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Rapid Report

A Null Mutation in a bZIP Factor Confers ABA-Insensitivity in *Arabidopsis thaliana*

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We have used a modification of the classical ABA-insensitive screen (Koornneef et al. 1984) to isolate novel mutations in the ABA signal transduction pathway of *Arabidopsis thaliana*. In our screen, mutants were recovered on the basis of their growth-insensitivity to ABA (GIA) rather than germination-insensitivity. Here we present the isolation of the *gia1* mutant as well as the identification of the *gia1* gene by positional cloning and complementation studies. *GIA1* is predicted to code for a bZIP transcription factor with high homology to previously characterized plant bZIP transcription factors (DPBF1, ABFs and TRAB1) known for their ability to bind ABA-responsive DNA elements. Our results provide in vivo evidence that a bZIP factor may indeed be involved in ABA signaling. Since *GIA1* turned out to be identical to *ABI5*, we designated *GIA1* as *ABI5* in the present paper.

Key words: Arabidopsis — Abscisic acid — Genetics — Positional cloning.

Abscisic acid (ABA), a phytohormone and growth regulator in higher plants, plays a multivariate and complex role in plant development (Reviewed in Himmelbach and Iten 1999). During early embryogenesis, ABA is essential for maintaining seed dormancy and preventing precocious germination. In the vegetative phase of the plant ABA also mediates plant stress responses to the environmental pressures of drought, cold and osmotic imbalances (Himmelbach and Iten 1999). As such, it is of vital importance to the survival of a plant and therefore understanding its mechanism of action could lead to valuable insights into plant stress response pathways.

One approach to elucidating the ABA signaling pathway is to isolate mutants that are insensitive to ABA. In *Arabidopsis*, ABA-insensitive (ABI) molecular genetic screens based upon positive selection of mutant plants capable of germinating on ABA have been performed (Kornneef et al. 1984, Finkelstein 1994). This has led to the identification of four different genes whose products affect embryonic as well as vegetative ABA responses (Giraudat et al. 1992, Leung et al. 1994, Meyer et al. 1994, Leung et al. 1997, Rodriguez et al. 1998). The *abi1* and *abi2* mutations primarily affect vegetative responses such as gene induction and stomatal closure after ABA exposure. Their wild type gene products ABI1 and ABI2 are two highly homologous protein serine-threonine phosphatases 2C. The *abi1* and *abi2* mutations are dominant and in both case the same amino acid (Gly) was substituted by Asp located in a homologous domain of the proteins (Leung et al. 1997). ABI1 and ABI2 have been suggested to be negative regulators of ABA responses (Gosti et al. 1999). Contrary to *abi1* and *abi2*, the *abi3* mutation is recessive (Kornneef et al. 1984) and its wild-type (wt) gene product, ABI3, is believed to be a transcriptional activator exerting its function through ABA-responsive elements (ABREs) located in the promoters of ABA-responsive genes (Hobo et al. 1999 and references therein). Its mode of action still needs clarification as no direct ABRE binding activity has been demonstrated. ABI3 operates in embryonic ABA signaling and its expression is restricted to embryonic tissues and rapidly declines upon germination (Parcy et al. 1994). Finally, ABI4 was recently identified (Finkelstein et al. 1998) as an AP2 domain transcription activator, which acts primarily in embryos as well. The *ABI5* gene disrupted in *abi5* (Finkelstein 1994) was not published at the time we started our project.

Clearly, more genes are likely to be involved in ABA signaling and their identification is required for a better understanding of the mode of action of this important phytohormone. We have hypothesized that additional genetic loci might be recovered using the classical ABA-insensitive screen (Kornneef et al. 1984). The difficulty with this approach is that one has to use relatively high ABA concentrations (5 to 10 μM) in order to significantly inhibit (that is, below 5%) wild type seeds from germinating. Indeed, at low ABA concentrations the germination criterion is no longer valid because too many false positives are recovered. Nevertheless, we chose to perform the same positive screen at lower ABA (2 μM) concentrations than

Abbreviations: ABA, abscisic acid; ABREs, ABA-responsive elements; BAC, bacterial artificial chromosome; CAPS, cleaved amplified polymorphic sequences; SSLPs, simple sequence length polymorphisms.

