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MicroRNAs in crop improvement: fine-tuners for complex traits

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One of the most common challenges for both conventional and modern crop improvement is that the appearance of one desirable trait in a new crop variety is always balanced by the impairment of one or more other beneficial characteristics. The best way to overcome this problem is the flexible utilization of regulatory genes, especially genes that provide more efficient and precise regulation in a targeted manner. MicroRNAs (miRNAs), a type of short non-coding RNA, are promising candidates in this area due to their role as master modulators of gene expression at the post-transcriptional level, targeting messenger RNAs for cleavage or directing translational inhibition in eukaryotes. We herein highlight the current understanding of the biological role of miRNAs in orchestrating distinct agriculturally important traits by summarizing recent functional analyses of 65 miRNAs in 9 major crops worldwide. The integration of current miRNA knowledge with conventional and modern crop improvement strategies is also discussed.

To meet the growing challenges stemming from rapid population growth, economic development and global climate change, crop production should be significantly increased in a sustainable manner in the future[1–3]. Genetic crop improvement or molecular breeding, which integrates advances in plant physiology, genetics, biotechnology and genomic research, can contribute towards achieving this goal. One of the critical requirements for genetic crop improvement is the introduction of new genetic material into crop lines of interest, which can be accomplished by introducing single or multiple genes via genetic engineering or marker-assisted selection[4]. In light of this, thousands of genes and tightly linked markers for various agronomic traits have been isolated and characterized in the past few decades[1,5]. Most notably, however, almost all the remarkable success achieved so far in genetic crop improvement is attributed to the introduction of a limited set of genes, whereas many other identified genes are inappropriate for direct use in the conventional or molecular breeding of crops with combined desirable traits due to the lack of natural trait-enhancing alleles and/or gene pleiotropism, which results in secondary issues despite correcting the primary defect[1,6]. Therefore, the further discovery of genetic modulators that provide precise regulation in a targeted manner, as well as a deeper understanding of complex plant biological processes, is essential for scientists to manipulate important agronomic traits more efficiently and accurately. Recent research has revealed a previously unrecognized layer of gene expression regulation exerted by microRNAs (miRNAs), which are endogenous single-stranded non-coding RNA molecules of approximately 22 nucleotides in length that bind to partially complementary sequences in target messenger RNAs (mRNAs)[7,8]. In plants, the majority of primary miRNA transcripts (pri-miRNAs) are transcribed from miRNA genes through RNA polymerase II. After the processing

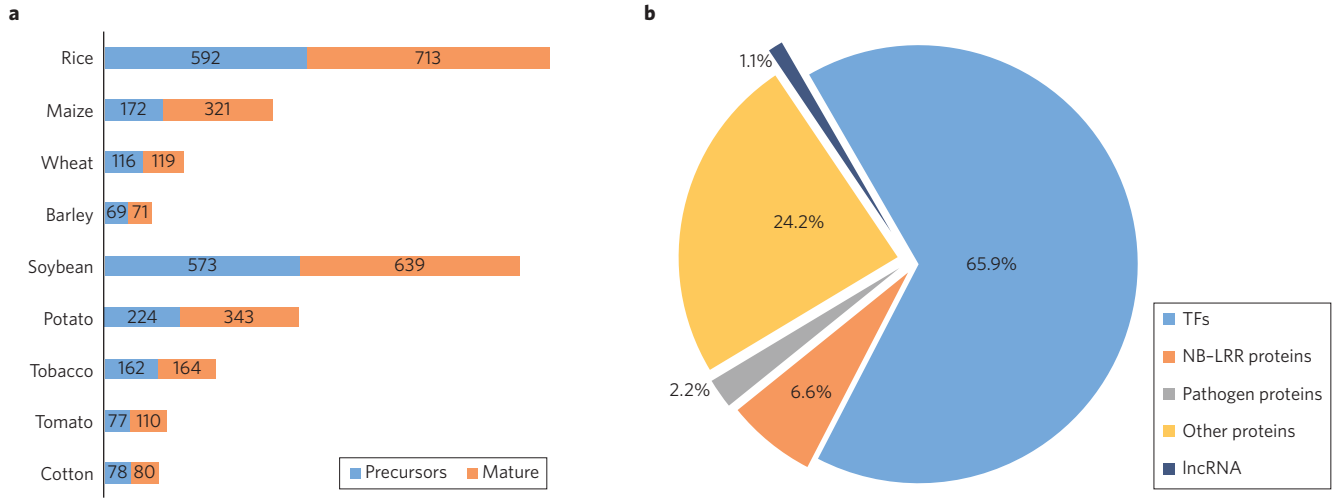

Figure 1 | Distribution of miRNAs and their target genes. a, Numbers of mature miRNAs and their precursors in nine major crops deposited in miRBase. **b**, Functional categories of the miRNAs' target genes. Target genes listed in Table 1, except for those marked with a question mark, are counted.

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Table 1 | Summary of functionally validated miRNAs and their targets in nine major crops.

