T-DNA insertion mutants reveal complex expression patterns of the aldehyde dehydrogenase 3H1 locus in Arabidopsis thaliana

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Abstract

The Arabidopsis thaliana aldehyde dehydrogenase 3H1 gene (ALDH3H1; AT1G44170) belongs to family 3 of the plant aldehyde dehydrogenase superfamily. The full-length transcript of the corresponding gene comprises an open reading frame of 1583 bp and encodes a protein of 484 amino acid residues. Gene expression studies have shown that this transcript accumulates mainly in the roots of 4-week-old plants following abscisic acid, dehydration, and NaCl treatments. The current study provided experimental data that the ALDH3H1 locus generates at least five alternative transcript variants in addition to the previously described ALDH3H1 mRNA. The alternative transcripts accumulated in wild-type plants at a low level but were upregulated in a mutant that carried a T-DNA insertion in the first exon of the gene. Expression of the transcript isoforms involved alternative gene splicing combined with an alternative promoter. The transcript isoforms were differentially expressed in the roots and shoots and showed developmental stage- and tissue-specific expression patterns. These data support the hypothesis that alternative isoforms produced by gene splicing or alternative promoters regulate the abundance of the constitutively spliced and functional variants.

Key words: abiotic stress, alternative first exon, alternative promoter, alternative splicing, nonsense-mediated mRNA decay.

Introduction

The Arabidopsis thaliana aldehyde dehydrogenase 3H1 (AT1G44170) gene (ALDH3H1) belongs to the aldehyde dehydrogenase (ALDH) superfamily. ALDHs are NAD(P)⁺-dependent enzymes (ALDH, EC 1.2.1.3), which are widely distributed in all organisms including human and plant genomes (Sophos and Vasiliiou, 2003; Stiti et al., 2011). The Arabidopsis genome contains 14 members of the ALDH gene family encoding proteins belonging to nine families (Kirch et al., 2004). ALDH3H1 belongs to family 3, which has two other members, ALDH3I1 and ALDH3F1. These three proteins share 60% amino acid homology. Phylogenetic analyses have shown that ALDH3H1, ALDH3I1, and ALDH3F1 are separated from other plant ALDHs and betaine aldehyde dehydrogenases (Kirch et al., 2004). ALDH3H1, ALDH3I1, and ALDH3F1 from A. thaliana and the family 3 ALDH from the desiccation-tolerant plant species Craterostigma plantagineum (Cp-ALDH) were found to be closely related to bacteria and animal ALDHs (Stiti et al., 2011). One feature of the ALDH3H1 locus is the presence of an intron larger than 1 kb. This distinguishes ALDH3H1 from all members of the Arabidopsis ALDH gene family. Whether this difference has any influence on the expression and function of the gene is so far unclear. The stress-related Cp-ALDH gene was found to be induced following abscisic acid (ABA) and dehydration treatments (Kirch et al., 2001). Similar to Cp-ALDH, ALDH3H1 is upregulated following ABA, NaCl, heavy metals, and dehydration treatments, and its ectopic expression in Arabidopsis confers abiotic stress tolerance to transgenic plants (Sunkar et al., 2003). Although

Abbreviations: ABA, abscisic acid; ALDH, aldehyde dehydrogenase; EST, expressed sequence tag; FAST, fast Agrobacterium-mediated seedling transformation; GUS, β-glucuronidase; MDA, malondialdehyde; MS, Murashige and Skoog; SR, serine/arginine-rich; UTR, untranslated region; WT, wild type.

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ALDH3F1 is constitutively expressed, plants overexpressing ALDH3F1 performed slightly better under stress conditions than wild-type (WT) plants (Stiti et al., 2011). Gene expression studies have shown that ALDH3H1 transcripts accumulate mainly in the roots of 4-week-old plants in response to ABA, dehydration, and NaCl (Kirch et al., 2001; Stiti et al., 2011), but the function of ALDH3H1 remains yet elusive.

Alternative gene splicing confers more flexibility to the transcriptome and has been studied extensively in animal species (Blencowe, 2006). About 20% of Arabidopsis and rice genes are predicted to undergo alternative splicing based on expressed sequence tag (EST)/cDNA analyses (Campbell et al., 2006; Wang and Brendel, 2006). More recently, Filichkin et al. (2010) reported that a minimum of 42% of intron-containing genes in Arabidopsis are alternatively spliced, as deduced from in silico analysis. Reports on genes with alternatively spliced transcripts in plants, and particularly in Arabidopsis, are likely to increase in the future. Several plant genes have been demonstrated experimentally to undergo alternative splicing. These genes belong to diverse pathways such as primary metabolism, photosynthesis, flowering, and homeostasis under biotic and abiotic stress conditions (Reddy, 2007; Mastrangelo et al., 2011). A subset of these genes encode transcription factors whereby they participate in the regulation of gene expression (Li et al., 2006). One example is the extensive alternative splicing events common to transcripts encoding members of the serine/arginine-rich (SR) protein family, which are known to function in splice site recognition and spliceosome assembly (Reddy, 2007, and references therein). Comprehensive analyses of alternative splicing of pre-mRNAs of Arabidopsis SR genes have indicated that 16 of the 20 SR genes displayed alternative splicing events (Palusa et al., 2007; Tanabe et al., 2007). About 95 transcripts are produced from 15 SR genes, thereby increasing the complexity of the SR gene transcriptome 6-fold (Palusa et al., 2007). Although alternative gene splicing has often been reported in plants as a principal component of gene regulation, it is difficult to address whether all alternatively produced transcripts have a biological function. Gene redundancy may further complicate functional analyses, if mutants of the gene do not have a visible phenotype. Careful analyses of alternatively spliced variants are therefore required to understand the biological relevance of splicing events and associated transcripts.

