Proteins of the 14-3-3 family have well-defined functions as regulators of plant primary metabolism and ion homeostasis. However, neither their function nor action mechanism in plant hormonal signaling have been fully addressed. Here we show that abscisic acid (ABA) affects both expression and protein levels of five 14-3-3 isoforms in embryonic barley roots. As ABA prolongs the presence of 14-3-3 proteins in the elongating radicle, we tested whether 14-3-3s are instrumental in ABA action using RNA interference. Transient co-expression of 14-3-3 RNAi constructs along with an ABA-responsive promoter showed that each 14-3-3 is functional in generating an ABA response. In a yeast two-hybrid screen, we identified three new 14-3-3 interactors that belong to the ABF protein family. Moreover, using a yeast two-hybrid assay, we show that the transcription factor HvABI5, which binds to cis-acting elements of the ABA-inducible HVA1 promoter, interacts with three of the five 14-3-3s. Our analyses identify two 14-3-3 binding motifs in HvABI5 that are essential for 14-3-3 binding and proper in vivo trans-activation activity of HvABI5. In line with these results, 14-3-3 silencing effectively blocks trans-activation. Our results indicate that 14-3-3 genes/proteins are not only under the control of ABA, but that they control ABA action as well.

Keywords: 14-3-3, barley, ABA responsive element binding factor (ABF/AREB), ABA, seed germination, yeast two-hybrid.

Introduction

During seed maturation, ABA establishes dormancy, prepares the seed for desiccation and controls the synthesis of seed storage reserves (Finkelstein et al., 2002). Furthermore, it inhibits precocious germination of the mature embryo and controls the transition from vegetative to reproductive growth (Rohde et al., 2000). In addition to these functions in developmental processes, ABA rearranges the molecular composition of the cell in order to adapt to environmental stress conditions, notably cold, drought and salinity (Verslues and Zhu, 2005).

Knowledge about how ABA functions at the molecular level is still fragmentary. The receptor for ABA has remained elusive until recently (Razem et al., 2006). The nature of the newly discovered receptor is not conventional – an RNA-binding protein (FCA in Arabidopsis, ABAP1 in barley) that regulates flowering time. Notably, FCA protein is not required for seed germination or the stomatal response to ABA. This supports the idea that there are multiple ABA response mechanisms (‘receptors’) operating at the same time, e.g. membranes that change fluidity when the lipophilic ABA is inserted, enzymes or ion transporters with allosteric sites for ABA binding (Rock, 2000), or binding proteins with receptor-like properties such as RPK1 (Osakabe et al., 2005).

Combined genetic, molecular, biochemical and biophysical approaches have succeeded in the identification and characterization of a wide range of genes with functions in ABA signaling (Finkelstein et al., 2002). Amongst these
genes are positive (e.g. OST1, RPK1 and RCN1) and negative (e.g. ABI1, ABI2, ERA1, ABH1, TPC1, ROP10 and AtRAC1) regulators of ABA signaling, as well as transcription factors belonging to the basic leucine zipper (bZIP) class proteins, designated ABA responsive element binding factor (ABF/AREB) (Choi et al., 2000; Hobo et al., 1999; Uno et al., 2000). Despite this wealth of signaling components, it is not clear what the physical or biochemical interactions amongst these network components are. Signaling networks often rely on the formation of dynamic protein complexes, with kinases/phosphatases determining the strength of protein–protein interaction through (de)phosphorylation. Scaffold proteins are important elements of such complexes, and one family of proteins known to fulfill an important scaffold function in all eukaryotic cells is the 14-3-3 protein family (Mackintosh, 2004; Sehnke et al., 2002; Thomas et al., 2005; Tzivion et al., 2001). Intriguingly, cells seem to have hundreds of proteins that are targeted by 14-3-3s, and many of these targets have a mutual relationship because they operate in networks, such as apoptosis, cell-cycle control and ion homeostasis (Van Hemert et al., 2001; Van den Wijngaard et al., 2005).

14-3-3 proteins are acidic regulatory proteins forming homo- and heterodimers. A 14-3-3 dimer forms a clampshape structure (Yaffe et al., 1997) that can interact with one protein at two different positions or bind two different target proteins (Braselmann and McCormick, 1995). The formation of a complex between 14-3-3 and one (or two) targets can have a range of context-dependent effects, including compartmental sequestration, conformational change, enzyme (in)activation, shielding, re-localization, and bridging between two molecules. Unlike in animal cells, where 14-3-3 proteins are major determinants of cellular signaling (Van Hemert et al., 2001), plant 14-3-3s are best characterized as regulators of proteins with a function in C and N metabolism (Huber et al., 2002; Sinnige et al., 2005a) and ion homeostasis (Bunney et al., 2002; Van den Wijngaard et al., 2005; Sinnige et al., 2005b).

In plants, so far only a small number of reports link 14-3-3s to proteins with a function in signal transduction. Camoni et al. (1998) reported the 14-3-3-induced activation of a calcium-dependent protein kinase (CDPK1). This kinase inhibits the calmodulin-stimulated Ca$^{2+}$ pump (ACA2) located in the endoplasmic reticulum of Arabidopsis through phosphorylation (Hwang et al., 2005).

