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ORIGINAL ARTICLE

Comprehensive genomic analysis and expression profiling of Argonaute gene family and examination of their regulatory roles in water-use efficiency and abiotic stress responses in apple

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Abstract Argonaute proteins are key players in small RNA-guided gene-silencing, which plays an important role in plant stress tolerance. However, little is known about how Argonaute genes affect the water-use efficiency (WUE) of apple (*Malus domestica*) or its responses to different abiotic stresses. We identified and characterized *MdAGOs* in apple and analyzed their chromosome locations, exon/intron structures, phylogeny, and the distribution of conserved motifs. We also examined the expression profiling of responses to abiotic stress and conducted molecular cloning of *MdAGO4.1*. In all, 16 *MdAGOs* were identified and characterized, then grouped into three separate clusters. Our qRT-PCR data demonstrated that these genes are induced by drought, salt, cold, and ABA treatments, indicating that they are good candidates for further analysis of their activities and functions. We have previously shown that, during long-term moderate drought, the abundance of MdAGO4.1 protein is increased in 'Qinguan' apple (a cultivar with high WUE) leaves, but not in 'Naganofuji No. 2'. These changes are in accordance with alterations in their WUE and expression of *MdAGO4.1* under the same test conditions as those used in the current study. Therefore, *MdAGO4.1* is a putative gene that positively regulates WUE. Our findings provide evidence that *MdAGOs* have a role in plant adaptations to abiotic stress and can be exploited to improve WUE. These results will serve as a framework for future functional studies of that gene family in apple.

Keywords Argonaute · Water-use efficiency · Apple · Abiotic stresses

Abbreviations

ABA	Abscisic acid
ABRE	Abscisic acid response element
AGO	Argonaute
EST	Expressed sequence tag
HSE	Heat shock element
LTR	Low-temperature-responsive element
MBS	MYB binding site
MdAGO	Argonaute of apple
miRNA	MicroRNA
ORF	Open reading frame
qRT-PCR	Quantitative real-time PCR
siRNA	Short interfering RNAs
WUE	Water-use efficiency
MeJA	Methyl jasmonate
GA	Gibberellic acid

Introduction

Argonaute proteins are core components of the small RNA-induced silencing complex (Wang et al. 2015). This complex contains specific microRNA (miRNA) scans for complementary mRNA transcripts and directs cleavage or translational repression at the target mRNAs. The eukaryotic Argonaute (AGO) family comprises AGO proteins and PIWI

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proteins, and plant Argonaute proteins are within the AGO clade (Meister 2013). Argonautes appear to be involved in all small RNA-guided gene-silencing processes. Research of these small RNAs has improved our understanding of gene regulation by revealing new details of transcriptional and post-transcriptional mechanisms as well as gene-silencing processes associated with the plant response to many environmental stresses (Du et al. 2015). For example, *AGO1* homeostasis in *Arabidopsis thaliana* is controlled transcriptionally by the miRNA MIR168 during abscisic acid (ABA) and other stress treatments (Li et al. 2012). Argonautes are highly conserved, with at least three subfamilies occurring in all eukaryotes (Meister 2013). The exception is *Saccharomyces cerevisiae*, which over time has lost its small RNA machinery (Drinnenberg et al. 2009). *Arabidopsis* has ten different Argonaute proteins that show preference to distinct classes of small RNAs. For example, AGO4 associates with heterochromatic short interfering RNAs (siRNAs) that participate in small RNA-directed DNA methylation (Du et al. 2015). When this protein is loaded with 24-nt siRNAs and assembled into an AGO4/siRNA complex by HSP90 in the cytoplasm, the mature complex is transported into the nucleus (Meister 2013; Ye et al. 2012).

The Argonaute proteins usually contain three conserved domains: PAZ at the N-terminus plus MID and PIWI at the C-terminus (Zhao et al. 2015). The PAZ domain anchors the 3′-end of the small RNA by bending it into a specific binding pocket. The MID domain anchors the 5′-end of the small RNA by providing a binding pocket (Jinek and Doudna 2009). The PIWI domain is structurally similar to RNase H. In fact, Argonaute proteins can function as endonucleases and cleave target RNA that is fully complementary to the bound small RNA (Jinek and Doudna 2009). The PIWI domain contains a catalytic triad composed of DDX (where X is D or H), and it has a critical role during the process of sequence-specific cleavage in the RNAi machinery (Gunter 2013). In some organisms, the Argonaute proteins may have lost the PAZ domain and retain only a PIWI domain (Hutvagner and Simard 2008).

The Argonaute proteins help maintain genome integrity, control protein synthesis and RNA stability, and function in the production of a specific set of small non-coding RNAs (Hutvagner and Simard 2008). Such proteins associate with nascent transcripts and guide small RNA-directed DNA methylation or chromatin modifications (Qi et al. 2006). However, little has been reported about how Argonaute genes affect plant responses to different abiotic stresses (Zhao et al. 2015).

Although various Argonaute functions have been identified, many are still poorly characterized in plant species. Apple (*Malus domestica* Borkh.) is one of the most economically important fruits worldwide. It is usually cultivated in arid and semi-arid areas. Water deficit is one of the most common challenges to apple production in these areas, and farmers require cultivars that are very tolerant to drought. Within a genetic/physiological context, drought tolerance refers to the ability of one genotype to yield 'better' under such stress (Bassett 2013), that is to say, shows greater water-use efficiency (WUE). Therefore, understanding the molecular mechanisms of drought tolerance and WUE in apple is critical for breeding more efficient cultivars that have higher productivity in dry climates. One step is to identify regulatory genes that can be used to enhance WUE.

We previously evaluated 31 apple cultivars and found that 'Qinguan' plants are more tolerant to drought and have the highest WUE under long-term moderate drought. Furthermore, imposition of such treatment improves WUE of that cultivar. In contrast, 'Naganofuji No. 2' apple is more sensitive, and its WUE under drought does not differ from that of well-watered plants (Liu et al. 2012). Using those two cultivars, we have also compared their leaf proteome patterns between well-watered and long-term moderate drought-treated plants (Zhou et al. 2015). Therefore, the abundance of MdAGO4.1 (a member of apple Argonaute) protein is increased by drought in 'Qinguan' but not in 'Naganofuji No. 2' leaves. All of these earlier results lead us to speculate that *MdAGO4.1* regulates WUE in apple. Therefore, we are now investigating the regulatory roles of *MdAGOs* (apple Argonaute genes) in conferring WUE and abiotic stress tolerance in apple. This study, described here, has involved comprehensive genomic analyses and expression profiling of *MdAGO*s under long-term moderate drought as well as in response to natural drought, salt, chilling, or ABA treatment.

Materials and methods

Identification and chromosome locations of apple Argonaute genes

We previously studied the mechanism of high WUE under long-term moderate drought in *Malus* cultivars (Zhou et al. 2015). When compared with levels in well-watered plants, the abundance of MdAGO4.1 (MDP0000176861) protein was increased 0.82 times by drought in leaves of 'Qinguan' apples, and its WUE was also improved by such stress. By contrast, the abundance of this protein was not enhanced in drought-exposed 'Naganofuji No. 2' leaves, and its WUE was not improved. This indicated that MdAGO4.1 may have a positive regulatory role in determining WUE under long-term moderate drought.