Transcription of some eukaryotic genes can be driven by multiple promoters (Landry et al., 2003; Chen et al., 2007). Each promoter determines a specific transcription start site with a first exon and, accordingly, generates a different alternative first exon transcript. Typically, an alternative first exon is defined as the first exon of one splice variant of a gene, which is either located downstream of a corresponding alternative first exon or absent from other variants (Luzi et al., 2000; Kimura et al., 2006). Using alternative first exons is a variant of alternative splicing that many eukaryotes employ to generate several different transcripts from a single gene (Kornblüth, 2005; Chen et al., 2007). Alternative first exons can be produced by alternative promoters, alternative splicing of gene transcripts or through a combination of both mechanisms. Reports on alternative first exons are derived mainly from mammalian genomes, especially mouse and human (Landry et al., 2003). Recently, a genome-scale analysis was performed on rice and Arabidopsis (Chen et al., 2007), which found that 5.9 and 5% of the total expressed genes in rice and Arabidopsis contained alternative first exons, respectively. About 58% of alternative first exon-containing gene structures are derived from alternative promoters. For instance, the Arabidopsis RAD21-like gene SYN1 is involved in meiosis and was shown to produce two transcripts with different 5' ends, with one transcript starting within the first intron of the other one (Bai et al., 1999). Likewise, the most upstream intron of a rice MADS box gene, OsMADS1, was reported to direct gene expression in flowers, whereas the 5' upstream promoter region lacking the first intron mediated the expression in both vegetative and reproductive tissues (Jeon et al., 2008). Koo et al. (2009) reported the existence of alternative promoters within the rice MAP kinase gene OsBWMK1 and their differential regulation. Although the mechanism is not well understood, the use of alternative promoters and/or alternative gene splicing appears to be a widespread mechanism to increase transcriptome complexity in all organisms. The generation of alternative transcripts is seen as an additional way of creating regulatory diversity and provides a mechanism to synthesize functionally related proteins acting together to mediate a specific biological response (Morello et al., 2002; Parsley and Hibberd, 2006; Qi et al., 2007).

In the current study, when using T-DNA insertion mutants to investigate the function of the A. thaliana ALDH3H1 gene, it was observed that one mutant line accumulated five alternatively spliced isoforms of ALDH3H1 (ALDH3H1-β, ALDH3H1-γ, ALDH3H1-δ, ALDH3H1-e and ALDH3H1-ε). These transcript isoforms were also found in WT plants at a lower level in addition to the major and previously known isoform ALDH3H1-a. Both the major and the alternative splicing isoforms showed tissue- and developmental stage-dependent expression patterns and were stress inducible. The study revealed the existence of an alternative promoter within the large intron located between the first and second exon. The integrity of this intron was necessary for the correct WT expression of the alternative isoforms. The observations indicated that ALDH3H1 expression may be regulated through both the use of the alternative promoter and gene splicing. The biological implications of these observations are discussed.

Materials and methods

Plant material and growth conditions

A. thaliana ecotype Col-0 was used throughout this study. WT and transgenic plants were grown on Murashige and Skoog (MS) agar plates (Murashige and Skoog, 1962) or in soil under white light. The soil was a 3:1 mixture of potting soil (Rolfs Gärtnereiência, 2000).
Siegburg, Germany) and vermiculite. The intensity of the light was approximately 120–150 μE m⁻² s⁻¹ with a 16 h photoperiod at 22 °C. Tissues of 14-day-old and 6-week-old plants were used for gene expression analyses. Inflorescence, leaf, and root tissues were harvested separately after stress treatment and frozen immediately in liquid nitrogen.

Isolation of homozygous T-DNA insertion mutants

Homozygous T-DNA insertion mutants were isolated by two consecutive PCR assays. The first assay involved the use of two gene-specific primers. In the second assay one gene-specific primer was combined with one T-DNA-specific primer. The T-DNA-specific primers S2, T1, and S3 were used for the lines 3h1-A, 3h1-B, and 3h1-C (see Results), respectively. The choice of the gene-specific primer pair was made according to the predicted insertion site and was such that the region to be amplified encompassed the insertion site. Thus, the primer pairs P1/P5 and P1/S2 were used for the line 3h1-A, primer pairs P1/P5 and P1/T1 for the line 3h1-B and primer pairs P1/P5 and P1/S3 for the line 3h1-C. All primers used in this work are listed in Table 1.

RNA isolation and first-strand cDNA synthesis

Isolation of total RNA and RT-PCR analyses were performed as described by Misshou et al. (2011). Gene-specific primers used to amplify first-strand cDNAs are described in Results. Transcript sequences of the A. thaliana Actin2 (AT3G18780) gene were used as reference (An et al., 1996) and amplified with the primers Ath_Actin_2_fwdM 5′-CCAATACCCAGAGACACCTATAAC-3′ and Ath_Actin_2_rev 5′-AGGATACTTCACTCAGAAATTTGTTTC-3′. The parameters of the standard PCR assay were as follows: 5 min at 94 °C, 30 cycles (30 s at 94 °C, 45 s at 62 °C, and 90 s at 72 °C), and 5 min at 72 °C, followed by holding at 4 °C.

