Functional regulation of Q by microRNA172 and transcriptional co-repressor TOPOLESS in controlling bread wheat spikelet density

Pan Liu†, Jie Liu†, Huixue Dong† and Jiaqiang Sun*  
National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China

Summary
Bread wheat (*Triticum aestivum*) spike architecture is an important agronomic trait. The Q gene plays a key role in the domestication of bread wheat spike architecture. However, the regulatory mechanisms of Q expression and transcriptional activity remain largely unknown. In this study, we show that overexpression of bread wheat tae-miR172 caused a speltoid-like spike phenotype, reminiscent of that in wheat plants with the q gene. The reduction in Q transcript levels in the tae-miR172 overexpression transgenic bread wheat lines suggests that the Q expression can be suppressed by tae-miR172 in bread wheat. Indeed, our RACE analyses confirmed that the Q miRNA is targeted by tae-miR172 for cleavage. According to our analyses, the Q protein is localized in nucleus and confers transcriptional repression activity. Meanwhile, the Q protein could physically interact with the bread wheat transcriptional co-repressor TOPOLESS (TaTPL). Specifically, the N-terminal ethylene-responsive element binding factor-associated amphiphilic repression (EAR) (LDLNVE) motif but not the C-terminal EAR (LDLDLR) motif of Q protein mediates its interaction with the CTLH motif of TaTPL. Moreover, we show that the N-terminal EAR motif of Q protein is also essentially required for the transcriptional repression activity of Q protein. Taken together, we reveal the functional regulation of Q protein by tae-miR172 and transcriptional co-repressor TaTPL in controlling the bread wheat spike architecture.

Introduction
MicroRNAs (miRNAs) are small noncoding RNAs of 21–24 nucleotides with a wide distribution in animals and plants (Bartel, 2005). Plant miRNAs show a high degree of sequence complementarity to, and are believed to guide the cleavage and/or translational suppression of their target messenger RNAs (Bartel, 2005). Plant miRNAs show a high degree of sequence complementarity to, and are believed to guide the cleavage and/or translational suppression of their target messenger RNAs (Bartel, 2005). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014). For instance, in Arabidopsis, miR172 promotes flowering mainly through the suppression of some floral repressor genes, that is a small subfamily of APETALA2 (AP2)-like transcription factor genes including AP2, TARGET OF EAT1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Mathieu et al., 2009; Wang et al., 2014).
Gene expression is regulated by gene-specific transcriptional activators and repressors through interaction with general co-activators and co-repressors. TOLPESS (TPL) and TOLPESS-related (TPR) proteins comprise a conserved family of plant transcriptional co-repressors (Causier et al., 2012). TPL proteins have a highly conserved N-terminal domain containing a Lissencephaly Homologous (LiSH) dimerization motif and a C-Terminal to LiSH (CTLiH) motif (Szemenyi et al., 2008). In plants, TPL/TPR co-repressors regulate development and hormone signalling through interaction with ethylene-responsive element binding factor-associated amphipathic repression (EAR) motifs found in diverse transcriptional repressors (Causier et al., 2012). In particular, previous studies showed that TPL-like proteins in maize and rice are both essential for proper establishment of spike/panicle architecture (Gallavotti et al., 2010; Yoshida et al., 2012). RAMOSA1 ENHANCER LOCUS2 (REL2) gene in maize encodes the TPL-like transcriptional co-repressor, and rel2 mutations increased the inflorescence primary branching phenotype of ramosa1 (ra1) and ramosa2 (ra2) (Gallavotti et al., 2010). Accordingly, REL2 is considered to participate in the determination of spikelet-pair meristems in maize inflorescence development through repressing the downstream genes expression (Gallavotti et al., 2010). In rice, the aberrant spikelets and panicle1 (asp1) mutant showed reduced number of panicle branches. In fact, ASPL encodes a TPL-related protein in rice, suggesting that TPL should be crucial in the regulation of rice panicle architecture (Yoshida et al., 2012).

In this study, we generated transgenic bread wheat plants, in which the tae-MIR172 precursor was overexpressed, to characterize the biological function of tae-miR172. We showed that overexpression of tae-miR172 caused a speltoid spike phenotype, reminiscent of the wheat with the q gene. Further analyses confirmed that the domestication gene Q was indeed the downstream target of miR172, and its expression was significantly suppressed by overexpression of miR172. Our results suggest that the Q protein is localized in nucleus and has transcriptional repression activity. Furthermore, we demonstrated that the Q protein could physically interact with the transcriptional co-repressor TaTPL and this interaction may be required for the transcriptional repression activity of Q protein. In brief, we report the functional regulation of Q protein by tae-miR172 and transcriptional co-repressor TaTPL in controlling bread wheat spike architecture variation.

Results
Identification of a tae-MIR172 precursor in bread wheat

Recent whole-genome shotgun draft sequencing of the bread wheat A-genome progenitor Triticum urartu revealed a group of scaffolds containing putative microRNA precursors (Ling et al., 2013). Among these scaffolds, scaffold7722 was predicted to contain a putative MIR172 precursor (Ling et al., 2013). In this study, we further verified this MIR172 precursor candidate by predicting its RNA secondary structure using the RNA-folding program Mfold (Zuker, 2003). Certainly, a stable stem-loop structure was obtained with low free energy (Figure 1a). More importantly, the miR172 mature sequence was contained in this structure within the 3’ arm of the hairpin (Figure 1a). These analyses suggest that the identified MIR172 precursor meets the biogenesis criteria as a miRNA gene locus (Ambros et al., 2003; Meyers et al., 2008). Based on the information of scaffold7722, we screened the database of hexaploid wheat survey sequences (http://wheat-urgi.versailles.inra.fr/) (Deng et al., 2007; International Wheat Genome Sequencing Consortium, 2014) and found three sequences separately derived from the 1A, 1B and 1D chromosomes of the hexaploid bread wheat cultivar Chinese Spring (CS) (Figure S1). Based on these sequences, we designed specific primers and finally obtained a MIR172 precursor derived from the 1B chromosome of the bread wheat cultivar Kenong199 (KN199) (Figure S1). Further analyses showed that all the identified MIR172 precursors from the hexaploid bread wheat could form stable hairpin stem-loop structures, resembling the MIR172 precursor from bread wheat A-genome progenitor T. urartu (Figure 1a). Meanwhile, the identified tae-miR172 mature sequence is identical to the miR172 sequences from different plant species (Figure 1b), suggesting that the miR172 sequence is evolutionarily conserved.