In the current experiments, we applied several methods to investigate the Argonaute genes in apple. The MdAGO4.1 protein sequence and all *Arabidopsis* Argonaute sequences were first taken as queries for performing a local Blastp against the apple genomics database (http://

genomics.research.iasma.it/local), using an E value threshold of $1E-46$. All searched protein sequences were then submitted to Pfam (http://pfam.sanger.ac.uk/), CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and SMART (http://smart.embl-heidelberg.de/) to verify the presence of the Argonaute catalytic domain. Each MdAGO protein sequence was also blasted against the apple non-redundant (nr) protein sequences database in NCBI to confirm the existence of the Argonaute catalytic domain. Proteins that lacked the conserved domain were not further examined. We then conducted a Blastn against the apple expressed sequence tag (EST) database at NCBI (http://www.ncbi.nlm.nih.gov/guide/) for additional confirmation of the accuracy of those genomic predictions. The coding sequences identified from those genome data were revised according to the information from the apple EST database. Signal peptides were analyzed by SignalP Version 3.0 program (http://www.cbs.dtu.dk/services/SignalP) (Bendtsen et al. 2004). Molecular weights and isoelectric point (pI) values of the proteins were calculated by the ExPASY Compute pI/Mw program (http://www.expasy.org/tools/pi_tool.html). Genes were then mapped to the chromosomes, and a sketch map was created with MapDraw (Liu and Meng 2003).

Phylogenetic analysis, determination of exon/intron structure, and multiple sequence alignments

To identify putative orthologs, we chose one monocot (rice; *Oryza sativa*) and four dicots (apple; grape, *Vitis vinifera*; *Arabidopsis thaliana*; and tomato, *Solanum lycopersicum*) and constructed an unrooted phylogenetic tree from alignments of their full-length protein sequences. All of those Argonaute sequences were aligned with the integrated MUSCLE program in MEGA7.0 (Tamura et al. 2011). The phylogenetic tree was also produced with MEGA7.0 software, using the maximum-likelihood (ML) method. In addition, a phylogenetic tree including only apple and *Arabidopsis* Argonaute sequences was developed. A diagram of the exon/intron organizations of all *MdAGO*s was generated by the online Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn). Multiple alignments of Argonaute protein sequences were performed with the DNAMAN program (Shao et al. 2014).

Plant materials and stress treatments

Several cultivars of apple were planted at Northwest A&F University, Yangling, China (34°20′N, 108°24′E). Buds of *Malus domestica* Borkh. 'Qinguan', 'Naganofuji No. 2', and 'Honeycrisp' (lowest WUE) (Liu et al. 2012) were grafted onto the apomixic rootstock of *M. hupehensis* Rehd. 'Pingyiensis' in early March. The plants were grown in pots (38 cm high, 23 cm diam.) containing a local 5:1 (v:v) mixture of loess and sand. All were placed in a greenhouse under conditions of ambient light, 20–35 °C, and 50–75 % relative humidity. At the end of May, uniform trees per cultivar were assigned to two different treatments (50 trees per cultivar per treatment type): (1) well-watered, irrigated daily to maintain 75–85 % field capacity; or (2) moderate drought, irrigated daily to maintain 45–55 % field capacity. These treatments continued for 3 months. We sampled after 1 week (short-term moderate drought) and at the end of the treatment period (long-term moderate drought). For gene expression analysis, mature leaves of equal age were removed from plants per cultivar per treatment, then quickly frozen in liquid nitrogen and stored at −80 °C. Each treatment had five biological replicates.

To investigate the regulatory roles of *MdAGOs* under other types of abiotic stress, we used one-year-old 'Golden Delicious' apple (*M. pumila* Mill. 'Jinguan', grafted onto *M. hupehensis* Rehd. 'Pingyiensis'). Natural drought stress was induced by irrigating plants once to 75–85 % field capacity and then withholding irrigation for up to 10 days. Leaf samples were harvested after 0, 2, 4, 6, 8, and 10 days of treatment. Plant responses to salinity, chilling, or exogenous ABA were also examined by irrigating to saturation with 200 mM NaCl, exposing the plants to 4 °C, or spraying the leaves with 100 μM ABA (Zhao et al. 2012), respectively. Leaves were sampled after 0, 2, 4, 8, 12, and 24 h for all of those experiments. Each treatment included five biological replicates.

Expression analysis of apple Argonaute genes

Total RNA was extracted by the CTAB method (Chang et al. 1993), and residual DNA was removed with RNasefree DNase I (Invitrogen, USA). Integrity of the RNA was checked via 1.2 % agarose gel electrophoresis. The first-strand cDNAs were synthesized with an SYBR Prime Script RT-PCR Kit II (TaKaRa, Japan), and specific primers for quantitative real-time PCR (qRT-PCR) were designed to amplify each gene (Table 1). All qRT-PCRs were conducted on an iQ5.0 instrument (Bio-Rad, USA), using SYBR Green qPCR kits (TaKaRa) according to the manufacturer's instructions. EF-1a served to standardize the cDNA samples of different genes. Conditions for qRT-PCR followed those described by Tan et al. (2014).

Search for cis-acting elements in the promoters of apple Argonaute genes

Upstream regions (1500 bp from the transcription start site) of the *MdAGOs* were used to search for cis-acting elements in the PlantCARE (http://bioinformatics.psb.ugent.be/

Table 1 Primers and sequences used for quantitative real-time PCR analysis

Gene	Primers (5′–3′)
MdAGO1.1	F: AAAACTCGCTACCAAGAAG
	R: TGTTAAACAACACTGGGAAA
MdAGO1.2	F: TACGCTGGCTTGGTCTG
	R: GTTGCTCTACGGAATGAAAT
MdAGO1.3	F: GGAGCCAAACTATCAGCC
	R: ACCGTGCCAGGTAAGAT
MdAGO5.1	F: GACCTGTATTCCCTCCAAC
	R: TTTAGCCCTAAGAACAACG
MdAGO5.2	F: GCCTTCGCTGAAATACC
	R: CCACTCGACCACCGTTA
MdAGO10.1	F: CTCTGGAACCCAACTATCA
	R: TGTCTATGCTGCTCCTGTC
MdAGO10.2	F: ACTTGCAGAACCCAGTGAA
	R: ATCAGACTTCCGACCACCT
MdAGO2	F: GGCGGTAGCGGTGGATA
	R: CTGATTCGGCTGTCGTT
MdAGO3	F: TTAAGCCAGAACACCCTG
	R: GGATCATCCGTGGATAACT
MdAGO7.1	F: AGAAAGCACAAAGGCTACG
	R: GTGGATGTGGATGGGTC
MdAGO7.2	F: GCTTGGTGATGGTGGTC
	R: CTTTGAGATAGCTGGCGTA
MdAGO4.1	F: ACCACTCGGAGTTAGGTG
	R: TTTGGACGCCGTAATCT
MdAGO4.2	F: CCCTACCACGCAACAAA
	R: GGACTCCCAGGACCATC
MdAGO6	F: TTATTGTCTTCAGGGATGG
	R: AAGCCTTTATGATTTGGTC
MdAGO8	F: GTCTTGAATGTGGAACTGG
	R: GGTGAATGCGTCTGGAA
EF-1a[a]	F: ATTCAAGTATGCCTGGGTGC
	R: CAGTCAGCCTGTGATGTTCC

[a] Internal control

webtools/plantcare/html/) (Lescot et al. 2002) and PLACE databases (Higo et al. 1999). Putative cis-elements were matched with those previously identified from other gene promoters.

Molecular cloning of *MdAGO4.1* sequence

Gene-specific primers (F: AAGAGTCCTCACGGCTGGAT, R: CGCCTGTCAAACGCACC; or F: CTCAG TCGTCGGTCTCAT, R: CCATAGTAGGGCTCAAGG; all sequences 5′–3′) for *MdAGO4.1* were designed according to the revised full-length *MdAGO4.1* sequence, using information from the NCBI apple EST database. For cloning the entire coding region of *MdAGO4.1*, first-strand 'Qinguan' cDNA was used as template. Gene-specific primers designed according to the predicted sequence were also used for cloning. The clone sequence was submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/submit).

