing little pieces of calli in 25 ml of liquid CIM media. The cultures were grown in 125 ml flasks with agitation (125 rpm) at 25°C and a 16 h/8 h light/dark photoperiod for 5 to 7 days. ABA (5 µM final concentration; higher concentrations inhibited cell growth) or water (control) was added and cultures were grown for two more days. To harvest the tissue, cultures were centrifuged 5 min at 2,000 rpm, the medium was discarded and the tissue was frozen in liquid nitrogen and stored at –80°C.

#### RNA isolation and RT-PCR analysis

RNA was isolated from *Arabidopsis* calli grown in CIM liquid media using the RNeasy Plant Minikit (Qiagen) according to the manufacturer's instructions. On-column DNase digestion was performed to avoid DNA contamination. A 1–2 µg of RNA was used to synthesize cDNA using the ThermoScript RT-PCR System (Invitrogen). PCR reactions were made with 2 µl of cDNA using Taq DNA Polymerase (Invitrogen). Usually 30–35 cycles of PCR were done and the temperature of annealing was set up according to the primers used. The primers used for RT-PCR were:

AtEm1-forward 5'-CGCAAAGCAACTGAGCA-3'  
 AtEm1-reverse 5'-TCCATCGTACTGAGTCCT-3'  
 AtEm6-forward 5'-TGGCGTCTCAACAAGAGAAG-3'  
 AtEm6-reverse 5'-TCTCCGGTGCTAAGACCA-3'  
 AtABI3-forward 5'-CTCCGACGTCAAATGTGGCA  
 AAT-3'  
 AtABI3-reverse 5'-ACTAGTTTTAACAGTTTGAGA  
 AGTTGGTG-3'  
 Ubiquitin-forward 5'-AAGATCCAGGACAAGGAG  
 GTATTCCT-3'  
 Ubiquitin-reverse 5'CATAACAGAGACGAGATTT  
 AGAAACCACCA-3'

#### Western blot

Whole cell extracts from embryonic calli were obtained by grinding calli in liquid nitrogen, incubating for 30 min on ice in extraction buffer containing 150 mM NaCl, 20 mM Tris pH 7.5, 250 mM sucrose, 1% Triton and a protease inhibitor tablet (ROCHE) and mixing. After centrifugation at 53,000g for 30 min, supernatant was used to determine protein content using the Bradford reagent (Bio-Rad). For Western blot analysis, protein (15 µg of whole cell extracts or 6 and 12 µg of nuclear extracts) was loaded onto a 12.5% SDS polyacrylamide gel and electroblotted to nitrocellulose membranes. Membranes were blocked with 5% milk in 1× TBST (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h. A 14-3-3 monoclonal antibody (Lu et al. 1992) was added at a 1:10,000 dilution and incubated overnight at 4°C. Washes were done using 1× TBST, and membranes were then incubated with an anti-mouse IgG peroxidase conjugate for 2 h at room temperature. Immunological detections were performed using the chemiluminescent method. This 14-3-3 monoclonal antibody was raised against a G box binding complex and 14-3-3 proteins were identified as antigens after immunoscreening a cDNA library (Lu et al. 1992). It is known to react with surface-accessible regions of complexed 14-3-3s (Lu et al. 1994; Sehnke et al. 2006).

#### Protein extracts and electrophoretic mobility shift assays

Partially purified nuclear proteins from *Arabidopsis* embryonic calli treated with 5 µM ABA for 48 h were obtained using the CellLytic PN, Plant Nuclei Isolation Extraction Kit (SIGMA) according to manufacturer's instructions. These extracts were loaded onto a DEAE-cellulose column equilibrated with buffer D (20 mM Hepes-KOH pH 7.6, 50 mM KCl, 0.5 mM EDTA, 20% v/v glycerol, 0.5 mM DTT and 0.2 mM PMSF) and proteins were eluted with the same buffer containing 500 mM KCl, dialyzed in buffer D and then used directly or stored at –80°C. An oligonucleotide probe was obtained by digesting with EcoRI the