Figure 1 Identification of the tae-MIR172 precursors in bread wheat. (a) The secondary structures of tae-MIR172 precursors from the wheat A-genome progenitor Triticum urartu, the bread wheat (T. aestivum) cultivar Chinese Spring (CS, chromosomes 1A, 1B and 1D) and cultivar Kenong199 (KN199, chromosome 1B). The mature tae-miR172 sequences are highlighted in red. ∆G describes the minimum free energy (mfe) of the RNA structure. (b) Sequences of mature miR172 from different plant species. The single nucleotide variants were highlighted in red. tae, T. aestivum; osa, Oryza sativa; zma, Zea mays; bdi, Brachypodium distachyon; ath, Arabidopsis thaliana; gma, Glycine max.
Over accumulation of tae-miR172 causes the speltoid spike phenotype in bread wheat

To define the biological function of tae-miR172 in bread wheat, we generated the transgenic bread wheat plants in KN199 background, in which the KN199 MIR172-1B precursor was overexpressed driven by the ubiquitin promoter. As shown in Figures 2a and S2, the whole plant morphology of pUbi:tae-MIR172 transgenic bread wheat plants, including plant height and tillering, is largely similar to those of wild-type (WT) KN199, even though more than two fold up-regulation of miR172 was detected in independent pUbi:tae-MIR172 transgenic lines (Figure 2b). Nevertheless, the spike architecture of the pUbi:tae-MIR172 transgenic plants was significantly altered, displaying the speltoid spike phenotype, that is a spear-shaped spike with elongated rachis (Figures 2c and S2). To quantitatively evaluate the spike phenotype, we determined the spikelet densities (the number of spikelets per centimetre of spike length) and revealed that the spikelet densities from different pUbi:tae-MIR172 transgenic lines were all significantly reduced compared with that in WT KN199 (Figure 2d). Together, these observations suggest that miR172 may be specifically involved in the regulation of bread wheat spike architecture.

The domestication gene Q is targeted by tae-miR172

To better understand the molecular basis for the tae-miR172 regulation on spike architecture, we focused on the identification of potential target genes of tae-miR172 in bread wheat. In this study, we predicted the targets of tae-miR172 using the web-based plant small RNA target analysis tool psRNATarget (Dai and Zhao, 2011) by setting the maximum expectation as 3.0 and maximum energy to unpair the target site (UPE) as 25.0. Finally, 22 nucleotide sequences were identified as the candidates of tae-miR172-targeting genes (Table S1).

Among these tae-miR172 targets, a certain number of sequences are described to encode the AP2-containing transcription factor Q in bread wheat (Table S1). To further experimentally confirm whether Q is the target of miR172, we first determined whether tae-miR172 could trigger cleavage on Q transcripts in vivo using the previously described rapid amplification of cDNA 5’ ends (5’ RACE) method (Liu et al., 2014). Our results showed that Q mRNA could indeed be cleaved in vivo at the specific cleavage site at the position 10–11 of tae-miR172 from its 5’ end (Figures 3a and S3), which was previously identified as a canonical miRNA cleavage site (Peters and Meister, 2007). To further validate the cleavage of tae-miR172 on Q mRNAs, we determined the Q transcript levels in WT KN199 as well as pUbi:tae-MIR172 transgenic plants and found that Q was significantly down-regulated upon the overexpression of tae-miR172 in pUbi:tae-MIR172 plants, compared with WT KN199 (Figure 3b). We next determined the suppression effect of tae-miR172 on Q by transient expression assays in Nicotiana benthamiana. As expected, co-expression of tae-MIR172 significantly decreased the accumulation of Q.

Figure 2 Over accumulation of tae-miR172 leads to the speltoid spike phenotype in bread wheat. (a) Whole plant view of wild-type (WT) KN199 (left) and pUbi:tae-MIR172 transgenic plants (right) at grain filling stage. Bar = 10 cm. (b) Quantification of tae-miR172 in WT KN199 and pUbi:tae-MIR172 transgenic plants by stem-loop quantitative reverse transcription PCR (qRT-PCR) at the heading stage. The accumulation levels of tae-miR172 were normalized against TaU6. Error bars represent standard deviations (SDs) among three independent replicates. (c) Spike morphology of KN199 (left) and pUbi:tae-MIR172 (right) at grain filling stage. Bar = 2 cm. (d) Spikelet densities (the number of spikelets per centimetre of spike length) of WT KN199 and pUbi:tae-MIR172 transgenic plants at the mature stage. Error bars denote SDs (n = 5). #1, #3, #4 and #5 represent independent transgenic lines; **p < 0.01 (Student’s t-test).
protein (Figure 3c, lane 2), compared with the empty vector (EV) control (Figure 3c, lane 1). By contrast, the alteration of protein (Figure 3c, lane 2), compared with the empty vector (EV) (a) Identification of cleavage site in Q
Figure 3
Pan Liu et al.
clones with matching 5'-RACE products from this site out of total clones identified by sequencing. mQ, tae-miR172-cleavage resistant form of Q. (b) Determination of Q transcript levels by qRT-PCR in WT KN199 and pUbi:tae-MIR172 lines at heading stage. The flag leaves were collected for the determination, and the transcript levels of Q were normalized against the internal control gene TaGAPDH. Error bars represent SDs among three independent replicates. **P < 0.01 (Student's t-test). (c) Transient expression assay in N. benthamiana confirming that Q is a target gene of tae-miR172. 35S:Q-Myc and 35S:mQ-Myc were separately co-expressed with 35S:tae-MIR172 in N. benthamiana leaves, and the protein levels of Q-Myc and actin were determined by Western blotting using α-Myc or α-Actin antibodies. Numbers below the blots represent relative protein levels as calculated by Image J software. In each experiment, four independent leaves were analysed, and three replicates were performed with similar results.

