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Full Length Research Paper

Cloning and molecular characterization of *TaAGO1*, a member of argonaute gene family in wheat (*Triticum aestivum* L.)

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Argonaute (AGO) proteins play important roles in RNA silencing processes through formation of complexes with the mature microRNAs. In this study, a wheat AGO gene referred to *TaAGO1*, which shares high similarities to *AtAGO1* in Arabidopsis and *OsAGO1* in rice, was characterized. As a cDNA full length of 3747 bp, *TaAGO1* encodes a 1099-aa polypeptide with a molecular weight of 122 kD and an isoelectric point (pI) of 9.52. Subcellular prediction analysis suggests that *TaAGO1* is to target onto the cytoplasm after endoplasmic reticulum (ER) sorted. Similar to AtAGO1 and OsAGO1, *TaAGO1* contains PIWI and DDH, two conserved domains in AGOs. Phylogenetic analysis indicated that *TaAGO1* was possibly derived from different progenitors with its homologous across diverse plant species. The transcripts of *TaAGO1* were significantly regulated by the stresses of phosphorus deprivation and dehydration, and exogenous treatment of abscisic acid (ABA), suggesting that this wheat AGO member also exerts roles on mediating above signaling transductions. Southern blotting analysis revealed that genome AA, SS, and DD, three diploids composing of the hexaploid wheat, all harbored two copies of *TaAGO1*. Down-regulation of *TaAGO1* in wheat led to conspicuously phenotypic alterations of the young plantlets, with a variety of abnormal growth features. Taking the results in this study together, it was implicated that *TaAGO1* exists as a subset of copies in wheat and plays critical roles on silencing of appropriate target genes via regulation of *TaAGO1*-MiRNAs complex formation.

Key words: Wheat (*Triticum aestivum* L.), argonaute (AGO) gene 1, cloning, molecular characterization, anti-sense expression.

INTRODUCTION

RNA silencing, generally known as post-transcriptional gene silencing (PTGS), refers to the expression of one or more genes to be downregulated or entirely suppressed based on the mediation via an antisense RNA molecule (Baulcombe, 2004). Thus far, the well-studied examples of RNA interference (RNAi) cases are microRNAs (miRNAs) and small interfering RNAs, derived from endogenous and exogenous, respectively. Both of them silence the target genes by degradation of complementary mRNAs as well as repression of the mRNA translation efficiencies (Baulcombe, 2004).

For silencing the genes mediated by miRNAs pathway, the pre-miRNAs are firstly transcribed from endogenous *MIR* genes and processed into mature miRNAs (generally 20 to 24 nt) by Dicer-like (DCL) 1 (Kurihara and Watanabe, 2004), which are further assembled into a protein complex referred to RNA-induced silencing complex (RISC). It has been confirmed that argonaute1 (AGO1) acts as a key component of the RISC by interactions of the conserved domains with the miRNAs (Vaucheret et al., 2004). With variations in sizes and compositions, AGO1 proteins generally contain two signature domains, PIWI and PAZ (Song et al., 2004; Ma et al., 2004; Lingel et al., 2003), and exhibit an

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associated ribonuclease activity (Slicer) that cleaves its substrate at sRNA-complementary sites (Pham, 2004; Nykanen et al., 2001; Hammond et al., 2000). Crystal structure analysis of AGO homologues reveals that the PIWI domain adopts a fold similar to RNase H (Song et al., 2004) and the PAZ domain functions to bind the miRNAs (Ma et al., 2004; Lingel et al., 2003), respectively. AGO proteins also harbor a DDH motif being functionally equivalent to the catalytic metal-coordinating triad DDE in RNase H, and this motif is essential for the Slicer activity in AGOs (Rivas et al., 2005; Liu et al., 2004).

In *Arabidopsis*, the AGO family comprises 10 members (Carmell et al., 2002; Fagard et al., 2000), of which the most studied is the member of *AGO1* (*AtAGO1*). *AtAGO1* is found to associate with the miRNA and transgene-silencing pathway (Fagard et al., 2000; Vaucheret et al., 2004). Mutants of loss-of-function with strong *AGO1* alleles showed the impaired silencing effects of foreign transgenes (cosupression) (Fagard et al., 2000) and to be hyper-susceptibility to *Cucumber Mosaic Virus* (CMV) (Morel et al., 2002), demonstrating that *AGO1* plays critical roles in affecting miRNA accumulation and miRNAs target regulation (Vaucheret et al., 2004). Until now, although quantity of studies on AGO proteins have been conducted in model plant *Arabidopsis* (Havecker et al., 2010; Baumberger and Baulcombe, 2005) and rice (Wu et al., 2009; Nonomura et al., 2007; Liu et al., 2005), the functional characterizations of AGO proteins in wheat as well as in other plant species have been largely unknown.

In this study, an *AGO1* gene in wheat referred to as *TaAGO1* has been characterized. Sharing high similarities to *AtAGO1* in *Arabidopsis* and *OsAGO1* in rice, TaAGO1 was revealed to harbor the conserved domains generally situated in *AGO1* proteins. The expression levels of *TaAGO1* are regulated by several of abiotic environmental stimuli, such as abiotic stresses of phosphorus deprivation and dehydration, and exogenous treatment of abscisic acid (ABA). Downregulation of *TaAGO1* led to significantly alterations in plantlet phenotypes, confirming that TaAGO1 plays crucial roles on sustainment of plant normal growth. Taking together the results in this study, it has been implicated that TaAGO1 is essential on regulation of plant growth and response of distinct environmental stimuli in wheat via modulation of the RNA-induced silencing complex (RISC).

MATERIALS AND METHODS

Obtaining an EST with similarities to the *AGO1* homologous in *Arabidopsis* and rice

Previously, a root suppression subtractive hybridization (SSH) cDNA library (cv. Shixin828) enriching differentially up-regulated expression genes in normal Pi condition (CK) was constructed using root samples derived from different Pi-supply treatments (2 mM of control and 24 h of 20 µM Pi-starvation treatment). Sequencing analysis of part cloned in the library and further BLAST searches revealed an expression sequence tag (EST, length in 356 bp) sharing a high similarity to *Arabidopsis AGO1* gene (*AtAGO1*, GenBank accession number ATU91995). Owing to the potential roles on regulation of gene silencing via miRNAs pathway and being scarcely reported, the EST, a putative AGO1 member of the ARGONAUTE gene family, was designated as *TaAGO1* EST and subjected to further molecular characterization analysis.

Identification of the full length cDNA corresponding to *TaAGO1* EST

Using the *TaAGO1*-EST as a query, BLAST search analysis against the full length cDNA database of wheat (TriFLDB: Triticeae FULL-Length CDS DataBase (http://trifldb.psc.riken.jp/index.pl)) was performed. As a result, a full length cDNA (number of tplb0006m04 in the database) sharing a perfect identity with the *TaAGO1* EST was identified. This full length cDNA was assumed to be a homolog of *AtAGO1*.

Cloning of the putative wheat *AGO1* gene *TaAGO1*

The *TaAGO1* EST was firstly identified in a SSH cDNA library in which the root cDNAs from 24 h of Pi-starvation stress (20 µM Pi) acted as the driver to subtract those from growing in normal Pi-supply condition (2 mM Pi). The library therefore enriched the differentially upregulated genes in the later condition. Thus, the root cDNAs derived from seedlings growing under normal-Pi (2 mM) condition were used. The polymerase chain reaction (PCR) template was used for cloning the *TaAGO1*. Based on sequence of the full length cDNA clone (tplb0006m04 in TriFLDB), a forward primer (5′-AGCGGCCGTCGTCTTTTC) and reverse primer (5′-GATGGTTACACAGAGTTTCACACG) were synthesized. The PCR was performed in a 20 µL volume composed of the following components: 2 µL of cDNA template, 1 µL of forward and reverse primer each with 20 µM concentration, 2 µL of 10 µM dNTP mixture, 2.5 U TaKaRa LA Taq HS (TaKaRa) and 2 µL 10 × buffer. The program for PCR was as follows: 95°C for 5 min, then 30 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 4 min. The PCR products were detected based on ethidium bromide staining after agarose electrophoresis analysis.

