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Accumulation of the transcription factor ABA-insensitive (ABI)4 is tightly regulated post-transcriptionally

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Abstract

ABA-INSENSITIVE (ABI)4 is a transcription factor implicated in response to ABA in maturing seeds, and seedling responses to ABA, salt, and sugar. Previous studies have shown that *ABI4* transcripts are high in seeds and in seedlings exposed to high concentrations of glucose and, to a lesser extent, osmotic agents and ABA, but that transcript levels are very low through most of vegetative growth. This study examined ABI4 protein accumulation indirectly, using transgenic lines expressing fusions to GFP and GUS. The GFP fusions were active, but undetectable visually or immunologically. Comparison of transcript and activity levels for GUS expression showed that inclusion of the ABI4 coding sequence reduced the ratio of activity to transcript ~40-fold when driven by the *CaMV 35S* promoter, and nearly 150-fold when controlled by the *ABI4* promoter. At least part of this discrepancy is due to proteasomal degradation of ABI4, resulting in a half-life of 5–6 h for the ABI4–GUS fusion. Comparison of the spatial localization of transcripts and fusion proteins indicated that the protein preferentially accumulated in roots such that transcript and protein distribution had little similarity. The components mediating targeting to the proteasome or other mechanisms of spatial restriction have not yet been identified, but several domains of ABI4 appear to contribute to its instability.

Key words: Abscisic acid, ABI4, *Arabidopsis*, post-transcriptional regulation, proteasome, protein stability.

Introduction

Production of healthy viable seedlings depends on a successful transition from seed maturation through developmental arrest to germination and seedling growth. These events are controlled by numerous regulators integrating response to internal signals such as abscisic acid (ABA) and gibberellins, and environmental factors including cold, light, and water availability. Early genetic studies identified the transcription factors ABA-INSENSITIVE(ABI)3, ABI4, and ABI5 as central mediators of this signalling (reviewed in Finkelstein *et al.*, 2002). All three of the *ABI* transcription factor genes are expressed throughout seed development, reaching their highest transcript levels at seed maturity, but decreasing during germination unless exposed to stresses that inhibit germination such as ABA or dehydrating conditions. Subsequent studies have placed

them in a much larger transcriptional hierarchy with extensive cross-regulation among the *LEAFY COTYLEDON (LEC)* loci, the *ABI* loci, additional B3-domain loci such as *ABI3/VP1*-like genes and *FUSCA3*, and genes encoding the *ABI5*-related bZIP factors such as the *ABF/AREBs* controlling the transition from embryogenesis to seed maturity and eventual seedling growth (Finkelstein *et al.*, 2005; To *et al.*, 2006; Suzuki *et al.*, 2007). Some of these factors are also regulated post-transcriptionally: activity of ABI5 and related factors depends on phosphorylation (reviewed in Cutler *et al.*, 2010), FUSCA3 is proteasomally degraded during embryo maturation and germination (Lu *et al.*, 2010), and both ABI3 and ABI5 are degraded via the proteasome in germinating seedlings (Lopez-Molina *et al.*, 2001; Zhang *et al.*, 2005).

Although the *ABI4* locus was initially identified on the basis of ABA-resistant germination of mutants (Finkelstein, 1994), additional *abi4* alleles have been isolated in screens for defects in salt or sugar signalling in seedlings (Arenas-Huertero *et al.*, 2000; Huijser *et al.*, 2000; Laby *et al.*, 2000; Quesada *et al.*, 2000), and retrograde regulation of plastids (Koussevitzky *et al.*, 2007). Consistent with roles in glucose signalling and expression of plastid proteins, *ABI4* expression has been shown to increase dramatically in response to growth-inhibiting concentrations of glucose (Arroyo *et al.*, 2003). Furthermore, the ABI4 protein binds to *cis*-acting elements mediating both sugar- and ABA-inducible gene expression (Bossi *et al.*, 2009; Reeves *et al.*, 2011) and sugar and ABA repression of photosynthetically active nuclear genes (Acevedo-Hernández *et al.*, 2005).

To analyse ABI4 function and determine whether ABI4 protein accumulation parallels its transcript accumulation, transgenic lines were constructed that overexpressed ABI4 with a variety of fusion tags. These studies revealed that ABI4 is also post-transcriptionally regulated.

Materials and methods

Transgene constructs and plant transformation

35S–GFP–ABI4 fusions were constructed by ligating an *Eco*RI cDNA fragment encoding all but the first two and last amino acids of ABI4 into the pEGAD vector (accession no. AF218816), as described in Reeves *et al.* (2011). *35S–ABI4–GR* fusions were constructed in pBI-ΔGR, a derivative of pBI121 in which the β-glucuronidase (*GUS*) gene is replaced with a fragment encoding amino acids (aa) 508–795 of the rat glucocorticoid receptor (Lloyd *et al.*, 1994). *35S–ABI4–GUS* and *35S–ABI4domain–GUS* fusions were constructed in pBI121 (accession no. AF485783) (Jefferson *et al.*, 1987). The 'full-length' *ABI4* fusion contains 30 bp of 5'UTR and all but the last two codons of *ABI4* (aa 1–326). The N-terminal fusion includes aa 1–224, the C-terminal fusion encodes aa 178–327. The various domains are delimited as follows:

ΔPEST	aa 51–326
Δ(PEST-AP2)	aa 101–326
PEST	aa 1–54
PEST-AP2	aa 1–103
AP2-ST	aa 51–187
ST	aa 101–187
Q	aa 178–213

Plasmids carrying the transgenes were introduced into *Agrobacterium tumefaciens* line GV3101 by direct transformation, followed by selection for growth on kanamycin. Transgenic lines were constructed by floral dip transformation (Clough and Bent, 1998), followed by selection of transformed seeds on the basis of kanamycin or BASTA resistance.

