The Evolution and Functional Roles of \( \text{miR408} \) and Its Targets in Plants

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Abstract: MicroRNA408 (\( \text{miR408} \)) is an ancient and highly conserved miRNA, which is involved in the regulation of plant growth, development and stress response. However, previous research results on the evolution and functional roles of \( \text{miR408} \) and its targets are relatively scattered, and there is a lack of a systematic comparison and comprehensive summary of the detailed evolutionary pathways and regulatory mechanisms of \( \text{miR408} \) and its targets in plants. Here, we analyzed the evolutionary pathway of \( \text{miR408} \) in plants, and summarized the functions of \( \text{miR408} \) and its targets in regulating plant growth and development and plant responses to various abiotic and biotic stresses. The evolutionary analysis shows that \( \text{miR408} \) is an ancient and highly conserved microRNA, which is widely distributed in different plants. \( \text{miR408} \) regulates the growth and development of different plants by down-regulating its targets, encoding blue copper (Cu) proteins, and by transporting Cu to plastocyanin (PC), which affects photosynthesis and ultimately promotes grain yield. In addition, \( \text{miR408} \) improves tolerance to stress by down-regulating target genes and enhancing cellular antioxidants, thereby increasing the antioxidant capacity of plants. This review expands and promotes an in-depth understanding of the evolutionary and regulatory roles of \( \text{miR408} \) and its targets in plants.

Keywords: evolution; \( \text{miR408} \); plant development; stress response; yield

1. Introduction

MicroRNAs (miRNAs) are small noncoding RNAs with 20 to 24 nucleotides (nt) that regulate gene expression post-transcriptionally through base pairing with their complementary mRNA targets [1]. MicroRNA (MIR) genes encoding miRNAs are transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II), and then these pri-miRNAs are processed into miRNA/miRNA* duplexes by the RNase III family enzyme DICER-LIKE1 (DCL1). Subsequently, these duplexes are 2′-O-methylated by the methyltransferase HUA ENHANCER1 (HEN1) at the 3′ end. One strand of the duplex is incorporated into the RISC. MicroRNA/target complementarity guides RISC to target the mRNA slicing [2,3]. Lin-4 was the first miRNA discovered in the nematode Caenorhabditis elegans in 1993 [4]. A second miRNA named let-7 was discovered in 2000 [5]. Since then, thousands of miRNAs have been quickly discovered in animals and plants, and their regulatory roles in many biological processes have been covered in [1,6–10]. In plants, miRNAs are observed to be closely related to transcriptional gene silencing (TGS), indicating that miRNAs play significant roles in plants [9].

MicroRNAs are the second most abundant plant sRNA class [11]. Many plant miRNA families have been conserved for a long time in the evolution of land plants [12]. Axtell and
Bartel [12] demonstrated that at least eight miRNA families remained basically unchanged before the emergence of seed plants, and at least two families (miRl60 and miR390) have remained unchanged since the last common ancestor of mosses and flowering plants. Based on a combination of experiments and computations, Fattash et al. [13] identified 48 novel miRNAs, including miR408, miR536, miR535, etc., in the moss Physcomitrella patens. Furthermore, the results of RNA degradome analysis revealed that miR408 was conserved between liverworts and other land plants [14]. In addition, miR408 was highly conserved in Oryza sativa, Medicago and Populus [10]. Nowadays, miR408 has been widely treated as an ancient and conserved miRNA in plants.

Although miR408 is ancient and conserved in plants, our understanding of the functions of miR408 and its targets has just begun. Since miR408 was first discovered in Arabidopsis thaliana, the crucial roles of miR408 in plants have been verified in many studies [10], and miR408 has been regarded as important regulator of plant vegetative growth and reproductive development. Because it can effectively avoid the artifacts caused by overexpression mutants, loss-of-function mutants are widely used to study the function of miRNAs. A loss-of-function mutant is regarded as an important prerequisite for understanding the biological function of miRNAs [15]. A recent study showed that the function loss of miR408 negatively regulated light-dependent seed germination [16]. Zou et al. [17] proved that the function loss of miR408 positively regulated the synthesis of Salvianolic acid B (SalB) and rosmarinic acid (RA) in Salvia miltiorrhiza. T-DNA insertion mutants of miR408 were used to explore how miR408 influences copper- and iron-dependent metabolism [18]. In addition, the function loss of miR408 also caused expression disorders of other miRNAs [19]. In contrast, overexpression mutants have been widely used to discover the function of the target genes of miR408. Overexpression of miR408, by down-regulating the target genes, can obviously promote numerous development processes, including the growth and development of leaves, flowers, roots, and seeds [20–26]. Similarly, overexpression of miR408 can also significantly increase photosynthetic activity, biomass and seed yield by down-regulating target genes [20–25]. The overexpression of miR408 also altered heading times in Triticum aestivum [24]. In addition, miR408 responds to various environmental factors, such as cold, salinity, drought, nutritional deprivation, oxidation, osmotic stress, heavy metals, lipopolysaccharide (LPS) and disease stress [22,27–32].

Although previous research has shown that miRNAs play multiple important regulatory roles, there are relatively few studies on the evolutionary pathway. In addition, previous studies either only used overexpression mutants or only loss-of-function mutants, lacking a systematic analysis of the functional effects of miR408 and its target genes.

2. Evolution Analysis of the miR408 Family in Plants

miR408 is an ancient and conserved miRNA in plants, a thorough and systematic understanding of its evolution process will help us understand and master the evolution and functional roles of miR408 in plants more comprehensively. Here, we analyzed the evolution of miR408 using bioinformatics methods.

2.1. Origin and Distribution of miR408 Family Members in Plants

As a typical multifunctional miRNA, miR408 has been annotated in more than 30 different plants [24]. In order to reveal the origin and distribution of miR408 family members in plants, we downloaded all miR408 precursor and mature sequences of known plants from the miRbase database (version 22.0) (http://www.mirbase.org/) (accessed on 31 October 2021) (Tables S1 and S2), and carried out biological classification. The results are summarized in Tables S3 and S4.

The sequence length of miR408 varies in different plants. As shown in Tables S1 and S2, the sequence length of the miR408 precursor varies greatly, ranging from 71 to 286 nt, while the sequence length of mature miR408 is between 20 and 22 nt, with little difference. In addition, the length of the precursor and mature miR408 are different in the same species.
Usually, the length of a miRNA precursor is about 70–80 nt, and it is very likely that the two arms will produce miRNA separately, so -5p/-3p will be added after the name to distinguish them. As shown in Tables S1 and S2, the miR408 family members include 55 precursor sequences and 72 mature sequences. Some miR408 precursors are produced separately in the two arms. For instance, the miR408 precursors produce miRNA in the two arms of *A. thaliana*, *O. sativa*, *Populus trichocarpa*, etc., separately. Among the 72 miR408 mature bodies, 17 were from the 3p arm and 17 were from the 5p arm. These 34 miR408 mature bodies came from 17 different angiosperms. In other miR408 mature bodies from bryophytes, ferns and gymnosperms, whether they were from the 3p arm or the 5p arm was not distinguished.

miR408 is widely distributed in different plants. As shown in Tables S3 and S4, miR408 family members have been identified in 40 different plants, and distributed in 18 orders and 22 families, most of which belong to Rosales and Gramineae. Among these 40 plants, dicots are the most common, and there are only 10 monocots. In addition, different species have different amounts of miR408. For instance, *Physcomitrella patens* has two precursors, while *Selaginella moellendorfii* has only one precursor. Furthermore, *A. thaliana* has two mature miR408, while *Saccharum officinarum* has five mature miR408. In addition, these 40 kinds of plants also include 1 species of bryophyte, 1 species of pteridophyte, 2 species of gymnosperms and 36 species of angiosperms. Interestingly, since bryophytes represent the first green plants to colonize terrestrial plants [33], it is clear that the miR408 family is an ancient and widely distributed family of miRNAs and bryophytes may be the evolutionary ancestors of the miR408 family and since then have been strongly conserved.