pGEMT vector (Promega) containing 177 bp of the *AtEm1* promoter, and then labeling it with  $^{32}\text{P}$ -dCTP by the Klenow fill-in reaction. Binding reactions (20–40  $\mu\text{l}$ ) contained 1–2 ng of radiolabeled probe, 1  $\mu\text{g}$  of poly-dIdC, 10 mM Tris-HCl pH 7.6, 50 mM KCl, 10% glycerol, 1 mM DTT and 6  $\mu\text{g}$  of protein extract, and were incubated in ice for 30 min. Competition assays were performed adding 5, 20 or 100-fold molar excess of unlabeled competitor. For super shift assays, extracts were preincubated with 1 or 2  $\mu\text{l}$  of a 1:10 dilution of the 14-3-3 monoclonal antibody (Lu et al. 1992) for 1 h on ice with or without previous incubation of extracts with 0.5 mM of the NR peptide (Sinnige et al. 2005). Binding reactions were loaded on a 4 or 5% polyacrylamide gel in  $0.5\times$  TBE buffer. Gels were run for 5 h, dried and exposed to a phosphorimager screen.

### Chromatin immunoprecipitation

A detailed protocol was described by Wang et al. (2002). Briefly, embryogenic culture tissue was equilibrated in MC buffer (10 mM potassium phosphate, pH 7.0, 50 mM NaCl, 0.1 M sucrose) with 2% formaldehyde for 4 h on ice. Formaldehyde was quenched by adding glycine to a final concentration of 0.125 M. After washing calli with MC buffer, tissue was frozen in liquid nitrogen. A 20 g of embryonic culture tissue was powdered with a mortar and pestle and liquid nitrogen. A crude nuclei isolation was performed using the CellLytic PN, Plant Nuclei Isolation Extraction Kit (SIGMA) as described above. The crude nuclear pellet was resuspended in 2 ml of sonication buffer (10 mM potassium phosphate, pH 7, 0.1 mM NaCl, 0.5% Sarkosyl, 10 mM EDTA and 1 mM PMSF) and glass beads and exposed to a probe sonicator (ten times 15 s pulses with cooling periods between pulses). Insoluble material was pelleted at 12,000g for 5 min at 4°C and chromatin was recovered. An equal volume of IP buffer was added (50 mM HEPES, pH 7.5, 150 mM KCl, 5 mM  $\text{MgCl}_2$ , 10  $\mu\text{M}$   $\text{ZnSO}_4$ , 1% Triton X-100, 0.05% SDS), and about 50  $\mu\text{l}$  saved for enrichment tests (input DNA). Solubilized chromatin was immunoprecipitated with anti-14-3-3 monoclonal antibody (Lu et al. 1992). After 2 h incubation at 4°C with gentle mixing, the samples were centrifuged at top speed for 5 min. The supernatant was removed and incubated with 25  $\mu\text{l}$  of a 50% slurry of protein A-Agarose for 1 h at 4°C with gentle mixing. Protein A-Agarose beads were pelleted and washed with IP buffer (5  $\times$  1 ml for 10 min) at room temperature. Cold glycine elution buffer (100  $\mu\text{l}$ , 0.1 M glycine, 0.5 M NaCl, 0.05% Tween-20, pH 2.8) was added to the beads, vortexed for 30 s and the beads were then pelleted at room temperature. The supernatant was removed to a tube with 50  $\mu\text{l}$  of 1 M Tris, pH 9 to neutralize the eluant. Elution and neutralization were repeated twice. The eluted sample was spun for 2 min at room tem-

perature and the supernatant was processed to recover DNA. DNA was extracted from the eluant and from the total DNA sample by treatment with RNase A (3 ng/ $\mu\text{l}$ , 20 min), and then with proteinase K (0.5 mg/ml, overnight at 37°C). After overnight incubation, a second aliquot of proteinase K was added and incubated at 65°C for 8 h to reverse the formaldehyde crosslink. Proteins were then extracted with phenol:chloroform and DNA was precipitated with 3 M NaOAc and ethanol. Recovered DNA was used for PCR amplification of an *AtEm1* promoter region using the oligonucleotides 5'-GGGGGAAACA-GAAAATGGATTAAG-3' and 5'-CTATGTTCCGACACAGATACGG-3' as primers. Thirty cycles of PCR were performed using KlenTaq1 and reactions were analyzed by agarose gel electrophoresis.