The *ABI4pro–GUS* construct was described in Söderman *et al.* (2000); additional lines with this transgene in the *rdr6* background were constructed for comparison with the *ABI4pro–ABI4–GUS* lines.

Plant growth conditions

Germination and seedling growth assays testing functionality of transgenes were performed as described in Söderman *et al.* (2000). For testing stability of fusion proteins, seedlings were grown initially on germination medium (GM: 0.5×MS salts and vitamins, 1% sucrose) solidified with 0.7% agar, then transferred to liquid GM in multiwell plates supplemented with cycloheximide, MG132 (Peptides International), or the appropriate solvent controls (EtOH and DMSO, respectively) at the concentrations indicated.

Measurement of GUS activity

GUS activity in intact plants was detected histochemically by vacuum infiltration with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), as described in Jefferson *et al.* (1987). Plant material was incubated in GUS staining solution containing 50 mM sodium phosphate pH 7.0, 0.1% Triton X-100, 0.5 mM K_3/K_4 FeCN, and 1 mM X-Gluc at 37 °C for 2–72 h depending on staining intensity. Tissues were cleared of chlorophyll in ethanol. Photographs of whole-mounted tissues were taken using a stereomicroscope.

Soluble extracts of seedlings were assayed fluorometrically for GUS activity, using 4-methylumbelliferyl glucuronide (Rose Scientific Ltd, Canada) as substrate, as described in Jefferson *et al.* (1987), and normalized relative to total protein content measured by Bradford assays (Bio-Rad).

RNA extraction and hybridization

RNA was extracted from seedling tissues by a modification of the procedure described in Verwoerd *et al.* (1989), and concentrations were estimated based on absorbance at 260 and 280 nm.

Total RNA was size fractionated on MOPS–formaldehyde gels, then transferred to Magna Nylon membranes (Osmonics, Westborough, MA, USA) using 20×SSPE as blotting buffer, and was bound to the filters by UV-crosslinking (120 mJ cm^{-2} at 254 nm) as previously described (Söderman *et al.*, 2000). Uniformity of loading and transfer was assayed qualitatively by methylene blue staining of the filters and eventually hybridization to an rDNA probe. Transgene transcripts were detected by hybridization to *ABI4* or *GUS* clones, labelled by random-priming to a specific activity of 10^8 cpm μg^{-1}. Hybridization conditions and washes were as described in Söderman *et al.* (2000). Hybridization was quantified by phosphoimager analysis; abundance of individual transcripts was normalized relative to rRNA present in each lane.

Results

Post-transcriptional regulation of ABI4

Initial studies of *ABI4* overexpression lines demonstrated that this transcription factor was sufficient to confer hypersensitivity to ABA and glucose resulting in reduced root growth, ABA-inducible vegetative expression of genes normally expressed only in seeds, and enhanced glucose-induced accumulation of anthocyanins (Söderman *et al.*, 2000; Finkelstein *et al.*, 2002). However, because the ABI4 protein was undetectable by immunoblotting with antibodies that had been raised against several different epitopes and the initial transgenic lines all inactivated their transgenes over a few generations (data not shown), new lines with fusion proteins that could be readily assayed by activity as well as immunologically were constructed. Function of these transgenes was assayed by their ability to confer hypersensitivity to ABA, salt, and glucose in a wild-type (Col) background and/or complement the ABA resistance of an *abi4* mutant. By these criteria, both *35S–GFP–ABI4* and *35S–ABI4–GUS* transgenes produced functional ABI4 proteins (Fig. 1 and Reeves *et al.*, 2011), although the overexpression phenotypes were less extreme
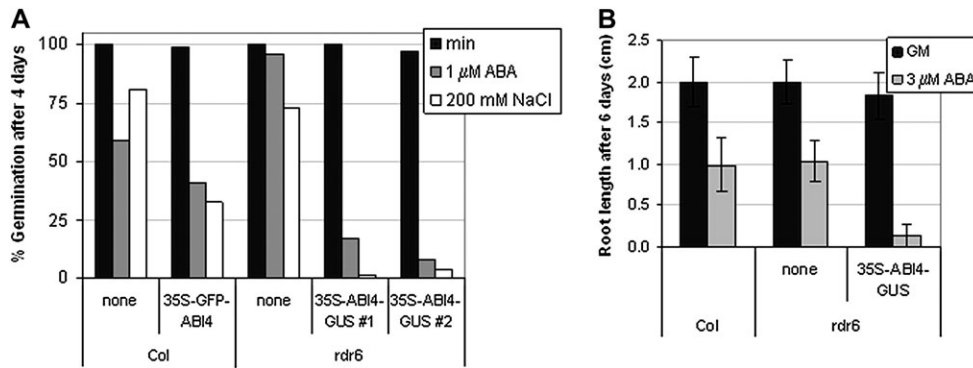
Fig. 1. *35S–GFP–ABI4* and *35S–ABI4–GUS* confer hypersensitivity to ABA and salt stress. (A) Hypersensitivity to ABA and NaCl inhibition of germination due to *35S–GFP–ABI4* and *35S–ABI4–GUS* transgenes in Col and *rdr6* backgrounds, respectively. Germination was scored as radicle emergence after 4 d of incubation on minimal nutrient salt medium (min), or min supplemented with 1 µM ABA or 200 mM NaCl. (B) *35S–ABI4–GUS* confers hypersensitivity to ABA for inhibition of root growth in *rdr6* background. Root lengths were measured 6 d after transfer from GM to fresh GM with or without 3 µM ABA. Genotypes are indicated by genetic background (Col or *rdr6*) and transgene present (none, *35S–GFP–ABI4*, or *35S–ABI4–GUS*).

