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ORIGINAL ARTICLE

Sequence and functional analyses of the *aldehyde dehydrogenase 7B4 gene* promoter in *Arabidopsis thaliana* and selected Brassicaceae: regulation patterns in response to wounding and osmotic stress

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Abstract
Key message **The core promoter of the antiquitin ALDH7B4 gene was compared between selected Brassicaceae. Conserved *cis* elements controlling osmotic stress and wound-induced expression were identified and analysed in *Arabidopsis thaliana* leaves and seeds.**
Abstract Aldehyde dehydrogenases metabolise a wide range of aliphatic and aromatic aldehydes, which become cytotoxic at high levels. Family 7 aldehyde dehydrogenase genes, often described as antiquitins or turgor-responsive genes in plants, are broadly conserved across all domains. Despite the high conservation of the plant ALDH7 proteins and their importance in stress responses, their regulation has not been investigated. Here, we compared *ALDH7* genes of different Brassicaceae and found that, in contrast to the gene organisation and protein coding sequences, similarities in the promoter sequences were limited to the first few hundred nucleotides upstream of the translation start codon. The function of this region was studied by isolating the core promoter of the *Arabidopsis thaliana ALDH7B4* gene, taken as model. The promoter was found to be responsive to wounding in addition to salt and dehydration stress. *Cis*-acting elements involved in stress responsiveness were analysed and two conserved ACGT-containing motifs proximal to the translation start codon were found to be essential for the responsiveness to osmotic stress in leaves and in seeds. The integrity of an upstream ACGT motif and a dehydration-responsive element/C-repeat—low temperature-responsive element was found to be necessary for *ALDH7B4* expression in seeds and induction by salt, dehydration and ABA in leaves. The comparison of the gene expression in selected *Arabidopsis* mutants demonstrated that osmotic stress-induced *ALDH7B4* expression in leaves and seeds involves both ABA- and lipid-signalling components.

Keywords ACGT motif · Aldehyde dehydrogenase · Antiquitin · DRE/CRT motif · Osmotic stress · Turgor-responsive gene · Wounding stress

Abbreviations

ALDH	Aldehyde dehydrogenase
FAST	Fast *Agrobacterium*-mediated seedling transformation
ABA	Abscisic acid
ABRE	ABA-responsive element
DRE/CRT	Dehydration-responsive element/C-repeat—low temperature-responsive element

Introduction

Aldehyde dehydrogenases (ALDHs) constitute the protein superfamily of $NAD(P)^+$-dependent enzymes (ALDH, EC 1.2.1.3) that metabolise a wide range of aliphatic and aromatic aldehydes, which are toxic, if their levels are not regulated. A steadily increasing number of ALDHs has been

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identified in almost all taxa (Sophos and Vasiliou 2003; Brocker et al. 2013). The *Arabidopsis* genome contains 16 members distributed over ten protein families (Kirch et al. 2004; Stiti et al. 2011). ALDH7B4 belongs to family 7 of the ALDH superfamily. Members of the ALDH7 protein family (EC 1.2.1.31) are also known as δ1-piperideine-6-carboxylate dehydrogenases, α-aminoadipic semialdehyde dehydrogenases or antiquitins, which reflects their evolutionary "antique" nature (Lee et al. 1994). The ALDH7 proteins are highly conserved in living organisms. A comparison of the amino acid sequences of plant and animal ALDH7 shows about 60 % sequence identity. The high degree of conservation between evolutionarily distant species implies that physiological functions are also conserved. The mammalian orthologous protein, ALDH7A1, plays a role in lysine catabolism by converting aminoadipic acid-δ-semialdehyde to its corresponding carboxylic acid α-aminoadipic acid using NAD^+ (Brocker et al. 2010). Mutations in *ALDH7A1* are the reasons for both pyridoxine-dependent epilepsy and folic acid-responsive seizures (Mills et al. 2006; Gallagher et al. 2009). Both disorders often result in status epilepticus and death, if left untreated. In pyridoxine-dependent epilepsy *ALDH7A1* mutations lead to α-aminoadipic semialdehyde accumulation in the body fluids (Mills et al. 2006). A condensation product is formed between α-aminoadipic semialdehyde and pyridoxal-5′-phosphate and causes cofactor inactivation and acute vitamin B6 depletion. Increased accumulation of α-aminoadipic semialdehyde in the brain of pyridoxine-dependent epilepsy patients was proposed to contribute to cerebral atrophy due to the toxicity of this aldehyde (Mills et al. 2006). In addition, Brocker et al. (2010) showed that human ALDH7A1 protects against hyperosmotic stress by generating osmolytes and metabolising toxic aldehydes. Purified recombinant ALDH7A1 proteins efficiently metabolised a number of aldehyde substrates, including the osmolyte precursor, betaine aldehyde, lipid peroxidation-derived aldehydes, and the intermediate lysine degradation product, α-aminoadipic semialdehyde. The ALDH7A1 protein was found in the cytosol, nucleus, and mitochondria.

Previous studies revealed essential functions of plant ALDHs in growth, development, and stress adaptation. The garden pea (*Pisum sativum*) turgor-responsive protein 26 g (now referred as ALDH7B1) was the first plant ALDH7 protein to be discovered and was expressed upon dehydration, low temperature, heat shock and ABA (Guerrero et al. 1990). An increase of the *ALDH7* gene transcripts was reported under osmotic stress in canola (*Brassica napus*) (Stroeher et al. 1995). In *Arabidopsis*, the ALDH7B4 protein showed a strong induction upon osmotic stress and ABA (Kirch et al. 2005; Stiti et al. 2011). Overexpression of ALDH7B4 in *Arabidopsis thaliana* has conferred osmotic and oxidative stress tolerance to transgenic plants (Kotchoni et al. 2006). T-DNA insertion mutants of *ALDH7B4* are more sensitive to NaCl and dehydration treatments (Kotchoni et al. 2006). Our results from transgenic tobacco (*Nicotiana tabacum*) plants overexpressing ALDH7B4 further confirmed the importance of the ALDH7B4 protein in plant stress responses (Raza 2010). Similarly, tobacco and *Arabidopsis* plants ectopically expressing the soybean (*Glycine max*) antiquitin-like *ALDH7* gene showed reduced malondialdehyde levels associated with improved drought and high salinity tolerance in addition to decreased sensitivity to hydrogen peroxide and methyl viologen (Rodrigues et al. 2006). The disruption of the rice *ALDH7* orthologous gene affected seed maturation and viability (Shin et al. 2009), which is due to the accumulation of malondialdehyde and of the yellow pigment oryzamutaic acid A, a product of aminoadipic semialdehyde polymerization, in mutant seeds (Shin et al. 2009; Shen et al. 2012).

Despite the high conservation and the importance of ALDH7 proteins in plant stress responses, the regulation of their expression has not been investigated. Here, we isolated the core promoter of the *Arabidopsis ALDH7B4* gene to examine whether osmotic stress-induced expression in vegetative tissues and in seeds share the same regulatory pathways. In addition to salt and dehydration stress, the promoter was found to be responsive to wounding, indicating that *ALDH7B4* may also be involved in response to plant pathogens. Like the homologous gene in rice, the protein was found to accumulate in seeds, thus showing that plant ALDH7 proteins are responsive to osmotic stress in both vegetative tissues and seeds. *Cis*-acting elements involved in stress responsiveness were analysed and two conserved ACGT-containing motifs proximal to the translation start codon were found to be essential for the responsiveness to osmotic stress in leaves and in seeds. A comparison of the gene expression in selected *Arabidopsis* mutants demonstrated that osmotic stress-induced *ALDH7B4* expression in seeds involves both ABA- and lipid-signalling pathways. Comparative analyses showed that the genomic organisation of the *ALDH7* gene locus and the promoter architecture are conserved between closely related Brassicaceae species endemic to different habitats.

Materials and methods

Plant material, growth conditions and stress treatments

Wild-type *Arabidopsis thaliana* ecotypes Col-0 (Columbia-0) and Ws (Wassilewskija) were used in this study. Ws, *opr3*, and *fad3-2fad7-2fad8* seeds were obtained from Dr. J. Browse (Institute of Biological Chemistry, Washington State University, USA). Seeds of *aos* and *oxi1* mutants

were obtained from Dr E. E. Farmer (Plant Molecular Biology Department, University of Lausanne, Switzerland) and Dr H. Hirt (INRA/CNRS—URGV, France), respectively. Seeds for Col-0 and all other mutants were obtained from the Nottingham Arabidopsis Stock Centre, UK. Seeds were germinated and plants were grown in plastic pots containing potting soil under short-day conditions (day/night cycle of 8/16 h) at 22 °C in white light of approximately 120–150 μE × m^{-2} × s^{-1}. All experiments were performed with approximately 6-week-old soil-grown plants, if not otherwise stated. For treatments, plants were removed from soil and incubated for the indicated time and solute concentration. Salt stress treatments were performed with up-rooted plants for 16 h using 250 mM NaCl if not stated otherwise. For dehydration experiments up-rooted plants were placed on filter paper at room temperature for 16 h, which leads to an average water loss of around 40 % per plant. For ABA treatments up-rooted plants were incubated in 100 μM *cis*-, *trans*-ABA (Sigma, St. Louis, MO, USA) solution at room temperature. Plants were sprayed with 100 μM jasmonic acid (Sigma) in 0.1 % (v/v) ethanol. Soil-grown plants were wounded by cutting the leaf with scissors or by treating the leaf surface area with abrasive sandpaper. All treated plant materials were frozen in liquid nitrogen and stored at −80 °C until further analyses.

Recombinant DNA techniques and gene expression analyses

Manipulation of nucleic acids by standard molecular techniques was performed according to Sambrook et al. (1989). RNA extraction and reverse transcription PCR were performed according to Missihoun et al. (2011). The following primers were used to amplify the *ALDH7B4* transcripts: 5′-GAAGCAATAGCCAAAGACACACGC-3′ and 5′-GATATCTCGATTATCGTAGGCTCC-3′. Densitometric analysis of the signal intensity of the *ALDH7B4* and *Actin-2* transcripts was done using the ImageQuant Version 5.2 software. The protein-blot analyses were performed according to Missihoun et al. (2012). The membrane was probed with 5,000-fold diluted ALDH7B4 antiserum according to Kotchoni et al. (2006). The immuno-detection assay was performed using the ECL Plus Western Blotting detection Kit (Amersham, Braunschweig, Germany). Signals were detected under a CCD camera (Intelligent Dark Box II, Fujifilm Corporation, Tokyo, Japan). DNA blots were performed according to Missihoun et al. (2011).

Promoter analysis and construction of promoter::GUS (β-glucuronidase) fusion plasmids

The promoter sequences were retrieved from the Phytozome v9.1 database (www.phytozome.net) then aligned using the Align X tool in the Vector NTI Advance® 11 software. The *ALDH7B4* promoter was amplified by PCR from *A. thaliana* Col-0 genomic DNA using the primer pair Aldh7B4-prom-5′ (5′-TCCCACTACTGAATTGACCTTCA-3′) and Aldh7B4-prom-3′ (5′-CTCTGCGCAAGAATTCACCCCA-3′), which contains an *Eco*RI site (underlined). The PCR product was digested with *Eco*RI and purified from an agarose gel. The resulting 0.64 kb *Eco*RI promoter fragment was subcloned into the pBT10-GUS plasmid (Sprenger-Haussels and Weisshaar 2000). One recombinant clone was digested with *Bam*HI and *Bgl*II to isolate the *ALDH7B4-promoter*::*GUS*::*nos_terminator* cassette, which was then subcloned into the unique *Bam*HI site of the binary vector pBIN19 (Bevan 1984; Frisch et al. 1995). Recombinant binary plasmids were transformed into *Agrobacterium tumefaciens* cells by electroporation.