### 2.2. Evolution Characteristics of the miR408 Family in Plants

In order to reveal the evolutionary characteristics of miR408 family member in plants, we respectively constructed the phylogenetic trees of the precursor sequences and the mature sequences of the miR408 family in Figures 1 and 2.

Species specificity is an important factor affecting the evolution of precursors in the miR408 family. As shown in Figure 1, except for a few species, the precursors of miR408 family members of most species have a closer aggregation at the family level, such as Gramineae and Leguminosae in monocots and Solanaceae in dicots. However, the precursors of miR408 family members in Brassicaceae are more dispersed, and they are distributed on different small branches.

Sequence conservation is one of the main factors affecting the evolution of the mature miR408 family. Pan et al. [20] showed that, except for the majority of the 5′ nucleotides, the mature miR408 sequence found in moss, angiosperms and gymnosperm species was unchanged. The results in Figure 2 show that the members of the mature sequences of miR408 are obviously divided into two branches. The sequences of miR408-5p members are more concentrated and form a single branch, and the miR408-3p members are gathered in another branch. In addition, as shown in Table S2, most of the miR408-3p mature member sequences are the same except for the first and last bases. However, most miR408-5p mature member sequences are quite specific, only a few mature miR408-5p family members of the same family or species have relatively conserved sequences. Therefore, the members of miR408-5p were quite specific, while the members of miR408-3p were conservative.

In addition, among 72 mature miR408s, 46 are distributed in dicots, 21 are distributed in monocots, 2 are distributed in coniferopsida, 2 are distributed in musci, and only 1 is distributed in lycopodiinae. In summary, ancient low-grade plants contain fewer miR408 species, and higher dicotyledonous plants contain more miR408 species, indicating that miR408 evolved gradually from bryophytes and played important roles in the evolution of plants to dicotyledonous plants. It may have been the emergence and change of miR408 that led to the evolution of various higher plants species. Members of the miR408 family coexist with conservatism and specificity in evolution, providing a valuable reference for exploring the various biological functions of the miR408 family in plants.
Figure 1. Phylogenetic relationships between all the 55 miR408 precursors (MIR408) from different plants. The colored branch shows the same family species. The dendrogram has been constructed using IQtree software by the Maximum-Likelihood method with 5000 bootstraps. According to IQ-Tree model evaluation, the best fitting model of the miR408 precursor sequence is TVMe+I+G4, and the best model of the miR408 mature sequence is JC. The Bryophyta Physcomitrella patens is the root of the evolutionary tree. aly, Arabidopsis lyrata; aqc, Aquilegia caerulea; ata, Aegilops tauschii; ath, Arabidopsis thaliana; ahy, Arachis hypogaea; bdi, Brachypodium distachyon; bra, Brassica rapa; cca, Cynara cardunculus; cme, Cucumis melo; cpa, Carica papaya; csi, Citrus sinensis; dpr, Digitalis purpurea; gma, Glycine max; hbr, Hevea brasiliensis; lja, Lotus japonicus; lus, Linum usitatissimum; mdm, Malus domestica; mes, Manihot esculenta; mtr, Medicago truncatula; nta, Nicotiana tabacum; osa, Oryza sativa; pta, Pinus taeda; ptc, Populus trichocarpa; ppt, Physcomitrella patens; rco, Ricinus communis; sbi, Sorghum bicolor; sof, Saccharum officinarum; stu, Solanum tuberosum; smo, Selaginella moellendorffii; tae, Triticum aestivum; vvi, Vitis vinifera; vun, Vigna unguiculata; zma, Zea mays; ssp, Saccharum ssp; vca, Vriesea carinata; sly, Solanum lycopersicum; ive, Fragaria vesca; pab, Picea abies; cas, Camelina sativa; aof, Asparagus officinalis.
Figure 2. Phylogenetic relationships between all 72 mature miR408 sequences (miR408) from different plants. The yellow circle and blue square represent the miR408 members formed from the 3p arm and the 5p arm, respectively. The dendrogram is constructed using IQtree software through the Maximum-Likelihood method using 5000 bootstraps. According to IQ-Tree model evaluation, the best fitting model of the miR408 mature sequence is JC. The bryophyta Physcomitrella patens is the root of the evolutionary tree.

3. Overview of the miR408-Regulated Target Genes

With the help of advanced genomic technologies such as next-generation sequencing, more and more miR408 target genes have been screened, some of which have been identified using the modified 5′ rapid amplification of cDNA ends (5′-RACE) method. Here, we have summarized the types and functions of the target genes regulated by miR408 in Figure 3. We have also sorted out and summarized the species distribution and splicing sites of these target genes in Table S5.
Figure 3. The function of miR408-regulated target genes in plants. Yellow, target genes; green, functions of the target genes.

3.1. Types of the miR408-Regulated Target Genes

Due to the imperfect complementarity required for binding, miRNAs may target hundreds of mRNAs, but only a small part of these interactions have been experimentally verified [34]. Although miR408 has different target genes in different species, all these target genes encode Cu proteins [25].

At present, the 5′-RACE technique has been used to validate miRNA targets, as cleavage occurs opposite, and at around the tenth position from the 5′-end of the miRNA [35]. Many miR408 target genes, such as plantacyanin (PLC), uclacyanin (UCL), cupredoxin, laccase...
(LAC), chemocyanin-like protein gene, timing of CAB expression 1 (TaToC1), DDB1/SK12/helY-like DEAD-box Helicase (DSHCT), 3-ketoacyl-CoA synthase 4 gene (KCS) and galacturonosyltransferase 7-like gene (GAUT) have been characterized in different plants and well verified experimentally using a modified version of the 5′-RACE polymerase chain reaction (PCR) (Table S5) [20–22,24,25,32,36–44]. In addition, miR408 also targets other genes encoding Cu proteins, such as copper P1B-ATPase in M. truncatula [42].

3.2. Functions of miR408-Regulated Target Genes

Many of the target genes regulated by miR408 belong to the Phytocyanins (PCs). PCs are blue Cu proteins that bind only one Cu ion. It is not only related to the activity of the electron carrier, but also has a great influence on the growth and stress resistance of plants [25]. Previous studies have shown that PCs are involved in a variety of plant activities, including pollen tube germination [45,46], anther pollination [45,46], reproductive potential determination [47] and apical bud organ development [48]. Most PCs are chimeric arabinogalactan proteins (AGPs), which are subdivided into the stellacyanins, PLCs, and UCLs based on their predicted Cu-binding ligands (His, Cys, His and Met/Gln) [49].

PLC is the most widely studied miR408 target gene in plants. It encodes a small extracellular matrix (ECM) protein, which belongs to the ancient plant-specific phytocyanin family [50], and the complementary sites are conserved in Spinacia oleracea, Zea mays, Cicer arietinum and O. sativa [21]. The first PLC with functional characteristics is Lily chemocyanin, which is secreted from the pistil and acts as an external signal to regulate pollen tube reorientation in vitro [45]. In addition, plant PLC is involved in the process of seed germination, electron transfer and shuttling between proteins, directing pollen tube growth, stress response, Cu homeostasis, plant reproduction, intercellular signal transduction, lignin formation, and so on [16,22,45,46,51,52].

UCL is a member of the ancient plant-specific phytocyanin family, which is a subfamily of blue Cu proteins [52,53]. Nersissian et al. [52] reported that UCLs were a mature form of chimeric proteins, consisting of a Cu-binding domain and a domain resembling a cell wall structural protein. Interestingly, recent research showed that UCL8 is localized in the cytoplasm and is associated with photosynthesis and grain yield, but many other phytocyanins are localized in the plasma membrane [25]. Further analysis showed that OsUCL8 is related to pollen tube growth, pollination and the seed setting rate of O. sativa [26]. In addition, UCL acts as a shuttle for electron transfer between proteins [52].