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previously used (Kornneef et al. 1984, Finkelstein 1994). At this concentration, even though wild type seeds eventually fully germinate after 5 d, greening and growth of the seedlings are dramatically inhibited thereafter. Therefore, by screening for not only germination but also vegetative growth at low ABA concentrations we might detect novel mutations in ABA signaling. We named the mutants recovered from this screen *gia* (growth-insensitive to ABA). We have been able to identify at least four recessive *gia* loci different from the known *abi* loci.

In the present study, we report the identification of the recessive mutant *gia1* and the identification of the *GIA1* gene by positional cloning and complementation analysis. During the preparation of this manuscript we learned that Finkelstein and Lynch (Finkelstein and Lynch (in press)) had simultaneously cloned *ABI5* by positional cloning and *GIA1* turned out to be identical to *ABI5* (Finkelstein personal communication). As *gia1* is a novel allele of *abi5* we designated it as *abi5-4*. Because Finkelstein (Finkelstein 1994) first isolated *abi5* mutants, we will now refer *GIA1* as *ABI5*. The *ABI5* ORF is predicted to code for a basic region leucine zipper (bZIP) transcription factor with sequemce homology to DPBF1 from sunflower (Kim et al. 1997), *Arabidopsis* bZIPs ABFs (Choi et al. 2000) and the rice TRAB1 (Hobo et al. 1999). The C-terminal bZIP domain of ABI5 is almost identical to those other bZIP factors which have been shown to bind ABA responsive elements (Kim et al. 1997, Hobo et al. 1999, Choi et al. 2000). We show that *ABI5* is expressed during embryonic development as well as in vegetative tissues. Moreover, two late embryonic abundant (*lea*) and ABA-inducible genes are strongly down regulated in *abi5-4* plants. Our results provide in vivo evidence that ABI5 plays a role in ABA signaling.

Materials and Methods

Plant material and mutant selection procedure—T-DNA INRA-Versailles lines available from the Nottingham Arabidopsis Stock Center (NASC) were used in the screening. *abi5-4* was obtained from a set of 2,400 lines (Stock numbers N5389 and N5455) and screened as pools of 100. 1,000 to 2,000 seeds were sown in 9 cm petri dishes containing 0.43% Murashige and Skoog salts (Murashige and Skoog 1962), 1% sucrose, 0,05% MES, 0.8% agar, pH 5.7) supplemented with 2 μM ABA (ABA, mixed isomers. Sigma,A7383). ABA was dissolved in methanol and control dishes without ABA received equal amounts of methanol as ABA supplemented ones. Plates were routinely kept 4 d in the dark at 4°C transferred thereafter to a growth chamber (22°C, 16 h photoperiod). Growth-insensitive to ABA plants were selected for their ability to green and develop roots 5 to 7 d after their transfer to the growth chamber and transferred to plates without ABA and finally transferred to soil after 2 weeks. The next generation was tested in the same manner in petri dishes containing 1, 2, 3 and 5 μM ABA. Only the plants that kept the ability to green and develop roots in 2 μM ABA were finally selected for further study.

Isolation of recombinant plants and molecular mapping—The *abi5-4* mutant was outcrossed with Columbia (Col) wild type plants and mapping populations were isolated by selecting ABA-