Results

Genome-wide identification of Argonaute gene family members in apple

In all, we found 16 candidate *MdAGOs* in the Argonaute family (Table 2). These genes coded for 93.28-to-121.50-kDa basic proteins, with pI values ranging from 8.83 to 9.60. At the level of gene structure, the number of introns varied from 2 to 32. The lengths of the open reading frames (ORFs) ranged from 2493 bp for *MdAGO6* to 3348 bp for *MdAGO1*, encoding potential proteins of 831 and 1116 amino acids, respectively (Table 2). Searches of the apple nr protein database in NCBI for conserved domains revealed that almost all MdAGOs share a C-terminus PIWI domain (besides MdAGO4.2), characteristic of other highly conserved plant Argonaute proteins. The MdAGO10.1, MdAGO3, MdAGO8, MdAGO9, MdAGO4.1, and MdAGO4.2 proteins lack the PAZ domain, as determined by the SMART tool. The result of our CDD online analysis also indicated that these proteins are Argonaute family members (Supplemental Table S1). Almost all *MdAGOs* matched at least one EST hit (Supplemental Table S2). The exceptions were MDP0000118779 and MDP0000159246.

Chromosomal location of apple Argonaute genes

To investigate the evolution of *MdAGOs* in apple, we analyzed their genomic distribution (Fig. 1). The 16 genes were distributed unevenly across 10 chromosomes, with Chr 3, 6, 7, 11, 13, and 16 each containing two *MdAGOs*, while Chr 5, 9, 10, and 14 carried single genes.

Phylogenetic analysis of the *MdAGOs*

The evolutionary relationships of Argonaute family members were compared between monocots and dicots (Supplemental Table S3). The phylogenetic analysis revealed that the Argonaute genes fell into three main clusters (Fig. 2). All had well-supported bootstrap values. Their uniform distribution across the three clusters indicated that they are highly conserved. The seven apple Argonaute genes in Cluster (Group) 1 include *MdAGO1.1*,

Table 2 Apple Argonaute genes identified in this study

Cluster	Gene	Locus	Description	Chromosome no.	Deduced polypeptide			Signal peptide
					Length (aa)	MW (kDa)	PI	
1	MdAGO1.1	MDP0000161046	PAZ and Piwi domain	6	1116	121.53	9.31	–
	MdAGO1.2	MDP0000069525	PAZ and Piwi domain	6	1093	121.50	9.36	–
	MdAGO1.3	MDP0000886537	PAZ and Piwi domain	14	1076	119.92	9.41	–
	MdAGO10.1	MDP0000191579	PAZ and Piwi domain	13	957	106.86	9.21	–
	MdAGO10.2	MDP0000071268	PAZ and Piwi domain	16	988	110.53	9.31	–
	MdAGO5.1	MDP0000232035	PAZ and Piwi domain	3	979	109.09	9.60	–
	MdAGO5.2	MDP0000774227	PAZ and Piwi domain	11	983	109.50	9.56	–
2	MdAGO7.1	MDP0000118779	PAZ and Piwi domain	16	1017	114.78	9.34	–
	MdAGO7.2	MDP0000159246	PAZ and Piwi domain	13	1025	116.22	9.36	–
	MdAGO2	MDP0000868788	PAZ and Piwi domain	10	1004	111.57	9.39	–
	MdAGO3	MDP0000260407	PAZ and Piwi domain	5	995	109.22	9.17	+
3	MdAGO6	MDP0000928339	PAZ and Piwi domain	9	831	932.84	9.21	–
	MdAGO8	MDP0000209079	PAZ and Piwi domain	7	889	99.85	9.34	–
	MdAGO9	MDP0000215105	PAZ and Piwi domain	7	846	94.72	9.36	–
	MdAGO4.1	MDP000017686	PAZ and Piwi domain	3	940	104.06	8.83	–
	MdAGO4.2	MDP0000272708	Piwi domain	11	975	108.01	8.97	–

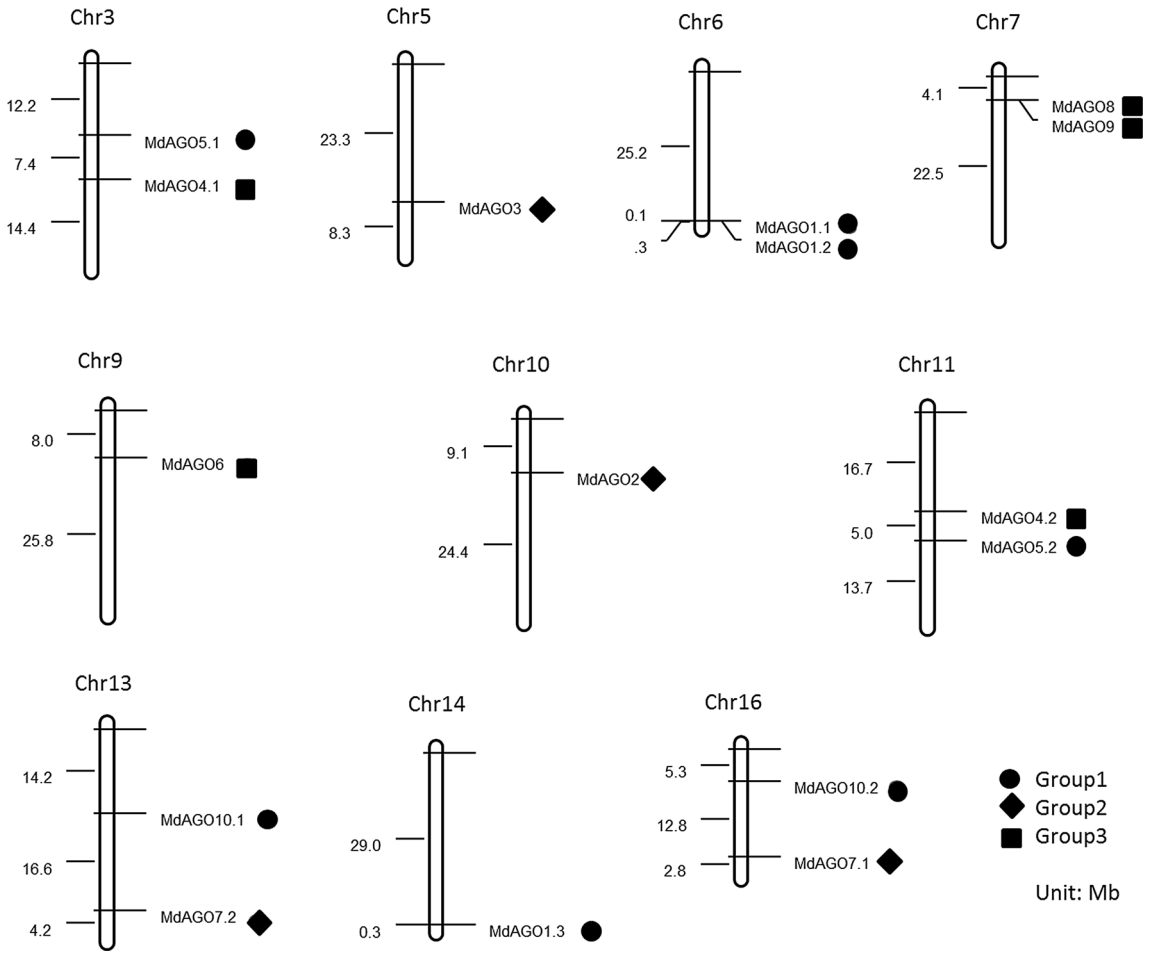

Fig. 1 Chromosomal mapping of apple Argonaute gene family. Clusters are indicated by different-shaped *dots*

