MiRNA-Encoded Peptide Discovery and Functions

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microRNAs (MiRNAs) are small endogenous single-stranded RNAs (20 to 22 nucleotides) that are involved in post-transcriptional gene silencing in eukaryotes. They allow the downregulation of target genes by specifically triggering the degradation of their messenger RNAs (mRNAs) or by inhibiting their translation. Most plant species have several hundred annotated miRNA genes. miRNA primary transcripts were recently shown to contain functional short Open Reading Frames producing regulatory peptides called miRNA-encoded Peptides (miPEPs).

miPEP plant animal

1. miRNA-Encoded Peptide Discovery

Peptides are known to be involved in many processes including developmental regulation, acclimation to abiotic stress, and defense against pathogens [1][2][3][4][5] (**Figure 1**). The majority of known regulatory peptides in plants are derived from precursor proteins [6]. However, peptides that are directly translated from short open reading frames (sORFs) have also been reported [1][3]. Among them, those located in the 5’ region of primary transcripts of miRNAs (pri-miRNAs), termed miRNA-encoded peptides (miPEPs), have recently received more attention [7][8][9]. Indeed, based on in-house and existing RACE-PCR-based annotations of pri-miRNAs of *M. truncatula* and *A. thaliana*, Lauressergues and colleagues (2015) performed an in silico analysis revealing the presence of at least one putative sORF in the 5’ region of *Mt*miR171b and *At*miR165a pri-miRNAs [10]. The functionality of these sORFs was validated for the first time in this research using *A. thaliana* and *M. truncatula* as model plants. Indeed, in both cases, the presence of endogenously expressed miPEPs was visualized by western blot and/or immunofluorescence using specific antibodies [10].

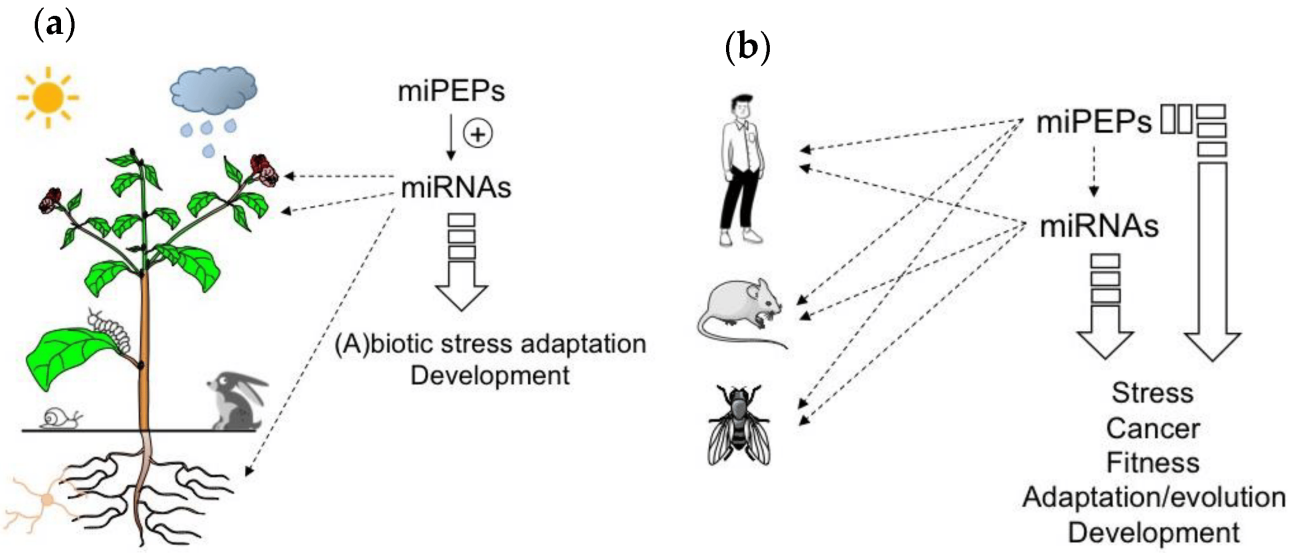

Figure 1. MicroRNA-encoded peptides (miPEPs) regulate many biological functions both in plants and animals. (**a**) The ability of plant miPEPs to positively regulate the expression of their respective pri-miRNAs is described for several miPEPs and plant species. (**b**) Conversely, in animals, the regulation of pri-miRNAs by miPEPs is less clear. MiPEPs frequently act independently.

Since their discovery, the existence of miPEPs has been extended to various pri-miRNAs in several plant species as listed in **Table 1** [11][12][13][14][15][16][17][18][19][20][21][22].

