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The genes *ABI1* and *ABI2* are involved in abscisic acid- and drought-inducible expression of the *Daucus carota* L. *Dc3* promoter in guard cells of transgenic *Arabidopsis thaliana* (L.) Heynh.

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Abstract. The *ABA INSENSITIVE1* (*ABI1*) and *ABI2* genes encode homologous type-2C protein phosphatases with redundant yet distinct functions in abscisic acid (ABA) responses. Results from Northern blot analysis showed that ABA- and mannitol-inducible expression of the *COR47* and *COR78*/*LTI78*/*RD29A* (*COR78*) genes was more impaired in the *abi2* mutant of *Arabidopsis thaliana* (L.) Heynh than in the *abi1* mutant. Furthermore, ABA-plus-mannitol treatments were additive towards *COR47* gene expression; however, the ABA-deficient *aba1* mutant showed reduced COR expression relative to the wild type in response to mannitol and ABA-plus-mannitol treatments. These results support the notion that drought- and ABA-signalling pathways are separate yet overlapping. To facilitate quantitative analysis of the genetic control of tissue-specific ABA- and desiccation-response pathways, we analyzed ABA- and mannitol-inducible expression of a carrot (*Daucus carota* L.) *Dc3* promoter:*uidA* (*β*-glucuronidase; GUS) chimaeric reporter (*Dc3-GUS*) in transgenic wild-type, ABA-deficient *aba1*, and ABA-insensitive *abi1* and *abi2* mutants. The *Dc3* promoter directed ABA- and mannitol-inducible GUS expression in *Arabidopsis* guard cells and the two treatments were additive. The *aba1*, *abi1*, and *abi2* mutant genotypes had reduced GUS expression in guard cells of cotyledons in response to mannitol, whereas *abi1* and *abi2* mutants were reduced in ABA-inducible GUS expression, consistent with overlapping ABA- and drought-response pathways. Quantitative fluorometric GUS assays of leaf extracts showed that *abi2* mutants responded less to exogenous ABA than did *abi1* mutants, and *abi2* mutants responded more to mannitol than did *abi1* mutants. We conclude that *Dc3-GUS Arabidopsis* is a tractable system in which to study tissue-specific ABA and drought signalling and suggest that *ABI2* functions predominantly over *ABI1* in *COR78* and *COR47* gene expression and guard-cell *Dc3-GUS* expression.

Key words: *Arabidopsis* – Cold-responsive gene – *Daucus* – Drought – Late-embryogenesis-abundant protein – Protein phosphatase

Introduction

The plant hormone abscisic acid (ABA) mediates a myriad of physiological processes in growth and development, including cell division, water-use efficiency, and gene expression during seed development and in response to environmental stresses such as drought, chilling, salt, pathogen attack, and UV light (Carrera and Prat 1998; Grill and Himmelbach 1998; Leung and Giraudat 1998; Albinsky et al. 1999). Despite the complex multitude of data, physiological, molecular, genetic, biochemical, and pharmacological, that implicate ABA in stress responses, the adaptive responses to ABA and stresses, and the pathways that trigger them, are largely unknown. Complex interactions have been reported for ABA-, salt- and cold-signaling pathways in *Arabidopsis* (Xiong et al. 1999). Cyclic ADP-ribose and phosphatidic acid have been shown to act as secondary messengers in ABA-regulated gene expression (Wu et al. 1997; Richie and Gilroy 1998). Genetic approaches in *Arabidopsis* have identified mutants that define overlapping signaling pathways for cold-, salt- and ABA-inducible gene expression (Foster and Chua 1999; Xiong et al. 1999) and resulted in cloning of novel genes involved in seed development and stomatal movements (Finkelstein et al. 1998; Luerssen et al. 1998; Pei et al. 1998).

The *COR* genes are *co*ld-, drought-, salt-, and ABA-*r*esponsive genes whose protein products are heat stable and hydrophyllic; some *COR* genes have structural

Abbreviations: ABA = abscisic acid; ADH = alcohol dehydrogenase; COR = cold-responsive gene; GUS = *β*-glucuronidase; LEA = late-embryogenesis-abundant; MU = methylumbelliferone; RFLP = restriction fragment length polymorphism

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similarities to the *l*ate-*e*mbryogenesis-*a*bundant (LEA) group-II and -III proteins (Dure 1993; Thomashow 1998). The *LEA* group-III homologues in wheat, maize, barley, carrot, and the resurrection plant *Craterostigma plantagineum* are induced by ABA and dehydration stress (Ingram and Bartels 1996). An ABA-inducible *LEA* group-III homologue has recently been cloned from *Arabidopsis* (Yang et al. 1997). The carrot LEA group-III *Dc3* gene is normally expressed in developing seeds and in vegetative tissues in response to drought, salt, and exogenous ABA (Seffens et al. 1990; Vivekananda et al. 1992; Thomas et al. 1997). The *cis*-acting elements for seed-specific and ABA-inducible *Dc3* gene expression have been characterized, and *Dc3* promoter-binding basic leucine-zipper proteins have been cloned (Kim and Thomas 1998; Siddiqui et al. 1998). The roles of *COR* and *LEA* genes in cold and desiccation tolerance are not yet known, but there is strong evidence that they have an adaptive function in desiccation, freezing, and osmotic tolerance (Xu et al. 1996; Steponkus et al. 1998; Swire-Clark and Marcotte 1999).

The genetic and functional relationships between *COR* and *LEA* gene expression and responses to freezing, ABA and drought have recently been elucidated with the cloning of *APETELA2*-domain-like transcription factors (*CBF* and *DREB*; Jaglo-Ottosen et al. 1998; Liu et al. 1998; Medina et al. 1999) that transactivate cold- and drought-inducible promoters. The cooperative action of at least two *cis* elements and the promoter configuration is critical for ABA-induced transcription (Ingram and Bartels 1996; Busk and Pages 1998; Leung and Giraudat 1998).

