Plant Physiology, September 1999, Vol. 121, pp. 81–87, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Differences in Spatial Expression between 14-3-3 Isoforms in Germinating Barley Embryos[1]

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The family of 14-3-3 proteins is ubiquitous in eukaryotes and has been shown to exert an array of functions. We were interested in the possible role of 14-3-3 proteins in seed germination. Therefore, we studied the expression of 14-3-3 mRNA and protein in barley (*Hordeum distichum* L.) embryos during germination. With the use of specific cDNA probes and antibodies, we could detect individual expression of three 14-3-3 isoforms, 14-3-3A, 14-3-3B, and 14-3-3C. Each homolog was found to be expressed in barley embryos. Whereas protein levels of all three isoforms were constant during germination, mRNA expression was found to be induced upon imbibition of the grains. The induction of *14-3-3A* gene expression during germination was different from that of *14-3-3B* and *14-3-3C*. In situ immunolocalization analysis showed similar spatial expression for 14-3-3A and 14-3-3B, while 14-3-3C expression was markedly different. Whereas 14-3-3A and 14-3-3B were expressed throughout the embryo, 14-3-3C expression was tissue specific, with the strongest expression observed in the scutellum and the L2 layer of the shoot apical meristem. These results show that 14-3-3 homologs are differently regulated in barley embryos, and provide a first step in acquiring more knowledge about the role of 14-3-3 proteins in the germination process.

Members of the highly conserved 14-3-3 protein family are capable of exerting a diverse array of functions. Various proteins involved in cell cycle regulation, differentiation, and signal transduction have been found to be associated with 14-3-3 proteins. In addition, the activity of several enzymes can be modified by 14-3-3 binding (Aitken, 1996).

All eukaryotes studied so far possess at least one 14-3-3 homolog. In plants, a number of functions have been demonstrated for 14-3-3 proteins (for review, see Ferl, 1996; Palmgren et al., 1998). In the plant nucleus, 14-3-3 proteins participate in a DNA-binding complex (Lu et al., 1992). In the cytosol, the best-documented action of 14-3-3 is its inhibition of nitrate reductase activity (Bachmann et al., 1996; Moorhead et al., 1996). 14-3-3 proteins associated with the plasma membrane H^+-ATPase can bind the fungal toxin fusicoccin (FC) (Oecking et al., 1997). Binding of the toxin stabilizes the association of 14-3-3 with the H^+-ATPase (Jahn et al., 1997; Oecking et al., 1997).

Binding of FC by 14-3-3 is restricted to plants, since in animal and yeast cells FC-binding activity could not be detected (Meyer et al., 1993). It has recently become clear that this specific function of 14-3-3 in plants is not due to specificity of the 14-3-3 isoforms, but is caused by the presence or absence of the plant PM H^+-ATPase. Baunsgaard et al. (1998) showed that animal and yeast 14-3-3 homologs can also bind FC when expressed together with a plant PM H^+-ATPase in yeast. This result and earlier work (Lu et al., 1994; van Heusden et al., 1996; Moorhead et al., 1996) suggest that 14-3-3 isoforms lack functional specificity. Instead, *14-3-3* genes seem to be differentially regulated at the expression level. In Arabidopsis, a distinct spatially and developmentally dependent expression pattern was observed for *GF14*χ (Daugherty et al., 1996), one of 10 *14-3-3* homologs of Arabidopsis (Wu et al., 1997).

We were interested in the role of 14-3-3 proteins in seed germination, as there are good indications that 14-3-3 proteins are involved in the signal transduction pathways that play a role in the germination process. First, FC, which binds to the 14-3-3-H^+-ATPase complex, can break seed dormancy and is a potent stimulator of seed germination (Marrè, 1979). In barley (*Hordeum distichum* L.) grains, it can promote germination without altering the endogenous level of the germination inhibitor ABA (Wang et al., 1998). ABA is an important factor in the induction and maintenance of dormancy during seed development (Wang et al., 1995; Bewley, 1997). Second, the transcriptional complexes associated with the G-box element in the promoters of several ABA-regulated genes (*osmotin*, *Adh*, and *Em*) contain 14-3-3 proteins (Lu et al., 1992; Liu et al., 1995; Schultz et al., 1998). The *Em*-promoter-associated GF14 (14-3-3) was shown to bind VP1, which is one of the effectors of ABA-induced maintenance of dormancy. This apparent involvement of 14-3-3 proteins in binding of FC and in ABA signal transduction prompted us to study the role of 14-3-3 in the germination of barley grains.