Protein-blot analysis

Protein expression was investigated by protein blotting followed by immunodetection using a polyclonal antiserum raised against the ALDH3H1 recombinant protein. Crude protein extracts were separated by 12% SDS-PAGE and electroblotted from the gel onto a nitrocellulose Protran BA-85 membrane (Whatman, Dassel, Germany) at 0.1 V for 1 h in pre-chilled protein-blot transfer buffer [25 mM Tris/HCl (pH 8.3), 192 mM glycerine, 20% (v/v) methanol] (Towbin et al., 1979). The membrane was stained with Ponceau Red solution (0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid) and then blocked for 1 h at room temperature or overnight at 4 °C. The blocking solution contained 0.1% (v/v) Tween 20 and 4% (w/v) non-fat dried milk powder dissolved in 1× TBS [20 mM Tris/HCl (pH 7.5), 150 mM NaCl]. The membrane was probed with ALDH3H1 antiserum diluted 1:5000. The antiserum was produced by BioGenes (Berlin, Germany) from affinity-purified recombinant ALDH3H1 protein and kindly provided by Dr Andrea Ditzer. The immunodetection assay was performed using an ECL Plus Western Blotting Detection Kit (Amersham, Braunschweig, Germany). Signals were detected under a CCD camera (Intelligent Dark Box II; FujiFilm Corp., Tokyo, Japan).

Molecular cloning and generation of transgenic plants

Amplons corresponding to the complete transcript or part of isogenes were obtained by PCR using different primer pairs (Table 1). First-strand cDNA was used as template for the PCR. The amplified cDNA fragments were purified directly from the PCR or from agarose gels after electrophoresis using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Purified fragments were cloned into the plasmid vector pJET1.2 provided in the CloneJET™ PCR Cloning Kit (Fermentas; St Leon-Rot, Germany). All fragments were sequenced from recombinant plasmids with the pJET1.2 primers or gene-specific primers. The sequences were aligned with the ALDH3H1 gene sequence (AT1G44170).

To generate the ALDH3H1 intron-β-glucuronidase (GUS) expression cassette, the region of intron 1 (+214 to +1457) between exon 1 and exon 2 of the ALDH3H1 gene locus (AT1G44170) was amplified from A. thaliana genomic WT (Col-0) DNA using the primers INT-Xba-fwd: 5′-GCGAGCTCAGAGAGGATTCTTTTTTCTCTC-3′ and INT-Xba-rev: 5′-CTGAGCTCTAGATATAAATGAGGAAAGGTCAGTG-3′. The primers were designed with an XbaI restriction site (underlined) to facilitate cloning. The expected 1244 bp PCR product was eluted from the agarose gel, digested with XbaI, and exchanged with the ALDH7B4 gene promoter fragment in the plasmid 7βG, a pBin19-derived plasmid, which contains the gene cassette pJET1.2-promoter::GUS::nos polyA. The resulting 3h1-intron::GUS::nos polyA gene was transformed into the reporter gene GUS driven by the ALDH3H1 intron 1 fragment. The construct was cloned into Agrobacterium tumefaciens cells. Recombinant clones were initially screened by PCR using the primers INT-Xba-rev and pBIN-HindIII: 5′-AGCTATGACCTGATTACGCCAACAG-3′. A. thaliana cells were used to transiently transform A. thaliana seedlings by a FAST (fast Agrobacterium-mediated seedling transformation) protocol (Ai et al., 2009).

To generate transgenic plants that ectopically expressed the ALDH3H1 protein, a BamHI cDNA (GenBank accession no. AY072122) fragment (1730 bp) containing the full-length ALDH3H1 coding region was isolated from plasmid pda06974 (RIKEN Institute) by BamHI restriction and subcloned into the BamHI site of the binary pROK2 vector (Baulcombe et al., 1986). The recombinant plasmid was introduced into A. tumefaciens. One A. tumefaciens clone harbouring the CuMV35S::ALDH3H1 fusion construct was used to transform A. thaliana WT plants (ecotype Col-0) by the floral dip method (Clough and Bent, 1998).

Histochemical detection of GUS activity

In situ detection of GUS activity was performed as described by Jefferson et al. (1987). Transiently transformed seedlings were incubated in GUS staining buffer [0.5 mg/ml X-Gluc, 50 mM

**Table 1.** Primers used for genotyping the T-DNA insertion mutants and characterizing the spliced isoforms.

| ALDH3H1   | Primer sequence (5′→3′) |
|-----------|------------------------|
| gene-specific primers |                        |
| P1        | AGAAGGTGTTTGGATCGGCGG   |
| P2        | ATGGTGTACCAACAGAAGATTAC |
| P3        | CAGCTAAGAAGAAGCAGTGGCCT |
| P4        | CGTTCGGGCAGCTAACGATAG   |
| P5        | TCAACCAATAGTGCTAGTTAG   |
| INT       | TCTTTGATTAGTCTCGTCCTTCC |
| F7        | TGTCCTGCGATTACTAGGAAG  |
| F6        | GAGAATACGATATGGGAGC    |
| E9R       | TGAAGAGAGGAGCCATAATTTTC |
| E3R       | GAGCCATCAGGCTTCTGAGGGA |
| I7        | GCCATACATCTAAGAGAAGAAAGAG |
| I5        | AACACACAAATAGCCGGAAATGTC |
| iR        | ACAAGCGCAAGAAAGAAGAC  |
| R1        | TAGTACTCTGCTGGTAAAGAA  |

a LB3 (Sessions et al., 2002).
NaH₂PO₄ 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 (SMZ-800; Nikon, Düsseldorf, Germany).

Stress treatments and measurement of malondialdehyde (MDA) content

For gene expression analysis, 15-day-old homozygous seedlings were removed from MS agar plates and either dehydrated on filter paper at room temperature for 1 h or incubated in 200 mM NaCl at room temperature for 4 h. Six-week-old plants were removed from soil and incubated in water or 250 mM NaCl for 6 h. Roots and shoots or leaves were harvested separately and analysed. To assay plant performance under stress, seeds were sown on MS agar containing different concentrations of NaCl. A germination test was performed as described by Jung et al. (2008) with minor modifications. Drought stress was imposed on 5-week-old plants by withholding watering for 14 d. Leaf samples were collected for biochemical analyses. To examine the salt tolerance of 5-week-old and soil-grown plants, they were watered every 2 d with 0–300 mM NaCl solutions for 14 d. The degree of peroxidation of membrane lipids was quantified by measuring MDA levels (Missihoun et al., 2011).