Molecular characterization analysis of *TaAGO1*

Online tools, referred to as ExPASy Proteomics Tools (http://www.expasy.org/tools), were adopted to assess the molecular characterization of *TaAGO1*. The tool that translates a nucleotide sequence to a protein sequence (http://www.expasy.org/tools/dna.html) was used to predict the putative translated polypeptide of *TaAGO1*. A computation tool of pI/Mw for Swiss-Prot/TrEMBL entries (http://www.expasy.org/tools/pi_tool.html) was used to calculate the isoelectric point (pI) and molecular weight (Mw) of TaAGO1. The TargetP 1.1 program that predicts the subcellular location of eukaryotic proteins (http://www.cbs.dtu.dk/services/TargetP/) was used to determine the subcellular location of TaAGO1. The conserved domains, catalytic domain harboring at the C-terminal region, conserved amino acid residues and Mg^{2+} coordinating residues situated in the DDH catalytic triad, were manually defined by referencing the descriptions of Baumberger and Baulcombe, (2005).

Phylogenetic analysis of *TaAGO1* and its plant homologous

For generation of a phylogenetic tree covering *TaAGO1* and its

plant homologous, the cDNA sequences similar to *TaAGO1* across diverse plant species were first obtained based on BLAST search against the GenBank in National Center for Biotechnology Information (NCBI) using the sequence of *TaAGO1* as a query. The cDNA sequences of *TaAGO1* and its homologous were subjected to establishment of a phylogenetic tree by using the package in DNAStar software (DNASTAR Inc., Madison, WI).

Expression analysis of *TaAGO1* under various abiotic stresses and treatment of abscisic acid (ABA)

Shixin828, a cultivar for generation of the root SSH library earlier mentioned, was used for the examination of the expression patterns of *TaAGO1* under diverse abiotic stresses and the treatment of abscisic acid (ABA). The seedlings for detection of the *TaAGO1* transcripts were hydroponically cultured in a growth chamber under the following conditions: photoperiod of 12 h (day)/12 h (night) with a light flux density of 400 µE m^{-1} s^{-1} during the daytime, temperature of 20°C (day)/15°C (night) and air humidity of 70%. During the culture process, the nutrient solution of MS medium was air-circulated with a mini pump and refreshed twice regularly in each week.

Abiotic stresses set up in this study included: deprivations of phosphorus (-P), nitrogen (-N), potassium (-K), and calcium (-Ca), and stresses of dehydration [polyethylene glycol (PEG)], low temperature (4°C) and salinity (NaCl). Exogenous treatment of ABA was also set up in addition to the aforementioned stress treatments. The treatments of deprivation of P, N, K and Ca were initiated by reducing the nutrient amount in the MS medium separately. Deprivation of P, N, K, and Ca was performed by reducing the amount of Pi, N, K^+, and Ca^{2+} from 2 mM to 20 µM, 5 mM to 20 µM, 5 mM to 50 µM, and 1 mM to 10 µM, respectively. For treatments of dehydration, salinity and ABA, the growth medium were supplemented with 10% (w/w) polyethylene glycol 6000 (PEG), 150 mM sodium chloride (NaCl), and 10 mg/L ABA, respectively while treatment of low temperature was carried out by transferring the seedlings to 4°C from the normal growth temperature. In all treatments, the roots were sampled at 1, 3, and 9 h after the treatment initiations. The roots harvested before the treatments were used as control (CK).

Total RNA was extracted using TRIzol reagents (Invitrogen) according to the product's instruction. After DNase treatment, the total RNA was transcribed into cDNAs by M-MLV reverse transcriptase (TaKaRa). The *TaAGO1* transcripts were detected based on semi-quantitative real-time polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR) using primers 5′-AGCCGGATACCTCTGACAGTG (forward) and 5′-CACACGGTACAACAATGAAAGTTT (reverse) for specifically amplification of the 3′-untranslated region (UTR). The reaction volume and program of RT-PCR was the same as that in cloning of the full length cDNA sequence of *TaAGO1*. For qRT-PCR analysis, the SYBR green dye (Bio-Rad) was added in the reaction solutions and the products were detected in iCycler iQ5 detection system (Bio-Rad). All the procedures, as well as the signal detections, were performed by following the company's description. The RT-PCR and qRT-PCR were all performed in triplicates with reproducible results. For normalization of the putative bias in the results, *tubulin*, a constitutively expressed gene in wheat, was employed as internal standard using primers 5′-AGAACACTGTTGTAAGGCTCAAC (forward) and 5′-GAGCTTTACTGCCTCGAACATGG (reverse).

Identification of the *TaAGO1* locations at the genome level

As a hexaploid, the wheat genome is derived from three homologous diploids which carry genome A, B, and D, respectively. For elucidating which diploid genome carries *TaAGO1*, PCR analysis was performed using DNA extracted from the three diploids as the template. The diploid materials used in this study were *Triticum urartu* 203 (genome AA), *Aegilops searsii* 4042 (genome SS), and *Ae. tauschii* 4857 (genome DD), respectively. Using the DNA extracted from these diploids by following the descriptions of Murray et al. (1980) as the templates, the PCR was performed with same reaction conditions as those in detection of *TaAGO1* transcripts mentioned previously.

For further examination of *TaAGO1* copies in diverse homologous diploids, Southern blotting analysis was performed by the standard procedure (Southern, 1975). Briefly, 10.0 µg of genomic DNA was digested with *Pst*I, *Not*I or *Nco*I, respectively. The fragments after digestion were separated on a 0.8% agarose Tris/acetate/EDTA gel and then transferred to positively charged nylon membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was then used to hybridize with *TaAGO1* 3'-UTR fragments that labeled with alkaline phosphatase (AlkPhos Direct, Amersham Biosciences) according to the manufacturer's instruction. The signals were detected by using CDP-*Star* chemiluminescent reagent (Amersham Biosciences).

Anti-sense plasmid construction and genetic transformation in wheat

The 5′- region of *TaAGO1* with anti-sense orientation was amplified by PCR from the plasmid using primers of 5′-AAGGATCCTGGAGGAACGTTACGTCCA (forward, *BamH*I site underlined) and 5'-AATCTAGAGGGCAGGGTGGTGGTGA (reverse, *Xba*I site underlined). After digested by *BamH*I and *Xba*I, the fragment of *TaAGO1* was cloned onto binary vector pBI121 (Clontech, USA) with anti-sense orientation positioning at the downstream of CaMV35S promoter. After verification by PCR analysis and sequencing, the binary plasmids were introduced into *Agrobacterium tumefaciens* EHA105 and then used to transform wheat immature embryo tissues (cv. Shixin828) by following the description of Wu et al. (2003). Briefly, the young seeds after 15 to 18 days of fertilization were collected from plants (var. Shixin 828) growing in the Experimental Station of Agricultural University, Baoding, China. After sterilization by 3 min of 70% ethanol followed by 10 min of 0.15% $HgCl_2$, the immature embryo was separated from the young seeds and induced to generate callus. The 4-week induced callus was subjected to transform the *A. tumefaciens* EHA105, which harbored the *TaAGO1* anti-sense expression construct. The induced callus that transformed the host harboring the empty vector (pBI121) was used as control. Pictures of the young plantlets generated from the callus that transformed the anti-sense *TaAGO1* construct and the binary empty vector were taken with digital camera, and the phenotypic features were compared based on visual analysis.