[1] This work was partially supported by European Community program no. PL962275 and by the Dutch Technology Foundation project no. 805.22.765.

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So far, three 14-3-3 homologs have been cloned from barley: *14-3-3A*, which is induced in barley leaf upon infection with *Erysiphe graminis* (Brandt et al., 1992), and *14-3-3B* and *14-3-3C* (accession nos. X93170 and Y14200). To study the roles of these different barley 14-3-3 isoforms in the physiological process of germination, it was necessary to first establish which of the isoforms are expressed in the embryo. Using specific probes and antibodies that each detect one of the barley 14-3-3A, 14-3-3B, or 14-3-3C isoforms, we demonstrated the presence of all three isoforms in barley embryos. In addition, we investigated the spatial expression of the three different 14-3-3 isoforms in the barley embryo. Since 14-3-3 proteins do not generally exhibit functional isoform-specificity, a possible differentiation in the function of 14-3-3 proteins during the germination process is likely to be reflected in the spatial distribution of the different 14-3-3 isoforms. In situ immunolocalization analysis using the isoform-specific antibodies did indeed reveal different expression patterns for 14-3-3A, 14-3-3B, and 14-3-3C in the germinating barley embryo. These results reinforce the idea that 14-3-3 isoforms are differentially regulated, and provide a starting point for elucidating the role of the different 14-3-3 isoforms in the germination process.

MATERIALS AND METHODS

Plant Material and Germination Tests

Barley (*Hordeum distichum* L. cv Triumph) grains were obtained from Heineken Technical Services (Zoeterwoude, The Netherlands). Ten to 20 intact grains were placed on two layers of Whatman no. 1 paper in a Petri dish (9 cm) containing 3 mL of distilled water. Plates were sealed with laboratory film to prevent evaporation, and then incubated in the dark at 20°C. Grains were scored as germinated when the radicle was ≥1 mm.

RNA Isolation and Northern Analysis

Embryos were dissected from the grain and ground in liquid nitrogen. Total RNA was isolated (Wang et al., 1998), separated on a glyoxal/DMSO/1% (w/v) agarose gel, and blotted to nylon membranes (Genescreen, DuPont). Blots were hybridized to the *14-3-3A, 14-3-3B*, and *14-3-3C* probes consisting of only the 3′ UTRs of the cDNAs (accession nos. X62388, X93170, and Y14200, respectively). For gene-specific probes, PCR products were used that were amplified from the full-length cDNAs using the following primers: 14-3-3A: F, TTGGCCCTCAAGAGTG and R, TGATGTTGAACATGTGGA; 14-3-3B: F, ACATTGTCTATGTGTCC and R, TGGAAAGGGTTCAGAAG; 14-3-3C: F, AGCCGGCTTTGCGAC and R, ACGATAAGAAGCAAC.

Production of Isoform-Specific Anti-14-3-3 Antibodies

Peptides corresponding to the C-terminal part of the 14-3-3A, 14-3-3B, and 14-3-3C proteins of barley were synthesized (CWTSDNAEEGGDEIK, CEEMKDAPKGESGDGQ, and CIREAPKHDSSEG, respectively) and used for immunization of rabbits (Eurogentec, Seraing, Belgium). For in situ immunolocalization studies, the antibodies were purified on an affinity column using the synthetic peptides. For 14-3-3C, preimmune serum was available, which was purified in the same way as the 14-3-3C antibody and used as a control in the immunolocalization experiments.

Cloning and Heterologous Expression of Barley 14-3-3 Protein in *Escherichia coli*

Isolation of the *14-3-3B* and *14-3-3C* clones (accession nos. X93170 and Y14200) was described previously by Brandt (1993) and Andersen (1997). The coding sequences of the *14-3-3* cDNAs were cloned into pET29b vector (Novagen) and transformed into *E. coli* strain BL21-DE3. Expression was induced with 1 mM isopropylthio-β-galactoside. Protein extracts were loaded on 15% (w/v) SDS-PAGE (Laemmli, 1970), blotted, and incubated with the isoform-specific anti-14-3-3 antibodies.