miRNA	miRNA targets		Agronomic traits	Refs
	Name	Classification		
Rice (*Oryza sativa*)				
osa-miR156	Os*SPL13*, Os*SPL14*, Os*SPL16*, and so on	SPL TFs	Grain size, grain yield, grain quality, panicle branching, tillering, plant height	13–17
osa-miR159	Os*GAMYB*, Os*GAMYBL1*	MYB TFs	Floral development, stem elongation	42
osa-miR160	*ARF16*	ARF TF	Immune response	25
	Os*ARF18*	ARF TF	Rice growth and development	24
osa-miR162	Os*TRE1*	Trehalase	Drought tolerance	116
osa-miR164	*OMTN1–OMTN6*	NAC TFs	Drought tolerance	47
osa-miR166	*RDD1*	DOF TF	Nutrient ion uptake and accumulation	58
osa-miR167	*ARF8*	ARF TF	Auxin response	78, 117
osa-miR168	*AGO1*	AGO1	Immune response	86
osa-miR172	*SNB*, Os*IDS1*, Os*TOE1*	AP2-like TFs	Spikelet determinacy, floral organ development, flowering time, panicle branching	17, 30, 31
osa-miR319	Os*PCF5*, Os*PCF6*, Os*PCF8*, *TCP21*	TCP TFs	Leaf morphogenesis, cold tolerance, immune response	38, 40
osa-miR390	Os*SRK*	Stress-responsive leucine-rich repeat receptor-like kinase	Cadmium tolerance and accumulation	118
osa-miR393	Os*TIR1*, Os*AFB2*	Auxin-signalling F-Box proteins	Nitrogen-promoted rice tillering, flowering time, salt and drought stress tolerance	76, 77, 79
osa-miR395	Nta*SULTR2*	Sulfate transporter	Sulfate homeostasis	119
osa-miR396	Os*GRF4*, Os*GRF6*, Os*GRF10*	GRFs	Grain size, grain yield, inflorescence development, salt-alkali stress tolerance	49–53
osa-miR397	Os*LAC*	Laccase-like protein	Grain size, grain number, grain yield	75
osa-miR398	*SOD2*	Superoxide dismutase2	Immune response	25
osa-miR399	*LTN1*	Protein containing a ubiquitin-conjugating domain	Phosphate-starvation response	80, 81
osa-miR444	Os*MADS23*, Os*MADS27a*, Os*MADS57*	MIKC^{C}-type MADS box TFs	Tillering, nitrate signalling, immune response	55–57
osa-miR528	*AO*	L-Ascorbate oxidase	Immune response	120
osa-miR529	*SPL14*, *SPL17*	SPL TFs	Tillering, panicle branching	17
osa-miR820	Os*DRM2*	Domains rearranged methyltransferase 2	Host genome–parasitic DNA interaction	121
osa-miR827	Os*SPX-MFS1*, Os*SPX-MFS2*	SPX-MFS proteins	Phosphate-starvation response	82
osa-miR1848	Os*CYP51G3*	Obtusifoliol 14α-demethylase	Phytosterol and BR biosynthesis during development and in response to stress	122
	Os*WS1*	Membrane-bound O-acyl transferase	Wax biosynthesis	123
osa-miR2118	*PMS1T*	lncRNA	Photoperiod-sensitive male sterility	74
osa-miR7695	Os*Nramp6*	Natural resistance-associated macrophage protein 6	Immune response	95
Maize (*Zea mays*)				
zma-miR156	*tga1*, *tsh4*, and so on	SPL TFs	Juvenile-to-adult phase transition, bract development, meristem boundary establishment	18, 21
zma-miR164	Zm*NAC1*	NAC TF	Lateral root development	45
zma-miR166	*rld1*	Class III homeodomain/leucine zipper TF	Leaf polarity	62
zma-miR172	*gl15*	AP2-like TF	Juvenile-to-adult phase transition	32
	ids1	AP2-like TF	Sex determination, meristem cell fate	33
Wheat (*Triticum aestivum*)				
PN-2013	Ta*MDHAR*	Monodehydroascorbate reductase	Immune response	124

continued

Table 1 | (continued)

miRNA	miRNA targets		Agronomic traits	Refs
	Name	Classification		
tae-miR159	Ta*GAMYB*	MYB TF	Anther development, heat response	43
tae-miR164	Ta*NAC21/22*	NAC TF	Immune response	48
tae-miR408	Ta*CLP1*	Chemocyanin-like protein	Response to abiotic and biotic stress	125
	Ta*TOC1*	Timing of CAB Expression 1 TF	Heading time	61
Barley (*Hordeum vulgare*)				
hvu-miR171	Hv*SCL*	SCARECROW-like TF	Phase transition, floral meristem determinacy	59
hvu-miR172	Hv*AP2*	AP2-like TF	Grain density, cleistogamous flowering	34, 36
hvu-miR398	Hv*SOD1*	Superoxide dismutase	Immune response	89
hvu-miR9863	*MLA1*	NB–LRR protein	Immune response	71
Soybean (*Glycine max*)				
gma-miR156	Gm*SPL1*? Gm*SPL2*?	SPL TFs	Level of nodulation	35
gma-miR160	Gm*ARF10/16/17*	ARF TFs	Soybean nodule development	27
gma-miR167	Gm*ARF8*	ARF TF	Soybean nodule development, lateral root development	28
gma-miR172	Gm*AP2-2*	AP2-like TF	Level of nodulation	35
Potato (*Solanum tuberosum*)				
stu-miR156	St*SPL3/6/9/13*, St*LG1*	SPL TFs	Plant architecture, tuber yield	19
stu-miR172	*RAP1*	AP2-like TF	Flowering time, tuberization time	37
stu-miR482	*NB–LRR* genes?	NB–LRR proteins	Immune response	69
Tobacco (*Nicotiana tabacum*)				
nta-miR6019	*N*	NB–LRR protein	Immune response	65
nta-miR6020	*N*	NB–LRR protein	Immune response	65
Tomato (*Solanum lycopersicum*)				
sly-miR156	Sly*SBP* genes?	SPL TFs	Vegetative and reproductive development	22, 23
sly-miR157	Le*SPL-CNR*	SPL TF	Fruit ripening	20
sly-miR159	*SGN-U567133*	Uncharacterized protein	Leaf and floral development	126
sly-miR160	Sl*ARF10A* etc.	ARF TFs	Ovary patterning, floral organ abscission, lamina outgrowth	26
sly-miR164	*GOB*	NAC TF	Boundary specification	46
sly-miR167	Sl*ARF6/8*	ARF TFs	Floral development, female sterility	29
sly-miR168	Sl*AGO1A*, Sl*AGO1B*	AGO1	Phase transition, leaf epinasty, fruit development	88
sly-miR169	Sl*NF-YA1/2/3*?	NF-YA TFs	Drought tolerance	90
	Sl*MRP1*?	Multidrug resistance-associated protein		
sly-miR319	*La*	CIN-TCP TF	Compound leaf development, immune response	39, 41
sly-miR482f	Solyc*08g075630*, Solyc*08G076000*	NB–LRR proteins	Immune response	68
sly-miR5300	Solyc*05g008650*, Solyc*09g018220*	NB–LRR proteins	Immune response	68
sly-miR858	Sl*MYB7*-like? Sl*MYB48*-like?	MYB TFs	Anthocyanin accumulation	127
sly-miR4376	Sl*ACA10*	Ca^{2+}–ATPase	Floral development, fruit yield	128
sly-miR6024	*I2* homologues?	NB–LRR proteins	Immune response	70
Cotton (*Gossypium hirsutum*)				
gh-miRNVL5	Gh*CHR*	Zinc finger domain-containing TF	Response to salt stress	60
gh-miR159	*HiC-15*	Isotrichodermin C-15 hydroxylase	Immune response	72
gh-miR166	*Clp-1*	Ca^{2+}-dependent cysteine protease	Immune response	72
gh-miR482	*NB–LRR* genes?	NB–LRR proteins	Immune response	76
gh-miR828	Gh*MYB2D*	MYB TF	Fibre development	44