The semidominant *abi1* and *abi2* mutations are the most pleiotropic of the ABA response mutants, affecting gene expression in seeds and vegetative tissues as well as rapid stomatal movements, seed dormancy and germination, and adaptive growth (Leung et al. 1997; Grill and Himmelbach 1998; Leung and Giraudat 1998). The *ABI1* and *ABI2* genes have been cloned by map-based methods and encode homologous type-2C protein serine/threonine phosphatases (Leung et al. 1997; Rodriguez et al. 1998). Remarkably, the sole mutant alleles, *abi1-1* and *abi2-1*, are both missense mutations of a conserved glycine to aspartate that result in a dominant negative phenotype in vivo and in vitro (Leung et al. 1997; Rodriguez et al. 1998; Sheen 1998). The *ABI1* (and probably *ABI2*) gene acts as a negative regulator of ABA signaling (Sheen 1998; Gosti et al. 1999). The *ABI1* gene affects an ABA-signaling pathway differentially over a drought- or cold-signaling pathway leading to *COR* gene expression (Gilmour and Thomashow 1991; Yamaguchi-Shinozaki and Shinozaki 1993; Mäntylä et al. 1995). Conversely, de Bruxelles et al. (1996) have proposed that *ABI2* specifically controls an *ABI1*-independent drought- and ABA-response pathway leading to alcohol dehydrogenase (*ADH*) gene expression. Finkelstein (1993) found that both *abi1* and *abi2* mutants showed a slight reduction in ABA-inducible LEA group I *AtEm6* transcripts in 8-d-old seedlings. Thus, the genetic and molecular evidence indicates that *ABI1* and *ABI2* have functional redundancy, yet their activities are not entirely overlapping (Leung et al. 1997; Leung and Giraudat 1998; Pei et al. 1997, 1998; Rodriguez et al. 1998; Gosti et al. 1999).

Our interest is to elucidate by genetic means the molecular mechanisms underlying the complex network of gene products that localize, perceive, and integrate ABA and stress signals. We have analyzed ABA- and desiccation-inducible expression of a carrot (*Daucus carota* L.) *Dc3* LEA group-III promoter:*uidA* (β-glucuronidase; GUS) chimaeric reporter (*Dc3-GUS*) in transgenic wild type and in ABA-deficient *aba1*, and ABA-insensitive *abi1* and *abi2* mutants of *Arabidopsis*. Here we show that *Dc3-GUS* transgenic *Arabidopsis* is an amendable system for molecular genetic analysis of tissue-specific ABA and drought signalling. Northern blot analysis suggests that *ABI2* may have a predominant role over *ABI1* in regulation of endogenous *COR78* and *COR47* expression. Quantitative and histochemical analysis of *Dc3-GUS* expression in mutants suggests that the *ABI1* and *ABI2* genes regulate guard-cell-specific *Dc3-GUS* gene expression in response to ABA and drought.

Materials and methods

Plant materials and growth conditions. The *Arabidopsis thaliana* (L.) Heynh. genotypes used in this study were the Landsberg *erecta* wild type (CS20), and the *aba1-1* (CS21), *aba1-4*, *abi1-1* (CS22) and *abi2-1* (CS23; Koornneef et al. 1984) mutants. Numbers in parentheses refer to the Arabidopsis Biological Resource Center catalogue number (Ohio State University, Columbus, Ohio, USA). Seed of the strong allele *aba1-4* was provided by Maarten Koornneef, Department of Genetics, Wageningen Agricultural University, Netherlands. Two independent *Dc3-GUS* transformant lines, 7-2 and 11-1 in the Landsberg *erecta* background, containing the full-length 1.5-kbp *Dc3* promoter fused to the *GUS* coding region (Seffens et al. 1990; Vivekananda et al. 1992; Thomas et al. 1997), were crossed and the F2 and F3 generations screened by restriction fragment length polymorphism (RFLP) analysis to identify a doubly homozygous *Dc3-GUS* line (P-series; see below).

Arabidopsis was grown in well-drained flats (27 cm × 38 cm × 7 cm depth) and humid (80% relative humidity) conditions at low irradiance (40–75 μmol photons m^{-2} s^{-1}). These conditions favor the growth of *aba1*, *abi1* and *abi2* mutants that are wilty under normal growth conditions. The potting medium was Metro Mix 300 from Hummert International (Earth City, Mo., USA) or Plantmate from Merlinder Ltd. (No. 5, 3/F; Wah Lai Industrial Center, Shatin, Hong Kong). For axenic seedling growth, seeds were surface-sterilized and plated on minimal medium (Scholl et al. 1998) petri dishes solidified with 1.2% phytagel (Sigma, St. Louis, Mo., USA). Plates were then stored at 4 °C for 4 d in the dark to break seed dormancy and transferred to a growth chamber [23 °C; 13 h light (120 μmol photons m^{-2} s^{-1}) and 11 h darkness daily] and grown vertically for 4 d.

Genetic analysis of aba1, abi1 and abi2 recombinants. Three physiological assays were used to select recombinant lines of *aba1*, *abi1* and *abi2* mutants. A dormancy assay with freshly harvested seeds was used to screen for the *aba1* homozygous recombinants from self-fertilized F1 *aba1*/+, *Dc3-GUS* lines. F2 seeds were sterilized, plated on minimal medium and placed immediately in a growth chamber without any cold treatment; the number of seeds germinated each day was counted. At 3 d post-imbibition, the wild-type germination percentage was 2.5 ± 0.7% (SE), while that for the *aba1* mutant was 17.7 ± 3% (SE, data not shown).

A germination assay in the presence of inhibitory concentrations of ABA was used to screen for *abi1* and *abi2 Dc3-GUS* F2 recombinants. Seeds of the wild type, *abi1*, *abi2*, and F2 recombinant *Dc3-GUS* lines were sterilized and germinated on 3 μM ABA minimal plates after a cold (4 °C) treatment for 4 d in the dark to break dormancy. After 2 d in a growth chamber, the percentage of germinated seed was determined. Wild-type (P-series) germination was 2.2 ± 0.6% (SE) and that of *abi1* and *abi2* mutants 36 ± 9% (SE, data not shown).

A transpiration assay was used to screen *aba1*, *abi1* and *abi2 Dc3-GUS* recombinants for the wilty phenotype. Individual seedlings from segregating lines identified in dormancy and germination assays were transplanted from plates to potting medium and grown until three or four pairs of mature rosette leaves had expanded. A mature leaf was cut at the petiole and placed in water to achieve full turgor. The leaf was then blotted dry and placed onto an analytical balance that was connected to the serial port of a personal computer. Weight was automatically recorded every 30 s for 20 min. The transpiration rate was calculated as the negative slope of percent of initial weight versus time. Wild-type (P-series) leaves lost 18 ± 8% (SE) of fresh weight after 20 min; *aba1*, *abi1* and *abi2* homozygous mutants lost 44 ± 10% (SE; Rock and Ng 1999).