Table 1. List of miPEPs (and their embedded miRs) described in the literature (and miRbase) both in plants and animals.

Organism	MiPEP (miR)	MiPEP Size	In Vivo miPEP Detection	Effect on the Corresponding Pri-miRNA	Regulation of miRNA Targets	Regulated Biological Functions	Ref
Plants							
Arabidospsis thaliana	AtmiPEP165a (ath-miR165a)	18	GUS reporter gene expression and wb	Upregulation	Downregulation of *HD-ZIP III PHAVOLUTA, PHABOLUSA, REVOLUTA*	Stimulation of main root growth; Acceleration of the inflorescence stem appearance and of the flowering time; Inhibitory effect on total root growth	[10] [11] [12]
Arabidopsis thaliana	AtmiPEP858a (ath-amiR858a)	44	GUS reporter gene	Upregulation	Downregulation of *MYB* transcription	Flavonoid biosynthesis and plant development	[13]

Organism	MiPEP (miR)	MiPEP Size	In Vivo miPEP Detection	Effect on the Corresponding Pri-miRNA	Regulation of miRNA Targets	Regulated Biological Functions	Ref
			expression and wb		factor *AtMYB12*		
Arabidopsis thaliana	AtmiPEP164b (ath-miR164b)	29	N/A	Upregulation	Downregulation of *NAC1*, *NAC4*, *NAC5*, *CUC1* and *CUC2*	Inhibitory effect on total root growth	[12]
Arabidopsis thaliana	AtmiPEP397a (ath-miR397a)	7	N/A	Upregulation	Downregulation of *LAC2*, *LAC4* and *LAC17*	Stimulation of total root growth	[12]
Dimocarus Longan Lour	N/A	50	N/A	Upregulation	Downregulation of *HD-ZIP IIIATHB15*	Embryogenesis	[14]
Glycine max	GmmiPEP172c (gma-miR172c)	16	N/A	Upregulation	Downregulation of *AP2* transcription factor *NODULE NUMBER CONTROL 1*	Increase in nodule number	[15]
Lotus japonicus	LjmiPEP171b (lja-miR171b)	22	N/A	Upregulation	N/A	Increase in mycorrhization rate	[16]
Medicago truncatula	MtmiPEP171b (mtr-miR171b)	20	GUS reporter gene expression and wb	Upregulation	Upregulation of *GRAS* transcription factor *LOST MERISTEMS 1* (*LOM1*)	Reduction of lateral root development and increase in mycorrhization rate	[10] [16]
Medicago truncatula	MtmiPEP171a (mtr-miR171a)	10	N/A	N/A	Downregulation of *LOM1*	Decrease in mycorrhization rate	[16]
Medicago truncatula	MtmiPEP171c (mtr-miR171c)	7	N/A	N/A	Downregulation of *LOM1*	Decrease in mycorrhization rate	[16]
Medicago truncatula	MtmiPEP171d (mtr-miR171d)	6	N/A	N/A	Downregulation of *LOM1*	Decrease in mycorrhization rate	[16]
Medicago truncatula	MtmiPEP171e (mtr-miR171e)	23	N/A	N/A	Downregulation of *LOM1*	Decrease in mycorrhization	[16]

Organism	MiPEP (miR)	MiPEP Size	In Vivo miPEP Detection	Effect on the Corresponding Pri-miRNA	Regulation of miRNA Targets	Regulated Biological Functions	Ref
						rate	
Medicago truncatula	MtmiPEP171f (mtr-miR171f)	5	N/A	N/A	Downregulation of *LOM1*	Decrease in mycorrhization rate	[16]
Oryza sativa	OsmiPEP171i (osa-miR171i)	31	N/A	Upregulation	N/A	Increase in mycorrhization rate	[16]
Solanum lycopersicum	SlmiPEP171e (slymiR171e)	19	N/A	Upregulation	N/A	Increase in mycorrhization rate	[16]
Vitiis vinifera	VvimiPEP171d1 (vvi-MIR171d1 *)	7	GUS reporter gene expression	Upregulation	Downregulation of *scarecrow-like VvSCL27*	Adventitious root formation	[17]
Vitis vinifera	VvimiPEP164c (vvi-miR164c)	16	N/A	Upregulation	Downregulation of *VvMYBPA1* grapevine transcription factor	Inhibition of proanthocyanidin synthesis and stimulates anthocyanin accumulation	[18]
Vitis vinifera	VvimiPEP172b (vvi-miR172b)	16	N/A	Upregulation	Downregulation of *VvRAP2-7-1*	Increase in cold tolerance in grapevine	[19]
Vitis vinifera	VvimiPEP3635b (vvi-MIR3635b *)	11	N/A	Upregulation	Downregulation of *VvENT3*	Increase in cold tolerance in grapevine	[19]
Barbarea vulgaris	BvmiPEP164b (bv-miR164b *)	8	N/A	Upregulation	Downregulation of *NAC1*, *NAC4*, *NAC5*, *CUC1* and *CUC2*	Inhibitory effect on main root growth and foliar surface	[12]
Brassica oleacera	BomiPEP397a (bo-miR397a *)	10	N/A	Upregulation	Downregulation of *LAC2*, *LAC4* and *LAC17*	Stimulation of main root growth and foliar surface	[12]
Brassica rapa	BrmiPEP156a (br-miR156a)	33	TAMRA-labeled peptide	Upregulation	N/A	Moderate stimulation of main root growth	[20]