than those of the original *35S–ABI4* lines (Söderman *et al.*, 2000). In addition, a *35S–ABI4–GR* fusion produced steroid-inducible ABI4 activity (Supplementary Fig. S1 available at *JXB* online), confirming that nuclear localization was required for function. To decrease the likelihood of transgene inactivation, these transgenes were also introduced into the siRNA-reduced *rdr6* background (Butaye *et al.*, 2004). Although all of these *ABI4* fusion transgenes were similarly highly expressed in a wild-type background, *ABI4–GUS* transcripts in the *rdr6* background were much higher (Supplementary Fig. S1 at *JXB* online). Consistent with this, the ABI4–GUS fusion in the *rdr6* background was detected both histochemically and fluorometrically, albeit at very low levels (Fig. 2 and Supplementary Fig. S2 at *JXB* online), but the ABI4–GFP fusion was undetectable by either fluorescence or immunoblotting with an anti-GFP antibody (data not shown).

The reduced activity of the ABI4 fusion proteins could reflect impaired expression at many levels, including transcription, mRNA stability, or translation of the transgene. To distinguish between these possibilities, relative levels of transcripts and GUS activity for *35S–GUS* and *35S–ABI4–GUS* lines in a wild-type background were compared, as were *35S–ABI4–GUS* expression in wild-type and *rdr6* backgrounds (Fig. 2). These studies showed at least 50-fold differences in transcript levels, but >300-fold differences in activity levels between *35S–GUS* and *35S–ABI4–GUS* transgenes in the wild-type background, indicating that transcript levels were not sufficient to explain the differences in activity. Although *35S–ABI4–GUS* transcripts in the *rdr6* background accumulated to levels similar to those of the *35S–GUS* transcripts, GUS activity was still ~40-fold lower in the *35S–ABI4–GUS* fusion line, again supporting regulation at a post-transcript stage. Although all lines showed multiple *GUS*-homologous degradation products, the differences in *ABI4–GUS* transcript levels between wild-type and *rdr6* lines suggested that the transgene was being aggressively silenced in the wild-type background. Interestingly,
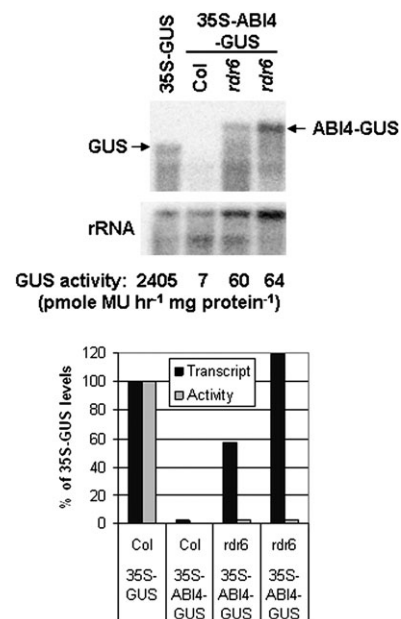

GUS activity: 2405 7 60 64
(pmole MU hr⁻¹ mg protein⁻¹)

Fig. 2. Post-transcriptional control of GUS activity in transgenic lines. (Top) Comparison of GUS transcript levels and GUS activities of *35S–GUS* and *35S–ABI4–GUS* lines in wild-type and *rdr6* backgrounds. The *rdr6* lines are derived from independent transformants. (Bottom) Transcript and activity levels are displayed normalized to the levels in the *35S–GUS* line.

the lines with the most active *35S–ABI4–GUS* transgenes grew very slowly and either failed to bolt and set seed, or inactivated their transgenes while doing so (data not shown). Lines with slightly lower transgene activity remained active, but homozygous progeny that could complete development and set seed could not be obtained. Consequently, even the lines with 'active' transgenes tend to have variable expression as they are comprised of mixtures of plants with different numbers of transgenes, some of which are inactivating.

Another possible cause of reduced GUS activity in the transgenic lines was reduced stability of the ABI4–GUS protein. This was tested by assaying GUS activity in the presence or absence of the protein synthesis inhibitor cycloheximide (Fig. 3). Our results showed that the ABI4–GUS fusion protein had a half-life of between 4 and 6 h (Fig. 3), ~10-fold less than the 50 h reported for GUS itself (Jefferson *et al.*, 1987). However, inclusion of the proteasome inhibitor MG132 largely reversed the effects of cycloheximide, indicating that ABI4 turnover is mediated at least partially by proteasomal degradation.

