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ORIGINAL PAPER

Ectopic-overexpression of an HD-Zip IV transcription factor from *Ammopiptanthus mongolicus* (Leguminosae) promoted upward leaf curvature and non-dehiscent anthers in *Arabidopsis thaliana*

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Abstract Several HD-ZIP IV transcription factors have been reported to play important roles in plant growth and development. However, the functions of most members remain unknown. In this study, an HD-ZIP IV transcription factor, *AmHDG1*, was identified from desert shrub *Ammopiptanthus mongolicus* (Leguminosae) by RACE PCR. *AmHDG1* consists of 2,508 bp, has an open reading frame of 2,292 bp, and encodes a predicted polypeptide of 763 amino acids. Phylogenic analysis with the HD-ZIP IV transcription factor family of *Arabidopsis* showed that it is clustered with the subfamily of AtHDG1 and AtANL2. AmHDG1 is localized in the nucleus and is able to activate transcription in yeast. In *A. mongolicus*, *AmHDG1* is preferentially expressed in young leaves. Constitutive overexpression of *AmHDG1* results in upcurved leaves and non-dehiscent anthers in *Arabidopsis thaliana*. In the flowers of *AmHDG1* overexpressors, the expression levels of two positive regulators of anther dehiscence, *AtNST1* and *AtNST2*, are down-regulated. On the other hand, the transcript level of another positive regulator, *AtMYB26* is not influenced. Taken together, our data demonstrate that *AmHDG1* plays a negative role in the regulation of anther dehiscence.

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Introduction

Numerous developmental events are under transcriptional control and involve a large variety of target gene-specific transcription factors (Khaled et al. 2005; Gao et al. 2010), which can be grouped into many different families based on the conserved structural domains that bind to specific DNA sequences in the regulatory regions of downstream target genes. The HD-Zip family of transcription factors constitutes one of the largest families of plant-specific transcription factors. Members of the HD-Zip family have a leucine zipper motif (LZ) immediately downstream of a homeodomain (HD) (Ariel et al. 2007). Based on sequence analyses, these proteins have been classified into four distinct groups, namely, HD-Zip I–IV (Elhiti and Stasolla 2009). HD-ZIP proteins play crucial roles in a variety of processes during plant growth and development (Elhiti and Stasolla 2009). HD-Zip I proteins generally participate in responses related to abiotic stress, abscisic acid (ABA), blue light, de-etiolation, and embryogenesis (Ariel et al. 2007; Elhiti and Stasolla 2009; Gago et al. 2002; Henriksson et al. 2005; Himmelbach et al. 2002; Olsson et al. 2004; Wang et al. 2003). HD-Zip II proteins are involved in response to illumination conditions, shade avoidance, and auxin signaling (Ariel et al. 2007; Delarue et al. 1998; Morelli and Ruberti 2000; 2002; Rueda et al. 2005; Sawa et al. 2002; Sessa et al. 2005). HD-Zip III proteins control embryogenesis, leaf polarity, lateral organ initiation, and vascular system development (Ariel et al. 2007; Baima et al. 2001; Emery et al. 2003; Kim et al. 2005; Mattsson et al. 2003; McConnell et al. 2001; Morelli and Ruberti 2000; Otsuga et al. 2001;

Prigge et al. 2005; Williams et al. 2005). HD-Zip IV proteins play crucial roles in anthocyanin accumulation, epidermal cell differentiation, trichome formation, root development, and cuticle development (Abe et al. 2003; Isaacson et al. 2009; Kubo et al. 1999; Luo and Oppenheimer 1999; Nakamura et al. 2006; Ohashi et al. 2003; Perazza et al. 1999). Recent research has discovered that up-regulating the expression of *HDG11*, one of the HD-Zip IV genes, allows *HDG11* to gain novel functions in drought tolerance (Cao et al. 2009; Yu et al. 2008). This finding may reveal how drought tolerance evolves, because changing the expression pattern of *HDG11* may be a way through which drought tolerance can evolve in nature (Yu et al. 2008).