RESULTS

Cloning and molecular characterization of *TaAGO1*

An expressed sequence tag (EST) derived from a root suppression subtractive (SSH) cDNA library was identified to share high similarities to *AGO1* genes in *Arabidopsis* (*AtAGO1*, GenBank accession number ATU91995) and in rice (*OsAGO1*, GenBank accession number AB081951), with identities of 77.1 and 75.9%, respectively. These results suggest that the gene corresponding to this EST is a member of argonaute (AGO) gene family in wheat and referred to *TaAGO1*-EST here

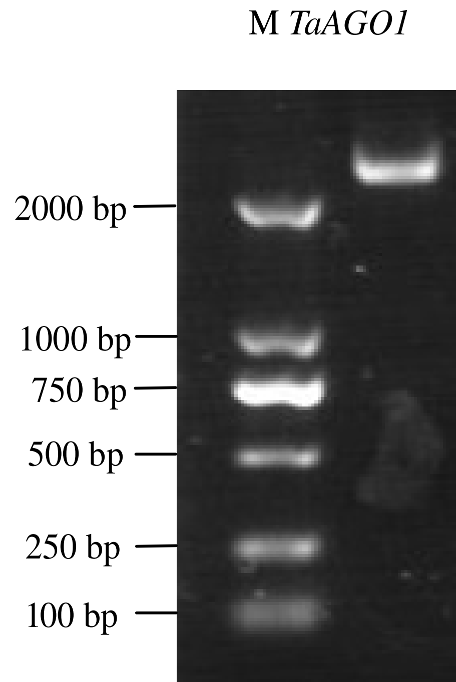

Figure1. *TaAGO1* RT-PCR products detected based on 1% agarose electrophoresis analysis. M, DL2000 DNA marker.

after. For identification of the putative full-length cDNA, a similarity search analysis was performed by scanning the *Triticeae* Full-Length CDS DataBase (TriFLDB, http://trifldb.psc.riken.jp/index.pl) using the EST as a query. Fortunately, a full-length cDNA (accession number of tplb0006m04) corresponding to *TaAGO1*- EST was identified. We designated the full-length cDNA as *TaAGO1*.

Based on the cDNA sequence of *TaAGO1*, the wheat AGO member was specifically amplified (Figure 1). Sequence comparison between the RT-PCR product (*TaAGO1*) and the wheat putative *AGO1* released in the database (tplb0006m04) revealed a base G, at position 1868 in tplb0006m04 was false and not of existence, possibly owing to the sequencing error. *TaAGO1* had a full-length cDNA of 3747 bp, containing an open reading frame (ORF) of 3300 bp and encoding a polypeptide of 1099 amino acids. The sequences of the full-length cDNA of *TaAGO1* and the corresponding translated polypeptide are shown in Figure 2. TaAGO1 has a molecular weight of 122 kD, with a isoelectric point (pI) of 9.52. Subcellular predication analysis did not detect conspicuous signals targeting to chloroplast, mitochondrion, and membrane system in TaAGO1, suggesting that TaAGO1 is targeted onto the cytoplasm after endoplasmic reticulum (ER) sorting and where to exert its biological functions.

The C-terminal region of AGO1 generally endows the argonaute protein catalytic roles (Rivas et al., 2005; Liu et al., 2004). Previously, the catalytic region, as well as the conserved domain PIWI of *AtAGO1* is defined to be located at the amino acid (aa) residues of 756 to 992. Based on alignment analysis, the C-terminal catalytic region and the PIWI domain in *TaAGO1*, as well as in *OsAGO1* and in barley *AGO1* (HvcDNA), were also identified in this study. The regions in the *AGO1* proteins are situated at aa residues of 810 to 1046, 616 to 852 and 830 to 1066, respectively (Figure 3). In addition, a total of three conserved sites for Mg^{2+} coordination, including the aa residue of 814 (aa residue of D), aa residues of 1000 to 1004 (aa residues of RDGVS) and aa residue of 1041 (aa residue of H) were identified in the catalytic triad of TaAGO1. Furthermore, two strongly conserved amino acid residues including the Gly-812 and His-852 were also identified in *TaAGO1* (Figure 3). Taken together, the highly shared conserved region, Mg^{2+} coordination sites, and amino acid residues in *TaAGO1* with other *AGO1* proteins across diverse plant species have implicated that this putative wheat *AGO1* member plays important roles on regulation of the target gene silencing via miRNA pathway.

Phylogenetic analysis of *TaAGO1*

Using the full-length cDNA sequence of *TaAGO1* as a query, the cDNA sequences sharing high similarities to this wheat argonaute member in diverse plant species were identified and all of them were subjected to