Recombinant lines that bred true for three generations as measured by high transpiration rates, high germination in the dormancy assay (for the *aba1* lines), and high germination on 3 μM ABA (for *abi1* and *abi2* lines) were concluded to be homozygous for the *aba1*, *abi1* and *abi2* mutations. These lines were further characterized for their *Dc3-GUS* insertion genotypes by RFLP analysis. The *abi1* and *abi2* recombinant lines were also confirmed as homozygous by the cleaved amplified polymorphic sequence assays described in Leung et al. (1997).

Southern and Northern analyses. It was considered important to use isogenic lines when quantifying the relative effects of *aba1*, *abi1* and *abi2* mutations on *Dc3-GUS* reporter gene activity. Therefore, RFLP analysis corresponding to the two *Dc3-GUS* reporter genes in the P-series wild-type line (7-2 and 11-1) was performed on the recombinant lines homozygous for *aba1*, *abi1* and *abi2* alleles. Manipulation of nucleic acids was by standard methods (Ausubel et al. 1994). Genomic DNA from individual segregant plants was prepared as described by Li and Chory (1998). The diagnostic *Hind*III RFLPs of 5.5 and 4.1 kbp for the 11-1 and 7-2 *Dc3-GUS* insertions, respectively, were confirmed in all of at least 16 individuals from the homozygous *aba1* and *abi2* lines. The *abi1* line used in the fluorometric assay was heterozygous for the *Dc3-GUS* 11-1 insertion (data not shown). Southern and Northern analysis was by chemiluminescence labeling and detection (Gene Images, Amersham, Buckinghamshire, UK) according to the supplier's protocol. Autoradiography was with pre-flashed X-ray film (Ausubel et al. 1994) in order to perform densitometry. The GUS coding region used as a probe of Southern blots was a 2.0-kbp *Nco*I-*Eco*RI fragment of the plasmid pBM207 (Hill et al. 1996). Plasmid pHH7.2 (Gilmour and Thomashow 1991) was digested with *Eco*RI to yield a 1.1-kbp fragment containing the *COR47* cDNA. Plasmid pRD29A (Yamaguchi-Shinozaki and Shinozaki 1993) was digested with *Bam*HI to give a 2.4-kbp fragment containing the entire coding sequence of *COR78* (*RD29A*) cDNA.

For Northern blot experiments, approximately 20 whole plants per sample of 4- to 5-week-old wild type (P-series), *aba1*, *abi1* and *abi2* genotype were used. For the ABA induction experiments, a solution of 100 μM ABA was sprayed on the plants as described by Yamaguchi-Shinozaki and Shinozaki (1993) and samples were frozen in liquid nitrogen after 6 h and stored at −70 °C until extraction of RNA. For the drought-stress-simulation [15% (w/v) D-mannitol (Sigma)] and synergy experiments [100 μM ABA plus 15% (w/v) mannitol], whole plants were floated on water (control) or given solutions for 6 h before freezing. Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Ausubel et al. 1994).

β-Glucuronidase assays. Histochemical and quantitative GUS analysis was as described by Jefferson (1987). Expanded 2nd and 3rd leaves from 3-week-old plants (15 per sample) were collected and floated in petri dishes with various treatments for 24 h in a growth chamber. Solutions of *cis*-(±) ABA (Sigma) were diluted from 100 mM stock solutions prepared in 90% ethanol; equivalent volumes of 90% ethanol were included in all treatments. After treatments, leaves were developed for 24 h with a 1 mM solution of indigogenic GUS substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Rose Scientific, Edmonton, Alberta, Canada) in 50 mM KH_2PO_4 (pH 7.0), 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.05% sodium azide, 0.1% Triton X-100. After staining, chlorophyll was photobleached from leaves by immersing tissue in 70% ethanol and placing under a fluorescent lamp (4×10^5 μmol photons m^{-2} s^{-1}) overnight. Plant tissue was mounted in 30% glycerol on microscope slides and photographed through a differential interference contrast microscope (Axiophot; Carl Zeiss, Oberkochen, Germany).

For quantitative GUS assays, 4-methylumbelliferone (4-MU) produced from the glucuronide precursor (4-methylumbelliferyl-β-D-glucuronide; Rose Scientific) was determined by a standard protocol (Jefferson 1987). The 4-MU fluorescence was measured with a Cytofluor II microplate spectrofluorometer (Molecular Dynamics, Sunnyvale, Calif., USA). Protein concentrations of plant extracts were determined by the dye-binding method (Bio-Rad Protein Assay; Bio-Rad, Hercules, Calif., USA). The GUS activity was calculated in units of nmol 4-MU produced $(\mu g \text{ protein})^{-1}$ h^{-1}.

Results

Previous work showed that *ABI1* and *ABI2* are involved in *COR* expression in response to ABA, but not to desiccation (for *COR78* in *abi1* mutants) or cold stress (Gilmour and Thomashow 1991; Yamaguchi-Shinozaki and Shinozaki 1993; Gosti et al. 1995; Mäntylä et al. 1995), suggesting the existence of ABA-independent stress-response pathways. In order to analyze the interaction of ABA- and desiccation-response pathways, we utilized 15% (w/v) mannitol [a non-penetrating osmolyte that mimics drought stress by inducing plasmolysis and ABA synthesis (Creelman and Zeevaart 1985)] as a medium for induction of gene expression alone or in combination with 100 μM ABA. Northern blot analysis showed that *COR78* and *COR47* expression in response to mannitol was lower in *aba1* and *abi2* mutant plants compared to the wild type, while *abi1* mutant plants showed only a slight reduction in *COR* transcripts relative to the wild type (Fig. 1C). Densitometry of Northern bands indicated the *aba1* and *abi2* mutations reduced expression of *COR78* and *COR47* in response to mannitol approximately 60–90% relative to the wild type, while the *abi1* mutation reduced *COR78* and *COR47* gene expression 60% and 30%, respectively (data not shown). In response to exogenous sprayed ABA, *aba1* and *abi1* mutants responded by accumulating *COR78* and *COR47* to almost wild-type levels (Fig. 1B), whereas *abi2* mutants did not induce *COR* gene expression. In synergy experiments with 100 μM ABA plus 15% mannitol, *COR78* had slightly elevated mRNA levels in *aba1* and *abi2* mutants, and *COR47* expression was higher in all genotypes relative to either ABA or mannitol treatments alone (Fig. 1D). Densitometry of *COR78* and *COR47* bands indicated that