The promoter sequences were screened for *cis*-acting elements using the plant *cis*-acting regulatory DNA elements (PLACE) database (http://www.dna.affrc.go.jp/PLACE/index.html) (Higo et al. 1999). Mutagenesis of *cis*-acting elements was done with the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). Mutations were introduced via a PCR reaction with mutagenesis primers (Suppl. Table S1). The recombinant pBT10-GUS plasmid containing the *ALDH7B4-promoter*::*GUS*::*nos_terminator* cassette was used as DNA template in each PCR. Transient transformation of *Arabidopsis* seedlings was performed by the fast *Agrobacterium*-mediated seedling transformation (FAST) assay (Li et al. 2009). Stably transformed *Arabidopsis* plants were obtained in Col-0 background by the floral dip method (Clough and Bent 1998).

Histochemical detection and measurement of the GUS activity

In situ detection of GUS activity was performed according to Jefferson et al. (1987). Transiently transformed seedlings were incubated in GUS-staining buffer (0.5 mg/ml X-Gluc; 50 mM NaH_2PO_4 buffer pH 7.2; 0.1 % (v/v) Triton X-100; 8 mM β-mercaptoethanol) at 37 °C for 14–16 h. The tissues were destained in 80 % (v/v) ethanol at 80 °C to remove the chlorophyll and kept in 10 % (v/v) glycerol. Photographs of the seedlings were taken under a dissecting microscope (Nikon SMZ-800, Düsseldorf, Germany). Fluorometric measurement of the GUS activity was done according to Jefferson et al. (1987). Protein concentrations were determined according to Bradford (1976) with a BioRad protein assay kit (Bio-Rad Laboratories GmbH, Munich, Germany). The absolute GUS activity in seeds was normalised to the activity in the leaves of the same line under non-stress growth conditions. This normalisation was necessary to account for the position effect related to the

site of insertion of the transgene into the genome, and for the possible effect of copy numbers of the reporter cassette in individual lines.

Results

ALDH7B4 genes in selected Brassicaceae species: conserved gene organisation and promoter architecture

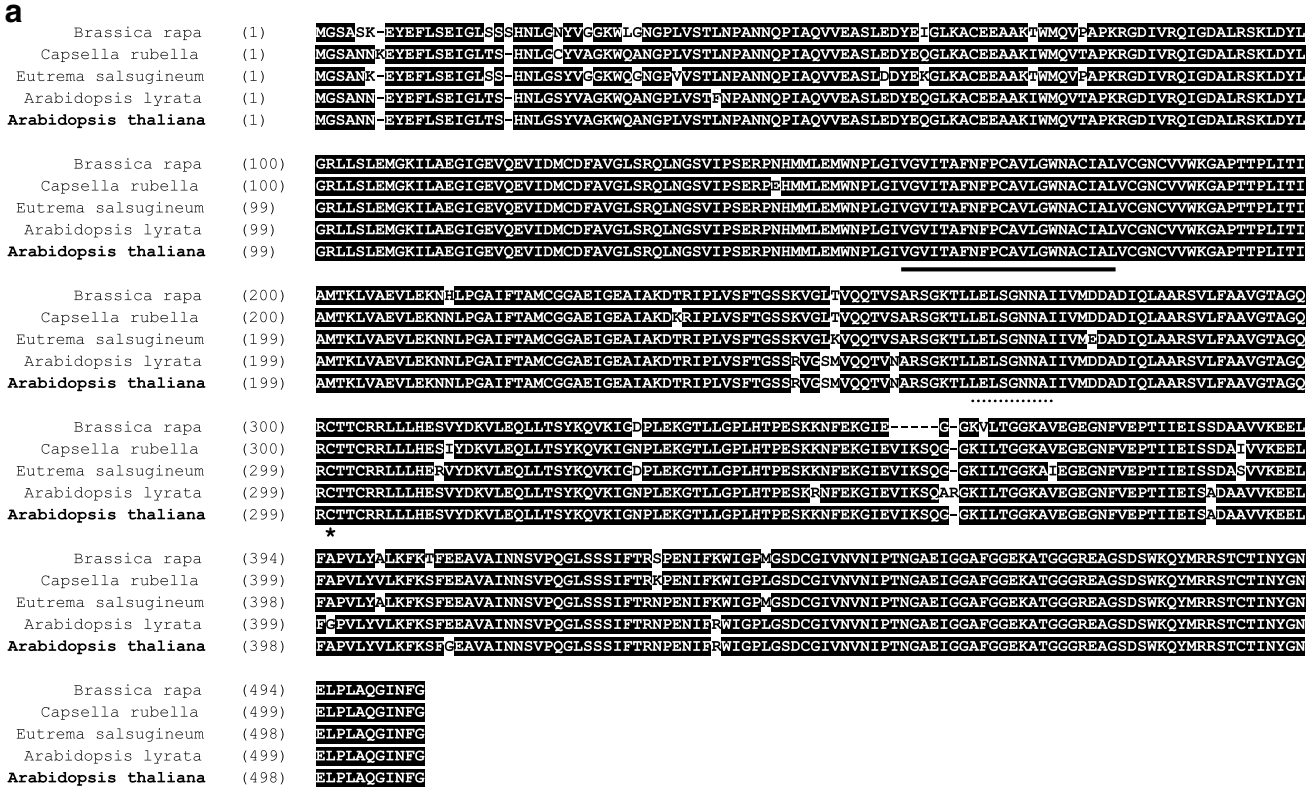
The amino acid sequences of ALDH7 proteins were reported to be well conserved between plants and animals within the ALDH protein superfamily (Wu et al. 2007; Brocker et al. 2013). We have studied the *ALDH7* genes in the Brassicaceae species *Brassica rapa*, *Capsella rubella*, *Eutrema salsugineum* (formally known as *Thellungiella halophila* or *T. salsuginea*), *Arabidopsis lyrata*, and *A. thaliana* to examine to which extend the genomic organisation has been conserved. Figure 1a shows that the ALDH7 amino acid sequences are nearly identical including the ALDH glutamic acid active site signature ^{265}LELSGNNA^{272} (PROSITE PS 00687; Perozich et al. 1999) and the hydrophobic region ^{158}VGVITAFNFPCAV LGWNACIAL^{179} in all five species (Guerrero et al. 1990; Lee et al. 1994). Also the intron exon structure is mostly conserved with a high level of conservation in the exons and more variation in the introns (Fig. 1b). Only *A. lyrata* has one exon less than the other four species. Although the intergenic region is longer, the gene structure is identical in *A. thaliana* and *A. lyrata* (Fig. 1b). Additional similarities were found in the spatial organisation of the *ALDH7* gene in the genome of the five species (Fig. 1b). A gene homologous to *cation calcium exchanger 4* is present in the opposite orientation upstream of the *ALDH7B4* gene in all five species. A gene encoding a membrane fusion protein (*use1*, *At1g54110* and *923976* in *A. thaliana* and *A. lyrata*, respectively) was found between the *cation calcium exchanger 4* gene and the *ALDH7* gene in *A. thaliana* and *A. lyrata* but not in *B. rapa*, *C. rubella* and *E. salsugineum*.

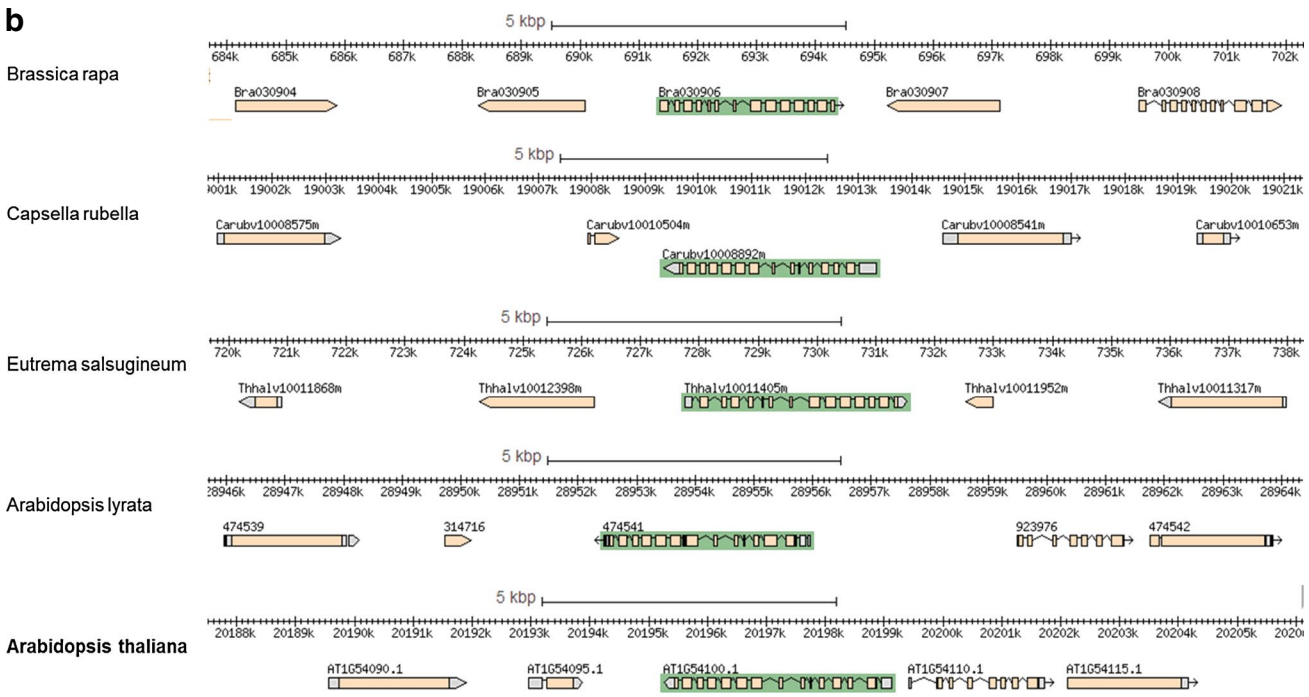
To investigate whether the sequence conservation extends to the non-transcribed promoter region, nucleotide sequences of the 2-kb region upstream of the ATG translation start codon were compared. The sequences were retrieved from the Phytozome v9.1 database (www.phytozome.net) and aligned using the Align X tool of the Vector NTI Advance® 11 software. The analysis showed a well-conserved promoter architecture for all five species within a region 0.6 kb upstream of the ATG of the *A. thaliana ALDH7B4* gene taken here as reference (Fig. 1c). Four blocks of high conservation were identified (block 1: AGC CAAAA; block 2: CAGCTCAGCTwTCGyyTT; block 3: AAGwCGTACACGTCTCTCTCwCTy; block 4: TTCTTy GATACsATC; w = A/T, y = C/T, s = C/G) (Fig. 1c). The conservation of the promoter sequences was not observed outside the 0.6 kb promoter region (Suppl. Fig. S1).