Barley Protein Isolation and Western Analysis

Embryos were ground in liquid nitrogen, and then 50 mM Tris, pH 7.5, was added and the extract was left for 30 min on ice and centrifuged for 10 min at 14,000 rpm. Soluble protein (20 μg) was analyzed on 15% (w/v) SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated overnight with the isoform-specific anti-14-3-3 antibodies (1:20,000 at 4°C), and bands were visualized by alkaline phosphatase-labeled goat anti-rabbit antibody, followed by incubation with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Promega).

In Situ Immunolocalization Studies

Intact barley grains were imbibed in water and one-half of the grain was fixed in methanol:acetone (1:1) at −20°C for 20 h. The grain halves were transferred to paraffin (Paraclean, Klinipath, Duiven, The Netherlands) through a graded ethanol and xylene series (10%, 30%, 50%, 70%, 90%, 96%, and 100% ethanol, followed by 3:1, 1:1, 1:3 ethanol:xylene, 100% xylene and 3:1, 1:1, 1:3 xylene:paraffin, each step 20 min). Sections (10 μm) were attached to Biobond coated slides (British Biocell International, Cardiff, UK) and rehydrated via xylene and ethanol. Sections were incubated in 0.4% (w/v) SDS, 3 mM 2-mercaptoethanol, 12 mM Tris, pH 6.8, for 20 min and blocked in 0.9% (w/v) NaCl, 0.1% (w/v) BSA-C (Aurion, Wageningen, The Netherlands) in 20 mM Tris, pH 8.2, for 3 min. Incubation with purified anti-14-3-3 antibody overnight at 4°C and secondary alkaline phosphatase-labeled goat anti-rabbit antibody at 20°C was in the same blocking buffer. The signal was visualized using NBT/BCIP substrate (Promega).

RESULTS

Northern-Blot Analysis of 14-3-3 Expression

Expression of the three different 14-3-3 isoforms, 14-3-3A, 14-3-3B, and 14-3-3C, was studied in embryos of ger-

minating barley grains. For this purpose, intact barley grains were imbibed in water for 0 to 48 h and the embryos were dissected for northern analysis. Specific probes that consisted of the 3′ UTRs of the *14-3-3* cDNAs were used to detect *14-3-3A*, *14-3-3B*, and *14-3-3C* expression with northern analysis. These 3′ regions did not show any homology to each other and negligible cross-hybridization was observed on dot blots (data not shown).

Expression of each homolog was induced upon imbibition of the grains (Fig. 1). In dry grains (t = 0), almost no *14-3-3* expression could be detected. Expression of *14-3-3A* increased until 24 h and decreased again at 48 h of imbibition. Induction of expression of *14-3-3B* and *14-3-3C* was different from *14-3-3A* in that it increased from 0 to 16 h and then stayed at a constant level. A slight increase in *14-3-3C* expression was observed at 16 h, which was not seen with *14-3-3B*, but, in general, expression of *14-3-3B* was similar to that of *14-3-3C*. The germination score for the same grains showed that the increase in *14-3-3A*, *14-3-3B*, and *14-3-3C* expression preceded visible germination (Fig. 1).

Production of Isoform-Specific Antibodies and Western Analysis of 14-3-3 Expression

To study the expression of each of the 14-3-3 isoforms at the protein level, specific polyclonal antibodies were raised against the C-terminal ends of each isoform. Figure 2 shows the specificity of these antibodies; no cross-reaction was observed with the other in *E. coli*-expressed barley 14-3-3 isoforms.

