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Abscisic acid-insensitive mutations provide evidence for stage-specific signal pathways regulating expression of an *Arabidopsis* late embryogenesis-abundant (*lea*) gene

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Abstract. An *Arabidopsis* homolog of the abscisic acid (ABA)-inducible cotton D19 and wheat *Em* genes was cloned and its expression assayed at two developmental stages in wild-type, ABA-deficient (*aba*) and three ABA-insensitive (*abi*) lines of *Arabidopsis thaliana.* Expression of this gene was reduced slightly in seeds of *aba* mutants and approximately ten-fold in *abi3* mutants, but seed expression was not decreased in either *abi1* or *abi2* monogenic mutants. In contrast, the *abi1* and *abi2* mutants showed a very slight reduction of ABA inducibility in 8-day-old plants, while the responses of *aba* and *abi*3 mutants were comparable to that of wild type. Although previous studies have shown that none of the *abi* mutations show completely stage-specific effects, the results reported here indicate that the importance of each of the *ABI* loci in regulating this single gene is stage-dependent. Furthermore, the fact that none of the *abi* mutations show more than minor effects on exogenous ABA inducibility of the *Arabidopsis D19/Em* homolog in young plants suggests that an additional ABA signalling pathway may be operating during vegetative growth.

Key words: *Arabidopsis thaliana* – Abscisic acid – Late-embryogenesis-abundant – ABA-insensitive mutants

Introduction

Maturing seeds of most plants undergo extreme desiccation, reaching a final water content of only 5–15% depending on the species and growth conditions (Adams and Rinne 1980). The ability to survive this dehydration, whether occurring during normal development or imposed experimentally, is referred to as desiccation tolerance. The timing of the onset of desiccation tolerance varies among species, but generally occurs during the desiccation phase of development, when embryo water content (mg/embryo) is decreasing (Long et al. 1981; Finkelstein et al. 1987; Fischer et al. 1988). This timing, encompassing the postabscission, predesiccation and desiccation stages described by Hughes and Galau (1991), coincides with accumulation of several families of proteins, most of which are characterized by an extremely hydrophilic amino acid composition (Baker et al. 1988). These families were initially identified as being major translation products of maturing embryos and became collectively known as *l*ate *e*mbryogenesis-*a*bundant (*lea*) gene products (Galau et al. 1986). Synthesis of some of these proteins is also induced in drought-, salt- or cold-stressed seedlings (reviewed in Skriver and Mundy 1990). In addition, related protein families are induced during drying of a desiccation-tolerant xerophytic species (Piatkowski et al. 1990). The consistent correlation between desiccation, tolerance of dehydrating conditions, and the accumulation of *lea*-like gene products, together with their unusual amino acid composition, has led to the hypothesis that these proteins function as desiccation protectants (Dure et al. 1989; reviewed in Skriver and Mundy 1990).

The diversity of environmental and developmental controls affecting *lea* expression would require either multiple signalling pathways or multiple initial signals feeding into a common regulatory pathway. Efforts to identify potential regulatory signals have indicated that drought, salt or cold stress can all induce accumulation of the phytohormone abscisic acid (ABA; reviewed in Zeevaart and Creelman 1988), which in turn can induce both synthesis of some *lea* family proteins and tolerance to these stresses (reviewed in Skriver and Mundy 1990). Such observations have led to the suggestion that ABA mediates stress tolerance, in part by inducing synthesis of these and other presumed osmoprotectants, but to date there is only weak evidence to support this hypothesis. Genetic studies directly testing the role of ABA itself or loci known to be required for ABA response have been limited, in part because few species exist for which multiple loci affecting ABA synthesis and response have been identified by mutation.

We have chosen to address the role of ABA action in regulation of a *lea* gene in *Arabidopsis thaliana* be-