Fig. 1 Multiple amino acid and nucleotide sequence alignments and genomic organisation overview of *ALDH7B4* orthologs in selected Brassicaceae. **a** Multiple amino acid sequence alignments of ALDH7B4 proteins from selected Brassicaceae species. The AlignX™ program (Vector NTI® 11) was used to align amino acid sequences of the ALDH7B4 protein from five Brassicaceae species: *B. rapa* (gene name: *Bra030906*), *C. rubella* (gene name: *Carubv10008892m*), *E. salsugineum* (gene name: *Thhalv10011405m*), *A. lyrata* (gene name: *Aly474541*) and *A. thaliana* (gene name: *At1g54100*). The sequences were retrieved from the Phytozome v9.1 database (www.phytozome.net), and shown by the name of the species from which they were derived. Amino acids identical in at least three species are shown in *black*. The ALDH glutamic acid active site signature ^{265}LELSGNNA^{272} (PROSITE PS 00687; Perozich et al. 1999) and the hydrophobic region ^{158}VGVI-TAFNFPCAVLGWNACIAL^{179} (Guerrero et al. 1990; Lee et al. 1994) are shown in *dashed* and *continued underlines*, respectively. **b** Genomic organisation of the *ALDH7B4* orthologs and their neighbouring genes in selected Brassicaceae viewed in Gbrowse environment (http://www.phytozome.net). The *ALDH7* genes are shown in green background in the middle along with the two 5′ and 3′ adjacent genes. *Arrows* indicate 5′ and 3′ orientation of the genes. **c** Multiple nucleotide sequence alignment of the putative promoter regions of *ALDH7B4* and its orthologs from selected Brassicaceae. The sequences were retrieved from the Phytozome v9.1 database (www.phytozome.net), and shown here by the name of the species from which they were derived. The alignment was performed as above and for the same species. Here a cut-off (0.6 kb) is shown of the alignment of 2-kb fragments upstream of the translation start codon of each gene. Thus, the last nucleotide of the 3′ end of each sequence is the one before the translation start codon ATG of the gene. Identical or conserved nucleotides between individual species are shown in black background. Ten to 20 consecutive and identical nucleotides determine *block 1* AGCCAAAA, *block 2* CAGCTCAGCTwTCGyyTT, *block 3* AAGwCGTACA CGTCTCTCTCwCTy, *block 4* TTCTTyGATACsATC (w = A/T, y = C/T, s = C/G). The nucleotide sequence between the nucleotides *underlined* by an *asterisk* (*) and the *plus sign* (+) represents the DNA fragment that was cloned from *A. thaliana* and tested for promoter activity. Nucleotides above P1, P2, and P3 represent the first nucleotide of the 5′ end of the promoter deletion fragments in *lines* P1, P2 and P3, respectively. The motifs DRE/CRT, ACGT1, ACGT2, and ACGT3 are shown by the *grey-shaded* nucleotides (see text for details). *Black*, *blue*, and *red lines* denote intron, 5′ UTR, and intergenic sequences, respectively

In silico analysis of a putative promoter region of *ALDH7B4* from *A. thaliana*

The *A. thaliana ALDH7B4* gene was chosen as a model to examine the nucleotide sequences of the *ALDH7* gene promoter in detail. The 0.6 kb region upstream of the translation start codon ATG was scanned using PLACE Web Signal Scan (Prestridge 1991; Higo et al. 1999). This region includes from 5′ end to 3′ end: the 5′ UTR of *At1g54110* which encodes a membrane fusion protein putatively involved in cation transport and is adjacent to *ALDH7B4* (*At1g54100*), the intergenic nucleotide sequence between





both genes, and the 5′ UTR of *ALDH7B4* (Fig. 1b, c). Several stress-related *cis* elements were identified (Suppl. Table S2), including one putative dehydration-responsive element/C-repeat—low temperature-responsive element (DRE/CRT motif: RYCGAC; R = A/G, Y = C/T; Svensson et al. 2006) and three ACGT motifs (Simpson et al. 2003): ACGT1, ACGT2 and ACGT3 (Fig. 1c). DRE/CRT and ACGT motifs form the core of G-boxes and

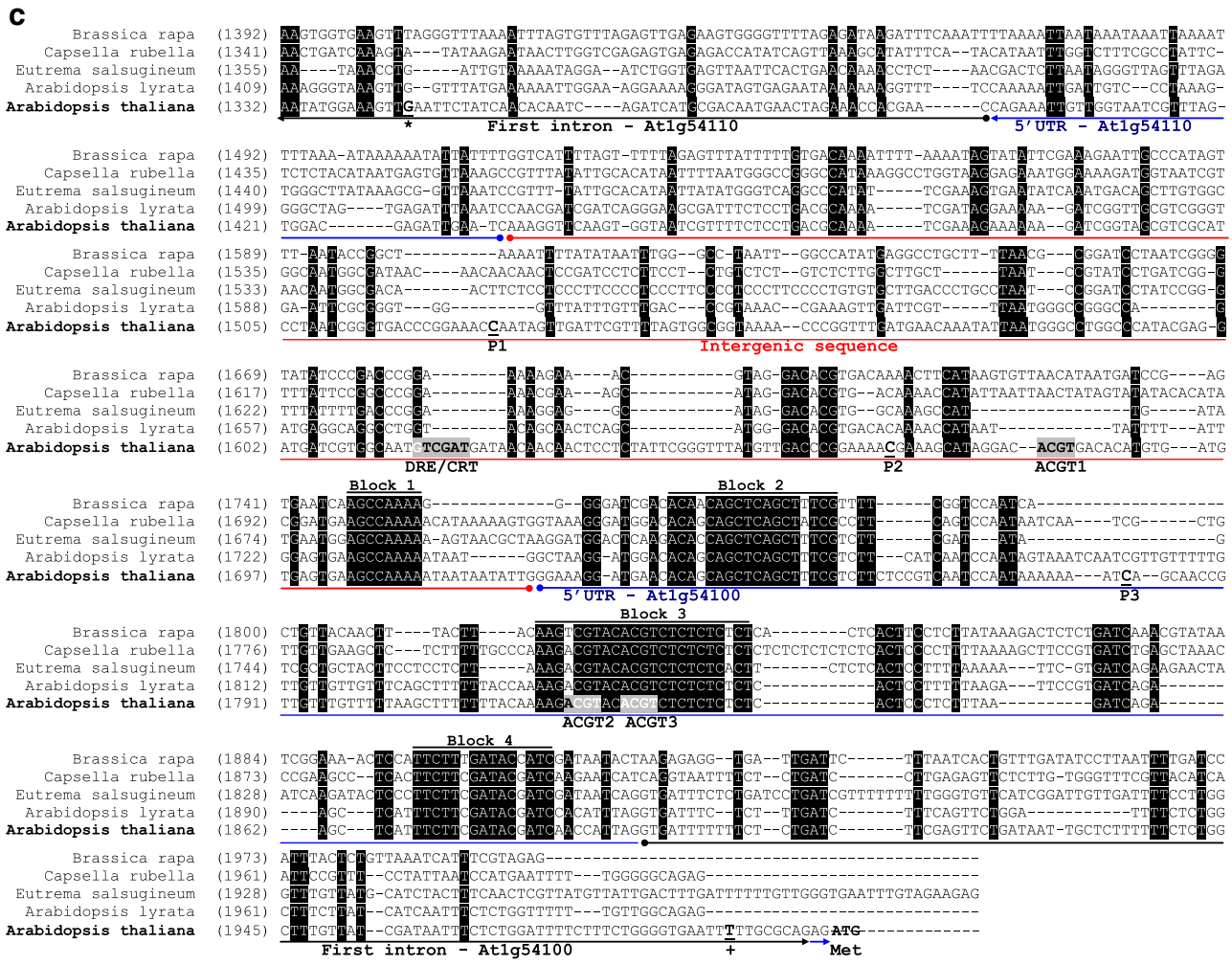


Fig. 1 continued

ABA-responsive *cis* elements (ABRE), respectively. The motifs ACGT2 and ACGT3 are both included in the block 3 of conserved nucleotides (AAGwCGTA CACGTCTCTCTCwCTy; w = A/T, y = C/T) (Fig. 1c). Two MYB1 recognition sequences (WAACCA; W = A/T; Abe et al. 2003) and three MYC recognition sequences (CANNTG; N = A/T/G/C; Abe et al. 2003; Chinnusamy et al. 2004) were also found. A list of stress-related *cis* elements within the 0.6-kb promoter region is provided in Suppl. Table S2.

Each DNA sequence was scanned using PLACE Web Signal Scan to compare the promoter sequences of the five Brassicaceae species. The DRE/CRT motif and the three ACGT motifs are present in similar spatial arrangements in all five promoters (Fig. 2). The motifs ACGT2 and ACGT3 are found towards the 3′ end of the sequence, and near the translation start codon (Fig. 2). The ACGT1-box is embedded in a MYC recognition sequence. Additional ACGT motifs are present in *B. rapa* but absent in the other species. The DRE/CRT motif was only found in *B. rapa* and *A. thaliana*. Except for the MYC recognition sequence harbouring the ACGT1 motif (indicated by the vertical dashed line in Fig. 2), no other MYC or MYB recognition sequences were found to be conserved within the examined Brassicaceae species.

Promoter analyses in transgenic *Arabidopsis thaliana* lines

The functional relevance of the 0.6 kb promoter region was investigated. A 646-bp DNA fragment from *A. thaliana* was chosen, transcriptionally fused to the reporter gene *β-glucuronidase* (*GUS*) coding sequence and integrated into the wild-type *A. thaliana* Col-0 genome. The 646-bp DNA fragment, which spans the region of −11 to −656 upstream of the translation start codon ATG (the A was set as +1; Fig. 1c), includes the 5′ UTR of *At1g54110*, the intergenic nucleotide sequence between both genes, and the 5′ UTR of *ALDH7B4* (except for the last 10 nucleotides) (Fig. 1b,

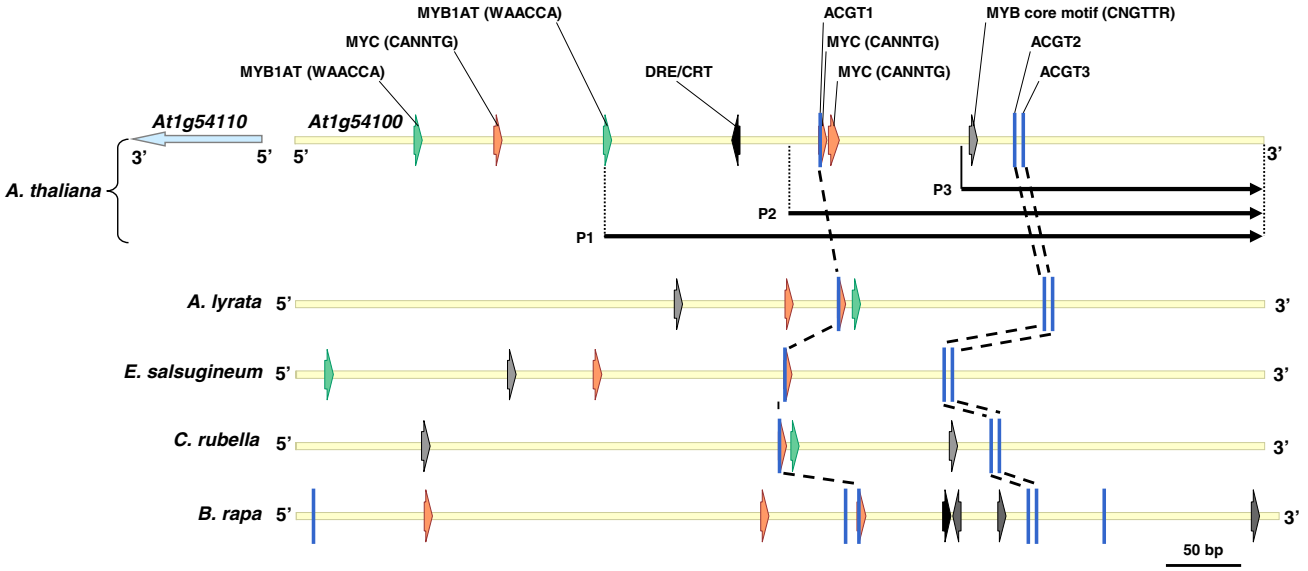


Fig. 2 Distribution of putative *cis*-acting regulatory elements within the promoter regions of *ALDH7B4* and orthologuous genes from selected Brassicaceae. Distribution of putative *cis*-acting regulatory elements associated with salt-, dehydration-, and ABA-induced gene expression within 0.6 kb of *A. thaliana*, *A. lyrata*, *E. salsugineum*, *C. rubella*, and *B. rapa ALDH7* promoter regions. Identical *cis* elements are shown with the same colour for all species whereas different *cis* elements are shown with *different colours*. The name of each *colour-coded cis* element is shown at the upper panel (*A. thaliana*). Conserved *cis*-acting regulatory elements within the *ALDH7* promoter regions are connected with *dashed lines*. The putative ACGT-containing ABA-responsive elements ACGT1, ACGT2, ACGT3, and drought-responsive element/C-repeat (DRE/CRT) within the *ALDH7* promoter fragments are displayed. MYB recognition sequences (WAACCA; W = A/T; Abe et al. 2003), MYB core motif (CNGTTR; Urao et al. 1993), and MYC recognition sequences (CANNTG; N = A/T/G/C; Abe et al. 2003; Chinnusamy et al. 2004) are also shown. The *horizontal black lines* with *arrows* P1, P2, and P3 represent the deletion constructs of the *ALDH7B4* promoter (see text for details)

c). Independent transgenic lines were selected on kanamycin and confirmed by PCR and DNA-blot analyses. The majority of kanamycin-resistant lines harboured more than one copy of the transgene (Suppl. Fig.S2). The segregation of the kanamycin resistance was monitored in the two lines B8 and B10, which have a single T-DNA insertion. The kanamycin resistance segregated as a single locus in the B8 and B10 siblings. No phenotypic difference was observed between these lines and the wild type with respect to germination rate, growth, flowering time and seed yield (data not shown). Offsprings from the lines B8 and B10 were used in the subsequent experiments. Measurement of the GUS activities in plant tissues indicated an increase of the GUS activities upon salt or dehydration treatments (Fig. 3a), which is in agreement with previously reported transcript and protein accumulation (Kirch et al. 2005; Kotchoni et al. 2006). Further characterisation of the *GUS* reporter lines led to the discovery that wounding increased the GUS activity strongly (Fig. 3b, c). Wounding activated *GUS* gene expression in the injured leaves and in leaves opposite to the wounded leaves, indicate a local and systemic expression of *ALDH7B4* in response to wounding (Fig. 3d, e).