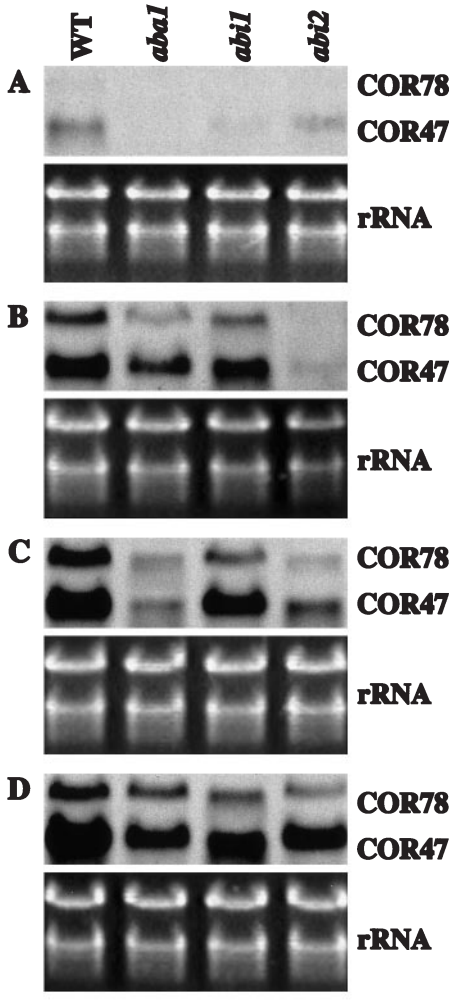

Fig. 1A–D. Northern blot analysis of *COR78* and *COR47* expression in wild-type (*WT*) *Arabidopsis* and in, *aba1*, *abi1* and *abi2* mutants in response to ABA, mannitol, or both. Whole plants were uprooted and treated for 10 h with either water (**A**), or sprayed with 100 μM ABA (**B**), 15% mannitol (**C**), or 100 μM ABA plus 15% mannitol (**D**). Lower panels show the ethidium bromide-stained formaldehyde-agarose gel of total RNA (10 μg)

treatment with ABA plus mannitol resulted in a 1.2- to 3.6-fold increase in *COR* transcript abundance relative to ABA treatment alone (data not shown).

To elucidate the genetic control of tissue-specific ABA- and desiccation-response pathways, the *abi1*, *abi2* and *aba1* mutant alleles were crossed into the isogenic wild-type *Dc3-GUS* genetic background and ABA- and mannitol-inducible *Dc3-GUS* expression was analyzed in homozygous *aba1*, *abi1* and *abi2* recombinant lines. The genetic approach of crossing the identical *Dc3-GUS* insertion lines into all genotypes effectively ruled out expression artifacts due to positional effects of random T-DNA insertions. No evidence of transgene silencing was ever seen in any of the *Dc3-GUS* lines (data not shown). Figure 2 shows guard-cell- and vascular-tissue-specific GUS staining in 4-d-old wild-type, *aba1*, *abi1*, and *abi2* mutant cotyledons treated with water (control), 100 μM ABA, 15% (w/v) mannitol, or ABA plus mannitol for 24 h. In control (water) treatments, there were trace levels of GUS expression in vascular tissues of wild-type, *abi1* and *abi2* cotyledons (Fig. 2A–D). Variability of vascular tissue GUS staining was observed in water-treated controls of each genotype (data not shown). Guard-cell-specific *Dc3-GUS* expression was observed in wild-type cotyledons in response to ABA, mannitol (to a lesser extent), and ABA-plus-mannitol treatments (Fig. 2E,I,M). Similar results were observed in young true leaves and the magnitude of the response decreased with leaf age (data not shown). Vascular tissue and guard-cell GUS staining was relatively enhanced in ABA-plus-mannitol treatments compared to either treatment alone (Fig. 2).

There was a slight reduction in the intensity of guard-cell staining between the wild type and *aba1* mutants treated with 100 μM ABA (compare Fig. 2E with 2F), and a stronger reduction in guard-cell staining in *abi1* and *abi2* versus the wild type (Fig. 2G and 2H versus 2E). In response to mannitol-induced desiccation, *aba1*, *abi1* and *abi2* mutant leaves all showed reduced guard-cell staining compared to the wild type (Fig. 2J,2K and 2L versus 2I). The *abi1* and *abi2* mutants showed attenuated *Dc3-GUS* expression (relative to wild type and *aba1*) in guard cells in response to mannitol plus ABA (Fig. 2O and 2P versus 2M or 2N), but the treatment appeared to give a slightly additive effect in *abi1* and *abi2* mutants compared with either treatment alone (Fig. 2O and 2P versus 2G/2K or 2H/2L, respectively).

In order to quantify the tissue-specific effects of the *aba1*, *abi1* and *abi2* genotypes on ABA- and drought-inducible *Dc3-GUS* expression, fluorometric assays of GUS activities in extracts of 3-week-old leaves were performed. Figure 3 shows the results of an ABA dose/*Dc3-GUS* response curve for the wild type and for the *aba1*, *abi1* and *abi2* recombinant lines. As observed by histology of cotyledons and leaves, the wild-type and *aba1* leaves responded to exogenous ABA as expected. The apparent saturation concentration of ABA for maximal *Dc3-GUS* expression in the wild type or *aba1* was about 100 μM, as assumed in the experiments on endogenous gene expression (Fig. 1). Consistent with the histology results on cotyledons, *abi1* and *abi2* leaves were significantly reduced in their response to exogenous ABA. The *Dc3-GUS* expression by *abi2* mutants relative to *abi1* was significantly lower at ABA concentrations of 100 μM and 320 μM, with *abi2* tissues responding to ABA at about 50% of the *abi1* response (Fig. 3). This result is consistent with the reduced *COR* expression observed in *abi2* in Northern blot analysis (Fig. 1B). Because of the limitations in solubility of ABA, it was not possible to determine if a maximal *Dc3-GUS* response could be achieved by the *abi* mutants at higher ABA concentrations.