Ammopiptanthus mongolicus is a relic of the Tertiary Period, distinctively distributed in the northwestern desert area of China marked by seasonally extreme drought and temperatures (over 40 °C in summer and under −30 °C in winter), poor soil quality with high salinity, and extraordinarily high ultraviolet irradiation (Wang et al. 2003). However, in the early part of the Tertiary Period, *A. mongolicus* was mainly distributed in the coast of the ancient Mediterranean, indicating that it once adapted to a wet and warm climate (Wei et al. 2011a). Therefore, the later evolvement of its extreme tolerance to a combination of abiotic stresses could be logically attributed to the gradual climate change (e.g., from warm and wet to extremely hot/cold and dry/salty) incurred by geological changes (Wei et al. 2011a). Its distinctive characteristics make *A. mongolicus* a valuable system for exploiting the mechanistic evolvement of abiotic stress tolerance in plants. To explore the genetic mechanism of its extremely abiotic tolerance, we set out to identify the HD-Zip transcription factors from *A. mongolicus*, which have crucial roles in plant abiotic stress. In this study, we report the isolation and functional characterization of an HD-Zip IV transcription factor, *AmHDG1*, from *A. mongolicus*.

Materials and methods

Plant materials and growth conditions

Arabidopsis plants and *A. mongolicus* were sown in 10-cm-side square pots with soil (peat soil:vermiculite:pearlite [v/v/v] 3:9:0.5 purchased from Shanghai Institute of Landscape Science) presoaked with plant nutrient medium, and grown in a 16-h light/8-h dark cycle at 24 °C.

Isolation of *AmHDG1*

Two primers, AmHDG11CF (5′-TTCAAGGAGTGTCC TCATCCAGA-3′) and AmHDG11CR (5′-ACCTTGGA ATATCCATTGGGC-3′), were designed for amplification of partial cDNAs of HD-Zip homologs from *A. mongolicus* based on multiple-alignment of the full-length mRNA sequences from different plant species. Two primers (forward: 5′-TGCTGGTTATGTTACCGAAGCTACAAGAG-3′; and reverse: 5′-TATCTAATGGTGTGGGTGGAACC-3′) were designed to perform 3′-RACE cDNA synthesis using the Smart™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Five primers [AmHDG15RRT (5′-T TCATCCTTGAGTCG-3′), AmHDG15RA1 (5′-GAAGCC ATGTCAAACCCAGTATG-3′), AmHDG15RS1 (5′-TGG CGTTCCAACTGGGTCT-3′), AmHDG15RA2 (5′-TTTG AGGAGCACCAAATTAGAAT-3′) and AmHDG15RS2 (5′-TCTTACTAAGATCCGCCCTTTG-3′)] were designed to perform 5′-RACE cDNA synthesis using the 5′-RACE Amplification Kit (Takara Japan). The clones obtained were sequenced, and the overlapping region with the first clone was confirmed. After re-construction of the open reading frame (ORF), a fragment containing the ORF was re-obtained via PCR with primer pair AmHDG1EL (forward: 5′-TGTTTTGGTCCTTTTACTTTGCTC-3′; and reverse: 5′-CAAAGACACTGAAATGAGATAACTGC-3′) from the root cDNA library, and then sequenced for further confirmation.

Plasmid construction

For the *AmHDG1* overexpression test, a pair of primers (forward: 5′-ATCGGATCCATGGAAGGCCATACTGAG-3′; and reverse: 5′-TGCGTCGACTCATACAATTCTGAGGG C-3′) were used to PCR amplify the whole open reading frame of *AmHDG1*. The PCR products were digested by *BamHI* and *Sal I*, and the resultant fragments were subcloned into the *BamHI and SalI* site of the pCHF3 vector.

For the subcellular localization analysis of AmHDG1, the full ORF of *AmHDG1* without the stop codon was amplified by primer AmHDG1S (forward: 5′-ATAGAGCT CATGGAAGGCCATACTGAG-3′; and reverse: 5′-ATAG GATCCTACAATTCTGAGGGCAGC-3′) and inserted into the *SacI* and *BamHI* sites of pCHF3-GFP vector.

