Polymorphic Homoeolog of Key Gene of RdDM Pathway, ARGONAUTE4_9 class Is Associated with Pre-Harvest Sprouting in Wheat (Triticum aestivum L.)

Manjit Singh1, Surinder Singh1, Harpinder Randhawa2, Jaswinder Singh1*

1 Department of Plant Science, McGill University, Quebec, Sainte-Anne-De-Bellevue, Canada; 2 Lethbridge Research Center, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada

Abstract

Resistance to pre-harvest sprouting (PHS) is an important objective for the genetic improvement of many cereal crops, including wheat. Resistance, or susceptibility, to PHS is mainly influenced by seed dormancy, a complex trait. Reduced seed dormancy is the most important aspect of seed germination on a spike prior to harvesting, but it is influenced by various environmental factors including light, temperature and abiotic stresses. The basic genetic framework of seed dormancy depends on the antagonistic action of abscisic acid (ABA) and gibberellic acid (GA) to promote dormancy and germination. Recent studies have revealed a role for epigenetic changes, predominantly histone modifications, in controlling seed dormancy. To investigate the role of DNA methylation in seed dormancy, we explored the role of ARGONAUTE4_9 class genes in seed development and dormancy in wheat. Our results indicate that the two wheat AGO4_9 class genes i.e. AGO802 and AGO804 map to chromosomes 3S and 1S are preferentially expressed in the embryos of developing seeds. Differential expressions of AGO802-B in the embryos of PHS resistant and susceptible varieties also relates with DNA polymorphism in various wheat varieties due to an insertion of a SINE-like element into this gene. DNA methylation patterns of the embryonic tissue from six PHS resistant and susceptible varieties demonstrate a correlation with this polymorphism. These results suggest a possible role for AGO802-B in seed dormancy and PHS resistance through the modulation of DNA methylation.

Citation: Singh M, Singh S, Randhawa H, Singh J (2013) Polymorphic Homoeolog of Key Gene of RdDM Pathway, ARGONAUTE4_9 class Is Associated with Pre-Harvest Sprouting in Wheat (Triticum aestivum L.). PLoS ONE 8(10): e77009. doi:10.1371/journal.pone.0077009

Editor: Keqiang Wu, National Taiwan University, Taiwan

Received June 19, 2013; Accepted August 28, 2013; Published October 9, 2013

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Funding: This work was supported by Natural Sciences and Engineering Research Council (NSERC) of Canada. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Jaswinder.Singh@mcgill.ca

Introduction

ARGONAUTE (AGO) proteins form a large family that regulates gene expression through transcriptional and post-transcriptional gene silencing; they are reviewed in [1]. In addition to their canonical role in heterochromatin silencing, recently, the involvement of the AGO4_9 class of AGO genes have been shown in the embryo as well as in reproductive and seed development [2–4]. The differential expression of AGO1003, an AGO4_9 class of Argonaute, in embryos of dormant vs non-dormant genotypes in barley provides further evidence of its possible role in maintaining the quiescent state of seeds, commonly known as seed dormancy [5]. Reduced seed dormancy is the most important aspect of seed germination on a spike prior to harvesting, or better known as pre-harvest sprouting (PHS).

In Canada, PHS in spring wheat causes significant economic losses due to a reduction in grain yield, end-use quality and viability of the seed for planting. The annual cost of PHS to the Canadian wheat industry is estimated at $100 million [6]. Seed dormancy is a complex trait which has a large component of genotype X environmental interactions, which are reviewed in [7–8]. The balance of the antagonistic action of abscisic acid (ABA) and gibberellic acid (GA) in promoting dormancy and germination constitutes the basic genetic framework of seed dormancy; nevertheless, it is influenced by various environmental factors including light, temperature and abiotic stresses [9–11].

Numerous studies have been conducted to understand the genetic control of seed dormancy and PHS. Several QTLs have been reported in different wheat populations since 1993; the majority are located on chromosome 3 and 4 [12–14]. Screening of mutants also contributes to our knowledge of ABA sensitivity and ultimately, PHS resistance in wheat [15–17] and barley [18].