Evidence for a function of 14-3-3 in the action of ABA is still fragmentary or indirect. For example, ABA signaling in guard cells antagonizes the blue-light-dependent H$^+$ pumping by dephosphorylation of the pump and release of 14-3-3 (Zhang et al., 2004), and in embryonic roots ABA inactives a 14-3-3-activated inward K$^+$ channel (Van den Wijngaard et al., 2005). At the level of transcriptional regulation, it has been reported that the ABA signaling effector VP1 (viviparous 1) interacts with 14-3-3 in a yeast two-hybrid assay (Schultz et al., 1998). VP1 is a transcriptional activator, but does not directly bind to the cis-acting elements (so-called ABREs) present in promoters of ABA-inducible genes (Suzuki et al., 1997). It has been suggested that 14-3-3 proteins function as adaptors between VP1 and the ABRE-binding trans-acting factors of the AREB/ABF/ABI5 family (Himmelbach et al., 2003). The ABF/AREB/ABI5 family is a key target of a conserved ABA signaling pathway in plants; their transcript and protein accumulation, phosphorylation state, stability and activity are highly regulated by ABA during germination and early seedling growth (Carles et al., 2002; Casaretto and Ho, 2003; Finkelstein and Lynch, 2000b; Furihata et al., 2006; Kagaya et al., 2002; Lopez-Molina et al., 2001).

In this study, we used the HvABI5- and VP1-dependent ABA-inducible transcription of the ABRC3 cis-acting elements derived from the HVA1 promoter to address the question of whether and how 14-3-3 proteins act as effectors in ABA signaling during seed germination. Our results show that silencing of each individual member of the barley 14-3-3 family (five genes identified) dramatically reduces the activity of ABRC3–GUS. We provide evidence that the transcription factors HvABF1, HvABF2, HvABF3 and HvABI5 interact with 14-3-3 proteins. Moreover, we show that the threonine residue, HvABI5-T$^{350}$, at the very C-terminus in a canonical 14-3-3 binding motif, is essential for interaction with HvABI5. Mutation of this HvABI5-T$^{350}$ reduces the activity of the HvABI5 for trans-activation of ABRC3–GUS. We discuss the function of 14-3-3 proteins in ABA-dependent signal transduction in seed dormancy and germination.

**Results**

Identification of two new barley 14-3-3 genes

Phylogenetic analysis, combining the three published barley 14-3-3 proteins (known as Hv14-3-3A, B and C in the GenBank database) with 14-3-3 proteins from three other monocotyledonous plants (rice, maize and wheat), showed that the proteins can be divided into five evolutionary clusters (Figure 1b). Two of the five clusters contain a barley ortholog, indicating that three barley genes remained to be identified. Therefore, a PCR-based screen was performed to identify these other barley 14-3-3 isoforms. Our search, which was based on the homology with other plant 14-3-3 proteins, yielded two novel barley 14-3-3 genes (Figure 1a). These two new 14-3-3 proteins, named Hv14-3-3D and Hv14-3-3E, show 75% similarity between each other, and >70% similarity with the other three 14-3-3 isoforms already known (amino acid level). A noticeable difference between Hv14-3-3E and the other four proteins is found in the loop between helices 2 and 3, where Hv14-3-3E contains four extra amino acids (Figure 1a). This extension of loop 1 is also present in the wheat and rice homologs, and is not found in any of the animal 14-3-3 proteins. Despite our extensive search, no sixth barley 14-3-3 has been identified so far.
ABA controls the expression and protein levels of Hv14-3-3 in the embryonic root of germinating barley seeds

As the embryonic root (radicle) is a good model system to study ABA action (Finkelstein and Lynch, 2000a; Van den Wijngaard et al., 2005), the 14-3-3 expression patterns were analyzed by quantitative PCR and corresponding protein levels by means of isoform-specific antibodies. Radicles were isolated from imbibed seeds 20 h after imbibition, just prior to emergence from the coleorhiza. Growth analysis of ABA-treated and control roots shows the viability of the isolated roots and their responsiveness to ABA (Figure 2f). The expression data show that four of the five 14-3-3 isoforms respond to ABA treatment (Figure 2a–e). The expression levels of the Hv14-3-3A isoform are not differently affected by the ABA treatment compared to control radicles, whereas ABA induces a transient up-regulation of the Hv14-3-3B and Hv14-3-3C isoforms, peaking at 6 h after the start of treatment (Figure 2a–c). ABA induces a strong and sustained up-regulation (6-8-fold) of the two newly identified Hv14-3-3D and Hv14-3-3E genes (Figure 2d,e). As for gene expression, the protein levels of four of the five 14-3-3 isoforms are also affected by ABA. The Hv14-3-3A isoform is post-translationally modified and is cleaved into a 28 kDa protein in untreated radicles (Figure 3). This truncation of Hv14-3-3A has been shown before in germinating barley embryos (Testerink et al., 2002). However, here we demonstrate that treatment with ABA prevents the proteolytic cleavage of Hv14-3-3A (Figure 3). Protein levels of Hv14-3-3B are not affected by ABA. Hv14-3-3C protein levels in untreated radicles show a transient increase around 28 h after imbibition and the protein has disappeared 46 h after imbibition. In ABA-treated radicles, Hv14-3-3C shows the same increase after 28 h after imbibition, but this level is maintained even after 46 h (Figure 3). Proteins of isoforms D and E disappear in untreated radicles within 1–1.5 days after imbibition, but ABA treatment prevents this reduction (Figure 3). Taken together, these results show that both 14-3-3 gene expression and 14-3-3 protein levels are controlled by ABA in an isoform-specific manner. In view of these responses to ABA, it is plausible that 14-3-3 proteins are instrumental in the action of ABA in maintaining seed dormancy.