Organism	MiPEP (miR)	MiPEP Size	In Vivo miPEP Detection	Effect on the Corresponding Pri-miRNA	Regulation of miRNA Targets	Regulated Biological Functions	Ref
Animals							
Human	miPEP200a (hsa-miR-200a)	187	wb; HA fused peptide over-expressed in cells	No regulation	Inhibit the expression of vimentin in cancer cells	Inhibition of the migration of prostate cancer cells	[23] [24]
Human	miPEP200b (hsa-miR-200b)	54	wb; HA fused peptide over-expressed in cells	N/A	Inhibit the expression of vimentin in cancer cells	Inhibition of the migration of prostate cancer cells	[23]
Human	miPEP155 (hsa-miR-155)	17	EGFP-fused ORF	No regulation	No regulation	Suppression of autoimmune inflammation by modulating antigen presentation	[24] [25]
Human	miPEP497 (hsa-miR-497)	21	N/A	No regulation	No regulation	N/A	[24]
Human	miPEP22 (hsa-miR-22)	57	wb	N/A	N/A	Tumor suppressor	[26]
Human	miPEP133 (hsa-miR-34a)	133	wb	[25][27][28][29][30][31][32] Up-regulation	N/A	Increase in p53 transcriptional activity by disrupting mitochondrial function	[27] [10][13][17][33]
Human	MISTRAV or MOCCI (hsa-miR-147b)	83	Wb; Immuno-fluorescence of over-expressed peptide	[24][31][34] No regulation	N/A	Viral stress response, inflammation and immunity	[28] [29]
Drosophila melanogaster	MSAmiP (dme-miR-iab-8)	9 to 20	EGFP-fused ORF	No regulation	N/A	Involved in sperm competition	[30]
Drosophila	DmmiPEP8	71	wb	No regulation	No regulation	Wing size	[31]

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eed, the overexpression of *At*miPEP165a in a heterologous species (*Nicotiana benthamiana*), or the application of its synthetic version, increased the expression of both its corresponding pri-miRNA and the mature miRNA, and correlatively decreased the expression of miRNA target genes in *A. thaliana*. Similarly, the *M. truncatula* miPEP171b was able to increase its *Mt*pri-miR171b expression, suggesting that the function of miPEPs is conserved and not limited to a few species [10]. The positive effect of miPEPs on the accumulation of their

Organism	MiPEP (miR)	MiPEP Size	In Vivo miPEP Detection	Effect on the Corresponding Pri-miRNA	Regulation of miRNA Targets [10]	Regulated Biological Functions	Ref
melanogaster	(dme-miR- 8)					reduction	
Mus musculus	MmmiPEP31 (mmu-miR-31)	44	EGFP-fused ORF and wb	down-regulation	N/A	Suppression of EAE by promoting the differentiation of Treg cells	[32]

*: miR not present in miRbase.