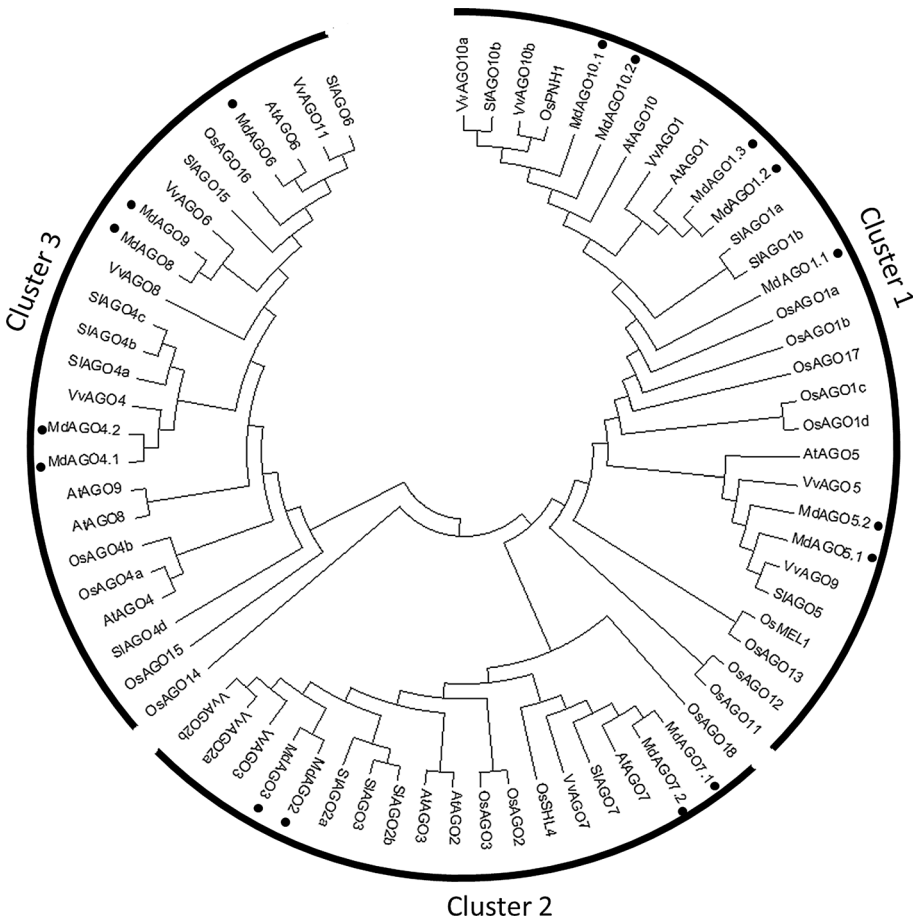

Fig. 2 Phylogenetic analysis of Argonaute proteins from *Malus domestica* (Md), *Vitis vinifera* (Vv), *Oryza sativa* (Os), *Solanum lycopersicum* (Sl), and *Arabidopsis thaliana* (At). Unrooted phylogenetic tree was constructed by ML method using MEGA 7.0 program. GenBank Accession Numbers are listed in Supplemental Table S3

MdAGO1.2, *MdAGO1.3*, *MdAGO5.1*, *MdAGO5.2*, *MdAGO10.1*, and *MdAGO10.2*, based on their high sequence homologies with *AtAGO1*, *AtAGO5*, and *AtAGO10*. Cluster 2 comprises four genes similar to *AtAGO2*, *AtAGO3*, and *AtAGO7*. Those apple members were named *MdAGO2*, *MdAGO3*, *MdAGO7.1*, and *MdAGO7.2*. Cluster 3 contains five apple Argonaute genes, similar to *AtAGO4*, *AtAGO6*, *AtAGO8*, and *AtAGO9*. They were named *MdAGO4.1*, *MdAGO4.2*, *MdAGO6*, *MdAGO8*, and *MdAGO9*.

Gene structures of *MdAGOs*

A separate phylogenetic tree was constructed from alignments of the full-length protein sequences for only apple and *Arabidopsis* (Fig. 3a). Similar to *Arabidopsis* (Meng et al. 2013), the apple proteins could be classified into three clusters. The exon/intron organization analysis for all *MdAGOs* indicated that those within the same cluster shared a similar structure. For example, members of Cluster 2 (*MdAGO2*, *MdAGO3*, *MdAGO7.1*, and *MdAGO7.2*) contained fewer introns than those in either Cluster 1 or Cluster 3 (Fig. 3b).

Multiple sequence alignments of apple Argonaute proteins

Multiple alignments were made to obtain detailed domain structures. All of these protein sequences contained conserved domains of Argonaute. The characteristic motifs of the PIWI domain in MdAGO proteins are shown in Fig. 4. Conserved Asp–Asp–His/Asp (DDH/D) motifs were found within the PIWI domains of *Arabidopsis* (Fig. 4a) and apple (Fig. 4b), as marked with red squares. Those motifs are thought to function as the catalytic triad and have critical roles during the process of sequence-specific cleavage in the RNAi machinery (Meng et al. 2013).

Ten MdAGO proteins (all Cluster 1 members plus MdAGO7.1 and MdAGO7.2) had conserved DDH/H798 residues. Both D760 and D845 were generally conserved between apple and *Arabidopsis*. The exception was MdAGO4.2, where D845 was replaced by phenylalanine (F). Although H798 was conserved in all Cluster 1 and Cluster 2 members, it was not in Cluster 3, where it was replaced by proline (P) in apple and by serine (S), proline (P), or arginine (R) in *Arabidopsis*. Whereas H986 was also conserved in most apple MdAGOs, it was replaced by aspartate (D) in MdAGO2 and MdAGO3 (Table 3).

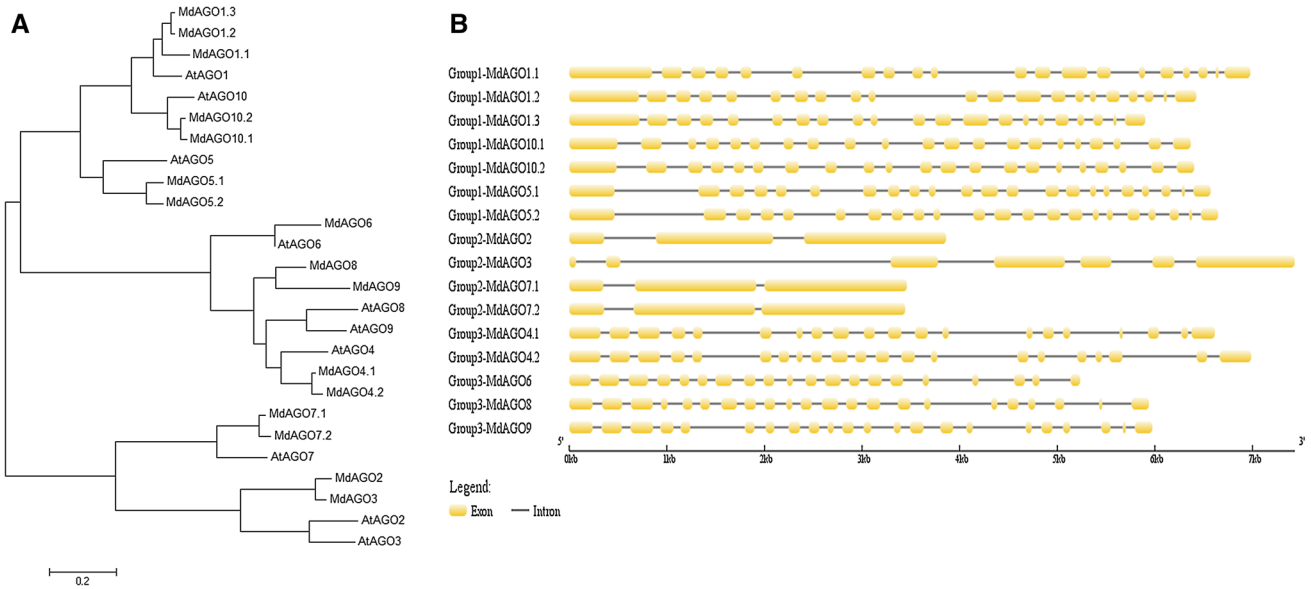

Fig. 3 **a** Phylogenetic analysis of apple and *Arabidopsis* Argonaute genes; **b** exon/intron organizations of apple genes

Effects of long-term and short-term moderate drought on *MdAGOs* expression for three apple cultivars

To evaluate the regulatory role of *MdAGOs* on WUE in apple, we developed expression profiles in response to long-term and short-term moderate drought. Here, the expression level of *MdAGO4.1* was upregulated by long-term drought in 'Qinguan' but not in 'Naganofuji No. 2' or 'Honeycrisp' leaves (Fig. 5a). These gene expression results were consistent with those for protein abundance and drought-related changes in WUE. *MdAGO1.2*, *MdAGO2*, and *MdAGO8* were upregulated by long-term moderate drought in the leaves of all three cultivars. The response for all other *MdAGOs*, whether induced or unchanged, was cultivar-dependent. Short-term moderate drought tended to induce more *MdAGOs* (Fig. 5b). *MdAGO4.1* was strongly upregulated by short-term drought in 'Qinguan' but not in 'Naganofuji No. 2' or 'Honeycrisp' leaves. *MdAGO10.1*, *MdAGO10.2*, and *MdAGO6* were upregulated in all three cultivars under short-term drought. For all other *MdAGO*s, the response was cultivar-dependent.