The table lists the vast majority of miRNAs from nine major crops that have been experimentally verified. It describes their targets and the agronomic traits that are regulated by them. Question marks indicate that these genes are partially validated targets from a big gene family and are not counted in Fig. 1b. See the main text for abbreviations.

of pri-miRNAs, precursor miRNAs (pre-miRNAs) are formed and are further processed by the Dicer-like 1 (DCL1) enzyme with the aid of HYPONASTIC LEAVES 1 (HYL1) and Serrate (SE) to yield the miRNA–miRNA* duplexes. The duplex is methylated by HUA ENHANCER 1 (HEN1) and the guide miRNA strand is then incorporated into ARGONAUTE (AGO) protein to form a functional RNA-induced silencing complex (RISC). Once a suitable pairing event between a miRNA and target mRNA occurs, the RISC complex triggers almost complete inhibition of protein expression by either destabilizing mRNA targets or by inhibiting protein translation[7,8]. In the past few years, extensive studies have demonstrated that miRNAs play versatile roles in almost all aspects of the intracellular processes associated with growth, development and stress responses in plants[7–9]. A growing body of evidence has been collected that supports the vital role of miRNAs in regulating diverse important agronomic traits in crops. According to records registered in miRBase (http://www.mirbase.org, release 21), 2,560 mature miRNAs encoded by 2,063 precursors have been identified through experimental or computational approaches from nine major crops, including rice, maize, wheat, barley, soybean, potato, tobacco, tomato and cotton, which are of paramount importance to satisfy world food, livestock and poultry feed, fibre and other economic requirements (Fig. 1a). The biological functions of dozens of crop miRNAs have also been well elucidated, which provides valuable information regarding the underlying biological processes of crop development, metabolism and stress response. This work strongly demonstrates that both miRNAs and their target genes can be exploited for crop improvement (Table 1). In this Review Article, we highlight recent advances in our understanding of miRNA-mediated regulation of important agronomic traits in these nine major crops, with an emphasis on outlining the targets by and the ways in which miRNAs orchestrate diverse agronomic traits. Furthermore, we use examples of individual miRNA–target modules to discuss their functional conservation and diversification among crops and the emerging utility of them as promising candidates for improving agronomic traits. Readers interested in miRNA-mediated control of specific agronomic traits should refer to recent reviews[10–12].

miRNAs fine-tune the expression of diverse regulatory genes

A preliminary statistical analysis shows that the functions of at least 65 miRNAs from 36 families have been experimentally validated through overexpression or loss-of-function analyses in the aforementioned nine major crops (Table 1). The verified targets of these miRNAs encode a diverse range of regulatory proteins. Notably, approximately 66% of these targets are transcription factors (TFs), clearly indicating that miRNAs play a role at the core of gene regulatory networks (Fig. 1b). These TFs have been established to be critical regulators of plant development or stress responses, including (1) SQUAMOSA-promoter binding protein-like (SPL) TFs being negatively regulated by miR156, miR157 or miR529, which function in the regulation of phase transition or vegetative and reproductive development[13–23]; (2) miR160 or miR167 targeting of auxin response factors (ARFs), which are involved in auxin or defence responses or play roles at many stages of development[24–29]; (3) the APETALA2 (AP2) family of TFs being dampened by miR172, which affects the fine-tuning of phase transition, organ development or sex determination[17,30–37]; (4) TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP) TFs being tuned by miR319, which affects leaf morphogenesis, cold tolerance or defence responses[38–41]; (5) MYB TFs being antagonized by miR159 in the control of floral development and heat response or in conjunction with miR828 to modulate fibre development[42–44]; (6) NAC (NAM, ATAF1/2 and CUC2) TFs, which are encoded by miR164 target genes, playing roles in lateral root development, boundary specification, drought tolerance or immune response[45–48]; (7) miR396-regulated growth-regulating factors (GRFs) being involved in the control of grain size and yield, inflorescence development or salt–alkali stress tolerance[49–54]; (8) MIKC^{C}-type MADS-box TFs being modulated by miR444 in the regulation of rice tillering, nitrate signalling and antiviral responses[55–57]; and (9) DNA-binding with one finger (Dof) TF RDD1, SCARECROW-like (SCL) TF HvSCL, zinc finger domain-containing TF GhCHR, Timing of CAB Expression 1 TF TaTOC1, and the class III homeodomain/leucine zipper (HD-ZIPIII) family member ROLLED LEAF1 (RLD1) being negatively regulated by miR166, miR171, miRNVL5, miR408 and miR166 to affect nutrient ion uptake and accumulation, phase transition and floral meristem determinacy, plant response to salt stress, heading time and leaf polarity, respectively[58–62].

The next gene class preferentially targeted by miRNAs is a major class of *R* (resistance) genes that encode proteins containing a central nucleotide-binding region and a C-terminal leucine-rich repeat (NB–LRR) (Fig. 1b). These proteins directly or indirectly recognize specific pathogen effectors and consequentially trigger *R*-gene-mediated immune responses[63]. Precise and timely control of *R* gene expression is crucial for plant fitness as the misregulation of *R* genes can result in the uncontrolled activation of immune responses, which is harmful to plant growth and development[64]. Recently, at least five miRNA families have been confirmed to target crop NB–LRR-encoding transcripts for posttranscriptional regulation, indicating a vital role for miRNAs in regulating plant effector-triggered immunity. In tobacco, two miRNAs, miR6019 and miR6020, were shown to guide cleavage of transcripts of the NB–LRR gene *N* and co-expression of both miRNAs resulted in the attenuation of *N*-mediated resistance against tobacco mosaic virus[65]. In potato, tomato and cotton, the miR482/2118 superfamily has been shown to regulate a subset of NB–LRR genes by targeting the conserved sequences encoding the P-loop motif in the mRNA sequences of R proteins[65–69]. Moreover, the homologues of the typical NB–LRR gene *I2* in tomato were shown to be targeted by miR6024 rather than by miR482[70]. Additionally, members of the *Triticeae*-specific miR9863 family were found to regulate a subset of barley *Mildew resistance locus a* (*Mla*), which encode NB–LRR proteins that have verified or anticipated race-specific disease resistance activity against the biotrophic fungal pathogen barley powdery mildew[71].