A study performed on grapevine was recently published in this context [17]. MiRNA171 family members are known to target genes involved in the formation and development of roots in different plants [10][35]. Chen and colleagues found that *VviMIR171* gene members were specifically expressed during the formation and development of grapevine (*Vitis vinifera*) adventitious roots [17]. When *Vvi*miR171d was overexpressed in *A. thaliana*, the plants displayed shorter primary roots, higher lateral root density, and earlier adventitious root development compared to wild-type (WT) plants. An in silico analysis predicted three putative sORFs in the 5' region of *Vvi*pri-miRNA171d. Their respective transient overexpression in grape tissue culture plantlets showed that only the first pri-miRNA sORF enabled an increase in *Vvi*miR171d expression. In addition, when a construct containing the region from the *Vvi*miR171d promoter to the ATG start site of this sORF fused to the GUS gene was expressed in *N. benthamiana* leaves or grape tissue culture plantlets, GUS activity was observed. These data demonstrate that this sORF encodes a peptide, which was named *Vvi*miPEP171d1. Similar to what was previously described, when grape tissue culture plantlets were treated with synthetic *Vvi*miPEP171d1, *Vvi*miR171d expression specifically increased while the expression of miRNA target genes correlatively decreased. In addition, when grape plantlets were grown on a medium containing synthetic *Vvi*miPEP171d1, the number of adventitious roots significantly increased, indicating that the miPEP is able to regulate the formation and development of grapevine adventitious roots. This property appears specific to grapevines since *Vvi*miPEP171d1 had no effect on *A. thaliana* roots.

More recently, the same group characterized the function of two other miPEPs in grapevines, namely *Vvi*miPEP172b and *Vvi*miPEP3635b [19]. First, the authors identified *Vvi*miRNAs in grape tissue culture plantlets, whose expressions were modified during cold stress (4°C). They then selected *Vvi*miR172b and *Vvi*miR3635b for further analysis. Using an in silico approach, they identified six and four putative sORFs, respectively, in the 5' region of the corresponding pre-miRNAs. They transiently expressed these sORFs in tissue culture plantlets independently and found that one ORF from each pre-miRNA was biologically active as it was able to increase the expression of its nascent pri-miRNA. They synthesized the corresponding miPEPs and, interestingly, their external application on grape tissue culture plantlets improved their tolerance to cold.

Another example illustrating the potentiality of miPEPs came from the study of the effect of *At*miPEP858a on *Arabidopsis* development [13]. *At*miR858 had previously been shown to downregulate the expression of different transcription factors such as *At*MYB11, *At*MYB12, and *At*MYB11, which regulate the phenylpropanoid pathway that sources the metabolites required for the biosynthesis of lignin and the production of many other important compounds such as flavonoids, coumarins, and lignans [36]. In addition, *At*miR858 modifies plant development by increasing root growth and accelerating flowering. By analyzing the region upstream of *At*pre-miR858a, the authors

found three putative sORFs, of which one was shown to be translated in planta using reporter gene fusion assays and western blot experiments. This peptide, named *At*miPEP858a, increased the expression of both *At*pri-miR858a and mature *At*miR858 when exogenously applied to *Arabidopsis* seedlings; this also correlated with a downregulation of the expression of *AtMYB12* and its target genes, and phenotypically with an increase in root length. The effect of *At*miPEP858a was then confirmed via genetic approaches using both transgenic *Arabidopsis* plants overexpressing the miPEP and Cas9-edited *At*miPEP858a mutant plants. Thus, *At*miPEP858a-overexpressing plants exhibited longer main roots than WT plants, while edited mutant lines showed an inverted phenotype. Interestingly, the exogenous treatment of *At*miPEP858a-edited mutant plants with *At*miPEP858a complemented this phenotype. Compared to WT plants, *At*miPEP858a-overexpressing plants exhibited a reduction in anthocyanin accumulation as well as an increase in lignin content, together with enhanced expression of lignin biosynthesis genes. The reciprocal phenotype was observed in *At*miPEP858a-edited plants [13]. Very recently, the same group showed that a disulfated pentapeptide, named Phytosulfokine4 (PSK4), plays a key role in the growth and development of *At*miR858-dependent *Arabidopsis*, through auxin [22]. Interestingly, *At*miPEP858a positively regulates the expression of *PSK4* via *At*miR858a. The expression of *At*miR858a and *PSK4* is also positively regulated by the *At*MYB3 transcription factor through the direct binding of *At*MYB3 to its target promoters. *At*MYB3, whose expression is regulated by *At*miPEP858a/*At*miR858a, is a key component in *At*miPEP858a/*At*miR858a-PSK4-dependent plant growth and development [22]. Concomitantly to this research, the same authors showed that light directly regulates *At*miPEP858a accumulation in *Arabidopsis* and is necessary for *At*miPEP858a action. This light-dependent miPEP regulation requires the shoot-to-root mobile, light-mediated transcription factor, *At*HY5 [37]. Overall, the data place *At*miPEP858a at the crossroads of several biological processes, most likely through the regulation of its corresponding miRNA.