Effects of ABA and abiotic stress on *MdAGOs* expression

When exogenous ABA was applied to 'Golden Delicious' apple, expressions of *MdAGO1.2*, *MdAGO1.3*, *MdAGO2*, *MdAGO3*, *MdAGO7.1*, *MdAGO7.2*, and *MdAGO4.1* were significantly induced after 6 h of exposure. The remaining genes all responded to varying degrees based on the length of the treatment period (Fig. 6).

When plants were exposed to 4 °C, most members of Cluster 1 (*MdAGO1.2*, *MdAGO1.3*, *MdAGO5.1*, *MdAGO5.2*, *MdAGO10.1*, and *MdAGO10.2*) and all in Cluster 3, plus *MdAGO3* and *MdAGO7.2* (Cluster 2), were upregulated. In particular, *MdAGO5.1* and *MdAGO3* were the most sensitive to cold stress, with transcript levels increasing by approximately 12-fold (Fig. 7).

Under natural-drought conditions, *MdAGO1.1*, *MdAGO10.1*, *MdAGO10.2*, *MdAGO2*, *MdAGO4.1*, and *MdAGO6* were significantly induced by 12.6-, 7.7-, 16.5-, 7.2-, 14.7-, and 9.8-fold, respectively, on Day 6 after irrigation was withheld. By Day 8 of such treatment, expression of *MdAGO7.2* was significantly induced by 12.6-fold. The remaining genes were induced to varying degrees over this treatment period (Fig. 8).

In response to 200 mM NaCl, *MdAGO5.2*, *MdAGO3*, *MdAGO7.2*, *MdAGO4.1*, *MdAGO6*, and *MdAGO8* in three-month-old seedlings were significantly induced. Especially, for *MdAGO7.2*, its expression increased by 27.1-fold after 6 h of exposure. In contrast, transcription was only slightly altered for *MdAGO1.1*, *MdAGO1.3*, *MdAGO10.1*, *MdAGO2*, and *MdAGO4.2* in plants undergoing salt stress (Fig. 9).

Promoter sequence analysis of selected *MdAGOs*

The cis-acting elements in the promoter regions of eight selected *MdAGOs* were identified (Supplemental Table S4). These cis-elements were of four different types:

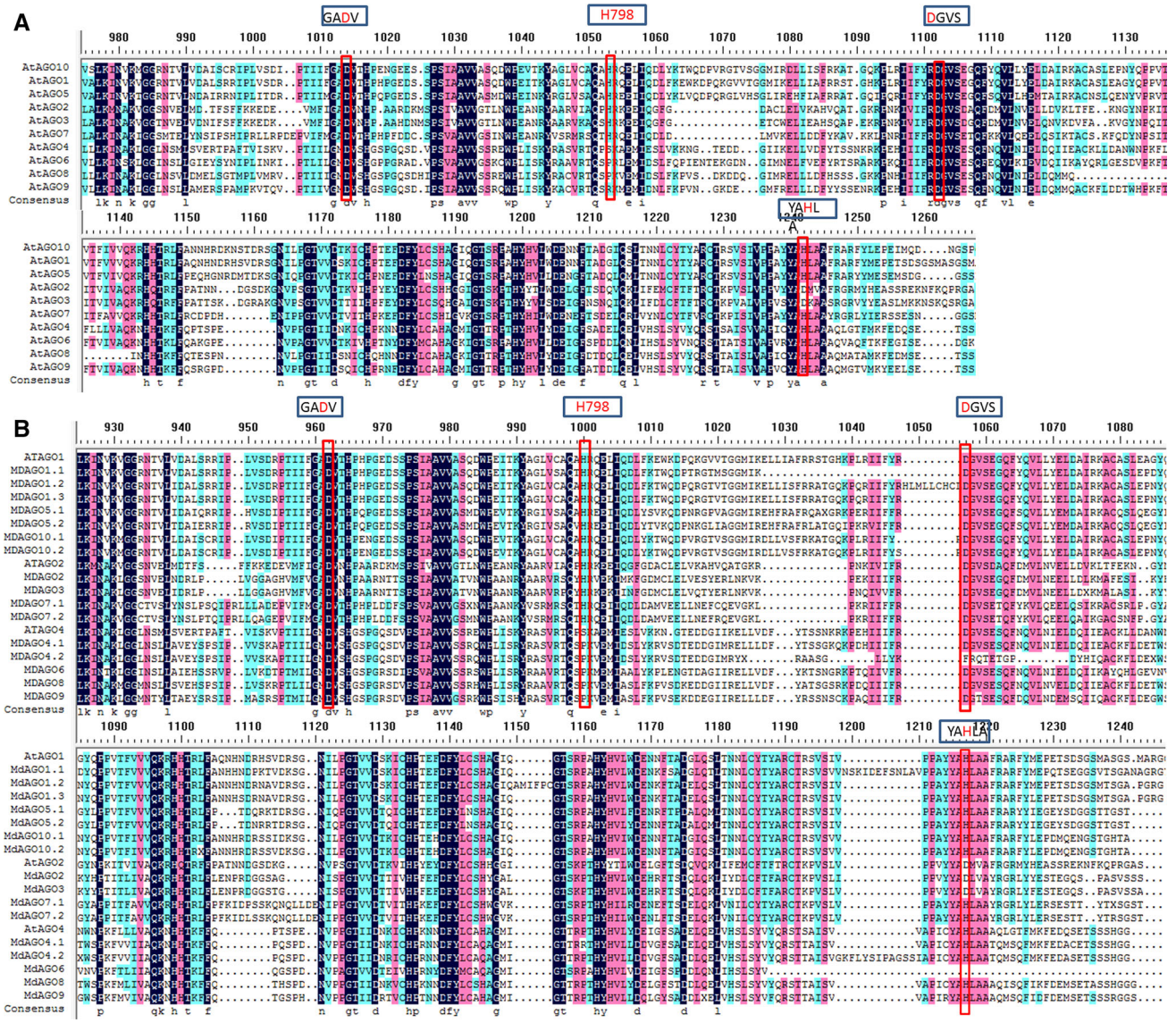

Fig. 4 a Multiple alignments by DNAMAN of PIWI domain in Argonautes of *Arabidopsis*; b Multiple alignments by DNAMAN of PIWI domain in apple Argonautes plus AtAGO1, AtAGO2, and AtAGO4 from *Arabidopsis*. *Squares* mark conserved metal-chelating amino acids DDH/D and H798

(1) plant hormone-responsive, including toward ABA, MeJA (methyl jasmonate), GA (gibberellic acid), and ethylene; (2) physiological and environmental responses to drought, cold, heat, wounding, pathogens, and, especially light; (3) temporal and spatial expression, such as in the seed, endosperm, and meristem; and (4) transcription activation, repression, or enhancement.

