Journal of Experimental Botany, Vol. 62, No. 2, pp. 487–495, 2011
doi:10.1093/jxb/erq295 Advance Access publication 15 October, 2010

Journal of Experimental Botany
www.jxb.oxfordjournals.org

FLOWERING NEWSLETTER REVIEW

Regulation of flowering time and floral patterning by miR172

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Received 15 July 2010; Revised 2 September 2010; Accepted 2 September 2010

Abstract

Since the discovery of miRNAs in plants it has become clear that they are central to the regulation of many aspects of plant development and responses to the environment. miR172 regulates expression of a small group of *AP2*-like transcription factors in an evolutionarily ancient interaction. miR172 functions in regulating the transitions between developmental stages and in specifying floral organ identity. These two roles are conserved across monocotyledons and dicotyledons. Investigations into the roles of miR172 and its targets in phase changes in the model plant *Arabidopsis* have illustrated that this process is governed by complex regulatory systems. In addition to its conserved roles, miR172 has also acquired specialized species-specific functions in other aspects of plant development such as cleistogamy and tuberization.

Key words: Floral organ identity, flowering time, miR172, post-transcriptional regulation.

Introduction

Reproductive success in plants is dependent on the timing of the switch from vegetative to reproductive growth coinciding with optimal environmental and developmental conditions. Plants have evolved an elaborate regulatory network that integrates endogenous and environmental signals to ensure that flowering commences when conditions are most favourable. Flowering time is controlled by several endogenous and environmental pathways, including the autonomous, photoperiod, vernalization, gibberellic acid (GA), thermosensory, and ageing pathways, with about 180 genes that have been shown to be involved in flowering time control based on loss-of-function mutants or transgenic analysis using *Arabidopsis* (Fornara *et al.*, 2010). The non-coding microRNA (miRNA) gene, *MIR172*, is one of these genes; it was one of the earliest plant *MIRNA* genes to be identified by small RNA cloning and sequencing in *Arabidopsis* (Park *et al.*, 2002). It generates a 21-nucleotide (nt) mature miR172 sequence that regulates the mRNA abundance and/or translation of the plant-specific transcription factor gene *APETALA2* (*AP2*) (Park *et al.*, 2002; Chen, 2004) and a small group of *AP2*-like genes, including *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3*, *SCHLAFMUTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) in *Arabidopsis* (Aukerman and Sakai, 2003; Schmid *et al.*, 2003; Schwab *et al.*, 2005), which contain sequences complementary to miR172. *AP2* is a floral organ identity gene (Bowman *et al.*, 1991; Drews *et al.*, 1991) while the five *AP2*-like genes mainly act as flowering repressors (Aukerman and Sakai, 2003; Jung *et al.*, 2007; Mathieu *et al.*, 2009). The subsequent identification of miR172 and its targets in many species (see Supplementary Table S1 at *JXB* online) has shown that miR172 has an ancient origin. It has been found in ferns, gymnosperms, and flowering plants, although not in lycopods and moss (Axtell and Bartel, 2005). Recent studies in maize, rice, and barley have shown that miR172 is also important in regulating phase transition and in the determination of floral organ identity in monocotyledons (Lauter *et al.*, 2005; Chuck *et al.*, 2007*b*; Zhu *et al.*, 2009; Nair *et al.*, 2010). These investigations have provided new insights into the functions of miR172 and its targets in both these processes and demonstrated that the interaction between miR172 and its *AP2* or *AP2*-like targets is deeply conserved and has an ancient role in developmental regulation in plants.

Expression profile of miR172 and its regulation

miR172 arises from *MIR172* loci that are transcribed by RNA polymerase II. The primary transcript of *MIR172* or

pri-miR172, has a 5' cap and a 3' poly(A) tail (Xie *et al.*, 2005) and is predicted to form a characteristic imperfect stem–loop hairpin structure that is processed twice by the RNase III-like enzyme DICER-LIKE1 (DCL1) acting in concert with the double-stranded RNA binding protein HYPONASTIC LEAVES1 and the zinc finger protein SERRATE (SE) to release a 21-nt miR172/miR172* duplex with 2-nt 3' overhangs. A 15-nt stem segment proximal from the miR172/miR172* duplex appears to be essential for accurate processing of pri-miR172 (Mateos *et al.*, 2010; Werner *et al.*, 2010). In the majority of plant species investigated so far, more than one *MIR172* locus has been identified; for example, *Arabidopsis*, maize, and rice have 5, 5, and 4 *MIR172* loci, respectively (see Supplementary Table S1 and Supplementary Fig. S1 at *JXB* online). In *Arabidopsis*, the expression of the *MIR172a*, *b*, and *c* genes is elevated as plants reach the reproductive growth stage, whereas the expression of the *MIR172d* and *e* genes is very low and does not alter as the plant develops (Jung *et al.*, 2007). In rice, *MIR172c* is expressed in seedlings but not in developing grains (Sunkar *et al.*, 2008; Zhu *et al.*, 2008). Thus the expression of different members of the *MIR172* family and, consequently, their corresponding mature miRNAs, depends on growth stages and tissue types. In addition, the expression level of miR172 is also affected by day length and temperature (Jung *et al.*, 2007; Lee *et al.*, 2010).