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1    GAGCGAGCTTCACTGCTGCTAGTTAACCGCAAGCGGCCGTCGTCTTTTCCCTCCTCAAAAAAGCAGCCGAGGGCAGGGTGGTGGTGACTGGAGGCGGAGACAGAGGGAGGAGCAGCGCCA
121  GTGCGAGAGATAGTGGGGGGGAGCTCGGACGAGGCCTAGGGCTTCCGGAGACCGCCTCCTCCCGCGCCGCCGTTCGCCGAGCTTGCTTGCTCAGCGGCCATCATGGTTAGGAAGAAAAGA
                                                                                                                       M  V  R  K  K  R
241  ACTGGTCCTGGCAGCCCTGGAGAGAGTTCTGGGGAAGCTTCAGGAGCTTCTGGGCAGGGTTCCTCGCAGCGAGCTGAGAGAGCTCCTCAACAGCATGGTGGAGGACGTGGTGTGGCACCT
      T  G  P  G  S  P  G  E  S  S  G  E  A  S  G  A  S  G  Q  G  S  S  Q  R  A  E  R  A  P  Q  Q  H  G  G  G  R  G  V  A  P
361  CAACAGCATGGCCGTGGTGGGGGCAACACCAGGGCCGCGGTGGGCACTATCAGGGCCATGGATCAGCTTCACACTATCCAGGTGGTGGCCCACCTGAATATCAACAGCGTGACTATCAG
      Q  Q  H  G  R  G  G  G  Q  H  Q  G  R  G  G  H  Y  Q  G  H  G  S  A  S  H  Y  P  G  G  G  P  P  E  Y  Q  Q  R  D  Y  Q
481  GGGCGGGGACATCCAGGTGGTGGTCCACCTGAATATCAACATCGTGACTATCAGGGGCGTGGACATCCGGGGGGTGGCCCACCTGAATATCAACCACGTGACTATCAGGGACGTGGCGGT
      G  R  G  H  P  G  G  G  P  P  E  Y  Q  H  R  D  Y  Q  G  R  G  H  P  G  G  G  P  P  E  Y  Q  P  R  D  Y  Q  G  R  G  G
601  CCACGCCCCAGAGGTGGTGGAGTGCCGCAGCCATACTATGGCGGGCATAGGGGAGGTAGTGCTGGACGTAACGTTCCTCCAGGTCCACCAAGAACAGTTCCCGAGCTGCACCAAGCCCCG
      P  R  P  R  G  G  G  V  P  Q  P  Y  Y  G  G  H  R  G  G  S  A  G  R  N  V  P  P  G  P  P  R  T  V  P  E  L  H  Q  A  P
721  TATGTCCAATATCAAGCCCCGGTGGTTTCACCATCCGCATCAGGAGCTGGCTCATCCTCTCAGCCTGTGTCAGAGGTGAGCAGTGGACAAGTCCAGCAACAGTTTCAGAAACTTGCGATT
      Y  V  Q  Y  Q  A  P  V  V  S  P  S  A  S  G  A  G  S  S  S  Q  P  V  S  E  V  S  S  G  Q  V  Q  Q  Q  F  Q  K  L  A  I
841  GTTGATCAAAGTTCAACAAGCCAATCCAGTCAACTGGCACCAGCATCAAGCAAATCAGTTAGATTTCCATTGCGCCCTGGAAAGGGTACACATGGAGATAGGTGTGTCGTGAAGGCAAAC
      V  D  Q  S  S  T  S  Q  S  S  Q  L  A  P  A  S  S  K  S  V  R  F  P  L  R  P  G  K  G  T  H  G  D  R  C  V  V  K  A  N
961  CATTTCTTTGCTGAGCTCCCTGATAAAGACCTTCACCAATATGATGTCTCCATAACTCCAGAGATTACATCACGTGGTGTCAATCGTGCTGTGATGGCAGAACTTGTTAGGCTCTATAGA
      H  F  F  A  E  L  P  D  K  D  L  H  Q  Y  D  V  S  I  T  P  E  I  T  S  R  G  V  N  R  A  V  M  A  E  L  V  R  L  Y  R
1081 CATTCTCAATTGGATGGACGCCTACCTGCGTATGATGGAAGGAAGAGCCTATATACAGCTGGGCCATTGCCATTTCCTTCAAAGACATTTGAAATTACTCTGCATGACGAAGAAGAAAGC
      H  S  Q  L  D  G  R  L  P  A  Y  D  G  R  K  S  L  Y  T  A  G  P  L  P  F  P  S  K  T  F  E  I  T  L  H  D  E  E  E  S
1201 CTTGTTGGTGGTCAAGTCGCACCAAGGCGTGAGAGACAATTCAGGGTGGTGATCAAATTTGCTGCTCGTGCTGATCTCCACCATTTGGCTATGTTCCTAGCTGGAAGGCAACCTGATGCA
      L  V  G  G  Q  V  A  P  R  R  E  R  Q  F  R  V  V  I  K  F  A  A  R  A  D  L  H  H  L  A  M  F  L  A  G  R  Q  P  D  A
1321 CCTCAAGAAGCTCTTCAGGTGCTTGACATTGTGCTGCGTGAACTGCCTACTGCCAGGTATTCTCCAGTTGGCCGGTCATTTTATTCTCCGAACTTAGGGAGACGTCAGAAACTTGGTGAT
      P  Q  E  A  L  Q  V  L  D  I  V  L  R  E  L  P  T  A  R  Y  S  P  V  G  R  S  F  Y  S  P  N  L  G  R  R  Q  K  L  G  D
1441 GGTTTGGAAAGCTGGCGTGGATTTTACCAAAGTATAAGACCTACACAGATGGGGCTTTCGCTGAATATTGATATGTCTTCTACTGCATTTATTGAGCCTCTTCCTGTGATTGATTTTGTT
      G  L  E  S  W  R  G  F  Y  Q  S  I  R  P  T  Q  M  G  L  S  L  N  I  D  M  S  S  T  A  F  I  E  P  L  P  V  I  D  F  V
1561 GCTCAGCTTCTGAGCAGAGACATCTCAGTTAGACCCCTATCTGATTCAGATCGTGTGAAGATTAAAAAAGCCCTAAGAGGTGTAAAGGTCGAGGTTACACATCGAGGAAACATGCGTAGG
      A  Q  L  L  S  R  D  I  S  V  R  P  L  S  D  S  D  R  V  K  I  K  K  A  L  R  G  V  K  V  E  V  T  H  R  G  N  M  R  R
1681 AAATACCGTATATCTGGACTAACTTCACAAGCAACACGAGAGCTATCATTCCCTGTTGATGATCGTGGTACTGTGAAGACTGTGGTGCAATACTTCCTGGAGACCTATGGTTTTAATATT
      K  Y  R  I  S  G  L  T  S  Q  A  T  R  E  L  S  F  P  V  D  D  R  G  T  V  K  T  V  V  Q  Y  F  L  E  T  Y  G  F  N  I
1801 CAGCACACAACTCTACCTTGCTTGCAAGTGGGCAATCAGCAAAGGCCTAATTATCTTCCTATGGAGGTTTGTAAGATTGTTGAAGGACAACGTTACTCGAAACGACTGAATGAGAAACAG
      Q  H  T  T  L  P  C  L  Q  V  G  N  Q  Q  R  P  N  Y  L  P  M  E  V  C  K  I  V  E  G  Q  R  Y  S  K  R  L  N  E  K  Q
1921 ATAACTGCTCTACTGAAAGTGACTTGCCAGCGCCCTCAAGAGCGTGAGAAGGACATCTTGCAGCCAGTGCATCACAATGCATACTATGAAGATCCATATGCACAGGAATTTGGCATAAAA
      I  T  A  L  L  K  V  T  C  Q  R  P  Q  E  R  E  K  D  I  L  Q  P  V  H  H  N  A  Y  Y  E  D  P  Y  A  Q  E  F  G  I  K
2041 ATTGATGAGCGACTTGCATCGGTTGAAGCTCGTGTTCTGCCTCCGCCAAGACTTAAATACCATGATAGTGGCAGAGAGAAGGATGTCTTGCCCAGGATCGGCCAATGGAACATGATGAAC
      I  D  E  R  L  A  S  V  E  A  R  V  L  P  P  P  R  L  K  Y  H  D  S  G  R  E  K  D  V  L  P  R  I  G  Q  W  N  M  M  N
2161 AAGAAAATGGTGAACGGTGGTAGGGTCAGTCACTGGGCATGTATAAACTTCTCTCGGAATGTGCAAGATAGTGCTGCCAGGGGTTTTTGCCATGAGCTTGCTATAATGTGCCAAATATCT
      K  K  M  V  N  G  G  R  V  S  H  W  A  C  I  N  F  S  R  N  V  Q  D  S  A  A  R  G  F  C  H  E  L  A  I  M  C  Q  I  S
2281 GGAATGGACTTGGCACCTGAACCTGTGCTGCCCCCACTTACTGCGAGACCTGAACATGTAGAAAGAGCACTAAAGGCGCGTTATCAAGATGCCATGAATATAATCAGACCCCAGGGAAGA
      G  M  D  L  A  P  E  P  V  L  P  P  L  T  A  R  P  E  H  V  E  R  A  L  K  A  R  Y  Q  D  A  M  N  I  I  R  P  Q  G  R
2401 GAACTTGACCTGCTAATTGTAATACTGCCTGACAATAATGGTTCTCTTTATGGGGATCTCAAAAGGATTTGCGAGACTGATCTTGGATTGGTATCTCAGTGTTGTCTGACAAAACATGTT
      E  L  D  L  L  I  V  I  L  P  D  N  N  G  S  L  Y  G  D  L  K  R  I  C  E  T  D  L  G  L  V  S  Q  C  C  L  T  K  H  V
2521 TTTAAGATGAGCAAGCAGTATCTTGCAAATGTAGCTCTTAAAATTAATGTTAAGGTGGGTGGAAGGAATACTGTACTTGTGGATGCCTTGACAAGGAGGATTCCCCTTGTCAGTGACAGA
      F  K  M  S  K  Q  Y  L  A  N  V  A  L  K  I  N  V  K  V  G  G  R  N  T  V  L  V  D  A  L  T  R  R  I  P  L  V  S  D  R
2641 CCAACAATAATCTTTGGTGCTGATGTTACACACCCTCATCCTGGAGAAGATTCCAGTCCTTCCATTGCTGCTGTGGTTGCTTCCCAAGACTGGCCTGAGGTCACTAAGTATGCAGGATTA
      P  T  I  I  F  G  A  D  V  T  H  P  H  P  G  E  D  S  S  P  S  I  A  A  V  V  A  S  Q  D  W  P  E  V  T  K  Y  A  G  L
2761 GTAGGCGCCCAAGCCCATAGGCAAGAGTGGATACAGGATCTTTTCAAAGTATGGCAAGATCCCCAAAGAGGAACTGTGACTGGTGGCATGATCAAGGAGCTTCTCATTTCTTTCAAGAGG
      V  G  A  Q  A  H  R  Q  E  W  I  Q  D  L  F  K  V  W  Q  D  P  Q  R  G  T  V  T  G  G  M  I  K  E  L  L  I  S  F  K  R
2881 GCAACTGGACAGAAACCCCAGAGAATCATATTTTACAGGGATGGTGTCAGCGAGGGACAGTTCTATCAAGTTCTTTTGTATGAACTCGATGCCATTAGAAAGGCATGTGCGTCCTTGGAA
      A  T  G  Q  K  P  Q  R  I  I  F  Y  R  D  G  V  S  E  G  Q  F  Y  Q  V  L  L  Y  E  L  D  A  I  R  K  A  C  A  S  L  E
3001 CCCAATTACCAGCCTCCAGTTACTTTTGTAGTCGTCCAGAAGCGTCATCACACCAGGCTCTTCGCTAATAACCACAATGATCAGCGTACTGTTGATAGAAGTGGAAACATACTGCCTGGC
      P  N  Y  Q  P  P  V  T  F  V  V  V  Q  K  R  H  H  T  R  L  F  A  N  N  H  N  D  Q  R  T  V  D  R  S  G  N  I  L  P  G
3121 ACTGTTGTTGATTCAAAGATTTGCCATCCGACTGAGTTCGATTTCTACTTGTGTAGCCACGCTGGCATTCAGGGAACTAGCCGTCCTGCTCATTATCACGTTCTATGGGACGAGAACAAA
      T  V  V  D  S  K  I  C  H  P  T  E  F  D  F  Y  L  C  S  H  A  G  I  Q  G  T  S  R  P  A  H  Y  H  V  L  W  D  E  N  K
3241 TTCACTGCTGATGAGTTGCAAACTCTAACAAACAACTTGTGCTACACGTATGCTAGGTGCACCCGCTCTGTATCAATTGTGCCTCCAGCATATTATGCACATTTGGCAGCCTTCCGGGCT
      F  T  A  D  E  L  Q  T  L  T  N  N  L  C  Y  T  Y  A  R  C  T  R  S  V  S  I  V  P  P  A  Y  Y  A  H  L  A  A  F  R  A
3361 AGGTTTTACATGGAGCCGGATACCTCTGACAGTGGGTCCATGGCAAGCGGTGCCCGTGGCCCTCCACAGGGTGGACGCAATACCAGGCCGTTTGGGAATGTTGCTGTCAGGCCCCTCCCC
      R  F  Y  M  E  P  D  T  S  D  S  G  S  M  A  S  G  A  R  G  P  P  Q  G  G  R  N  T  R  P  F  G  N  V  A  V  R  P  L  P
3481 GCCCTGAAGGAAAACGTGAAGCGGGTCATGTTTTACTGTTAAGGTCGTGGTGGTTATTGTTCACCCTAATATGCAAACCTGAAGATATTCGACTGAGTTTGGGTGTGTTAAAAGTATTTG
      A  L  K  E  N  V  K  R  V  M  F  Y  C
3601 CACTGCATTGGTGGCGCTGGGAGAACACCGCCATGCAAACTTTCATTGTTGTACCGTGTGAAACTCTGTGTAACCATCTACCTATCTGTGGTCGTATTTGAACTACCCTGAAATCATTTC
3721 TGGTTTGGGAAAAAAAAAAAAAAAAAA
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Figure 2. Sequences of the full length cDNA and the translated polypeptide of *TaAGO1.* The translation start codon ATG and the translation termination codon TAA are boxed. The arrows with directions of forward and reverse show the positions of primers for detection of the transcripts of *TaAGO1* when subjected to various abiotic stresses and treatment of abscisic acid (ABA)