In extracts of barley embryos (Fig. 3), 14-3-3B and 14-3-3C antibodies detected a single band of approximately 31 kD. The 14-3-3A antibody detected a 30-kD protein (Fig. 3), and a slightly lower molecular mass band of approximately

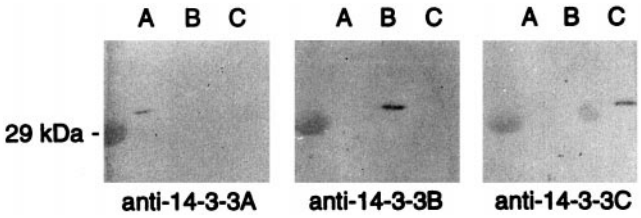

Figure 2. Specificity of polyclonal anti-14-3-3 antibodies. Extracts were prepared from *E. coli*, producing either 14-3-3A, 14-3-3B, or 14-3-3C (lanes A, B, and C, respectively). The proteins were separated on 15% (w/v) SDS-PAGE and blotted on a nitrocellulose membrane. Blots were incubated with anti-14-3-3A, anti-14-3-3B, or anti-14-3-3C antibody (as indicated below the blots).

29 kD was occasionally detected as well (data not shown). These molecular masses are in accordance with the predicted molecular masses of the 14-3-3 isoforms as judged from the cDNA sequences.

All three 14-3-3 proteins were already present in dry barley embryos and no clear increase in expression of either isoform could be observed in the embryo in the first 24 h of imbibition of intact grains (same germination experiment as used for northern analysis). Therefore, the marked increase in steady-state mRNA early during germination was not reflected in the protein level as measured by western analysis. Only after 24 h was a slight increase in the expression of 14-3-3A and 14-3-3C and, to a lesser extent, 14-3-3B protein observed (which was more clear in other experiments than in the one shown in Fig. 3).

In Situ Immunolocalization of 14-3-3 Proteins

Next, analysis of the localization of the three different 14-3-3 isoforms in barley embryos was undertaken. The barley embryo in the dry grain is highly differentiated,

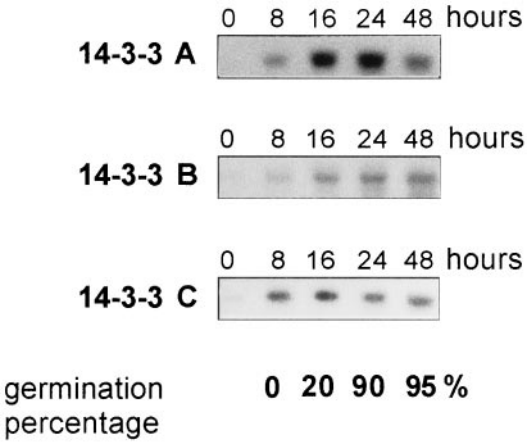

Figure 1. *14-3-3A*, *14-3-3B*, and *14-3-3C* steady-state mRNA in germinating barley embryos. Intact barley grains were imbibed in water on two layers of filter paper in a Petri dish. Germination was scored and the embryos were dissected from the grains. mRNA was isolated from the embryos and analyzed for *14-3-3A*, *14-3-3B*, and *14-3-3C* expression using isoform-specific probes. Equal amounts of RNA were loaded (15 μg). One representative result of three independent experiments is presented.

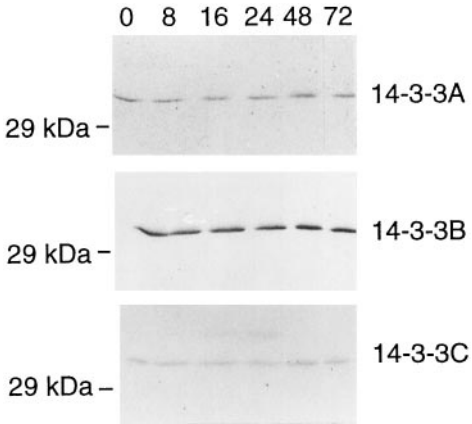

Figure 3. Expression of 14-3-3A, 14-3-3B, and 14-3-3C proteins in germinating barley embryos. Intact barley grains were imbibed in water on two layers of filter paper in a Petri dish. Germination was scored (Fig. 1) and the embryos were dissected from the grains at the designated times as indicated above the lanes (hours). Protein was isolated from the embryos and 20 μg was used for western analysis. Blots were incubated with anti-14-3-3A, anti-14-3-3B, or anti-14-3-3C antibody.

with the first leaves already present and the radicle being further developed than in dicots. The scutellum, the single cotyledon of a monocot, has a dual function: the secretion of hydrolytic enzymes to the starchy endosperm and the absorption of nutrients from the endosperm in a later stage.