In both dicotyledons and monocotyledons, the expression level of miR172 increases as plants grow and after flowering it accumulates in leaves and floral buds (Aukerman and Sakai, 2003; Lauter *et al.*, 2005; Chuck *et al.*, 2007*b*; Jung *et al.*, 2007; Zhu *et al.*, 2009). The expression pattern of miR172 is inversely correlated with that of miR156 (Fig. 1; Chuck *et al.*, 2007*a*; Wu *et al.*, 2009). Studies in *Arabidopsis* have identified a regulatory link between miR156 and miR172. miR156 acts upstream of miR172 and regulates miR172 expression through the miR156 target *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*9 (*SPL9*), which promotes the expression of miR172 by directly activating its transcription (Wu *et al.*, 2009). In transgenic *Arabidopsis* plants that express a miR156-resistant *SPL9* transcript under the control of its native promoter, the pri-miR172b level was consistently higher throughout shoot development. This is in keeping with the presence of multiple copies of SPL binding elements in the miR172b promoter, at least one of which is strongly bound by SPL9 based on a ChIP assay (Wu *et al.*, 2009). In addition to *SPL9*, *SPL10* and several other *SPL* genes, such as *SPL11* and *SPL15*, probably act redundantly in the transcriptional regulation of miR172 (Wu *et al.*, 2009). This SPL-mediated regulatory module allows the sequential action of miR156 and miR172, which contributes to the regulatory network that governs developmental timing (Wang *et al.*, 2009; Wu *et al.*, 2009). Furthermore, the expression levels of miR172 and the miR172b precursor (pre-miR172b) reduced ~50% in the *toe1 toe2* double mutants and the level of pre-miR172b was slightly elevated in plants over-expressing the miR172 target *TOE1*

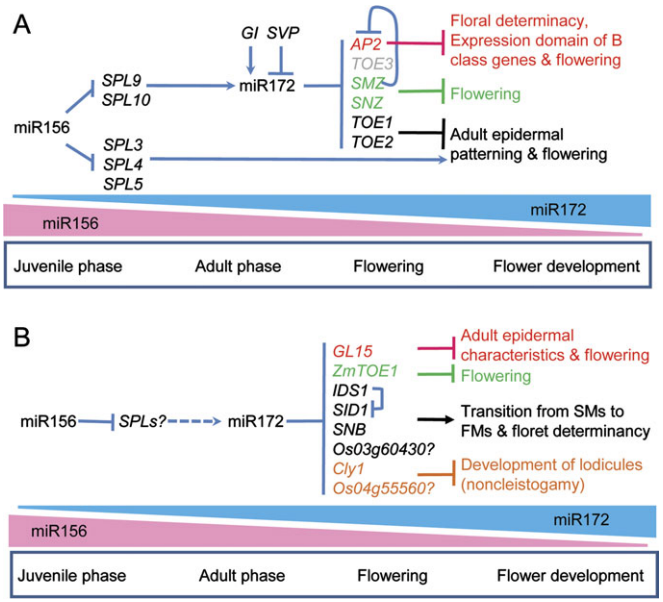

Fig. 1. Conserved regulatory roles of miR156 and miR172 in developmental phase change and flower development in *Arabidopsis* (A) and monocotyledons (B). The expression level of miR172 is inversely correlated with that of miR156 during the plant life cycle. The miR172 targets shown are experimentally confirmed except *ZmTOE1*. See main text for detailed functions of these miR172 targets. The proposed functions of *Os03g60430* and *Os04g55560* are yet to be confirmed. The miR156 regulation of miR172 via *SPL* genes in monocotyledons (indicated by a dashed arrow) has not been determined to date.

(Wu *et al.*, 2009), suggesting the existence of a possible feedback loop in which a miRNA-regulated transcription factor regulates the transcription of its cognate miRNA.

The flowering time of plants is affected by ambient temperature. In *Arabidopsis*, this is achieved by regulating the thermosensory pathway genes *FVE*, *FCA*, and *SHORT VEGETATIVE PHASE* (*SVP*) (Blazquez *et al.*, 2003). The expression level of miR172 was up-regulated at 23 °C compared with 16 °C based on a recent microarray experiment (Lee *et al.*, 2010). In the *svp-32* mutant, expression of miR172a was increased at both 16 °C and 23 °C, and a significant reduction of the expression levels of the miR172 targets *AP2*, *SMZ*, *SNZ*, *TOE1*, and *TOE2* was observed at 23 °C; reduced levels of *TOE1* and *TOE2* were also observed at 16 °C (Lee *et al.*, 2010). This is consistent with the early flowering time phenotype of the *svp-32* mutant, suggesting a role of the interaction between *SVP* and miR172 in the control of flowering time in *Arabidopsis*, although over-expressing miR172 leads to *Arabidopsis* plants insensitive to temperature for flowering (Lee *et al.*, 2010). Meanwhile, miR172 abundance is also influenced by the autonomous pathway genes in *Arabidopsis* (Jung *et al.*, 2007). These results suggest that the expression level of miR172 is transcriptionally regulated by a network with multiple layers of controls.