Root growth inhibition assay

Approximately 50 seeds were germinated on MS agar containing 2%/ (w/v) sucrose and grown for 6 d. Seedlings were then transferred to fresh MS agar plates containing 100 mM NaCl. The plates were kept in a vertical position for 3 d. The increase in root length was measured for seedlings of each genotype. Root growth inhibition was estimated as a percentage of the increase in root length on the medium containing NaCl compared with the medium not supplemented with NaCl.

Results

Gene models of ALDH3H1

The ALDH3H1 (AT1G44170) gene, a member of the aldehyde dehydrogenase family, is composed of ten exons and nine introns. According to the Arabidopsis genome resource database TAIR (http://gbrowse.arabidopsis.org/), three protein-coding gene models can be assigned to the gene: AT1G44170.1, AT1G44170.2, and AT1G44170.3. Each gene model would generate a different transcript. The primary transcripts corresponding to the gene models AT1G44170.1 and AT1G44170.2 differ in their 5' untranslated (5' UTR) sequences (Fig. 1A). An additional intron in the 5' UTR splits the 5' UTR of AT1G44170.1 into two parts, the sequence of which overlaps with the intronless 5' UTR sequence of AT1G44170.2 (Fig. 1A). The protein-coding sequences of AT1G44170.1 and AT1G44170.2 are identical, each initiating at a single translational start codon (ATG) in exon 1, and they will therefore yield the same protein (53 kDa). The transcript model AT1G44170.3 predicts a shorter transcript, which lacks the first exon of the locus and would therefore code for a smaller protein (46 kDa) with a different N terminus. The gene model AT1G44170.3 suggests the existence of a short exon (exon 2') of 24 bp at the 5' end including a small 5' UTR in the first intron of the locus (Fig. 1A). Previous analyses did not focus on transcripts associated with each of these gene models and therefore the transcripts corresponding to AT1G44170.1 and AT1G44170.2 were studied as a single ALDH3H1 transcript encoding a 53 kDa protein (Kirch et al., 2001, 2005). The Arabidopsis EST clone DR182505 (Alexandrov et al., 2006) confirms the in silico prediction for AT1G44170.3. The use of different T-DNA insertion mutants allowed us to detect and analyse the expression patterns of several ALDH3H1 transcript isoforms including the one predicted by the gene model AT1G44170.3. For a better coherence of the text, transcripts corresponding to the models AT1G44170.1 and AT1G44170.2 are considered as the major isoform and are hereafter named ALDH3H1-a, whereas that corresponding to AT1G44170.3 is termed ALDH3H1-b.

T-DNA insertions reveal complex expression patterns of the ALDH3H1 gene

Two T-DNA insertion mutants (depicted in Fig. 1B) were identified from the Syngenta Arabidopsis Insertion Library collection (SAIL; McElver et al., 2001; Sessions et al., 2002) and seeds were obtained from the European Arabidopsis Stock Centre (NASC). The line SAIL_832_A05, hereafter named 3h1-A, carried a T-DNA insertion in exon 1 and the line SAIL_828_D05, hereafter named 3h1-C, had an insertion in exon 5 (red triangles in Fig. 1B). The lines 3h1-A and 3h1-C carried the same T-DNA (4763 bp) derived from plasmid pDAP101 (McElver et al., 2001; Sessions et al., 2002). A third ALDH3H1 T-DNA insertion, the line KO69, hereafter renamed 3h1-B, was obtained from the collection described by Rios et al. (2002) and carried a T-DNA fragment (7355 bp) in the first intron of ALDH3H1 (yellow triangle in Fig. 1B). The T-DNA fragment in the line 3h1-B was derived from plasmid pPCV6NFHyg (Mathur et al., 1998). Homozygous plants were isolated for each T-DNA mutant (Fig. 2A); transcripts from the lines were examined by RT-PCR with different primer combinations (for primers, see Fig. 1B and Table 1). The homozygous 3h1-A plants were initially screened using the gene-specific primers P1 and P5 to amplify the full-length transcript ALDH3H1-a. The results showed that line 3h1-A did not accumulate the expected transcript ALDH3H1-a. Putative transcripts among the RT-PCR products were detected as a single band on agarose gels when primer P1 was replaced by primer P4, which is located downstream of the T-DNA insertion site (Figs 1B and 2B). This observation was examined in detail. We hypothesized that this amplicon could correspond to the transcript variant ALDH3H1-b based on the gene model predictions on the TAIR webpage. Two additional primers were designed for RT-PCR experiments to identify the transcripts. One primer (P2) covered the first 22 nt of exon 2' and the other primer (P3) was located in exon 2 (Fig. 1B). Primer P2 was designed to amplify specifically the transcript ALDH3H1-b, whereas primers P3 and P4 would not discriminate ALDH3H1 transcript isoforms with variants upstream of exon 2.
Consistent with our hypothesis, an amplicon of the expected size was obtained from the homozygous \(3h1-A\) mutant using primers P2 and P5 (Fig. 2C). To verify that the amplicon was also present in the WT, the primer pairs P2/E9R and P2/P5 were used in RT-PCR experiments using total RNA from WT plants with varying PCR cycle numbers. The results showed that the same amplicons were produced in WT and \(3h1-A\) plants (Fig. 2D).