The in situ protein localization of 14-3-3 protein was studied at 0, 24, and 48 h of imbibition (Figs. 4 and 5). After 24 h of imbibition, 14-3-3A and 14-3-3B expression were similar and were observed throughout the embryo (Fig. 4, A and B), whereas 14-3-3C expression was limited to specific tissues (Fig. 4C). 14-3-3A and 14-3-3B expression was strongest in the foliage leaves, radicle, coleoptile, and scutellum; the tissues surrounding these organs were less intensely stained. In the foliage leaves, strong expression of both 14-3-3A and 14-3-3B could be detected in the vascular bundles. For 14-3-3B, this was less clear because of the more intense staining, but a pattern similar to that of 14-3-3A was observed for 14-3-3B in other samples. In the scutellum, the strongest expression of 14-3-3A and 14-3-3B protein was observed in the scutellar epithelial cells.

The expression pattern of 14-3-3C was clearly different from that of 14-3-3A and 14-3-3B. The scutellum tissue and the shoot apical meristem were most strongly labeled with 14-3-3C antibody (Fig. 4C). In the shoot apical meristem (Fig. 4F), expression was restricted to the second layer of the tunica (the L2 layer). Almost no expression was detected in the mesocotyl and coleorhiza, and even less in the coleoptile, radicle, and leaves. 14-3-3C expression in the