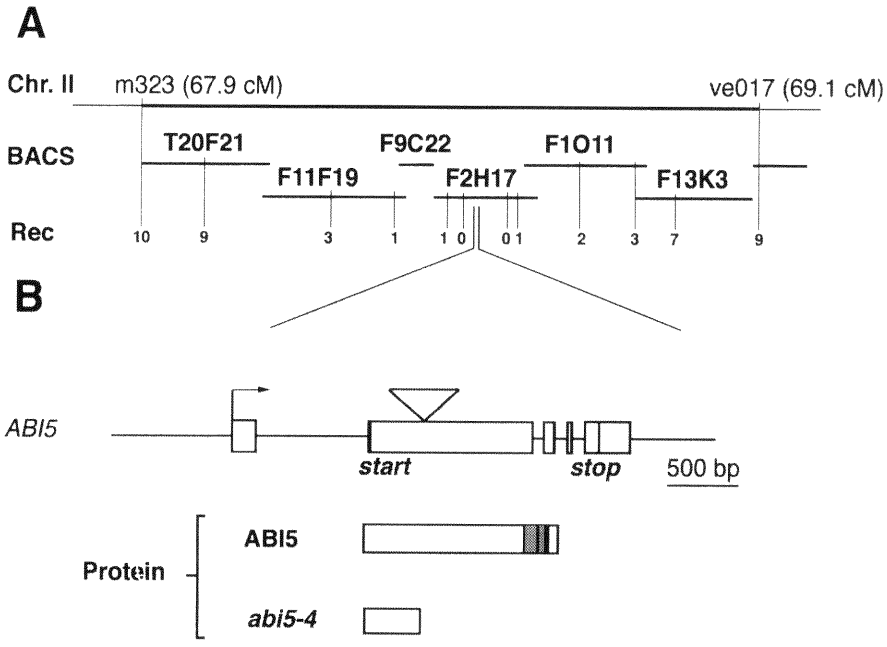

Fig. 1 (A) Fine mapping of *ABI5* locus on chromosome 2. 19 recombinants were isolated between CAPS markers m323 and ve017. New CAPS and SSLP markers were generated and vertical lines indicate their relative position in the BAC contig. Numbers below marker lines indicate the recombination events (REC). (B) Structure of the ABI5 gene. Boxes indicate exons. The triangle indicates the site of the 37 bp insertion in the *abi5-4* mutant. Translation start and stop sites are indicated as well as the predicted protein products in both Wt and abi5-4 plants. The shaded portions represent the predicted location of the basic and leucine-zipper domain.

Table 1 *abi5-4* complementation with single insertion independent transgene (T2 generation)

Plant	Kanamycin sensitivity	Germination and growth in 3 μM ABA
Ws	100% (n=251)	1.3% (n=232)
abi5-4	100% (n=204)	95.2% (n=208)
abi5-4/P208,1	25.3% (n=182)	24.8% (n=205)
abi5-4/P208,2	24.8% (n=149)	23.5% (n=229)
abi5-4/P208,3	27.8% (n=115)	23.2% (n=151)
abi5-4/P208,4	23.8% (n=88)	20.3% (n=153)

Complementation analysis by single transgene insertion in four independent *abi5-4* lines. Ws, wild type. Percentages indicate frequencies.

insensitive F2 families. *abi5-4* was initially mapped to the lower arm of chromosome 2 using 28 F2 progeny plants. For mapping, a combination of cleaved amplified polymorphic sequences (CAPS) markers (Konieczny and Ausubel 1993, Glazebrook et al. 1998) and simple sequence length polymorphisms (SSLPs) markers (Bell and Ecker 1994) was used. DNA preparation and PCR conditions were exactly as Glazebrook et al. 1998. For fine mapping, novel CAPS and SSLPs were generated between CAPS markers m323 and Ve017 as indicated on Figure1. The sequences of the oligonucleotides and restriction enzymes for these markers are available upon request.

Complementation studies—The bacterial artificial chromosome F2H17 was obtained from the Arabidopsis Biological Resource Center (ABRC, http://aims.cps.msu.edu/aims/). A *Bam*HI sub-fragment of F2H17 (Accession number: AC006921, positions +31670 to +37348) containing 1.5 kbp and 2 kbp of upstream and downstream *ABI5* sequences, respectively, was subcloned into binary vector pBin19 (Bevan 1984). This complementation vector (called P208) was electroporated into *Agrobacterium tumefaciens* and *abi5-4* plants were vacuum-infiltrated as described (Bechtold and Pelletier 1998). Four independent T2 families were selected for antibiotic and ABA sensitivity (see Table 1).

cDNA isolation and 5′ RACE analysis—A cDNA library (a gift of Dr. Hiroharu Banno) was constructed in the Ziplox™ system (Life Technologies, Inc) from whole seedlings Columbia mRNA. The library was screened according to the manufacturer instructions. 5′ RACE was performed from Columbia leaf total RNA using the SMART™ PCR cDNA synthesis kit according to the manufacturer instructions.