Another RT-PCR-based approach was used to map the length of the transcript from which the amplicon was derived. Primers F6 and F7 are further upstream of exon 2\(^\#\) and were included in the transcript analyses. The results revealed the presence of additional amplicons (Fig. 3). Whether these amplicons represented some alternative transcripts generated through alternative splicing or were PCR artefacts was examined. Primers were designed to obtain cDNA fragments spanning the gene from exon 1 to a few nucleotides beyond exon 10 (Fig. 1B; Table 1). RT-PCR experiments were conducted using total RNA from WT and \(3h1-A\) plants, and the amplicons were cloned and sequenced. The alignment of DNA sequences of cDNA fragments obtained by RT-PCR to the gene sequence confirmed the existence of additional alternative splicing variants of the \(ALDH3H1\) gene, mostly generated through intron-retention-type alternative splicing events. As shown in Fig. 4, five novel splicing variants of the \(ALDH3H1\) primary mRNA were identified and named: \(ALDH3H1-\beta\), \(ALDH3H1-\gamma\), \(ALDH3H1-\delta\), \(ALDH3H1-\epsilon\) and \(ALDH3H1-\zeta\). Because clones were chosen randomly during the cloning procedure, it could not be excluded that additional splicing variants occurring at low frequency were not detected.

**Only full-length \(ALDH3H1\) protein accumulates in \(A.\ thaliana\)**

The nucleotide sequences of the five newly isolated isoforms were analysed \textit{in silico}. Four of them contain at least one premature termination codon that would yield a truncated version of the \(ALDH3H1\) protein. The locations of these premature termination codons are indicated by asterisks in Fig. 4. Only isoform \(ALDH3H1-\beta\) corresponding to the gene model AT1G44170.3 did not harbour any premature termination codon and would therefore encode a truncated \(ALDH3H1\) protein (\(ALDH3H1-\beta\)). Depending on the translation start codon used, the putative protein \(ALDH3H1-\beta\) is predicted to be 421 aa (46.2 kDa) or 395 aa (43 kDa). The accumulation of \(ALDH3H1-\beta\) was investigated. Equal amounts of protein extracts from the WT, the homozygous \(3h1-A\), \(3h1-B\), and \(3h1-C\) mutants, and two independent lines overexpressing the protein \(ALDH3H1-\alpha\) were analysed by protein blots followed by immunodetection using antibodies raised against \(ALDH3H1\). In comparison with the WT and the \(ALDH3H1\) overexpressors, no expression of the \(ALDH3H1\) protein (53.2 kDa) was found in the T-DNA insertion mutants (Fig. 5). In addition, no truncated protein of the size of \(ALDH3H1-\beta\) was identified. The extra band
marked by an asterisk in Fig. 5 was intrinsic to the antiserum and not to ALDH3H1 because this band was specific to ALDH3H1-α, whereas P2/P5 and P2/E9R allowed amplification of ALDH3H1-β. Primer pairs P3/P5 and P4/P5 could not discriminate between the variants. Genomic WT DNA (Col-0) was used as control (G), while (−) denotes a control reaction without nucleic acid. M, DNA size marker. See Fig. 1B for primer locations.

An alternative promoter and alternative splicing regulate expression of the ALDH3H1 gene at the transcription level

No amplicon was obtained from the RT-PCR assays when primer F7 was replaced by primer INT, which is located about 400 bp upstream of F7 (Fig. 1B). This suggests that either the 5’ end of the newly identified alternative ALDH3H1 transcripts locates between these primers or other splicing events occur in the region between the primers INT and F7. In agreement with this, the existence of a 5’-end EST clone (DR182505; Alexandrov et al., 2006) that corresponds to the isoform ALDH3H1-β described in this work suggests that isoforms may exist that are produced from an alternative promoter located within intron 1 upstream of exon 2’ (Fig. 1B). To investigate whether a functional alternative promoter is present within the ALDH3H1 locus, the expression pattern of the major isoform ALDH3H1-α was first compared with that of the alternative isoforms. Primer P2 (located in exon 2’; Fig. 1B) was used to discriminate between ALDH3H1-α and the alternative variants because exon 2’ is present only in the latter. When homozygous and heterozygous 3h1-A plants were examined by RT-PCR, the heterozygous plants (3h1-3h1-Aa) expressed both ALDH3H1-α and the alternative transcripts, in contrast to homozygous (3h1-3h1-AA) plants, which expressed only the alternative transcripts (Fig. 6A). The strength of the signal obtained from 3h1-3h1-Aa samples was about half of that derived from the homozygous 3h1-3h1-AA plants, suggesting that the presence of the T-DNA in exon 1 could have enhanced expression of the alternative transcripts. This is supported by the observation that the WT accumulated lower levels of the alternative transcripts than the homozygous 3h1-A plants (Fig. 2D). Transcription of the major and alternative transcript variants may be differentially activated; therefore, the relative abundance of the isoforms was studied in a subsequent experiment. Semi-quantitative RT-PCR assays were conducted using total RNA from WT and homozygous 3h1-A plants. The primer pairs P1/E9R and P2/E9R were used to amplify ALDH3H1-α and the alternative isoforms, respectively. As shown in Fig. 6B (reactions R1 and R2), the WT accumulated more of the isoform ALDH3H1-α than the alternative isoforms. In contrast, higher levels of the alternative isoforms were present in 3h1-A mutant plants than in the WT (Fig. 6C).