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cause mutants affecting three distinct loci required for ABA response (*ABI1, ABI2*, and *ABI3*, for *AB*A-*i*nsensitive) and one locus required for ABA biosynthesis (*ABA*) are available (Koornneef et al. 1982, 1984). All three response mutants produce non-dormant seeds which are insensitive to exogenous ABA inhibition of germination (Koornneef et al. 1984). However, other than this similarity in phenotype of mature seeds, the *ABI* mutants differ in the extent of their ABA insensitivity at other stages in the life cycle, such as embryogeny and vegetative growth (Koornneef et al. 1984; Finkelstein and Somerville 1990). Furthermore, genetic studies suggest that the products of the three *ABI* loci act in at least two overlapping response pathways (Finkelstein and Somerville 1990). Therefore, these mutants provide a good system in which to test whether a single ABA-inducible gene expressed at more than one developmental stage is subject to regulation by the same response pathways at all stages when it is expressed. In addition, the ABA biosynthetic mutant (*aba*) allows us to test the role of endogenous ABA in regulating this gene at the relevant stages. The *lea* gene we have chosen as our marker is an *Arabidopsis* homolog of the cotton D19/wheat *Em* family. These genes are known to be responsive to exogenous ABA during both embryogenesis and seedling growth, although the induction in germinating mature cotton and barley seeds is much weaker than in wheat seedlings (Williamson and Quatrano 1988; Berge et al. 1989; Hughes and Galau 1991; Espelund et al. 1992). Extensive analysis of promoter sequences required for developmental and hormonal control of the wheat gene has shown that its regulation is maintained in heterologous transgenic systems, implying that the signalling pathways are conserved (Marcotte et al. 1988, 1989). This paper reports the cloning of a D19/*Em* homolog from *Arabidopsis thaliana* (ecotype Landsberg) and compares its expression in seeds and ABA-treated seedlings of wild-type, ABA-deficient and ABA-insensitive mutants. The homologous gene and another family member have been simultaneously isolated from the Columbia ecotype. Characterization of their structure and expression is reported in the accompanying paper (Gaubier et al. 1993).

Materials and methods

Plants. Arabidopsis thaliana seed stocks carrying the following mutant alleles were a generous gift of M. Koornneef: *abi1* (isolation number AII), *abi2* (EII), *abi3* (CIV) and *aba-1* (A26). All are ethyl methanesulfonate-induced mutants derived from the Landsberg *erecta* line (Koornneef et al. 1982, 1984). Plants were grown to maturity under continuous fluorescent illumination at 22° C on a mixture of vermiculite, perlite and sphagnum (1:1:1) irrigated with mineral nutrients (Haughn and Somerville 1986). All seeds were incubated for at least 20 h at 4° C after sowing before transfer to growth chambers.

Plant culture. Seed of appropriate genotypes was surface-sterilized and plated on cheesecloth overlying minimal medium (Haughn and Somerville 1986) solidified with 0.5% agar. Approximately 700 seeds were used per treatment. All experimental treatments were performed at least twice. Plates were incubated overnight at 4° C to break any residual dormancy, then transferred to 22° C in continuous light for 8 days. At this point, plantlets had 2 to 4 true leaves expanded. Eight-day-old plants were either harvested immediately or transferred on their cheesecloth supports to fresh minimal medium solidified with 0.7% agar and supplemented with ABA (0, 1, 10, or 30 μM, mixed isomers (Sigma)) or sorbitol as an osmoticum (0.2, 0.45, or 0.7 M) for an additional 24 h culture. All tissue was flash-frozen in liquid nitrogen, then stored at −70° C until used for RNA extraction.

Library screening. An *Arabidopsis* (ecotype Landsberg) genomic library in the vector EMBL4 was purchased from Promega. The library was plated on CES200 host cells in NZM top agarose as described (Sambrook et al. 1989). Plaques were transferred to Nitropure (Micron Separations) filters where the DNA was denatured and immobilized as described (Sambrook et al. 1989). The 0.9 kb cotton D19 cDNA clone (Galau et al. 1986) was gel-purified, then labeled by random priming to a specific activity of 10^8–10^9 cpm/μg for use as a probe (Hodgson and Fisk 1987). Hybridization conditions for library screening were 40% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS, 200 μg/ml sheared calf thymus DNA at 43° C. Filters were washed twice at room temperature in 2×SSC, 0.1% SDS and once at 50° C in 0.2×SSC, 0.1% SDS for 30–60 min. All hybridization reagents were prepared as described in Sambrook et al. (1989).

Following plaque purification, *Eco*RI fragments of the phage insert DNA were subcloned into pBS-SK (Stratagene) and the region containing homology to D19 was mapped by Southern analysis of the 4 kb subclone.

Genomic reconstruction Southern analysis. Genomic (1 μg/lane) and plasmid (37 pg/*Arabidopsis* haploid copy equivalent (Leutwiler et al. 1984)) DNAs were digested with *Eco*RI and size-fractionated on agarose gels run in 1×TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8). Gels were soaked in 0.25 N HCl, then denatured, neutralized and transferred to Nitropure filters as described (Sambrook et al. 1989), using 1 M ammonium acetate, 0.02 N NaOH as transfer buffer (Rigaud et al. 1987). The DNA was bound to the membranes by baking under vacuum at 80° C and filters were probed with a random-primed genomic fragment containing the entire coding sequence. Hybridization conditions were 5×SSPE, 5×Denhardt's, 0.5% SDS, 100 μg/ml calf thymus DNA and 35 or 45% formamide at 45° C (T_m-30° C and $T_m-19.5$° C, respectively). Both filters were washed at T_m-24° C.