As the wheat Q gene has been reported to influence a number of domestication-related traits such as the rachis fragility and threshability (Simons et al., 2006; Zhang et al., 2011), we tested whether these domestication-related traits were affected in our pUbi:tae-MIR172 transgenic plants. Indeed, not like the loosely held seeds in WT KN199, we observed a non-free-threshing phenotype in all the tested pUbi:tae-MIR172 transgenic plants when grown in the field conditions (Figure S4). More importantly, the spikes from the pUbi:tae-MIR172 transgenic plants were more easier to break into individual spikelets due to more fragile rachis (Figure S4).

In summary, our data strongly suggest that tae-miR172 targets Q gene, and directs the cleavage of Q transcripts in bread wheat.

The Q protein acts as a nuclear transcriptional repressor

The Q gene encodes a member of the AP2 family transcription factors, which contains an atypical EAR motif (LDLNR) on the N-terminal region, two AP2 domains in the middle region and a typical EAR motif (LDLDR) on its C-terminus (Figure 4a). To determine the subcellular localization of Q protein, we fused green fluorescent protein (GFP) with Q for the transient expression assay in N. benthamiana. As shown in Figure 4b, the fluorescence signal of GFP could be observed exclusively in the nuclei of N. benthamiana epidermal cells, confirming that Q functions as a nucleus-localized transcriptional regulator. Further, we determined the transcriptional activity of Q in a transient dual-luciferase expression system using N. benthamiana protoplasts. In this assay, the effector luciferase (LUC) gene was fused to a 5′ GAL4 binding site to generate the reporter, and the renilla luciferase (REN) gene driven by 3SS promoter was used as the internal control (Figure 4c). Meanwhile, the effector plasmid was constructed by fusing the Q coding sequence to the GAL4 DNA binding domain (GAL4-BD) (Figure 4c). Biosensor determination revealed that the expression of Q led to an obvious down-regulation of the relative luciferase activity, compared to the EV control (Figure 4d). These results indicate that Q potentially acts as a transcriptional repressor in plant cell nuclei.

Molecular characterization of the transcriptional co-repressor TaTPL in bread wheat

Our sequence analysis revealed that the Q protein contains two EAR motifs (Figure 4a). Thus, we hypothesized that Q might physically interact with the transcriptional co-repressors of TPL/TRPs. To this end, we identified the TaTPL genes from bread wheat cultivar KN199. First, we used the coding sequence (CDS) of OsTPL (LOC_Os08g0162100) as a query to search the wheat survey sequences using BLAST (http://wheat-urgi.versailles.inra.fr/) (Deng et al., 2007; International Wheat Genome Sequencing Consortium, 2014) and identified one putative TPL-like gene that shares high-level identity with OsTPL. Based on this sequence, we next designed gene-specific primers and cloned its homologous genes from bread wheat cultivar KN199. Totally, two highly conserved TPL-like sequences with single nucleotide polymorphisms (SNPs) were obtained from KN199 (Figures S5 and S6). By sequence comparison, the two TPL-like sequences were separately located on chromosomes 4A and 4D, which show ~71% and ~72% similarities with OsTPL and AtTPL, respectively. Thus, we named these two coding sequences as TaTPL-4A and TaTPL-4D. Further analysis revealed that the protein sequences encoded by TaTPL-4A and TaTPL-4D are completely identical and share ~67% and ~72% identity with OsTPL and AtTPL at the protein level (Figure 5a), suggesting that TPL proteins are highly conserved in different plant species.

More significantly, similar to the TPL family proteins in Arabidopsis and rice (Ke et al., 2015; Szemenyei et al., 2008), TaTPL also contains the conserved LiSH and CTLH motifs in its N-
Figure 4  Q encodes an AP2 family transcription factor with transcription repression activity. (a) Schematic diagram of the domain structure of Q protein. The EAR (ethylene-responsive element binding factor-associated amphiphilic repression) motifs located at the N- or C-terminus of Q were annotated as EAR1 (LDLNVE) and EAR2 (LDLDLR), respectively. (b) Subcellular localization of Q. The 35S:Q-GFP was expressed in N. benthamiana leaves by Agrobacterium-mediated infiltration. GFP signal was detected 48 h post infiltration (hpi). In each experiment, four leaves were analysed. Three replicates were performed independently with similar results. BF, bright field. Scale bars, 20 μm. (c) and (d) Transient expression assay in N. benthamiana protoplasts illustrating the transcriptional repression activity of Q. The reporters and effectors used in the assay were generated as shown in (c). The activities of firefly luciferase (LUC) and renilla luciferase (REN) were determined 16 h post-transformation. The relative luciferase activities in control and Q-expressed samples as shown in (d) were calculated by normalizing the LUC values against REN. Error bars indicate SDs among three independent replicates. **P < 0.01 (Student’s t-test).

terminus and WD40 repeats in the C-terminal region (Figure 5b), indicating the conserved molecular structure of TaTPL as the putative transcriptional co-repressor in bread wheat.