When different organs were examined, both development-dependent and tissue-specific expression patterns were observed among the variants (Fig. 6D). The major isoform ALDH3H1-α was present but at a lower level in WT seedlings than in fully expanded leaves and flowers. The WT contained lower amounts of the alternative isoforms in seedlings and leaf tissues than in flowers. In contrast, the
alternative isoforms were upregulated in all examined tissues of the 3h1-α line. Differences in expression were also seen among isoforms. For example, amplicon 1 appeared to be downregulated in flowers, whereas amplicon 2 was constitutively expressed in all tissues (Fig. 6D, panel P2/E9R). A similar differential expression was observed for the isoforms δ, ε and ζ (Fig. 6D, panels P2/I5 and P2/I7). These observations indicate that isoforms are differentially regulated and support the hypothesis that these transcripts are expressed from different promoters.

To test the activity of a putative alternative promoter, the entire intron region (+214 to +1457) between exon 1 and exon 2' was amplified from WT A. thaliana (Col-0) genomic DNA and subcloned in front of the reporter gene GUS to generate the expression cassette 3h1-intron::GUS::nospolyA. This construct was functionally analysed in A. thaliana
seedlings. Transiently transformed seedlings were found to express the reporter protein in roots and cotyledons (Fig. 7). In silico analysis of intron 1 of the ALDH3H1 locus using PLACE Web Signal Scan (Prestridge, 1991; Higo et al., 1999) revealed the presence of several cis elements. Two dehydration-responsive element/C-repeat–low temperature-responsive elements (DRE/CRT) and one ACGT box (a putative ABA responsive element) were found at 366, 314, and 333 nt upstream of the predicted translational start codon ATG in exon 2’, respectively. Using the PlantCare tool for in silico analysis of promoter sequences (Lescot et al., 2002), the closest predicted TATA box and the corresponding transcription start signal were found at 133 and 103 nt, respectively, upstream of the predicted translational start codon ATG in exon 2’. Only two nucleotides separate the predicted transcription start site from the first nucleotide of the 5’ EST clone DR182505 of ALDH3H1 (Alexandrov et al., 2006). This indicates that the intronic fragment contains functional cis elements able to direct the transcription of a downstream protein-coding DNA fragment. Analysis of the genomic DNA sequence in this locus revealed that the classical plant mRNA splicing acceptor site does not exist at the junction between the intron sequence and exon 2’. The two nucleotides TA terminate the intronic region upstream of the exon 2’ instead of AG found in the consensus sequence (GT...AG) at the intron-exon junction of plant protein-coding genes (Reddy, 2007). Our findings support the existence of an alternative promoter within intron 1. This promoter may direct expression of the ALDH3H1 alternative transcripts, and particularly that of ALDH3H1-β.

Examination of dehydration and salt stress responses in the ALDH3H1 mutants

Several Arabidopsis ALDH genes have been shown to be stress responsive and their ectopic expression in transgenic plants leads to stress tolerance (Sunkar et al., 2003; Kirch et al., 2005; Kotchoni et al., 2006). Although no protein corresponding to the alternative transcript ALDH3H1-β had been identified in WT or mutant plants so far, it is possible that ALDH3H1-β, in addition to the other alternative transcript variants, is functional at the RNA level. To test whether the alternative ALDH3H1 transcript isoforms were stress responsive, the accumulation patterns were examined in 3h1-A plants in response to dehydration or NaCl treatment. Fifteen-day-old homozygous 3h1-A seedlings or 6-week-old heterozygous 3h1-A plants were used to simultaneously monitor ALDH3H1-x (the major isoform) and the alternative ALDH3H1 isoforms. Primers P1 and P2 were used in combination with primer P5 in the RT-PCR assays as described above. Primer pair P1/P5 amplified the major variant, P2/P5 the alternative isoforms, and P4/P5 all isoforms. As shown in Fig. 8A, the alternative isoforms accumulated at a low level in seedling roots following NaCl treatment but decreased in the shoot. In adult plants, they were upregulated by salt treatment in both roots and leaves. In contrast, ALDH3H1-x did not increase in the roots but increased in the leaves following salt treatment (Fig. 8B).

Based on these observations, the contribution of the alternative isoforms was investigated further with respect to abiotic stress. The salt stress response was examined in the mutant line 3h1-A, which accumulates high levels of the alternative isoforms compared with the WT. Line 3h1-C, which is a null-mutant of ALDH3H1, was also included in the experiment. Inhibition of root growth was assayed for all genotypes. As shown in Fig. 9A, homozygous 3h1-A (3h1-AA) and 3h1-C mutants were affected in their root growth to the same extent, and this was more severe than in the WT and the heterozygous 3h1-Aa, which showed similar phenotypes. These observations indicated that the alternative transcript variants do not functionally compensate for the lack of the major isoform ALDH3H1-x under salt stress, at least for root growth inhibition by salt. The results also showed that mutants lacking ALDH3H1-x are sensitive to salt stress.

Because the major transcript ALDH3H1-x was also responsive to stress, it was ectopically expressed in stably transformed A. thaliana plants. Several independent transgenic lines that overexpress the full-length ALDH3H1 protein were obtained. Results of the protein-blot analysis for two representative lines (S10 and S13) are shown in Fig. 5. The WT and transgenic plants were sown on MS agar medium containing different concentrations of NaCl and the germination rate was scored. The WT and transgenic plants were similarly affected in their germination rate and seedling growth (Fig. 9B). No difference was seen between 3h1-B and WT plants with regard to seed germination rate on medium supplemented with NaCl.
However, line S13 similar to the other tested ALDH3H1-overexpressing lines, accumulated less MDA, a measure of the extent of membrane peroxidation, than the WT and significantly less than the mutant 3h1-B (Fig. 9C). The salt stress response was also examined in adult WT and ALDH3H1-overexpressing plants. Similar MDA contents were found in all lines after 14 d of salt stress (Fig. 9D). When the plants were subjected to drought stress for 14 d, the ALDH3H1 overexpressors accumulated less MDA than the WT and the 3h1-B mutant (Fig. 9E). However, the fitness of the overexpressors was not improved, as they all dried out, like the WT. No difference was seen between the WT and transgenic plants when treated with methyl viologen, a reactive oxygen species propagator (data not shown). In summary, overexpression of the ALDH3H1 protein did not confer abiotic stress tolerance to *Arabidopsis*. The results,
variants if most isoforms are generated from random