RNA isolation and analysis—Silique and dry seed RNA was isolated according to Vicient and Delseny 1999. RNA from vegetative tissues was isolated using the Qiaqen Plant Mini Kit (Qiagen Inc.). For Northen Blot analysis, RNA was size fractionated on formaldehyde agarose gels and transferred to nylon membranes (Duralon, Stratagene) according to standard procedures (Sambrook et al. 1989). Hybridization conditions were as Church and Gilbert (Church and Gilbert 1984). Ribonuclease assays were performed as described (Lopez-Molina et al. 1997).

Hybridization probes—*AtEm6* and *AtEm1* specific DNA probes (Gaubier et al. 1993) were generated by PCR amplification of Ws genomic DNA using the following primers: 5′ CAAG TGATAGTAGTTACGAGCTAC and 5′ ACATACTATCAC AAGTAAGACACG (*AtEm1*), 5′ GAAGGAGTCGAGATAG ACGAATCC and 5′ CAATTCTAGTCGAATTAGCTCTACC (*AtEm6*). The *Cor47* and *rab18* probes were from Foster and Chua (Foster and Chua 1999). The ABI5 probe consisted of the largest ABI5 cDNA insert (see Results). For RNase protection a riboprobe encompassing position +34410 to +34555 of BAC F2H17 was used.

Computer software—The following internet sites were used for sequence comparison and phosphoaminoacid prediction: BLAST, http://www.ncbi.nlm.nih.gov/blast/blast.cgi; PROSCAN, http://pbil.ibcp.fr/cgi-bin/npsa automat.pl?page=/NPSA/npsa proscan.html; NETPHOS, http://www.cbs.dtu.dk/services/NetPhos.

The alignment shown in Figure 2 was produced with the program Megalign (DNASTAR Inc. 1997).

Results

abi5-4 identification and positional cloning of ABI5—*abi5-4* was isolated from a set of 2400 INRA-Versailles T-DNA lines available from the Nottingham Arabidopsis Stock Center (NASC). The T-DNA lines were screened as pools of 100 and putative mutants were selected for their ability to germinate and grow in a medium supplemented with 2 μM ABA. Putative mutant plants were transferred to soil and progeny from the next generation was tested again at different ABA concentrations. *abi5-4* plants were capable of germinating in concentrations as high as 5 μM ABA (90% of the plants having full radical tip emergence after 3 d of breaking dormancy) although their growth was limited. At 3 μM ABA they could germinate and grow normally but slower than in the absence of ABA. In 3 μM ABA wild type plants germinated 5 d after breaking dormancy but the seedling failed to green.

Having established the germination and growth-insensitivity of *abi5-4* plants we attempted to directly identify genomic DNA flanking the site of the presumed T-DNA insertions generated by the plant transformation vector pGKB5 used in these lines. We failed to detect any insertion by southern blot analysis and growth on kanamycin (data not shown). We therefore used a positional cloning approach to discover the responsible genetic lesion of *abi5-4*.

The *abi5-4* mutant, which is in a Wassilewskija (Ws) ecotype background, was backcrossed into Columbia (Col) and Ws wild type background. The F2 generation from these crosses established that *abi5* was a monoallelic, recessive mutation (data not shown). We used cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993) and simple sequence length polymorphisms (SSLPs) (Bell and Ecker 1994) developed for *Arabidopsis* as genetic markers. Initial analysis of 28 individual F2 *abi5-4* plants from the *abi5-4* × Col backcross established closed linkage with CAPS marker m429 and SSLP marker nga168 on chromosome 2. Because *ABI4* is also closely linked to these markers, we amplified its open reading frame by polymerase chain reaction (PCR). Sequencing of independent PCR fragments showed that the *ABI4*