DNA Sequencing. Both strands of the region encompassing the D19 homology were sequenced using [^{35}S]dATP and dideoxy chain termination reagents provided in the Sequenase system (US Biochemicals). The templates for these reactions were double-stranded plasmids; some

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D19h  GTAAGGCACAAATTCAATGACGATTACCAACATTTCCAATCATGAATCCATCCGGGATTTCTTCCTCAGGTATTCATATT  -390
GEA6  ................................................................................
D19h  TACTTTCATACAGTATAGTCAAATGATTGAAAGATTTTATGAATACAAAGGCAACACACAAAATAATTGCGGCGGTATAG  -310
GEA6  ................................................................................
D19h  TTAAAGAACACGCGGCGAAGAAGTACGGCCACTACCACGTTCAAAGTATACGTCAGCTAATAAAGTCAGACACGTGGCAT  -230
GEA6  ................................................................................
D19h  GTCACCAAGAAAGAAGAGCATACGTATGACGTACCGATTGTCCCTGAGTCACCACGTCGAACATCCACGATGCAACACAA  -150
GEA6  ................................................................................
D19h  CCAAAACGCCTCCGGATTGAACTCGCTATATATAGAGCGAGCTACTATAGTCTTCTTCGTATATCATCATCAAATCGTTG   -70
GEA6  .........................................................*...............G......
D19h  CGAGTTTGTTTGGTTTTTACTTGTCTAGTGTTTTATATAAACTTGTGTTGTGTAAGTAACTAAAGAGCCATGGCGTCTCA    11
GEA6  ...........*....................................................................
                                                                             M  A  S  Q
D19h  ACAAGAGAAGAAGCAGCTGGATGAGAGGGCAAAGAAGGGCGAGACCGTCGTGCCAGGTGGTACGGGAGGCAAAAGCTTCG    91
GEA6  ......................................C.........................................
       Q  E  K  K  Q  L  D  E  R  A  K  K  G  E  T  V  V  P  G  G  T  G  G  K  S  F  E
D19h  AAGCTCAACAGCATCTCGCTGAAGGTCATTCTTTCTCTTATTCATGGTTGCGTATGTTTCAAGTTAGGTATTTAGGCTAG   171
GEA6  .....................................................................C..........
        A  Q  Q  H  L  A  E  G
D19h  AGTAACGAGATTAATGTTTGTGGAATATTTATTATATGAAGGGAGGAGCCGAGGAGGGCAAACTCGAAAGGAGCAGTTAG   251
GEA6  ................................................................................
                                                 R  S  R  G  G  Q  T  R  K  E  Q  L  G
D19h  GAACTGAAGGATATCAGCAGATGGGACGCAAAGGTGGTCTTAGCACCGGAGACAAGCCTGGTGGGGAACACGCTGAGGAG   331
GEA6  .........................................................................C......
        T  E  G  Y  Q  Q  M  G  R  K  G  G  L  S  T  G  D  K  P  G  G  E  H  A  E  E
D19h  GAAGGAGTCGAGATAGACGAATCCAAATTCAGGACCAAGACCTAAATCAAACTTCCCCACACGGATGTAATATTAATCCG   411
GEA6  ................................................................................
      E  G  V  E  I  D  E  S  K  F  R  T  K  T
D19h  GTTTAAATCTTTCCATGTAGTTATATCTTGTGTAGTTTCCTGTGCGTACTTAAGTTCTTGTTTAGTT                478
GEA6  ...................................................................
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Fig. 1. DNA and predicted amino acid sequences of the *Arabidopsis* homolog of the cotton D19 gene. The abscisic acid response element (ABRE) at position −237 is *underlined*, the TATA box is in *boldface type*. Base alterations and gaps (*asterisks*) relative to the *GEA6* sequence reported by Gaubier et al. (1993) are indicated

were nested deletions and others were specific restriction fragment subclones. The nested deletions were constructed by exonuclease III and mung bean nuclease digestion followed by religation, as described by Stratagene. The primers were the universal, reverse, KS and SK primers described by Stratagene and one specific internal primer (purchased from Operon Technologies). Sequencing reaction products were fractionated on denaturing 5% and 6% acrylamide gels, then fixed, dried and autoradiographed. Sequence alignments were performed using the UWGCG package (University of Wisconsin).