Regulation of miR172 abundance by photoperiod is achieved through a clock-independent (or *CONSTANS*

independent) *GIGANTEA* (*GI*) pathway (Jung *et al.*, 2007). miR172 abundance was reduced significantly in the *gi-2* mutant but was unaffected in the *constans-2* (*co-2*) mutant. In addition, the expression of miR172 was at a very low level under short-day conditions (SDs) and at a higher level under long-day conditions (LDs) (Jung *et al.*, 2007). The expression levels of miR172 increased in both control and the *gi-2* mutant upon transferring the plants from SDs to LDs, but the pri-miR172 levels were higher in the *gi-2* mutant than in the control plants when grown under SDs and reduced slightly in both plants upon exposure to LDs. This suggests that *GI* regulates miR172 maturation rather than transcription of pri-miR172 (Jung *et al.*, 2007), i.e. post-transcriptional regulation also has a role in governing the expression of miR172. Consistently, DCL1 and SE, two enzymes required for miRNA processing, were also reduced in the *gi-2* mutant (Jung *et al.*, 2007); how *GI* achieves this regulatory role is still unclear. In addition, based on high throughput mapping data for miRNA cleavage sites, *Arabidopsis* pri-miR172b seems to be cleaved by miR172 at the miR172* region. This observation was further confirmed by conventional 5' RACE (German *et al.*, 2008), suggesting that the expression of miR172 could be regulated by self-cleavage. Whether this is an active regulatory mode or a passive consequence resulting from the relatively unstable stem-loop structure of pre-miR172b because of its low free energy ($\Delta G = -36.4$ kcal mol^{-1}, the lowest among the five pre-miR172) needs further investigation.

Dual modes of miR172-mediated gene regulation

miRNAs repress the expression of protein-coding genes through targeting their messenger RNAs (mRNAs) for cleavage or translational repression (Jones-Rhoades *et al.*, 2006; Voinnet, 2009). The mode of regulation adopted by a particular miRNA generally depends on where its target site is located in the mRNA and the extent of sequence complementarity between the miRNA and the target site (Rhoades *et al.*, 2002; Schwab *et al.*, 2005). miRNAs with perfect or near perfect complementary to their targets are predicted to cause RNA cleavage, whereas miRNAs that have a significant number of mismatches with their target sequence or that have target sites located in 3' UTR regions are predicted to cause translational repression. In plants, the majority of miRNAs have perfect or near perfect complementarity with their targets which are usually located in the coding region and thus their main mode of action is thought to be via mRNA cleavage. The high complementarity between miRNAs and their target sites allows relatively confident prediction of plant miRNA targets by bioinformatics approaches.

The miR172 target site is located in the coding region, but close to the 3' end, of a group of *AP2*-like transcription factors that contain two AP2 domains (Fig. 2; Kim *et al.*, 2006; Shigyo *et al.*, 2006). In *Arabidopsis*, cleavage of mRNAs of *AP2*, *TOE1*, *TOE2*, *SMZ*, and *SNZ* by miR172 has been reported (Aukerman and Sakai, 2003; Kasschau *et al.*, 2003; Schwab *et al.*, 2005; Jung *et al.*, 2007; Mathieu *et al.*, 2009) but translational repression appears to be the major mode of action of miR172 (Aukerman and Sakai, 2003; Chen, 2004). miR172 was the first plant miRNA that was shown to regulate its targets mainly via translational repression. In the miR172b over-expresser, *early activation tagged-Dominant* (*eat-D*, an activation-tagged mutant), AP2 protein abundance was dramatically reduced, but the *AP2* transcript accumulated to the normal level. *TOE1* and *TOE2* transcripts were also not reduced in *eat-D* (Aukerman and Sakai, 2003). Similarly, *AP2* transcript abundance in *hua enhancer1* (*hen1*) and *dcl1* mutants