phylogenetic analysis. It revealed that *TaAGO1* and its plant homologous could be classified into five subgroups based on sequence identities, of which *TaAGO1* was cladded into same subgroup with the unknown function cDNAs from barley (*Hordeum vulgare* cDNA, accession No. AK250484), rice (*Oryza sativa* cDNA, accession No. AK111587) and maize (*Zea Mays* CL857 cDNA, accession No. AY109385) with a range of identity percentages of 74.1 to 94.8% at nucleic acid level. *TaAGO1* was also shown to share high similarities with

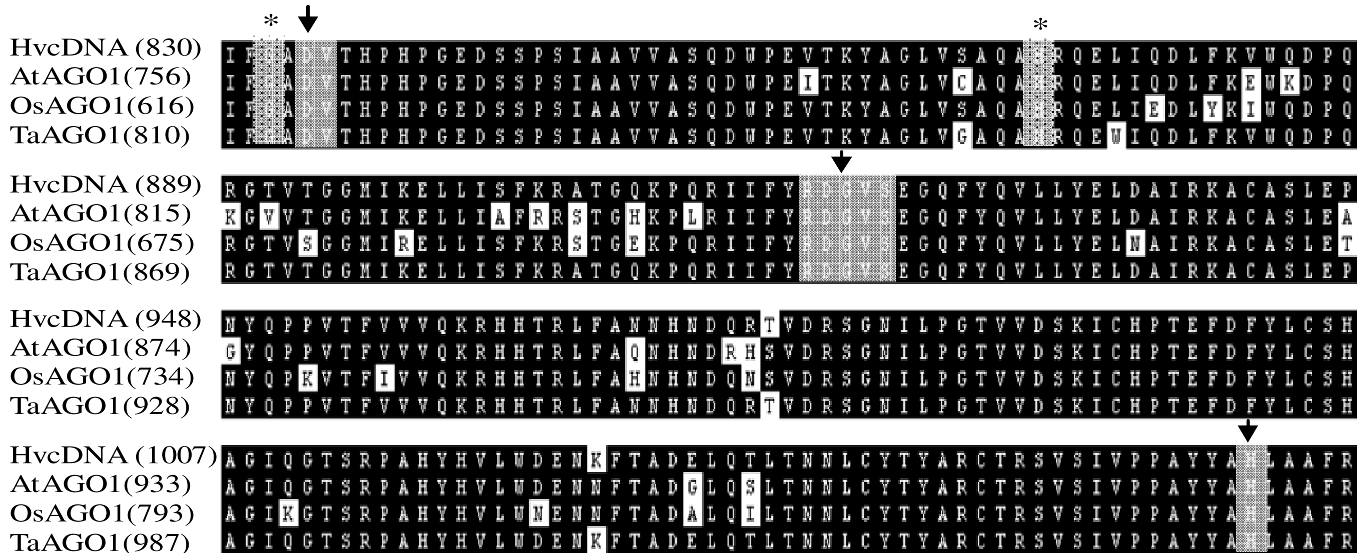


Figure 3. Alignment of the conserved PIWI domains, which act as the catalytic centers in AGO1 proteins across *TaAGO1*, *AtAGO1*, *OsAGO1*, and a function unknown protein of from a *H. vulgare* cDNA. The positions of Mg^{2+} coordinating residues in the DDH catalytic triad are indicated as arrows. The Gly-812 and His-852 in *TaAGO1*, corresponding to Gly-758 and His-798 in AtAGO1, two amino residues strongly conserved throughout AGOs, are labeled by symbol (*).

AtAGO1 and *OsAGO1*, showing the identities of 62.7 and 57.8%, respectively (Figure 4). Therefore, the wheat *AGO1* member *TaAGO1* is possibly derived from same progenitor with the function unknown cDNAs from barley, rice, and maize mentioned previously.

Expression patterns of *TaAGO1* under the conditions of various abiotic stresses and ABA treatment

The expression patterns of *TaAGO1* in roots under conditions of normal growth (CK) and various abiotic stresses were assessed. The treatments set up in this study covered the abiotic stresses such as deprivations of phosphorus (-P), nitrogen (-N), potassium (-K), and calcium (-Ca), and stresses of dehydration (PEG treatment), low temperature (4℃) and salinity (NaCl treatment), and treatment of exogenous ABA. Of these treatments, the expression levels of *TaAGO1* were not altered under the conditions of -N, -K, -Ca, 4℃ and salt stress based on semi-quantitative RT-PCR and qRT-PCR analysis. In a 9-h regime of above stress conditions, no variations of the *TaAGO1* transcripts were detected in these treatments and those in control (Figure 5). However, *TaAGO1* transcripts were dramatically regulated by stresses of -P, PEG and ABA treatment. During a 9-h duration treatment, the expression levels of *TaAGO1* were downregulated under stresses of -P and PEG and upregulated under ABA treatment (Figure 5). Therefore, *TaAGO1* has been implicated to exert extra roles on responses to part of external signals, such as stress signaling of phosphorus starvation, dehydration and ABA, in addition to its conserve functions on formation of RISC that mediates the target gene silencing via miRNAs pathway.

Location of *TaAGO1* at the genome level

Using the DNAs derived from genome AA, SS and DD, three homologous diploid genomes comprising the hexaploid genome of wheat PCRs were performed to determine the location of *TaAGO1* at the genome level. It is shown that *TaAGO1* could be specifically amplified in all the tested diploids (Figure 6A), suggesting that the genome AA, SS and DD all contained *TaAGO1* copies. For examination of exact copy numbers of *TaAGO1* in the three homologous diploids, Southern blotting analysis was performed using the labeling of *TaAGO1* 3′-untranslated region (UTR) as the probe. In consistent with the mentioned PCR results, all of the three homologous diploids harbored *TaAGO1*, with two copies in each (Figure 6B). Based on the PCR and Southern blotting analysis results, it was confirmed that *TaAGO1*, an *AGO1* member of the argonaute proteins, is exist as a small family in wheat.