The other 22 bona fide protein-coding targets of the 19 miRNA families cannot be clearly classified (Fig. 1b). These targets encode regulatory proteins, metabolic enzymes, transporters or uncharacterized proteins involved in modulating many agronomic traits such as grain or fruit yield, fibre or floral development, nutrient homeostasis, environmental stress tolerance, resistance to fungi or viruses, and so on (Table 1). Notably, crop miRNAs not only regulate endogenous genes but also target exogenous genes. A recent study revealed that, in response to infection with *Verticillium dahliae*, a soil-borne fungal pathogen responsible for a devastating and widespread wilt disease affecting many crops worldwide, cotton plants increase the production of miR166 and miR159 and export both to the fungal hyphae for specific silencing of two *V. dahliae* genes: *Clp-1*, which encodes a Ca^{2+}-dependent cysteine protease, and *HiC-15*, which encodes an isotrichodermin C-15 hydroxylase, respectively. Both *V. dahliae* genes are essential for fungal virulence[72]. These findings described a novel plant defence strategy against pathogenic fungi through the export of specific miRNAs from the colonized host plants to induce specific cross-kingdom silencing of fungal virulence genes. This study is also a reminder that it is necessary to extend the scope of plant miRNA target searches to pathogens. Correspondingly, a previous research illustrated that *Botrytis cinerea*, an aggressive fungal pathogen, has the capability to generate and deliver small RNAs into hosts to suppress host immunity[73]. Both findings disclosed an interesting, important and possibly common plant–microorganism interaction model in

which small RNAs have been used by both sides as weapons to fight each other.

The most recent study shows that osa-miR2118 triggered the cleavage of a long non-coding RNA (lncRNA), *PMS1T* (*Photoperiod-sensitive genic male sterility 1*), rather than protein-coding genes in rice. This miRNA–lncRNA interaction leads to the generation of 21-nucleotide phased small-interfering RNAs (phasiRNAs), and the elevation of phasiRNAs in young panicles under long-day conditions eventually results in male sterility in rice[74].

miRNAs coordinate development and stress responses

Recent studies on the 65 miRNAs listed in Table 1 have borne out claims that most miRNAs do not work independently, but are involved in complex regulatory networks to coordinate varied developmental processes and stress responses. For illustration purposes, a preliminary miRNA network in rice is described here as an example by mining the literature of experimentally verified miRNA–trait relationships (see Fig. 2 for a simplified summary, and Fig. 3 for a more detailed version), which is composed of, but not limited to, three layers of interaction.

Synergy or antagonism between distinct target genes of one miRNA. When a miRNA targets multiple mRNAs, the targeted genes usually belong to a single gene family and can govern the given traits in a synergistic or antagonistic manner. For example, osa-miR156 has been suggested to be involved in controlling rice grain size and panicle branching by targeting several *SPL* genes, including Os*SPL13*, Os*SPL14* and Os*SPL16* (refs 13–16) (Fig. 3). Os*SPL13* positively increases grain length and grain thickness without changing grain width, while Os*SPL16* decreases grain length and increases grain width. Moreover, Os*SPL13* and Os*SPL14* promote panicle branching, but Os*SPL16* functions as a negative regulator of panicle branching. Thus, a subtle balance among these osa-miR156 target genes is crucial to establish rice grain size and panicle architecture.

Functional convergence of distinct miRNAs on one trait. Although we are still at a very early stage in understanding the overall impact of miRNAs on rice development and stress responses, a general consensus is emerging that a specific developmental event is usually subject to modulation by diverse miRNA families. For example, osa-miR156, osa-miR396 and osa-miR397 are involved in regulating rice grain size[14,16,49,50,75]; these three miRNA families, along with osa-miR172 and osa-miR529, have been linked to the control of rice panicle branching[13–17]; osa-miR156, osa-miR393 and osa-miR444 have been demonstrated to regulate rice tillering[13,15,17,55,76,77]; osa-miR160, osa-miR167 and osa-miR393 converge on auxin responses[24,76–79]; and osa-miR399, osa-miR444 and osa-miR827 play crucial roles in phosphate-starvation responses[56,80–82] (Figs 2 and 3). Based on these results, the ways in which miRNAs functionally converged can be grouped into three categories: (1) different miRNA families regulate the same target genes, such as miR156 and miR529, both of which coordinately regulate panicle branching through maintaining the expression of *SPL* genes, including Os*SPL14* and Os*SPL17*, at optimal levels[17] (Fig. 3); (2) one miRNA mediates the expression of another miRNA. In rice, osa-miR172 regulates reproductive branching by targeting AP2-like genes, such as *SNB*, Os*IDS1* and Os*TOE1*. Intriguingly, the expression of osa-miR172 and its precursors is suggested to be directly regulated by Os*SPL14*, the target gene of osa-miR156, which coincides with complementary expression patterns of osa-miR156 and osa-miR172 in a range of tissues, and supports the conclusion that osa-miR156 and osa-miR172 coordinately regulate rice vegetative and reproductive branches[17] (Fig. 3); (3) miRNAs functionally converged via direct or indirect interaction between their targets. To date, a number of auxin signalling genes have been confirmed to be the targets of several miRNAs. osa-miR393 was shown to target the auxin receptor genes, Os*TIR1* and Os*AFB2*, both of which encode F-box proteins and are required for the ubiquitin-mediated degradation of specific substrates during auxin signalling[76,77,79]. Furthermore, osa-miR160 and osa-miR167 target the