LAC is another widely studied target of miR408, and was first discovered in 1883 [54]. LAC belongs to the blue oxidases and also belongs to the superfamily of multicopper oxidases (MCOs). It contains a group of enzymes including many proteins with different substrate specificities and multiple biological functions [25,54]. LAC combines four Cu ions and is widely distributed in Anacardiaceae and other higher plants, including Pinus taeda, Acer pseudoplatanus, Nicotiana tabacum, P. trichocarpus, Liriodendron tulipifera, Lolium perenne, A. thaliana, Z. mays, O. sativa, Sacchorum officinarum, Brassica napus, and Brachypodium distachyon [54]. In addition, it is reported that plant LACs are involved in the process of lignin synthesis [55,56], the maintenance of cell wall structure and integrity [53], responses to environmental stresses [57], wound healing [36], iron metabolism [58] and the polymerization of phenolic compounds [54].

In addition to PLC, LAC and UCL, miR408 also targets many other genes. Plastocyanin, cupredoxin and chemocyanin are considered to be the electron transfer shuttles between proteins [51,59,60]. TaTOC1s are members of the phytocyanin family [24]. Zhao et al. [24] reported that miR408 regulates the heading time of T. aestivum by controlling the transcription of TaTOC1s. In addition, TaTOC1s are involved in the clock’s evening loop [61]. IbGAUT plays a role in the cell wall structure for wound healing [62]. A chemocyanin-like protein gene (TaCLP1), plays positive roles in the response of wheat to high-salinity, heavy Cu stress and stripe rust [32].
3.3. Species Distribution and Splicing Sites of miR408-Regulated Target Genes

Plant miRNA-mediated transcript cleavage is usually located at the 10th and 11th nucleotides of the 5′ end of miRNA [63]. The cleavage site may also lie outside the targeted complementary region, but this probability is very low [41]. miR408 has multiple prediction targets, and miR408-mediated transcript cleavage is mainly located at the tenth and eleventh nucleotides (Table S5). In some cases, miR408-mediated transcript cleavage site may also be located outside the target complementary region (Table S5).

In *A. thaliana*, miR408-mediated transcript cleavage is relatively complex. The cleavage site is not only located at the 9th–10th nucleotides, 10th–11th nucleotides, 11th–12th nucleotides, but also outside the complementary region. For instance, miR408 cleaved LAC3 at the 9th-10th nucleotides, and cleaved PLC, LAC12 and UCC2 at the 10th–11th nucleotides at the 5′ end [16,20,36]. Abdel-Ghany and Pilon [36] showed that miR408 cleaves LAC12 at the 11th–12th and 14th–15th nucleotides at the 5′ end, respectively [37]. In addition, they demonstrated that miR408 targeting LAC13 has two cleavage sites, the most typical cleavage site is the 10th–11th nucleotide site, and another cleavage site is outside the complementary region [36]. But Song et al. [21] and Zhang et al. [37] reported that miR408 cleaves LAC13 at the 10th–11th nucleotides and 14th–15th nucleotides from the 5′ end, respectively. For PLC, miR408 cleaves at the 9th-10th nucleotides and 12th–13th nucleotides from the 5′ end [21].

In *O. sativa*, miR408-mediated transcript cleavage is different from that in *A. thaliana*. For instance, miR408 cleaves UCL8 and LAC3-like1 at the 10th–11th nucleotides at the 5′ end [46]. The PLC-like1, UCL30, and DSHCT cleavage sites guided by miR408 are all located at the 11th–12th nucleotides from the 5′ end [25]. In addition, miR408 targets PLC with three cleavage sites, which are at the 10th–11th, 14th–15th, and 17th–18th nucleotides [38].

In *N. tabacum*, miR408 cleaves UCC-like1 at the 10th–11th and 11th–12th nucleotides at the 5′ end [20]. In contrast to the interaction between UCC-like1 and miR408, the PLC-like1 and LAC12-like1 cleavage sites guided by miR408 are located at the 10th-11th nucleotides of the 5′ end [20]. In *T. aestivum*, miR408 cleaves TaTOC-A1, TaTOC-B1, and TaTOC-D1 at the 10th-11th nucleotides from the 5′ end [20]. In *S. miltiorrhiza*, miR408 cleaves LAC3 at the 7th–8th and 10th–11th nucleotides from the 5′ end [41]. In contrast to the interaction between LAC3 and miR408, the LAC18 cleavage site guided by miR408 is located at the 10th–11th nucleotides from the 5′ end [20].

In *Ipomoea batatas*, miR408 cleaves *IbPCL* (*Plantacyanin*) directly at the 10th nucleotide from the 5′ end [44]. In contrast to the interaction between *IbPCL* and miR408, *IbKCS* (3-ketocycl-CoA synthase 4) is a typical target gene of miR408 that contains two mismatches within the miR408 sequence. The *IbKCS* cleavage sites guided by miR408 are located at the 5th and 10th nucleotides from the 5′ end, which are non-canonical and canonical cutting sites, respectively [44]. In *S. officinarum*, the *Diphenol oxidase laccase* cleavage sites guided by miR408 are located at the second to third nucleotides from the 5′ end [64]. In *M. truncatula*, miR408 cleaved PCL at the 10th–11th nucleotides from the 5′ end [41]. In *Dimocarpus longan*, the miR408-3p targeting *DiLAC12* has two cleavage sites, the most typical cleavage site is the 10th site AAG/AGG, and the other cleavage site is outside the complementary region [65].

All these results indicate that miR408 cleavage sites for the target gene are different in different species, and the transcript cleavage is usually located at the 10th and 11th nucleotides from the 5′ end of miR408. In addition, the cleavage site of miR408 on the target gene may be different in the same species.

4. The Roles of miR408 and Its Targets in Plant Development

The expression of miR408 is significantly affected by a variety of developmental and environmental conditions; however, its biological function is unknown [21]. It has been shown that miR408 is important for regulating the growth and development of plant leaves, flowers, seeds and roots. Two models for plant biology research are *O. sativa* and *A. thaliana*. We summarized the roles of miR408 and its targets in *O. sativa* and *A. thaliana* in Figure 4. In addition, we also summarized the main regulatory roles of miR408 and its targets in plant growth and development (Table S6).
4.1. Leaf Development

The shape of leaves is spectacularly diverse. miR408 and its targets are involved in the regulation of the development of leaves, including leaf size, petiole length and leaf flag angle [20–24]. In *A. thaliana*, the function loss of miR408 decreased leaf size [21], while the overexpression of miR408 significantly increased leaf size [21–23]. The overexpression of miR408 in *N. tabacum* also significantly increased leaf size [20]. In addition, the overexpression of miR408 in *A. thaliana* significantly increased the petiole length [21]. The mechanism by which overexpression of miR408 induces increased leaf area and petiole length is summarized as follows: miR408 down-regulates target genes, which belong to PLC, LAC, UCL and cupredoxin (Figure 4q) [20], and then directly and/or indirectly modulates cytoplasmic growth and/or gibberellic acid (GA) biosynthesis, which promotes the increase of leaf size by enhancing cell expansion (Figure 4r,s) [21].

Physiological and morphological traits of the flag leaf angle play important roles in determining crop grain yield and biomass. The overexpression of miR408 decreased the flag leaf angle in *O. sativa* and *T. aestivum* [20,24]. TaTOC1s (TaTOC-A1, TaTOC-B1, and TaTOC-D1), the targets of miR408, have been identified in *T. aestivum* [24]. The function loss of TaTOC1s significantly decreased the flag leaf angle in *T. aestivum*. Further research discovered that...
miR408 regulates the angle of flag leaf by down-regulating its target genes, which belong to PLC, LAC and TOC1 family members (Figure 4i) [20,24].