Transcriptional expression analyses of Q and TaTPL

To investigate the biological correlation between Q and TaTPL, we examined their expression patterns in different tissues/stages in bread wheat. For this experiment, the root tips (R), the stem (St) and the fully expanded leaf (L) from 1-month-old bread wheat plants, as well as the flag leaf (FL) from adult plants, and young spikes at different developmental stages (1, 2 and 5 cm in length) were individually collected. Interestingly, our quantitative real-time PCR analyses revealed that the transcriptional expression patterns of Q and TaTPL were quite similar (Figure 6). Although the two genes were constitutively expressed in all tested tissues, their transcripts were predominantly accumulated in flag leaves and spikes, with highest levels in 1-cm spikes (Figure 6). In addition, we noticed that the transcript levels of Q and TaTPL in spikes gradually decreased during the development of spike (Figure 6), indicating that Q and TaTPL might be involved in the early-stage development of bread wheat spike.

Q physically interacts with the co-repressor TaTPL

Further, we determined whether Q protein could physically interact with the identified transcriptional co-repressor TaTPL. First, we performed yeast two-hybrid (Y2H) assays. Considering that the TaTPL-4A and TaTPL-4D genes encode the same protein sequence, here we used TaTPL-4D for the assays. As shown in Figure 7a, an obvious interaction was indeed observed between Q and TaTPL in the AH109 yeast (Saccharomyces cerevisiae) cells. To further confirm this interaction in planta, we carried out firefly luciferase (LUC) complementation imaging (LCI) assay in N. benthamiana (Song et al., 2011). Results showed that strong LUC activity was exclusively observed in nLUC-Q and cLUC-TaTPL co-expressed samples, but not in the negative controls (Figure 7b), indicating that Q and TaTPL could physically associate with each other in vivo. Moreover, parallel biomolecular fluorescence complementation (BiFC) assays in N. benthamiana also confirmed that Q could directly interact with TaTPL in the nuclei of plant cells (Figure 7c).

Together, these data strongly suggest that Q physically associate with the transcriptional co-repressor TaTPL in the nuclei, which is consistent with the above finding that Q functions as a functional transcription factor with transcriptional repression activity (Figure 4d).

The N-terminal EAR motif of Q protein mediates the interaction with TaTPL

As described above, Q contains two different EAR motifs (Figure 4a). To further define which EAR motif is essentially required for the interaction with TaTPL, we generated truncated forms of Q. As shown in Figure 8a, Q-N and Q-C separately represent the N (1–199 amino acids)- and C (276–447 aa) terminal domains of Q, which contain the EAR1 and EAR2 motifs, respectively; while Q-M denotes Q middle domain (120–275 aa) containing the two conserved AP2 domains. The LCI assays revealed that similar to the full-length Q protein, Q-N, but not Q-M or Q-C, reserved the interaction signal (Figure 8b), indicating that the EAR1 motif on the N-terminus of Q might be essential for the physical interaction with TaTPL. To further confirm this conclusion, we generated a mutated Q form (Q\textsuperscript{mEAR1}), in which the EAR1 motif (LDLNVE) was substituted by the tandem Alanine (AAAAAA). As expected, Q\textsuperscript{mEAR1} failed to interact with TaTPL, illustrating that the N-terminal EAR1 motif of Q is required for its interaction with TaTPL.
The N-terminal CTLH motif of TaTPL mediates the interaction with Q protein

Based on the highly conserved domains contained in TaTPL, we generated TaTPL derivatives, including TaTPL-N (1–339 aa) and TaTPL-C (340–1135 aa), to map the domain responsible for its interaction with Q (Figure 9a). Interestingly, a strong interaction signal was only observed between Q and TaTPL-N (Figure 9b). Further, we deleted the conserved CTLH motif to create a TaTPL-NΔCTLH fragment. Results showed that the deletion of CTLH motif in TaTPL abolished its interaction with Q (Figure 9b). Together, these results suggest that the CTLH motif of TaTPL is essentially needed for the physical interaction with Q protein.
The TaTPL-interacting EAR motif of Q is required for its transcriptional repression activity

Based on our findings that Q function as a transcriptional repressor and physically interacts with the transcriptional co-repressor TaTPL through its N-terminal EAR motif (Figures 4 and 8), we speculated that the N-terminal EAR motif of Q might be responsible for its transcriptional repression activity through recruiting the transcriptional co-repressor TaTPL. To test this hypothesis, we determined the transcriptional activity of QmEAR1, recruiting the transcriptional co-repressor TaTPL. To test this hypothesis, we determined the transcriptional activity of QmEAR1, recruiting the transcriptional co-repressor TaTPL through its N-terminal EAR motif (Figures 4 and 8). Consistent with our above results (Figure 4d), the expression of Q effector led to a repression of the relative luciferase activity (Figure 10b). However, not like Q, the QmEAR1 effector did not show any repressive activity when compared with the control (Figure 10b), suggesting that the transcriptional repression activity of Q was abolished when the N-terminal EAR motif was mutated. These results indicate that the N-terminal EAR motif of Q is essentially required for its transcriptional repression activity and the interaction with the transcriptional co-repressor TaTPL.