RNA quantification. RNA was isolated by hot phenol extraction as described previously (Finkelstein et al. 1985). Total RNA from seeds or seedlings (0.1–1 μg and 25 μg, respectively) was size fractionated on 1% or 1.5% agarose MOPS-formaldehyde gels (Sambrook et al. 1989), then transferred to Nytran (Schleicher and Schuell) membranes using 20 × SSPE as blotting buffer. RNA was bound to the filters by UV crosslinking (120 mJ/cm^2 at 254 nm). Uniformity of loading and transfer was assayed qualitatively by methylene blue staining of the filters (Herrin and Schmidt 1988). The *D19h* mRNA was detected by hybridization to a nearly full-length antisense riboprobe, labeled with [^{32}P]UTP using T3 polymerase (Stratagene) as described by the manufacturer. Hybridization was at 60° C in 50% formamide, 0.25 M sodium phosphate buffer (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 7% SDS, 10% PEG8000 as described (Amasino 1986). The final wash was in 0.1 × SSPE, 0.1% SDS at 75° C.

Gene-specific expression was determined by RNase protection experiments using the reagents and protocol provided by Ambion. Total RNA from seeds or seedlings (0.1–1 μg and 10 μg, respectively) was hybridized overnight in solution to a gel-purified antisense riboprobe complementary to 82 nt of coding sequence and 203 nt of upstream sequence. Following RNase digestion, the remaining nucleic acids were precipitated and size-fractionated on a denaturing (8 M urea in 90 mM Tris-borate buffer, 2 mM EDTA, pH 8) 8% polyacrylamide gel which was subsequently fixed, dried and autoradiographed. Figures 3, 4 and 5 display representative results taken from at least two replicates of each experimental treatment or tissue type. Figures 4 B and 5 were compiled from parts of replicate gels; autoradiogram exposure times were adjusted to give equivalent intensity bands for samples common to all gels represented in a given figure.

Results

Cloning and sequence of a conserved D19 homolog

An *Arabidopsis* genomic library was screened at a low stringency ($T_m - 24°$ C) with a 0.9 kb cDNA clone encoding the cotton D19 gene product. We selected those phage showing the strongest hybridization and sub-

cloned a 4 kb *Eco*RI fragment containing the entire region of homology. After further mapping of the homologous regions within this 4 kb fragment, we sequenced the transcribed region and several hundred nucleotides of 5′ and 3′ sequence (Fig. 1). This sequence has been deposited in the EMBL database under the accession number X66023. Comparison of the predicted amino acid sequence of the *Arabidopsis* gene (*D19h*) with the cotton D19 and wheat *Em* genes shows a high degree of conservation: 73% and 68% identity, respectively (Gaubier et al. 1993). This is comparable to the 69% identity seen between the predicted amino acid sequences of the cotton and wheat coding regions. In addition, the genomic sequence appears to contain an intron whose location is conserved in cotton (Baker et al. 1988). While we have not isolated a cDNA clone, the coding sequence of our genomic clone matches that of the *GEA6* clone (Gaubier et al. 1993) and their cDNA sequence demonstrates that we have correctly predicted the splice sites for the intron. It is worth noting that although the predicted amino acid sequences are identical, several single nucleotide differences are observed between the Landsberg and Columbia ecotypes (indicated in Fig. 1). The *Arabidopsis D19h* gene also shows the conserved ABA response element (ABRE) heptanucleotide ACGTGGC (reviewed in Hetherington and Quatrano 1991) at position −237 relative to the translation start site, but we have not addressed its functional significance in regulation.

Expression in seeds

As their name implies, *lea* genes are most highly expressed late in embryogenesis (Hughes and Galau 1991). The D19-like family belongs to a class whose mRNA is ABA-inducible in cultured embryos and remains abundant in mature dry seeds (Galau et al. 1986). Therefore, we compared *D19h* mRNA levels in dry seeds of wild-type *Arabidopsis* with those in seeds of ABA response and biosynthesis mutants. Northern blot analysis (Fig. 2A), using an antisense riboprobe spanning all but 30 nt of 5′ coding sequence (Fig. 2C), shows high levels of *D19h* mRNA accumulation in all genotypes except those homozygous for a mutant allele of *ABI3*. Seeds of *abi3* plants all show approximately ten-fold lower levels of this transcript while those of the double mutants *abi1 abi3* or *abi2*/+*abi3*/*abi3* plants are reduced even further. The ABA-deficient seeds show only a slight reduction in *D19h* mRNA levels. Gaubier et al. (1993) have shown that in *Arabidopsis* this is actually a two-member gene family. Although we find that we must decrease the hybridization stringency ($T_m - 24°$ C vs. $T_m - 19.5°$ C) in order to detect the other family member on genomic Southern filters (Fig. 3), we have used a gene-specific probe in an RNase protection assay to definitively quantify expression from *D19h* only (Fig. 2B). The probe is a 335 nt fragment including 82 nt of coding sequence and 203 nt of upstream sequence (Fig. 2C). Assuming initiation at approximately −80 nt (40 nt downstream of a TATA box, consistent with the start