Microarray data (http://www.genevestigator.com) (Zimmermann et al. 2004) indicated the increase of the *ALDH7B4* expression during seed maturation and in mature dry seeds. The expression of the *ALDH7B4* gene was therefore analysed in reproductive organs and seeds. Floral buds, opened flowers, siliques, and seeds were assayed for *ALDH7B4* promoter GUS activity. The activity strongly increased during the maturation of siliques and stayed at a high level in mature seeds, which is consistent with protein-blot analysis of seeds (Fig. 4a–f). These findings demonstrate that the 646-bp DNA fragment contains all necessary *cis* elements directing the *ALDH7B4* expression in a tissue-specific manner and in response to salt, dehydration and wounding stress.

Functional analysis of the *cis* elements within the *Arabidopsis thaliana ALDH7B4* promoter in response to different stress factors

To identify the *cis* elements that confer stress and tissue-specific gene activation, a mutational analysis of the *ALDH7B4* promoter was performed. Point mutations were introduced in the ACGT motifs and the putative DRE/CRT-box by site-directed mutagenesis. Each of the ACGT motifs was changed to ATTT, whereas the ATCGAC in the DRE/CRT-box was changed to ATATTT. The construct A and the construct D lack the motifs ACGT1 and DRE/CRT, respectively. The construct AD lacks both the motifs ACGT1 and DRE/CRT and the construct AB the motifs ACGT2

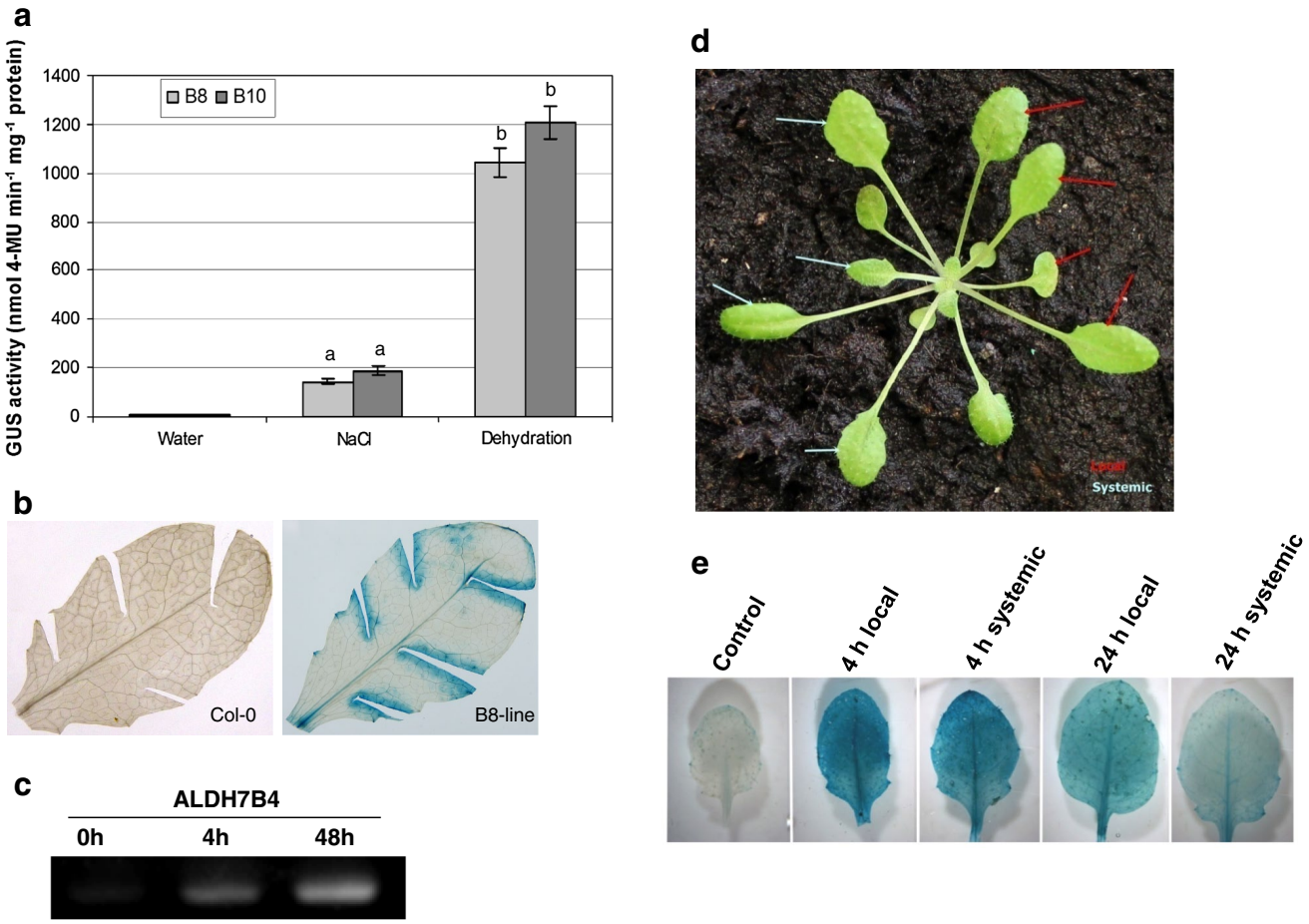


Fig. 3 Salt-, dehydration-, and wounding-induced activity of the 0.6 kb *ALDH7B4* promoter measured as enzymatic activity of the β-glucuronidase (GUS) reporter protein. **a** Promoter activity in leaves of 4-week-old transgenic plants (*lines* B8 and B10) following 200 mM NaCl or 16 h dehydration treatments. *Error bars* with *different letters* are significantly different from each other and from the control treatment (water); $P \leq 0.05$, Student *t* test. **b** In situ detection of the promoter activity in wounded wild-type (Col-0) and transgenic leaves (B8 *line*). Leaves of 6-week-old plants were wounded with scissors and were detached from the plant after 4 h for GUS staining. Pictures were taken after destaining the tissues in 80 % (v/v) ethanol. **c** Agarose gel showing the accumulation of *ALDH7B4* transcripts after wounding. Transcript accumulation is shown at 0 h in control non-wounded leaves and in leaves 4 and 48 h after wounding leaves with abrasive sandpaper. **d**, **e** Local and systemic expressions of the *ALDH7B4* gene in wounded and non-wounded leaves: *light blue arrows* indicate the wounded leaves and *red arrows* indicate the opposite non-wounded leaves; wounding was done with abrasive sandpaper (**d**). GUS expression was examined for local and systemic responses in these leaves (**e**)

and ACGT3 (Fig. 5a). The GUS activity of the promoter constructs A, D, AD, and AB *GUS* was first examined in young seedlings of *A. thaliana* by transient transformation (Fig. 5b). A strong decrease was observed for the construct AB carrying mutated motifs ACGT2 and ACGT3.

In addition to the constructs carrying mutated *cis* elements, deletion constructs were generated and fused to the *GUS* gene: P1 (−11 to −474), P2 (−11 to −335), and P3 (−11 to −219) (Fig. 2). All mutated promoter constructs were analysed in stably transformed fully developed *A. thaliana* plants and compared to the wild-type promoter using the B8 line as reference. Salt and dehydration treatments induced higher GUS activity than wounding or ABA treatment in the wild-type B8 line (Fig. 6). The mutations affected the responsiveness to the various stimuli differently. The simultaneous mutation of the motifs ACGT2 and ACGT3 (line AB) abolished the induction of the promoter upon salt or dehydration treatment, which is consistent with the transient transformation of seedlings (Figs. 5b, 6). A strong decrease was observed following the mutation of the motif ACGT1 (line A), particularly upon dehydration (Fig. 6). The mutation of the DRE/CRT motif (line D) decreased the amplitude of induction upon dehydration, but less than the mutation of the motif ACGT1 (line A) (Fig. 6). A similar trend was observed for salt, but the decrease was not significant. Lines A and AB (mutated ACGT motif) as well as the lines P2 and P3 (deletion constructs P2 and P3) showed a low responsiveness to ABA similar to the wild

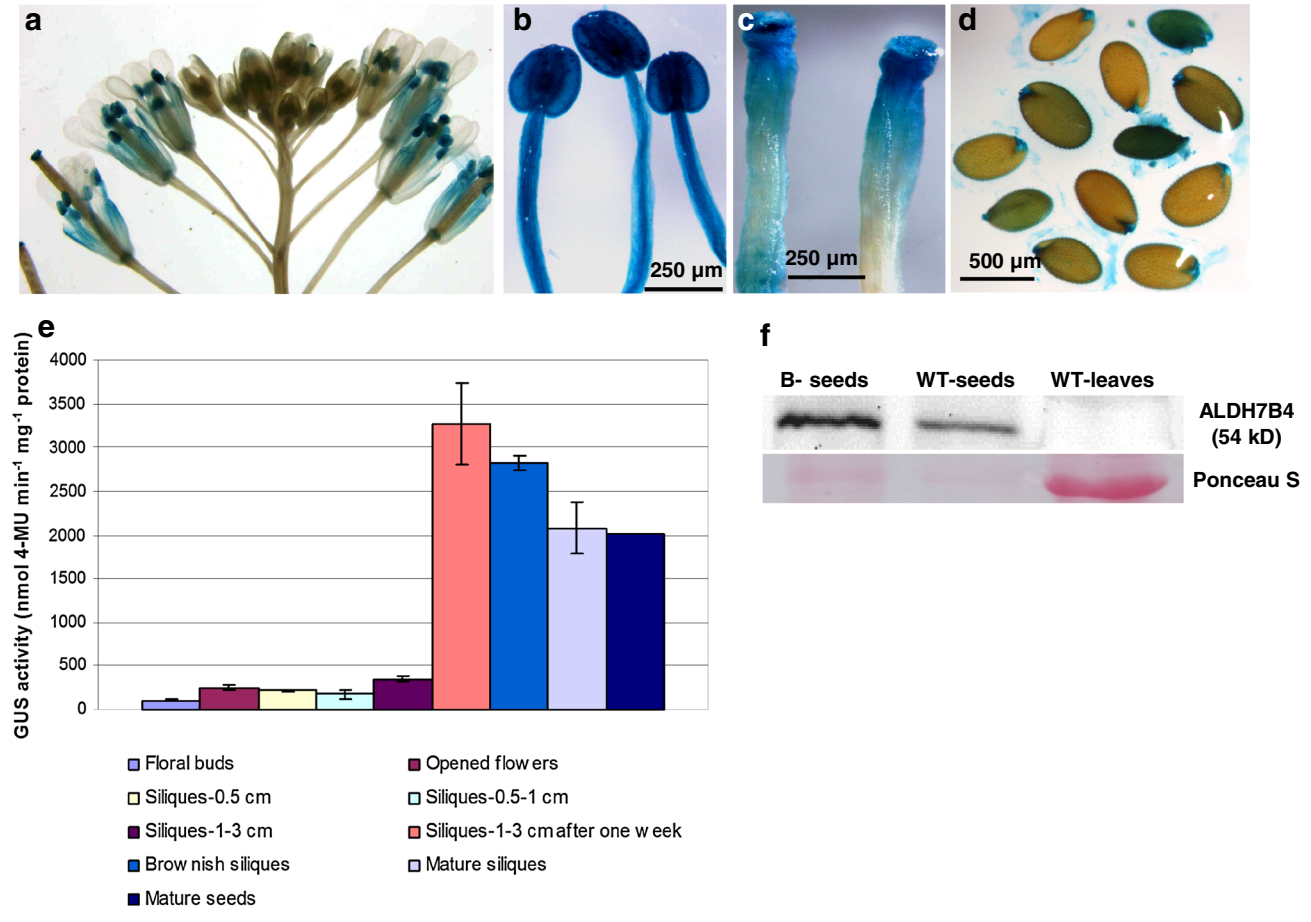


Fig. 4 Tissue-specific activity of the 0.6 kb *ALDH7B4* promoter measured as the enzymatic activity of the β-glucuronidase (GUS) reporter protein. **a** flowers, **b** stamens, **c** pistil, **d** seeds. **e** Measurement of the promoter activity in different organs. **f** Immuno-detection of the ALDH7B4 protein (54 kD) by protein-blot analysis of total protein extracts from the transgenic B-*line* and wild-type (WT) *A. thaliana* leaves and dry seeds

type (Fig. 6). However, the mutation of the DRE/CRT motif (line D) or the deletion of the first 172 nucleotides (line P1) strongly increased the activity of the promoter in response to ABA (Fig. 6). GUS activity in line P1 was not only higher in response to ABA but also in response to salt and dehydration (Fig. 6).