Generation of transgenic *Arabidopsis* plants

The above constructs were introduced into GV3101 *Agrobacterium tumefaciens* by the freeze-and-thaw method (Holsters et al. 1978). *Arabidopsis* was transformed using the floral dip method (Clough and Bent 1998). Putative transgenic plants were selected on plates supplemented with 50 mg L^{-1} Kanamycin, and further verified by PCR.

Transcriptional activation analysis in yeast

The ORF of *AmHDG1* was first amplified by a pair of primers AmHDG1AY (forward: 5′-GCGGAATTCATG

GAAGGCCATACTGAG-3′; and reverse: 5′-TGCGTCGA CTCATACAATTCTGAGGGC-3′). The PCR products were then subcloned into the *EcoRI* and *SalI* sites of pGBKT7. The resulting constructs as well as pGBKT7 were then transformed into the yeast strain, AH109, harboring the *HIS3* reporter gene. After three days of incubation on synthetic defined medium (SD/Trp$^-$ or SD/His$^-$ medium) at 30 °C, the growth status of the transformants was evaluated.

Real-time PCR

Real-time PCR was performed according to Wei et al. (2011a, b). Specific primers for real-time PCR of the respective genes were as follows: *AmHDG1* (forward: 5′-TCTGGATGTGTT GTACAGGATATGCC-3′; and reverse: 5′-GAGGGTGGC GATCCATCTATGAG-3′), *AtACTIN2* (forward: 5′-CGCT CTTTCTTTCCAAGCTC-3′; and reverse: 5′-AACAGCCCT GGGAGCATC-3′), *AmACTIN2* (forward: 5′-TTCCTCACG CTATTCTTCGGTTGG-3′; and reverse: 5′-GCTCATAATC AAGGGCAACATAGGC-3′), *AtMYB26* (forward: 5′-CC TGGAAGAACAGATAACGAGGTCAA-3′; and reverse: 5′-TTGAATCCATTGTGATAAGGAAGGTTT-3′), *AtNST1* (forward: 5′-ACGGGAACGAGAACTAACAGAGC-3′; and reverse: 5′-ATCAGATTTTTGGCCGTGAGG-3′), *AtNST2* (forward: 5′-GTGATAGAATCGGGATGCGAAAGAC-3′; and reverse: 5′-CCACCCATCCTTCGTCACTTCCTA-3′).

Results

Isolation and molecular characterization of *AmHDG1*

A cDNA library was constructed using mRNA isolated from the radicle of *A. mongolicus*. A cDNA 2,508 bp nucleotides in length, with an open reading frame of 2,292 bp, was then obtained by RACE PCR from the cDNA library. BLAST analysis revealed that it belonged to the HD-Zip IV gene family and shared the highest identity (88 %) with a predicted HD-Zip IV factor from soybean (Accession No. XP_003 536477). It was therefore tentatively named *AmHDG1* (Fig. 1). The overall expression pattern of *AmHDG1* is broad, but *AmHDG1* is preferentially expressed in young wrapped leaves (Fig. 2).

AmHDG1 localizes to the nucleus

To investigate the sublocation of AmHDG1, its C terminus was fused to green fluorescent protein (GFP), and the resulting fusion protein was introduced into Col-0. Figure 3 shows that GFP fluorescence in transgenic plants was predominantly observed in the nucleus. By comparison, GFP in the 35S-GFP control plants was present in both the cytoplasm and nucleus. These data confirmed that AmHDG1 is localized in the nucleus.