14-3-3 proteins are regulators of ABA-inducible ABRC3 derived from the HVA1 promoter

We next investigated whether some, or all, of these 14-3-3 isoforms have a function in the signal transduction cascade of ABA that results in gene activation. The presence of 14-3-3 proteins in G-box binding complexes has been shown previously (Lu et al., 1992; Schultz et al., 1998; de Vetten et al., 1992), and supports our hypothesis that 14-3-3 proteins may be important for ABA signal transduction. However, the molecular mode of action of 14-3-3 proteins has not been shown in these protein–DNA complexes. To address this question, we studied the ABA signal transduction pathway...
by biolistic transformation of embryoless half seeds and silencing the 14-3-3 isoforms using RNA interference. To follow ABA signaling, the promoter activity of a well-characterized ABA-inducible gene, HVA1, was measured as output of the ABA signal transduction pathway. Aleurone layers of barley seeds have been shown to be a good model for biolistic transformation and RNA interference studies (Zentella et al., 2002). To show that the ABA responsiveness of HVA1 in the aleurone layer of embryoless half seeds corresponds to that in our radicle system, the expression levels of HVA1 were followed in radicles by quantitative PCR. As shown in Figure S1, HVA1 responds quickly and is highly upregulated in response to ABA in barley radicles.

The ABRC3–GUS reporter construct contains the ABA-responsive cis-acting elements from the HVA1 promoter that are necessary and sufficient for ABA induction fused to GUS (Shen et al., 1996). The ABRC3–GUS fusion is used to measure ABA induction of gene expression in vivo (Casaretto and Ho, 2003). Silencing of the 14-3-3 isoforms was performed using isoform-specific RNA interference constructs designed against the 3' UTR sequence of the 14-3-3 genes in untreated and ABA-treated barley radicles.

Figure 2. Growth and expression profiling of 14-3-3 genes in untreated and ABA-treated barley radicles. (a–e) ABA affects the expression levels of four of the five 14-3-3 isoforms. (a–e) correspond to isoforms A, B, C, D and E, respectively. The relative expression levels of the five 14-3-3 isoforms in radicles were followed in untreated radicles (○) and ABA-treated radicles (●, 10 μM). Time is indicated as hours after imbibition. Expression levels are normalized on the housekeeping gene Actin (n = 3; ±SE). The stability of the expression of the Actin gene in growing radicles was tested using a second housekeeping gene GAPDH (data not shown).

(f) Relative growth (fresh weight increase) of isolated radicles is followed over time (normalized to the fresh weight of starting material). Growth of untreated radicles (○) and ABA-treated radicles (●, 10 μM) is shown (n = 2; ±SE). Radicles were isolated 20 h after imbibition and treatments were started at 22 h after imbibition.

Figure 3. ABA stabilizes 14-3-3 proteins in isolated radicles. Western blots of total protein extracts (15 μg) of untreated and ABA-treated (10 μM) radicles. Time is shown in hours after imbibition. Radicles were isolated 20 h after imbibition and treatments started at 22 h after imbibition. Three independent experiments were performed and representative results are shown. Western blots were stained using Ponceau S (Bio-Rad), prior to hybridization with primary antibody, to check for equal loading.

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genes (Figure S5). The isoform-specific 14-3-3 RNAi constructs were individually co-bombarded with the ABRC3–GUS construct. In order to minimize variations among particle bombardments, luciferase under the control of the ubiquitin promoter (UBI–Luc) is used as an internal control. As 14-3-3 proteins have multiple targets and are capable of inhibiting enzymes, we first ruled out the possibility that 14-3-3 RNAi has an effect on the luciferase enzyme activity by co-bombarding the UBI–Luc and UBI–GUS constructs with and without 14-3-3 RNAi constructs. In this way, the luciferase activity could be normalized on the GUS activity. The 14-3-3 RNAi constructs did not have an effect on the luciferase activity (data not shown).

Figure 4(b) shows that the ABRC3–GUS construct is up-regulated by ABA. Bombardment of the effector construct targeted to \(Hv14-3-3A\) resulted in a reduction of 40% of the ABRC3–GUS expression in the presence of ABA. Silencing of the other four 14-3-3 isoforms induced a reduction of ABRC3–GUS activity in the presence of ABA of at least 70%. 14-3-3E RNAi showed the strongest reduction of ABRC3–GUS activity – up to 90%. ABRC3–GUS activities of non-treated seeds also showed reduced activity in response to the 14-3-3 RNAi construct. We hypothesize that this is due to the endogenous ABA that triggers the ABA signal transduction in aleurone cells, which is reduced due to the lack of 14-3-3 proteins. The same effect was observed when similar experiments were performed with the transcription factor HvABI5 (data not shown).

Figure 4. Transient transformation of barley aleurone cells with 14-3-3 RNAi-generating constructs suppresses the ABA induction of ABRC3–GUS. (a) Schemes of reporter and effector constructs. RNA interference constructs of the \(Hv14-3-3\) genes were targeted to the 3’ UTR region of each gene. (b) ABA responsiveness of ABRC3–GUS was followed in the aleurone layer of embryoless barley half seeds by biolistic transformation with the ABRC3–GUS fusion. Untreated barley seeds (solid bars) and ABA-treated (20 \(\mu M\)) barley seeds (white bars) are shown (\(n = 4\); \(\pm SE\)).