Effects of downregulation of *TaAGO1* on differentiation and growth of the genetic transformants

For examination of the putative biological functions of *TaAGO1*, transgenic wheat plantlets integrated *TaAGO1* 3′- UTR with the anti-sense orientation were generated based on *Agrobacterium*-mediated transformation

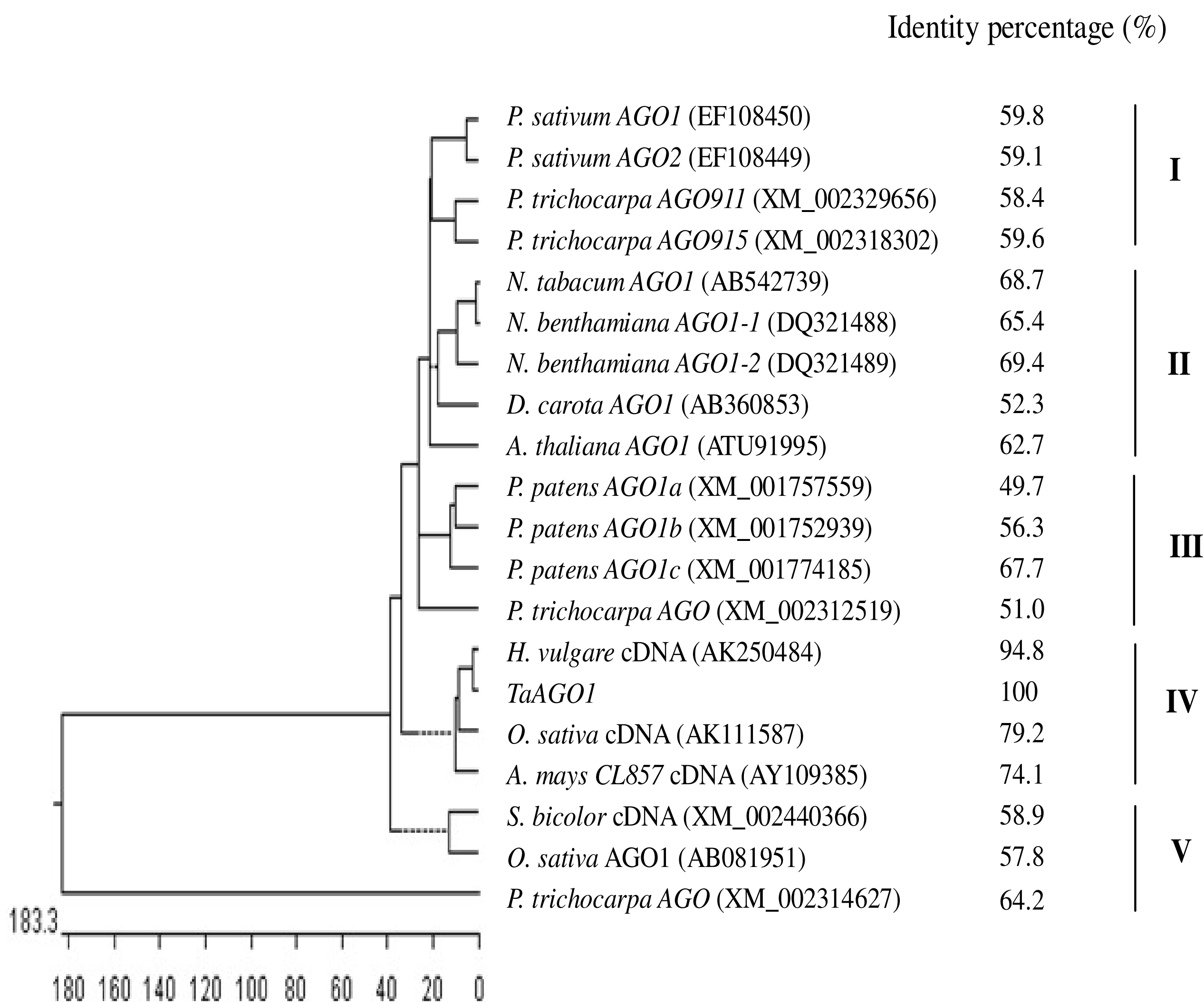


Figure 4. Phylogenetic analysis of *TaAGO1* and its homologs across diverse plant species at the nucleic acid level. I to V represent the subgroups classified based on similarity divergence.

approach. It was observed that downregulated expression of *TaAGO1* could result in conspicuous alterations on the shoots phenotype, showing the generated young plantlets with more stunted growing rate, more frequently abnormal growth features and much more adventitious roots compared with the control (shoots generated from the transformation of empty binary vector) (Figure 7). These results implicate that *TaAGO1* acts as an important effecter on regulation of plant growth via miRNA-mediated pathway.

DISCUSSION

Members of the AGO class of proteins among eukaryotes have been demonstrated to act as effectors in RNA silencing, a conserved mechanism that involves the processing of double-stranded RNA (dsRNA) into small RNA (sRNA) species of 21 to 26 nucleotides (Hannon, 2002; Bartel, 2004). Among the 10 predicted AGO proteins in *Arabidopsis*, the number of *AGO1* (*AtAGO1*) is verified to act as a RNA slicer that selectively recruit sRNA species to direct the cleavage of target mRNA in RISC (Liu et al., 2004; Baumberger and Baulcombe, 2005; Rivas, et al., 2005; Qi et al., 2005). In *Arabidopsis* and rice, the AGO1 is confirmed to act as the founding member of the AGO family and is required for normal plant development (Bohmert et al., 1998). *AGO1* mutants were shown to confer pleiotropically effects on plant architecture (Morel et al., 2002) and null alleles behaved elevation of miRNA target mRNAs (Vaucheret et al., 2004), which are in agreement with AGO1 being the slicer component of RISC. In addition, *AGO1* was observed to play a central role in the posttranscriptional