There is increasing evidence that epigenetic modification can also influence seed dormancy. Recently, Zheng et al. [19] demonstrated that histone methylation plays an important role in establishing seed dormancy. Knockout mutants for the histone methyltransferase KYP/SUVH4, which mediates H3K9 dimethylation, exhibit increased dormancy; plants which overexpress KYP/SUVH4 show reduced seed dormancy [19]. It was further shown by Zheng et al. [19], that KYP/SUVH4 is regulated by ABA and GA. Sensitivity of seed germination to ABA and paclobutrazol, an inhibitor of GA synthesis, is enhanced slightly in the mutant. Evidence of the role of chromatin modifiers in seed dormancy was further proven by Liu et al. [20] through cloning of the REDUCED DORMANCY 4 (RDO4) locus in Arabidopsis. RDO4 encodes a C3HC4 RING finger E3 ligase that catalyzes histone H2B monoubiquitination and therefore, was renamed...
HISTONE MONOUBIQUITINATION 1 (HUB1). Histone H2B monoubiquitination is associated with the activation of gene transcription whereas deubiquitination is linked to repression [21]. Consistent with this role, expression of several dormancy-related genes involved in ABA metabolism and signalling, such as NCED9 and ABAH, are reduced in freshly harvested seeds of hub1 mutants as compared to wild-type plants. Apart from these two studies, there is also a large body of evidence concluding the indirect influence of epigenetic modification on seed dormancy. ABA, which plays a central role in the induction of dormancy, is able to induce chromatin modification and is itself regulated by chromatin modification. ABA has been shown to induce acetylation of H3-K14 and methylation of H3-K4 [22]. Histone deacetylation is also known to affect seed germination through ABA induced gene expression [23–25].

Evidence for the role of DNA methylation in seed dormancy has been suggested previously. H3K9 methylation mediated by SUVH4/KYP and SUVH5, another histone methyltransferases, is also required for the maintenance of CNG methylation by the CMT3 DNA methyltransferase [26–27]. ARGAONUTE (AGO) proteins of the AGO4_9 class are key players in DNA silencing in plants through RNA dependent DNA methylation (RdDM; this was reviewed in [1]). Our recent investigation of AGO1003, an AGO4_9 class of AGO genes in barley embryos, indicates that these genes are differentially expressed in dormant and non-dormant genotypes, where the expression of AGO1003 is significantly reduced in embryos of dormant varieties [5]. Altogether, in this study, we have explored AGO4_9 class genes from wheat, and conducted an expression analysis of AG0802 and AGO804 in developing embryos of wheat. Expression of AGO802 homolog AGO802-B was also investigated in embryos of PHS resistant and susceptible varieties of wheat. Further, we analyzed the DNA methylation status in embryos of PHS resistant and susceptible varieties.

Results

Identification of AGO4_9 wheat homoeologs

It has been previously suggested that there are two AGO4_9 class genes in barley, a diploid species closely related to wheat [5]. Thus, bread wheat, a closely related allohexaploid species with three subgenomes, is expected to have three homoeolog genes corresponding to each barley homolog. Sequence information of one AGO4_9 homolog AGO0802 corresponding to the barley homolog, AGO1003, was previously available in the chromatin database ChromDB (www.chromdb.org). To obtain sequences of the remaining five homoeologs, we performed BLAST searches in three different wheat EST databases i.e. Graingenes [http://wheat.pw.usda.gov/ GG2/index.shtml], JCVI [http://www.jcvi.org/], TGI [http://compbio.dfci.harvard.edu/tgi/software/] and PGGDB [http://www.pygresql.org/pgdb.html]. Through BLAST search utilizing the AG0802 sequence, we obtained forty-five singleton ESTs or contigs assembled from ESTs and mRNA sequences corresponding to the wheat AGO4_9 genes. These forty-five sequences were assembled into six distinct contigs which represented the six homoeologs in wheat corresponding to the two barley AGO4_9 genes. For 3 of these assembled mRNA sequences, the complete 3’ UTR sequences including the polyA tail were obtained. For the remaining two genes corresponding to homologs of AGO1003 and homoeologs of AGO802, we performed a 3’ RACE with gene-specific primers designed from AG0802 and related sequences on cDNA synthesized from embryos 10 DAP. Two distinct sequences were obtained through 3’ RACE which are homologous to AG0802. Thus, utilizing the sequences obtained from different databases and 3’ RACE, we were able to assemble the six transcripts of wheat AG04_9 genes. Three of these sequences are homologous to the barley AG01002 gene and thus represent the three wheat homoeologs of this gene. The remaining three sequences which include AG0802 are the wheat homoeologs of the barley AG01003 gene. In addition to the ESTs assembled in six homoeologs, we identified an EST which probably is a paralog of the wheat AG0004 gene. Two more ESTs were identified which are the splice variants of an AGO802 homoeolog.