Discussion

Q is a critical domestication gene in polyploid wheat that is responsible for the widespread cultivation of the bread wheat (Fans and Gill, 2002; Muramatsu, 1963; Simons et al., 2006; Zhang et al., 2011). Studies reveal that Q affects a series of domestication characters in wheat, including the seed freethreshing and rachis fragility (Zhang et al., 2011). More significantly, Q is involved in the control of spike morphology, considering that the q allele in wild wheat causes a speltoid spike phenotype that displays elongated rachis and spear-shaped spike morphology. In this study, we reproduced the speltoid spike phenotype by overexpression of tae-miR172 in bread wheat plants KN199 (Figures 2c and S2), suggesting that tae-miR172 should be involved in same signalling pathway with Q in the regulation of bread wheat spike architecture.

Several lines of evidence indeed show that the domestication gene Q is targeted and regulated by tae-miR172. First, the mature tae-miR172 sequence is highly complementary to the target region of Q mRNA (Figure 3a). Second, the Q mRNA levels are markedly reduced in the tae-miR172 overexpression bread wheat plants (Figure 3b). Third, 5’ RACE assays showed that the Q mRNAs are cleaved in a specific site by tae-miR172 (Figure 3b). Fourth, the Q accumulation could also be significantly suppressed by the expression of tae-miR172 in the transient expression system (Figure 3c). Taken together, our present data support the hypothesis that tae-miR172 may regulate wheat spike architecture, especially the spikelet density mainly through the repression of Q. Interestingly, during the review of our study, a study reported that the nucleotide mutations in the miR172-targeting site of Q interfere with the miR172-mediated suppression and thus lead to Q accumulation, resulted in increased spikelet densities in bread wheat (Xu et al., 2017). This report, from another point of view, well supports our conclusion that the miR172-Q module is crucial in the regulation of wheat spike architecture.

Besides the Q gene, several other genes encoding AP2-containing transcription factors were also predicted to be potential targets of tae-miR172 (Table S1). Generally, these AP2-like transcription factors may function redundantly in planta to influence multiple signalling pathways. So far, we could not rule out the possibility that besides Q, other AP2-like transcription factors might also participate in the modulation of bread wheat spike architecture. Thus, it would be intriguing to test the biological functions of these transcription factors in bread wheat, and also their genetic relationship with the miR172-Q module. Transcriptional degradation and translational inhibition are two major mechanisms for miRNAs to direct the regulation of their target genes. Previous study in Arabidopsis thaliana revealed that miR172 likely suppresses its target gene APETALA2 mainly through translational suppression, as the AP2 mRNA abundance was not affected by the overexpression of miR172 (Chen, 2004). However, in this study, we clearly observed the miR172-guided cleavage of Q transcripts by 5’ RACE in our assays (Figure 3a), which was also supported by very recent studies (Debernardi et al., 2017; Greenwood et al., 2017; Xu et al., 2017). More importantly, the Q mRNA levels were dramatically decreased in tae-miR172 overexpression bread wheat plants, compared with those in the WT control (Figure 3b), suggesting that the transcriptional degradation is the major mechanism employed by tae-miR172 in suppression of Q in bread wheat. In consistent with our findings, some works in Arabidopsis and rice also...
detected the cleavage event of AP2 mRNA directed by miR172 (Kasschau et al., 2003; Varkonyi-Gasic et al., 2012; Zhu et al., 2009). These reports, together with our results, promote us to propose that miR172 can regulate its target genes through both mechanisms of transcript cleavage and translational inhibition, but chose one as the major mode of action, depending on the specific conditions and plant species.

Although it has been shown that Q gene plays important roles in the domestication of bread wheat (Debernardi et al., 2017; Greenwood et al., 2017; Simons et al., 2006; Xu et al., 2017; Zhang et al., 2011), the underlying mechanisms of Q protein action remain unclear. In this study, we showed that Q protein is a transcription repressor with two EAR motifs (Figure 4). Previous study in Arabidopsis has reported that AP2 transcription factor functions as the transcriptional repressor through the recruitment of the co-repressor TOPLESS (Krogan et al., 2012). Thus, we asked whether the transcriptional repressor Q could also physically associate with TaTPL. Using LCI, BIFC and Y2H analyses, we confidently demonstrated that Q could indeed interact with the transcriptional co-repressor TaTPL (Figure 7). Interestingly, our

Figure 7  Q directly interacts with TaTPL
(a) Yeast two-hybrid (Y2H) assay showing the interaction between Q and TaTPL. SD-L/W, synthetic dextrose medium lacking Leu and Trp; SD-L/W/H/A, synthetic dextrose medium lacking Leu, Trp, His and Ade. (b) Luciferase (LUC) complementation imaging (LCI) assay illustrating that Q could interact with TaTPL in N. benthamiana. Luciferase signals were detected 48 hpi. (c) Bimolecular fluorescence complementation (BIFC) assay confirming the physical interaction of Q with TaTPL in N. benthamiana. YFP fluorescence was detected 48 hpi. BF, bright field. Scale bars, 20 μm. In each experiment in (b) and (c), six leaves were employed for the analyses. All the above experiments were independently repeated for three times with similar results.
Figure 8. The N-terminal EAR motif of Q mediates its interaction with TaTPL. (a) The truncated or mutated versions of Q employed in the interaction assays in *N. benthamiana*. Q-N, 1–199 amino acids (aa); Q-M, 120–275 aa; Q-C, 276–447 aa; QmEAR1, the full-length Q with mutation in EAR1 motif. (b) LCI assays mapping the interaction domain of Q with the full-length TaTPL. The interaction signals were detected at 48 hpi. In each experiment, six *N. benthamiana* leaves were infiltrated for analysis, and similar results were observed. Three independent replicates were performed. EV, empty vector.