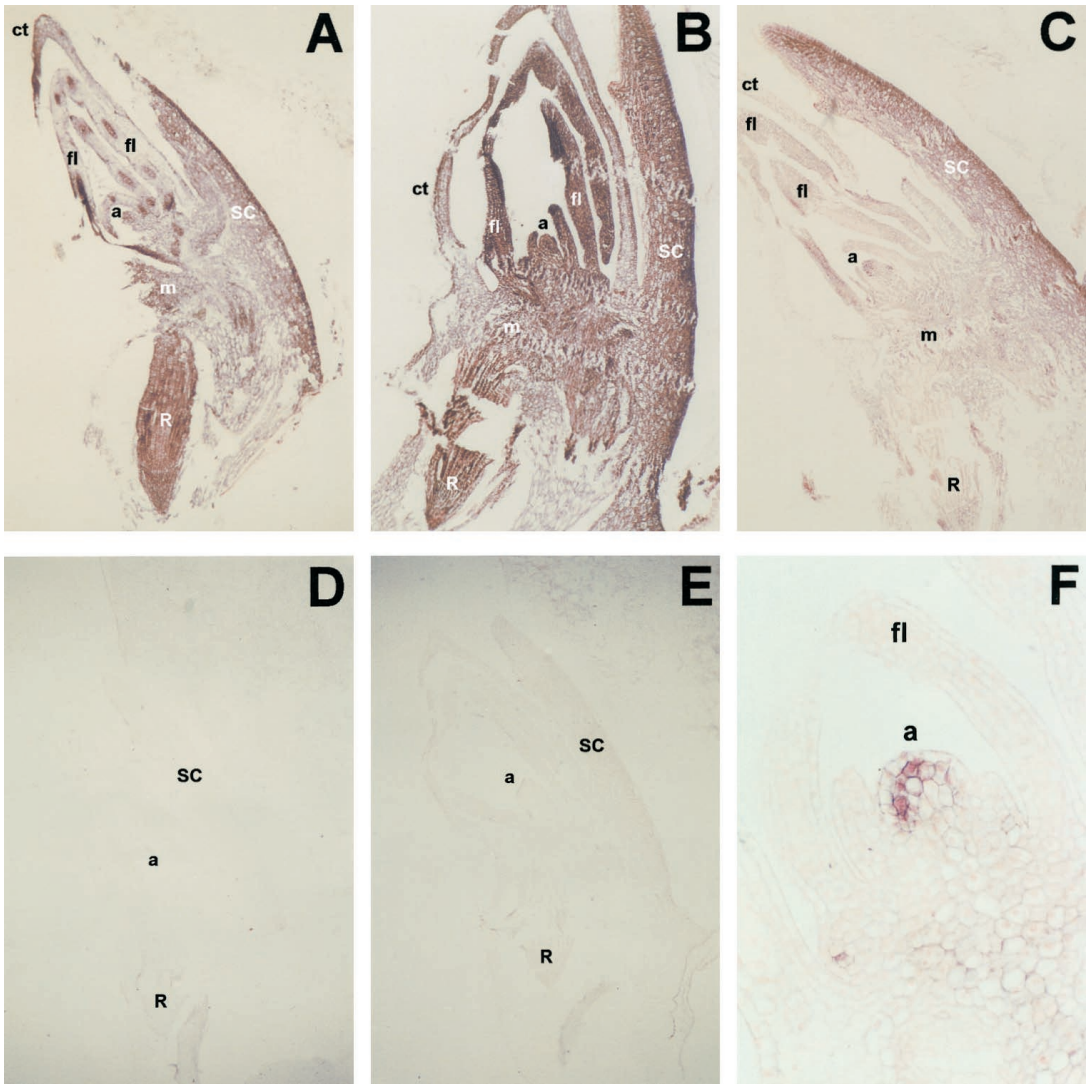

Figure 4. Immunolocalization of 14-3-3 proteins in longitudinal sections of 24-h-imbibed barley embryos. Protein was detected using a secondary alkaline phosphatase-conjugated antibody followed by incubation with NBT/BCIP. A, Detection of 14-3-3A protein with anti-14-3-3A antibody. B, Detection of 14-3-3B protein with anti-14-3-3B antibody. C, Detection of 14-3-3C with anti-14-3-3C antibody. D, Control incubated only with secondary antibody. E, Control incubated with preimmune serum of the rabbit used for immunization with 14-3-3C peptide. F, Close-up of 14-3-3C protein detected in the L2 layer of the shoot apical meristem. Localization of 14-3-3A, 14-3-3B, and 14-3-3C protein was studied in at least three embryos and one representative example is shown here. sc, Scutellum; a, shoot apex; fl, foliage leaf; ct, coleoptile; R, radicle; m, mesocotyl.