In order to quantify the contribution of *ABA1*, *ABI1* and *ABI2* genes to ABA- and drought-signaling pathways, fluorometric GUS assays were performed on *Dc3-GUS* mutant leaves treated for 24 h with water (control), a saturating dose (100 μM) of ABA, mannitol (15% w/v), or ABA plus mannitol. The results are shown in

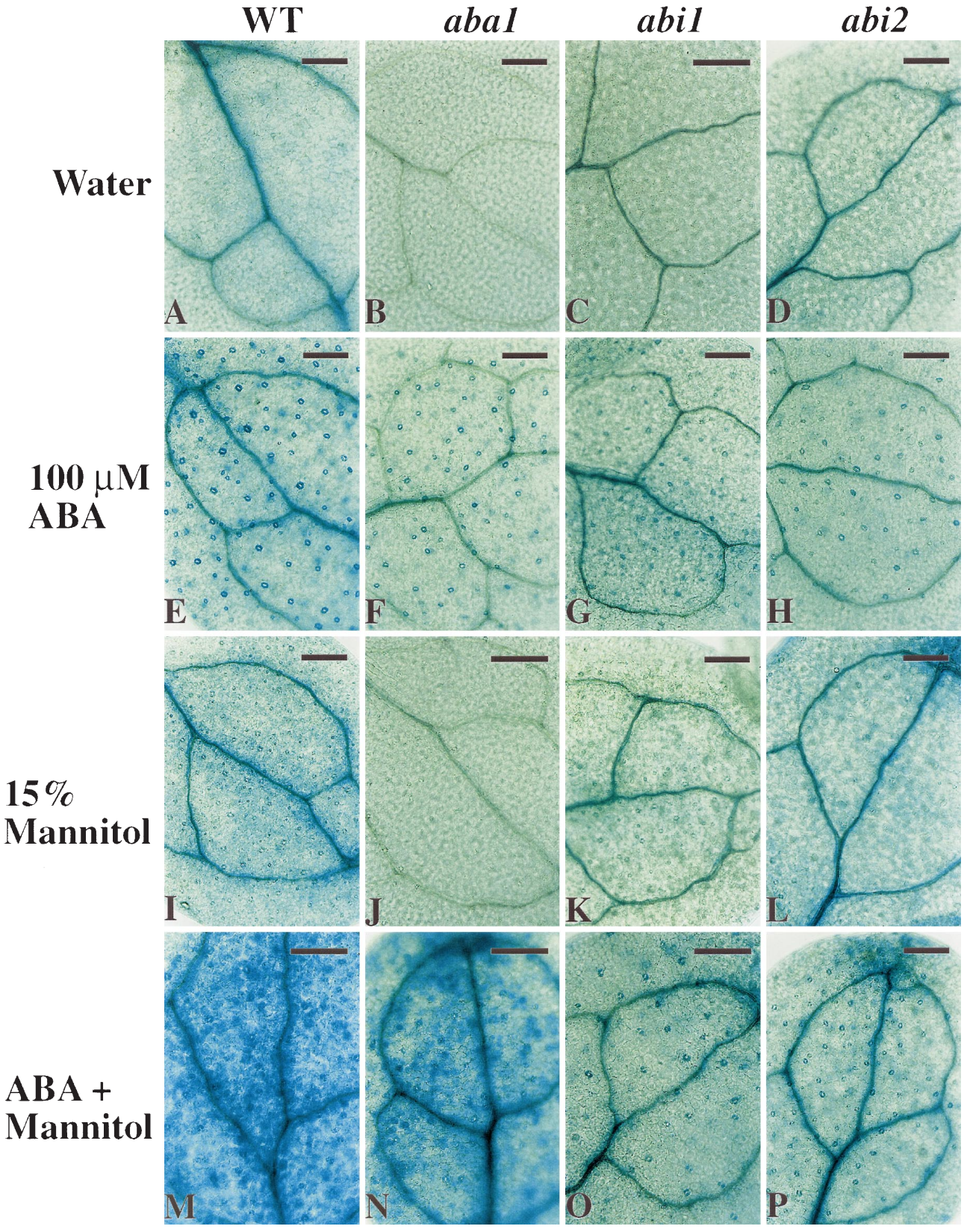

Fig. 2A–P. Guard-cell-specific expression of *Dc3-GUS* in cotyledons of 4-d-old wild-type (*WT*), *aba1*, *abi1* and *abi2 Arabidopsis* seedlings treated with water (**A–D**), 100 µM ABA (**E–H**), 15% mannitol (**I–L**), or ABA plus mannitol (**M–P**) for 24 h and visualized by X-Gluc staining. Results are representative of 8 replicates. Bar = 160 µm

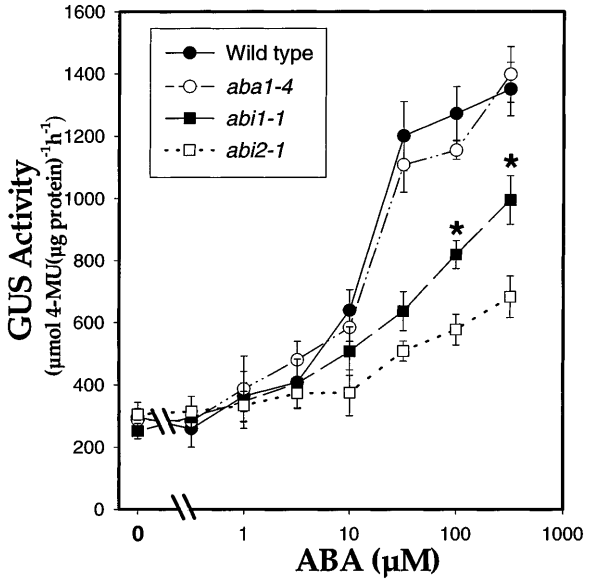

Fig. 3. Abscisic acid dose-response of *Dc3-GUS* by 3-week-old leaves of wild-type *Arabidopsis*, and *aba1*, *abi1* and *abi2* mutants treated 16 h. Results are the average of 3–7 replicates, ±SE. Asterisk (*) denotes a significant difference between *abi1* and *abi2*, $P < 0.02$ (Student's two sided *t*-test, equal variance assumed)

Fig. 4. In young wild-type leaves, GUS expression driven by the *Dc3* promoter was induced to a similar extent (over 4-fold) by a saturating dose of ABA or 15% (w/v) mannitol. Consistent with the histology results in cotyledons, *aba1* mutant leaves showed a statistically significant 25% reduction in GUS expression in response to mannitol, but not ABA, compared with the wild type (Fig. 4). Expression of GUS in *abi1* and *abi2* recombinants was lower than in the wild type following mannitol and/or ABA treatments. Consistent with the *COR* Northern results (Fig. 1B), the *abi2* mutant was significantly more impaired than *abi1* (about 45%) in ABA-inducible *Dc3-GUS* expression (Fig. 4). However, *abi1* was more impaired than *abi2* (about 35%) in drought-inducible expression. An additive effect of ABA-plus-mannitol treatments was observed in all genotypes (Fig. 4).