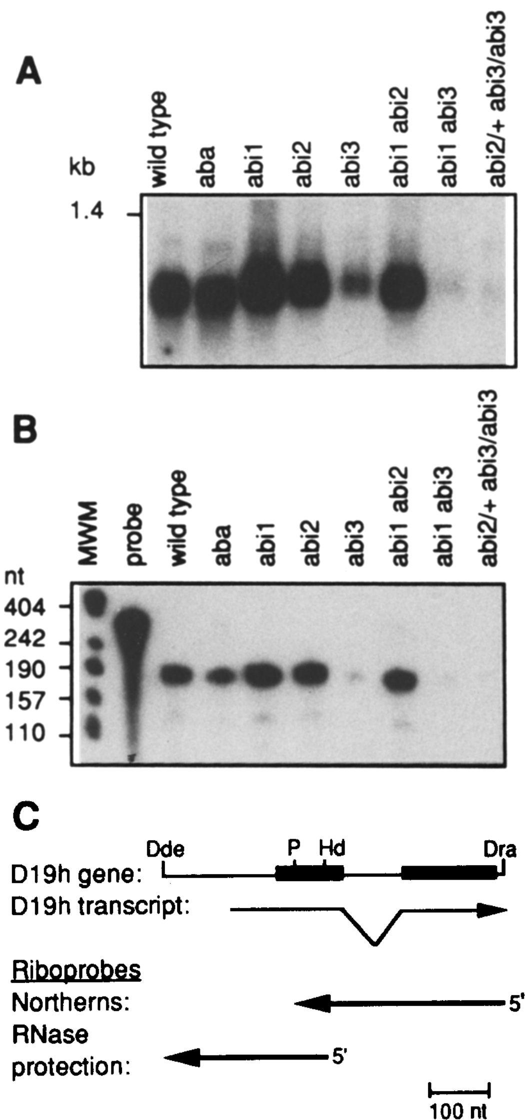

Fig. 2A–C. Expression of *D19h* in *Arabidopsis* seeds. **A** Northern blot analysis of total RNA from mature dry seeds (1 µg/lane) of the indicated genotypes, hybridized to a nearly full-length coding sequence probe. **B** RNase protection assay with samples identical to those in **A** Markers are end-labeled fragments from *Hpa*II-digested pBS-SK(−). **C** Schematic diagram of *Arabidopsis D19*/*Em* homolog indicating extent of transcript and probes used for Northern and RNase protection analyses. Open reading frames are designated by *black boxes*. P, *Pvu*II; Hd, *Hin*dIII

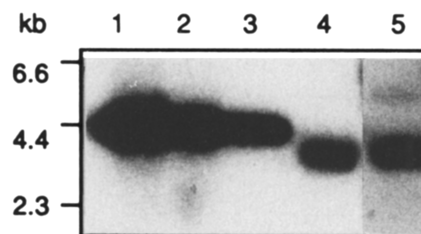

Fig. 3. Genomic reconstruction Southern blot of *Eco*RI-digested genomic (lanes 4 and 5) and plasmid (lanes 1–3) DNA. Plasmid DNA was loaded in quantities equivalent to 1, 2 or 5 copies per genome (lanes 3, 2, and 1, respectively). Lanes 1–4 were hybridized at $T_m - 19.5°$ C, lane 5 at $T_m - 30°$ C; all were washed at $T_m - 24°$ C

sites mapped by Gaubier et al. 1993) the protected fragment should be 160 nt. Given that RNA fragments migrate more slowly than DNA in this gel system, resulting in a difference of 5–10% in relative mobilities (Sambrook et al. 1989), the observed migration is reasonable for a 160 nt fragment. The RNase protection assays show the same relative expression levels as Northerns hybridized with the nearly full-length probe, demonstrating that either our Northern hybridization conditions are selective for this gene product or both family members show similar regulation in these genetic backgrounds.

Expression in seedlings

The *Em* gene promoter has been shown to be induced by exogenous ABA and water stress in wheat seedlings (Berge et al. 1989) and by exogenous ABA in rice protoplasts (Marcotte et al. 1988). Previous studies with the *Arabidopis abi* mutants had shown a degree of stage specificity in the effects of these mutations, in that the *abi3* mutation primarily affected seed development while *abi1* and *abi2* exerted their major effects during vegetative growth (Koornneef et al. 1984; Finkelstein and Somerville 1990). Therefore, we were interested in determining whether the *abi3* effect on *D19h* expression was limited to seeds and if the *abi1* and *abi2* mutations affected expression in vegetative growth. Preliminary experiments with seedlings and young plantlets at a variety of ages indicated that the plants became less responsive to ABA over time (data not shown). We chose to use 8-day-old seedlings because this was the latest stage tested that was still responsive. The effects of exogenous ABA and sorbitol (as osmoticum) on *D19h* transcript accumulation are shown in Fig. 4. The riboprobe nonspecifically binds rRNA, providing an internal standard of the uniformity of sample loading. Accumulation is enhanced by exogenous ABA over the range of 1–30 μM. However, unlike the wheat *Em* gene, the *Arabidopsis D19h* gene does not appear to be induced by water stress imposed by sorbitol at concentrations whose effects ranged from growth inhibition to complete loss of turgor. Comparison with mRNA levels in dry seeds indicates that expression, even when induced by 10–30 μM ABA, is 100 to 200-fold lower in seedlings than in seeds. The apparent decrease in mobility of the transcript in seedling RNA is due to the additional 24 μg RNA per lane in these samples (data not shown). Furthermore, RNase protection assays with a gene-specific probe again confirm that the Northern analysis accurately reflects abundance of the *D19h* transcript (Fig. 4B).