Domains involved in instability

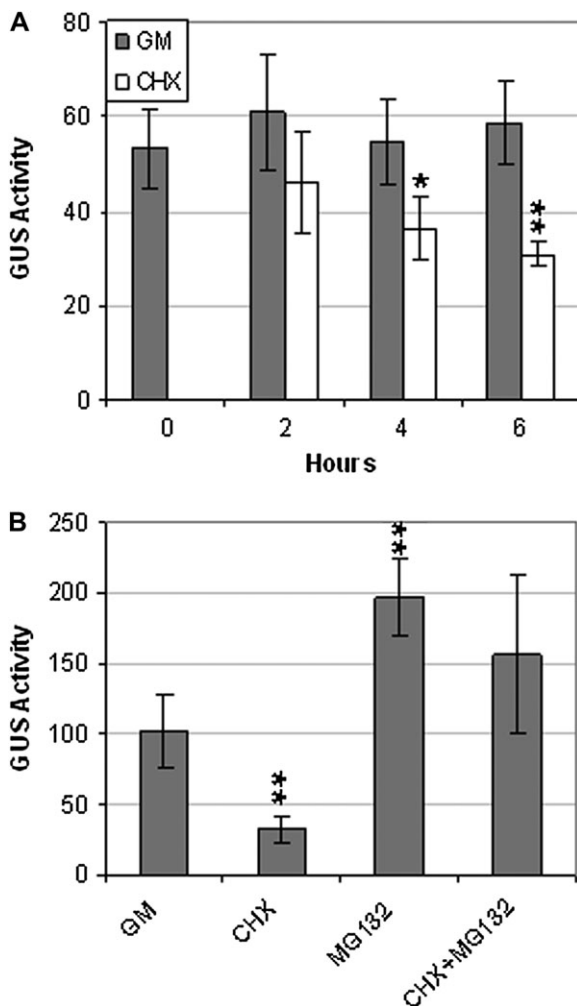Analysis of the predicted amino acid sequence of ABI4 revealed no clear degradation-associated motifs other than

Fig. 3. *35S–ABI4–GUS* activity in *rdr6* background. (A) Comparison of GUS activities during 6 h incubation in GM, with or without cycloheximide (CHX). (B) Comparison of GUS activities after 5 h exposure to the indicated treatments. Seedlings were incubated in GM, supplemented with CHX and/or MG132, or the appropriate solvent controls. GUS activity units are pmol MU h^{-1} mg protein^{-1}. ** and * indicate statistically different from activity in GM ($P<0.01$ and $P<0.02$, respectively, based on two-tailed Student's *t*-test).

a possible PEST domain near the amino terminus (aa 22–40, PESTfind score: +13.48) and two poor PEST sequences in the carboxy half (aa 218–236, PESTfind score –2.65, and aa 274–311, PESTfind score –1.51) (Rechsteiner and Rogers, 1996). To test the relative stability of different domains of the protein, a series of *35S-(ABI4domain)–GUS* fusion lines was constructed (Fig. 4A). Comparison of GUS activity levels in these lines showed that fusions containing either the amino or carboxy halves of the protein were more active than those with the full-length protein, but still much less active than GUS alone (Fig. 4B). Differences in GUS fusion transcript levels were not sufficient to account for the different activities (Fig. 4B), indicating that fusion accumulation was still regulated at a post-transcript stage. Cycloheximide treatment for 5 h reduced all three of these fusions to ~50% of their levels in control treatments. However, MG132 suppressed this effect only for fusions containing the N-terminal half of ABI4, suggesting that proteasomal degradation depended on motif(s) in this half of the protein (Fig. 4C).

The GUS activities of the fusion lines varied over several orders of magnitude, even for a single construct, as is common for independent transformants. Part of this variability was due to differences in transcript level, but several of the fusions containing smaller regions of ABI4 also had higher ratios of activity to transcript (Supplementary Fig. S3 at *JXB* online). The fusions with the highest activity were those containing just the potentially destabilizing PEST domain or the Q-rich domain, and these remained at high levels in the presence of cycloheximide (Fig. 4D). Fusion proteins lacking the PEST and AP2 domains were slightly more stable than the full-length or N-terminal fusions, retaining ~80% of their activity after 5 h exposure to cycloheximide. Although this suggested that the AP2 domain contributed to instability, fusions containing both the AP2 and ST-rich domains were not significantly less stable than GUS.