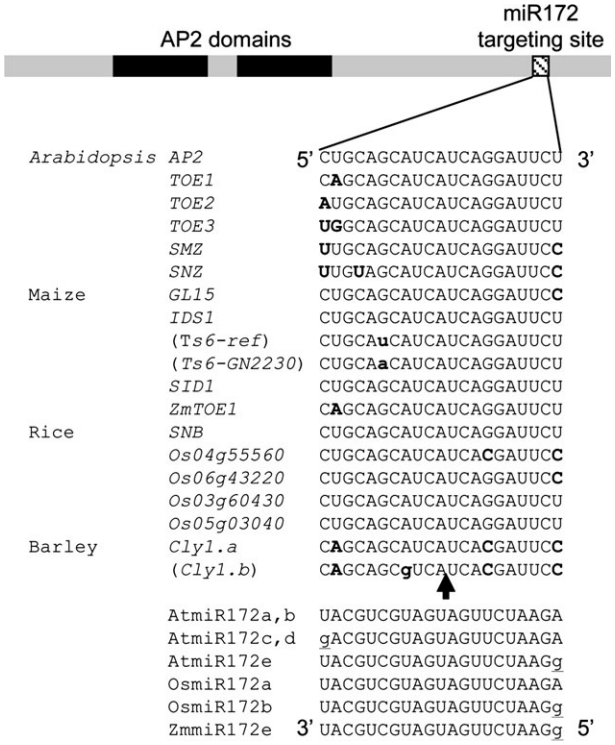

Fig. 2. Functionally characterized miR172 members and their targets in *Arabidopsis*, maize, rice, and barley. The grey bar represents the coding region of miR172 targets with the positions of the two AP2 domains indicated by black bars and the miR172 targeting site indicated by a hatched bar. Nucleotides that differ from *Arabidopsis AP2* are shown in bold. The point mutations that affect miR172-mediated cleavage or translational repression in *Ts6-ref, Ts6-GN2230*, and *Cly1.b* are shown in bold lower case letters. The expected cleavage position in the miR172 targeting site is indicated by a black arrow. Nucleotides of the mature miR172 sequences that are different from that of *Arabidopsis* miR172a and b are shown in underlined lower case letters. Functions of *Arabidopsis* miR172a, b, d, and e, rice miR172a and b, and maize miR172e were analysed by over-expressing their precursors or using knock-out mutants (Aukerman and Sakai, 2003; Chen, 2004; Chuck *et al.*, 2007*b*; Jung *et al.*, 2007; Wu *et al.*, 2009; Zhu *et al.*, 2009). Plants over-expressing AtmiR172e and OsmiR172a did not shown visible phenotypes (Chen, 2004; Zhu *et al.*, 2009). At: *Arabidopsis thaliana*; Os: *Oryza sativa*; Zm: *Zea mays*.

was similar to that in the wild type (WT), whereas AP2 protein amounts in these two mutants were ~2–3-fold higher (Chen, 2004). *AP2* transcript accumulation in 35S::miR172 plants was also similar to that in control plants, but AP2 protein was undetectable in 35S::miR172 plants (Chen, 2004). miR172 and *TOE1* function through the photoperiod pathway to promote early flowering; miR172 levels were changed in *gi-2* and photoreceptor mutants but the levels of *TOE1* transcript were unaltered in these mutants (Jung *et al.*, 2007). These results all indicate that miR172 represses its targets at the level of translation. Recent evidence from *Arabidopsis* suggests that many plant miRNAs function at least partly by translational repression (Broderson *et al.*, 2008).

In maize, using an antibody targeting the C-terminus of INDETERMINATE SPIKELET1 (IDS1), it was found that IDS1 was more abundant in *tasselseed4* (*ts4,* a loss-of-function mutant of maize *MIR172e*, caused by transposon insertion) tassels than in WT during floral meristem initiation (Chuck *et al.*, 2007*b*). However, unlike *Arabidopsis* where miR172-mediated cleavage appears to be a minor mode of action, cleavage of miR172 targets appears to be as important as miR172-mediated translational repression. The expression of the maize *GLOSSY15* (*GL15*) mRNA has an inverse relationship with that of miR172 during the vegetative growth stage. *GL15* transcripts rapidly decrease after the onset of miR172 accumulation and miR172-mediated cleavage was detected by 5' RACE (Lauter *et al.*, 2005). This miR172-mediated degradation of *GL15* seems to be sufficient for transition from the juvenile phase to the adult phase (Lauter *et al.*, 2005). These results suggest that *GL15* is targeted by miR172 for mRNA degradation, although regulation of *GL15* transcription cannot be ruled out, as changes in *GL15* expression have not been analysed in miR172 loss-of-function mutants. In barley, a synonymous nucleotide substitution at the miR172 targeting site in *Cleistogamy1* (*Cly1*) abolishes miR172-mediated cleavage of *Cly1* (Fig. 2) and results in the failure of flower (spikelet) opening when flowering, producing a cleistogamous phenotype (Nair *et al.*, 2010).

Studies in *Arabidopsis* found that cleavage of *AP2* and *TOE1* transcripts were substantially increased in the miR172 over-expresser but the steady-state levels of these two mRNAs did not show a commensurate decrease (Schwab *et al.*, 2005). The accumulation of the *SUPERNUMERARY BRACT* (*SNB*) mRNA in rice plants over-expressing miR172 (Zhu *et al.*, 2009) was also observed. A possible explanation for these findings is that these miR172 targets are under direct or indirect feedback regulation by their own products to increase transcription of the target genes when miR172 is acting to reduce their protein levels. This was first shown by an elegantly designed experiment, in which the wild-type *AP2* transcript was distinguished from the miR172-resistant *AP2* (*mAP2*) transcript by digestion with restriction enzyme (Schwab *et al.*, 2005). Wild-type *AP2* mRNA abundance was increased in *35S::AP2* plants as expected, but strongly decreased in plants over-expressing *mAP2*, which presumably over-produce AP2 protein as they are no longer targeted by miR172 (Schwab *et al.*, 2005). It was also proposed that the efficiency of the feedback regulation differs for different target genes, which could explain the different responses observed for different miR172 targets (Schwab *et al.*, 2005). These results suggest that the amount of miR172 relative to its target transcripts, the degree of complementarity between miR172 and its targets, and feedback regulation of gene expression each contribute to whether transcript degradation or translational repression appears to predominate.

miR172 and developmental phase transition

Flowering plants undergo three primary post-embryonic developmental stages, juvenile, adult, and reproductive. The juvenile phase is characterized by a variety of morphological traits, such as leaf shape, leaf size, and epidermal cell pattern and characteristics. The adult and reproductive phases are marked by an increase in reproductive competence and production of flowers (Poethig, 2009). The transition from the adult to the reproductive phase is quite obvious and has been studied intensively, but less is known about the juvenile-to-adult transition. It is now apparent that miR172 and miR156 play a crucial role in these phase transitions in plants (Fig. 1).