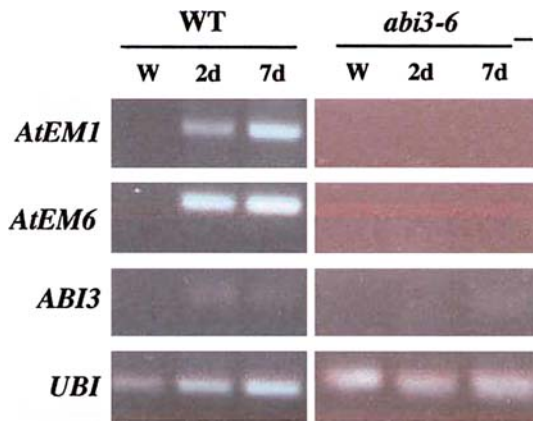
### Results

*AtEm* genes can be induced by ABA in embryogenic calli

To investigate the expression of *Em* genes and their regulation by ABA and ABI3, calli from *Arabidopsis* embryos were generated. After 2 or 7 days of hormone treatment in liquid cultures, callus tissue was harvested and used for RNA extraction. None or very low expression of *AtEm1*, *AtEm6* or *ABI3* was detected by RT-PCR in control calli. However, expression of *AtEm1* and *AtEm6* was clearly induced after ABA treatment of embryo callus (Fig. 1). Consequently, the ability to induce *Em* genes by ABA is maintained in this undifferentiated tissue. Furthermore, no expression of these genes was observed when callus derived from *abi3-6* embryos was treated with 5  $\mu\text{M}$  ABA for 2 or 7 days (Fig. 1) or up to 10  $\mu\text{M}$  ABA (data not shown). These data confirmed that ABI3 protein is also necessary for the induction of *AtEm1* and *AtEm6* genes by ABA in callus tissue derived from *Arabidopsis* embryos. In addition, this undifferentiated tissue proved to be a convenient source of tissue to study the expression and regulation of these and perhaps other embryo specific genes.

14-3-3 proteins are present in nuclear proteins that interact with the *AtEm1* promoter

Electrophoretic mobility shift assays (EMSA) were performed with a region of the *AtEm* promoter that responds to ABA (Fig. 2a) (Carles et al. 2002). Nuclear extracts from ABA treated embryo calli were able to recognize the radiolabeled probe of the *AtEm1* promoter (Fig. 2b, lane 2). Competition reactions with increasing amount of unlabeled probe caused a reduction of the shifted bands intensity, demonstrating that this interaction was specific (Fig. 2b, lanes 3–5).



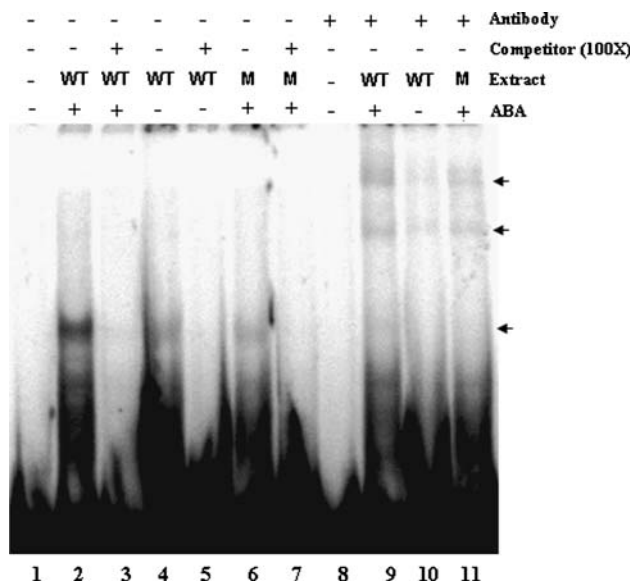
**Fig. 1** Expression of *AtEm1* and *AtEm6* is induced by ABA in embryo calli. RT-PCR experiment using RNA extracted from water-treated (W) or ABA-treated calli derived from wild type (WT) or *abi3-6* mutant embryos. Tissue was induced with 5 μM ABA for 2 and 7 days. Reactions were run in 2% agarose and visualized under UV light