Developmental and environmental regulation

ABI4 transcripts have been shown to be highly expressed in seeds and in seedlings exposed to stresses such as high glucose, and to a lesser extent ABA and osmoticum (Arroyo *et al.*, 2003). However, if the ABI4 protein is unstable, these major fluctuations in transcript levels may not result in substantial changes in ABI4 activity. To determine whether any of the environmental signals inducing ABI4 transcript accumulation could also enhance protein stability, the effects of ABA, glucose, and sorbitol on GUS activity were tested (Fig. 5A). None of these signals stabilized the 35S–ABI4–GUS fusion product in 8-d seedlings to the same extent as seen with MG132. ABA and glucose effects on the stability of the 35S–ABI4–GUS product at up to 2 d post-stratification were also tested because previous studies had shown that seedlings are most sensitive to ABA and stress-induced growth arrest during the first 48 h post-imbibition (Gibson *et al.*, 2001; Lopez-Molina *et al.*, 2001). Although both ABA and glucose reduced germination and growth of these seeds, only glucose-treated seedlings had slightly
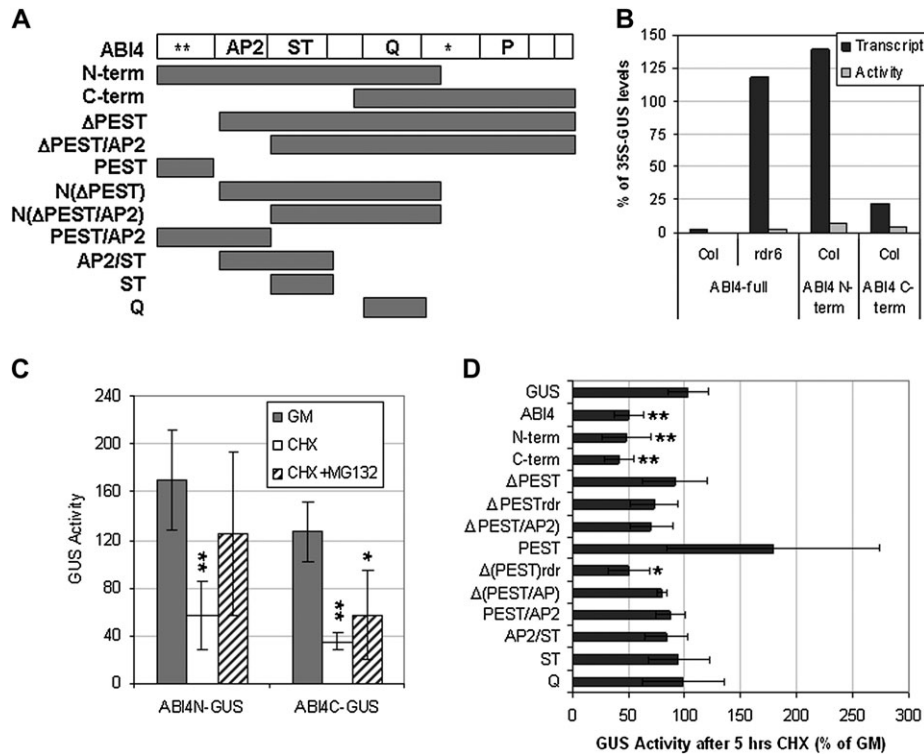
Fig. 4. Mapping ABI4 domains contributing to instability. (A) Domain structure and subclones; * potential PEST domains; AP2, APETALA2 domain; ST, serine/threonine-rich domain; Q, glutamine-rich domain; P, proline-rich domain. (B) Comparison of *GUS* transcript and activity in full-length, N-terminal, and C-terminal domain fusions relative to *35S–GUS* expression. (C) Comparison of GUS activity (pmol MU h^{-1} mg protein^{-1}) of N-terminal and C-terminal domain fusions following 5 h exposure to the indicated treatments. Seedlings were incubated in GM, supplemented with cycloheximide (CHX) with or without MG132, or the appropriate solvent controls. ** and * indicate statistically different from activity in GM (*P*<0.01 and *P*<0.03, respectively, based on ANOVA). (D) Effect of CHX treatment on GUS activity of all deletion transgenes. ** and * indicate fusions with statistically different stability in CHX compared with *35S–GUS* (*P*<0.01 and *P*<0.02, respectively, based on ANOVA).

higher ABI4–GUS activity (Fig. 5B). However, transfer to media for a 6-h exposure to ABA or glucose did not significantly stabilize the ABI4–GUS fusion, which was still substantially degraded in the presence of cycloheximide (Fig. 5C). This suggests that the large increase reported for *ABI4* transcript levels in 3-d seedlings exposed to 7% glucose (Arroyo *et al.*, 2003) might not actually result in a comparable increase in ABI4 protein.

It is possible that plants can tolerate only a limited amount of ABI4, such that all *35S*-driven expression exceeds this level and they are unable to stabilize fusions to such high levels except by pharmacological inhibition of the degradation machinery. To test this possibility, transgenic lines with *ABI4–GUS* under control of the *ABI4* promoter were constructed. *ABI4pro–ABI4–GUS* transgenes partially complemented an *abi4* mutant (Supplementary Fig. S4 at *JXB* online), but the GUS activity was undetectable. The levels were higher in an *rdr6* background, permitting comparison of glucose-induced transcript and activity levels in *ABI4pro–GUS* and *ABI4pro–ABI4–GUS* lines. *ABI4pro–GUS* lines in the *rdr6* background had similar activities to those in the Col background (data not shown). Although seedlings with either transgene had ~8-fold higher GUS activity after 6 d on 5% glucose than when grown on 1%

glucose, they differed in that these levels were ~20-fold higher in the *ABI4pro–GUS* lines (Fig. 6A). This might reflect the stronger expression of *ABI4pro–GUS* in the shoots, or a higher total protein concentration in the *ABI4pro–ABI4–GUS* seedlings due to their minimal growth on glucose (Supplementary Fig. S5 at *JXB* online). Two possible explanations for the limited activity of the ABI4–GUS fusion protein are limited transcript accumulation or limited protein accumulation. To distinguish between these, *GUS* transcript levels were measured and, surprisingly, showed that the *ABI4pro–ABI4–GUS* transcripts were actually ~7-fold higher than the *ABI4pro–GUS* transcripts (Fig. 6B). Consequently, the GUS activity per transcript was nearly 150-fold higher for *ABI4pro–GUS* than for the *ABI4pro–ABI4–GUS* transgene.