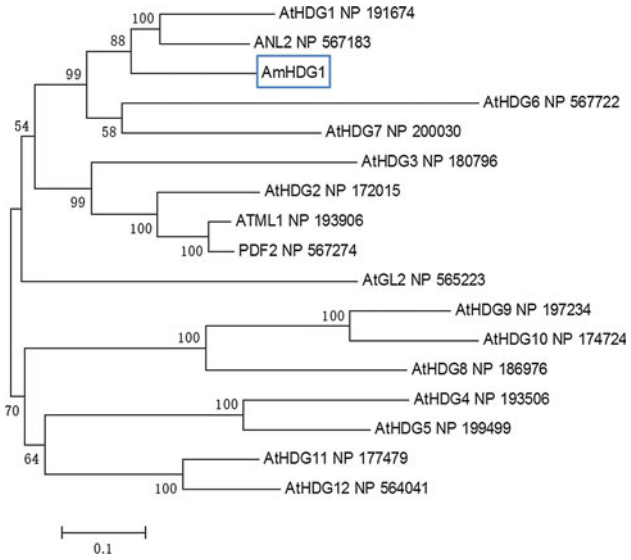

Fig. 1 Phylogenetic analysis of AmHDG1 with 16 *Arabidopsis* HD-Zip IV transcription factors. A phylogenetic tree was constructed with the neighbor-joining method using MEGA; Bootstrap analysis was performed with 1,000 replicates excluding positions with gaps. *Numbers* in *branches* indicate bootstrap values (*percent*). HD-Zip IV protein names are followed by their accession numbers; The protein AE007838_10 is used as an outlier

Transcriptional activation activity of AmHDG1 in yeast

The ORF of *AmHDG1* was fused to the GAL4 DNA-binding domain to examine whether it has transcriptional activation activity. The resultant construct, as well as the negative vector control pGBKT7, were expressed in yeast cells. Only AmHDG1 could promote yeast growth in the absence of histidine (Fig. 4).

Overexpression of *AmHDG1* in Col-0 resulted in upcurved leaves and non-dehiscent anthers

To functionally characterize *AmHDG1 in planta*, its open reading frame (ORF) driven by a 35 s promoter was introduced into Col-0 using the floral dip method (Clough and Bent 1998). Twenty-four transgenic lines were obtained and verified by PCR. Ten T_1 lines exhibited variations of the upcurved leaf phenotype, and four exhibited the non-dehiscent anthers phenotype. Four plants were then randomly selected for detecting the expression level of *AmHDG1* (Fig. 5). The expression levels of *AmHDG1* in these lines were well correlated with the severity of the upcurved leaf phenotype. Furthermore, the two lines displaying non-dehiscent anthers exhibited relatively higher expression levels of *AmHDG1* (Figs. 5b, 6a–c).