sequence was unaffected in the *abi5-4* mutants. This prompted us to further analyze a larger number of individual segregants demonstrating that the *abi5* locus was in fact between CAPS markers m323 and ve017 (REF) about 2 Mb proximal of *ABI4* (see Fig. 1). In order to perform a fine mapping of the *abi5-4* mutation we randomly amplified 1 kbp fragments using the sequence information from the bacterial artificial chromosome (BAC) contig covering the interval (see Fig. 1). Single nucleotide polymorphisms were found between Col and Ws ecotypes for each BAC which were used to generate novel CAPS markers (see Fig. 1). Analysis of 1920 meiotic events allowed us to identify 19 recombination events between markers m323 and ve017 (Fig. 1). These recombinants enabled us to finally narrow down the interval containing the *abi5* mutation within approximately 60 kbp on BAC

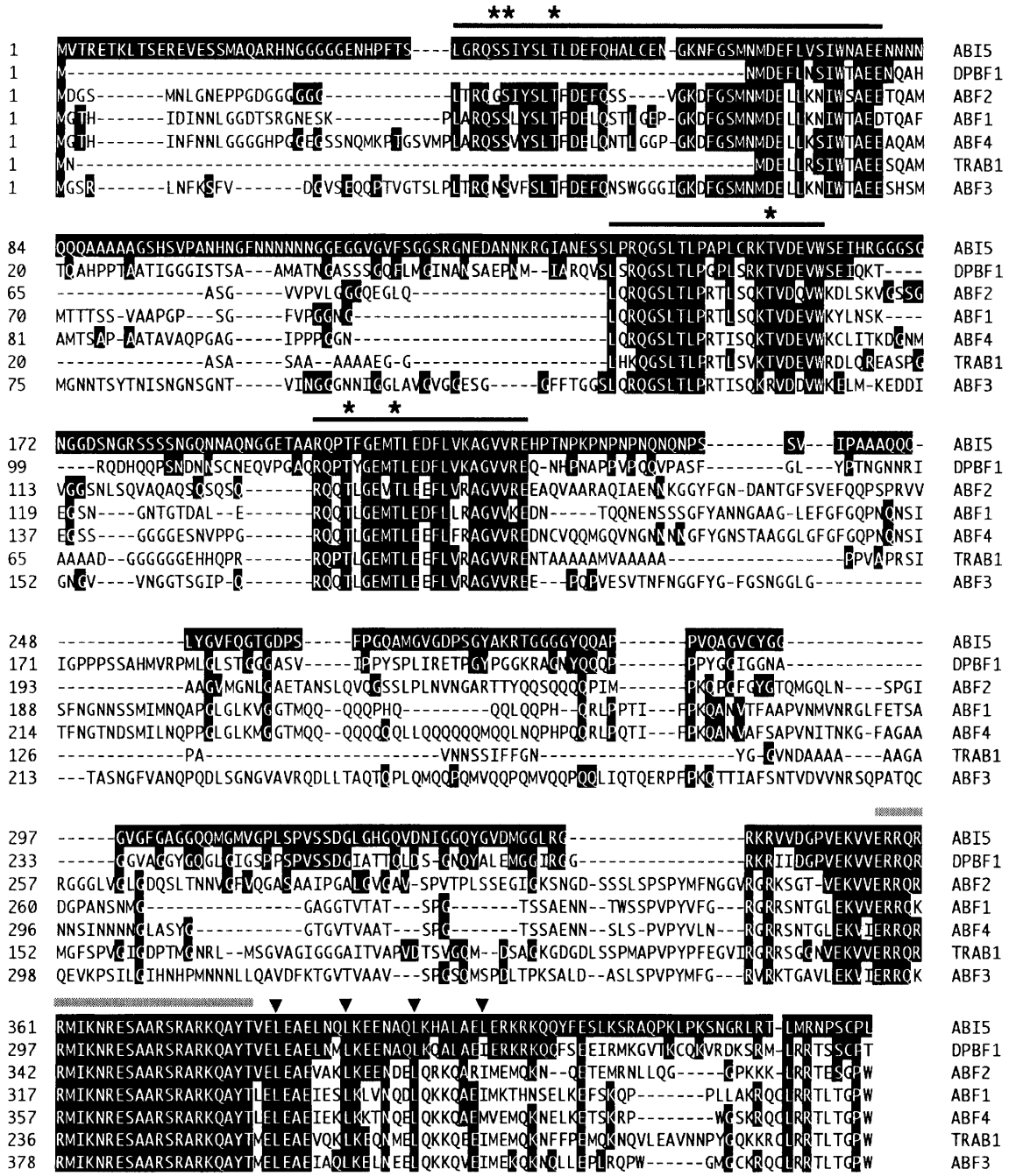

Fig. 2 Sequence comparison of ABI5 with known similar plant bZIP proteins. Potential conserved phosphoaminoacids are indicated with (*). The three highly homologous domains are overlined in black. The conserved basic region is overlined in gray. Arrowheads indicate the predicted positions of the ABI5 zipper leucines.

F2H17 (GenBank accession number: AC006921). PCR amplification of *abi5* genomic DNA within this interval followed by sequencing analysis showed that predicted gene F2H17.12 (accession number AC006921.5) had a 37 bp insertion 330 bp downstream of the start codon of the first exon (Fig. 1). As this insertion causes a premature translation stop (Fig. 1) the *abi5-4* mutant gene product is predicted to be one third the length of the ABI5 protein. The F2H17.12 ORF encodes a putative bZIP transcription factor containing at the C-terminus, the basic domain signature followed by a leucine zipper domain.

To demonstrate that the mutated gene was indeed responsible for the *abi5* phenotype we constructed a complementation vector (called P208) consisting of the pBIN19 binary plasmid with a 5.6 kbp *Bam*HI fragment from BAC F2H17 encompassing F2H17.12. P208 was used to transform *abi5* plants and the ability of the transformants to germinate and grow on ABA was tested. Analysis of T2 seeds from four independent lines carrying single insertion transgene showed conclusively that P208 was indeed sufficient to complement the *abi5-4* mutation, since both kanamycin sensitivity and ABA-insensitivity segregated with Mendelian ratios (see Table1).