Comparison of *ABI4pro–GUS* and *ABI4pro–ABI4–GUS* function after only 2 d showed similar GUS activities for the transcriptional fusion with or without high glucose or NaCl, but both stresses induced a 3- to 4-fold increase in fusion protein activity (Fig. 6C), suggesting that they primarily affect protein accumulation. In contrast, exposure to 2 μM ABA induced mild (~1.5-fold) increases in GUS activity of both fusion lines, indicating that ABA primarily affected transcript accumulation. However, the fusion
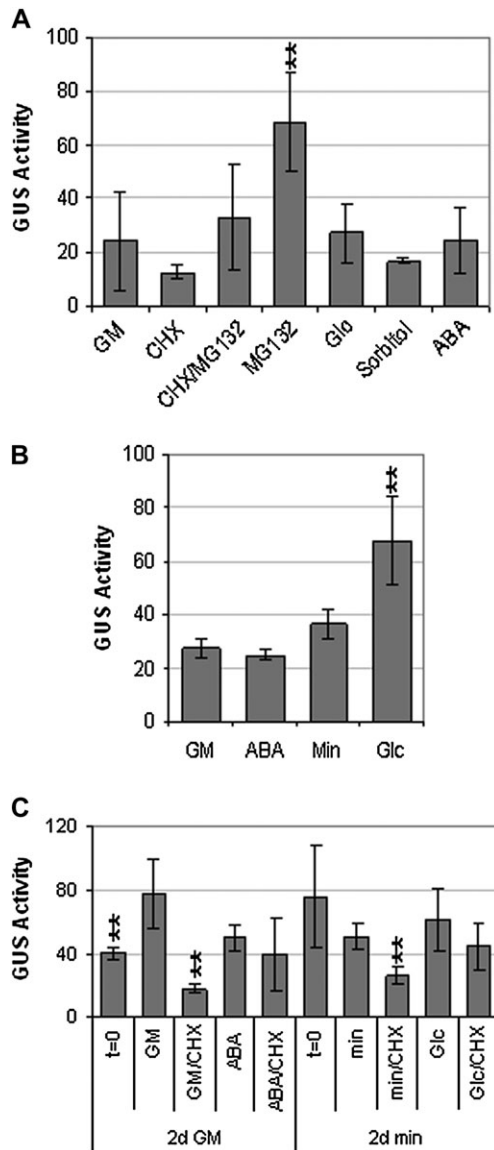
Fig. 5. Developmental or stress regulation of *35S–ABI4–GUS* activity. (A) GUS activity in 8-d seedlings exposed to the indicated treatments for 5 h (100 µM CHX, 100 µM MG132, 6% glucose (Glc), 6% sorbitol, 100 µM ABA); (B) GUS activity in seedlings stratified and incubated for an additional 2 d on indicated medium (GM, GM + 3 µM ABA, min, min + 6% glucose), (C) GUS activity in 2 d seedlings germinated on either GM or min medium, then transferred for an additional 6 h to the indicated medium (as in B, with or without 100 µM CHX). GUS activity units, media, and treatment abbreviations as described in Figs 1, 3. ** indicates statistically different from activity on GM or minimal medium (*P*<0.01, based on ANOVA).

protein activities were ~15-fold lower than those of the transcriptional fusion under all conditions. As at 6 d, the promoter was active in both shoots and roots, even without glucose, but the ABI4–GUS fusion protein did not accumulate in unstressed shoots (Supplementary Fig. S5 at *JXB* online).

Histochemical staining of *35S–(ABI4domain)–GUS* transgenic lines also revealed non-uniform expression, with

GUS activities higher in roots than shoots for many lines (Supplementary Fig. S6 at *JXB* online), in contrast to the constitutively high expression of *35S–GUS* fusions throughout the plant. Comparison of transcript levels shows that the *ABI4–GUS* fusion transcripts are often 2- to 3-fold more abundant in shoots (Fig. 7), indicating that the GUS activity disparities are due to tissue-specific differences in translation or protein stability.

Discussion

Numerous transgenic lines with *ABI4* fusions under control of either the *CaMV 35S* promoter or the *ABI4* promoter have transgene expression levels sufficient for complementation of the *abi4* mutation, yet are often undetectable by GFP or GUS activity. Lines that achieve higher levels of transgene expression display very low ratios of activity relative to the transcripts encoding these fusions. In fact, even constitutive expression via the *CaMV 35S* promoter was not sufficient to raise ABI4–GUS activity levels above those produced by glucose-inducible expression via the *ABI4* promoter. Although these experiments do not exclude the possibility of poor translation or improper folding, the fact that similar physiological phenotypes have been produced by *35S*-driven *ABI4–GUS*, *GFP–ABI4*, and *GR–ABI4* fusions, as well as *35S–ABI4*, yet most are undetectable immunologically and these transgenes tend to inactivate rapidly, suggests that these proteins are simply accumulated to low levels. Our studies show that in the case of the GUS fusions the low activity reflects protein instability, at least partly via the proteasome. The instability of ABI4 is reminiscent of similar regulation of ABI3 and ABI5, but differs in that ABA can stabilize those transcription factors (Lopez-Molina *et al.*, 2001; Zhang *et al.*, 2005), but not ABI4. However, high glucose is a more effective inducer of *ABI4* expression than ABA (Arroyo *et al.*, 2003) and also promotes ABI4 accumulation within 2 d after stratification, as do growth-inhibiting levels of NaCl and ABA.