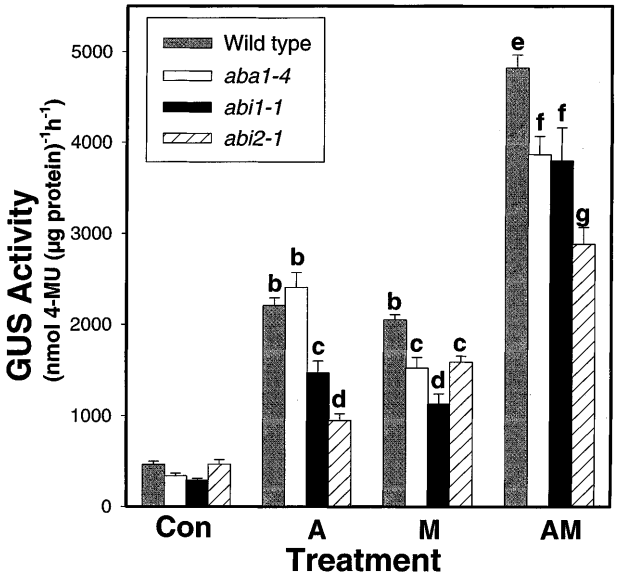

Fig. 4. Additive effects of ABA and mannitol on *Dc3-GUS* expression in leaves of wild-type *Arabidopsis*, and in *aba1*, *abi1* and *abi2* mutants. Three or more leaves per sample were floated for 24 h on water (*Con*), 100 µM ABA (*A*), 15% mannitol (*M*), or 100 µM ABA plus 15% mannitol (*AM*). Results are the average of 4–9 replicates, ±SE. A different letter denotes a significant difference among genotypes, $P < 0.04$ (Student's two sided *t*-test, equal variance assumed)

Discussion

Because the *aba1* mutant is impaired in ABA biosynthesis (Rock and Zeevaart 1991), our results showing reduced mannitol-induced marker gene expression in the *aba1* mutant demonstrate that ABA is partially involved in drought-inducible *COR*- and guard-cell-specific *Dc3* gene expression. Furthermore, the additive effects of ABA plus mannitol on *COR* gene expression relative to ABA alone (Fig. 1D versus 1B) and *Dc3-GUS* expression (Fig. 4) support the existence of separate yet overlapping ABA- and drought-signaling pathways (Yamaguchi-Shinozaki and Shinozaki 1993; Gosti et al. 1995; Mäntylä et al. 1995). Quantitative differences between our Northern results and those of others may be related to tissue-specific expression of *COR* genes or environmental conditions. Taken together, the reduced additive effects of ABA and mannitol on *Dc3-GUS* and *COR* expression in *aba1*, *abi1* and *abi2* mutants establishes that ABA biosynthesis and the *ABI1* and *ABI2* gene products are involved in overlapping drought- and ABA-response pathways. Similar additive effects have been reported for independent salt- and ABA-response pathways leading to *COR78*/*RD29A* transcript accumulation (Xiong et al. 1999).

Our results with *COR78* and *COR47* (Fig. 1) and *Dc3-GUS* (Figs. 3, 4) distinguish *ABI2* function from that of *ABI1*, which has a minimal effect on drought-inducible *COR78* expression (Yamaguchi-Shinozaki and Shinozaki 1993; Mäntylä et al. 1995). The histology studies of *Dc3-GUS* expression suggest that, in guard cells, *ABI1* and *ABI2* are important for ABA and drought signaling to the nucleus as well as for ion channel activities and stomatal movements (Pei et al. 1997, 1998). The histology and quantitative experiments reported here were performed on different tissues (cotyledons versus leaves) and differences in tissue sensitivity and/or specificity of ABA and *ABI1*/*ABI2* activities cannot be ruled out. Taken together, our data suggest the *ABI1* and *ABI2* genes may act differentially on ABA- and drought-response pathways, with *ABI2* acting primarily on ABA signaling, and *ABI1* and *ABI2* distinctly (and/or redundantly?) affecting drought signaling in guard cells and possibly other target tissues. There is circumstantial evidence in the literature consistent with this model: (i) the ABA- and drought-inducible *Athb-7*, *AtDi21* and *RAB18*/*AtDi8* transcripts are expressed in *abi2* but not *abi1* mutant plants in response

to drought (Gosti et al. 1995; Söderman et al. 1996); (ii) *ABI1*, but not *ABI2*, has been implicated in drought rhizogenesis, a morphogenetic adaptive response of drought-stressed roots (Vartanian et al. 1994); (iii) Leung et al. (1997) showed that *ABI2* transcript accumulation is more severely impaired than that of *ABI1* in the ABA-deficient *aba1* mutant, consistent with autoregulation of *ABI2* expression by ABA (Gosti et al. 1999). De Bruxelles et al. (1996) concluded that *ABI2*, but not *ABI1*, primarily controls drought- and ABA-induction of the *ADH* gene in roots. In contrast, Jarillo et al. (1993) concluded that *ABI1*, not *ABI2*, controls cold-, ABA- and, to a lesser extent, drought-induced expression of the same *ADH* gene in leaves. *ABI1* and *ABI2* are expressed in all tissues thus far examined (Leung et al. 1997). Taken together, these diverse gene expression patterns suggest the existence of tissue-specific regulatory factors that modulate ABI1 and ABI2 activities and *COR* gene expression.