ABI5 analysis—To verify the gene structure of *ABI5* we screened a cDNA library constructed from RNAs made from 3 week old wt Columbia seedlings. Sequence analysis of three independent cDNA clones indicated the presence of at least two different polyadenylation sites separated by 15 nucleotides. The longest cDNA insert (1,561 bp) confirmed the occurrence of the predicted introns and exons but revealed the presence of an additional exon-intron junction (Fig. 1B) located seven nucleotides upstream of the presumed start codon. This 5′ intron is 782 bp long. 5′ RACE analysis confirmed the presence of this intron and extended the total length of the first exon to 145 bp. Figure 1B depicts the genomic structure of *ABI5* along with the position of the insertion generating the new *abi5-4* allele. Despite the presence of this additional exon, the predicted start codon is likely to be correct since there is an upstream ATG which is immediately followed by a stop codon (ATGTGA).

ABI5 protein structure analysis—Comparison of ABI5 with other proteins in the database using BLAST software shows that it has the highest homology to five bZIP plant transcription factors: DBPF-1 (Kim et al. 1997), ABFs (Choi et al. 2000) and TRAB1 (Hobo et al. 1999) from sunflower, *Arabidopsis* and rice respectively. The highest homology is found with DBPF1 (49% identities, 57% positives, 8% gaps), followed by ABF2 (38%, 47%, 27%), ABF1 (35%, 42%, 23%), ABF4 (34%, 42%, 23%), TRAB1 (34%, 43%, 26%) and ABF3 (31%, 42%, 19%). An alignment of the six proteins is shown in Figure 2. Highest homology is observed in the C-terminal part of the protein within the basic region, which is either identical (24/24) or only differs by a single amino acid (23/24). Three highly homologous domains are found in the N-terminal region (see Fig. 2) with minimum amino acid identities of 60% for the first (43 aa), 63% for the second (22 aa) and 81% for the third domain (22 aa). PROSCAN and NETPHOS (see material and methods) software analysis of these domains predict potential conserved serines and threonines as possible targets for phosphorylation (see Fig. 2). This suggests that the activity of these proteins may be regulated post-translationally by phosphorylation and dephosphorylation from upstream ABA signal-transduction pathway components. In addition, ABI5 contains two bipartite nuclear localization signals located at amino acid positions 344 and 366. This finding is not surprising since ABI5 is presumed to play a role as a transcription factor.