Having identified conditions in which ABA inducibility of the *D19h* gene could be assayed in seedlings, we tested the effects of the ABA response and biosynthetic mutations on *D19h* expression at this stage (Fig. 5). We used 10 μM ABA for all genotypes because it was effective for induction in wild-type plants, yet did not appear to be saturating and was therefore not likely to mask a shift in sensitivity in any of the mutants. RNase protection assays showed that *D19h* mRNA accumulation was

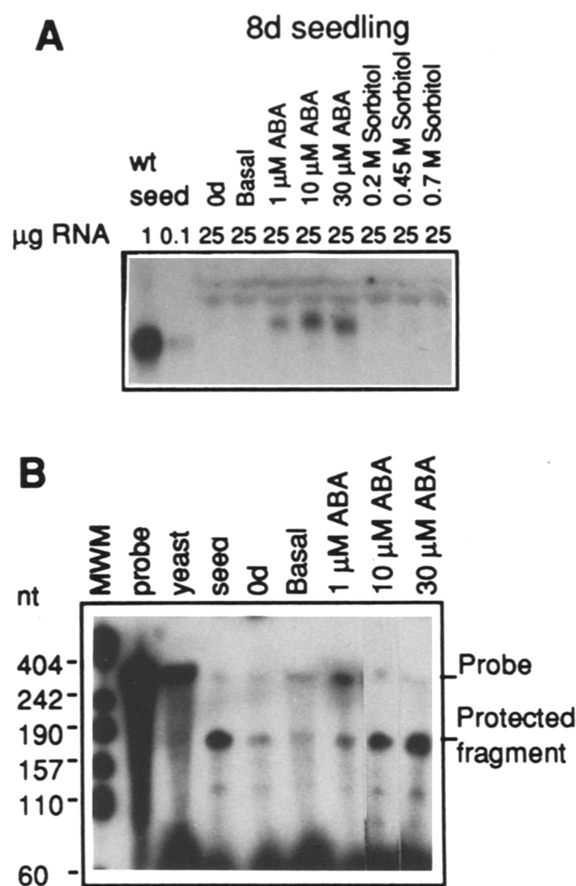

Fig. 4A, B. Dose response for ABA induction of *D19h* in *Arabidopsis* seedlings. **A** Northern blot of total RNA (amounts as shown) from wild-type seeds and seedlings following the indicated treatments (no culture or 24 h culture on 0, 1, 10 or 30 μM ABA or 0.2 M, 0.45 M or 0.7 M sorbitol). **B** RNase protection assay with RNA isolated from seedlings ±ABA-treatment (10 μg each), seeds (0.1 μg) or yeast (10 μg). Probes are same as in Fig. 2

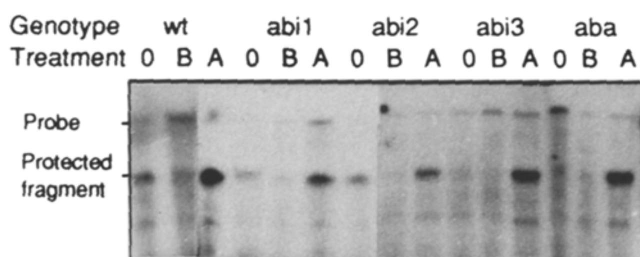

Fig. 5. ABA inducibility of *D19h* in wild-type, *abi* and *aba Arabidopsis* seedlings. RNase protection assay with total RNA from seedlings of the indicated genotypes harvested immediately (0) or following 24 h culture on basal medium (B) or 10 μM ABA (A). Probe is the same as in Fig. 2B

the same in wild-type and *abi3* seedlings, indicating that the ten-fold decrease in *D19h* transcript levels in *abi3* seeds was a seed-specific alteration. Conversely, *abi1* and *abi2* showed a very slight, but reproducible, seedling-specific decrease in exogenous ABA induction of this transcript.