In summary, miR408 plays crucial roles in leaf development by regulating target genes encoding Cu binding proteins (including PLC, LAC, UCL and TaTOC1s family members) (Figure 4 and Table S6). In addition, these target genes directly and/or indirectly regulate cytoplasmic growth and/or GA biosynthesis to promote cell expansion and increase leaf size and petiole length.

4.2. Flower Development

Flowers are important organs of plants, and the timing of flowering is not only an interesting topic in developmental biology, but it also plays a significant role in agriculture for its effects on the maturation time of seeds [24]. It has been confirmed that flower development, including flower initiation and flowering morphogenesis, is regulated by miR408 and its targets (TaTOC1s, UCL8 and LAC) [24–26].

The correct timing of the switch from vegetative to reproductive growth, namely the floral transition, is critical for reproductive success in flowering plants. In T. aestivum, the overexpression of miR408 and the function loss of TaTOC1s (target genes of miR408) caused an earlier and shorter heading time than in the wild type (WT), but the overexpression of TaTOC1s led to later flowering [24]. These results suggested that the miR408 functions in the wheat by mediating TaTOC1s expression. Further analysis revealed that TaFT1, the key gene involved in the flowering time, was up-regulated in both miR408 overexpressing and TaTOC1s knockdown transgenic wheat, while the expression of TaCO1 was reduced [25]. All these results suggested that the miR408 regulation of wheat heading time and duration are dependent on its TaTOC1s mRNA cleavage activity and this enhances the expression of TaFT1 [24–26].

miR408 and its target gene UCL8 also regulate flowering morphogenesis, including the flower size, pollen wall, stigmas, anthers, peduncle diameter and peduncle vascular bundles. The overexpression of miR408 significantly increased the flower size in A. thaliana [21]. In O. sativa, the overexpression of miR408 significantly increased the diameter of the peduncle, but with the function loss of UCL8 it formed vigorous pollen with a higher germination rate [26]. In contrast, the overexpression of UCL8 decreased the diameter of the peduncle in the main panicle, caused a thinner pollen intine, smaller stigmas, twisted anthers and an abnormal germination of pollen grains [25].

Further research discovered that miR408 regulates flower size (Figure 4k), the pollen wall (Figure 4g), the peduncle diameter and peduncle vascular bundles (Figure 4h) by down-regulating its target genes PLC, LAC and UCL8 [21,25,26]. Among these, UCL8 regulates the production of vitamin B1 (VB1) components through the interaction with OsPKIWI, and leads to significant irregularities in pollen tube growth and pollination, which then affects the seed setting rate in O. sativa (Figure 4f) [26].

All these results indicate that miR408 and its targets play active roles in flower development, the control of flowering morphogenesis, pollen germination and pollen tube growth, and ultimately affect the fertility and seed setting rate. Therefore, miR408 and its targets are closely related to seed development and grain yield in plants.

4.3. Seed Development and Grain Yield

As an important complex agronomic trait, grain yield is dependent upon numerous factors such as seed size, seed weight and seed number.

Seed size is one of the most important factors affecting grain yield in plants. In A. thaliana and N. tabacum, the overexpression of miR408 significantly increased seed size and seed weight compared with the WT [20,22]. In O. sativa, the overexpression of miR408 and the function loss of UCL8 also increased the seed size and 1000-grain weight, which was manifested by a substantial increase in seed length and width and a slight increase in thickness [20]. In contrast, the overexpression of UCL8 in O. sativa significantly decreased
the 1000-grain weight [25]. These observations indicate that the seed size regulated by miR408 is highly conserved both in monocots and dicots.

Silique length is an important yield trait, which positively correlates with the number of seeds per silique and seed weight. In *A. thaliana*, the overexpression of miR408 resulted in a significant increase in silique length and increased grain yield, but the overexpression of PLC and LAC13 resulted in a significant decrease in silique length and a decreased grain yield [21]. In addition, the overexpression of PLC and LAC13 also significantly increased the number of siliques in the inflorescence and increased grain yield [21]. Interestingly, the overexpression of miR408 in *A. thaliana* also significantly reduced the seed coat color and root length (Figure 4p), and the mechanism remains unclear.

The number of grains per panicle is largely associated with the morphologies of the panicle, including the number of primary and secondary branches in the panicle. In *O. sativa*, miR408 positively regulates grain yield by increasing the panicle branches and grain number through cleaving its target gene UCL8 [25]. In *O. sativa*, the overexpression of miR408 or the function loss of UCL8 significantly increased the number of panicle branches and the number of grains per panicle, but the overexpression of UCL8 dramatically reduced the number of panicle branches. In contrast, the overexpression of UCL8 dramatically reduced the effective number of grains per panicle [25]. In conclusion, miR408 negatively regulates UCL8 to affect seed size, seed weight and seed number to ultimately control grain yield in *O. sativa*. Similarly, miR408 negatively regulates PLC and LAC13 to affect seed size, seed weight, silique length and seed number to ultimately control grain yield in *A. thaliana*.

Further research discovered that miR408 regulates seed size (Figure 4c,n), seed weight (Figure 4a,o), silique length (Figure 4s), panicle branching (Figure 4e) and grain number (Figure 4d) by regulating its targets and plastocyanin (PC). The target genes of miR408 encode the blue Cu proteins associated with electron carrier activity in photosynthesis, which affects Cu homeostasis in the plant cell. Similarly, PC can transfer electrons from the cytochrome b6f complex to the photosystem I in photosynthesis [66]. miR408 down-regulates these target genes and transports Cu to PC, thereby affecting photosynthesis and ultimately controlling yield-related traits, and promoting grain yield (Figure 4a,b,m).

4.4. Seed Germination

miR408 not only controls seed morphogenesis, but also regulates seed germination [16]. The GA/abscisic acid (ABA) ratio is critical for seed germination. In *A. thaliana*, the overexpression of miR408 or the function loss of PLC or PIF1 increased GA but decreased ABA content. In contrast, the function loss of miR408 or overexpression of PIF1 decreased GA and maintained ABA content. Further research demonstrated that PIF1 binds to the miR408 promoter and represses the accumulation of miR408, which then silences PLC after transcription, thereby forming a PIF1–miR408–PLC repression cascade. In addition, the PIF1–miR408–PLC cascade regulates germination by modulating the ratio of GA/ABA [16].

5. Functional Roles of miR408 and Its Targets in Response to Stresses

Biotic and abiotic stresses greatly affect the growth and development of plants. In order to cope with these challenges, plants have evolved complex strategies to deal with these unfavorable environments and minimize damage as much as possible, so as to produce adaptive responses through physiological and morphological changes [63,67]. In recent years, an increasing number of studies have shown that in addition to regulating plant growth and development, miR408 is also stress responsive in many plant species. Among the identified roles of miR408 and its targets in response to biotic and abiotic stress, some have been verified by performing overexpression, T-DNA insertion, and RNAi experimentation. Here, we have summarized the regulating roles and modified expression of miR408 in response to biotic and abiotic stress (Tables 1 and 2).
Table 1. Roles of miR408 and its targets in abiotic and biotic stress in plants.