Figure 9. The CTHL motif of TaTPL is required for the physical association with Q protein. (a) The truncated versions of TaTPL used in the interaction assays. TaTPL-N, 1–339 aa; TaTPL-C, 340–1135 aa; TaTPL-ΔCTHL, the TaTPL-N domain without the CTHL motif (34–92 aa). (b) LCI assays illustrating the interaction of truncated versions of TaTPL with the full-length Q protein in *N. benthamiana*. Signals were collected at 48 hpi. Six *N. benthamiana* leaves were analysed in each experiment, and three independent replicates were performed with similar results.
data showed that the N-terminal atypical EAR motif (LDLNVE), but not the C-terminal typical EAR motif (LDLDLR) of Q protein is essentially required for the interaction with the conserved CTLH motif of TaTPL. More importantly, our assays confirmed that the N-terminal EAR motif of Q is essentially required for the Q-mediated transcriptional repression activity (Figure 10). These results further support the hypothesis that TPL is indispensable for the repression activity of Q. Previous studies indeed showed that TPL-like proteins are involved in the appropriate establishment of spike/panicle architecture (Gallavotti et al., 2010; Yoshida et al., 2012). In maize, REL2 was identified as a TPL-like transcriptional co-repressor and could physically interact with RA1 to repress the downstream genes that are involved in the spikelet-pair meristem determinacy pathway (Gallavotti et al., 2010). In support of this, the rel2 mutant plants showed enhanced inflorescence primary branching phenotype (Gallavotti et al., 2010). Our data, together with the above reports, lead us to propose a molecular mechanism in regulating bread wheat spike architecture: the miR172-regulated Q, functions as a transcription factor with transcriptional repression activity, should negatively regulate the expression of numerous downstream target genes through the recruitment of TOPLESS co-repressors, and subsequently suppresses the speltoid phenotype in bread wheat. Nevertheless, to further experimentally confirm the potential relationship between Q and TaTPL in spike architecture determination, more works are eagerly needed to identify the downstream genes that are directly regulated by Q, which will further deepen our knowledge with respect to the bread wheat spike architecture regulatory network.

Experimental procedures

Gene transformation, plant materials and growth conditions

In this study, the bread wheat (T. aestivum) cultivar Kenong199 (KN199), which is one of the major bread wheat cultivars in China and also easy for gene transformation, was employed as the receptor material to generate the transgenic plants. The 1-month-old embryogenic calli of KN199 were selected as the receptor materials, and the gene transformation was performed using a PDS1000/He particle bombardment system (Bio-Rad, Hercules, CA) with a distance of 6.0 cm from the stopping plate at helium pressure 1100 psi, as described previously (Shan et al., 2013).

For observation of bread wheat spike phenotypes, seeds of the wild-type (WT) KN199 and transgenic bread wheat were first germinated at 22 °C, and transferred into 4 °C cold room for a 1-month vernalization. Then, the seedlings were transplanted in the experimental field under natural conditions. The spike phenotypes were collected at grain filling and mature stages, and the spikelet densities (the number of spikelets per cm of spike length) were determined at the mature stage.

Nicotiana benthamiana was grown in glasshouse at 22 °C with a 16-h light/8-h dark cycle.

Prediction of miRNA targets and MIRNA secondary structure

In this study, the web-based plant small RNA target analysis tool psRNATarget was used for the prediction of potential target genes of tae-miR172 in bread wheat (Dai and Zhao, 2011). The wheat unigene library (DFCI Gene Index, version 12, released on 2010.04.18) was employed as the database, and the mature tae-miR172 was used as the query. Maximum expectation was set as 3.0, length for complementarity scoring 20 and maximum energy to un-pair the target site (UPE) as 25.0.

The secondary structure of MIRNA was predicted using the RNA-folding program Mfold (Zuker, 2003), and the minimum free energy (mfe) of the RNA structure, described as ΔG, was calculated simultaneously.

RNA extraction and gene expression analysis

Total RNA was extracted using Trizol (Invitrogen) reagent. For microRNA reverse transcription, the stem-loop primer was designed according to the sequence of mature miR172 (Chen et al., 2005). About 2 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (M-MLV, Invitrogen) were used for the reverse transcription. The TaU6 gene was reversely transcribed simultaneously as the internal control gene. cDNA was obtained using 2 μg total RNA and All-In-One RT MasterMix (Applied Biological Materials) following the manufacturer’s instruction. For real-time quantification, SYBR® Premix Ex Taq™ (Perfect Real Time, TaKaRa) was used. The expression levels of coding genes and miRNAs were separately normalized to TaGAPDH and TaU6.

Validation of miRNA cleavage by 5’ RACE assay

The 5’ RACE assay was performed using the RLM-RACE kit (TaKaRa, Code D315), following the procedure reported before (Liu et al., 2014). Briefly, about 2 μg of total RNAs of KN199 was used to directly ligate the RNA oligo adaptor, followed by reverse
transcription using random primer. The 5' RACE outer primer together with gene-specific outer primer and the 5' RACE inner primer coupled with gene-specific inner primer were separately used for the first and second round nested PCR. The PCR product was extracted by gel purification and ligated to cloning vector (pEASY-Blunt, Transgen biotech CB101) for sequencing.

Confirmation of the miRNA target by transient expression assay

Transient expression assay in N. benthamiana was performed to confirm the miRNA target (Liu et al., 2014). Briefly, Agrobacterium tumefaciens suspension expressing the miRNA precursor was first infiltrated into N. benthamiana leaves, meanwhile, A. tumefaciens harbouring empty vector was infiltrated as the negative control. After 24 h, A. tumefaciens containing the constructs expressing the target genes of interest were infiltrated into the same position of N. benthamiana leaves. Samples were collected 36–48 h post infiltration (hpi). Total proteins were extracted using 2 x Laemmli buffer (Laemmli, 1970) and detected by immunoblotting using anti-Myc antibody (1 : 5000; Roche, 11667149001) and anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1 : 75 000; Sigma, A9044-2ML). The levels of actin were detected by anti-β-actin antibody (1 : 5000; CWBIO, CW0264) and anti-mouse IgG as the internal control.