Surprisingly, none of the deletion constructs showed a significant decrease in GUS activity upon wounding. The lines A and AB showed a slightly lower GUS activity than the wild type (Fig. 6). The analysis of the wounding response in the different promoter GUS lines did not reveal a direct correlation between *cis* elements and wounding.

Regulatory roles of the *cis* elements within the *ALDH7B4* promoter in mature seeds

To analyse whether the same regulatory mechanisms are active in response to environmental factors and during seed dehydration, the effects of the promoter mutations were examined in seeds. The relative induction of the GUS activity driven by each mutated promoter construct in seeds was determined as the ratio of the GUS activity in seeds to the activity measured in leaves of the same line under normal growth conditions. Mutations represented by the lines A and AB did not affect the promoter activity in seeds substantially and only a small, non-significant increase of GUS activity was seen in seeds of the lines D and AD (Fig. 7). In contrast, the deletion constructs P2 and P3 abolished the GUS expression in seeds, whereas the deletion in P1 led to an increase (Fig. 7). This indicates that sequences in front of the fragments P2 and P3 are necessary for the *ALDH7B4* promoter activation in seeds.

Summary of promoter activities

The analysis of the deletions and point mutations revealed that the motifs ACGT2 and ACGT3 are required for the promoter induction by salt and dehydration but not by

ABA. The integrity of motifs ACGT1 and DRE/CRT is required for induction by salt, dehydration, ABA and for *ALDH7B4* expression in seeds. The mutations of the motifs ACGT1, ACGT2 and ACGT3 affected the promoter activity upon wounding stress weakly. An overview is presented in Table 1.

Mutations affecting the *ALDH7B4* expression upon wounding and in seeds

The magnitude of changes by wounding was not very large except for the mutations A and AB. Therefore *ALDH7B4* expression was analysed in selected mutants by monitoring the gene expression in response to wounding. The mutants included those affected in ABA and jasmonic acid biosynthesis as well as signalling, as wounding response involves both phytohormones. A list of the mutants is shown in Table 2. The *ALDH7B4* expression was examined in mature leaves without wounding and four hours after wounding. The *ALDH7B4* gene expression substantially increased as early as 4 h upon injury in the wild type and in the *aba3*, *fad2*, *opr3*, and *pld* double mutants. Only a minor increase of the expression was observed in the other mutants (Fig. 8a). Because of the involvement of jasmonic acid in the wounding response, we also verified the induction of *ALDH7B4* transcripts in response to jasmonic acid. The expression level of *ALDH7B4* in response to jasmonic acid alone or to combined application of wounding stress and jasmonic acid was similar to that of wounding in all tested mutants (data not shown).

Fig. 6 Induction of the GUS activity driven by the mutated promoter fragments in leaves of stably transformed *A. thaliana* plants. The *lines* A, D, AD, AB, P1, P2, and P3 denote transgenic plants expressing the corresponding mutated promoter fragment. The relative induction is the ratio of measured GUS activity from leaves treated by NaCl, dehydration, or ABA for 16 h, and wounding for 4 h to the GUS activity measured from leaves of untreated plants. Data represent means from four independent *lines* expressing each wild-type or a mutated promoter fragment. Nine biological replicates were used for each independent *line*. *Error bars* with *different letters* are significantly different from each other and from the wild-type non-mutated promoter of the B8 *line*; $P \leq 0.05$, Student *t* test

The comparison of the ALDH7B4 protein level in mutant seeds indicated that ALDH7B4 accumulation was impaired in *abi1*, *abi5*, *PLDδ3*, and *PLDα1/δ3* seeds (Fig. 8b).

Discussion

The amino acid sequence identity among members of the family 7 of ALDH proteins is about 60–80 %, making them besides histone H2A proteins one of the most evolutionarily conserved eukaryotic proteins (Lee et al. 1994; Fong et al. 2006; Wu et al. 2007). The proportion of identical amino acid residues is even higher between the selected Brassicaceae species. A decrease in nucleotide sequence conservation was expected in the putative gene promoter region, and this was confirmed when the DNA sequences upstream of the translation start codon (ATG) were aligned

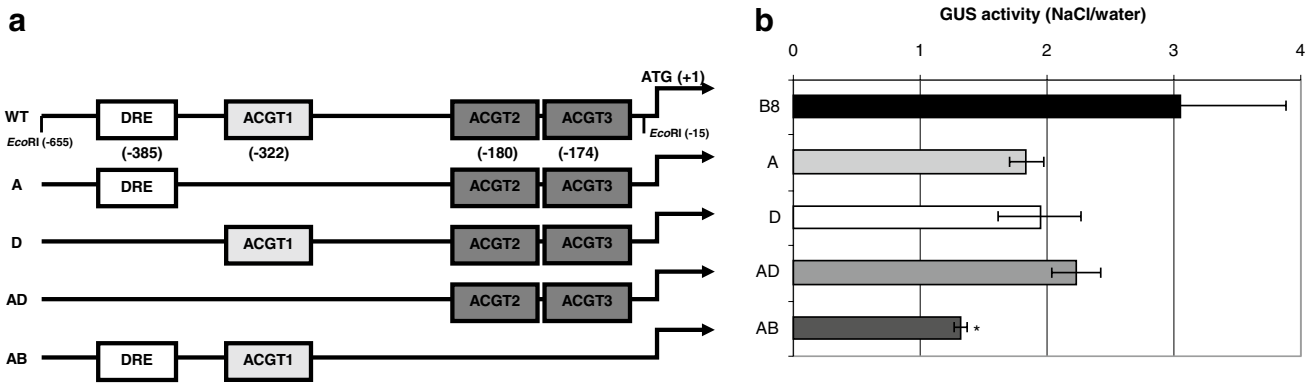
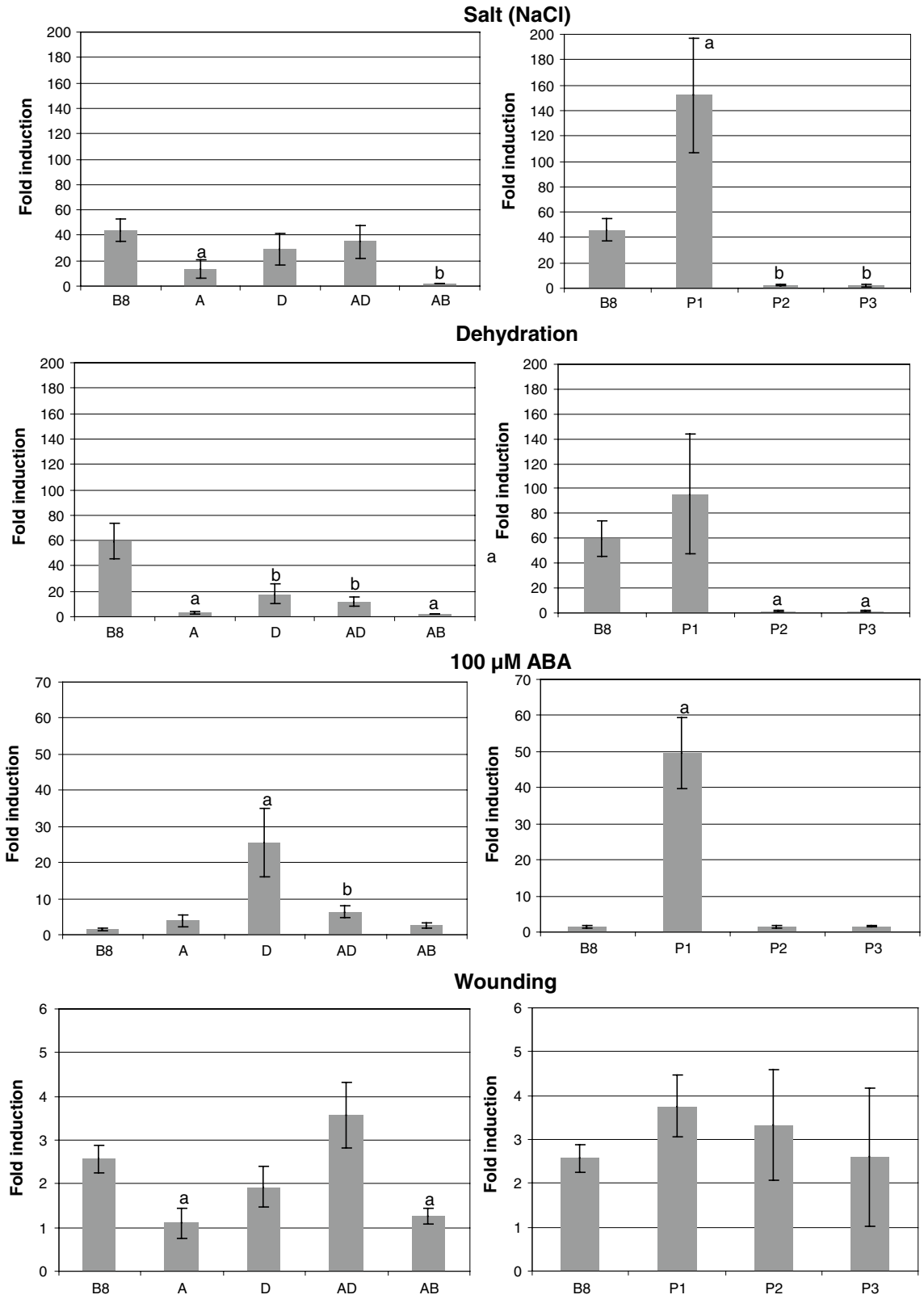


Fig. 5 Schematic representation and activity of the mutated versions of the 0.6 kb *ALDH7B4* promoter region. **a** Schematic representation of the mutated versions of the 0.6 kb *ALDH7B4* promoter region. WT denotes the 0.6 kb wild-type promoter fragment used to generate the *line* B8 and B10. The mutated versions of the wild-type fragment are A, D, AD, AB, which are characterised by point mutations within the ACGT- or DRE/CRT-containing motifs. P1, P2 and P3 derived from sequential 5′ end deletions of the wild-type promoter. The locations of the ACGT- or DRE/CRT-containing motifs relative to the translation start codon ATG are shown in *brackets*. **b** GUS activities derived from the mutations in the promoter fragments A, D, AD and AB. The activities were assayed upon transient transformation of *A. thaliana* seedlings and were normalised to the activity of the 0.6 kb wild-type promoter (in the *line* B8). The GUS activities of NaCl-treated samples were divided by the activities of water-treated samples for each promoter fragment. This ratio represents the magnitude of the induction and is shown as the mean of three independent transformations. A solution of 100 mM NaCl was used. Data are mean ± SE from three biological replicates. Significant difference between B8 and AB is shown with (*asterisks*); $P \leq 0.05$, Student *t* test



for selected monocot and dicot species, for which genome sequences are available (Fig.S1). However, blocks of conserved nucleotide sequences were identified within the *ALDH7* promoters of the examined Brassicaceae species, which reflects their close relationship and conservation of functional units within the promoters. The *Arabidopsis*