| Stresses       | Species                        | miRNA       | Target Genes                                | References |
|---------------|--------------------------------|-------------|---------------------------------------------|------------|
| ABIOTIC       |                                |             | Plastocyanin, Copper ion binding protein    | [68]       |
| Mild drought  | Prunus persica                 | miR408      | Plastocyanin                                |            |
|               | Prunus dulcis                  | miR408      | Plantacyanin                                |            |
| Severe drought| Interspecific Prunus persica--| miR408      | Plantacyanin                                |            |
|               | Armeniaca Prunus dulcis        |             |                                             |            |
| Drought       | Convolvulaceae (tolerant wild  | miR408      | Plantacyanin, plastocyanin-like domain,     | [39,71]    |
|               | Ipomoea campanulata)           |             | containing proteins                         |            |
|               | Convolvulaceae (sensitive      | miR408      |                                             | [69]       |
|               | Cultivated Jacquemontia       |             |                                             |            |
|               | Pentantha)                     |             |                                             |            |
|               | Oryza sativa (tolerant Cultivars N22, dana) | miR408-3p | Plastocyanin                                | [70]       |
|               | Oryza sativa (sensitive cultures PB1, IR64) | miR408-3p | Plastocyanin                                | [70]       |
|               | Triticum aestivum              | miR408      | Plastocyanin                                | [72]       |
|               |                               | miR408      |                                             |            |
|               | Lycopersicon esculentum (sensitive) | miR408a-3p | Plastocyanin                                | [73]       |
|               | L.esculentum var.              | miR408      |                                             |            |
|               | Cerasiforme                    | miR408      |                                             |            |
|               | Pisum sativum                 | miR408      |                                             | [72]       |
|               | Oryza sativa                  | miR408      | plastocyanin genes (Os01g53880 and Os09g20930) | [74]       |
|               | Arabidopsis thaliana          | miR408      | Cupredoxin                                  | [22]       |
|               |                               |             | Plantacyanin                                |            |
|               |                               |             | LAC3                                        |            |
|               | Cicer arietinum                | miR408      |                                             | [75]       |
| Water         | Ipomoea campanulata (tolerant) | miR408      | Plantacyanin                                | [70]       |
| deficit       | Jacquemontia Pentantha        | miR408      |                                             |            |
|               | (sensitive)                   | miR408      |                                             |            |
|               | Pisum sativum                 | miR408      | P1B-ATPase                                  | [76]       |
|               | Medicago truncatula           | miR408      | Plantacyanin                                | [42]       |
| Dehydration   | Hordeum vulgare               | miR408      | Plantacyanin                                | [77]       |
| Salinity      | Oryza sativa                  | miR408      | DSHCT Plastocyanin-like                     | [40]       |
|               | Salvia miltiorrhiza            | miR408      |                                             | [78]       |
|                |                               |             | Cupredoxin                                  |            |
|                |                               |             | Plantacyanin                                |            |
|                |                               |             | LAC3                                        |            |
| Cold          | Arabidopsis thaliana          | miR408      | Cupredoxin                                  | [22]       |
| Osmotic       |                               |             | Plantacyanin                                |            |
|                |                               |             | LAC3                                        |            |
| Oxidative     |                               | miR408      | Cupredoxin                                  | [22]       |
### Table 1. Cont.

| Stresses                             | Species               | miRNA   | Target Genes                        | References |
|--------------------------------------|-----------------------|---------|-------------------------------------|------------|
| Low dose rate γ-ray                  | *Oryza sativa*        | miR408  | DSHCT                               | [27]       |
| High dose rate γ-ray                 |                       | miR408  |                                     |            |
| **Nutrient deprivation**             |                       |         |                                     |            |
| Nitrogen deficiency                  | *Zea mays*            | miR408  | Cupredoxin                           | [79,80]    |
|                                     |                       |         | SOD1A                               |            |
| Carbon, nitrogen, and sulfur deficiency |                     | miR408  | LAC3                                | [28]       |
|                                     |                       |         | LAC12                               |            |
|                                     |                       |         | Plantacyanin                         |            |
| Copper deficiency                    | *Arabidopsis thaliana*| miR408  | LAC3                                | [22,36]    |
|                                     |                       |         | LAC12                               |            |
|                                     |                       |         | LAC13                               |            |
|                                     |                       |         | Plantacyanin                         |            |
| Iron deficiency                      | *Arabidopsis thaliana*| miR408  | LAC3                                | [18]       |
|                                     |                       |         | LAC12                               |            |
|                                     |                       |         | LAC13                               |            |
|                                     |                       |         | Plantacyanin                         |            |
| Boron deficiency                     |                       | miR408  | Plantacyanin                         | [82]       |
|                                     |                       |         | LAC3                                |            |
|                                     |                       |         | LAC13                               |            |
| Excess fertilizer                    | *Linum usitatissimum* | miR408  | No                                  | [83]       |
| Phosphorus deficiency                | *Arabidopsis thaliana*| miR408  | No                                  | [84]       |
|                                     | *Glycine max*         | miR408  | No                                  | [85]       |
|                                     | *Triticum aestivum*   | miR408  | No                                  | [86]       |
| Zinc deficiency                      | *Sorghum bicolor*     | miR408  | Plantacyanin                         | [87]       |
| Potassium deficiency                 | *Triticum aestivum*   | miR408  | No                                  | [88]       |
| **Heavy metals**                     |                       |         |                                     |            |
| Cadmium                              | *Triticum aestivum*   | miR408  | Chemocyanin-like protein             | [89]       |
|                                     | (12h, leaves)         |         |                                     |            |
|                                     | miR408 (24h, leaves)  |         |                                     |            |
|                                     | miR408 (6, 12, 24 and 48h, roots) |         |                                     |            |
|                                     | *Oryza sativa*        | miR408  | hZIP, ERE, MYB, SnRK1, and HSPs     | [29]       |
| Arsenate and arsenite                | *Oryza sativa*        | miR408  | No                                  | [30]       |
| Manganese                            | *Phaseolus vulgaris*  | miR408  | No                                  | [90]       |
| BIOTIC                               | *Puccinia graminis*   | miR408  | Plantacyanin                         | [91]       |
| P. triticici                          | *Triticum aestivum*   | miR408  |                                     |            |
| Rhizoctonia solani                   | *Oryza sativa*        | miR408  | No                                  | [92]       |
| Lipopolysaccharide                   | *Arabidopsis thaliana*| miR408  | Plantacyanin                         | [31]       |

Green: increased miR408 abundance; red: decreased miR408 abundance. DSHCT, DOB1/SK12/helY-like DEAD-box Helicase; LAC, laccase.
Table 2. Modified expression of plant miR408 and its targets in response to abiotic and biotic stress.

| Stresses | Species         | Approach          | Phenotype                                                                                                                                       | References |
|----------|-----------------|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Cold     | Arabidopsis thaliana | miR408 overexpression | More tolerant to cold tolerant, Lower electrolyte leakage, Higher Fv/Fm value, Lower MDA, Higher chlorophyll, Enhanced cold sensitivity, Enhanced electrolyte leakage, Lower Fv/Fm value, MDA were elevated, Lower chlorophyll | [22]       |
|          | Oryza sativa     | miR408 overexpression | Lower ion leakage, Enhanced SOD activity, Enhanced proline content                                                                         | [74]       |
| Salinity | Arabidopsis thaliana | miR408 overexpression | Root development was better, Lower ROS, Inhibited root development, Enhanced ROS, Improved root growth, Significantly higher fresh weights, Higher germination rates, Lower growth inhibition, Reduced ROS Accumulation, Lower accumulations of H₂O₂, Higher POD, SOD, CAT activities, Lower levels of O₂⁻ and H₂O₂ | [22]       |
|          | Triticum aestivum | TaCLP1 overexpression | More tolerant to oxidative stress, Higher biomass, Higher total root length, Increased CSD1, CSD2, CCS1, GST-U25 and SAP12 Reduced ROS Accumulation, Reduced ROS, Higher POD, SOD, CAT activities, Lower levels of O₂⁻ and H₂O₂ | [32]       |
| Oxidative| Arabidopsis thaliana | miR408 overexpression | More tolerant to oxidative stress, Higher biomass, Higher total root length, Increased CSD1, CSD2, CCS1, GST-U25 and SAP12 Reduced ROS Accumulation, Reduced ROS, Higher POD, SOD, CAT activities, Lower levels of O₂⁻ and H₂O₂ | [22]       |
|          | T-DNA miR408 mutant |                 | Retarded growth, Lower FW, Death rate lower, Higher height, Death rate lower, High stress tolerance, Lower height, Increasing number of leaves, BHLH23 down-regulated, ERF/AP reduced expression, DREB2A/1A genes were increased, Rd17 and Rd29a were increased, Rd22 up-regulated Narrower leaves of similar length, Less vein number, More closely folded leaves, Greener, Higher chlorophyll, More bristle-like trichomes on the leaf surface, Relatively smaller and more sunken stomata, Lower stomatal conductance, Less tissue damage, Higher leaf RWC, Lower water loss rate, Higher leaf electrolyte leakage (EL), Higher activities of SOD, CAT and POD, Lower accumulation of H₂O₂ and MDA | [75] [93] |
Table 2. Cont.