In *Arabidopsis*, miR156 is required for the expression of all juvenile leaf characteristics, and regulates the timing of the juvenile-to-adult transition by co-ordinating the expression of several pathways that control different aspects of this process (Wu and Poethig, 2006; Wu *et al.*, 2009). miR156 achieves its role by repressing the expression of a group of *SPL* transcription factors that possess distinct functions necessary for vegetative phase change. In turn, miR172 is activated by *SPL9*, and possibly by *SPL10* as well, to promote adult phase identity via the repression of its targets *TOE1* and *TOE2* (Wu *et al.*, 2009). Expression of *TOE1* and *TOE2* is high in young seedlings and gradually decreases throughout the life cycle, which is inversely correlated with the expression of miR172 (Aukerman and Sakai, 2003; Jung *et al.*, 2007). Plants expressing 35S::miR172a or 35S::miR172b produce leaves with abaxial trichomes (an adult leaf trait) earlier than controls; similarly *toe1* or *toe2* single mutants display accelerated generation of leaves with abaxial trichomes; this acceleration is more obvious in the *toe1 toe2* double mutants (Wu *et al.*, 2009). By contrast, both the *MIR172a* knock-out mutant and the *35S::TOE1* plants show delayed production of abaxial trichomes (Wu *et al.*, 2009).

In maize, *GL15* is required for the expression of juvenile leaf epidermal cell traits (such as epicuticular waxes and lack of leaf hairs). The onset of miR172 expression between 6 d and 12 d after sowing coincides with the period when the identity of the first leaf expressing adult traits is specified; indicating that increased miR172 expression is associated with the transition to the adult vegetative phase. miR172-mediated down-regulation of *GL15* mRNA may play a role during this transition (Lauter *et al.*, 2005).

miR156 and miR172 also have complementary expression patterns in maize (Chuck *et al.*, 2007*a*), but whether this is mediated by *SPL* genes has not been established.

In addition to a role in the juvenile-to-adult transition, the miR156-*SPL9*-miR172-*TOE1*/*TOE2* cascade also contributes to the transition from the adult to the reproductive phase in *Arabidopsis* as gain- and loss-of-function mutations in *TOE1* and *TOE2* have been shown to affect flowering time (Aukerman and Sakai, 2003; Jung *et al.*, 2007). The *toe1 toe2* double mutant flowers earlier than either individual mutant, suggesting that these two genes are functionally redundant (Aukerman and Sakai, 2003; Jung *et al.*, 2007), but it is not as early flowering as the miR172 over-expresser, suggesting other *AP2*-like genes also act as flowering repressors. More recently, *SMZ* and *SNZ* have been shown to act redundantly with *TOE1* and *TOE2* as floral repressors with over-expression of *SNZ* or *SMZ* causing late flowering (Mathieu *et al.*, 2009). However, the *toe1 toe2 smz snz* quadruple mutant still flowers significantly later than plants that constitutively express miR172 (Mathieu *et al.*, 2009), supporting a previous finding that *AP2* acts as a flowering repressor (Chen, 2004). This has been confirmed by the detection of direct binding of *AP2* to key flowering time promoting genes, such as *APETALA1* (*AP1*), *FRUITFULL* (*FUL*), and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Yant *et al.*, 2010). The function of *TOE3* remains to be uncovered as its expression profile is different from that of other *AP2*-like genes and over-expression of *TOE3* does not delay flowering time (Jung *et al.*, 2007).