Proteasomal regulation of transcriptional regulators has been well-characterized for the AUX/IAA repressors of auxin response, the JAZ repressors of jasmonate response, the DELLA protein repressors of GA response, the EIN3 regulator of ethylene response factors, and two ABA response factors: ABI3 and ABI5 (reviewed in Vierstra, 2009). For most of these, the half-lives have been documented to be as little as an hour or less, which is substantially shorter than that observed for ABI4. F-box subunits of the E3 ligases required for ubiquitination leading to degradation are known for the auxin, jasmonic acid, gibberellin and ethylene regulators, and specific conserved domains have been identified as essential for instability in the DELLA and AUX/IAA proteins. Two RING E3 ligases involved in ABI factor degradation have also been identified: KEEP ON GOING (KEG), which ubiquitinates ABI5, and an ABI3-interacting protein (AIP2) (Zhang *et al.*, 2005; Stone *et al.*, 2006). AIP2 is highly
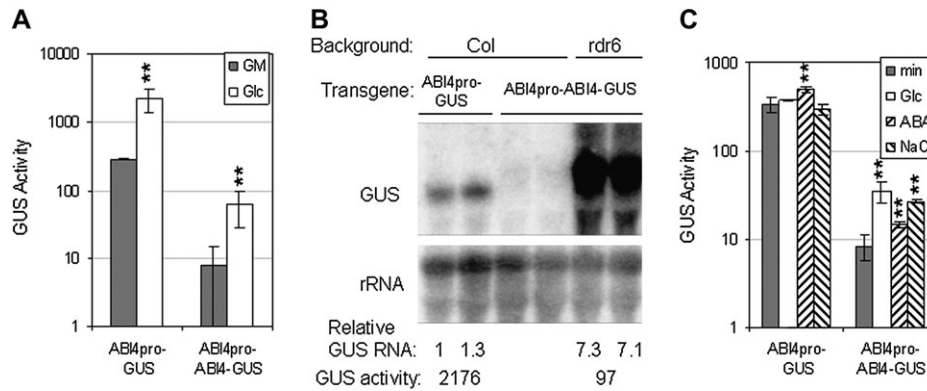
Fig. 6. Glucose regulation of transcriptional and translational ABI4–GUS fusions. (A) GUS activity of *ABI4pro–GUS* and *ABI4pro–ABI4–GUS* seedlings after 6 d incubation on GM with or without 5% glucose (Glc). (B) GUS transcript levels in seedlings grown and harvested in parallel with those used for GUS assays in (A). (C) GUS activity of *ABI4pro–GUS* and *ABI4pro–ABI4–GUS* seedlings after 2 d incubation on minimal medium with or without 6% glucose, 2 µM ABA, or 200 mM NaCl. GUS activity units, displayed on a log scale, are pmol MU h^{-1} mg protein^{-1}. ** indicates statistically different from activity on minimal medium ($P<0.01$, based on two-tailed Student's *t*-test).
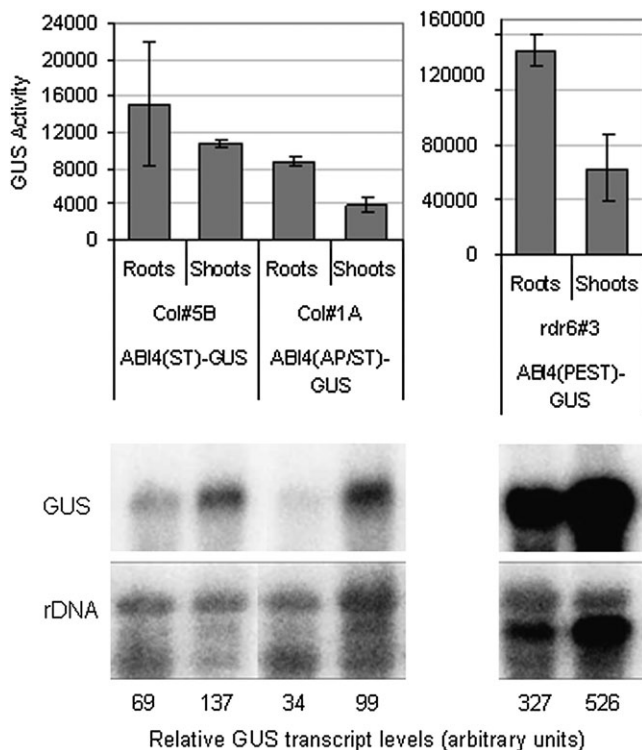