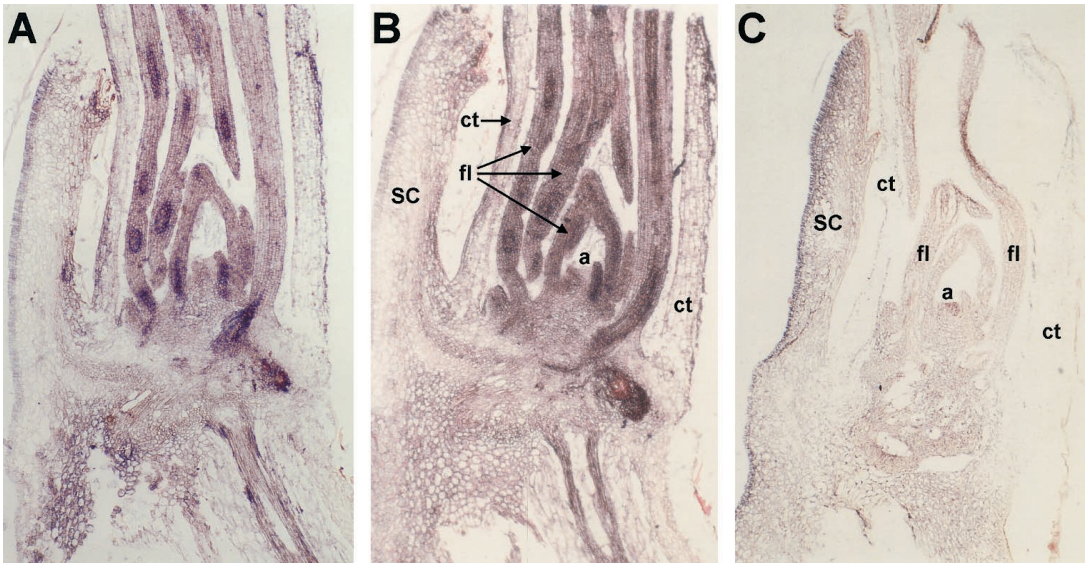

Figure 5. Immunolocalization of 14-3-3 proteins in longitudinal sections of 48-h-imbibed barley embryos. Protein was detected using a secondary alkaline phosphatase-conjugated antibody, followed by incubation with NBT/BCIP. A, Detection of 14-3-3A protein with anti-14-3-3A antibody. B, Detection of 14-3-3B protein with anti-14-3-3B antibody. C, Detection of 14-3-3C with anti-14-3-3C antibody. Localization of 14-3-3A, 14-3-3B, and 14-3-3C protein was each studied in at least three embryos and one representative example is shown here. sc, Scutellum; a, shoot apex; fl, foliage leaf; ct, coleoptile.

scutellum was comparable to that of 14-3-3A and 14-3-3B, with the strongest expression in the epithelial cells.

No signal was detected in the control sections that were only incubated with the secondary antibody (Fig. 4D), nor was any signal detected in preimmune-serum-incubated tissue, showing specificity of the 14-3-3C signal (Fig. 4E). In addition, the 14-3-3B and 14-3-3C signal could be competed by the synthetic peptides that were used for raising the 14-3-3B and 14-3-3C antibodies, respectively (data not shown; the 14-3-3A peptide was no longer available).

In dry grains, in situ immunolocalization was difficult to perform, since the tissue was very rigid. However, the results obtained were consistent with the expression patterns of 14-3-3A, 14-3-3B, and 14-3-3C at 24 h after imbibition (data not shown).

At 48 h after the start of imbibition, both the root and shoot of the embryo had grown out of the grain and had to be cut off before making sections of the remaining embryo. Therefore, only the scutellum and the newly developed leaves could be studied at this time (Fig. 5). In general, expression of 14-3-3A, 14-3-3B, and 14-3-3C was similar to the expression at 24 h of imbibition. For both 14-3-3A and 14-3-3B protein, expression, as detected by antibodies, had decreased in the coleoptile compared with expression at 24 h. No expression could be detected in the secondary antibody control or with preimmune serum of the 14-3-3C antibody (data not shown).

DISCUSSION

Tissue-Specific Expression of 14-3-3

We studied the expression of three 14-3-3 isoforms in germinating barley embryos as an initial part of a study on the roles of the different 14-3-3 isoforms in the germination of barley. Since FC, which can be bound by 14-3-3 proteins, stimulates germination of barley grains and can break dormancy of these grains, we expected 14-3-3 proteins to be involved in the regulation of the germination process.

Using specific cDNA probes and antibodies against the barley 14-3-3 isoforms 14-3-3A, 14-3-3B, and 14-3-3C, we could demonstrate differential mRNA expression of these isoforms in the germinating embryo. Northern and western analysis demonstrated that all three isoforms were expressed in the barley embryo, so at present none of these can be ruled out for performing a function (e.g. FC binding) during germination. When temporal regulation of 14-3-3A, 14-3-3B, and 14-3-3C mRNA production was studied, it appeared that 14-3-3A mRNA expression was correlated with germination. The peak in expression of 14-3-3A mRNA coincides with visible germination of the embryo, and expression decreases after the process is completed. 14-3-3B and 14-3-3C mRNAs, on the other hand, are induced upon imbibition of the grain, and their amount stays rather constant until 48 h, like numerous other mRNAs that are produced upon imbibition of a grain (Bewley, 1997).

Unlike the mRNA levels, the 14-3-3 protein levels did not change during the first 24 h of germination (Figs. 1 and 3). A possible explanation for this observation could be that activity of the 14-3-3 protein during the germination process increases its turnover. At this point, we do not know the function of 14-3-3 mRNA up-regulation during imbibition. However, the mRNA expression patterns do show us that there is a difference in temporal expression of 14-3-3 homologs, in addition to a difference in spatial regulation of 14-3-3 proteins, during germination of barley embryos.