Phylogenetic analysis of AGO4_9 genes

Plant Argonaute genes are divided into three clades based on sequence and functional homology with Arabidopsis AGO proteins. AGO4_9 clade, which consists of the Arabidopsis AG04 and AG09 function in the DNA silencing pathway. Phylogenetic analysis of wheat AGO4_9 Argonautes based 224 aa partial protein sequences including the PIWI domain, grouped the wheat homoeologs of AG0802 and AG0804 into two distinct groups with the expected closest homology to the two barley AGOs. The three wheat AG0802 homoeologs are almost identical for the sequence compared with the B genome homoeolog differing by only one amino acid from A and D genomes. The AG0802 homoeologs have a 97% amino acid identity to the barley AG01003. The DNA sequence homology for this region and the 3’UTR between the wheat homoeologs is 95–97% (Fig. 1). In contrast the sequence homology with AG01003 is 85%. The AG0804 homoeologs have 90–99% amino acid identity with each other and to the barley AG01002. The DNA sequence homology is 91–93% among the AG0804 homoeologs and 81% to AG01002 of barley (Fig. 1). When compared with AG04_9 proteins from other cereals, wheat AG0804 homoeologs and AG01002 clustered in the AGO group which includes maize AG0104 and sorghum AG02618 (Fig. 2). AG0802 homoeologs and AG01003 cluster into a separate group distant to AG0804 and AG01002, though are more closely related to maize AG0105 and AG02608.

AGO802 and AG0804 map to chromosome 3S and 1S

The six wheat AG04_9 genes were assigned to the specific chromosome arms of wheat utilizing the Chinese Spring nullisomic-tetrasomic lines and diletosomic lines [28]. Gene-specific primers were designed from the 3’ UTR sequences to discriminate between the homoeologs of AG0802 and AG0804. Initial PCR was performed on nullisomic-tetrasomic lines to assign the genes to a specific chromosome. Second PCR was then performed on a subset of diletosomic lines specific to the chromosome assigned through screening of nullisomic lines. AG0802 was assigned to chromosome 3S and the gene-specific primers were able to discriminate the three homoeologs for A, B and D genome for this gene (Fig. 3). Similarly AG0804 was assigned to chromosome 1S and the gene-specific primers were able to discriminate the A, B and D genome homoeologs for this gene (Fig. 3).

Expression of AG0802 and AG0804 homoeologs during seed development

To study the expression of six wheat AG04_9 genes in the developing seeds, homoeolog-specific primers described for mapping were used. QRT-expression analysis was performed at six different stages consisting of ovaries at 5 DAP stages and embryos at 10, 15, 20, 25 and 30 DAP (Fig. 4). The expression patterns varied amongst the homoeologs for both of these genes. Overall the expression of AG0802 was 2–3 times higher than AG0804.
AGO802 in these tissues. Amongst the AGO802 homoeologs AGO802A and AGO802B showed higher expression than AGO802D albeit in different stages. AGO802A showed the highest expression at 5 and 10 DAP decreasing in subsequent stages of embryo development. AGO802B expression is lower at 5 DAP but subsequently increases to a maximum level at 20 DAP. Between 20 and 30 DAP, AGO802B expression decreases dramatically to less than half the level at 20 DAP. Although the expression of AGO802D was lower, compared to AGO802A, the expression pattern is similar being highest at 5 DAP and decreasing in subsequent stages. Amongst the AGO804 homoeologs, AGO804D has the highest expression while AGO804A has the lowest. AGO804B has an intermediate level of expression compared to its counter-homoeologs.