Discussion

The cotton *lea* D19 and wheat *Em* genes were initially cloned as highly abundant gene products expressed late in embryogenesis (Williamson et al. 1985; Galau et al. 1986). These and subsequent studies showed that they were regulated by exogenous ABA and osmoticum as well as developmental stage (Williamson and Quatrano 1988; Berge et al. 1989; Hughes and Galau 1991). To date, homologous genes have been isolated from radish (Raynal et al. 1990), carrot (Ulrich et al. 1990), maize (Williams and Tsang 1991) and barley (Espelund et al. 1992). Deletion analysis of the *Em* promoter in heterologous systems (rice protoplasts and transgenic tobacco) has identified *cis*-acting domains required for ABA and developmental stage regulation and led to the cloning of a DNA-binding protein which specifically binds the conserved ABRE element (Marcotte et al. 1988, 1989; Guiltinan et al. 1990). Although analysis of mutants affecting upstream signalling events has not been possible in these systems, recent studies have shown that expression of the maize *Em* homolog is greatly reduced in *vp5* (ABA-deficient) and *vp1* (ABA-insensitive) mutant embryos (McCarty et al. 1991). In contrast, another exogenous ABA-inducible maize embryo protein gene, *Glb1*, shows nearly wild-type mRNA accumulation in four different ABA-deficient *vp* mutants, but its expression is drastically reduced in the ABA-insensitive *vp1* mutant (Kriz et al. 1990). The *Glb1* gene is expressed earlier than *Em* in normal seed development, so it is not surprising that it might be subject to different regulation. However, this difference illustrates the importance of monitoring regulation of multiple ABA-responsive genes in many genetic backgrounds affecting ABA synthesis and response before constructing a model for the role of ABA in regulation of such genes.

This is the first description of the expression of a single *lea* gene at different developmental stages in both ABA-deficient and multiple ABA-insensitive backgrounds. Such studies have been limited in part because *Arabidopsis* is the only flowering plant in which multiple loci required for ABA response have been identified mutationally (Koornneef et al. 1984) and few ABA-regulated genes expressed at multiple stages have been cloned from this species. We have cloned *D19h*, one of the two *Arabidopsis* homologs of the D19/*Em*-like family, and compared its expression in ABA-deficient and ABA-insensitive lines of *Arabidopsis* with that in wild-type plants. Our studies indicate that the *abi3* mutation dramatically decreases accumulation of this transcript in seeds, but does not affect *D19h* expression in seedlings treated with exogenous ABA. It is worth noting that, unlike the maize *vp5* mutant, an ABA-deficient mutant of *Arabidopsis* accumulates nearly wild-type levels of this transcript in seeds.

The observation that only the *abi3* mutation significantly reduces *D19h* expression in seeds is similar to that previously reported for the expression of the major seed storage protein cruciferin in wild-type, *aba* and *abi* seeds (Finkelstein and Somerville 1990). However, *D19h* expression differs from that of cruciferin in that *D19h* mRNA accumulation begins during the desiccation phase of seed development, when cruciferin mRNA levels are declining and the siliquae start to lose chlorophyll (unpublished observations, Gaubier et al. 1993). These events follow the decline of the large peak of endogenous ABA supplied by maternal tissue, but coincide with accumulation of the smaller ABA fraction contributed by the embryo (Karssen et al. 1983). This embryonic ABA is known to be required for induction of dormancy, a significant aspect of embryo maturation, and its accumulation correlates with the onset of *D19h* expression, making it a plausible signal for regulation of the *D19h* gene. However, even though the *D19h* transcript is inducible by exogenous ABA in immature *Arabidopsis* embryos (Gaubier et al. 1993), the nearly wild-type expression of this gene in ABA-deficient seeds implies that endogenous ABA is not an essential control signal for expression of the *D19h* gene in seeds of this species. Taken together, these results support previous suggestions that *abi3* acts via an ABA-independent pathway affecting responses that may also be subject to regulation by a pathway responsive to exogenous ABA (Finkelstein and Somerville 1990).

In contrast, the *abi1* and *abi2* mutations do not decrease *D19h* expression in seeds of monogenic mutant lines, but appear to increase slightly the severity of the effects of the *abi3* mutation on seed expression of *D19h*. This is consistent with our previous suggestion that the *ABI* loci may be controlling seed development through different regulatory pathways (Finkelstein and Somerville 1990). However, it is somewhat surprising that the *abi1* and *abi2* mutations have no obvious effects on seed *D19h* expression in the absence of the *abi3* mutation.