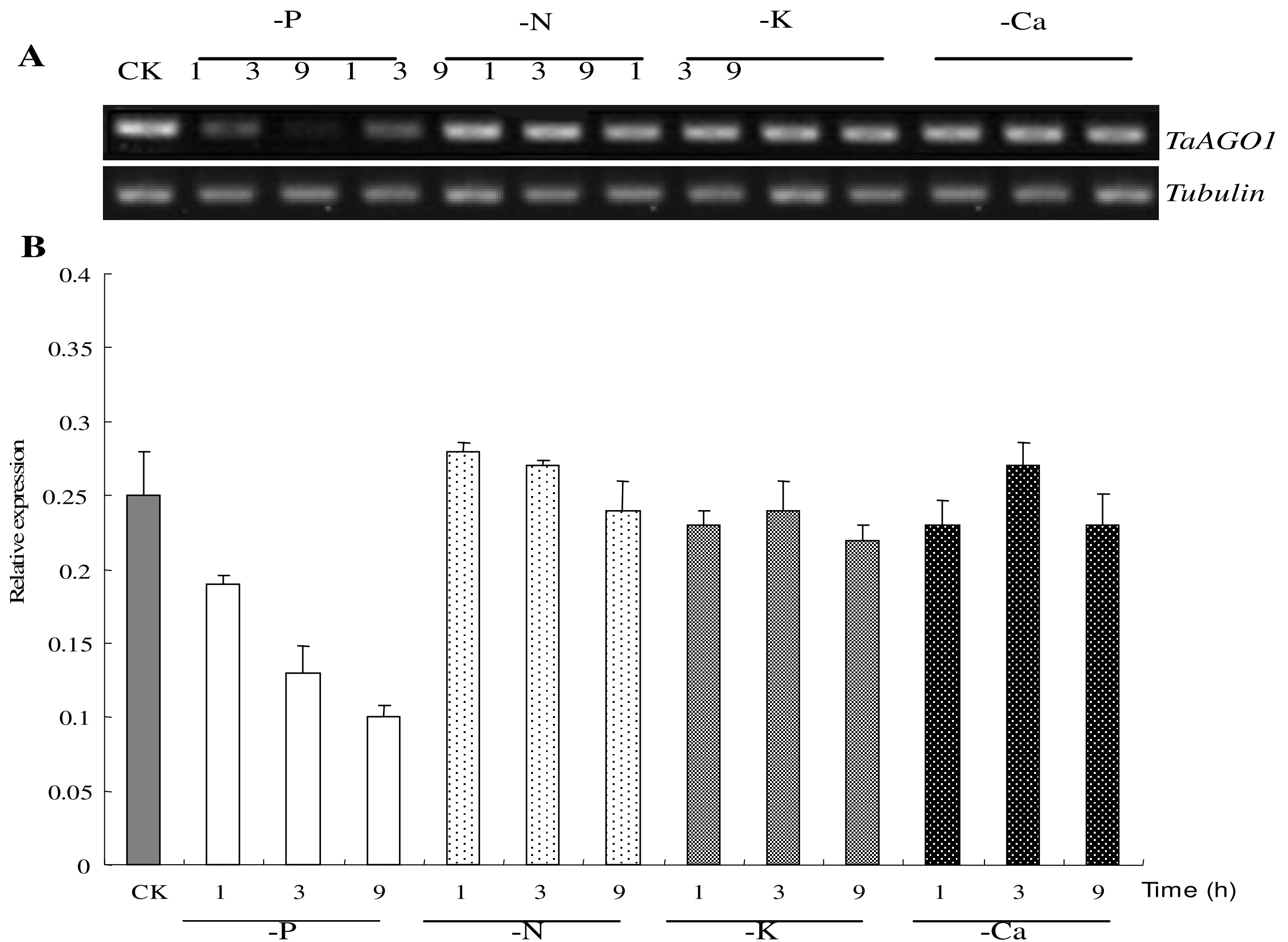
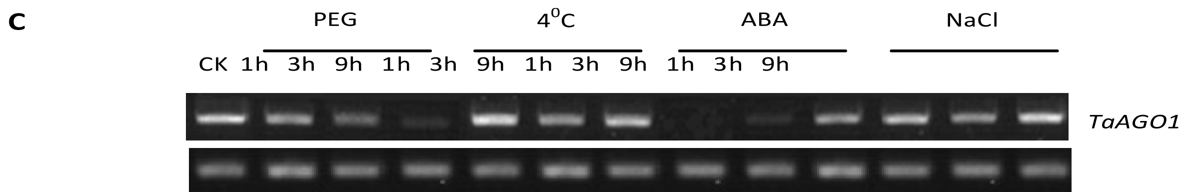


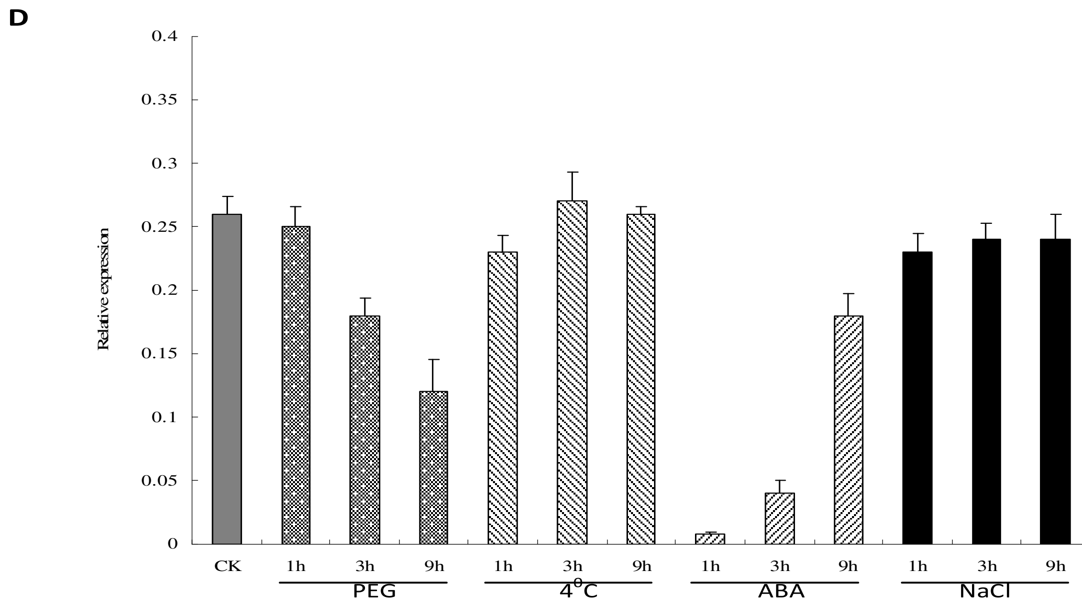
Figure 5. Expression patterns of *TaAGO1* under various abiotic stresses and exogenous treatment of abscisic acid (ABA). The abitic stresses include deprivations of phosphorus (-P), nitrogen (-N), potassium (-K), and calcium (-Ca), and drought (PEG treatment), low temperature (4℃), and salinity (NaCl treatment). A,C. *TaAGO1* transcripts detected based on semi-quantitative RT-PCR analysis. B,D. *TaAGO1* transcripts detected based on qPCR analysis. In B and D, the data were shown mean± Se from three independent biological replicates. The constitutive expressed gene *tubulin* was used the internal standard for normalization of the *TaAGO1* transcripts.

gene silencing of *CLF*, a gene encoding a Polycomb group protein that maintains the repression of both *KNOX* genes and the homoerotic genes *AG* and *AP3* in vegetative leaves (Goodrich et al., 1997; Katz et al., 2004), and was involved in regulation in pollen development (Kidner and Martienssen, 2005).

So far, the *AGO1* homologs have been functionally characterized in model plant Arabidopsis, as well as in monocot specie rice (Morel et al., 2002; Nonomura et al., 2007), few investigations on *AGO* genes have been conducted in wheat and other diverse plant species. In this study, we cloned a putative AGO1 homologous in wheat referred to as *TaAGO1*, based on an EST identified in a root suppression subtractive hybridization (SSH) cDNA library. *TaAGO1* shared high similarities with *AtAGO1* and *OsAGO1*, the *AGO1* members in Arabidopsis and rice, respectively at the nucleic acid level. Similar to *AtAGO1*, *TaAGO1*, as well as other AGO1 homologous in rice and barley, all contain the AGO signature domain PIWI that plays critical roles on binding and cleaving of RNA (Ma et al., 2005; Cerutti et al., 2000). Previously, crystal structure analysis revealed that the PIWI domain harboring at the AGO proteins possesses an RNase H activity and the core of the PIWI domain has a tertiary structure belonging to the RNase H family of enzymes (Cerutti et al., 2000). In addition, TaAGO1 also contains the DDH catalytic triad identified in AGO1 proteins and three positions in the DDH catalytic triad for Mg^{2+} coordination. These results implicate that TaAGO1 is one of AtAGO1 homologous and plays critical roles on RNA silencing via miRNAs pathway in wheat. Subcellular location analysis revealed that TaAGO1 is a cytoplasmic protein in accordance with the findings that argonaute proteins in eukaryotes have been identified in high concentrations in regions of the cell's cytoplasm known as cytoplasmic bodies, to which mRNA

Figure 5 Contd





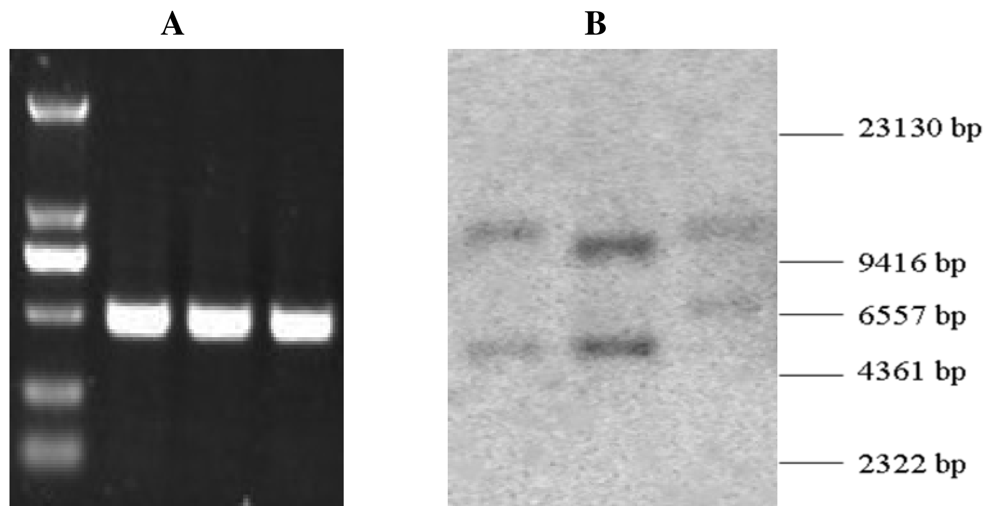


Figure 6. Identification of *TaAGO1* location in various diploid species. A: PCR analysis; B: Southern blotting analysis. AA, SS and DD represent genome AA, genome SS, and genome DD, respectively.

decay is also localized (Sen and Blau, 2005).