miR172 over-expressing plants exhibit early flowering under both LDs and SDs (Jung *et al.*, 2007). In miR172 over-expressers and *toe1* mutants, *FLOWERING LOCUS T* (*FT*) and floral homeotic genes, such as *LEAFY* (*LFY*) and *AP1*, are up-regulated, whereas expression of *CO*, *FLOWERING LOCUS C* (*FLC*) and other genes functioning in the autonomous and GA pathways is unaltered. Furthermore, miR172 abundance is substantially reduced in the cryptochrome mutants and *gi-2*, but unaffected in *co-2* and increased significantly in *phyB-9* and *hy1* (Jung *et al.*, 2007). These results indicate that miR172 exerts its role by regulating expression of *FT* through a genetic pathway other than the autonomous and GA pathways (Jung *et al.*, 2007). Based on these results, a working model in which miR172 mediates light signals from *GI* and promotes flowering by inducing *FT* through *TOE1* but independent of *CO* has been proposed (Jung *et al.*, 2007). More recently, a direct interaction between *SMZ* and *FT* chromatin has been demonstrated using the ChIP-chip approach (Mathieu *et al.*, 2009). The binding site of *SMZ* is located approximately 1.5 kb downstream of the *FT* protein coding sequence. This is consistent with the observation that no *FT* transcription was detected in *smz-D* (a dominant late-flowering mutant) or *35S::SMZ* plants, and that *FT* expression reached a higher level in the *toe1 toe2 smz snz* quadruple mutant (Mathieu *et al.*, 2009). In addition to *FT*, other flowering time regulators, such as *SOC1* and *AP1*, were also repressed by direct binding of *SMZ* and *AP2* upstream of their transcription start site (Mathieu *et al.*, 2009; Yant *et al.*, 2010). Interestingly, significant *SMZ* and *AP2* binding signal was found less than 2 kb upstream of the transcription start site of *TOE3* (Mathieu *et al.*, 2009; Yant *et al.*, 2010), which suggests that *TOE3* could also be a flowering time regulator and achieve its role in an *SMZ* and *AP2* dependent manner. This may explain why the role of *TOE3* in flowering time regulation was not identified previously. Furthermore, *SNZ* and *AP2* are *SMZ* targets (Mathieu *et al.*, 2009), and *SMZ*, *TOE1*, and *SNZ* are targets of *AP2* (Yant *et al.*, 2010). *TOE1* is not a target of *SMZ* but plants over-expressing *SMZ* show reduced *TOE1* levels (Mathieu *et al.*, 2009), suggesting expression of *TOE1* is also regulated by *SMZ*. Meanwhile, both *AP2* and *SMZ* seem to be self-regulated (Mathieu *et al.*, 2009; Yant *et al.*, 2010). These results strongly suggest a complex negative feedback regulatory mechanism among the miR172 targets. The role of miR172 in regulating flowering time in other plant species is not yet clear, although over-expressing miR172 promotes flowering in potato and over-expression of miR172 targets (*GL15* or *ZmTOE1*) delays flowering time in maize (Lauter *et al.*, 2005; Salvi *et al.*, 2007). This complex regulation by miR172 and its targets being uncovered in *Arabidopsis* may inform further investigations in other species.

miR172 and floral organ identity

Arabidopsis flowers have four whorls of floral organs. From outer to inner, they are sepals (whorl 1), petals (whorl 2), stamens (whorl 3), and carpels (whorl 4). The identities of these floral organs are specified by three classes of regulatory genes, the A, B, and C class genes (Causier *et al.*, 2010). *AP2* is an A class gene; it specifies sepal identity in whorl 1, and together with B class genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), petal identity in whorl 2. The stamen identity in whorl 3 is specified by the B class genes together with the C class gene *AGAMOUS* (*AG*). *AG* determines carpel identity in whorl 4. *AP2* and *AG* act antagonistically to restrict each other's activities to their proper domains of action within the flower meristem to specify the identities of the perianth and reproductive organs (Bowman *et al.*, 1991; Drews *et al.*, 1991; Zhao *et al.*, 2007). Loss of *AP2* function converts sepals and petals into carpels due to the expansion of *AG* activity into the outer two whorls of the flower (Bowman *et al.*, 1991). The first indication of miR172 playing a role in the determination of floral identity in *Arabidopsis* was the identification of the miR172 activation-tagging mutant *eat-D*, which not only flowers very early but shows floral defects that are virtually identical to those observed for strong *ap2* mutant alleles (Bowman *et al.*, 1991), including the complete absence of petals and the transformation of sepals to carpels (Aukerman and Sakai, 2003). The same or similar floral defects were observed in plants over-expressing other members of the miR172 family (Chen, 2004).

In situ hybridization detected strong miR172 signals in stage 1 floral primordia; the signal persisted in all four floral whorls until stage 7, and after that stage the miR172 signal appears to be concentrated in the inner two floral whorls. This expression profile of miR172 may cause the AP2 protein to accumulate in the outer two floral whorls to specify perianth identities (Chen, 2004). Meanwhile, expression of a miR172-resistant version of the *AP2* cDNA (*35S::AP2m3*), but not the WT *AP2* cDNA (*35S::AP2*), leads to dramatic floral defects, indicating that miR172 acts in the flower to repress *AP2* (Chen, 2004). The more precise roles of miR172-mediated repression of *AP2* in floral patterning were revealed by over-expressing *AP2* under the control of its own promoter (Zhao *et al.*, 2007). It was discovered previously that *AP2* promotes the expression domain of the B class genes, *AP3* and *PI*, by antagonizing *AG* (Jack *et al.*, 1992). It is now clear that the function of *AP2* is restricted by miR172 to define the inner boundary of the expression domain of the B class genes to ensure proper floral patterning (Zhao *et al.*, 2007). Furthermore, miR172-mediated repression of *AP2* is crucial for maintaining floral meristem size and for floral determinacy, which is achieved through both *AG*-dependent and *AG*-independent pathways (Zhao *et al.*, 2007).