DNA constructs and primers

DNA constructs used in the study were generated based on construction methods following the classic molecular biology protocols and Gateway technology (Invitrogen). All details of DNA constructs used in this study were listed in Table S2. All the primers used for generation of these constructs were shown in Table S3.

Luciferase transient transcriptional repression assay

Two kinds of reporters were used in the assay. One contains the firefly luciferase (LUC) gene fused with 5 x GAL4 binding site, and the other is a plasmid expressing the renilla luciferase (REN) gene as the internal control. The effector plasmid (GAL4-BD-Q) was co-transformed with the two reporters, and the activities of LUC and REN were separately determined 24 h post-transformation using Dual-Luciferase® Reporter Assay System (Promega, E1910).

Yeast two-hybrid assay

For yeast two-hybrid analysis, the GAL4-AD and GAL4-BD derivatives were generated by fusing the coding sequences of TaTPL and Q and co-transformed into the yeast (Saccharomyces cerevisiae) strain AH109. The yeast cells were first selected on synthetic dextrose medium lacking Leu and Trp (SD-L/W) and then transferred to the SD medium lacking Leu, Trp, His and adenine (SD-L/W/H/A) for interaction analysis.

LCI assay

The LCI assays were performed in N. benthamiana leaves (Sun et al., 2013). The full-length or truncated versions of target proteins were separately fused with the N- and C-terminal part of the firefly luciferase LUC, and expressed in N. benthamiana leaves by A. tumefaciens-mediated infiltration. The LUC activity was analysed at 48 hpi.

Subcellular localization analysis

The coding sequence of Q was cloned into the pGWBS vector (Nakagawa et al., 2007) and then was transformed into A. tumefaciens GV3101. The Agrobacterium was infiltrated into N. benthamiana leaves, and the fluorescence signal of green fluorescent protein (YFP) was observed at 48 hpi.

BiFC assay

BiFC was performed in N. benthamiana leaves to detect Q-TaTPL interaction (Lu et al., 2010). Briefly, the full-length coding sequences of TaTPL and Q were separately ligated into the pEarleygate201 and pEarleygate202 vectors, and co-expressed in N. benthamiana leaves through A. tumefaciens-mediated transient expression. The fluorescence signal of yellow fluorescent protein (YFP) was observed at 48 hpi.

Acknowledgements

We thank the Biotechnology Facility of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences for assistance in generation of transgenic bread wheat plants, and Hao Lin for providing plasmids used for transient expression assay in N. benthamiana protoplasts. This research was supported by the Ministry of Agriculture of China (grant no. 2016ZX08009003–003), the National Key Research and Development Program of China (grant no. 2016YFD0100302), Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), the Agricultural Science and Technology Innovation Program of CAAS, and Youth Talent Plan of CAAS.

Conflict of interest

The authors declare no conflict of interests.

Author contributions

JS designed research; PL, JL and HD performed the experiments; JS, PL and JL analysed the data and wrote the manuscript.

References

Ambros, V., Bartel, B., Bartel, D.P., Burge, C.B., Carrington, J.C., Chen, X., Dreyfuss, G. et al. (2003) A uniform system for microRNA annotation. RNA, 9, 277–279.

Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell, 15, 2730–2741.

Bartel, D.P. (2009) MicroRNAs: target recognition and regulatory functions. Cell, 136, 215–233.

Causier, B., Ashworth, M., Guo, W. and Davies, B. (2012) The TOPLESS interactome: a framework for gene repression in Arabidopsis. Plant Physiol. 158, 423–438.

Chen, X. (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science, 303, 2022–2025.

Chen, X. (2009) Small RNAs and their roles in plant development. Annu. Rev. Cell Dev. 25, 21–44.

Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M. et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33, e179.

Chuck, G., Meeley, R.B. and Hake, S. (1998) The control of maize spikelet meristem fate by the APETALA2-like gene indeterminate spikelet1. Gene Dev. 12, 1145–1154.

Chuck, G., Meeley, R., Irish, E., Sakai, H. and Hake, S. (2007) The maize tasseled4 microRNA controls sex determination and meristem cell fate by targeting Tasseledseed5indeterminate spikelet.1. Nat. Genet. 39, 1517–1521.

Dai, X. and Zhao, P.X. (2011) psRNAtarget: a plant small RNA target analysis server. Nucleic Acids Res. 39, W155–W159.

© 2017 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 16, 495–506.
Debernardi, J.M., Lin, H., Chuck, G., Faris, J.D. and Dubcovsky, J. (2017) microRNA172 plays a crucial role in wheat spike morphogenesis and grain threshability. Development, 144, 1966-1975.

Deng, W., Nickle, D.C., Leem, G.H., Maust, B. and Mullins, J.J. (2007) ViroBLAST: a stand-alone BLAST web server for flexible queries of multiple databases and user’s datasets. Bioinformatics, 23, 2334-2336.

Faris, J.D. and Gill, B.S. (2002) Genomic targeting and high-resolution mapping of the domestication gene Q in wheat. Genome, 45, 706–718.

Gallavotti, A., Long, J.A., Stanfield, S., Yang, X., Jackson, D., Vollbrecht, E. and Schmidt, R.J. (2010) The control of axillary meristem fate in the maize ramosa pathway. Development, 137, 2849-2856.