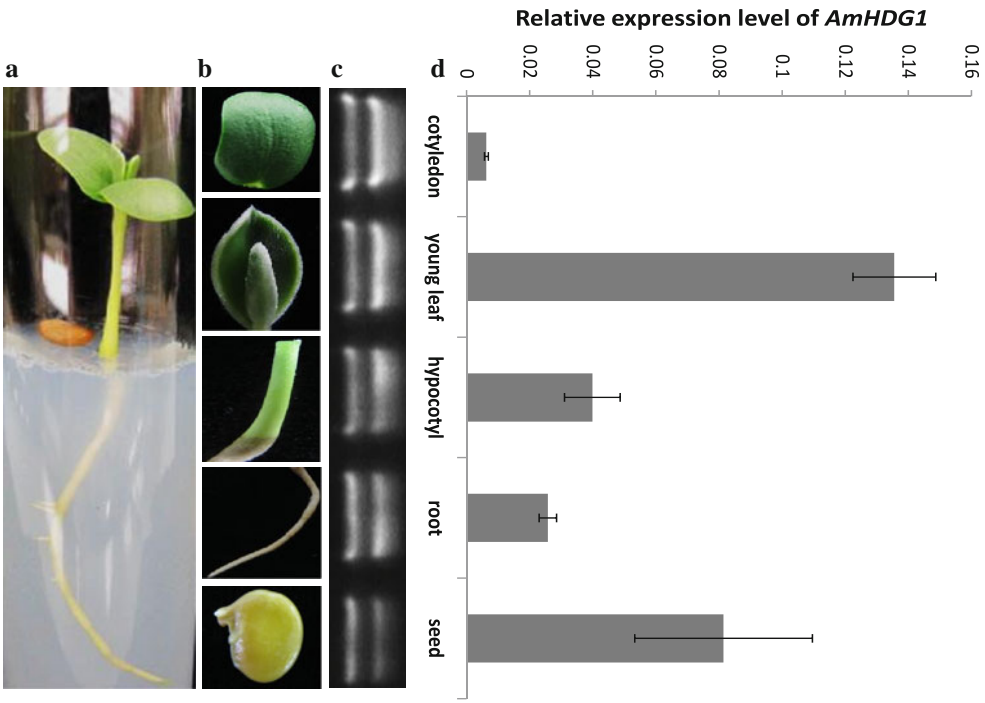

Fig. 2 Expression pattern of *AmHDG1*. **a** A typical 10-day old plant grown on MS-medium. **b** Various tissues from the plant depicted in **a**. **c** Expression level of *AmHDG1* in the tissues depicted in **b**. Values are means of three replicates. *Error bars* indicate SD

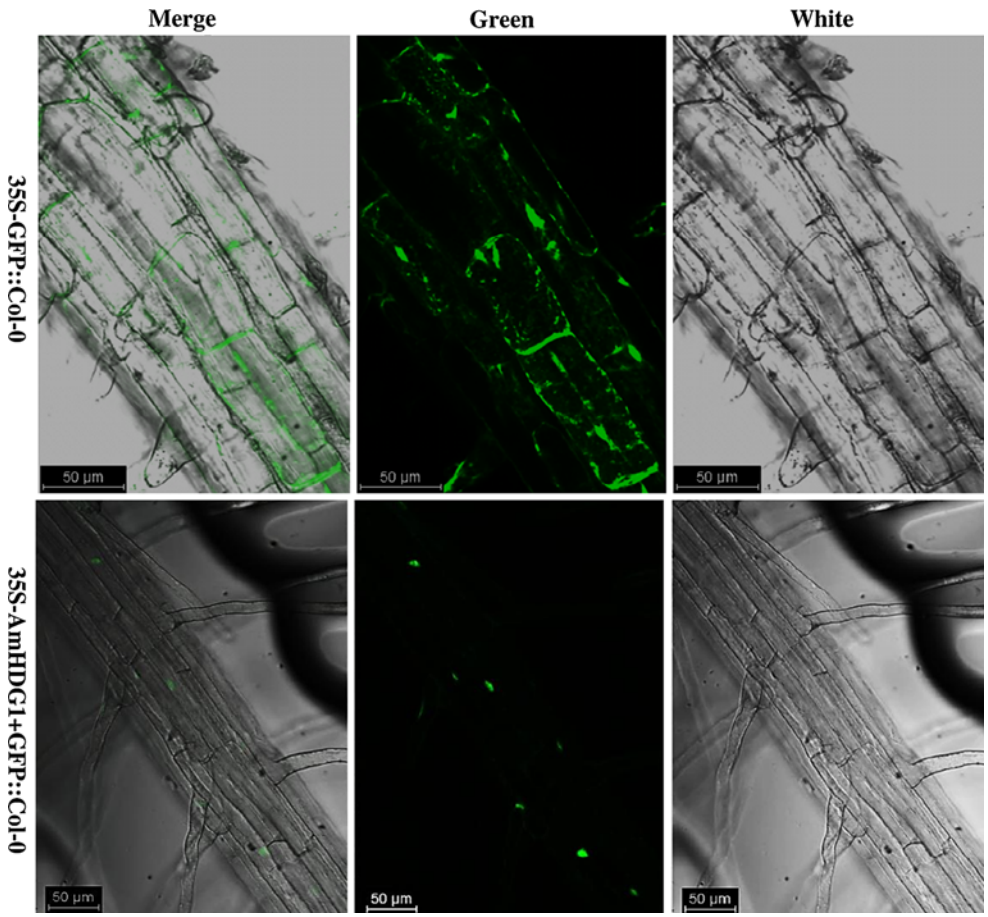

Fig. 3 Localization of AmHDG1 to the nucleus. Confocal micrographs of root tissue from Col-0 expressing the 35S-GFP reporter (*top*) and Col-0 harboring the 35S-AmHDG1-GFP construct (*bottom*). *Green* confocal micrographs of fluorescence from GFP (*green channel*). *White* transmitted light image. *Merge* merged micrographs of *white* and *green* images. *Bar* 50 µm