Among these cis-elements, we focused on those associated with hormone- and stress-related responses. For example, abscisic acid-responsive elements (ABREs) (Hobo et al. 1999) were present at one to two copies in the promoter regions of *MdAGO4.1*, *MdAGO8*, and *MdAGO10.2*. A MYB binding site (MBS), involved in drought-inducibility (Xiong et al. 2002), existed in the promoter regions of four genes. Only two of our selected *MdAGOs* lacked heat shock elements (HSEs) (Rieping and Schoffl 1992) in their promoter regions. Low-temperature-responsive elements (LTRs) (Jiang et al. 1996) were carried at different copy numbers in five cold-inducible genes. The presence of these motifs showed that the responsiveness of *MdAGOs* to adverse abiotic stresses may be regulated by these cis-acting elements within the promoter, as well as by corresponding transacting factors.

Cloning of *MdAGO4.1* from 'Qinguan' apple leaves

The variability in WUE values, *MdAGO4.1* expression, and MdAGO4.1 protein abundance between 'Qinguan' and

Table 3 Argonaute proteins with missing catalytic residue(s) in PIWI domains of apple and *Arabidopsis*

Apple		*Arabidopsis*[b]	
Argonaute	Motif[a]	Argonaute	Motif[a]
MdAGO2	DDD/H	AtAGO2	DDD/H
MdAGO3	DDD/H	AtAGO3	DDD/H
MdAGO4.1	DDH/P	AtAGO4	DDH/S
MdAGO4.2	DFH/P		
MdAGO6	DD./P	AtAGO6	DDH/P
MdAGO8	DDH/P	AtAGO8	DDH/P
MdAGO9	DDH/P	AtAGO9	DDH/R

D aspartate, *H* histidine, *S* serine, *P* proline, *R* arginine, the '.' in 'DD./P' represents none amino acid there

[a] Motifs correspond to conserved D760, D845, H986/H798 of *Arabidopsis* AGO1

[b] Reviewed by Bai et al. (2012)

'Naganofuji No. 2' under long-term moderate drought indicated that this particular gene has a positive regulatory role for WUE of apple. To confirm the predicted cDNA sequence, we cloned the full length of *MdAGO4.1*, using first-strand 'Qinguan' cDNA as template. The cloned sequence showed high similarity (>99 %) with the revised full-length sequence.

Different gene-specific primers were used to amplify the entire coding region of *MdAGO4.1* according to either the revised full-length sequence or its predicted sequence. The latter was not successful in our amplification. The ORF was 2601 bp for the predicted sequence and 2820 bp for the cloned full sequence. The two were 75.4 % similar. The clone sequence was submitted to NCBI GenBank with Accession Number KU167607.

Discussion

Genome-wide identification and analysis of Argonaute gene family members in apple

RNA-silencing plays an important role in regulating gene expression during periods of abiotic stress (Zhao et al. 2015; Bai et al. 2012). Although that process requires Argonautes, little has been known about the characteristics and functions of related genes and their regulatory roles for WUE and stress tolerance in apple. Here, we identified 16 Argonaute genes in apple and analyzed their chromosomal distribution within the genome, phylogenetic relationships with other species, and gene structure. Our qRT-PCR data analysis provided evidence that these proteins contribute to WUE and abiotic stress responses.

A previously released annotation of the apple genome showed that the 16 putative, complete *MdAGO* sequences encode 16 deduced proteins (Velasco et al. 2010). As these data continue to be updated, additional Argonaute members will probably be found. The number currently known for apple is similar to the 15 for tomato and the 17 for rice. By contrast, the *Arabidopsis* genome contains only 10 Argonautes (He et al. 2010). This variation in numbers suggests that this gene family may have expanded along with whole-genome duplication after those lineages were separated. Furthermore, paleo-duplication events and a relatively recent genome-wide duplication (approximately 60–65 Mya) may have occurred in the apple genome and had a great impact on the amplification of those genes (Velasco et al. 2010).

The apple Argonaute genes are distributed on 10 of 17 chromosomes. None of them appears to have originated from tandem duplications. Our unrooted phylogenetic tree demonstrated that some putative MdAGO proteins share high sequence similarity with those from *Arabidopsis*, rice, tomato, and grape, indicating that the Argonaute proteins are highly conserved between monocot and dicot crops. A phylogenetic tree obtained previously for 13 grape Argonautes (VvAGO1 to VvAGO11) revealed four functional clusters, i.e., AGO1 Cluster, AGO5 Cluster, AGO7 Cluster, and AGO4 Cluster (Zhao et al. 2015). In contrast, the phylogenetic tree that we developed here for apple, grape, *Arabidopsis*, rice, and tomato presented three clusters, with AGO5 Cluster being consisted in AGO1 Cluster. This finding of three clusters is in accord with that reported by Meng et al. (2013) for Argonautes from *Arabidopsis*.

Characterization of the main conserved domain

Our multiple alignments of the MdAGOs suggest strong conservation of the characteristic motifs involved in interactions with small RNAs (Jinek and Doudna 2009; Simon et al. 2011). They also indicate that Argonaute is a highly conserved protein family. The PIWI domain of Argonaute is highly homologous to RNaseH, binding the 5′-end of siRNA to the target RNA (Hock and Meister 2008), and cleaving the target RNAs that exhibit sequence complementarity to small RNAs (Zhao et al. 2015). An aspartate–aspartate–histidine/aspartate (DDH/D) motif, and a conserved histidine at position 798 (H798) in the PIWI domain are the active sites (Kapoor et al. 2008). This DDH/D motif and H798 histidine are critical for in vitro endonuclease activity of AGO1 in *Arabidopsis* (Kapoor et al. 2008). To determine whether MdAGOs possess these conserved catalytic residues and can act as slicer components for silencing effector complexes, we used DNAMAN to align the PIWI domains from all of the MdAGOs and AtAGOs. Except for MdAGO4.2, all MdAGO proteins contain a PIWI domain and a DDH/D motif, thereby indicating their endonuclease activity. Moreover, H798 in Cluster 3 members from *Arabidopsis* and apple is replaced