Nearly all intron-containing genes could produce splice

splicing therefore requires careful experimental approaches.

as transcriptome junk. Examination of alternative gene

isoforms with biological properties can easily be mistaken

relevant splicing variants. Conversely, alternative transcript

spliced transcripts (due to splicing errors) as biologically

et al.

to the increasing number of alternatively spliced genes

ALDH3H1-

to the major variant,

ALDH3H1-

alternatively spliced transcript isoforms, namely

gene utilizing T-DNA insertion mutants. Five

This study has provided an analysis of the

Discussion

However, support a role of the ALDH3H1 protein in

alleviating stress injuries related to membrane lipid perox-

idation under moderate stress conditions.

reportedly (Reddy, 2007). Co-purification of some

intron-retaining mRNAs with ribosomes has supported

intron retention as a valid splicing mechanism and has

underlined the importance of alternative splicing in plant

gene regulation (Ner-Gaon et al., 2004). Alternative splicing

of some genes is found to be conserved between evolution-

arily distant plant species. A comparison of rice and

Arabidopsis showed that about 27% of alternatively spliced

genes have the same alternative splicing type in both species

(Wang and Brendel, 2006). However, not all splicing events

are conserved; some may have evolved later in evolution

and, as such, they are species specific. This is probably the

case for ALDH3H1, which structurally differs from other

Arabidopsis ALDH genes. A unique feature of ALDH3H1 is

the 1.2 kb intron that separates exons 1 and 2 (Fig. 1).

Alignment of the sequence of intron 1 with sequences

deposited in the plant DNA sequence databases did not

identify any related sequences, which points to a recent

evolutionary event. The position and size of intron 1 are

likely to influence expression of the ALDH3H1 gene. Whether

the size of intron 1 has facilitated alternative splicing is unclear.

Among the five alternative ALDH3H1 isoforms described

here, only ALDH3H1-β does not derive from intron

retention-type alternative splicing (Fig. 4). It is also unlikely

that it is generated through skipping of exon 1, because it was

detected in both the WT and 3hl-A, despite the presence of T-DNA in the latter (Fig. 1B). The reading

frame of ALDH3H1-β is identical to the spliced isoform

ALDH3H1-α starting downstream of exon 2 (Figs 1A and

4). These features argue for ALDH3H1-β being the product of the gene model AT1G44170.3 and being identical to the

5'-end EST clone DR182505 reported by Alexandrov et al.

(2006). The 5'UTR sequence of ALDH3H1-β and that of

the other alternative splicing variants have not been examined. Such an analysis would not be informative

because of the extensive overlap upstream of exon 2' between ALDH3H1-β and the other isoforms (Fig. 4). The

5'UTR of ALDH3H1-β and ALDH3H1-γ, -δ, -ε, and -ζ could be partially overlapping.

A protein derived from ALDH3H1-β was not detected, although ALDH3H1-β does not contain any premature

termination codon in the protein-coding region. This can be

attributed to a lower stability of ALDH3H1-β transcripts

undergoing a post-transcriptional degradation that prevents

their translation into protein. Another reason could be that the

translation efficiency of ALDH3H1-β is poor and, accordingly, the concentration of ALDH3H1-β would be

below the detection limit. Alternatively, the absence of ALDH3H1-β protein could indicate that the occurrence of

the alternative isoforms has functions other than the diversification of protein variants. The four other isoforms

(ALDH3H1-γ, -δ, -ε, and -ζ) were found to contain at least

one premature termination codon, which makes them

putative targets of the nonsense-mediated mRNA decay

surveillance mechanism (Belgrader et al., 1994; Maquat,

2004; Palusa and Reddy, 2010). Accumulating reports

support the idea that the accumulation of unproductive

Fig. 8. Expression patterns of the ALDH3H1 transcript isoforms in response to stress conditions. The expression pattern of the major

transcript (ALDH3H1-α) was compared with that of the alternatively

spliced ALDH3H1 isoforms (β, γ, δ, ε, and ζ) following dehydration

and NaCl treatments in seedlings (A) and in adult plants (B). Primer

pairs that were used in the PCR assays are shown for each panel

to refer to the major (ALDH3H1-α) and the alternative isoforms.

Primer pair P1/P5 was specific to ALDH3H1-α whereas P2/P5

allowed simultaneous amplification of ALDH3H1-β and the other

alternative isoforms. Primer pair P4/P5 could not discriminate

between the variants (see Fig. 1B for the primer locations).

however, support a role of the ALDH3H1 protein in

alleviating stress injuries related to membrane lipid perox-

idation under moderate stress conditions.
splicing transcripts and their subsequent targeting to the nonsense-mediated mRNA decay pathway is a widespread mechanism to fine tune the abundance of functional transcripts (Maquat, 2004; Lareau et al., 2007; Barbazuk et al., 2008; Saltzman et al., 2008; Palusa and Reddy, 2010).