Figure 5. Silencing constructs are specific and show no signs of cross-silencing. (a) Scheme of the reporter and effector constructs. Part of the 14-3-3E cDNA sequence was fused to the 3’ end of the GUS reporter gene. 14-3-3 isoform-specific RNA interference constructs were used. (b) Relative GUS activities after the bombardments are shown. The control bombardment was set to 100%, showing that the 14-3-3E RNAi construct is the only construct that reduces the GUS activity of the GUS–14-3-3E fusion.
the RNAi construct derived from Hv14-3-3E silences the GUS–14-3-3E with an efficiency of approximately 60%. Moreover, the RNAi constructs directed against the other 14-3-3 isoforms do not reduce the GUS activity, indicating that the RNAi constructs are specific. This provides evidence that every individual member of the barley 14-3-3 protein family has a function in ABA-induced activation of the ABRC3 promoter elements in barley aleurone cells.

Yeast two-hybrid screen with 14-3-3 proteins as bait to identify molecular targets in the ABA signal transduction pathway

A main hallmark of 14-3-3 action is that 14-3-3 proteins act as master regulators in specific signaling networks, such as the cell cycle, apoptosis and hormonal signaling (Van Hemert et al., 2001). As the ABA signal transduction pathway forms a complex network with many signal mediators (Rock, 2000), it is not unlikely that there are multiple 14-3-3 targets within this network. HvABI5 and VP1 are two transcription factors that have been shown to be important mediators of the ABA signal in barley aleurone cells (Casaretto and Ho, 2003), and one of these (ZmVP1) was shown to interact with rice 14-3-3 proteins GF14a, GF14b and GF14c in a yeast two-hybrid assay (Schultz et al., 1998). In a yeast two-hybrid screen, using a cDNA library constructed from RNA derived from 7-day-old barley leaves and all five individual barley 14-3-3 isoforms as bait, we identified 132 new 14-3-3 target proteins (Schoonheim and De Boer, unpublished data). Amongst these targets were three AREB/ABF/ABI5-like proteins (Figure 6a,b). We named the newly identified AREB/ABF/ABI5 proteins after their Arabidopsis homologs, i.e. HvABF1,
HvABF2 and HvABF3 (Figure S2A). In view of this result, we addressed the question of whether the seed-specific ABF-homolog, HvAB15, might interact with 14-3-3 proteins as well. The HvAB15 protein has two putative 14-3-3 interaction motifs located around T160 (REIPT160AP) and T350 (RRTLT350GP); the latter motif is conserved in most of the ABF proteins (Figure S2B). A yeast two-hybrid assay using the five 14-3-3 isoforms as prey and HvAB15 as bait showed that HvAB15 interacts with three of the five 14-3-3 isoforms: Hv14-3-3C, Hv14-3-3D and Hv14-3-3E (Figure 6c,d). The in vivo interactions of the 14-3-3 proteins with the AREB/ABF/ABI5-like proteins were confirmed in vitro by performing far-Western analysis using recombinant proteins (Figure 6e).

This interaction between 14-3-3 proteins and HvAB15 might explain why 14-3-3 silencing prevents activation of ABRC3–GUS in response to ABA (Figure 4). Therefore, we decided to focus on the role of HvAB15/14-3-3 interaction in vivo, by mutating the 14-3-3 binding site in the HvAB15 protein and follow its activity in the trans-activation of ABRC3–GUS. To identify the 14-3-3 binding site in the HvAB15 protein, we introduced point mutations in two putative 14-3-3 interaction motifs. The T160 and T350 amino acids of HvAB15 were substituted by an alanine amino acid, resulting in three mutant forms of HvAB15: HvAB15T160A, HvAB15T350A and the double mutant HvAB15T160A + T350A (HvAB15dm). These mutants were tested in the yeast two-hybrid assay for interaction with all five 14-3-3 isoforms. Mutation of both putative interaction motifs (double mutant) in HvAB15 resulted in a complete loss of interaction with Hv14-3-3C, D and E (Figure 6d). Assays with the individually mutated threonine residues showed an interesting isoform-specific interaction with the HvAB15 protein. It was found that the T160 mutation (HvAB15T160A) disrupts only the interaction with the Hv14-3-3C isoform, whereas isoforms Hv14-3-3D and E were still capable of binding to the HvAB15T160A protein. In contrast to the T160 mutation, the T350 mutation (HvAB15T350A) disrupted the interaction with all three interactors, Hv14-3-3C, D and E (Figure 6d). From this we conclude that the canonical RRTLT350GP motif is essential for interaction between HvAB15 and Hv14-3-3C, D and E. However, both 14-3-3 binding sites, i.e. REIPT160AP and RRTLT350GP, are necessary for interaction with the Hv14-3-3C protein.