**Fig. 2** 14-3-3 Proteins are present in embryonic calli nuclear extracts that recognize the *AtEm1* promoter in vitro. **a** Partial nucleotide sequence of the *AtEm1* promoter used as probe and competitor in the electrophoretic mobility shift assay. ABA response elements are *underlined*. **b** Electrophoretic mobility shift assay with nuclear extracts from embryonic calli treated with 5 μM ABA for 2 days. A 177-bp probe and 6 μg of nuclear proteins were used in each binding reaction. The molar excess of the competitor is indicated at the top of each lane. In the last four reactions the extracts were pre-incubated with 1 or 2 μl of a 1:10 dilution of a 14-3-3 monoclonal antibody for 1 h on ice (4°) or room temperature (RT). Reactions were resolved in a 4% acrylamide gel. *Arrows* indicate shifted and super-shifted bands

To test whether 14-3-3 proteins are also present in *Arabidopsis* nuclear extracts that bind to the *AtEm1* promoter, a 14-3-3 monoclonal antibody was included in the EMSA. When the antibody was pre-incubated with the extracts for different time periods or at different temperatures, two super-shifted bands were detected (Fig. 2b, lanes 6–8), indicating the presence of a 14-3-3 epitope in the protein preparation that recognized the *AtEm1* promoter. The presence of 14-3-3 proteins in the nuclear extracts used for EMSA was also verified by Western blot employing the same amount of protein used in the binding assays of EMSA (S1). The supershifted bands represent the interaction between the monoclonal Ab and the 14-3-3 proteins, since the proven specificity of the monoclonal 14-3-3 antibody in previous studies (Liu et al. 1995; Sehnke et al. 2006) indicates that a non-specific cross-reaction with other proteins in the extract is highly improbable. Similar shift and super-shift pattern was observed using nuclear extracts derived from wild type calli without ABA or ABA-treated *abi3-6* calli, suggesting that neither *ABI3* nor ABA is essential for the DNA–protein complex formation in vitro (Fig. 3), although they are critical for the expression of the gene, as deduced from the RT-PCR experiments (Fig. 1). Nuclear protein extracts from untreated wt. calli or from *abi3-6* mutant calli were able to recognize the probe of *AtEm1* promoter, producing a shifted band in the EMSA; though to a lesser extent than ABA-treated wt. calli (Fig. 3, lane 2 vs. lanes 4 and 6). When unlabeled probe was added, these shifted bands disappeared, demonstrating the specificity of the interaction (Fig. 3, lanes 5 and 7). The EMSA reactions using the 14-3-3 monoclonal antibody together with the wt. untreated and the ABA-treated *abi3-6* extracts demonstrated that these proteins are also present in these complexes. The same two supershifted bands appeared in the ABA-treated or untreated wt. callus and in the ABA-treated mutant callus (Fig. 3, lanes 9, 10 and 11).

To further demonstrate the presence of 14-3-3 proteins in these DNA–protein complexes, a specific competition assay with a peptide that binds 14-3-3 proteins was performed (Sinnige et al. 2005). Two versions of a 14-residue peptide (NR) with a 14-3-3-specific binding site were used: a phosphorylated form that has high affinity for 14-3-3 proteins and a non-phosphorylated form with low affinity. Figure 4 shows an EMSA where nuclear extracts from ABA-treated callus produced the expected shift of the probe (lane 2). Addition of 14-3-3 antibody to the binding reaction produced a much slower band and disappearance of the small upper band in lane 2 (lane 4). Intensity differences of shifted and supershifted bands between Figures 2 and 4 may be due to different nuclear protein extractions used in both assays. When the extracts were pre-incubated with the phosphorylated peptide, the shifted bands were greatly reduced (Figure 4, lane 6). However, when the non-phosphorylated