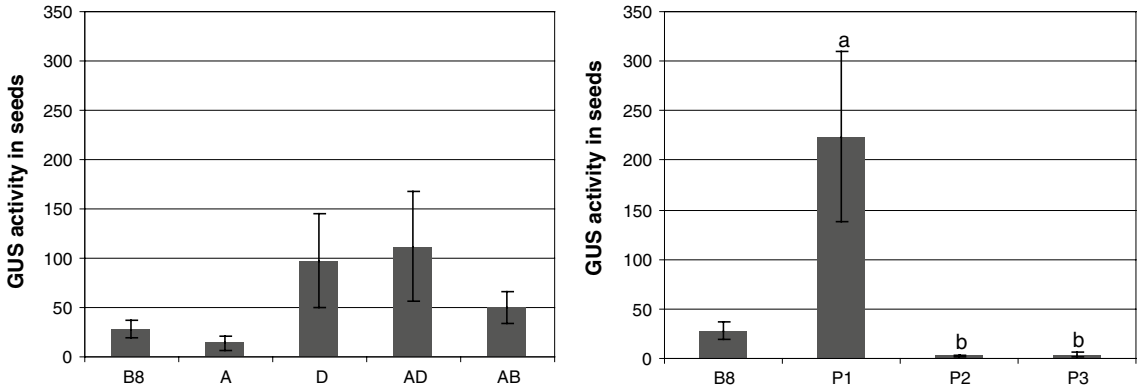


Fig. 7 Relative induction of the GUS activity driven by the mutated promoter fragments in seeds of stably transformed *A. thaliana* plants. The *lines* A, D, AD, AB, P1, P2, and P3 denote transgenic plants expressing the corresponding mutated promoter fragment. The activity in seeds is shown as the ratio of the measured GUS activity in seeds to the activity measured in the leaves of the same *line* under non-stress growth conditions. Data represent means from four independent *lines* expressing each the wild-type or a mutated promoter fragment. Three biological replicates were used for each independent *line*. *Error bars* with *different letters* are significantly different from each other and from the wild-type non-mutated promoter of the B8 *line*; $P \leq 0.05$, Student *t* test

Table 1 Summary of the promoter activity resulted from the mutations

	NaCl	Dehydration	ABA	Wounding	Seeds
A (ACGT1)	↓	↓	→	↓	→
D (DRE/CRT)	→	↓	↑	→	→
AD (ACGT1 + DRE/CRT)	→	↓	↑	→	→
AB (ACGT2 + ACGT3)	↓	↓	→	↓	→
P1	↑	→	↑	→	↑
P2	↓	↓	→	→	↓
P3	↓	↓	→	→	↓

The modified *cis* elements are shown in brackets for the fragments A, D, AD, and AB. See Fig. 1 for further details for the fragments P1, P2, and P3

↑, up-regulation with reference to wild-type promoter; ↓, down-regulation with reference to wild-type promoter); →, no change with respect to wild-type promoter

ALDH7B4 gene was taken as a model to investigate the function of conserved promoter sequences.

Osmotic stress is triggered in plant vegetative tissues by environmental stresses such as high salinity or dehydration and it is physiologically induced by internal cues during seed and pollen maturation. Consistently ALDH7 proteins have been shown to be induced by osmotic stress in several plant species (Guerrero et al. 1990; Stroeher et al. 1995; Kirch et al. 2005; Kotchoni et al. 2006; Rodrigues et al. 2006; Raza 2010), and in seeds of rice and Arabidopsis (Shin et al. 2009; Shen et al. 2012; this work). It has so far been unclear whether osmotic stress-induced ALDH7 expression is regulated in vegetative tissues and in seeds through similar signalling pathways. To examine this question *cis* elements and signalling pathways were analysed for the Arabidopsis *ALDH7B4* gene. We demonstrated here that wounding is a main trigger besides osmotic stress for *ALDH7B4* induction. Therefore, it was investigated whether wounding and osmotic stress share signalling pathway or are independent from each other.

Promoter analysis of genes that are differentially expressed in response to abscisic acid (ABA) or osmotic stresses identified a number of ACGT-containing *cis* motifs (Zhang et al. 2005). Stress signalling was shown to be regulated by both ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki 2007). Particularly, ABA-responsive elements (ABREs; PyACGTGGC), G-box elements (CACGTGGC) and DRE/CRT (A/GCCGAC) motifs were found to mediate the ABA-dependent and the ABA-independent gene expression, respectively (Fujita et al. 2011). Our results from mutated *ALDH7B4* promoter constructs indicate that the ACGT motifs (ACGT2 and ACGT3) near to the translational start codon are relevant for salt and dehydration stress responses (Fig. 6). The presence of the motifs ACGT2 and ACGT3 within the block 3 of conserved nucleotides in all tested Brassicaceae *ALDH7* promoters suggests that these motifs determine salt and dehydration responses within Brassicaceae species. Our results indicate that these motifs would only be of minor importance in response to ABA, since mutating them did not alter the response of the promoter to ABA. In a previous study, we reported that the induction of *ALDH7B4* by dehydration and salt occured in an ABA-dependent and ABA-independent manner, respectively (Kirch et al. 2005). Although both salt and dehydration stress trigger changes in the cell turgor, the signalling

Table 2 List of *A. thaliana* mutants used in this study

Mutants	Gene or pathway affected	References
aba1-1	Zeaxantin epoxidase; ABA biosynthesis	Koornneef et al. (1982), Marin et al. (1996)
aba3-2	Xanthosin oxidase; ABA biosynthesis	Koornneef et al. (1982), Léon-Kloosterziel et al. (1996)
abi1-1	Protein phosphatase 2C; ABA signalling	Koornneef et al. (1984), Leung et al. (1994)
abi2-1	Protein phosphatase 2C; ABA signalling	Koornneef et al. (1984), Leung et al. (1997), Rodriguez et al. (1998)
abi5-1	Protein phosphatase 2C; ABA signalling	Finkelstein (1994)
PLDα1	Phospholipase D, isoform α1; lipid signalling	Bargmann et al. (2009)
PLDδ3	Phospholipase D, isoform δ3; lipid signalling	Bargmann et al. (2009)
PLDα1/δ3	Phospholipase D, isoforms α1 and δ3; lipid signalling	Bargmann et al. (2009)
fad2-1	Phosphatidylcholine 18:1 desaturase; fatty acid biosynthesis	Lemieux et al. (1990), Miquel and Browse (1992)
fad3-2fad7-2fad8	Phosphatidylcholine 18:2 desaturase (FAD3)—chloroplast 16:2/18:2 desaturase (FAD7)—chloroplast 18:2 desaturase (FAD8); fatty acid biosynthesis	Lemieux et al. (1990), McConn and Browse (1996)
aos	Allene oxide synthase; jasmonic acid biosynthesis	Park et al. (2002)
opr3	12-oxo-phytodienoic acid reductase; jasmonic acid biosynthesis	Stintzi and Browse (2000)
oxi1	Oxidative signal-inducible1 encoding a serine/threonine kinase; responsive to oxidative stress and wounding	Rentel et al. (2004), Anthony et al. (2004)

through an ABA-dependent or ABA-independent pathway may discriminate between *cis* elements involved in the activation. This likely explains why we observed different effects for the same mutation depending on the treatment applied to the tissues. The observation that mutations of the ACGT2 and ACGT3 motifs did not affect the ABA response suggests that these motifs may not function as ABREs to mediate the dehydration response. ACGT-containing sequences that do not function as ABREs have been reported (Kao et al. 1996; Busk et al. 1997). Mehrotra and Mehrotra (2010) showed that two copies of ACGT motifs separated by five nucleotides imparted salicylic acid induction to a basal promoter whereas ABA-responsiveness was obtained when the distance was at least 25 nucleotides between both ACGT elements. Other data indicated that several plant *cis*-acting elements actually contain the core ACGT-sequence but the flanking sequences are required for correct functions (Guiltinan et al. 1990; Salinas et al. 1992; Busk et al. 1997). Therefore, it could be that the region mediating the ABA response is not restricted to the ACGT2 and ACGT3 motifs but includes surrounding nucleotides in the *ALDH7B4* promoter. Alternatively, additional more distant *cis* elements could be required to modulate the promoter activity in response to ABA, salt and dehydration. Often, a single copy of the ABRE element is not sufficient for ABA-responsive transcription, and ABREs require coupling elements such as CE1 and CE3 for ABA-induced gene transcription (Shen et al. 1996; Fujita et al. 2011). In some cases an adjacent copy of ABRE or DRE/CRT was found to function as a coupling element (Narusaka et al. 2003). Most of the known coupling elements are similar to ABREs and contain an A/GCGT motif (Hobo et al. 1999). In contrast to ACGT2 and ACGT3, ACGT1 (fragment A) functions as an ABRE and is involved in the activation of the *ALDH7B4* gene promoter by salt and dehydration, given that deletion of ACGT1 suppressed activation of *ALDH7B4* by dehydration or salt. The GUS activities obtained with the promoter deletion fragments P1, P2, and P3 indicated that both *cis* elements DRE/CRT and ACGT1 are essential together with ACGT2 and ACGT3 for the induction by salt and dehydration. Therefore, ACGT2 and ACGT3 could function as coupling elements for the motifs DRE/CRT or ACGT1 to modulate promoter activation by salt and dehydration.

Neither the mutations of the ACGT motifs (constructs A and AB) nor the deletions in the constructs P2 and P3 affected the promoter responsiveness to ABA. We, however, found that the mutation of the DRE/CRT motif (fragment D) has increased the activity of the promoter in response to ABA. This suggests the presence of *cis*-acting elements, which attenuate the *ALDH7B4* promoter activity. These elements must be present in the region upstream of the ACGT1-box including the DRE/CRT-box, and would negatively modulate the response of the promoter to the ABA signal. This conclusion is further supported by the data from seeds. The comparison between P1, P2 and P3 showed that the DRE/CRT and ACGT1 motifs are essential for the promoter activity in seeds. Thus, *cis*-acting elements that enhance the *ALDH7B4* promoter activity are also active in seeds, as suggested by the strong increase of the GUS expression in P1 lines compared to the wild-type promoter B8 line. It is unclear which elements upstream of the DRE/