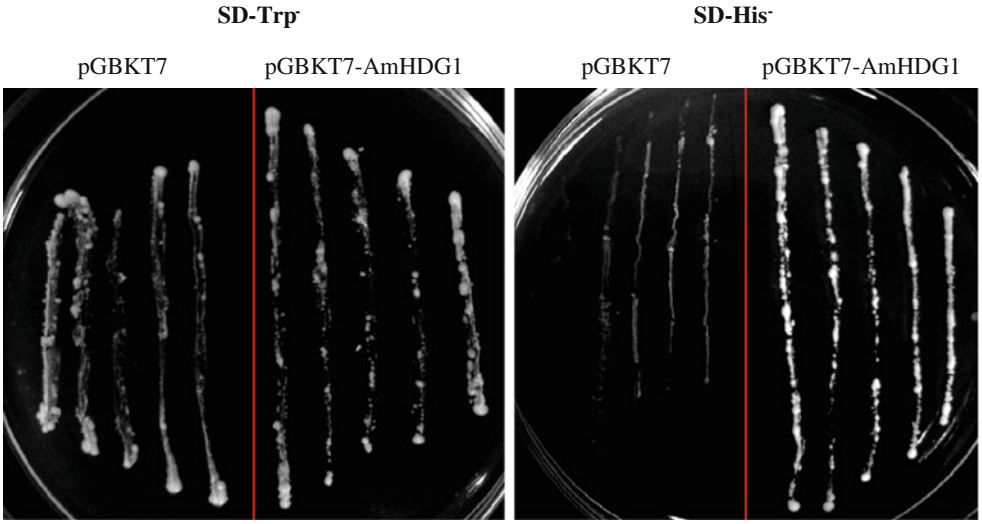

Fig. 4 Trans-activation analysis of AmHDG1 in yeast. Fusion proteins of pGBKT7-AmHDG1 or the vector (pGBKT7) were expressed in the yeast strain AH109. Transformants were streaked onto SD/Trp$^-$ and SD/His$^-$ plates. Plates were incubated for 3 days to identify transactivation activity of the transformants

Discussion

The class IV HD-ZIP family is also known as HD-GL2 after the first identified gene GLABRA2 (GL2) (Nakamura et al. 2006). In the *Arabidopsis* genome, there are 16 HD-Zip IV members, mostly with unknown functions. Using loss-of-function mutants to explore their functions is difficult, possibly because of the functional redundancy among these genes. Alternatively, the ectopic or increased expression of these genes may cause developmental abnormalities, thus providing new insights into their functions (Li et al. 2007). In this study, we reported the isolation and functional characterization of a nuclear-located HD-Zip IV protein, *AmHDG1*, from a desert shrub. Ectopic overexpression of *AmHDG1* gave rise to leaf morphological changes and male sterility in *Arabidopsis*.

Protein BLAST against the NCBI database indicated that AmHDG1 belongs to the HD-Zip IV family. Further phylogenetic analysis with 16 *Arabidopsis* HD-Zip IV proteins revealed that AmHDG1 clustered with the subgroup of ANL2 and AtHDG1. Previous work has reported that ANL2 is involved in anthocyanin accumulation and