Greenwood, I.R., Finnegan, E.J., Watassek, N., Trevaskis, B. and Swain, S.M. (2017) New alleles of the wheat domestication gene Q reveal multiple roles in growth and reproductive development. Development, 144, 1959–1965.

Jung, J.H., Seo, Y.H., Seo, P.J., Reyes, J.L., Yun, J., Chua, N.H. and Park, C.M. (2007) The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. Plant Cell, 19, 2736–2748.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A. and Carrington, J.C. (2003) PHHC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. Dev. Cell, 4, 205–217.

Ke, J., Ma, H., Gu, X., Thelen, A., Brunzelle, J.S., Li, J., Xu, H.E. et al. (2015) Structural basis for recognition of diverse transcriptional repressors by the TOPLESS family of corepressors. Sci. Adv., 1, e1500107.

Krogan, N.T., Hogan, K. and Long, J.A. (2012) APETALA2 negatively regulates the TOPLESS family of corepressors and the histone deacetylase HDA19. Development, 139, 4180-4190.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lauter, N., Kampani, A., Carlson, S., Goebel, M. and Moose, S.P. (2010) Draft genome of the wheat A-genome progenitor Triticum urat. Nature, 496, 87-90.

Liu, J., Cheng, X., Liu, D., Xu, W., Wise, R. and Shen, Q.H. (2014) The miR9863 family regulates distinct Mla alleles in barley to attenuate NLR receptor-triggered disease resistance and cell-death signaling. PLoS Genet. 10, e1004755.

Lu, Q., Tang, X., Tian, G., Wang, F., Liu, K., Nguyen, V., Kohalmi, S.E. et al. (2010) Arabidopsis homolog of the yeast TREX-2 mRNA export complex: components and anchoring nucleoporin. Plant J. 61, 259–270.

Matheu, J., Yant, L.J., Murdter, F., Kuttner, F. and Schmid, M. (2009) Repression of flowering by the miR172 target SMZ. PLoS Biol. 7, e1000148.

Meyers, B.C., Axtell, M.J., Bartel, B., Bartel, D.P., Baalcombe, D., Bowman, J.L., Cao, X. et al. (2018) Criteria for annotation of plant microRNAs. Plant Cell, 30, 3186–3190.

Muramatsu, M. (1963) Dosage effect of the spelta gene Q of hexaploid wheat. Genetics, 48, 469–482.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyoooka, K. et al. (2007) Development of series of gateway binary vectors, pGWBS, for realizing efficient construction of fusion genes for plant transformation: J. Biosci. Bioeng. 104, 34–41.

Peters, L. and Meister, G. (2007) Argonauta proteins: mediators of RNA silencing. Mol. Cell, 26, 611-623.

Rubio-Somoza, I. and Weigel, D. (2011) MicroRNA networks and developmental plasticity in plants. Trends Plant Sci. 16, 258–264.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K. et al. (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31, 686-688.

Simons, K.J., Fellers, J.P., Trick, H.N., Zhang, Z., Tai, Y.S., Gill, B.S. and Faris, J.D. (2006) Molecular characterization of the major wheat domestication gene Q. Genetics, 172, 547–555.

Song, S., Qi, T., Huang, H., Ren, Q., Wu, D., Chang, C., Peng, W. et al. (2011) The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect Jasmonate-regulated stamen development in Arabidopsis. Plant Cell, 23, 1000–1013.

Sun, Q., Li, Y., Zhai, Q. and Li, C. (2013) pKF4 and pKF5 transcription factors link blue light and auxin to regulate the phototropic response in Arabidopsis. Plant Cell, 25, 2102-2114.

Szemenyei, H., Hannon, M. and Long, J.A. (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science, 319, 1384–1386.

The International Wheat Genome Sequencing Consortium (IWGSC) (2014) A chromosome-based draft sequence of the hexaploid bread wheat (Triticum aestivum) genome. Science, 345, 1215788.

Varkonyi-Gasic, E., Lough, R.H., Moss, S.M., Wu, R. and Hellens, R.P. (2012) Kiwifruit floral gene APETALA2 is alternatively spliced and accumulates in aberrant indeterminate flowers in the absence of miR172. Plant Mol. Biol. 78, 417–429.

Wang, Y., Wang, L., Zou, Y., Chen, L., Cai, Z., Zhang, S., Zhao, F. et al. (2014) Soybean miR172C targets the repressive AP2 transcription factor NAC1 to activate ENOD40 expression and regulate nodule initiation. Plant Cell, 26, 4782–4801.

Xu, B.-J., Chen, Q., Zheng, T., Jiang, Y.-F., Qiao, Y.-Y., Guo, Z.-R., Cao, Y.-L. et al. (2017) An overexpressed Q allele leads to increased spike density and improved processing quality. bioRxiv, doi: http://dx.doi.org/10.1101/098558. [Epub ahead of print].

Yoshida, A., Ohmori, Y., Kitano, H., Taguchi-Shiobara, F. and Hirano, H.Y. (2012) Aberrant spikelet and panicle1, encoding a TOPLESS-related transcriptional co-repressor, is involved in the regulation of meristem fate in rice. Plant J. 70, 327–339.

Zhang, Z., Belcam, H., Gornicki, P., Charles, M., Just, J., Huneau, C., Magdelenat, G. et al. (2011) Duplication and partitioning in evolution and function of homologous Q loci governing domestication characters in polyploid wheat. Proc. Natl Acad. Sci. USA, 108, 18737–18742.

Zhu, Q.H., Upadhyaya, N.M., Gubler, F. and Helliwell, C.A. (2009) Over-expression of miR172 causes loss of spikelet determinacy and floral organ abnormalities in rice (Oryza sativa). BMC Plant Biol., 9, 149.

Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res., 31, 3406-3415.