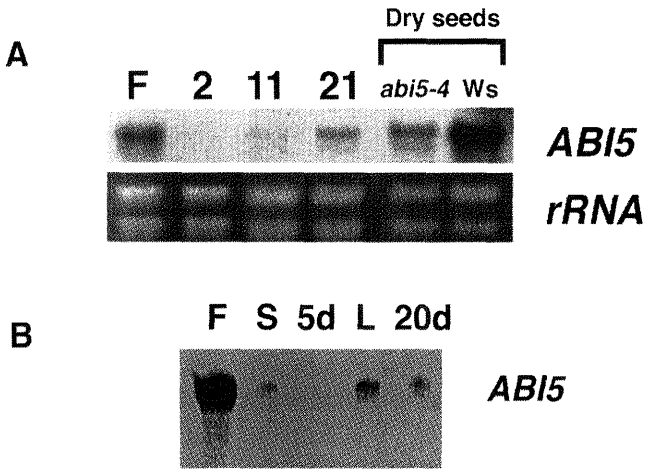

Fig. 3 *ABI5* expression. (A) *ABI5* expression by Northen blot analysis in flowers (F), siliques 2, 11 and 21 d after flowering. Dry seed expression is shown for abi5-4 and wild type (Ws). 2 μg per lane of total RNA was used. (B) *ABI5* by RNase protection analysis in vegetative wild type (Ws) tissues. F, flowers; S, stem; 5d, five d old seedlings; L, leaves; 20d, 20 d old plants. 3 μg of input total RNA were used per lane.

ABI5 mRNA expression—We studied the tissue distribution of *ABI5* transcripts by Northen blot analysis and RNase protection assay. Figure 3 shows that *ABI5* was expressed in both vegetative and embryonic tissues, throughout most stages of silique development. From almost undetectable levels 0 to 2 d after pollination (DAP) ABI5 expression levels showed a slow and gradual increase culminating in dry seeds where the highest levels were observed. High expression levels were also observed in flowers (Fig. 3). In contrast to flowers and dry seeds, lower transcript levels were found in vegetative tissues such as stem and leaves of 20 d old seedlings. ABI5 transcript levels were undetectable in 5 d old seedlings.

ABI5 target genes—DBPF-1 has been cloned by yeast one-hybrid screen for its ability to bind to the promoter region of Dc3 (Kim et al. 1997), a carrot *lea* gene highly

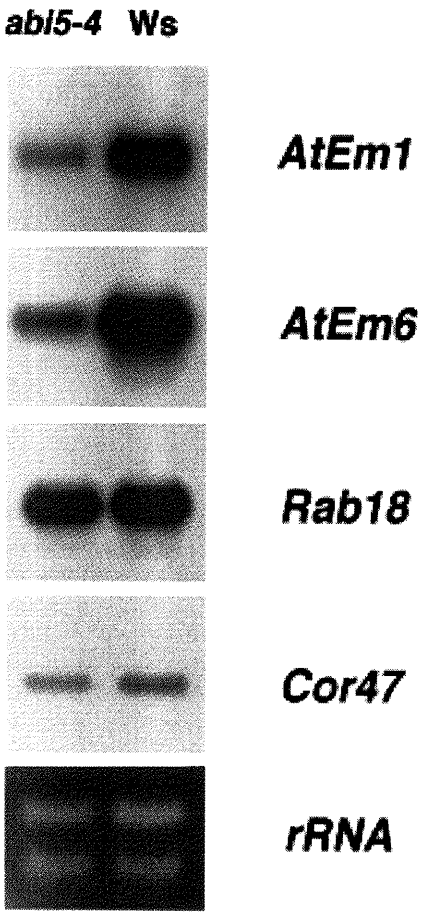

Fig. 4 Gene expression in *abi5-4* dry seeds. Northern blot analysis using 2 μg of total RNA from dry seeds per lane.