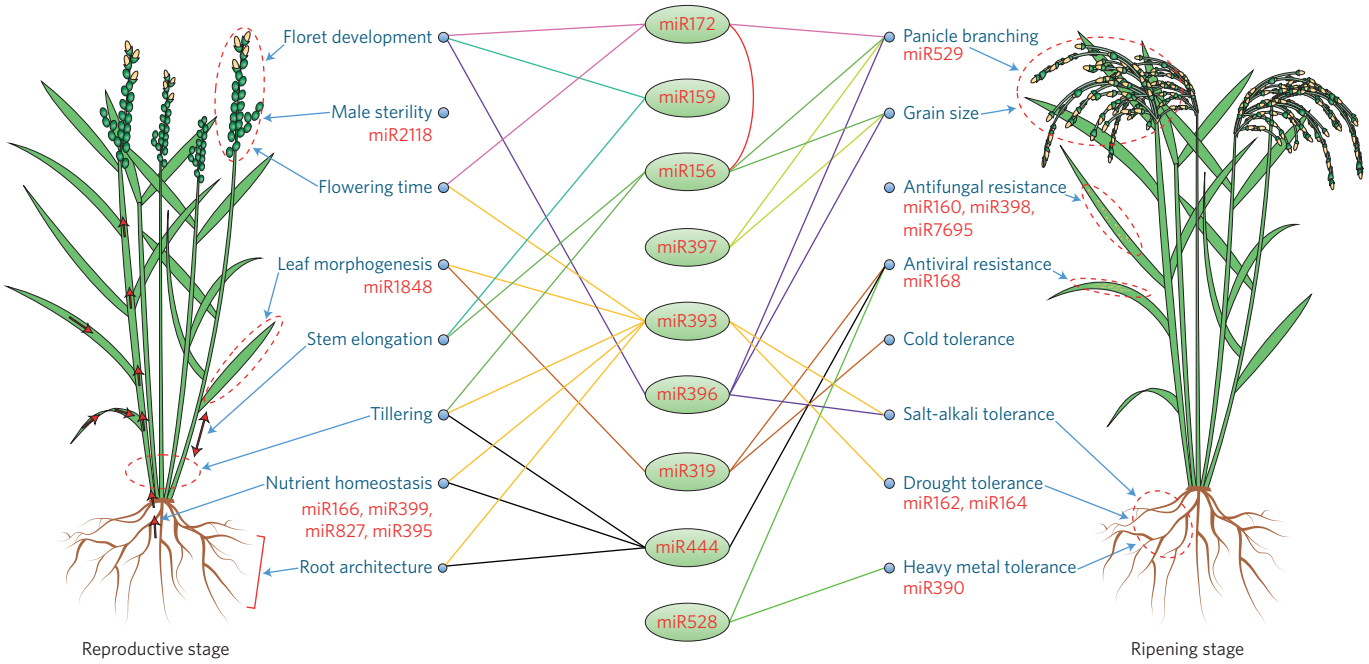

Figure 2 | Experimentally verified miRNA:trait relationships in rice. Nine miRNAs that coordinate different developmental events and/or stress responses are listed in the middle of the figure (green). miRNAs (red) that regulate specific traits are listed under the names of respective traits (blue). miR160, miR167 and miR820, which affect many aspects of rice growth and development (Table 1), are not included in this figure. Coloured lines illustrate the relationships between miRNAs and traits or between different miRNAs. Rice plants at two developmental stages are shown to illustrate various agronomic traits of rice. This does not indicate that the miRNA–target modules act specifically or even primarily at these stages.

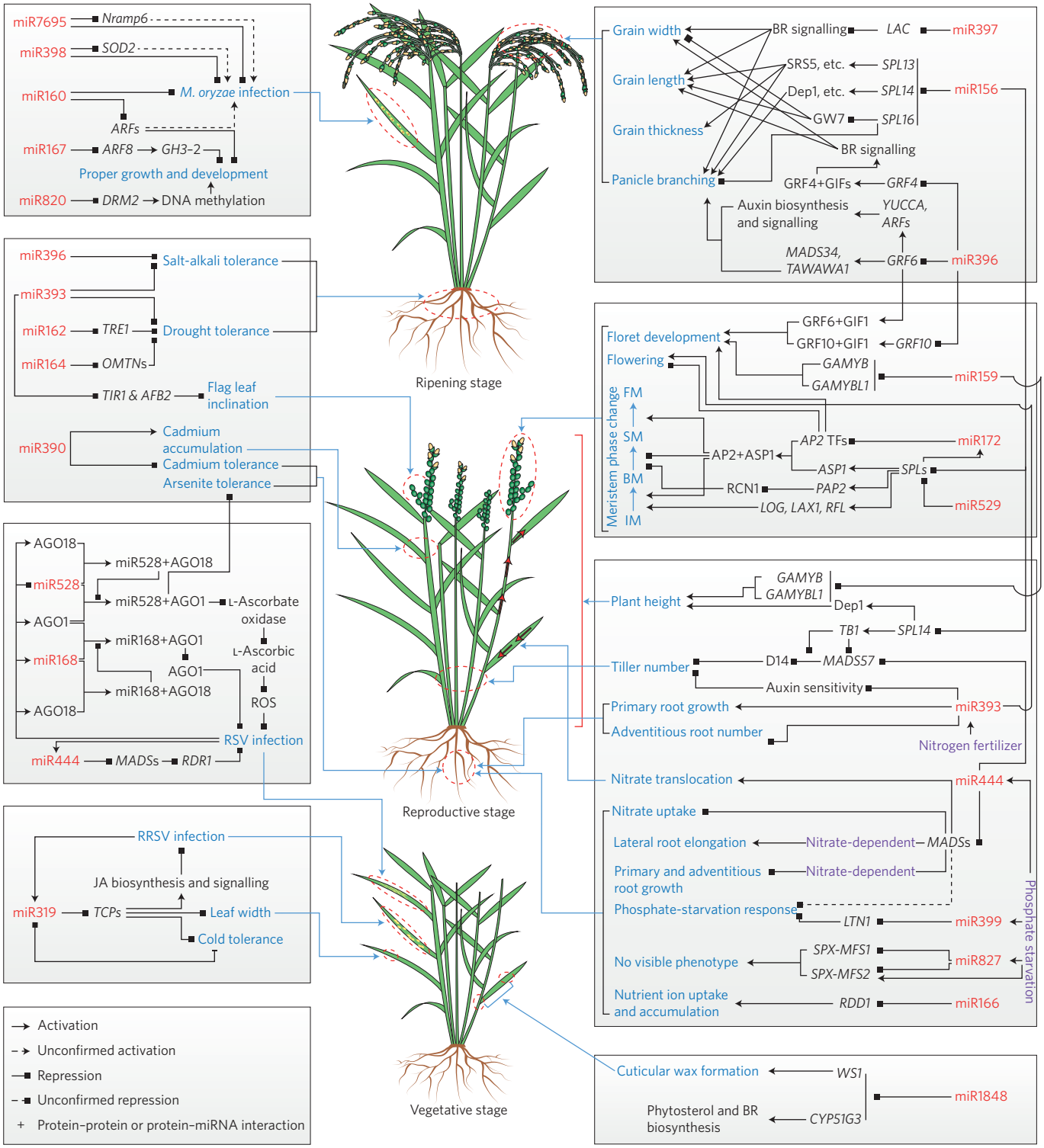

Figure 3 | Regulatory circuits orchestrated by miRNAs in rice. An overview of the current understanding of miRNA-mediated regulatory networks involved in rice growth, development and adaptation to abiotic and biotic stresses (based on the literature listed in Table 1). Rice plants at three developmental stages are shown to illustrate various agronomic traits of rice that are affected. This does not indicate that the miRNA-target modules act specifically or even primarily at these stages. IM, inflorescence meristem; BM, branch meristem; SM, spikelet meristem; FM, floral meristem; ROS, reactive oxygen species; RRSV, rice ragged stunt virus; RSV, rice stripe virus.