AGO802B gene shows polymorphism for retrotransposon insertion in Canadian wheat varieties

Previous studies in our laboratory have shown that AGO1003 is differentially expressed in the 20 DAP embryos of pre-harvest sprouting (PHS) resistant and susceptible barley varieties [5].
expression of this gene in PHS resistant varieties was significantly lower than in PHS susceptible genotypes. To test this expression pattern in wheat, we analyzed the transcripts of AGO802B in ten Canadian wheat varieties. Interestingly, we observed polymorphism in the length of the fragments amplified from the genomic DNA of these varieties. Seven of the ten wheat varieties analyzed contained an insertion of 160 bp in the 3' UTR which is inserted 30 base pairs downstream of the stop codon (Fig. 5; Table. 1). This polymorphism was also detected in the transcripts amplified through RT-PCR suggesting that the retrotransposon insertion is a stable part of the 3' UTR and is not spliced from the mRNA. Although the BLAST analysis did not show homology to any known sequence, a nine base duplication is present at both ends of this sequence suggesting a retroelement insertion. This element likely represents a short, interspersed nuclear element (SINE). This insertion is present in the genotypes RL4137, Snowbird, AC Domain, AC Karma, AC Vista, Thatcher and CDC Teal (Table 1, Figure 5). It was absent in AC Andrew, Sadash and SC 8021-V2. With the exception of SC 8021-V2, the retro-element insertion is present in PHS resistant/tolerant varieties and is absent in PHS-susceptible varieties suggesting its correlation with the PHS trait in the wheat varieties tested. CDC Teal, a variety with a medium PHS reaction, also carried this insertion.

PHS-susceptible and resistant wheat varieties are differentially methylated in 5S rDNA repeats

Argonaute proteins of AGO4_9 clade are components of DNA methylation pathways in plants with an important role in silencing repetitive DNA and transposons. Mutants of the Arabidopsis AGO4 and maize AGO104 genes exhibit reduced DNA methylation in 5S and centromeric repeats [3], [29–30]. To test if the wheat varieties with a retro-transposon insertion in the AGO802B genes and variable PHS reaction, exhibit any differences in methylation of repetitive DNA, we analyzed the non-CG methylation of 5S rDNA repeats using DNA Blot analysis. The DNA from embryos of mature seeds was analyzed due to the embryo-preferential expression of AGO802B and the critical role of
embryos in determining the PHS reaction of genotypes. DNA from six wheat varieties, namely, RL4137, Snowbird, AC Domain, AC Karma, CDC Teal and AC Andrew, was digested with methylation sensitive restriction enzyme \( MspI \) and probed with 5S rDNA (Fig. 6a). The six wheat varieties showed a variable pattern for non-CG DNA methylation at 5S rDNA repeats. PHS resistant varieties RL4137, Snowbird, AC Domain, AC Karma show reduced methylation as compared to AC Andrew, a PHS susceptible variety. DNA methylation in CDC Teal, a variety with medium PHS reaction, was also less when compared to AC Andrew but more than the PHS resistant varieties. Among the four PHS resistant varieties, Snowbird and AC Domain showed the least DNA methylation. Similarly, most of PHS resistant varieties show low level of AGO 4 protein concentration while comparing with susceptible varieties (Figure 7).