In situ immunolocalization showed that while the 14-3-3A and 14-3-3B proteins are expressed uniformly throughout the embryo, the 14-3-3C protein is expressed in

distinct tissues. The strongest 14-3-3C protein signal was detected in the scutellum and the shoot apical meristem (Fig. 4, C and F). These results are in accordance with earlier work on tissue specificity of 14-3-3 isoforms. For animal 14-3-3 proteins it has been shown for several 14-3-3 homologs that there is a high degree of tissue specificity (Watanabe et al., 1994; Wang and Shakes, 1997). In plants, only the expression pattern of one of the Arabidopsis 14-3-3 homologs, *GF14χ*, has been reported (Daugherty et al., 1996). In that study, mRNA expression of GF14χ was shown to be restricted to specific tissues. It is not known whether any of the other Arabidopis 14-3-3 isoforms show a tissue-specific expression pattern.

In barley embryos, we studied the expression of three isoforms and can therefore conclude that different expression patterns exist for the individual 14-3-3 isoforms. The spatial regulation of the 14-3-3 isoforms might provide an explanation for the large number of biochemically interchangeable 14-3-3 isoforms. Possibly, different 14-3-3 homologs are required to allow the plant to specifically express 14-3-3 proteins where and when they are required to function in the plant.

Role of 14-3-3 Proteins in Seed Germination

The expression of 14-3-3C proteins in the scutellum and shoot apical meristem of the germinating barley embryo provides us with some interesting leads for the role of 14-3-3 in germination. The scutellum tissue has a dual function: first it produces hydrolytic enzymes to degrade the endosperm, and then it absorbs the breakdown products to sustain further growth of the embryo. H^+ pumping is likely to be involved in these processes, and 14-3-3 proteins may have a function in the regulation of H^+-ATPase in the scutellum that requires expression of 14-3-3C specifically in this tissue.

The shoot apical meristem of Hordeae usually consists of three layers (Brown et al., 1957): the L1 and L2, which together form the tunica layer and probably give rise to the epidermis and mesophyll, respectively, and the L3 layer (corpus), which lies beneath the tunica and produces the pith and vascular tissues (Clark et al., 1997). Because the direction of cell division in the tunica is anticlinal, it can be distinguished as a discrete layer that is different from the corpus, which divides in different directions. Expression of 14-3-3C in the shoot apical meristem is restricted to the L2 layer, suggesting a role for this 14-3-3 isoform in the development of the mesophyll.

As 14-3-3A and 14-3-3B are also present in the L2 layer and the scutellum, it is not obvious why extra expression of 14-3-3C might be required specifically in these tissues. Possibly, 14-3-3C can perform additional functions that the other 14-3-3 isoforms cannot. Although differences in functional specificity of plant 14-3-3 proteins could not be demonstrated in vitro or in yeast (Baunsgaard et al., 1998), in planta posttranslational modification might confer an ability to the different 14-3-3 isoforms to perform specific functions.

In summary, the three barley 14-3-3 homologs, 14-3-3A, 14-3-3B, and 14-3-3C, were all shown to be expressed in barley embryos. 14-3-3A and 14-3-3B showed a similar expression pattern in protein localization studies of germinating embryos, while 14-3-3C expression was clearly different. Strong expression of 14-3-3C was observed in the scutellum and the L2 layer of the shoot apical meristem. In northern analysis, 14-3-3A mRNA expression proved to be different from 14-3-3B and 14-3-3C in that it decreased after 24 h of imbibition. These results show that the expression of the 14-3-3 isoforms in barley embryos is differentially regulated, which might reflect a difference in function of the individual isoforms in the germination process. Since a possible role of 14-3-3 in the control of germination is likely to be connected with its binding of FC (Marrè, 1979; Wang et al., 1998), it will be interesting to investigate where in the embryo FC is bound.

ACKNOWLEDGMENTS

We are grateful to David Collinge and his group for generously providing the barley 14-3-3 clones. We thank Bert van Duijn for critical reading of the manuscript and Tom Bunney, Wessel Holtman, Henrie Korthout, and Karin Visser for stimulating discussions and support. Our thanks are also due to Gerda Lamers and Dave Geerlings for technical assistance and to Peter Hock and Adri't Hooft for their assistance in preparing the figures.

Received December 21, 1998; accepted May 27, 1999.

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