Ectopic expression of *35S::AP2m3* in *N. benthamiana* induces floral patterning defects and the severity of the defects correlates with the *AP2* mRNA and protein levels. Meanwhile, ectopic expression of *Arabidopsis* miR172 in *N. benthamiana* also causes early flowering as in *Arabidopsis*, indicating that miR172-mediated repression of *AP2* in dicotyledons is conserved (Mlotshwa *et al.*, 2006).

miR172-mediated regulation of *AP2*-like genes in the establishment of proper floral patterns is also important in monocotyledons. Maize has two distinct types of inflorescences. The male inflorescence, the tassel that has long branches, is apical, whereas the female inflorescence, the ear that does not have long branches, is formed at the axils of leaves on shortened lateral shoots. The inflorescence meristems (IMs) of the tassel and the ear each produce spikelet pair meristems (SPMs). Each SPM bears two spikelet meristems (SMs) that, in turn, produce a pair of floral meristems (FMs). Several *tasselseed* mutants that show partial to total sex reversal have been described in maize. *ts4* mutants have a highly branched male inflorescence, in which pistils fail to abort and male floral organs do not develop, resulting in complete feminization of the male flowers. *ts4* SPMs produce several SMs rather than two; the SM of *ts4* is also indeterminate and produces multiple florets in a random pattern (Chuck *et al.*, 2007*b*). Database searches and genetic analysis found that *IDS1* is the major target of *ts4* as the loss-of-function mutation of *IDS1* suppresses the sex determination and branching defects in the *ts4* mutants and the IDS1 protein expression domain is expanded in the *ts4* mutants compared to that in WT (Chuck *et al.*, 2007*b*). Furthermore, *Ts6*, a dominant *tasselseed* mutant caused by a G-to-A transition mutation within the miR172 targeting site, is an allele of *IDS1* (Fig. 2) and phenocopies the mutant phenotypes of the *ts4* mutants (Chuck *et al.*, 2007*b*). Loss-of-function mutants of *IDS1* delay the timely conversion of the spikelet meristem to the floral meristem and initiate extra florets instead of two (Chuck *et al.*, 1998, 2007*b*). Based on analysis of spatial expression patterns of *IDS1* transcript and protein in combination with the morphological changes in the *ts4* and *ids1* mutants, it appears that miR172 acts to define the determinacy of SPMs and SMs by restricting IDS1 protein activity at the base of the spikelet meristem. The formation of extra florets because of expansion of IDS1 expression in the *ts4* mutant indicates that *IDS1* functions in promoting the transition from SMs to FMs (Chuck *et al.*, 2007*b*).

The *ids1* mutant does not completely suppress the *ts4* mutant phenotype (the ear of *ids1 ts4* double mutants resembles that of *ts4* single mutants), suggesting there is another miR172 target that contributes to the residual phenotypes observed in *ids1 ts4* double mutants (Chuck *et al.*, 2007*b*). This candidate miR172 target was later found to be *SISTER OF INDETERMINATE SPIKELET1* (*SID1*) as the *sid1* mutation enhances suppression of the *ts4* mutant phenotype by the *ids1* mutation (Chuck *et al.*, 2008). The *SID1* single mutant does not show an obvious phenotype and *SID1* functions redundantly with *IDS1* to specify the fate of several lateral meristems in the inflorescence (Chuck *et al.*, 2008). This function of *IDS1* and *SID1* could be achieved by restricting expression of maize *AG*-like MADS-box transcription factors within the lateral organs of the spikelet, similar to the relationship between *AP2* and *AG* in *Arabidopsis* (Bowman *et al.*, 1991; Drews *et al.*, 1991; Chuck *et al.*, 2008).

Rice *SNB* is closely related to maize *SID1* and *IDS1* (Fig. 3). Loss-of-function mutants of *SNB* delayed the transition from spikelet to floral meristem and produced extra glumes before initiating florets (Lee *et al.*, 2007), consistent with the loss-of-function mutant phenotypes of *IDS1* (Chuck *et al.*, 1998). In addition, in some spikelets of the *snb* mutants, the empty glumes and lodicules were transformed into lemma/palea-like organs (Lee *et al.*, 2007). Transgenic rice plants over-expressing miR172b phenocopied the T-DNA insertion mutant of *SNB*; the severity of the mutant phenotypes observed in the miR172b over-expressers correlated with the expression level of miR172; furthermore, miR172-mediated cleavage of *SNB* mRNA was detected in panicles (Zhu *et al.*, 2009). These results provide evidence that miR172-mediated regulation of *SNB* is important for the development of rice spikelets and florets. In addition to the mutant phenotypes of the *snb* mutant, the miR172b over-expressers displayed additional floral defects, suggesting that at least one other *AP2*-like target gene is required for correct floral development (Zhu *et al.*, 2009). Based on phylogenetic analysis (Fig. 3) and expression profiles (Zhu *et al.*, 2009), a possible candidate is *Os03g60430*. Based on *in situ* hybridization, *SNB* is initially expressed in the branch meristem and spikelet meristem, and is then primarily restricted to the boundary region of the spikelet and glume primordia. Once the spikelet meristem is converted into a floret meristem, a decreased expression of *SNB* is observed (Lee *et al.*, 2007). Both miR172 and *SNB* were highly expressed in <1 cm long