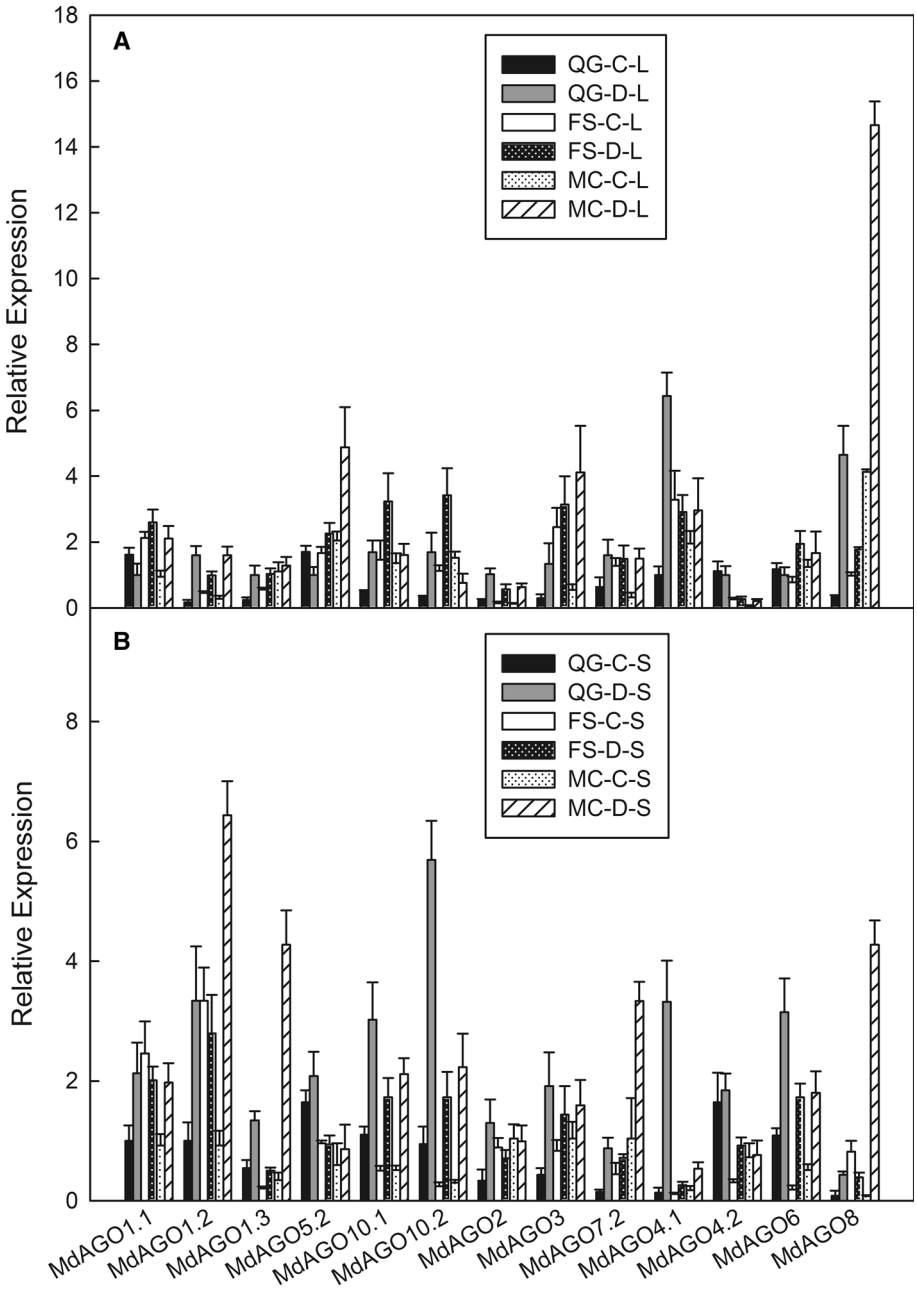

Fig. 5 Quantitative real-time PCR analysis of response by selected *MdAGO* genes to long-term moderate drought (**a**; treatment L) and short-term moderate drought (**b**; treatment S) in mature leaves from 'Qinguan', 'Naganofuji No. 2', or 'Honeycrisp' apple. *QG-CK* well-watered 'Qinguan', *QG-D* moderately drought-stressed 'Qinguan', *CF-CK* well-watered 'Naganofuji No. 2', *CF-D* moderately drought-stressed 'Naganofuji No. 2', *MC-CK* well-watered 'Honeycrisp', *MC-D* moderately drought-stressed 'Honeycrisp'. Data were normalized to apple EF1-a expression level. Value for each sample is mean of three replicates. *Vertical bars* indicate standard deviation

by serine, proline, or arginine. The absence of conserved catalytic residues in MdAGO4.2 may have led to a loss of function in targeting RNA-processing as well as modulating subsequent regulatory pathways (Kapoor et al. 2008). This might explain why MdAGO4.2 did not respond to our experimental treatments.

The response of *MdAGOs* to different abiotic stresses and cloning of *MdAGO4.1*

Our expression profile showed that 13 of the 16 Argonaute genes are expressed, to varying degrees, in response to long-term and short-term moderate drought in our three apple cultivars. The response to short-term water stress is more intense, possibly because a result of sudden environmental change (short-term drought) may cause more unstable biological reaction in plants, but the plants are better able to adapt as the drought period became prolonged. The response to long-term drought is more meaningful for WUE. Under such long-term stress, *MdAGO1.2*, *MdAGO2*, and *MdAGO8* are upregulated in all three cultivars. However, *MdAGO4.1* is upregulated only in 'Qinguan', a pattern that is consistent with its adjustments to WUE and MdAGO4.1 protein abundance, implying that *MdAGO4.1* positively regulates WUE in apple.

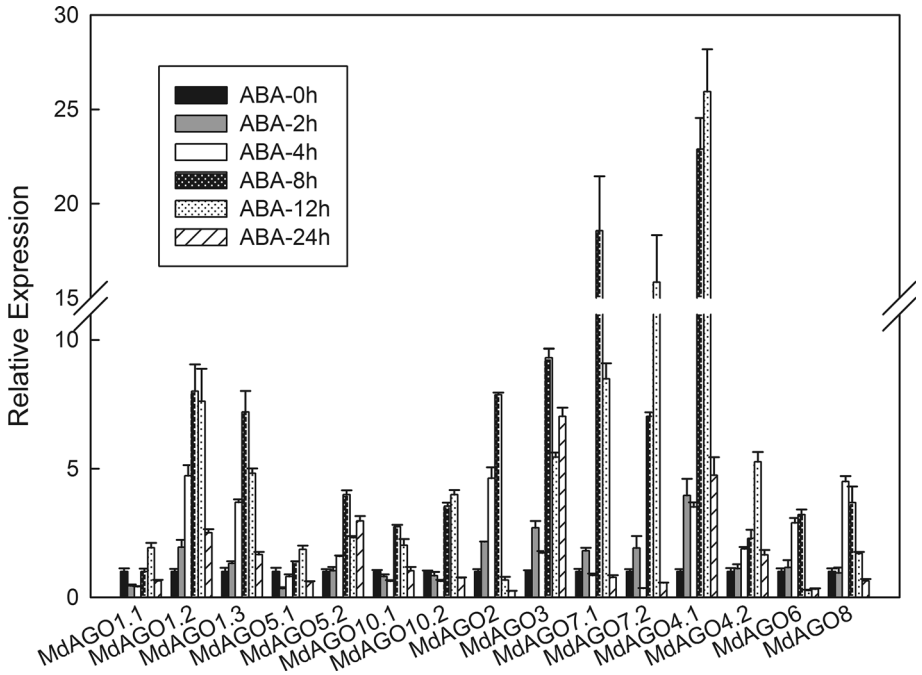

Fig. 6 Quantitative real-time PCR analysis of *MdAGOs* in response to ABA treatments in mature leaves from 'Golden Delicious' apple. Data were normalized to apple EF1-a expression level. Value for each sample is mean of three replicates. *Vertical bars* indicate standard deviation

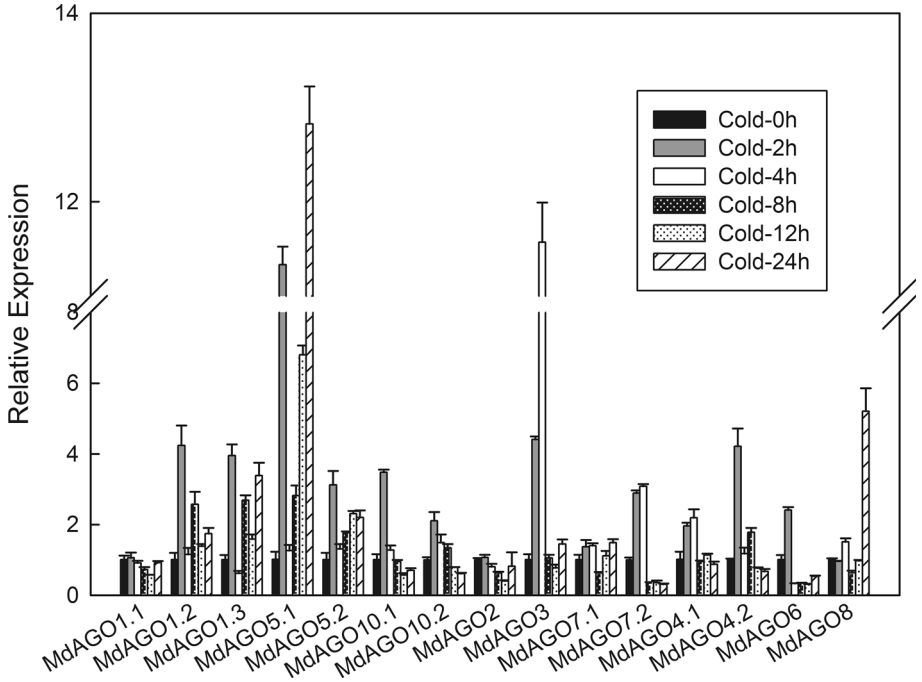

Fig. 7 Quantitative real-time PCR analysis of *MdAGOs* in response to cold treatments in mature leaves from 'Golden Delicious' apple. Data were normalized to apple EF1-a expression level. Value for each sample is mean of three replicates. *Vertical bars* indicate standard deviation