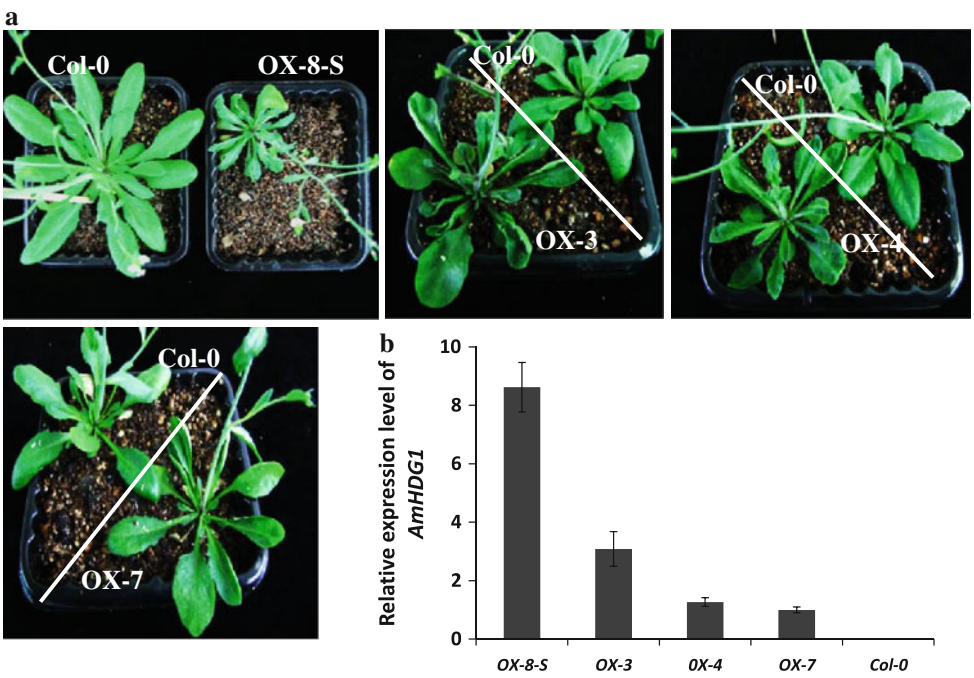

Fig. 5 *AmHDG1* transcript level-correlated phenotypes in overexpressing *Arabidopsis lines*. **a** Four representative T1 transgenic plants with the upcurved leaf phenotype. **b** Real-time PCR analysis of *AmHDG1* transcripts in the *above lines*, with *AtACTIN2* as the reference. Values are means of three replicates. *Error bars* indicate SD. Col-0, wild type