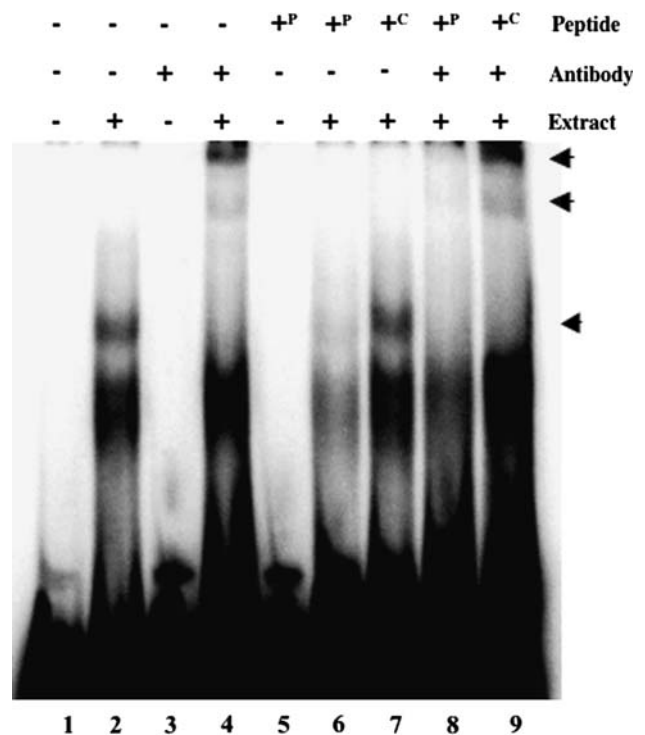


**Fig. 3** Complexes with 14-3-3 proteins are formed in vitro with extracts from untreated wild type and ABA-treated *abi3-6* mutant calli. Electrophoretic mobility shift assay with nuclear extracts from wild type (WT) or *abi3-6* (M) embryonic calli treated with or without 5  $\mu$ M ABA for 2 days. A 177-bp probe with a partial sequence of the *AtEm1* promoter and 6  $\mu$ g of nuclear proteins were used in each binding reaction; 100-fold excess of the competitor was used in some reactions as indicated at the top of each lane. In the last four reactions the extracts were pre-incubated with 1  $\mu$ l of a 1:10 dilution of a 14-3-3 monoclonal antibody for 1 h on ice (4 $^{\circ}$ ). Reactions were resolved in a 4.5% acrylamide gel. Arrows indicate shifted and super-shifted bands

peptide was used, the intensity of the shifted bands was similar to the bands observed in the reaction without the peptide (Fig. 4, compare lane 2 to lane 7). The NR peptide was also able to compete against the 14-3-3 antibody interaction when added to the binding reaction. As seen in Fig. 4, lane 8, the shifted and supershifted bands almost disappeared. In contrast the non-phosphorylated peptide did not show the same effect, in accordance with its less binding affinity towards 14-3-3 proteins (Fig. 4, lane 9). These results demonstrate the participation of 14-3-3 proteins in the maintenance of this complex in vitro, because the shifted and super-shifted bands cannot be detected when 14-3-3 proteins are captured by the phosphorylated peptide.

#### 14-3-3 proteins are associated with the *AtEm1* promoter in vivo

To investigate if 14-3-3 proteins are connected to the *AtEm1* promoter in vivo, chromatin immunoprecipitation (ChIP) was performed. A PCR reaction that employed immunoprecipitated and crosslinked DNA generated the expected 241-bp band (Fig. 5, CHIP lane). Subsequent sequencing of the amplified product confirmed the promoter identity (not shown). Control reactions with non-crosslinked chromatin or non-immunoprecipitated proteins

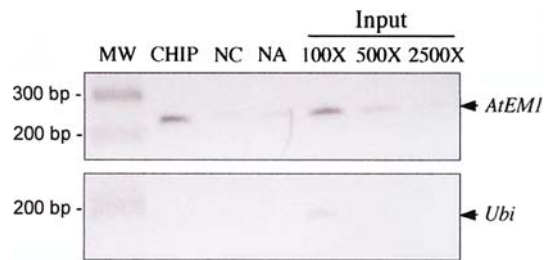


**Fig. 4** Specific recognition of 14-3-3 proteins present in nuclear extracts from embryonic callus. Electrophoretic mobility shift assay with nuclear extracts from embryo calli treated with 5  $\mu$ M ABA for 2 days. A 177-bp probe and 6  $\mu$ g of nuclear proteins were used in each binding reaction. Extracts were pre-incubated with or without 0.5 mM of phosphorylated (P) or nonphosphorylated (C) NR peptide for 30 min and then with or without 4  $\mu$ l of a 1:10 dilution of a 14-3-3 monoclonal antibody for 1 h on ice. Reactions were resolved in a 5% acrylamide gel. Arrows indicate shifted and super-shifted bands