Exogenous ABA-induced expression of the *D19h* gene in wild-type seedlings is 100–200 fold lower than in seeds, reflecting developmental differences in regulation reminiscent of those seen in cotton (Hughes and Galau 1991). A further difference from expression in seeds is that only the *abi1* and *abi2* mutations alter exogenous ABA inducibility of *D19h* mRNA accumulation in seedlings and the reduction in exogenous ABA response of these mutants is very slight. Seedling expression of the *Arabidopsis D19h* gene differs from that of the wheat *Em* gene (Berge et al. 1989) in that it is far less responsive to exogenous ABA and even less responsive to water stress. We have not done a complete timecourse of stress- or exogenous ABA-dependent induction of *D19h* expression in seedlings, so it is possible that we are measuring non-peak levels in a transient rise in transcript accumulation. Alternatively, the failure of water stress induction may be an indication that endogenous ABA is not an important regulatory signal for *D19h* expression in seedlings either, but this conclusion requires confirmation that endogenous ABA levels are elevated in water-stressed *Arabidopsis* seedlings.

Studies of other ABA-inducible genes in ABA response and synthesis mutants fall into two classes: (1) comparison of expression at different stages in only wild type and a single response or synthesis mutant background and (2) expression in several mutant backgrounds at a single developmental stage. An example

of the first class is a recent study of the role of ABA in regulation of the maize late embryogenesis-abundant ABA-responsive gene, *rab28*. *rab28* expression was compared in seeds and seedlings of wild-type, *vp1* (ABA-insensitive) and *vp2* (ABA-deficient) mutant plants (Pla et al. 1991). These experiments showed that *rab28* expression in seeds is normally subject to developmental, but apparently endogenous ABA-independent, regulation by *Vp1*. An additional level of control in seeds can be provided by exogenous ABA, implying the existence of an exogenous ABA response pathway separate from that involving *Vp1*. Although such a pathway has not yet been identified mutationally in maize, evidence for multiple pathways of exogenous ABA response in mature seeds has been provided by studies of the *abi* mutants of *Arabidopsis* (Finkelstein and Somerville 1990). At the second developmental stage tested by Pla et al. (1991), seedlings, *rab28* is not expressed unless exposed to exogenous ABA or environmental stresses such as water stress. The dehydration induction is dependent on the ability to synthesize ABA (Neill et al. 1986), but the role of *Vp1*-dependent regulation at this stage was unclear because *vp1* and wild-type seedling responses to exogenous ABA and dehydration were not compared directly.

ABA regulation of gene expression at a single developmental stage has been investigated for several cold-induced (*cor* and *lti*, for *co*ld-*r*egulated and *l*ow *t*emperature-*i*nduced, respectively) genes in wild-type and multiple mutant backgrounds of *Arabidopsis* (Gilmour and Thomashow 1991; Nordin et al. 1991). These genes showed similar patterns of regulation in that all were inducible to various degrees by cold and exogenous ABA, but were not dependent on either endogenous ABA or functional *ABI* gene products for cold-induction. Unlike *D19h*, the cold-induced genes showed severely reduced exogenous ABA inducibility in the *abi1* mutant, but wild-type ABA induction in *abi2* and *abi3* mutants. Only the *lti* gene was tested under water stress conditions and it was found that this stress induction was partly mediated by endogenous ABA and only partly dependent on *ABI1* function. Together, these experiments provide evidence for several overlapping pathways of response to environmental stresses, only some of which are mediated by endogenous ABA or require the activity of any of the known *ABI* loci.

In summary, accumulation of the *D19h* transcript is most strongly affected by the *abi3* mutation, but this effect is confined to expression in seeds. It is possible that *D19h* expression in seeds is normally controlled by an endogenous ABA-independent stage-specific pathway, but can also be induced by an exogenous ABA-dependent pathway. The *abi3* mutation could affect *D19h* expression by blocking either of these pathways, but any effects on sensitivity to exogenous ABA would not be relevant during normal seed development. We did not test whether the *abi3* mutation actually blocked exogenous ABA induction of this gene, as was observed for the cruciferin genes (Finkelstein and Somerville 1990), or if *D19h* remained ABA-inducible in *abi3* embryos, analogous to the regulation of the *rab28* gene in *vp1* mutants of maize (Pla et al. 1991). The relatively minor effect of both the *abi1* and *abi2* mutations on exogenous ABA inducibility in seedlings of the *D19h* gene might be an indication that ABA regulation of this gene is dependent on still another ABA signalling pathway, which has not yet been identified by mutation. Alternatively, the subtlety of the effect could reflect leaky activity through an *ABI1*- and/or *ABI2*-dependent pathway, with the *D19h* gene having a lower threshold requirement for exogenous ABA induction than the *lti* and *cor* genes. Comparison of such classes of genes and their expression in the various genetic backgrounds should provide a powerful system for molecular genetic analysis of *cis*-acting elements required *in vivo* for ABA-induced vs. seed-specific or stress-induced expression.

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