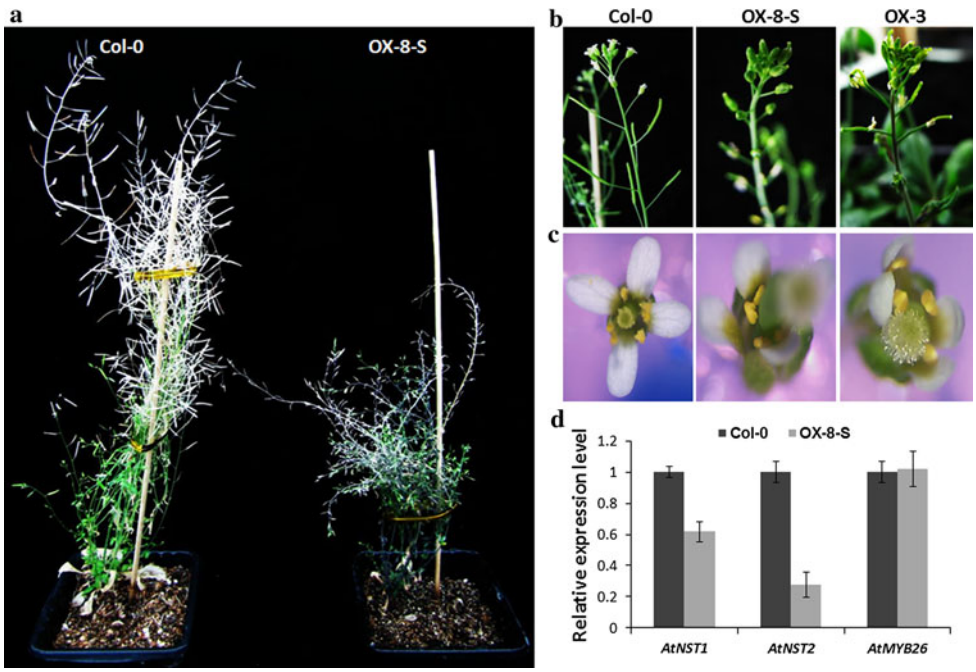

Fig. 6 Non-dehiscent anthers of *Arabidopsis* plants expressing high levels of *AmHDG1*. **a** A representative sterile *AmHDG1* overexpressors. **b** Inflorescences of wild type and transgenic plants. **c** Anthers of plants depicted in **b**. **d** Expression of genes involved in anther dehiscence. Values are means of three replicates. *Error bars* indicate SD

root development (Kubo et al. 1999; Li et al. 2007). Recently, a gene annotated as a homolog of *ANL2* was identified in tomato, and the mutation of this gene altered the cutin content (Isaacson et al. 2009). *AtHDG1* which is negatively regulated by CFL1 has been found to regulate cuticle development by affecting the downstream genes *BDG* and *FDH* (Wu et al. 2011). However, there has been no report so far about the phenotypes of *AtHDG1/ANL2* overexpressors. In our work the overexpression of an *AtHDG1/ANL2* homolog, *AmHDG1* could causes obvious upcurved leaf and non-dehiscent anther phenotypes. These phenotypes are similar to those of the overexpressors of *AtHDG3* (Li et al. 2007), which belongs to the subgroup of *AtHDG2/AtHDG3* (Nakamura et al. 2006). This finding indicates that *AtHDG1/ANL2* and *AtHDG2/AtHDG3* may share similar functions. Aside from the two subgroups with overlapping expression patterns, this phenomenon could also explain why double mutants of *hdg2/hdg3* do not display an abnormal phenotype (Nakamura et al. 2006).

Pollen release achieved through a process called anther dehiscence (Sanders et al. 2000) requires careful timing and regulation for synchronized development of the anther and the flower, thus ensuring that pollen release occurs at the optimal time to maximize either cross- or self-fertilization (Wilson et al. 2011). In *Arabidopsis*, three genes identified by genetic studies, namely, *AtMYB26*, *AtNST1* and *AtNST2*, are required for anther dehiscence (Mitsuda et al. 2005; Yang et al. 2007). Mutants of *AtMYB26* or double mutants of *AtNST1* and *AtNST2* completely fail to undergo anther dehiscence due to loss of secondary wall thickening in the anther endothecium (Mitsuda et al. 2005; Yang et al. 2007). *AtMYB26* acts upstream of the two NAC genes that act redundantly, namely, *AtNST1* and *AtNST2* (Mitsuda et al. 2005). In turn these genes stimulate thickening in the endothecium (Yang et al. 2007). In *AmHDG1* overexpressors, female fertility appears unaffected (data not shown) indicating that the style is fully functional. However, the anthers fail to dehisce resulting in male sterility. In flowers of transgenic plants the transcript levels of *AtNST1* and *AtNST2* are down-regulated, whereas the expression of *AtMYB26* is not influenced, indicating that *AmHDG1* negatively regulates anther dehiscence by controlling the expression of *AtNST1* and *AtNST2*. It is unclear at present whether this regulation occurs directly or indirectly. However, analysis of the promoter regions of *AtNST1* and *AtNST2* suggests the absence of the L1 box, an HD-ZIP IV transcription factor binding site discovered in previous studies (Abe et al. 2001, 2003), in the 5′ upstream regions. Interactions between *AmHDG1* and the promoters of these two genes need further investigation to elucidate the regulatory mechanisms involved.

Controlling male fertility is an important goal for plant reproduction and selective breeding (Wilson et al. 2011). Male sterility is associated not only with the lack of viable pollen, but also with the failure of pollen release (Wilson et al. 2011). In such instances, failure of anther dehiscence has the advantage of producing viable pollen that can be used for subsequent rescue of fertility (Wilson et al. 2011). The work reported in this study provides an efficient method to produce male sterility lines via overexpressing *AmHDG1*.

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