Trans-activation of ABRC3–GUS by ZmVP1 and HvAB15 is dependent upon the 14-3-3 protein binding site of the HvAB15 protein

In the next step, we tested whether the intact C-terminal 14-3-3 interaction motif (around T350) of the HvAB15 protein is essential for the in vivo action of HvAB15. To do so, we performed transient trans-activation experiments to analyze the effect of amino acid substitutions and deletions on its trans-activation activity. Ectopic expression of HvAB15 alone has no or very little effect on the activity of ABRC3–GUS (Casaretto and Ho, 2003). However, when HvAB15 is co-bombarded together with ZmVP1, ABRC3–GUS is trans-activated and ABA treatment does not have an additional effect on the activity of ABRC3–GUS (Figure 7). We tested three mutant forms of HvAB15 for their ability to trans-activate ABRC3–GUS together with ZmVP1: T350A, T350D and C4Del, where the last four C-terminal amino acids (including T350) are missing. The rationale behind the choice of these mutations is that T350 is necessary for 14-3-3 binding, as shown in the yeast two-hybrid assays (Figure 6c,d), so both HvAB15T350A and C4Del will have a reduced capacity to interact with 14-3-3 proteins. Substitution of T350 with an aspartic acid (D) provides a negative charge at this position, thus mimicking the phosphorylated form of threonine. As a phosphorylated interaction motif has a much higher affinity for 14-3-3 binding, we expected that trans-activation activity would be retained by the HvAB15T350D protein. As shown in Figure 7(b), the mutant proteins HvAB15T350A and HvAB15C4Del have a reduced capacity (50%) to trans-activate ABRC3–GUS. In contrast, the HvAB15T350D mutant has the same activity as the wild-type form of HvAB15, providing support for our conclusion that an intact 14-3-3 binding motif at the C-terminus is important for the in vivo function of the HvAB15 protein.
Finally, if the reduced trans-activation capacity of the HvABI5 proteins with a non-functional 14-3-3 binding motif is indeed the result of reduced 14-3-3 interaction, we hypothesize that 14-3-3 silencing will also have a negative effect on the trans-activation activity of wild-type HvABI5 (in combination with ZmVP1). Figure 8 shows that co-expression of RNAi of all 14-3-3 isoforms negatively affects trans-activation of ABRC3–GUS (by up to 70%) by co-bombardment of HvABI5 and ZmVP1. Untreated barley seeds (solid bars) and ABA-treated (20 μM) barley seeds (white bars) are shown (n = 4; ±SE).

Discussion

When a seed embryo has matured, it is important that the seed remains quiescent until conditions for germination become favorable. Seeds from some cereal crops have the tendency to germinate when still in the head (pre-harvest sprouting or vivipary), whereas others may show too much dormancy at harvest, which results in uneven germination. Both traits are undesirable from an agronomic viewpoint and may lead to serious economic losses. Abscisic acid biosynthesis and responses are clearly involved in both vivipary and dormancy (Koornneef et al., 2002). Although many elements of the ABA signal transduction cascade have been discovered in recent years (Finkelstein et al., 2002; Himmelbach et al., 2003; Rock, 2000; Schroeder et al., 2001), the picture is still far from complete. One new putative element, the 14-3-3 protein family, has come to the foreground in recent years as regulators of proteins that are important for ABA action during seed germination and stomatal regulation: the transcription factor VP1 (Schultz et al., 1998), plasma membrane K⁺ channels (Van den Wijngaard et al., 2005), the vacuolar SV channel (Peiter et al., 2005; Van den Wijngaard et al., 2001) and the plasma membrane H⁺-AT-Pase (Brault et al., 2004; Kinoshita and Shimazaki, 2002). We used the barley primary root (radicle) and aleurone cells to obtain a better insight in the 14-3-3/ABA relationship.

Barley 14-3-3 genes and proteins respond to ABA

Our search for additional barley 14-3-3 genes resulted in the identification of two new isoforms, Hv14-3-3D and Hv14-3-3E. The deduced amino acid sequence of the new 14-3-3 proteins showed highest homology between each other (75% identity), and Hv14-3-3D and Hv14-3-3E are orthologs of the wheat TaWIN2 and TaWIN1, respectively (Figure 1b). Barley radicle protrusion is an ideal system to study seed germination. Barley seeds are large seeds (approximately 8–9 mm), and thus radicles can easily be isolated from the germinating seed and can be studied as an isolated system. After a seed is imbibed in water, the plant maintains a post-germination developmental checkpoint for 2–3 days. When the seed experiences drought or salt stress within this period, it can respond with a growth arrest through the production of ABA (Lopez-Molina et al., 2001). When ABA is produced or applied outside this time window, there is no growth response. Therefore, we started the ABA treatments 22 h after seed imbibition and measured growth and followed gene expression and protein levels of the 14-3-3 family. Expression of the Hv14-3-3A isoform is unaffected by ABA, but the protein shows an interesting ABA-dependent post-translational modification (Figure 8). Whereas in control radicles during the second day after imbibition, the 30 kDa Hv14-3-3A protein is fully truncated to a 28 kDa protein, ABA completely prevents proteolytic cleavage (Figure 3). The 28 kDa form of Hv14-3-3A lacks 10 or 12 amino acids at the non-conserved C-terminus of the protein (Testerink et al., 2002). Although Hv14-3-3B and Hv14-3-3C are closely related genes (Figure 1b), their protein levels...
during development of the radicle differ greatly. Whereas the Hv14-3-3C protein appears only during a rather narrow time window, peaking at around 30 h, the Hv14-3-3B protein remains stable throughout the second day (Figure 3). Moreover, ABA does not affect Hv14-3-3B protein levels, but prevents the breakdown of the Hv14-3-3C protein that starts around 34 h (Figure 3). Isoforms D and E show similar patterns of expression and protein levels during normal development and in response to ABA treatment (Figures 2 and 3). ABA ensures that the D and E expression and protein levels are highly upregulated in the course of the 2nd day after imbibition. Clearly, there is a strong developmental and hormonal regulation of the barley 14-3-3s in an isoform-specific manner, and it should be noted that the level of mRNA does not necessarily correlate with the corresponding protein level.