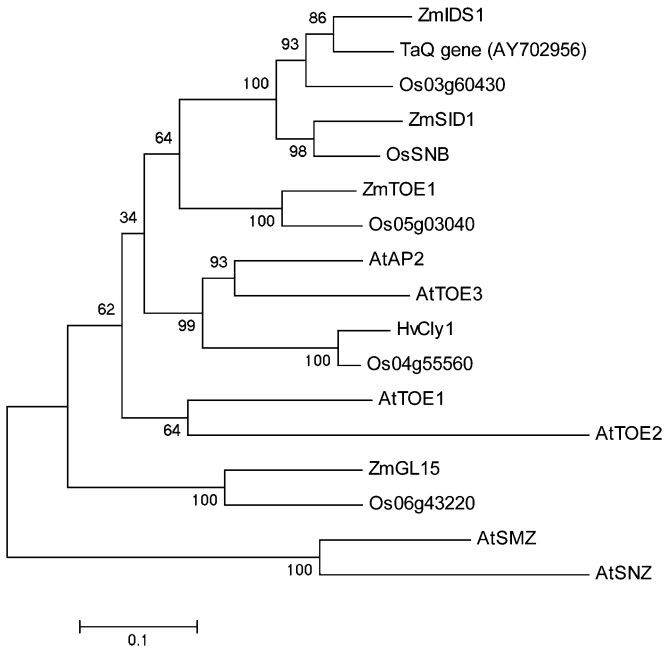

Fig. 3. Phylogenetic tree of miR172 targets from *Arabidopsis* (At), rice (Os), maize (Zm), barley (Hv), and wheat (Ta). The phylogenetic tree and molecular evolutionary analyses were performed by MEGA version 4 using full-length protein sequences. Local bootstrap probabilities are shown and the scale bar represents 0.1 amino acid substitutions per residue.

panicles (Zhu *et al.*, 2009); therefore miR172 could act to restrict the expression domain of *SNB* during floret development in the same manner that maize miR172 restricts the expression domain of *IDS1* (Chuck *et al.*, 2007*b*).

Lodicules are grass family specific floral organs, which develop at the base of the carpel and stamens, and are considered to be homologous to petals in eudicots (Bommert *et al.*, 2005). They swell when flowering to open the floret. A recent study in barley has shown that a single nucleotide substitution in the miR172 binding site in *Cly1* results in smaller lodicules and cleistogamous (or closed) flowering (Nair *et al.*, 2010). In transgenic rice plants over-expressing miR172b, an enlarged and/or increased number of lodicules was frequently observed and, in most cases, lodicules were elongated and transformed into lemma/palea-like organs; consequently, these spikelets were unable to close after flowering and resulted in naked grains (Zhu *et al.*, 2009). It is hypothesized that this might be a result of increased suppression of expression of *Os04g55560,* the most closely related of the five rice miR172 targets to *Cly1* (Fig. 3). These results suggest that miR172-mediated cleavage of *Cly1* and *Os04g55560* (the most frequently cleaved among the five miR172 targets in WT rice plants; Zhu *et al.*, 2009) is essential for normal flower opening in barley and rice. Both non-cleavage and over-cleavage results in defects in development of the lodicules; therefore, normal non-cleistogamy, an ancestral and dominant character in barley (perhaps in all grass species), seems to be maintained by a balance of miR172-mediated repression. How this balance is achieved warrants further investigation. In addition, the wheat domestication gene *Q*, which confers reduced plant height, a free-threshing character and spike compactness to cultivated wheat, is also a miR172 target although it is not clear whether miR172-mediated regulation had a role in the domestication course of the gene (Simons *et al.*, 2006).

Does miR172 act as a long-distance signal?

There is increasing evidence that small interfering RNAs can act as mobile silencing signals (Dunoyer *et al.*, 2010; Molnar *et al.*, 2010). In most cases miRNAs are thought to act in a cell autonomous manner; however, miR399 has been shown to be a long-distance signal involved in the regulation of plant phosphate homeostasis (Pant *et al.*, 2008) which suggests there may be circumstances in which miRNAs act as mobile signals. In potatoes, miR172 expression increases under tuber-inducing short days and is present in vascular bundles and phloem (Martin *et al.*, 2009). Over-expression of miR172 promotes tuberization, an effect which is graft transmissible from scion to stock. This raises the possibility that miR172 can act as a mobile signal in this system, although to date this has not been directly demonstrated.

Conclusion

miR172 is one of the more ancient miRNAs in plants and regulates a small group of AP2 domain-containing plant-specific transcription factors whose functions are broadly conserved although with differing morphological outcomes in different species (Fig. 1). The detection of miR172 in the majority of plants investigated and the demonstrated regulation of predicted target mRNAs indicates that the miR172-AP2 family interaction has been retained throughout plant evolution. This suggests that the regulation of the AP2-domain genes by miR172 has long been a critical component of plant development. The functions of miR172 targets in *Arabidopsis* have been characterized in detail using knockout mutants or transgenic plants over-expressing miR172, providing clues for the functional analysis of miR172 targets in other species, in particular rice, maize, barley, and wheat, where *AP2*-like targets of miR172 are likely to play an important role in reproductive development and the formation of floral organs. Understanding the role of miR172 and its targets in these species could provide new ways for manipulating reproductive development to improve crop productivity. This is exemplified by the recent demonstration that alteration of the miR156 regulation of *SPL14* expression can be used to change plant architecture and improve grain yield in rice (Jiao *et al.*, 2010; Miura *et al.*, 2010).

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. miR172 family and its AP2-domain containing targets in plants.

Supplementary Fig. S1. Alignment of published miR172 sequences in plants.

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