The data obtained for ALDH3H1 point to the existence of an alternative promoter within intron 1 that directs expression of the alternative transcript variants. The presence of alternative promoters or enhancers within introns has been reported for many plant species (Kim et al., 2006; Morello and Breviario, 2008). Several examples of introns directing the expression of a promoterless gene have been shown in rice and Arabidopsis (Morello et al., 2002, 2006; Rose, 2008; Fojtová et al., 2011). The observation that a fragment of ALDH3H1 intron 1 is capable of directing the expression of the GUS reporter gene is consistent with the conclusions above. Our data indicate that intron 1 of ALDH3H1 interferes with expression of the gene.

The alternative promoter may also direct expression of the alternative transcripts, because the alternative isoforms

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**Fig. 9.** Root lengths, germination rates, and MDA levels of the ALDH3H1 T-DNA insertion mutants and of the ALDH3H1-overexpressors subjected to salt or dehydration stress. (A) Root growth inhibition by salt. Root growth was measured as the increase in root length on MS agar medium supplemented with 100 mM NaCl. The growth on medium not supplemented with salt was taken as the reference for calculating the percentage increase in root length. 3h1-Aa and 3h1-AA refer to the heterozygous and homozygous knockout T-DNA insertion mutant 3h1-A, respectively. Columns with different letters (a, b) are significantly different (Student’s t-test; *P* < 0.05). (B) Germination rate. The percentage of seeds that germinated on salt is shown for each genotype. (C) MDA contents in 2-week-old seedlings grown on MS agar supplemented with NaCl. (D, E) MDA content in adult plants grown on soil and irrigated with different concentrations of NaCl (D) or subjected to dehydration (E). Line 3h1-B is an ALDH3H1 knockout T-DNA insertion mutant, while S10 and S13 are ALDH3H1-overexpressors. FW, fresh weight.
are present not only in the WT but also in the T-DNA mutant 3h1-A (Fig. 1B). Otherwise, all alternative isoforms should have been absent in this mutant line. In agreement with this, no transcript was detected in line 3h1-B, which has a T-DNA insertion within intron 1, suggesting that the integrity of intron 1 is required for generation of the alternative isoforms. The finding that intron 1 contains an alternative promoter also supports the existence of the 5’ EST clone DR182505 of ALDH3H1 (Alexandrov et al., 2006). Our observations point to a coupling of the alternative promoter with alternative splicing events in fine tuning ALDH3H1 gene expression. The necessity of such an alternative promoter and the function of the alternative transcripts are not understood. Chen et al. (2007) observed that genes involved in enzymatic reactions and cellular processes were enriched in alternative first exon transcripts in rice and Arabidopsis, indicating that the transcriptional regulation mediated by alternative first exons may be important for adapting to environmental changes.

The expression pattern of ALDH3H1-α differs substantially from that of ALDH3H1-β, -γ, -δ, -ε, and -ζ in roots of adult plants, which indicates that the two promoter regions are differentially activated. Alternative promoters are often responsible for tissue- or developmental stage-specific gene expression (Morello et al., 2002; Kim et al., 2006). Likewise, our RT-PCR analyses revealed that stress-mediated induction of ALDH3H1-α was restricted to leaves of adult plants, whereas induction of the alternative isoforms ALDH3H1-β, -γ, -δ, -ε, and -ζ occurred in both roots and leaves. The diversification of transcription start sites and/or transcripts evolved from alternative promoters or alternative splicing contributes to variations of gene expression patterns (Wang et al., 2002; Tanaka et al., 2009).

The comparative analysis between the 3h1-A mutants, the WT and the 3h1-C mutants indicated that 3h1-A plants were affected similarly to 3h1-C mutants in their root growth in response to NaCl. The overexpression of ALDH3H1-α diminished the accumulation of toxic aldehydes, measured as MDA in plants under stress, but the transgenic plants were as sensitive as WT plants. Compared with the results obtained from transgenic plants overexpressing ALDH3H1 (Sunkar et al., 2003; Kotchoni et al., 2006), this indicates that ALDH3H1 protein has a moderate role in response to drought or salt stress and may have a different function.

A general observation was the overall low expression of the alternative ALDH3H1 isoforms in WT plants. This can be attributed to the strength of the promoters. Alternative promoters that were 100-fold different in their strength have been documented for mammalian α-amylase genes (Schibler et al., 1983). The dominance of the ALDH3H1 promoter upstream of exon 1 is probably suppressed in the T-DNA insertion mutant 3h1-A. Insertion of the T-DNA possibly affected the integrity of some cis elements in the upstream promoter.

Alternative promoters and gene splicing provide additional flexibility in the control of gene regulation. It is interesting to understand why evolution has favoured the occurrence of both an alternative promoter and gene splicing in the ALDH3H1 locus and why the activity of the alternative promoter is maintained at a low level in the WT. Generation of the alternative promoter may be an isolated event that has occurred during evolution. Landry et al. (2003) proposed several events that can explain the origin of alternative promoters. First, they may occur through progressive mutations, which create over time new functional motifs that can recruit the transcriptional machinery and that are in a favourable genomic position to serve as a promoter. Alternatively, they can result from a recombination event duplicating a promoter region, and subsequent mutations would affect the strength and tissue specificities of the promoters. Other possibilities are the insertion of a transposable element or genomic rearrangements near the gene, thereby creating a novel promoter. Considering that the occurrence of a novel promoter would be accompanied by the generation of novel transcription start sites, downstream promoters or transcription start sites may produce a truncated protein whose function is deteriorated or lost. In contrast, the generation of upstream mutations would affect downstream transcriptional signals and gene function. Therefore, Landry et al. (2003) suggested that upstream mutations would have been negatively selected during evolution and such modifications would only persist if they could resist selective pressure and become beneficial or neutral. Accordingly, the alternative promoter in the ALDH3H1 gene probably occurred after ALDH gene diversification in A. thaliana.

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