showed almost undetectable *AtEm1* product (Fig. 5, NC or NA). Reactions with diluted amounts of input DNA demonstrated the correct amount of specific product. Primers for a Ubiquitin gene were used as a negative control for the ChIP experiment and only generated the expected product in the low diluted input DNA sample (Fig. 5). Therefore, the presence of 14-3-3 proteins in the *AtEm1* promoter protein complex was not only demonstrated by in vitro assays but also in vivo.

#### Discussion

*AtEm* genes have been used to monitor gene regulation by the transcriptional activator ABI3 and the phytohormone ABA during *Arabidopsis* late embryo development. Biochemical studies using developing embryos in *Arabidopsis* are difficult due to the small size of embryos and lack of easy access to embryonic tissue. In this study, we demonstrated that *Arabidopsis* callus produced from embryos retained the same regulation of the *AtEm1* and *AtEm6* genes as intact embryos, at least in their requirement for



**Fig. 5** 14-3-3 proteins are associated to the *AtEm1* promoter in vivo. Embryo calli treated with 5  $\mu$ M ABA for 2 days were crosslinked with formaldehyde and chromatin immunoprecipitation was performed using 5  $\mu$ l of a 14-3-3 monoclonal antibody. PCR amplification was done for a 241-bp region of the *AtEm1* promoter or a ca. 200-bp segment of the *Ubiquitin* gene (*Ubi*). Samples not treated with formaldehyde served as non-crosslinked control (NC) and crosslinked samples not immunoprecipitated served as control for antibody specificity (NA). Total DNA before immunoprecipitation (input) was used at 100-, 500- and 2,500-fold dilutions for enrichment tests of linear amplification of the genes. MW Molecular weight marker

both ABA and ABI3. When liquid cultures of calli that exhibited the embryonic regulation of the *AtEm* genes were treated with ABA, both *AtEm1* and *AtEm6* were clearly induced compared to untreated cultures. However, neither *AtEm1* nor *AtEm6* were induced in *abi3-6* mutant callus cultures by ABA (Fig. 1). Increasing ABA concentrations did not result in different levels of expression of *AtEm1* or *AtEm6* in wild type or *abi3-6* callus lines (data not shown).

To use these callus lines to study biochemical interactions between the *AtEm1* promoter and the proteins that may regulate its expression, we used a 177 bp promoter fragment which contains two ABRE boxes that, when mutated, completely suppressed the response to ABA (Delseny et al. 2001). Nuclear extracts from ABA treated embryo calli were able to specifically bind the *AtEm1* promoter in vitro (Fig. 2b). The bZIP transcription factor ABI5 is likely to be in the protein complex since Carles et al. (2002) have shown that ABI5 is able to bind the *AtEm* promoters in vitro. Studies using *abi5* mutants, as well as ABI5 antibodies, would help to verify whether ABI5 is necessary for the formation of these complexes.

In vitro interaction of ABRE-responsive promoter complexes with 14-3-3 proteins has been shown for a wheat *Em* promoter, also positively regulated by ABA and VP1 (Schultz et al. 1998). A monoclonal antibody against *Arabidopsis* 14-3-3 proteins confirmed the presence of 14-3-3 proteins in the extracts and was able to further retard one of the two shifted bands observed with nuclear extracts in the EMSA (Fig. 2b). To demonstrate the occurrence of 14-3-3 proteins in the formation of these DNA–protein complexes, a specific competition assay was performed with a peptide that is a target of 14-3-3 proteins (Sinnige et al. 2005; Fig. 4). The DNA–protein complex almost disappeared

when the phosphorylated form of this peptide, captured the 14-3-3 proteins, demonstrating their participation in the establishment of this interaction.