Induction of the *Dc3-GUS* transgene in *Arabidopsis* by ABA and drought stress is similar to that in carrot and tobacco (Seffens et al. 1990; Vivekananda et al. 1992; Siddiqui et al. 1998). Expression of *Dc3-GUS* in *Arabidopsis* guard cells is subject to the action of the *ABA1*, *ABI1* and *ABI2* genes. Other ABA-regulated and ABA-signaling genes are expressed in guard cells of *Arabidopsis*, pea, and broad bean (Shen et al. 1995; Taylor et al. 1995; Hey et al. 1997; Pei et al. 1998). Therefore, it is likely that *Dc3* promoter activity in *Arabidopsis* reflects interaction with conserved, physiologically important overlapping ABA- and stress-signaling pathways. We conclude that transgenic *Dc3-GUS Arabidopsis* is a tractable system in which to study tissue-specific ABA- and stress-inducible gene expression.

A conservative model of ABA signaling is that there is a single response pathway in plants, but different elements are rate-limiting in different species or in the same species in different tissues. Evidence in support of this model can be drawn from diverse experiments: (i) *cis*- and *trans*-controlling elements of ABA responses function in species as divergent as mosses and angiosperms (Yamaguchi-Shinozaki and Shinozaki 1993; Vilardell et al. 1994; Knight et al. 1995; Hill et al. 1996; Carrera and Prat 1998; Sheen 1998); (ii) ectopic expression of *ABI3* in vegetative tissues of *Arabidopsis* potentiates ABA responses (Parcy and Giraudat 1997); (iii) cyclic ADP ribose and protein phosphorylation/dephosphorylation regulate both gene expression and guard-cell movements (Hey et al. 1997; Wu et al. 1997; Leckie et al. 1998). However, there is evidence against this model, since some ABA- and stress-inducible sequences are expressed differently in different species (Vilardell et al. 1994; Taylor et al. 1995). Restoration of ABA responses by kinase inhibitors in *abi1*-expressing guard cells requires ABA (Armstrong et al. 1995; Pei et al. 1997), which suggests there are parallel *ABI1*-dependent and independent branches of ABA responses.

It has been proposed that overlapping combinations of regulatory proteins co-opted by *ABI1* and *ABI2* phosphatases would present a versatile system highly responsive to subtle changes in environmental and cellular signals (Leung et al. 1997; Rodriguez 1998). Differential cellular localization and/or substrate specificities of the *ABI1* and *ABI2* phosphatases or other factors may integrate environmental (e.g. drought) and cellular (e.g. ABA) signals, respectively. The *DREB2A* transcription factor has a conserved serine/threonine-rich region adjacent to the DNA-binding domain (Liu et al. 1998) and it is possible that protein kinases and/or phosphatases such as ABI1 and ABI2 may act in environmental or cellular response modules that include targets such as DREBs. Consistent with this notion is the fact that expression of *DREB2A*, *ABI1* and *ABI2* is induced by dehydration (Leung et al. 1997; Liu et al. 1998). We are currently taking a genetic approach to identify upstream effectors and downstream targets of *ABI1* and *ABI2* with a tissue-specific mutant screen for ectopic expression of *Dc3-GUS*. One advantage of such a screen is that it can identify mutations which otherwise have no visible phenotype, which may be the case in redundant genetic systems such as ABA and drought signaling in *Arabidopsis*.

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References

Albinsky D, Masson JE, Bogucki A, Afsar K, Vass I, Nagy F, Paszkowski J (1999) Plant responses to genotoxic stress are linked to an ABA/salinity signaling pathway. Plant J 17: 73–82

Armstrong F, Leung J, Grabov A, Brearley J, Giraudat J, Blatt MR (1995) Sensitivity to abscisic acid of guard-cell K^+ channels is suppressed by *abi1-1*, a mutant *Arabidopsis* gene encoding a putative protein phosphatase. Proc Natl Acad Sci USA 92: 9520–9524

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1994) Current protocols in molecular biology. Wiley, New York

Busk PK, Pages M (1998) Regulation of abscisic acid-induced transcription. Plant Mol Biol 37: 425–435

Carrera E, Prat S (1998) Expression of the *Arabidopsis Abi1-1* mutant allele inhibits proteinase inhibitor wound induction in tomato. Plant J 15: 765–771

Creelman RA, Zeevaart JAD (1985) Abscisic acid accumulation in spinach leaf slices in the presence of penetrating and nonpenetrating solutes. Plant Physiol 77: 25–28

de Bruxelles GL, Peacock WJ, Dennis ES, Dolferus R (1996) Abscisic acid induces the alcohol dehydrogenase gene in *Arabidopsis*. Plant Physiol 111: 381–391

Dure L III (1993) A repeating 11-mer amino acid motif and plant desiccation. Plant J 3: 363–369

Finkelstein RR (1993) Abscisic acid-insensitive mutations provide evidence for stage-specific signal pathways regulating expression of an *Arabidopsis* late embryogenesis-abundant (*lea*) gene. Mol Gen Genet 238: 401–408

Finkelstein RR, Wang ML, Lynch TJ, Rao S, Goodman HM (1998) The *Arabidopsis* abscisic acid response locus *ABI4* encodes an APETALA 2 domain protein. Plant Cell 10: 1043–1054

Foster R, Chua N-H (1999) An *Arabidopsis* mutant with deregulated ABA gene expression: implications for negative regulator function. Plant J 17: 363–372

Gilmour SJ, Thomashow MF (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol Biol 17: 1233–1240

Gosti F, Bertauche N, Vartanian N, Giraudat J (1995) Abscisic acid-dependent and-independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*. Mol Gen Genet 246: 10–18

Gosti F, Beaudoin N, Serizet C, Webb AAR, Vartanian N, Giraudat J (1999) The *ABI1* protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell 11: 1897–1909

Grill E, Himmelbach A (1998) ABA signal transduction. Curr Opin Plant Biol 1: 412–418

Hey SJ, Bacon A, Burnett E, Neill SJ (1997) Abscisic acid signal transduction in epidermal cells of *Pisum sativum* L. Argenteum: both dehydrin mRNA accumulation and stomatal responses require protein phosphorylation and dephosphorylation. Planta 202: 85–92

Hill A, Nantel A, Rock CD, Quatrano RS (1996) A conserved domain of the *viviparous-1* gene product enhances the DNA binding activity of the bZIP protein EmBP-1 and other transcription factors. J Biol Chem 271: 3366–3374

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 377–403

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) Arabidopsis *CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science 280: 104–106

Jarillo JA, Leyva A, Salinas J, Martinez-Zapater JM (1993) Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidopsis thaliana*, a chilling-tolerant plant. Plant Physiol 101: 833–837