transcripts of many ARF genes, including Os*ARF8*, Os*ARF16* and Os*ARF18*[24,25,78]. Incidentally, in *Arabidopsis*, the miR164-directed cleavage of *NAC1* mRNA affects the auxin regulation of lateral root development[83]. In addition, miR390 was found to mediate a regulatory pathway involved in auxin signalling by directing the cleavage of *TAS3* (ref. 84). As another example, two studies indicated that the osa-miR396- and osa-miR397-mediated regulatory pathway may converge on the brassinosteroid (BR) signalling pathway to establish rice grain size (Fig. 3). Overexpression of osa-miR397 increases grain size by downregulating its target, Os*LAC*, which

encodes a laccase-like protein that negatively regulates the sensitivity of plants to BR signalling[75]. Our recent work revealed that when it escapes from suppression by osa-miR396, Os*GRF4*, a GRF gene, accumulates to a proper extent and increases grain size by activating specific BR responses[49]. Although both miRNAs appear to play opposite roles in regulating specific BR signalling to control grain size, subtle details about their crosstalk remain elusive.

Harmonization of different traits. To better adapt to their complicated and changeable environment, plants have evolved intricate regulatory networks to promptly and tightly coordinate different developmental events and stress responses. Increasing evidence shows that miRNAs are fully integrated in such genetic networks to play essential roles in coordinating the multitude of physiological processes relating to diverse agronomic traits. In rice, several functionally characterized miRNAs have been demonstrated to be involved in different developmental processes (Figs 2 and 3). For instance, the overexpression of osa-miR156 in rice promotes leaf and tiller initiation, decreases inflorescence meristem activity and suppresses the initiation of reproductive branching[17]. Correspondingly, a point mutation in Os*SPL14* that blocks the cleavage of osa-miR156 results in rice plants with ideal plant architecture characteristics, including low tiller numbers, increased panicle branches, improved lodging resistance and enhanced grain yield[13,15]. The role of osa-miR172 was also established in rice, which demonstrates the involvement of osa-miR172 in controlling the phase transition from spikelet meristem to floret meristem, inflorescence branching, floral and seed development, and flowering time[17,30,31]. In addition, some miRNAs play multiple roles in coordinating the relationship between development and stress-resistance processes (Figs 2 and 3). Expression of the rice miRNA, osa-miR444, can be induced under nitrate- or phosphate-starvation conditions. Analyses of osa-miR444 overexpression lines suggested that osa-miR444 regulates nitrate signalling in rice root growth, nitrate accumulation and phosphate-starvation responses[56]. A previous study demonstrated that osa-miR444 also participates in the modulation of rice tillering by targeting Os*MADS57*, which encodes a MADS box protein that interacts with its suppressor, OsTB1 (Teosinte Branched1), to maintain the balanced expression of *D14* (*Dwarf14*) for tillering[55]. The most recent study further revealed that osa-miR444 can be induced by infection with rice stripe virus (RSV). The activation of osa-miR444 diminishes the repressive roles of OsMAD23, OsMAD27a, and OsMADS57 on Os*RDR1* (RNA-dependent RNA polymerase1) transcription, and then activates the OsRDR1-dependent antiviral RNA-silencing pathway, leading to resistance to RSV infection[57]. Nitrogen supply promotes the upregulation of osa-miR393 in rice, which alleviates sensitivity to auxin in axillary buds by decreasing the expression of Os*TIR1* and Os*AFB2*, thereby promoting rice tillering[76]. Overexpression of osa-miR393 also leads to early flowering, enlarged flag leaf inclination, altered primary and crown root growth, and decreased tolerance to salt and drought stress[77,79]. Similarly, the induction or overexpression of osa-miR319 reduces the expression of miR319-regulated TCP genes, including *TCP21* and Os*PCF6*, which consequently impacts leaf morphogenesis, resulting in enhanced tolerance to cold stress, and suppressed jasmonic acid (JA)-mediated defence to facilitate the infection of rice ragged stunt virus (RRSV)[38,40]. Notably, the direct use of miRNAs such as osa-miR393 or osa-miR319 in crop breeding may be problematic as manipulation of such miRNAs may improve one trait while also impairing the other desired traits.

Conservation and diversification of miRNAs among crops

The majority of the 36 functionally validated miRNA families are evolutionarily conserved among distantly related plant species[85]. miRNAs that are conserved between species also tend to have conserved targets[9]. The high conservation of these miRNA–target regulatory modules among species suggests a strong selective pressure and is consistent with their pivotal roles in multiple aspects of plant growth, development and adaptation. Therefore, much focus has been placed on studying these conserved miRNAs. The characteristics of miRNA conservation make some of these miRNAs more convenient for use in trait improvement in different species. For example, the miR319–TCPs module was reported to be involved in root-knot nematode resistance by affecting JA synthetic genes and the endogenous JA level in tomato[41]. The action of miR319 in serving as a systemic defensive regulator can be transplanted for rice immunity improvement. In rice, osa-miR319 overexpression or *TCP21* knockdown resulted in a decrease in endogenous JA, while overexpressing a miR319-resistant form of *TCP21* resulted in increased levels of endogenous JA and conferred resistance against RRSV[40].