Function of barley 14-3-3 protein in ABA signaling

As both 14-3-3 transcript and protein levels are strongly ABA-responsive, as described above, and in view of the well-documented function of 14-3-3 proteins in animal hormonal signaling (Thomas et al., 2005), we hypothesized that 14-3-3 proteins may be important for ABA signaling. To test this hypothesis, we took advantage of the model system for studying the ABA signal transduction pathway in seeds: the barley aleurone layer (Lovegrove and Hooley, 2000). The cis-acting elements of the ABA-inducible \textit{HVA1} gene (ABRC3) were chosen to report the effectiveness of ABA action and thus ABA signaling (Casaretto and Ho, 2003, 2005). From each 14-3-3 isoform, RNAi constructs were prepared (Figure 4a) and aleurone layers were co-bombarded with ABRC3-GUS constructs and 14-3-3 RNAi constructs. The outcome was rather surprising – silencing of each isoform resulted in a reduction of the ABRC3-GUS activity, which indicates that each individual 14-3-3 isoform has a key position in the pathway leading from ABA perception to ABA-induced gene expression.

A hallmark of 14-3-3 action is the interaction with other proteins. Our observation that silencing of the 14-3-3s negatively affects the progression of ABA signaling towards activation of gene expression suggests that 14-3-3s interact with important mediators of ABA signaling. A yeast two-hybrid screen using the five barley 14-3-3 isoforms as bait to conduct a yeast two-hybrid assay (Figure 6c,d). Both the quantitative as well as the qualitative assay clearly show that HvA15 interacts with three of our 14-3-3s: Hv14-3-3C, Hv14-3-3D and Hv14-3-3E. Despite the high degree of homology between the B and C isoforms (92% identity at amino acid level), Hv14-3-3B does not show an interaction with HvA15 in our assay, which emphasizes the high degree of isoform specificity. A similar observation was made with the enzyme sucrose phosphate synthase (SPS) (Bornke, 2005), and from deletion analysis these authors concluded that differences in affinity for a certain target were mediated by the variable C-terminus of the 14-3-3s. Indeed, half of the last 20 amino acids of the 14-3-3B and 14-3-3C C-terminal ends are different (Figure S3). We focused on the bZIP transcription factor HvA15 as putative target for a number of reasons: (i) HvA15 has been shown to interact with the ABRC3 (Casaretto and Ho, 2003), (ii) the HvA15 protein sequence contains two canonical 14-3-3 interaction motifs (REIPT160AP and RRTL350GP) in which phosphorylation of T160 and/or T350 may control 14-3-3 interaction, and (iii) related bZIP-type transcription factors such as AtA15 and AtAREB1 are phosphorylated upon ABA treatment (Furuhata et al., 2006; Lopez-Molina et al., 2001).

The serine or threonine residue in the canonical 14-3-3 binding motif (K/R(x)xxS/TxP) is important because (de)phosphorylation of these residues determines the affinity for 14-3-3 proteins. To investigate whether the two putative 14-3-3 binding motifs in the HvA15 protein are indeed functional for 14-3-3 binding, we used site-directed mutagenesis to substitute the T160 and T350 by alanine residues. The site of interaction for the two strongest binding partners, Hv14-3-3D and Hv14-3-3E, is located at the C-terminal end around amino acid T350. Intriguingly, substitution of T160 by an alanine only affects interaction with Hv14-3-3C and not with Hv14-3-3D or E. This suggests that Hv14-3-3C binds the HvA15 protein in two different places. This two-site interaction resembles the way 14-3-3 proteins interact with AANAT (arylalkylamin N-acetyltransferase), the penultimate enzyme in melatonin biosynthesis (Ganguly et al., 2005). HvA15 and AANAT also share the recently identified mode III binding motif (Coblitz et al., 2006) located at the very C-terminal end: RRTLTPW-COOH and RRN350D-COOH respectively. However, here we show that different 14-3-3 isoforms interact with one and the same target (HvA15), but that they bind the target in a different fashion. It can be speculated that different modes of interaction between HvA15 and the three 14-3-3s are functionally relevant, as HvA15 is also involved in a positive regulatory loop up-regulating its own expression (Casaretto and Ho, 2005). These different binding modes, the fact that 14-3-3 proteins act both as homo- and heterodimers, and the possibility that the 14-3-3 proteins ensure coordina-
ted regulation of the ABA signaling network through interaction with multiple intermediates, may be the reason that our silencing data suggest little redundancy in 14-3-3 action. In order to identify the complete 14-3-3 interactome from germinating barley seeds, we are currently undertaking a 14-3-3 affinity purification approach, using the recombinant 14-3-3 proteins and extracts from control and ABA-treated treated.

The intact C-terminal 14-3-3 interaction motif is important for the trans-activation activity of HvABI5

Investigating the in vivo function of HvABI5 is possible through ectopic expression of the two transcription factors HvABI5 and ZmVP1, which results in trans-activation of ABRC3-GUS in the absence of ABA. Using this transient trans-activation method, we studied the relevance of the 14-3-3 interaction site (RRTL786GP) for in vivo activity. Mutant versions of HvABI5 (HvABI5ST286A and C4Del) that do not contain the 14-3-3 binding site have a reduced ability to trans-activate the ABRC3-GUS (50%). This provides indirect evidence that 14-3-3s are important for the in vivo trans-activation activity of HvABI5, and this idea was corroborated by the negative effect of 14-3-3 silencing on the trans-activation activity of HvABI5 (Figure 8).