| Stresses          | Species              | Approach                                      | Phenotype                                      | References |
|-------------------|----------------------|-----------------------------------------------|-----------------------------------------------|------------|
| Osmotic           | Arabidopsis thaliana | miR408 overexpression                         | Retarded growth, Lower FW                      | [22]       |
|                   |                      | T-DNA miR408 mutant                           | Grow better, Higher height                     |            |
| Copper deficiency | Triticum aestivum    | TaCLP1 overexpression                         | Higher tolerance to copper deficiency          | [32]       |
|                   |                      | T-DNA miR408 mutant                           | Lower chlorophyll-a content, Lower lignin content, LAC3, LAC12, LAC13 and plantacyanin (ARP1) up-regulated, Lower lignification-related genes (F6'H1', CCR1, B-GLU23, LAC17), Lower hLH39, Higher phenoloxidase activity, Higher H2O2 levels, Lower MCO3 and CAT2, Lower chlorophyll-a content, Lower lignin content, LAC3, LAC12, LAC13 and plantacyanin (ARP1) down-regulated, lignification-related genes (F6'H1', B-GLU23, LAC17) significantly increased, Lower FIT, Lower H2O2 levels, lower MCO3 and CAT2, Higher Copper levels, miR408 expression decreased, LAC3, LAC12, LAC13 and plantacyanin (ARP1) up-regulated, Lower FRO2, FRO3, IRT1, COPT2, Lower phenoloxidase activity, Lower ferroxidase activity, Higher H2O2 levels, Higher lignin staining of vascular cylinder, Higher lignin staining of vascular cylinder, Lignification of the vascular bundles was more evident in the aerial part | [18]       |
| Iron deficiency   | Arabidopsis thaliana | Wild type                                     | Decreased stripe rust resistance               | [94]       |
|                   |                      | Pre-OsmiR408 and OsmiR408 overexpression      |                                               |            |
|                   |                      | RNAi TaCLP1 mutant                            |                                               |            |

The same color symbol represents the same abiotic or biotic stress. MDA, malondialdehyde; ROS, Redox and reactive oxygen species; POD, peroxidase; SOD, superoxide dismutase; CAT, catalase; SODs, Cu/Zn superoxide dismutase CSD1 and CSD2; CCS1, copper chaperone; GST-U25, glutathione-S-transferase; SAP12, stress-associated regulatory protein fresh weight; BHLH23, transcription factor; ERF/ AP2, Apteala2/Ethylene Response Factors; DREB, Dehydration-Responsive Element Binding Protein; bHLH39 and FIT transcriptional activators; MCO3, ascorbate oxidase; CAT2, catalase; FRO2, Ferric Reductase 2; IRT1, Iron Regulated Transporter 1.

5.1. Cold Stress

Low temperature is one of the most common environmental stresses which seriously affects the growth and development of plants [95]. Sunkar and Zhu [10] first discovered miRNAs in response to cold stress by Small RNA Sequencing. Then Ma et al. [22] verified the roles of miR408 and its targets in response to cold stress through an overexpression technology.

In A. thaliana under cold stress, the expression of miR408 increased under low temperature stress, while the expression of its target genes cupredoxin and LAC3 decreased. In addition, the overexpression of miR408 increased the survival rate, maximum quantum efficiency (Fv/Fm) values and chlorophyll fluorescence, while it decreased malondialdehyde (MDA), electrolyte leakage and luminescence [22]. In addition, an increased expression of CSD1 and CSD2 were also observed [22].

In O. sativa, the expression of pre-OsmiR408 and OsmiR408 increased under cold stress. As expected, the expression of its target genes (plastocyanin genes, Os01g53880 and Os09g29390) were down-regulated by cold treatment. The overexpression of miR408 in O. sativa improved cold tolerance and it exhibited better growth than the WT [74]. In addition, the overexpression of miR408 decreased ion leakage, and increased SOD.
activity and proline content [74]. Cuproproteins (such as cupredoxin) that are reduced with the overexpression of miR408, may increase the endogenous availability of Cu for other cuproproteins (such as Cu/Zn superoxide dismutases (CSDs)), and these cuproproteins are involved in the mediation response to abiotic stress [22]. It has also been proved that the CBF-independent nuclear protein, Tolerant to Chilling and Freezing 1 (TCF1), which is related to Blue-Cu-Binding Protein (BCB), can regulate lignin biosynthesis in A. thaliana [96]. The reduction of lignin deposition in the cell wall increases its permeability and enhances its elasticity, allowing it to adapt to the growth of ice crystals, which may reduce or prevent damage to dehydrated cells and cell walls [96]. In loss-of-function TCF1 mutants and BCB knockouts there was a decreased lignin content and increased freezing tolerance.

All the results of these studies clearly show that miR408 helps plants improve their tolerance to cold stress by regulating the genes related to Cu homeostasis, oxidative stress, and lignin biosynthesis.

5.2. Salinity Stress

Salinity stress is one of the main environmental stresses that limit plant growth and productivity [97]. Previous studies have discovered that miR408 plays functional roles in A. thaliana, S. miltiorrhiza, O. sativa and T. aestivum under salinity stress [22,32,40,78].

In A. thaliana under salinity stress, the expression of miR408 was up-regulated, while its target gene cupredoxin was down-regulated. In addition, the overexpression of miR408 reduced oxidative stress by increasing the length of the main root and lateral root and increasing the expression of CSD1 and CSD2, thereby enhancing the salinity tolerance of A. thaliana [22]. Similarly, when S. miltiorrhiza was exposed to salinity, the expression of miR408 was up-regulated [78]. The overexpression of miR408 promoted seed germination and reduced the accumulation of ROS under salinity stress by increasing the expression of antioxidative enzyme genes, i.e., NbSOD, NbPOD, and NbCAT and increasing their enzyme activities [78].

However, in O. sativa under salinity stress, the expression of miR408 was down-regulated, while the expression of its target genes helicase DSHCT and plastocyanin-like were up-regulated [40]. TaCLP1, the gene target of miR408, plays a positive role in the response of T. aestivum to high-salinity. The overexpression of TaCLP1 in yeast significantly increased cell growth under high salinity, but the mechanism remains unclear [32].

In summary, although increasing the expression of antioxidant enzyme genes is considered to be one of miR408’s strategies to improve plant salinity tolerance, many other mechanisms still need to be further studied. In particular, the up-regulation or down-regulation of miR408 expression in different plants under salinity stress is inconsistent. The reason for these opposite results is not clear, indicating that different plants have different salt stress resistance mechanisms.

5.3. Drought Stress

Drought stress is a major constraint to agricultural productivity worldwide [98]. Using miRNA high-throughput sequencing, researchers have identified that the expression of miR408 was significantly up-regulated or down-regulated under drought stress, as shown in Table 1. Further analysis showed that the up-regulation or down-regulation of miR408 expression depends on the species, variety, tissue distribution and the degree of drought stress.

The response of miR408 to drought stress varies in different species of plants. When exposed to drought stress, the expression of miR408 was up-regulated in Prunus dulcis [68], Prunus persica [68], C. arietinum [75], Hordeum vulgare [77], T. aestivum [72], Ipomoea campanulata [69], Jacquemontia Pentantha [70] and M. truncatula [42]. On the contrary, when exposed to drought stress, the expression of miR408 was down-regulated in P. dulcis [68], Lycopersicon esculentum [73], Pisum sativum [76], A. thaliana [22] and I. Campanulata [70].