**Discussion**

**Wheat homologs of AGO4_9 clade**

Bread wheat (\( Triticum aestivum \)), an allohexaploid with three different genomes (A, B and D), arose as a result of two
AGO1002, barley homologue to homoeologs, AGO4_9 class genes are preferentially expressed in embryos during seed development. AGO802 is preferentially expressed in embryos during the Triticum ployploidization event between the ancestral species of polyploidization events involving three diploid species. An ancient ployploidization event between the ancestral species of Triticum monococcum and Aegilops ventricosa created Triticum urartu, a tetraploid species which hybridized to Aegilops tauschii in a more recent hybridization event to produce hexaploid bread wheat [31]. Barley, a diploid species closely related to wheat, has two genes of the AGO4_9 clade [5]. Utilizing the sequence information from barley AGO1002 and AGO1003 genes and wheat AGO802 gene homoeologs, AGO4_9 class genes were identified. AGO802 is homologous to AGO1003 and AGO804 is homologous to AGO1002. AGO802 maps to chromosome 3S whereas AGO804 maps to chromosome 1S based on the homoeolog-specific PCR primers. DNA and protein sequence comparisons with AGO4_9 genes of related cereals and Arabidopsis showed that the wheat homoeologs have the maximum homology amongst themselves followed by homology to barley. The triticeae (wheat and barley) AGO4_9 class genes tend to cluster separately from other related cereals. Particularly, AGO802 and AGO1003 form a cluster divergent from all other AGOs suggesting a triticeae specific evolution of these genes. The triticeae family is distinctive from related cereals by large genomes; the majority of which are composed of transposons and repetitive DNA [32]. The role of AGO4_9 genes in silencing of transposons and repetitive DNA is well known [33]. The triticeae-specific AGO4_9 genes, AGO802 and AGO1003 may have a specific role in managing the large genomes of these species.

AGO802 is preferentially expressed in embryos during seed development

Argonautes of AGO4_9 class have been shown to possess an important role in pre- and post-fertilization development. AGO104 is in maize, required for meiosis and knockout mutations in this gene, results in aberrant meiosis leading to the production of unreduced gametes [3]. The AGO9 class has been implicated in pre-meiotic megaspore differentiation and also in transposon silencing during gametogenesis in Arabidopsis [2]. Previously, we have shown that the two barley AGO4_9 genes, AGO1002 and AGO1003, are preferentially expressed during sporogenesis and post-fertilization embryo development [5].

Studies in Arabidopsis have also shown the expression pattern of AGO9 in reproductive tissues and they have been shown to be necessary for female gamete formation [2]. The authors observed a high expression of AGO genes in the pre-fertilization stages, specifically in ovules and anthers, but none in embryos. Another instance where Pup1 cDNA, corresponding to the AGO9 gene family, demonstrates an early expression of this gene is found specifically in the tissues of ovules and anthers [34]. To understand the function of AGO4_9 genes in wheat reproductive development and seed dormancy, we studied their expression in embryos at different stages after fertilization. In general, the relative expression of AGO802 homoeolog was higher than AGO804 homoeologs in all of the tissues examined.

Among AGO802 homoeologs, the expression of AGO802A and AGO802B was significantly higher than AGO802D, which had an expression similar to AGO804 homoeologs. The two highly expressed genes, AGO802A and AGO802B, have a converse expression pattern. AGO802A has a higher expression in the stages immediately following fertilization, which decreases as the embryo matures. On the other hand, the expression of AGO804B is low following fertilization but increases during embryo development reaching a maximum level at 20 DAP and then decreasing during seed maturity and desiccation. In barley, AGO1003 is expressed at higher levels than AGO1002 which is consistent in wheat; however, two wheat homoeologs, AGO802A and AGO802B, appeared to acquire a different pattern of
expression. It is possible that the role played by AGO1003 in barley is assumed by these two homologs in wheat, but with a differentiation in function between the early, and late, stages of embryo development.

**AGO802B retro-transposon insertion may be related to PHS resistance in wheat**

There is increasing evidence that epigenetic changes may play a role in seed dormancy and hence, may determine the PHS reaction of cultivars and varieties. In Arabidopsis *HUB1*, a histone mono-ubiquitination factor is required for seed dormancy and its mutant exhibit reduced seed dormancy [20]. Recently, it has been shown that histone demethylation caused by a mutation in the gene shows increased seed dormancy in Arabidopsis [19]. In barley, the expression of the AGO1003 gene is reduced in developing embryos in the seeds of PHS resistant varieties [5]. In wheat, we observed that AGO802B mimics the expression of AGO1003 more closely with respect to a role in modulating seed dormancy. Similar to AGO1003, the maximum expression of AGO802B is in the embryos at 20 DAP and thereafter declines as the seed matures (30 DAP). This stage is particularly important as reprogramming of seed dormancy activates during that time. In wheat, when we analyzed the expression of AGO802B in embryos of PHS resistant and susceptible varieties, we observed polymorphism in the size of the amplified region in both the genomic and RT-PCR. The polymorphism is due to the insertion of a retroposon-like DNA sequence, which produced 9 bp terminal end duplication but does not have homology to any known retroposon in the databases.