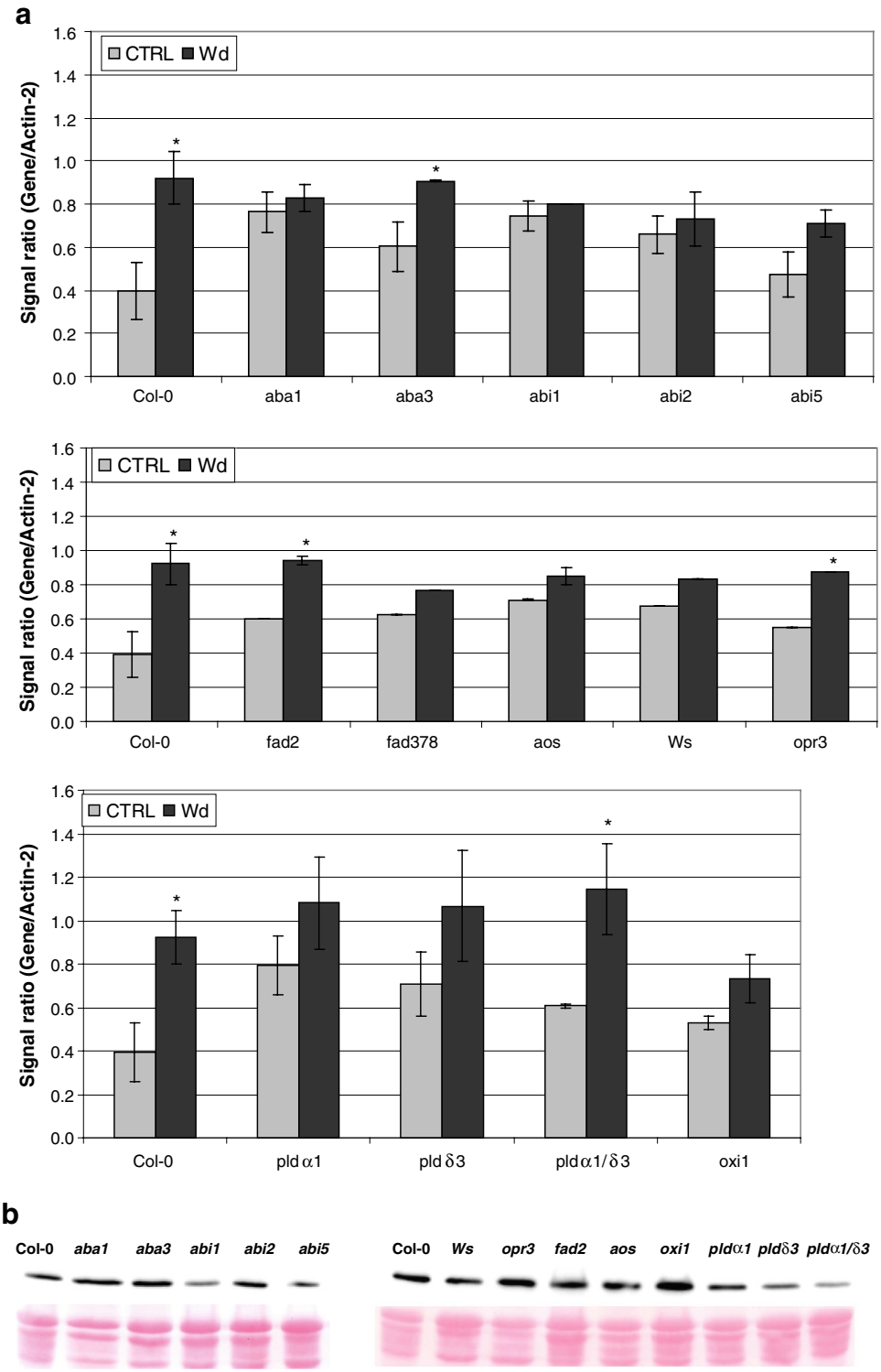


Fig. 8 Analysis of the *ALDH7B4* gene expression in selected *A. thaliana* mutants. **a**, **b** Semi-quantitative analysis of the *ALDH7B4* gene expression. The expression was assayed by RT-PCR and the intensity of the signal was related to that of the *ACTIN-2* gene amplified from the same tissue. All plants were kept in soil and were either *left* untreated (CTRL) or wounded (Wd). Leaf samples were harvested 4 h upon the treatment. All mutants are *in A. thaliana* Col-0 background except for *opr3*, which is in Ws background. Data are mean ± SE from three biological replicates. Significant difference between control and wounded samples is shown with (*asterisk*); $P \leq 0.05$, Student *t* test. **c** Accumulation of the ALDH7B4 protein in the wild type and mutants. Fifteen micrograms crude protein extracts from seeds was analysed by immunoblot. Antiserum raised against the ALDH7B4 protein (Kotchoni et al. 2006) was used for the immuno-detection assay. The *upper panel* indicates the signal from the ALDH7B4 protein obtained by chemo-luminescence. The *lower panel* shows the *red* Ponceau S staining of the membrane to monitor equal load of proteins

CRT motif in the analysed 646-bp DNA region could be responsible for the promoter activity. This part of the 646-bp DNA fragment (−475 to −656; Fig. 1c) contains from the 5′ end sequences of intron 1 of *At1g54110*, the 5′ UTR of *At1g54110*, and the last 90 nucleotides of the intergenic sequence between *ALDH7B4* and *At1g54110*. Although our reporter gene analyses showed that the 646-bp DNA fragment retained the activation pattern of the chromosomal *ALDH7B4* gene, the actual promoter may be limited to the length of the P1 fragment. Consistent with this conclusion, we often observed that the endogenous level of *ALDH7B4* transcripts was higher than that of the *GUS* transcripts in

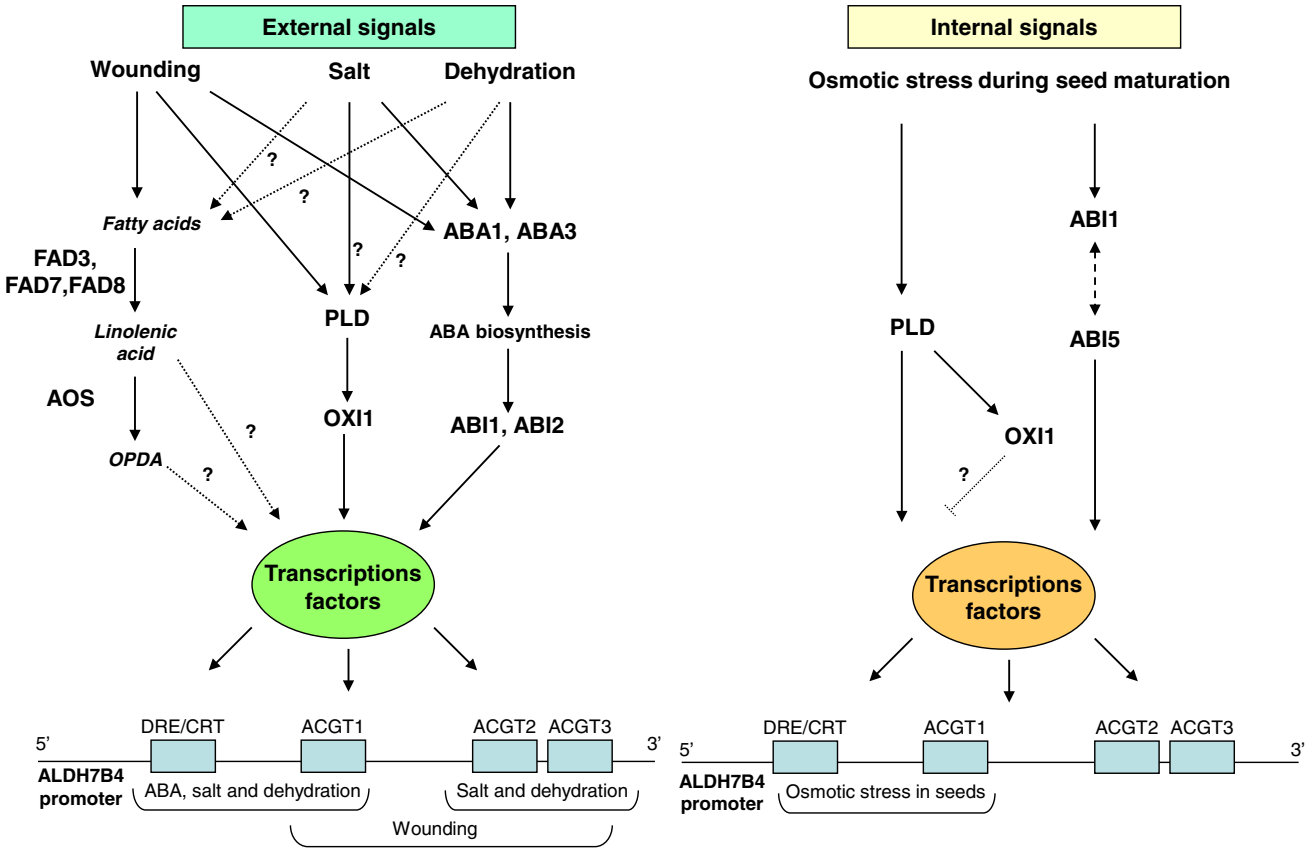


Fig. 9 Proposed regulatory pathways for the osmotic stress-induced *ALDH7B4* expression in leaves upon external stimuli and in seeds. In this model, *ALDH7B4* is induced by osmotic stress generated from either outside (wounding, dehydration, salt) or endogenous (seed desiccation) to the plant. The expression of *ALDH7B4* in response to wounding, dehydration, salt and in seeds is mainly mediated by PLD- and ABA-signalling components. However, the expression in leaves and seeds is likely to involve different transcription factors. At the promoter level, the DRE/CRT, ACGT1, ACGT2 and ACGT3 motifs are required for the induction by salt and dehydration. DRE/CRT is essential for the activation by ABA whereas ACGT1, ACGT2 and ACGT3 are relevant for the wounding response. The DRE/CRT and ACGT1 motifs are particularly important for the expression in seeds. *Continued arrows* indicate steps or routes for which evidence has been provided either from our previous works (Kirch et al. 2005), this study, or in the literature. *Dotted arrows* with a question mark indicate hypothetical routes or steps, which require experimental verification. The *discontinued line* with a *question mark* indicates a probable inhibition. Compounds are in *italics*. Proteins are in *capital letters* and *regular font*. *OPDA* 12-oxo-phytodienoic acid, *ABA* abscisic acid, *FAD* fatty acid desaturase, *ABA1, ABA3* ABA biosynthesis genes, *ABI1, ABI2, ABI5* ABA insensitive/ABA-signalling genes, *PLD* phospholipase D, *OXI1* oxidative signal-inducible 1, *AOS* allene oxide synthase (see Table 2 for mutant details)

the B8 line (data not shown). The findings suggest that The endogenous gene expression likely depends on the P1 fragment only instead of the longer fragment in the B8 line. It is difficult to guess which sequences correspond to the P1 fragment in the promoter region of the other Brassicaceae *ALDH7* genes. This is mainly due to the absence of the DRE/CRT motif from these promoter regions and the dissimilarities in the occurrence of stress-related *cis* elements upstream of the ACGT1 motif (Fig. 2). The existence of conserved blocks between the Brassicaceae *ALDH7* gene promoters, however, suggests that the P1 fragment from *A. thaliana* may also be functional and similarly activated in the other Brassicaceae species.

It is still unclear how the *ALDH7B4* promoter is activated by wounding stress. Wounding response was shown to be orchestrated by jasmonic acid or the jasmonic acid-related compound 12-oxo-phytodienoic acid, and by oligogalacturonides released from the cell wall components following injury (Howe 2004). Because the wounding response was impaired in the *aos* mutant but not in the *opr3* mutant, we hypothesised that 12-oxo-phytodienoic acid could be a signalling molecule activating the expression of *ALDH7B4* upon wounding. Other oxylipins including volatile aldehydes could also be involved, since *ALDH7B4* expression was also impaired in the triple mutant *fad3-2fad7-2fad8,* which does not accumulate linolenic acid, a precursor of oxylipins (Berger 2002; Matsui 2006). In contrast to jasmonic acid and 12-oxo-phytodienoic acid, little is known about the signalling by oligogalacturonides. Because our results from the mutant analyses did not

indicate a dependence on jasmonic acid of the promoter activation by wounding (data not shown), it remains to be tested which volatile aldehydes, 12-oxo-phytodienoic acid, and oligogalacturonides derived from cell wall degradation could activate the *ALDH7B4* promoter. In a microarray study, ethylene and ABA-signalling components were found to be involved in the local wounding response in Arabidopsis, regulating the repression of photosynthetic genes and expression of drought-responsive genes, respectively (Delessert et al. 2004). G-box-related motifs were found in significant proportion of the promoters of genes affected by wounding (Delessert et al. 2004). A substantial overlap was previously shown between wounding- and water stress-responsive genes (Reymond et al. 2000), and wounded plants were found to accumulate ABA in the region surrounding the wound site (Peña-Cortés et al. 1995; Birkenmeier and Ryan 1998). These previous data indicate that responses to water stress and wounding significantly overlap, and they support our observations that the deletions of either ACGT1 or both ACGT2 and ACGT3 led to the decrease of the promoter activity upon wounding, even though weaker than upon dehydration or salt (Fig. 6). In addition to the local response, we found that the *ALDH7B4* promoter is systemically induced in non-wounded leaves. While genes induced at the wound site are thought to play a role in wound healing, protection against water loss and pathogen invasion, systemically induced genes are proposed to prime the plant against further attack by herbivores and pathogens (Bowles 1993; Delessert et al. 2004). We therefore hypothesise that *ALDH7B4* may be induced by pathogen and herbivore attacks and involved in plant biotic stress response too. The rice homolog, *ALDH7B7*, was indeed found to be induced in rice leaves infected by the incompatible race of the blast fungus *Magnaporthe grisea* (Wu et al. 2007).