MiPEPs can also modulate rhizospheric plant-microorganism interactions. For instance, an exogenous application of *Gm*miPEP172c specifically increases nodule numbers in soybean (*Glycine max*) when inoculated with *Bradyrhizobium diazoefficiens* and leads to an increase in *Gm*miR172c transcripts [15]. These results are in agreement with those previously observed by Wang et al., (2014), which show that *Gm*miR172c overexpression positively regulates nodulation in soybean through the repression of its target gene—the Apetala 2 (*Gm*AP2) transcription factor Nodule Number Control 1 (*Gm*NNC1)—which directly binds to the promoter of Early Nodulin 40 (*Gm*ENOD40) to repress its transcription [38]. Another example is the role played by *Mt*miPEP171b in arbuscular mycorrhizal symbiosis in *M. truncatula* [16]. Unlike other members of the *Mt*miPEP171 family, *Mt*miPEP171b stimulates arbuscular mycorrhizal symbiosis and positively regulates the expression of its corresponding *Mt*miR171b as well as the expression of *Mt*miR171b target *Mt*LOM1 (Lost Meristems 1). *Mt*miR171b is specifically expressed in root cells containing arbuscules and protects *Mt*LOM1 from being silenced by other *Mt*miR171 members through its mismatched cleavage site [16].

2.2. In Animals

With the miPEP description within *miR34a, miR31, miR155, and miR147b* genes in mammals and *miR8* and *iab8* genes in *Drosophila* (see above), it is now well established that pri-miRNAs can encode miPEPs in animal cells and, for some of them, their function and biology have even been documented. However, whether and how

miPEPs regulate their corresponding pri-miRNA expression remains contradictory. To date, the only example in the animal literature describing a positive effect of a miPEP on the expression of its corresponding pri-miRNA is that of *Hs*miPEP133. *Hs*miPEP133 is a 133 amino acid peptide encoded by *Hs*pri-miR34a. *Hs*miPEP133 induces the expression of *Hs*pri-miR34a/miR34a which leads to the downregulation of *Hs*miR34a-targeted genes [27]. *Hs*miPEP133 is expressed in various healthy tissues but is downregulated in cancer cell lines and tumors. The overexpression of *Hs*miPEP133 indicates that the peptide acts as a human tumor suppressor *in cellulo* and in vivo by inducing apoptosis and inhibiting the migration and invasion of cancer cells. However, *Hs*MiPEP133 is mainly localized in mitochondria and not in nuclei as reported for plant miPEPs. It modulates a yet-to-be-defined signaling cascade that increases p53 transcriptional activity by disrupting mitochondrial function. Since *miR34a* is a direct target gene of the transcription factor p53, the latter upregulates both *Hs*miPEP133 and its corresponding *Hs*miR34a, most likely among a plethora of other p53 target genes. In addition, the authors showed that the positive feedback regulation of *Hs*miR34a by *Hs*miPEP133 can occur in both a p53-dependent and -independent manner, suggesting that miPEP133 can act through other molecular players [27]. More recently, Zhou and colleagues (2022) showed, in mice (*Mus musculus*), that *Mm*miPEP31 promotes the differentiation of regulatory T cells by repressing the expression of *Mm*miR31 in a sequence-dependent manner [32]. Interestingly, the authors showed that miPEP31 enters cells spontaneously and localizes to nuclei. The authors also demonstrate that miPEP31 negatively controls the expression of miR31, providing the first evidence that a miPEP can negatively control the expression of a miRNA gene. However, the mechanism involved seems different from that of miPEP133. Indeed, *Mm*miPEP31 binds to the *Mm*pri-miR31 promoter, induces the deacetylation of histone H3K27 (likely through the recruitment of a cofactor), and competes for the binding of an unknown transcription factor [32].

Although these two examples show that mammalian miPEPs are able to regulate their corresponding pri-miRNAs, either positively or negatively, animal miPEPs likely play other functions, which remain to be identified. The mechanism described in plants is probably not a general mechanism conserved in animal pri-miRNAs. Indeed, *Hs*miPEP200a, *Hs*miPEP155, *Hs*miPEP497, *Hs*MOCCI/MISTRAV, *Dm*miPEP8 and *Dm*MSAmiP do not reveal any effect on their corresponding pri-miRNA [24][25][28][29][30][31]. Furthermore, these miPEPs exhibit regulatory and biological functions uncoupled from their miRNA activity, acting either antagonistically to [25], in parallel with [31], or independently of [30], the miRNA pathway.

To conclude this part, the studies described above show that while positive feedback regulation has been found in all plant miRNA genes studied so far, diverse miPEP effects have been reported in different animal model systems (**Figure 1**b), indicating that miPEP-dependent positive feedback regulation of miRNA genes is not a general mechanism that can be extended to all organisms.

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