The response of miR408 to drought stress also depends on cultivars. When convolvulacee was exposed to drought stress, the expression of miR408 was up-regulated in the
tolerant wild Ipomoea campanulata, and down-regulated in the sensitive cultivated J. Pentantha [70]. Contrarily, another study illustrated that under drought stress, the expression of miR408 was down-regulated in tolerant wild I. campanulate, and up-regulated in sensitive cultivated J. Pentantha [69]. In rice, during drought the expression of miR408 was up-regulated in the fag leaves of the tolerant cultivars, Nagina 22 (N22) and Vandana, but down-regulated in the sensitive cultivars, Pusa Basmati 1 (PB1) and IR64 [39,71].

In addition, the responses of miR408 to drought stress also depends on different tissues. In T. aestivum exposed to drought stress, miR408 was down-regulated in root, but up-regulated in leaf [72].

The responses of miR408 to drought stress also depends on the degree of drought. In P. persica, the expression of miR408 was up-regulated under mild drought stress, while it was down-regulated under severe drought stress [68].

The functional roles of miR408 and its target genes in response to drought stress are shown in Table 2. When A. thaliana was exposed to drought stress, the expression of miR408 was up-regulated, while the expression of its target genes cupredoxin, PLC and LAC3 were down-regulated. In addition, the overexpression of miR408 increased the sensitivity to drought stress in A. thaliana [22]. On the contrary, a loss-of-function mutant of miR408 had a decreased death rate and increased height under drought stress [22].

In L. perenne, the overexpression of miR408 changed the leaf morphology and increased the antioxidant capacity, which in turn improved the ability of plants to cope with drought stress [93].

In C. arietinum, the overexpression of miR408 showed a high ability to provide drought tolerance [75]. It is known that Element Binding Protein 1A (DREB1A) and Dehydration-Responsive Element Binding Protein 2A (DREB2A) are transcription factors involved in drought tolerance, and the overexpression of miR408 increased the expression of DREB2A, DREB1A and Rd17/29A. Moreover, the expression of PLC (a target of miR408) was significantly decreased with the overexpression of miR408. Further research demonstrated that the overexpression of miR408 leading to PLC transcript repression, caused the regulation of DREB and other drought responsive genes. All these results confirm that the DREB1A and DREB2A transcription factors and their target genes RD17 and RD29 provide plant tolerance allowing them to survive under drought stress [75].

In O. sativa, the overexpression of miR408 decreased drought tolerance. In addition, after recovering from drought stress, the overexpression of miR408 decreased the survival rate and increased water loss rates [74]. When exposed to drought stress, the tolerant cultivar exhibited a higher amount of transcription factor SPL9, higher ROS, lower Cu transporters (COPT1, COPT2, COPT4, COPT5, COPT7 and ATPases) and lower internal Cu levels than the sensitive cultivars PB1 and IR64 [71]. Further research showed that miR408 was regulated by OsSPL9 and directly interacted with the promoter via the GTAC motif [71]. In addition, miR408 was up-regulated to decrease the transcript levels of plastocyanin-like in an ABA and Ca2+-dependent manner [39].

In total, the mechanisms of miR408 in improving drought tolerance are summarized as follows (Figure 5): (1) Under drought stress, the internal Cu content of flag leaves decreases, which may be due to differences in Cu transporters. Then, lower Cu leads to the up-regulation of miR408 via the transcription factor OsSPL9. Elevated miR408 levels down-regulate target genes encoding Cu-containing proteins (PLC), and then cause cellular Cu to be preferentially distributed to PCs, thereby maintain PC activity and electron transport. The electron transport pathway is one of the main ROS production pathways. Finally, ROS promotes the closure of stomata in response to drought stress (Figure 5a) [39,71]. (2) miR408 enhances the activities of SOD, CAT and POD, leading to a decrease in ROS accumulation, thereby improving drought tolerance (Figure 5b) [93]. (3) miR408 down-regulates the target gene PLC, leading to an accumulation of Cu levels, and DREB levels increase when Cu is excessive. Subsequently, the DREB2A target gene RD29B is down-regulated, while the DREB1A and DREB2A transcription factor target genes RD17 and RD29A are up-regulated.
Finally, DREB1A and DREB2A and their target genes RD17 and RD29 provide plant survival tolerance under drought stress [75].

Figure 5. The potential regulatory mechanism model of miR408 and its targets in plant tolerance to drought stress. In brief, drought stress decreases internal copper (Cu) levels by inhibiting the expression of Cu transporters. Then a lower concentration of Cu activates the expression of SQUAMOSA promoter binding protein (SBP)-like 9 (SPL9) and then up-regulates miR408. a, miR408 targets several Cu containing proteins. These targets include several member proteins containing plantacyanins/plastocyanin-like domains. miR408 down-regulates target genes, saves Cu for plastocyanin, and maintains a stable plastocyanin level, thereby increasing reactive oxygen species (ROS). At the same time, high concentrations of ROS promote stomata closure, thereby enhancing drought tolerance [75]. b, miR408 leads to higher activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), and leads to lower hydrogen peroxide (H$_2$O$_2$) and malondialdehyde (MDA). c, miR408 down-regulates the target gene PLC and then increases Cu accumulation. Then the DREB level is increased when there is excess Cu. Subsequently, the target gene RD29B of DREB2A is down-regulated. The target genes of DREB1A and DREB2A, RD17 and RD29A are up-regulated. All these results confirm that DREB1A and DREB2A transcription factors and their target genes RD17 and RD29 provide plants with tolerance for survival under drought stress. Dark blue: transcription factors; yellow: miR408 target genes.
5.4. Nutrient Deficiency

miR408 plays important roles in regulating the response of plants to nutrient deficiencies. Nitrogen (N), phosphorus (P) and potassium (K) are the three most important macronutrients required for plant growth and crop yield. The expression of miR408 was repressed in A. thaliana under N starvation, while the expression of its target genes LAC and PLC increased [81]. Similarly, the expression of miR408 was repressed in A. thaliana under C, N, and S deficiency, while the expression of its target genes LAC3 and LAC13 increased [28]. When Z. mays was exposed to N deficiency, the expression of miR408 was down-regulated, while the expression of its target gene cupredoxin and SOD1A were up-regulated [79,80]. miR408 has different regulatory roles in different species of plants under P deficiency. In A. thaliana and Glycine max, miR408 was significantly up-regulated under P deficiency [84,85]. However, in Taestivum, miR408 was down-regulated under P deficiency stress [86]. When Taestivum was exposed to K deficiency, the expression of miR408 was down-regulated [88]. In Linum usitatissimum, the expression of miR408 was induced under excessive fertilization treatments [83]. In Citrus sinensis, B-deficiency induced the down-regulation of miR408 and the up-regulation of PLC, LAC3 and LAC13 [82].

Although nutrients are essential elements required for the completion of plant growth and development [99], high concentrations of some mineral nutrients are harmful to plants. Cu plays essential roles in photosynthesis, oxidation responses and other physiological processes in plants [100]. Cu deficiency leads to an abridged photosynthetic electron transport, decreased pigment synthesis, and a disintegrated thylakoid membrane [101]. miR408 interacts with genes encoding Cu-containing proteins such as cupredoxin, PLC, UCL and LAC, all of which belong to the phytocyanin family [52,60,102,103]. In addition, an increasing number of studies have shown that miR408 is responsive to Cu deficiency in plants. It is inferred that miR408 is the main regulator in Cu homeostasis.