14-3-3 proteins most probably interact with other factors present in this complex, as 14-3-3 proteins usually bind to phosphoserine-containing proteins and are unlikely to interact directly with DNA in a transcriptional complex. Nevertheless, some human 14-3-3 proteins have been shown to bind cruciform DNA in replication origins (Alvarez et al. 2002). As mentioned earlier, the monoclonal antibody against 14-3-3 is specific for this family of proteins, and has been extensively used to prove the presence of 14-3-3 proteins in complexes (Lu et al. 1992, 1994; Schultz et al. 1998). In addition, this antibody was recently shown to specifically recognize 6 of the 13 14-3-3 isoforms reported in *Arabidopsis* (Sehnke et al. 2006). Consequently, we can argue that at least one of these six isoforms ( $\psi$ ,  $\nu$ ,  $\omega$ ,  $\zeta$ ,  $\phi$ ,  $\nu$ ) is present in the *AtEm1* promoter complex, not excluding the possibility that other isoform(s) not recognized by this antibody may also be in the complex.

Occurrence of 14-3-3 proteins in other complexes with promoters that also respond to ABA has been described for the tobacco *osmotin* gene (Liu et al. 1995) and the *Arabidopsis* and maize *Adh* genes (de Vetten et al. 1992; Lu et al. 1992). Even though these genes are related to ABA signals, they are not regulated by similar signalling events, which points out that different proteins or transcription factors are targets of 14-3-3 proteins. Moreover, 14-3-3 proteins were also identified to participate in promoter complexes of some maize 14-3-3 promoters (de Vetten and Ferl 1994). Recently, it was demonstrated that 14-3-3 proteins play an important role in ABA signalling during seed germination. They interact with several barley bZIP transcription factors, amongst them one that activates a *LEA* gene promoter (Schoonheim et al. 2007).

When nuclear extracts from ABA-treated *abi3-6* mutant calli or wild type untreated calli were used, similar patterns of shifted and super-shifted bands were detected (Fig. 3), even though the *AtEm1* promoter was not actively making transcripts. It appears that although a functional ABI3 protein is absent in the mutant extract, the transcriptional complex of the *AtEm1* promoter is still capable of forming, at least in vitro. It is still possible that a truncated protein, or protein with internal deletion that derives from the translation of the *abi3-6* allele, still has the ability to interact within the complex. However, the transcription of the *Em1* gene depends on a functional ABI3 that most probably interacts with other transcription factors present in this complex. Recently, it was demonstrated that the *Arabidopsis* ABI3 and FUS3 transcription factors are able to bind DNA through its B3 domain, in particular to the RY element present in promoters of seed-specific genes (Mönke et al. 2004) which is not present in the *AtEm1* promoter

used in this study. Future analysis using antibodies against ABI3 or other putative transcription factors could establish their presence in these complexes.

Regarding the wild type untreated callus, we hypothesize that the complex is still assembled *in vitro*, but ABA may be essential (for example by inducing a transcription factor) for initiating the transcription of *Em* genes in *Arabidopsis*. This same hypothesis could be applicable for the mutant calli where a functional ABI3 would be essential for transcription.

The participation of 14-3-3 proteins in the transcriptional complex in the *AtEm1* promoter was also analyzed by chromatin immunoprecipitation (ChIP) analysis. The 14-3-3 antibody recovered a specific region of DNA corresponding to the *AtEm1* promoter fragment used in EMSA. This result clearly demonstrated that 14-3-3 proteins are part of this transcriptional complex not only *in vitro* but also *in vivo* (Fig. 5). Future studies using ChIP assays and the 14-3-3 antibody could lead to a more comprehensive identification of promoters containing 14-3-3 proteins as part of the transcriptional complexes, as well as promoters linked to ABA signalling.

The callus lines generated for this investigation represent a useful system to use biochemical approaches to study transcription regulation of *Em* genes during *Arabidopsis* embryo development. The presence of 14-3-3 proteins in the transcriptional complex associated with the *AtEm1* promoter was clearly shown. Analysis of 14-3-3 knock out mutants or RNAi-silenced plants could facilitate the identification of specific 14-3-3 isoforms and their exact role in the formation of this complex, despite the redundancy that these proteins might present. This culture system may also allow us to identify other proteins in the complex and their role in the transcription of ABA and ABI3 regulated genes during embryogenesis. Finally, these callus lines can be tested for retention of other seed-specific regulatory pathways.

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