In general, separate but parallel studies of a given conservative miRNA–target module in different crops can rapidly expand the understanding of its biological function. For instance, in rice and *Nicotiana benthamiana*, the miR168–AGO1 module has been shown to play an essential role in antiviral immunity[86,87], while a study in tomato provides new insights into the role of the miR168-AGO1 module in determining phase transition, leaf epinasty and fruit development[88]. In addition to their conserved roles, the diverse roles of many conserved miRNA–target modules in plant development and adaptation have been uncovered among crops. The miR159-GAMYB system is conserved between monocots and eudicots for the vegetative-to-reproductive phase transition. However, the overexpression of At*GAMYB* in *Arabidopsis* resulted in dramatic and pleiotropic developmental defects due to its broad action throughout the whole plant, whereas wheat Ta*GAMYB1*-overexpressing rice lines develop normally except for a delay in heading time and an increased number of primary branches[43]. Apart from their conserved roles in influencing the expression of meristem identity genes as in *Arabidopsis*, miR171–SCL modules are proposed to execute monocot specific functions in modulating vegetative phase transitions in barley by activating the miR156 pathway[59]. Moreover, miR398–SOD might act to positively regulate rice defence responses to blast fungus infection, but it appears to impede the effector-triggered hypersensitive reaction to powdery mildew fungus in barley[25,89]. Another extreme case shows that the conserved miR169–nuclear factor-YA (NF-YA) module plays opposite roles in regulating the response to drought stress in tomato and *Arabidopsis*[90,91]. Taken together, these findings suggest that the functional divergence of conserved miRNAs should be taken into consideration when they are chosen as candidates for crop improvement. In addition to conserved miRNAs, which are functionally important in crop development and adaptation, the majority of miRNAs present in any plant species appear to be species-specific[85]. These species-specific miRNAs usually have low but organ- or tissue-specific expression activity, show improper processing, and tend to evolve neutrally[85,92]. As a few of these species-specific miRNAs acquire solid and specialized functions and relating research remains in infancy[93,94], further functional analysis of them in crops is favourable to broaden our understanding on both miRNA evolution and miRNA-mediated regulation of complex agronomic traits and will also provide valuable resources for crop improvement.

Integration of miRNA knowledge to crop improvement

The discovery of miRNAs as the natural pivotal regulators of gene expression has led to the development and application of several miRNA-derived approaches for crop improvement. One simple and straightforward approach is to modulate agronomic traits by constitutively overexpressing a specific miRNA. For instance, overexpression of the rice-specific miRNA osa-miR7695, which downregulates expression of an alternatively spliced transcript of *Natural resistance-associated macrophage protein 6* (*Nramp6*), confers resistance

to infection by the fungal pathogen *Magnaporthe oryzae*[95], whereas tomato plants overexpressing sly-miR169c display reduced stomatal opening, decreased transpiration rate, lower leaf water loss and enhanced drought tolerance[90]. In cases in which miRNAs function as negative regulators of the traits of interest, overexpressing a miRNA-resistant form of the target that escapes cleavage by its miRNA or using an artificial miRNA–target mimic that inhibits the activity of a given miRNA might be effective strategies for improving crop traits[96]. Additionally, the new layer of gene expression regulation mediated by miRNAs raises the possibility of developing artificial miRNAs to specifically and effectively silence any given genes, which should have broad applicability for crop trait modulation[97]. The details of the development and application of the aforementioned miRNA-based strategies in molecular breeding for crop improvement have been reviewed extensively[10,98]. For completeness, we mainly discuss the integration of the knowledge of miRNAs with current crop improvement strategies from three aspects.

Exploitation of natural variation in miRNAs and their targets. At present and in the near future, conventional and molecular marker-assisted selection and pyramiding of natural genetic variation remain the most commonly used breeding programmes in practice. Although many plant miRNAs are evolutionarily conserved, miRNA loci, as with protein-coding genes, could also be targets of domestication selection and play crucial roles in crop domestication and improvement[99,100]. Indeed, sequence polymorphisms, especially single-nucleotide polymorphisms (SNPs) in their genes, widely exist and may affect miRNA biogenesis and function via modifying transcriptional patterns or miRNA–target interactions[7,99]. For instance, a GG/AA polymorphism in the terminal loop region of the osa-miR2923a precursor was found to be significantly associated with grain length and japonica/indica rice seed type differentiation[101]. A naturally occurring shortening of the 3′-untranslated region (UTR) polyadenylation tail of the osa-miR156h precursor transcript increases the abundance of osa-miR156h, resulting in reduced plant height, enhanced lodging resistance, increased tiller numbers and increased rice grain yield[102]. On the other hand, natural variation within miRNA-binding sites has also been observed to give rise to remarkable phenotypic changes. In barley, a synonymous nucleotide substitution at the miR172-targeting site in Hv*AP2* suppresses hvu-miR172-guided Hv*AP2* mRNA cleavage, producing the cleistogamous phenotype[36]. In wheat, an SNP in the 3′-half of the miR172-binding site in the AP2-like domestication gene *Q* is assumed to have had a role in wheat inflorescence architecture formation and domestication[33]. In rice, plant lines of certain varieties such as Shaoniejing and Aikawa 1 have a single nucleotide change from C to A at the osa-miR156-targeting site in Os*SPL14*, resonating with an ideal plant architecture as described before[13,15]. Moreover, a rare natural variant of Os*GRF4* with a two-base-pair substitution mutation in the osa-miR396-binding site impedes the osa-miR396-directed regulation of Os*GRF4*, and was shown to have enormous potential in breeding high-yield rice[49,50]. Collectively, all these findings show that natural variants of miRNAs and their respective target genes are valuable sources of beneficial traits for crop improvement.

Utilization of miRNAs in hybrid crop breeding. Hybrid breeding is a rapid, highly efficient and popular breeding strategy used to boost crop yields and enhance yield stability via systematically exploiting hybrid vigour, a complex phenomenon also called as heterosis in which the F_1 hybrid progeny exhibits better agronomic performance than its homozygous parental lines in terms of yield and/or adaptation to environmental stresses. Although heterosis is extensively utilized in agriculture, the underlying molecular mechanisms remain largely elusive despite extensive investigation. Genome-wide approaches to analyse heterosis have identified changes in miRNA expression profiles in hybrids of rice, maize and other species, indicating the potential contributions of miRNA-mediated regulatory networks to heterosis[103–106]. Generally, most of the identified miRNAs are expressed non-additively and tend to be downregulated in hybrids compared to their parental lines[103–105]. Global downregulation of miRNA expression in hybrids could lead to the enhanced expression of important target genes, which might contribute to the superior phenotypic performance of hybrid lines. For instance, zma-miR164 was suppressed significantly in the elite maize hybrid Xundan 20, which indicated that one of its target genes, *NAC1*, might be upregulated, and led to enhancement of auxin and gibberellin signalling, resulting in the elongation of internodes[103]. In wheat, non-additive repression of grass-specific miRNAs, such as tae-miR9009 and tae-miR5200, may contribute to the robustness of nascent allohexaploid wheat in disease resistance by upregulation of *R* gene analogues and flowering-related adaptations by upregulation of *FLOWERING LOCUS T* (*FT*) homologues, respectively[104]. Notably, a recent study revealed that blocking osa-miR396, 1 of 17 miRNAs that is significantly differentially expressed between the widely cultivated three-line hybrid, Yuetai-A/9311, and either of its parents, Yuetai-A and 9311, significantly increases rice grain yield by shaping inflorescence architecture through the direct induction of the Os*GRF6* gene[51]. This finding provides an indicative case for establishing an intrinsic connection between miRNAs and heterosis for specific traits. Building more such connections will help crop breeders efficiently select the best combinations of parent inbred lines for generating commercially available F_1 hybrids. Furthermore, exploitation and flexible manipulation of miRNA–target modules, such as osa-miR2118-*PMS1T*[74], which play vital roles in anther/pollen development and genic male sterility occurrence, or others that are involved in the formation of cytoplasmic male sterility[107,108], will facilitate the development of male sterile lines and the production of more valuable hybrids for agricultural production.