At first sight, the dependence of ABA signaling in seeds upon five individual 14-3-3 proteins seems bewildering. However, now that studies in the field of animal 14-3-3 biology have identified hundreds of 14-3-3 target proteins, a picture is emerging with a key position for 14-3-3 proteins in network means for the processes going on within the network and for cross-talk with other signaling processes.

Experimental procedures

Plant material

Two different varieties of barley (Hordeum vulgare L.) have been used for these studies. For expression profiling and Western blots, variety Alexis was used (Saatzucht Jozef Breun GdBR, Herzogenaurach, Germany), and for biolistic transformation of aleurone layers, the variety Himalaya was used (harvest 1998, Pullman Crop and Soil Sciences, Washington State University, Pullman, WA, USA).

Isolation and growth of barley radicles

The isolation and growth of the barley radicles were performed as described by Van den Wijngaard et al. (2005).

Identification of Hv14-3-3D and Hv14-3-3E genes

To identify new barley 14-3-3 isoforms, a degenerated primer (5'-RGAYTC5AC5YT5ATATG-3'; UIB coding, '5' denotes an inosine) was designed against the homologous part of the 3' end of most of the known plant 14-3-3s. PCR was performed using this degenerated primer as the forward primer and the 3' RACE primer as the reverse primer (5'-AAGCAGTGTAAACACCGAAGTAGTTTTTTTTTTTTTTTTTATGC(TG)-3'). The amplified fragments were cloned into a pGEM-T (Promega, Madison, WI, USA) vector. Colonies were screened using filter lifting, and filters were hybridized with a radiolabelled probe directed against the conserved region of the Hv14-3-3C cDNA. Positive clones were sequenced using the T7 promoter and Bigdye sequencing kit (Applied Biosystems, Foster City, CA, USA). The full-length cDNA sequence was identified by 5' RACE. The full cDNA sequence was cloned in-frame into the pPinpoint Xa vector (Promega).

RNA isolation and quantitative PCR

The isolation of total RNA from radicles and subsequent gene expression profiling by quantitative PCR were performed as described by Van den Wijngaard et al. (2005). Primers that were used for the quantitative PCR are given in Table S4.

Determination of 14-3-3 protein levels

The isolation of total protein and Western blotting were performed as described by Van den Wijngaard et al. (2005).

Preparation of DNA constructs

RNA interference constructs were designed to target the 3' UTR sequence of the five barley 14-3-3 isoforms. The five barley 14-3-3 sequences were aligned and primers were designed to the unique part of the 3' UTR. The primers for the 14-3-3A, B and C constructs were designed to introduce EcoRI restriction sites at the 5' end and BamHI restriction sites at the 3' end, and vice versa for 14-3-3D and E. For 14-3-3A, B and C, part of the coding region was used as loop DNA, for 14-3-3D and 14-3-3E part of the vector was used as loop DNA (pPinpoint Xa). Amplicons were digested with EcoRI and ligated, and the ligated PCR fragments (short-long) were inserted into a BamHI-digested UB1-driven vector (UBILuc described by Zentella et al., 2002), resulting in the RNA interference construct. The primers used are shown in Table S1. The reporter constructs UBILuc, ABRC3-GUS and UBI-GUS have been described previously (Casaretto and Ho, 2003). The 14-3-3E–GUS fusion was prepared by digesting the 14-3-3E–Pinpoint construct and UBI–GUS with KpnI and SacI. The 14-3-3E insert was ligated at the 3' end of the construct between the GUS ORF and the nos terminator. The ABF–His constructs were prepared...
by digesting the pACT2 vector (Clontech, Maintain View, CA, USA) containing the ABF insert with BamHI and BglII. The digested insert was subsequently ligated into a BamHI-digested pSET-C vector (Invitrogen, Carlsbad, CA, USA). HvAB15 mutant constructs were prepared by PCR using primers that introduce the mutations. For the C-terminal four amino acid deletion mutant (C4Del), a TGA stop codon was inserted behind the L249. To minimize PCR mistakes, we digested the PCR fragment for the T245 mutants with SacII and KpnI, and the digested PCR fragment was ligated into the SacI–KpnI-digested UBI-HvAB15. For the HvAB15T160 mutants, a two-step PCR was performed to introduce the SacII restriction site into the PCR fragment. The primer combinations used are shown in Table S2. To prepare the double mutant HvAB15, we digested the UBI-HvAB15-T245 A and UBI-HvAB15T160 A with BamHI and SacII. The HvAB15T160 A BamHI–SacII fragment was ligated into the BamHI–SacII-digested UBI–HvAB15T245 A vector. For yeast two-hybrid constructs, pGAL4-AD and pGAL4-BD (Stratagene, La Jolla, CA, USA) were digested with the appropriate restriction enzymes. The inserts for most of the constructs were prepared by using PCR; for HvAB15, the constructs were prepared by digestion with SmaI and EcoRV and ligated into the SmaI site of the pBD-GAL4 vector. The primers used are shown in Table S3. All constructs were checked by sequencing.