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5: 387–405

Kim SY, Thomas TL (1998) A family of novel basic leucine zipper proteins binds to seed-specification elements in the carrot *Dc3* gene promoter. J Plant Physiol 152: 607–613

Knight CD, Sehgal A, Atwal K, Wallace JC, Cove DJ, Coates D, Quatrano RS, Bahadur S, Stockley PG, Cuming AC (1995) Molecular responses to abscisic acid and stress are conserved between moss and cereals. Plant Cell 7: 499–506

Koornneef M, Karssen CM, Reuling G (1984) The isolation and characterization of abscisic acid insensitive mutants of *Arabidopsis thaliana*. Physiol Plant 61: 377–383

Leckie CP, McAinsh MR, Allen GJ, Sanders D, Hetherington AM (1998) Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose. Proc Natl Acad Sci USA 95: 15837–15842

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Biol 49: 199–222

Leung J, Merlot S, Giraudat J (1997) The *Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2)* and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell 9: 759–771

Li J, Chory J (1998) Preparation of DNA from *Arabidopsis*. In: Martínez-Zapater JM, Salinas J (eds) Arabidopsis protocols. Humana, Totowa New Jersey, pp 55–60

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10: 1391–1406

Luerssen K, Kirik V, Herrmann P, Misera S (1998) *Fusca3* encodes a protein with a conserved *Vp1*/*Abi3*-like B3 domain which is of functional importance for the regulation of seed maturation in *Arabidopsis thaliana*. Plant J 15: 755–764

Mäntylä E, Lang V, Palva ET (1995) Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of *LT178* and *RAB18* proteins in *Arabidopsis thaliana*. Plant Physiol 107: 141–148

Medina J, Bargues M, Terol J, Pérez-Alonso M, Salinas J (1999) The *Arabidopsis CBF* gene family is composed of three genes encoding *AP2* domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiol 119: 463–470

Parcy F, Giraudat J (1997) Interactions between the *ABI1* and the ectopically expressed *ABI3* genes in controlling abscisic acid responses in *Arabidopsis* vegetative tissues. Plant J 11: 693–702

Pei ZM, Ghassemian M, Kwak CM, McCourt P, Schroeder JI (1998) Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science 282: 287–290

Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and *abi1* and *abi2* mutants. Plant Cell 9: 409–423

Richie SM, Gilroy S (1998) Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. Proc Natl Acad Sci USA 95: 2697–2702

Rock CD, Ng PPF (1999) Dominant Wilty mutants of *Zea mays* (Poaceae) are not impaired in abscisic acid perception or metabolism. Am J Bot 86: 1796–1800

Rock CD, Zeevaart JAD (1991) The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci USA 88: 7496–7499

Rodriguez PL (1998) Protein phosphatase 2C (PP2C) function in higher plants. Plant Mol Biol 38: 919–927

Rodriguez PL, Benning G, Grill E (1998) *ABI2*, a second protein phosphatase 2C involved in abscisic acid signal transduction in *Arabidopsis*. FEBS Lett 421: 185–190

Scholl R, Rivero-Lepinckas L, Crist D (1998) Growth of plants and preservation of seeds. In: Martínez-Zapater JM, Salinas J (eds) Arabidopsis protocols. Humana, Totowa New Jersey, pp 1–12

Seffens WS, Almoguera C, Wilde HD, Haar RAV, Thomas TL (1990) Molecular analysis of a phylogenetically conserved carrot gene: developmental and environmental regulation. Dev Genet 11: 65–76

Sheen J (1998) Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. Proc Natl Acad Sci USA 95: 975–980

Shen LM, Outlaw WH, Epstein LM (1995) Expression of an mRNA with sequence similarity to pea dehydrin (Psdhn-1) in guard cells of *Vicia faba* in response to exogenous abscisic acid. Physiol Plant 95: 99–105

Siddiqui NU, Chung H-J, Thomas TL, Drew MC (1998) Abscisic acid-dependent and -independent expression of the carrot late-embryogenesis-abundant class gene *Dc3* in transgenic tobacco seedlings. Plant Physiol 118: 1181–1190

Söderman E, Mattsson J, Engström P (1996) The *Arabidopsis* homeobox gene *ATHB-7* is induced by water deficit and by abscisic acid. Plant J 10: 375–381

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the *COR15a* gene on the freezing tolerance of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 95: 14570–14575

Swire-Clark GA, Marcotte WR Jr (1999) The wheat LEA protein Em functions as an osmoprotective molecule in *Saccharomyces cerevisiae*. Plant Mol Biol 39: 117–128

Taylor JE, Renwick KF, Webb AA, McAinsh MR, Furini A, Bartels D, Quatrano RS, Marcotte WR Jr, Hetherington AM (1995) ABA-regulated promoter activity in stomatal guard cells. Plant J 7: 129–134

Thomas T, Chung HJ, Nunberg AN (1997) ABA signalling in plant development and growth. In: Aducci P (ed) Signal transduction in plants. Birkhauser, Basel, pp 23–43

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol 118: 1–7

Vartanian N, Marcotte L, Giraudat J (1994) Drought rhizogenesis in *Arabidopsis thaliana*: differential responses of hormonal mutants. Plant Physiol 104: 761–767

Vilardell J, Martinez-Zapater JM, Goday A, Arenas C, Pages M (1994) Regulataion of the *rab*17 gene promoter in transgenic Arabidopsis wild-type, ABA-deficient and ABA-insensitive mutants. Plant Mol Biol 24: 561–569

Vivekananda J, Drew MC, Thomas TL (1992) Hormonal and environmental regulation of the carrot *lea*-class gene *Dc3*. Plant Physiol 100: 576–581

Wu Y, Kuzma J, Maréchal E, Graeff R, Lee HC, Foster R, Chua N-H (1997) Abscisic acid signaling through cyclic ADP-ribose in plants. Science 278: 2126–2130

Xiong L, Ishitani M, Zhu J-K (1999) Interaction of osmotic stress, temperature, and abscisic acid in the regulation of gene expression in *Arabidopsis*. Plant Physiol 119: 205–211

Xu D, Duan X, Wang B, Hong B, Ho T-HD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol 110: 249–257

Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol Gen Genet 236: 331–340

Yang H, Saitou T, Komeda Y, Harada H, Kamada H (1997) *Arabidopsis thaliana ECP63* encoding a LEA protein is located in chromosome 4. Gene 184: 83–88