Altogether, the results from previous and present studies indicate that *ALDH7* genes code for versatile osmotic stress-responsive proteins involved in both biotic and abiotic stress responses. We propose a model, which combines our previous results (Kirch et al. 2005) and the data from this study with the currently available literature, to summarise our understanding of *ALDH7B4* regulation in response to osmotic stress in vegetative tissues and seeds (Fig. 9). In this model, *ALDH7B4* is induced by osmotic stress generated from both outside (wounding, dehydration, salt) and inside (seed desiccation) the plant. The expression of *ALDH7B4* in response to wounding, dehydration or salt is likely to be mediated by PLD- and ABA-signalling components. The dehydration response may additionally be through lipid signalling (Kirch et al. 2005). At the promoter level, the DRE/CRT, ACGT1, ACGT2 and ACGT3 motifs are required for the induction by salt and dehydration. DRE/CRT is essential for the activation by ABA whereas ACGT1, ACGT2 and ACGT3 are relevant for the wounding response. As in leaves, the expression of *ALDH7B4* in seeds appears to involve both PLD- and ABA-signalling pathways. Different transcription factors in leaves and seeds may, however, be involved. For *cis*-regulation, the DRE/CRT and ACGT1 motifs in the promoter are particularly important for the expression in seeds.

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References

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78

Anthony RG, Henriques R, Helfer A, Meszaros T, Rios G et al (2004) A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in Arabidopsis. EMBO J 23:572–581

Bargmann BOR, Laxalt AM, ter Riet B, Testerink C, Merquiol E, Mosblech A, Leon-Reyes AH, Pieterse CM, Haring MA, Heilmann I, Bartels D, Munnik T (2009) Reassessing the role of phospholipase D in the Arabidopsis wounding response. Plant Cell Environ 32:837–850

Berger S (2002) Jasmonate-related mutants of Arabidopsis as tools for studying stress signaling. Planta 214:497–504

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res 12:8711–8721

Birkenmeier GF, Ryan CA (1998) Wound signaling in tomato plants. Evidence that aba is not a primary signal for defense gene activation. Plant Physiol 117:687–693

Bowles DJ (1993) Local and systemic signals in the wound response. Semin Cell Biol 4:103–111

Bradford M (1976) A rapid and sensitive method for the quantification of proteins utilizing the principle of protein-dye binding. Anal Biochem 2:248–254

Brocker C, Lassen N, Estey T, Pappa A, Cantore M, Orlova VV, Chavakis T, Kavanagh KL, Oppermann U, Vasiliou V (2010) Aldehyde dehydrogenase 7A1 (ALDH7A1) is a novel enzyme involved in cellular defense against hyperosmotic stress. J Biol Chem 285:18452–18463

Brocker C, Vasiliou M, Carpenter S, Carpenter C, Zhang Y, Wang X, Kotchoni SO, Wood AJ, Kirch HH, Kopečný D, Nebert DW, Vasiliou V (2013) Aldehyde dehydrogenase (ALDH) superfamily in plants: gene nomenclature and comparative genomics. Planta 237:189–210

Busk PK, Jensen AB, Pagès M (1997) Regulatory elements in vivo in the promoter of the abscisic acid responsive gene *rab17* from maize. Plant J 11:1285–1295

Chinnusamy V, Schumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot 55:225–236

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Delessert C, Wilson IW, Van Der Straeten D, Dennis ES, Dolferus R (2004) Spatial and temporal analysis of the local response to wounding in *Arabidopsis* leaves. Plant Mol Biol 55:165–181

Finkelstein RR (1994) Mutations at two new *Arabidopsis* ABA response loci are similar to the *abi3* mutations. Plant J 5:765–771

Fong WP, Cheng CH, Tang WK (2006) Antiquitin, a relatively unexplored member in the superfamily of aldehyde dehydrogenases with diversified physiological functions. Cell Mol Life Sci 63:2881–2885

Frisch DA, Harris-Haller LW, Yokubaitis NT, Tomas TL, Hardin SH, Hall TC (1995) Complete sequence of the binary vector BIN 19. Plant Mol Biol 27:405–409

Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. J Plant Res 124:509–525

Gallagher RC, Van Hove JLK, Scharer G, Hyland K, Plecko B, Waters PJ, Mercimek-Mahmutoglu S, Stockler-Ipsiroglu S, Salomons GS, Rosenberg EH, Struys EA, Jakobs C (2009) Folic acid-responsive seizures are identical to pyridoxine-dependent epilepsy. Ann Neurol 65:550–556

Guerrero FD, Jones JT, Mullet JE (1990) Turgor-responsive gene-transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol 15:11–26

Guiltinan MJ, Marcotte WR Jr, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250:267–271

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. Nucl Acids Res 27:297–300

Hobo T, Asada M, Kowyama Y, Hattori T (1999) ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant J 19:679–689

Howe GA (2004) Jasmonates as signals in the wound response. J Plant Growth Regul 23:223–237

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kao CY, Cocciolone SM, Vasil IK, McCarty DR (1996) Localization and interaction of the *cis*-acting elements for abscisic acid, VIVIPAROUS1, and light activation of the *C1* gene of maize. Plant Cell 8:1171–1179

Kirch H–H, Bartels D, Wei Y, Schnable PS, Wood AJ (2004) The ALDH gene superfamily of *Arabidopsis*. Trends Plant Sci 9:371–377

Kirch H–H, Schlingensiepen S, Kotchoni OS, Sunkar R, Bartels D (2005) Detailed expression analysis of selected genes of the aldehyde dehydrogenase (ALDH) gene superfamily in *Arabidopsis thaliana*. Plant Mol Biol 57:315–332

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet 61:385–393

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

Kotchoni SO, Kuhns C, Ditzer A, Kirch HH, Bartels D (2006) Over-expression of different aldehyde dehydrogenase genes in *Arabidopsis thaliana* confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress. Plant Cell Environ 29:1033–1048

Lee P, Gelbart T, Kuhl W, Kamimura T, West C, Beutler E (1994) Homology between a human protein and a protein of the green garden pea. Genomics 21:371–378

Lemieux B, Miquel M, Somerville CR, Browse J (1990) Mutants of *Arabidopsis* with alterations in seed lipid fatty acid composition. Theor Appl Genet 80:234–240

Léon-Kloosterziel KM, Gil MA, Ruijs GJ, Jacobsen SE, Olszewski NE, Schwartz SH, Zeevaart JA, Koornneef M (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. Plant J 10:655–661

Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F, Giraudat J (1994) Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science 264:1448–1452

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 JF, Park E, von Arnim AG, Nebenführ A (2009) The FAST technique: a simplified *Agrobacterium*-based transformation method for transient gene expression analysis in seedlings of Arabidopsis and other plant species. Plant Methods 5:6

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Hugueney P, Frey A, Marion-Poll A (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. EMBO J 15:2331–2342

Matsui K (2006) Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr Opin Plant Biol 9:274–280

McConn M, Browse J (1996) The critical requirement for linolenic acid is pollen development, not photosynthesis, in an Arabidopsis mutant. Plant Cell 8:403–416

Mehrotra R, Mehrotra S (2010) Promoter activation by ACGT in response to salicylic and abscisic acids is differentially regulated by the spacing between two copies of the motif. J Plant Physiol 167:1214–1218

Mills PB, Struys E, Jakobs C, Plecko B, Baxter P, Baumgartner M, Willemsen MA, Omran H, Tacke U, Uhlenberg B, Weschke B, Clayton PT (2006) Mutations in antiquitin in individuals with pyridoxine-dependent seizures. Nat Med 12:307–309

Miquel M, Browse J (1992) Arabidopsis mutants deficient in polyunsaturated fatty acid synthesis. Biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase. J Biol Chem 267:1502–1509

Missihoun TD, Schmitz J, Klug R, Kirch HH, Bartels D (2011) Betaine aldehyde dehydrogenase genes from *Arabidopsis* with different sub-cellular localization affect stress responses. Planta 233:369–382

Missihoun TD, Kirch HH, Bartels D (2012) T-DNA insertion mutants reveal complex expression patterns of the *aldehyde dehydrogenase 3H1* locus in *Arabidopsis thaliana*. J Exp Bot 63:3887–3898

Narusaka Y, Nakashima K, Shinwari ZK, Sakuma Y, Furihata T, Abe H, Narusaka M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis rd29A* gene in response to dehydration and high-salinity stresses. Plant J 34:137–148

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J 31:1–12

Peña-Cortés H, Fisahn J, Willmitzer L (1995) Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. Proc Natl Acad Sci USA 92:4106–4113

Perozich J, Nicholas HB Jr, Wang BC, Lindahl R, Hempel J (1999) Relationships within the aldehyde dehydrogenase extended family. Prot Sci 8:137–146

Prestridge DS (1991) SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. Comput Appl Biosci 2:203–206

Raza H (2010) Functional characterization of transgenic *Arabidopsis thaliana* plants co-overexpressing aldehyde dehydrogenases and genes for soluble osmolytes. Ph.D. thesis, University of Bonn, Bonn, Germany

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Petersen L, Okamoto H, Knight H, Peck SC, Grierson CS, Hirt H, Knight MR (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. Nature 427:858–861

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 12:707–720

Rodrigues SM, Andrade MO, Gomes APS, Damatta FM, Baracat-Pereira MC, Fontes EPB (2006) *Arabidopsis* and tobacco plants ectopically expressing the soybean antiquitin-like ALDH7 gene display enhanced tolerance to drought, salinity, and oxidative stress. J Exp Bot 57:1909–1918

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

Salinas J, Oeda K, Chua NH (1992) Two G-Box-related sequences confer different expression patterns in transgenic tobacco. Plant Cell 4:1485–1493

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York (ISBN:0-87969-309-6)

Shen Q, Zhang P, Ho TH (1996) Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell 8:1107–1119

Shen Y, Zhang Y, Yang C, Lan Y, Liu L, Liu S, Chen Z, Ren G, Wan J (2012) Mutation of *OsALDH7* causes a yellow-colored endosperm associated with accumulation of oryzamutaic acid A in rice. Planta 235:433–441

Shin JH, Kim SR, An G (2009) Rice aldehyde dehydrogenase7 is needed for seed maturation and viability. Plant Physiol 149:905–915

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227

Simpson SD, Nakashima K, Narusaka Y, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Two different novel *cis*-acting elements of *erd1*, a *clpA* homologous *Arabidopsis* gene function in induction by dehydration stress and dark-induced senescence. Plant J 33:259–270

Sophos NA, Vasiliou V (2003) Aldehyde dehydrogenase gene superfamily: the 2002 update. Chem Biol Interact 143–144:5–22

Sprenger-Haussels M, Weisshaar B (2000) Transactivation properties of parsley proline-rich bZIP transcription factors. Plant J 22:1–8

Stintzi A, Browse J (2000) The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci USA 97:10625–10630

Stiti N, Missihoun TD, Kotchoni SO, Kirch H–H, Bartels D (2011) Aldehyde dehydrogenases in *Arabidopsis thaliana*: biochemical requirements, metabolic pathways, and functional analysis. Front Plant Sci 2:65

Stroeher VL, Boothe JG, Good AG (1995) Molecular cloning and expression of a turgor-responsive gene in *Brassica napus*. Plant Mol Biol 27:541–551

Svensson JT, Crosatti C, Campoli C, Bassi R, Stanca AM, Close TJ, Cattivelli L (2006) Transcriptome analysis of cold acclimation in barley *Albina* and *Xantha* mutants. Plant Physiol 141:257–270

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis *myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5:1529–1539

Wu C, Su S, Peng Y (2007) Molecular cloning and differential expression of an aldehyde dehydrogenase gene in rice leaves in response to infection by blast fungus. Biologia 62:523–528

Zhang W, Ruan J, Ho TH, You Y, Yu T et al (2005) Cis-regulatory element based targeted gene finding: genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. Bioinformatics 21:3074–3081

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. Plant Physiol 136:2621–2632