RNA silencing via miRNAs acts as an important pathway at the post-transcriptional level. MiRNAs responding to abiotic stresses in Arabidopsis, including miR393 and miR159 to be regulated by ABA and gibberellins (Sunkar and Zhu, 2004; Achard et al., 2004) and miR399 upregulated by phosphorus deprivation stress (Chiou et al., 2005) have been verified. The modified transcripts of

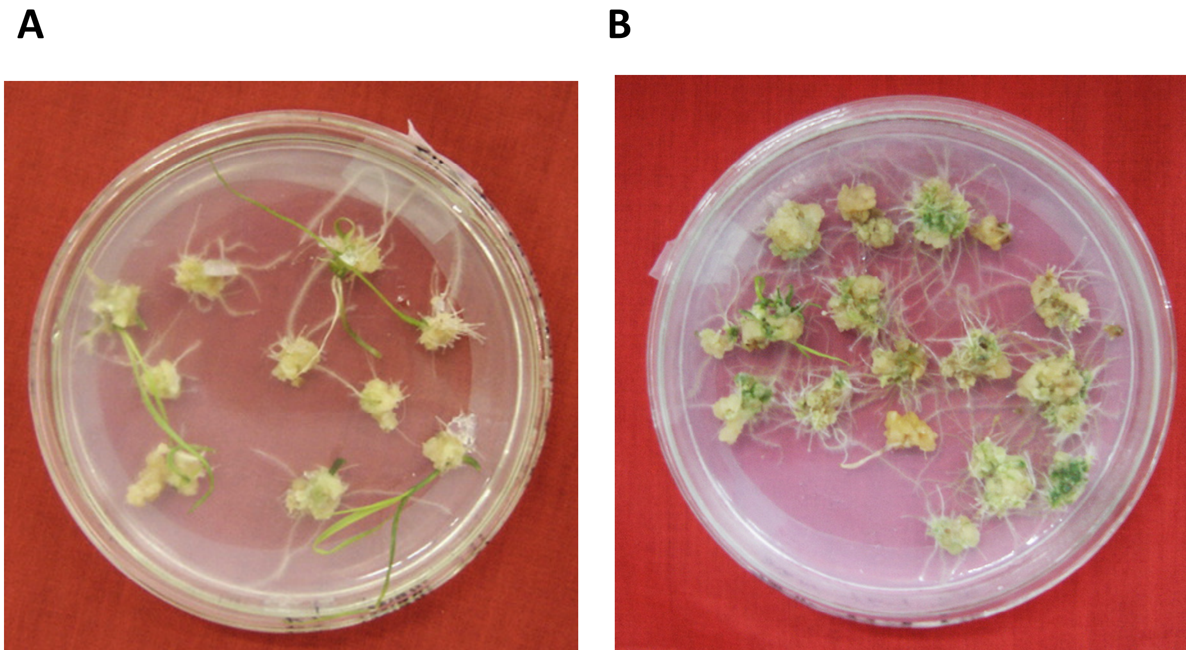


Figure 7. Phenotypic features of young plantlets generated from the callus that were transformed the anti-sense *TaAGO1* construct. A: Young plantlets generated from the callus that were transformed empty binary vector pBI121; B: Young plantlets generated from the callus that were transformed the anti-sense *TaAGO1* construct.

distinct miRNAs were further functional on the target gene silencing process (Chiou et al., 2005). However, although 10 and 18 numbers of AGO genes have been identified in Arabidopsis (Morel et al., 2002) and rice (Nonomura et al., 2007), respectively, the expression patterns and transcriptional regulation mechanisms regulated by the external signals have been rarely reported. In this study, the expression profiles of *TaAGO1* under diverse external treatments such as deprivations of P, N, K and Ca, and abiotic stresses of dehydration (PEG treatment), low temperature, salinity and exogenous treatment of ABA, have been investigated. It was revealed that the expression levels of *TaAGO1* were dramatically modulated by stresses of phosphorus deprivation, PEG and treatment of ABA, showing patterns to be downregulated by stresses of phosphorus deprivation and PEG, and downregulated by treatment of ABA, respectively. Therefore, the biological functions of *TaAGO1* exerted extra roles by responding to distinct stress signaling such as stresses of low-Pi and dehydration, and treatment of ABA, in addition to playing roles on regulation of target gene silencing via miRNAs pathway. The molecular mechanism of RISC formation and further regulation effects on the target gene silencing via altered *TaAGO1* transcripts under distinct environmental stimuli need to be further explored.

As a hexaploid, wheat is derived from three homologous diploids which possess the genome AA, SS and DD, respectively. Based on PCR and Southern blotting analysis, it was confirmed that all the diploid genomes harbored *TaAGO1* and each one contains two copies. These results suggest that *TaAGO1* exists as a small family in the wheat genome. Considering the critical roles of *AGO1* gene in post-transcriptional regulation via miRNA-mediated pathway, relative more copies of *TaAGO1* in wheat genome seems to be valuable and necessary for a long evolution process of the hexaploid specie.

Mutation of *argonaute1* (*AGO1*) was first characterized as a leaf developmental defectiveness (Bohmert et al., 1998). In Arabidopsis, *ago1* mutants were shown to confer pleiotropic abnormalities in plant phenotype such as small, unexpanded cotyledons and narrow, and polarity altered of bladeless leaves (Kidner and Martienssen, 2004). The axillary meristems in *AGO1* mutants were absent. In the inflorescence, it was observed that only a short shoot initiated and flowers altered organ morphology. The flowers are completely radialized in the more severe cases (Bohmert et al., 1998; Kidner and Martienssen, 2004). Meanwhile, the *AGO1* mutants are generally sterile (Morel et al., 2002). Strong alleles caused a lack of the shoot apical meristem, suggesting that *AGO1* plays a crucial role for stem cell maintenance. Phenotypic and double mutant analyses also indicate that *AGO1* may regulate stem cell function via shoot meristemless, because AGO1 was also observed to be necessary for normal expression of the determinacy regulators LEAFY, APETALA1, and AGAMOUS. Further-more, the Polycomb group gene *CURLY LEAF* (*CLF*) was found to be overexpressed in

the *AGO1* background, and mutations in *AGO1* can partially suppress *clf*-induced defects in floral meristem identity, indicating that RNAi-based mechanisms may regulate the function of Polycomb group factors (Kidner and Martienssen, 2005).

In this study, transgenic wheat seedlings with down-regulated expression of *TaAGO1* were generated based on *Agrobacterium*-mediated transformation approach. Compared with the plantlets derived from those transformed, the empty binary vector and the plantlets generated from the callus that were transformed, the anti-sense *TaAGO1* construct showed conspicuous alterations in architecture, having more stunted growth rate, more frequently abnormal growth features and much more adventitious roots. These results are similar to those obtained in Arabidopsis *AGO1* mutants, which have been implicated that a subset of endogenous mRNA targets of RNA interference may be regulated by *TaAGO1* via miRNA-mediated pathway in wheat.

The mechanisms in which TaAGO1 exerts its biological roles on regulation of plant growth as well as the other putative functions of this wheat AGO protein are necessary to be further characterized.

Acknowledgements

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