expressed in embryos and whose expression is ABA-inducible (Kim et al. 1997). The ability of DBPF-1 to bind ABRE containing promoters of a *lea* gene and the increased *ABI5* expression in late embryogenesis prompted us to analyze the expression of similar Arabidopsis *lea* genes in the *abi5-4* seeds. Figure 4 shows that the ABA-responsive *lea* genes *AtEm6* and *AtEm1* (Gaubier et al. 1993) expression was strongly diminished (but not eliminated) in *abi5-4* dry seeds as compared to Ws control seeds. By contrast, the expression of two other ABA-responsive genes *Cor 47* (Gilmour et al. 1992) and *Rab18* (Lang and Palva 1992) were identical in *abi5-4* and wt. Therefore, our results suggest *abi5-4* affects a subset of ABA-regulated genes late in embryogenesis. Interestingly, *ABI5* expression was strongly diminished in *abi5-4* seeds suggesting autoregulation of *ABI5* and/or a lower stability of the *abi5-4* transcript.

Discussion

Here we report the identification of a novel *abi5* allele (Finkelstein and Lynch, in press) as well as the identification of the *ABI5* gene by map-based cloning and complementation analysis. *ABI5* encodes a protein highly homologous to bZIP transcription factors known to bind ABRE elements. *abi5-4* mutant plants were isolated for their ability to germinate and grow on medium supplemented with ABA. Moreover, we have shown that *AtEm1* and *AtEm6*, two ABA regulated genes (Gaubier et al. 1993) were strongly down regulated in seeds. Thus, our results provide in-vivo evidence that this class of transcription factors plays a role in ABA signal transduction pathway. This novel allele likely generates a null-mutation of *ABI5* as only a third of the protein would be translated without the capability of dimerizing and binding to DNA.

The role of *ABI5* in ABA signaling remains to be further explored and placed in the context of similar factors such as the ABF family recently identified (Choi et al. 2000). It remains to be investigated how these different factors interact and mediate ABA-induced gene responses and also other environmental responses such as drought, cold and high salinity. As members of bZIP transcription factor family, they might heterodimerize in order to execute specific functions. In vegetative tissues some functional redundancy is expected. For example, the induction of *kin2* and *rd29a*, two ABA responsive genes (Kurkela and Borg-Franck 1992, Yamaguchi-Shinozaki and Shinozaki 1993) with ABREs in their promoters, appeared to be normal in *abi5-4* plants (data not shown).

Another area for future investigation is the regulation the activity of these bZIP factors by ABA and other environmental cues. Potential sites of phosphorylation are found in the homologous regions in the N-terminal part of these proteins. Phosphorylation and dephosphorylation are very likely to play an important role in ABA signaling as suggested by microinjection experiments (Wu et al. 1997) and the fact that ABI1 and ABI2 are PP2Cs.

In embryos, *ABI5* is apparently not completely functionally redundant since it could be recovered from the mutant screen and shows altered gene expression in candidate target genes. The relationship of ABI5 and ABI3 needs to investigated further as well. First, *abi3* mutants also show down regulation of *AtEm1* and *AtEm6* in seeds (Parcy et al. 1994). Second, TRAB1, which is homologous to ABI5, has been shown to interact with OSVP1 (ABI3 ortholog in rice) by a yeast two-hybrid assay (Hobo et al. 1999). It is, therefore, tempting to speculate that such an interaction may exist between ABI3 and ABI5. ABI5 could mediate the known ABI3 activation effect of ABRE-containing promoters by binding to ABI3 and to ABREs simultaneously, and these processes might be ABA regulated. Indeed, experiments done by Parcy et al. (Parcy et al. 1994) suggest that ABI3 activity is ABA-dependent. They have shown that embryo-specific genes (such as *AtEm1*) could be induced in vegetative tissues in an ABA-dependent manner. It will be interesting to repeat the experiment

in an *abi5-4* background to see if these gene inductions are ABI5-dependent.

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