Interestingly, this insertion was present in six of the seven PHS resistant genotypes and absent in the two PHS susceptible genotypes that were tested. The seven PHS resistant varieties tested were RL4137, Snowbird, AC Karma, AC Domain, AC Vista, Thatcher and SC8021-V2. With the exception of SC8021-V2, all other genotypes have insertion in the AGO802B gene. This insertion was also present in the variety CDC Teal which has a medium PHS reaction. Incidentally, the varieties Snowbird, AC Karma, AC Domain, AC Vista, which contain a DNA insertion, are related. These varieties possess RL4137 in their pedigree which is derived from a cross involving Thatcher [35]. It is quite possible that the DNA insertion in the AGO802B gene is derived from the Thatcher variety. SC8021-V2 on the other hand was derived from a cross that did not involve either Thatcher or RL4137 [36]. Previous studies in our laboratory suggest a role for DNA methylation in modulating seed dormancy and components of the RdDM pathway, including AGO1003, are down regulated in the developing embryos of seed dormant genotypes [5]. The strong correlation of the insertion in AGO802B with the PHS resistant genotypes suggests that it may be involved in modulating seed dormancy in these genotypes similar way to the reduced expression of AGO1003 in barley seeds [5]. The insertion in the AGO802B gene is in the 3’ UTR 30 bp downstream of the stop codon and it may have a regulatory effect on this gene. The role of 3’ UTRs in post-transcriptional gene regulation is well known [37–38]. It is possible that the insertion in 3’ UTR in AGO802B in PHS resistant genotypes has a negative post-translational effect which is similar to transcriptional down regulation of AGO1003 in barley. This can be verified through western blot analysis if specific antibodies are raised to distinguish not only between the two AGO4_9 class genes, but also between the three homologs of AGO802. However, this may be complicated due to high homology between homologs.

In the absence of a suitable tool to assess the differences in post-transcriptional regulation among the different wheat varieties, we tested to determine if there is a change in methylation of DNA repeats in the embryos of mature seeds, which could be indicative of differences in AGO802B activity. AGO4_9 genes are components of the RdDM pathway which has a role in the methylation of the repetitive DNA, particularly the 3S rDNA and the centromeric repeats [3,30]. Our observation of methylation pattern at 3S rDNA repeats among six varieties with the variable PHS reaction, indicate that the PHS resistant varieties (RL4137, Snowbird, AC Domain and AC Karma) are hypomethylated at these repeats compared to AC Andrew, a PHS susceptible variety. Consequently, low protein concentration of AGO 4 in PHS resistant varieties is found to be consistent with methylation status (Figure 7).

CDC Teal showed an intermediate methylation status which is consistent with its medium PHS reaction. Further, the methylation pattern is consistent with the polymorphism in AGO802B gene suggesting a strong correlation between the DNA insertion, DNA methylation and PHS reaction of wheat varieties. methylated DNA repeats, as such 3S rDNA, is indicative of a global methylation status and therefore, the above results suggest methylome differences between the embryos of wheat varieties which could underlie the differences in gene activity and their reaction to PHS. In Arabidopsis, histone methylation is also impaired in ago4-2 mutants, suggesting the close interplay between DNA and histone methylation [29].

RL4137 has been a source of PHS resistance for Canadian wheat and several varieties have been developed using RL4137 as a parent [35]. The PHS resistance of RL4137 has been attributed to two different mechanisms – one associated with the red seed coat color and a second yet unknown mechanism independent of seed coat color [35]. Several white seed coat PHS resistant varieties have been derived from RL4137 although their resistance was not as strong as RL4137 [35,39]. The pool of PHS resistant varieties investigated in this study had both red and white seed coat color, and all these genotypes suggest that seed color does not have any correlation with polymorphism or methylation status. Our results imply that DNA methylation could be one of the contributors to PHS resistance in RL4137 and its derivatives, which is independent of seed coat colour.