**Transient expression assays**

Particle bombardment of embryoless barley half seeds was performed according to the method described by Shen et al. (1993). Briefly, embryos of the barley cultivar Himalaya were cut off. The embryoless barley seeds were imbibed in the dark at 20°C for 2 days on top of moist filter paper. After 2 days, the testa and pericarp layers were removed using a dissecting microscope. M17 tungsten particles (Bio-Rad, Hercules, CA, USA) were coated with reporter and effector cDNA (1:1) using 1 m CaCl₂ and 0.1 m spermine (free base). An UBI–Luc construct was also co-bombarded (1:1) using 1M CaCl₂ and 0.1 M spermine (free base). An UBI–Luc construct was also co-bombarded (1:1) with the reporter for the quantitative ade4 reporter. The inserts were sequenced using the pACT2 sequencing primer (TaqMan, Foster City, CA, USA). HvAB15 mutant constructs were prepared by PCR using primers that introduce the mutations. For the HvAB15T160 mutants, a two-step PCR was performed to introduce the SacII restriction site into the PCR fragment. The primer combinations used are shown in Table S2. To prepare the double mutant HvAB15, we digested the UBI-HvAB15-T245 A and UBI-HvAB15T160 A with BamHI and SacII. The HvAB15T160 A BamHI–SacII fragment was ligated into the BamHI–SacII-digested UBI–HvAB15T245 A vector. For yeast two-hybrid constructs, pGAL4-AD and pGAL4-BD (Stratagene, La Jolla, CA, USA) were digested with the appropriate restriction enzymes. The inserts for most of the constructs were prepared by using PCR; for HvAB15, the constructs were prepared by digestion with SmaI and EcoRV and ligated into the SmaI site of the pBD-GAL4 vector. The primers used are shown in Table S3. All constructs were checked by sequencing.

**Yeast two-hybrid assay**

The Stratagene GAL4 two-hybrid phagemid vector kit was used. The improved yeast strain PJ69-4A was used (James et al., 1996), which contains an extra reporter selecting for adenine auxotrophy (ADE2). The ADE2 is a more stringent reporter compared to HIS3. Transformation of the yeast cells was performed by the lithium acetate method as described by Gietz et al. (1992). Yeast two-hybrid screens were performed using a barley leaf yeast two-hybrid cDNA library that was a kind gift from Masumi Robertson (CSIRO Plant Industry, Canberra, Australia). DNA was isolated from interacting clones and inserts were sequenced using the pACT2 sequencing primer (Table S1). To double check interactions from the screen, DNA that was isolated from the interacting clones was re-transformed into PJ69-4A yeast. Double-transformed yeast clones were grown up in 1 ml liquid medium (SD) lacking the amino acids leucine and tryptophan (-LWHA) and grown for 4 days at 30°C. For the quantitative β-galactosidase assays, 1 ml of yeast culture was spun down and used for activity assays (three individual replicates). To check whether the clones contain the correct DNA constructs, DNA was isolated from 3 ml of full-grown cultures. PCR was performed using vector- and gene-specific primers. The β-galactosidase activity protocol is described at http://www.fh crc.org/labs/gottsching/yeast/Bgal.html. All yeast two-hybrid data presented here were reproduced in at least three experiments.

**Preparation of recombinant proteins and far-Western analysis**

Preparation of the recombinant proteins was performed as described previously (Van den Wijngaard et al., 2005). Far-Western experiments were performed according to the method described by Moorhead et al. (1999): briefly, 1 μg of recombinant ABF–HIS protein was run on SDS–PAGE. Proteins were transferred to PVDF membranes using the Bio-Rad semi-dry blotting system (15 V for 2 h). Membranes were incubated with 5% Marvel (Bio-Rad, Hercules, CA, USA) for 16 h at room temperature; subsequently membranes were incubated with 3 μg/ml recombinant 14-3-3 for 2 h. As secondary antibody, we used avidin–HRP conjugate (Pierce, Rockford, IL, USA), and HRP activity was visualized using the ECL detection kit (Amersham, Uppsala, Sweden).

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**Supplementary Material**

The following supplementary material is available for this article online:

Figure S1. ABA induction of HVA1 gene expression in barley radicles.

Figure S2. Sequence analysis of the C-terminus of the ABF transcription factor family.

Figure S3. Sequence analysis of the barley 14-3-3 proteins B and C.

Figure S4. Specificity of 14-3-3 isoform-specific antibodies tested on recombinant proteins.

Figure S5. DNA sequences of the 14-3-3 3' UTR region used for the RNAi constructs.

Table S1 Primers used for 14-3-3 RNA interference constructs

Table S2 Primers used for HvAB15 mutations

Table S3 Primers used for yeast two-hybrid constructs

Table S4 Primers used for quantitative PCR

This material is available as part of the online article from http://www.blackwell-synergy.com.

**References**


Accession numbers: The sequences of the newly identified Hv14-3-3D, Hv14-3-3E, HvABF1, HvABF2 and HvABF3 were deposited in the NCBI database. GenBank accession numbers of the genes used in this study are as follows: AY150676 (HvABI5), X62388 (14-3-3A), X93170 (14-3-3B), Y14200 (14-3-3C), DQ295785 (14-3-3D), DQ295786 (14-3-3E), DQ786408 (HvABF1), DQ786409 (HvABF2), DQ786410 (HvABF3), AY145451 (Actin) and X78205 (HVA1).