Fig. 7. Organ-specific differences in ABI4–GUS activity. (Top) GUS activity levels (pmol MU h^{-1} mg protein^{-1}) in roots and shoots of the indicated transgenic lines. AP/ST- and PEST-domain fusions have statistically different activity in roots and shoots ($P=0.00024$ and $P= 0.0028$, respectively, based on two-tailed Student's *t*-test) (Bottom) RNA gel blots showing GUS-fusion transcript levels in parallel samples aligned with their activity levels.

expressed in freshly stratified seeds, where it can induce destruction of ABI3 as part of dormancy release. In addition, AIP2 levels increase in vegetative tissues exposed to ABA, leading to ABI3 degradation and decreased ABA signalling at later stages. Surprisingly, ABA has the opposite effect on KEG; by promoting self-ubiquitination and degradation of KEG, it inhibits destruction of ABI5

(Liu and Stone, 2010). ABI5 action is also regulated by sumoylation, which both represses its activity and increases its stability (Miura *et al.*, 2009). An additional class of ABI5-interacting proteins, the AFPs, have been implicated in altering stability of ABI5, but the mechanism is not clear (Garcia *et al.*, 2008; Lopez-Molina *et al.*, 2003) and recent studies suggest that they may actually function as transcriptional co-repressors (Pauwels *et al.*, 2010). ABA sensitivity of germination, seedling sugar sensitivity, and lipid breakdown are also regulated by the N-end rule pathway of protein degradation, but the specific substrates involved have not yet been identified (Holman *et al.*, 2009). A recurring theme is the existence of multiple regulators responsible for controlling stability of a given protein or protein family in a variety of tissues or conditions.

Superficially one might expect reciprocal abundance of destabilizing factors and their targets, but many (e.g. AIP2, EBF1 and EBF2, AFP1 and AFP2), are components of negative feedback loops such that their accumulation is induced by the signals whose action they will inhibit. Furthermore, many of the destabilizing factors are posttranscriptionally regulated themselves. For example, the auxin receptor F box genes are broadly transcribed, but protein accumulation is under miRNA control (Parry *et al.*, 2009). Consequently, it is not possible to predict candidate regulators based on expression patterns.

Our current study implicates several regions contributing to the instability of ABI4, none of which resemble previously characterized destabilizing domains. Although the susceptibility to proteasomal degradation is likely to involve ubiquitination, some proteins are targeted by ubiquitinindependent mechanisms. The targeting mechanism for ABI4 has not yet been identified.

Previous studies of *ABI4* regulation have shown strong induction by glucose in seedlings, with preferential promoter activity in shoots and root tips (Arroyo *et al.*, 2003; Bossi *et al.*, 2009). The current study confirms this result by histochemical staining of *ABI4pro–GUS* seedlings, but *ABI4-pro–ABI4–GUS* lines show stronger activity in roots than

shoots. Similarly, *CaMV 35S*-driven expression generally resulted in higher *ABI4–GUS* transcript levels in shoots, based on RNA gel blot analyses, yet GUS activity was usually higher in roots. This difference in the ratio of activity to transcript between roots and shoots implies preferential translation or stability in roots such that the levels of functional ABI4 do not reflect the transcript levels. To date, searches of small RNA databases (available at http://asrp.cgrb.oregonstate.edu/db/) have not shown any likely candidates for regulators of ABI4. However, a variety of RNA-binding proteins have been implicated in stress responses (reviewed in Lorković, 2009), including a zinc finger-containing glycine-rich RNA-binding protein, atRZ-1a, with mutant and over-expression phenotypes very similar to those for *ABI4* (Kim *et al.*, 2007). Although *ABI4* transcript levels are unaffected in these loss- and gain-of-function lines, this does not preclude the possibility of effects on translation.

In summary, these studies show that ABI4 is subject to stringent post-transcriptional regulation that prevents the protein from accumulating to high levels, and restricts its action to a subset of the tissues where the gene is expressed. The specific regulatory components remain unknown, but at least part of the mechanism involves proteasomal degradation.

Supplementary data

Supplementary Fig. S1. *35S–ABI4–GR* transgenes confer dexamethasone (Dex)-dependent hypersensitivity to ABA inhibition of germination and root growth, and glucose (Glc) inhibition of germination and seedling growth. Transcript levels for these *ABI4* fusion transgenes are similar to those for the *GFP–* and *–GUS* fusions in a wild-type background.

Supplementary Fig. S2. *35S–ABI4–GUS* activity in Col (left) and *rdr6* (right) backgrounds. Fluorometrically assayed GUS activity is ~10-fold higher in the *rdr6* background.

Supplementary Fig. S3. Comparison of GUS transcript and activity levels shows that all ABI4 domain fusion constructs displayed except that containing only the PEST domain accumulate fusion proteins relatively inefficiently.

Supplementary Fig. S4. *ABI4pro–ABI4–GUS* weakly complements the *abi4* mutation, suppressing the glucose resistance of this background.

Supplementary Fig. S5. Histochemical staining of GUS activity in seedlings of the indicated genotypes (*ABI4pro–GUS* and *ABI4pro–ABI4–GUS*) grown for 6 d on GM with or without 5% glucose, or 2 d on minimal medium with or without 6% glucose.

Supplementary Fig. S6. Histochemical staining of GUS activity in a variety of *35S-(ABI4domain)–GUS* transgenic seedlings. Activity varied substantially between independent transgenic lines for each fusion, and even between individual progeny of each line, but the shoots were much more likely to lose activity than the roots.

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