Modification of miRNAs or their targets via CRISPR–Cas9 technology. CRISPR–Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) is a recently developed breakthrough technology for genome editing[109]. Using specifically designed single-guide RNAs (sgRNAs), Cas9 can be directed to almost any desired sequence in the genome, generating DNA double-strand breaks that usually result in gene silence due to the introduction of small random insertions or deletions at the target site during DNA repair via error-prone, non-homologous end joining (NHEJ)[110]. Over the past few years, although CRISPR–Cas9-mediated single or multiple protein-coding gene knockouts have been successfully performed in many plants[110], no literature is available on CRISPR–Cas9 editing of non-coding RNA genes, including miRNA and lncRNA genes, in plants. As many miRNAs are transcribed from multiple loci, manipulation of a certain loci may not result in an intended functional change. In addition to this, using CRISPR–Cas9 to knock down or knock out miRNA genes, especially when it is located in introns of protein-coding genes, is thought to be complicated due to the limited design space for targeting non-coding genes without affecting nearby or host genes[111]. Most recently, the successful application of two kinds of derivative approaches to achieve gene/base replacement in rice would defuse the above embarrassment to some extent and, more importantly, broadens vastly the scope of CRISPR–Cas9 application for crop improvement. The first approach is to use one or a pair of sgRNAs that target adjacent introns and a donor DNA template that includes the same pair of sgRNA sites to implement intron-mediated site-specific gene insertion or replacement via NHEJ[112]. The second approach is based on the use of fusions of a nicked Cas9 with only the Asp10Ala mutation and a cytidine deaminase enzyme, which retains the ability to be programmed with a guide RNA without the induction of double-stranded DNA breaks, and mediates the direct conversion of

cytidine to uridine, thereby resulting in C-to-T (or G-to-A) substitutions within a window of approximately five nucleotides[110,113,114]. As many agriculturally important traits in crops are conferred by point mutations in miRNAs or their respective target genes, as described in the previous two sections[13,33,36,49,74,101], similar successes in efficient and precise gene/base replacement in these target loci will probably be achieved in the near future.

Conclusions and perspectives

Hitherto, the biological significance of many miRNA–target modules from diverse crop species has been revealed. It is now abundantly clear that, in addition to the fundamental role of miRNAs in the suppression of target gene expression, miRNAs in crops have more complicated and diverse functions in the complex regulatory networks mentioned above. A deeper understanding of the function of miRNAs in these networks will not only broaden our understanding of plant biological processes, but will also probably allow breeders to choose appropriate targets for modulating agriculturally important traits. One of the most important challenges in the future will be to functionally identify additional miRNA–target modules in such networks. As functional studies focus on conserved miRNAs, information regarding the essential roles of many species- and tissue-specific miRNAs is extremely limited. Moreover, the involvement of miRNAs in several agriculturally important traits such as heat tolerance and seed dormancy has largely remained a mystery in crops. Notably, more than half of the 65 miRNAs come from rice and tomato, and very few miRNAs from other crops have been investigated in detail (Table 1). For these crops, much more work is needed to decipher the preliminary miRNA-mediated regulatory network.

To date, only a few genetic variations in miRNAs and target genes have been functionally characterized, and these were found to be valuable for crop trait enhancement due to their regulatory function in gene expression and/or gene product activity[13,36,49,102]. Further exploitation and functional verification of genetic variation in other miRNAs and target genes are necessary and will provide more-natural trait-enhancing alleles for crop breeding and shed new light on their evolution and functions during crop domestication.

The heritable and desirable changes in crops created by CRISPR–Cas9 in a more rapid, efficient and precise manner are essentially identical to the natural variation or mutations selected via conventional breeding. Moreover, the exogenous DNA sequences used for gene editing are easy to remove in subsequent segregating generations of the edited plants, turning them into transgene-free lines that are favourable for dispelling public safety concerns over transgenic crops. These features make CRISPR–Cas9 an attractive genome-editing strategy and a widely adopted crop improvement technology. Therefore, it is urgent and important to determine the editing feasibility and efficiency of current CRISPR–Cas9 systems or to develop other CRISPR–Cas9-derived systems that target plant miRNA loci and miRNA-binding sites. A highly efficient miRNA-related genome-editing system will significantly expand the range of miRNA applications for crop breeding. In addition to knockout or base replacement of a given miRNA or target gene, such a system may be able to replace the seed region of a miRNA to change its targets or to replace the miRNA promoter region with an artificially designed promoter to manipulate its expression profile. Genome editing is also expected to be able to knock in a miRNA-binding site to a specific gene, placing it under the regulation of the given miRNA. For instance, knocking in a target site of a rice blast fungal elicitor-specific responsive miRNA in an appropriate site (for example, 3′-UTR) of *Pi21*, a gene that negatively regulates blast resistance of rice[115], will make it possible to obtain pathogen-triggered disease resistance in rice. Whether it is integrated into a specific target-gene-mediated regulatory pathway, or integrated into a complex regulatory network, miRNA has huge potential to serve as a powerful lever to improve one or several traits. Undoubtedly, further identification, validation and analysis of more miRNA modules, as well as further development of miRNA-based breeding strategies, will bring the application of miRNAs for crop improvement a more promising future.

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J.T. and C.C. wrote this article.

Additional information

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Competing interests

The authors declare no competing financial interests.