**Materials and Methods**

**Plant Material**

Plants from ten different wheat varieties with varying degree of seed dormancy along with Chinese Spring (CS) were grown in a growth chamber at 20°C/18°C under a 16-h light/8-h dark cycle. RL4137 [35], Snowbird [7], AC Domain [40], AC Karma [41], SC8021-V2 [36], Thatcher and AC Vista [42] are PHS resistant or tolerant varieties whereas AC Andrew [43] and Sadash [44] are PHS susceptible varieties. The CDC Teal variety has a medium PHS reaction [45]. Samples for RNA extraction were collected at 5 DAP (days after pollination) for ovaries and 10, 15, 20, 25 and 30 DAP for embryos.

Complete sets of nullisomic and di-telosomic lines were obtained from Wheat Genomics Resource Center (WGRC), Kansas State University and grown in the greenhouse with similar growing conditions as described above. Primers used to map the homologs of AGO802 were AGO802-A, AGO802-B and AGO802-D and for AGO804 were AGO804-A, AGO804-B and AGO804-D (Table 2).

**DNA Extraction and PCR**

Genomic DNA was extracted using standard protocols. The PCR reaction consisted of 160 nmol of each primer, 2×
Table 2. List of primers used in mapping studies.

| Primer | orientation | Sequence (5’→3’) |
|--------|-------------|------------------|
| AGO802-A | F | CCGTGGACGTCTCGGAGATGTTG |
| R | AGTAAAGGTTCAGCT CGACGACAGTG |
| AGO802-B | F | CCGTGGACGTCTCGGAGATGTTG |
| R | AGTAAAGGTTCAGCT CGACGACAGTG |
| AGO802-D | F | TCGGAGACGTCGCGACGAGAG |
| R | GAAAGAATGCTGCTGCGGAC |
| AGO804-A | F | GAACATGACGTCGCGGACG |
| R | AACGCGACGAGAGGTAACAT |
| AGO804-B | F | GATGGGACGGAAATTCATGCTGT |
| R | CGCCCAACACACACAGGGTAAAT |
| AGO804-D | F | AGCTGATGCTGGTGCTTAG |
| R | AGCTTCAAGGGTTCAGTCC |

doi:10.1371/journal.pone.0077009.t002

DNA Gel Blot Analysis

To analyze the methylation of 5S rDNA repeats 15 μg DNA was digested with MspI restriction enzyme, electrophoretically separated on 0.8% agarose gels, transferred to Hybond N* nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), hybridized with 32P-labeled barley 5S rDNA probe and washed according to manufacturer’s instructions. 5S rDNA probe was obtained through amplification from barley genomic DNA with 5S primers (Hv5S-F: CGAAGTTAAGCGTGCTTGG and Hv5S-R: GCATCCGACATGCAACTATC). Amplified products were cloned and sequenced to verify the correct amplification. Labeling of the probe was performed according to manufacturer’s instructions, using Ready-To-GoTM DNA Labeling Beads (Amersham Bioscience, Buckinghamshire, England, UK).

Protein extraction and immunoblotting

Proteins were extracted from embryos of six wheat varieties using modified protocol of Singh et al. [48]. One gram of ground embryos were mixed with 2 ml of a buffer (pH 7.4) containing 50 mM 2-amino-2-[hydroxymethyl]-1,3-propanediol (TRIS)-HCl, 200 mM NaCl, 10 mM, CaCl2, 0.1% triton and 1% EDTA-free protease inhibitor. The extract was centrifuged at 14000 rpm and supernatant was collected for further analysis. Equal amounts of protein were loaded on 8% SDS-PAGE gels. Proteins were transferred to a PVDF membrane, blocked in 5% skim milk powder and incubated with Anti-AGO4 antibody (Agrisera AB,
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Author Contributions

Conceived and designed the experiments: JS MS. Performed the experiments: MS SS. Analyzed the data: MS SS JS. Contributed reagents/materials/analysis tools: JS HR. Wrote the paper: MS JS JR.