In A. thaliana, the expression of miR408 was up-regulated under Cu deficiency, while the expression of the target genes cupredoxin, PLC, UCL, LAC3, LAC12 and LAC13 were down-regulated [22,36]. The regulation of miR408 under Cu deficiency was mediated by SQUAMOSA promoter binding protein-like 7 (SPL7) by binding to the GTAC motifs of the miR408 promoter [23]. Another identified transacting factor that directly mediates miR408 expression via promoter area binding is HY5 (elongated hypocotyl 5). In A. thaliana, by directly interacting with the promoter through GTAC and G-box motifs, SPL7 and HY5 act in coordination to transcriptionally regulate miR408, which results in the differential expression of miR408 and its target genes in response to changing light and Cu conditions [37]. This regulation makes sense, since in the light required for photosynthetic growth, which has a high demand for Cu, HY5 mediates gene expression.

In addition to the regulation of lignin biosynthesis, miR408 may regulate the iron deficiency response in complex ways [18]. In A. thaliana, when exposed to Fe deficiency, the expression of miR408 was up-regulated in the WT, while its target genes LAC3, LAC12, LAC13 and plantacyanin (ARP) were down-regulated. The overexpression of miR408 leads to enhanced Fe sensitivity [18]. It increased the expression of lignification-related genes F6′H1′, B-GLU23 and LAC17 under Fe deficiency, and at the same time decreased the expression BHLH29 and its target genes LAC3, LAC12, LAC13 and PLC.

Zinc (Zn) is an essential micronutrient in plants and miR408 is an important regulator of the response to Zn deficiency in plants. In Sorghum bicolor, miR408 in leaves was significantly up-regulated by Zn deficiency, while the expression of its target gene plantacyanin was decreased [87]. However, the regulatory mechanism of miR408 in Zn deficiency still needs further study.

5.5. Other Abiotic Stresses

The expression of miR408 not only changes under cold, salinity, drought and nutrient stresses, but also alters under methyl viologen (MV)-induced oxidative, osmotic and heavy metals stresses.
miR408 plays a positive function in enhancing the oxidation tolerance of *A. thaliana*. The expression of miR408 in *A. thaliana* was up-regulated under oxidative treatment, while its target gene PLC was repressed [22]. In addition, when exposed to MV-induced oxidative stress, the overexpression of miR408 increased the biomass and root length, while the function loss of miR408 decreased the root length in *A. thaliana*. Further research showed that elevated levels of miR408 can increase tolerance to MV-induced oxidative stress by reducing the accumulation of ROS [22]. In contrast, the overexpression of miR408 reduces the tolerance of plants to osmotic stresses. Under osmotic stress, in *A. thaliana* the overexpression of miR408 showed growth retardation and a decreased fresh weight, while the function loss of miR408 showed better growth and an increased fresh weight [22].

Interestingly, when exposed to metal stresses, the expression of miR408 changes with time and varies in different organs. In *T. aestivum* under cadmium (Cd) stress, the expression of miR408 in the leaves was down-regulated, reaching their lowest expression level at 12 h, and then reaching a relatively high expression level at 24 h. The expression of its target gene, *chemocyanin-like protein*, was down-regulated in leaves, gradually decreasing at 6 h, increasing at 12 h, and then slightly decreasing at 24 h. In addition, miR408 was up-regulated and maintained extremely high expression levels at all time points in the roots (6, 12, 24 and 48 h). The expression of *chemocyanin-like protein* in roots was up-regulated after 24 and 48 h, reaching the highest expression at 48 h [89]. However, in *O. sativa*, miR408 family members were up-regulated under Cd stress and play important roles in reducing Cd accumulation by down-regulating the expression of candidate genes, such as *bZIP*, *ERF*, *MYB*, *SnRK1* and *HSPs* [29].

In addition, using miRNA arrays for miRNA analysis, researchers found that miR408 family members were up-regulated in *O. sativa* under Arsenic (As) (III) and As (V) stress [30]. miR408 was strongly inhibited in *Phaseolus vulgaris* leaves or roots under manganese (Mn) toxicity [90]. In addition to responding to Cu deficiency, miR408 also responds to high Cu stress [43].

Overall, the results demonstrate a significant involvement of miR408 in abiotic stress responses, emphasizing the central function of miR408 in plant survival [22], but the detailed mechanisms are still poorly understood and further studies are needed.

### 5.6. Biotic Stress

miR408 not only plays important roles in the tolerance to various abiotic stresses, but also plays vital roles in the tolerance to biotic stresses such as LPS stress, *Puccinia striiformis f. sp. Triticum* (Pst) infection, *Puccinia graminis*sp*tritici* infection and *Rhizoctonia solani* infection. When *A. thaliana* was exposed to LPS stress, the expression of miR408 was up-regulated, while its target gene PLC was inhibited [31]. Wheat stripe rust caused by Pst is one of the most destructive diseases in the world, and it can greatly reduce or even completely destroy *T. aestivum* yields in epidemic years [32]. TaCLP1 is the target of miR408, which positively regulate stripe rust resistance in *T. aestivum* [32]. Compared with the WT control, the function loss of TaCLP1 in *T. aestivum* decreased its resistance to stripe rust, showing some fungal sporulation, larger necrotic areas and a larger hyphal length [32]. *Puccinia graminis*sp*tritici* is a rust fungus causing serious disease to *T. aestivum* [91]. Gupta et al. [94] reported that miR408 in *T. aestivum* was involved in the defense response to stem rust infection. During Pst infection, the up-regulation of miR408 may trigger the lignin biosynthetic pathway as a hypersensitive response (HR) response to prevent epidermis rupture. Sheath blight disease, caused by *Rhizoctonia solani*, is one of the most destructive *O. sativa* diseases, resulting in an estimated yield loss of up to 50% [92]. In *O. Sativa*, a late responsive miR408 might be involved in *R. solani* infection in rice plants. In addition, the expression of miR408 was up-regulated under *R. solani* infection [104].

As shown in Tables 1 and 2, miR408 is involved in various biotic and abiotic stresses, but the detailed regulation mechanism of miR408s in response to stresses has not yet been fully explored. Existing studies have shown that miR408 has a great relationship with the antioxidant system, but no studies have shown that miR408 directly regulates the
expression of specific genes involved in the antioxidative system. Ma et al. [22] proposed that increasing the expression of miR408 can reduce the level of ROS and regulate the target genes encoding Cu-containing proteins, thereby increasing the endogenous availability of Cu for other Cu proteins involved in response to abiotic and biotic stress. In addition, Zhang et al. [37] showed that miR408 is an important component part of the HY5–SPL7 gene network and can mediate a coordinated response to light and Cu. Peñarrubia et al. [105] proposed that the interaction between Cu homeostasis and plant hormones (mainly ABA and ethylene) is related to abiotic stress. Therefore, the mechanism of miR408 in response to stress may go beyond a simple interaction between a single miR408 and its target genes. More attention should be paid to the interaction of miR408 and target genes with other transcription factors and hormones.

6. Conclusions

Evolutionary analysis shows that the miR408 family is an ancient and widely distributed miRNA family, and bryophytes might be the evolutionary ancestors of the miR408 family. There are fewer miR408 species in ancient and lower plants, and more miR408 species in higher dicotyledonous plants, indicating that miR408 evolved gradually from bryophytes and may play an important role in the evolution of plants to dicotyledonous plants. It may be that the emergence and changes of miR408 led to the evolution of various higher plants. A number of studies have shown that miR408 plays significant roles in regulating plant growth, development and stress response. miR408 affects the growth and development of plant leaves, flowers, seeds and roots by down-regulating the target genes encoding blue Cu proteins; by transporting Cu to PC, it regulates photosynthesis and ultimately promotes the increase of grain yield. In addition, miR408 can also enhance the content of antioxidants in cells, improve the antioxidant capacity of plants, and thereby improve the tolerance to various stresses. All these results greatly expand our understanding of the evolution and functional roles of miR408 and its targets in plants.

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