Almost all *MdAGO*s appear to respond to natural drought, NaCl, cold stress, and ABA treatment. Most members, especially *MdAGO5.1* and *MdAGO3*, are upregulated by chilling. When apple plants are exposed to NaCl, most genes in Clusters 2 and 3, as well as *MdAGO5.2*, are upregulated, indicating their involvement in the salt-stress response. Under ABA and drought treatments, more genes are induced and upregulated by a larger degree. These results demonstrate that various members participate in different abiotic stresses, and that *MdAGOs* are more sensitive to ABA or drought than to cold or NaCl. Overall, *MdAGO1.1*, *MdAGO10.2*, *MdAGO7.2*, and *MdAGO4.1* are the most responsive to drought.

MdAGO4.1 is most strongly upregulated by ABA or drought, but only slightly by cold or NaCl, indicating that this gene functions primarily in the drought–response pathways and ABA-signaling processes. Considering the crosstalk between those two, it appears that the induction

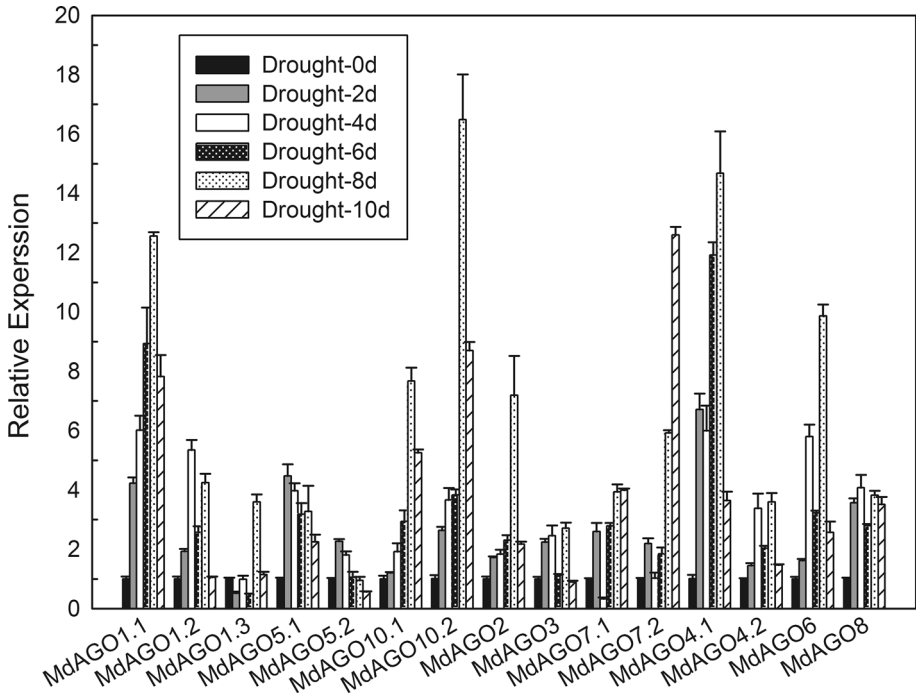

Fig. 8 Quantitative real-time PCR analysis of *MdAGOs* in response to natural drought treatments in mature leaves from 'Golden Delicious' apple. Data were normalized to apple EF1-a expression level. Value for each sample is mean of three replicates. *Vertical bars* indicate standard deviation

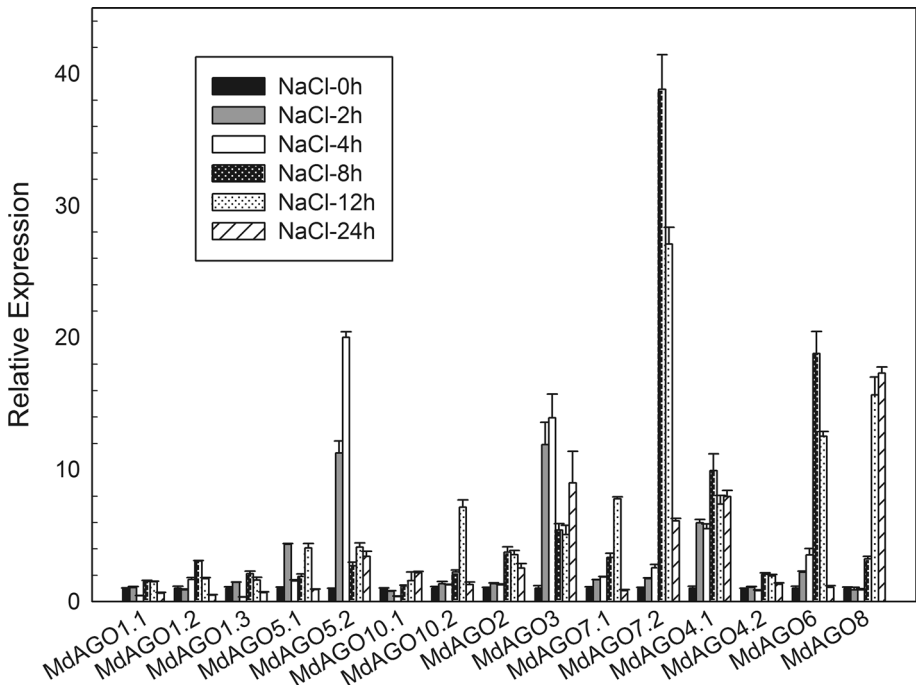

Fig. 9 Quantitative real-time PCR analysis of *MdAGOs* in response to NaCl treatments in mature leaves from 'Golden Delicious' apple. Data were normalized to apple EF1-a expression level. Value for each sample is mean of three replicates. *Vertical bars* indicate standard deviation

of *MdAGO4.1* by ABA is somehow related to the drought response. Our proteome data showed that MdAGO4.1 protein is increased by drought in the high WUE 'Qinguan' apple (Zhou et al. 2015). Together with the qRT-PCR results, we might conclude that *MdAGO4.1* confers drought tolerance and enhances WUE in apple. When we cloned its full-length ORF, we found a vast difference between the experimentally determined sequence and the corresponding predicted coding sequence. However, this was more likely due to assembly errors rather than to intrinsic differences between 'Qinguan' and 'Golden Delicious' cultivars.

The promoter regions of selected apple Argonaute genes contain ABREs, MBS, HSEs, and W-boxes. Because so many of these cis-elements are involved in responses to abiotic stresses and ABA treatment, we believe that this is

the reason why those genes are more highly expressed when plants are exposed to such conditions.

Conclusions

We performed a genome-wide analysis of the Argonaute gene family in apple and examined expression patterns under different abiotic stress conditions. These Argonautes present interesting gene pools for developing stress-tolerant apple cultivars. Further research is necessary to understand the biological functions and regulatory mechanisms of *MdAGOs* when plants are exposed to drought, and to explore how *MdAGO4.1* is involved in determining water-use efficiency in fruit crops.

Author contribution statement Shasha Zhou performed the experiments and wrote the manuscript. Shuangxun Ma contributed to the bioinformatic analysis. Mingjun Li, Cuiying Li, Xiaoqing Gong, and Qingmei Guan supervised the research design and reframed the manuscript. Yanxiao Tan, Yun Shao along with Chao Li discussed the results and commented on the manuscript. Fengwang Ma conceived and designed the study. There were no potential conflicts of interest between all authors.

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Compliance with ethical standards

This research had no potential conflicts of interest and did not involve any human participants or animals.

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