

Review

Plant microProteins: Small but powerful modulators of plant development

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SUMMARY

MicroProteins (miPs) are small and single-domain containing proteins of less than 20 kDa. This domain allows microProteins to interact with compatible domains of evolutionary-related proteins and fine-tuning the key physiological pathways in several organisms. Since the first report of a microProtein in mice, numerous microProteins have been identified in plants by computational approaches. However, only a few candidates have been functionally characterized, primarily in Arabidopsis. The recent success of synthetic microProteins in modulating physiological activities in crops makes these proteins interesting candidates for crop engineering. Here, we comprehensively summarise the synthesis, mode of action, and functional roles of microProteins in plants. We also discuss different approaches used to identify plant microProteins. Additionally, we discuss novel approaches to design synthetic microProteins that can be used to target proteins regulating plant growth and development. We finally highlight the prospects and challenges of utilizing microProteins in future crop improvement programs.

INTRODUCTION

MicroProteins (miPs) are small proteins of 5–20 kDa in size and contain only a single domain for protein-protein interaction. MicroProteins are predicted to be evolutionarily related to the proteins they interact with. They originated after duplications and deletions in genes encoding large multidomain proteins resulting in the evolution of single-domain proteins which interact with their ancestral protein (Straub and Wenkel, 2017). Therefore, miPs generally do not contain any activation or DNA-binding domain and hence are not expected to be involved in regulating transcriptional changes directly. However, the presence of the protein-protein interaction (PPI) domain suggests their ability to interact with the homologous proteins post-translationally. They are generally involved in the inactivation of the proteins by making heterodimers through interaction with similar PPI domains.

There are numerous putative and functional small proteins identified in plants through *in silico* as well as functional studies. However, only a few of them have been characterized as microProteins based on their size, structure, origin, and functional mechanisms (Bhati et al., 2018). Many proteins that are categorized as miPs, were identified as interacting partners of functional proteins, mostly transcriptional regulators. The first miP identified in living organisms was DNA binding Inhibitor (Id) protein. Id was isolated about three decades ago from a cDNA library obtained from murine erythroleukemia cells. It is a 16 kDa tiny helix loop helix (HLH) domain protein that interacts with the transcriptional regulator MyoD (Myoblast Determination Protein 1) which is a regulator of muscle differentiation (Benezra et al., 1990). The first miP family reported in plants was LITTLE ZIPPER (ZPR) family of proteins (ZPR1-4) (Wenkel et al., 2007). ZPR has a leucine zipper domain through which it interacts and post-translationally regulates other leucine zipper-containing proteins to modulate plant development. It governs developmental processes like stem cell maintenance in shoot apical meristem (SAM) and leaf development by interacting with HD-ZIPIII/REV. An miP named TRYPTYCHON (TRY) was identified and functionally characterized much earlier than ZPRs but was not classified as an miP at that time (Szymanski and Marks, 1998). Later, based on its size and function, it was classified as a microProtein (Eguen et al., 2015).

Based on their origin, microProteins are classified as *cis*-miPs and *trans*-miPs. *Cis*-miPs are encoded by mRNA isoforms through alternative splicing as well as alternate translation start and stop site selections. *Trans*-miPs, in turn, have evolved through genome amplification and subsequent domain loss event

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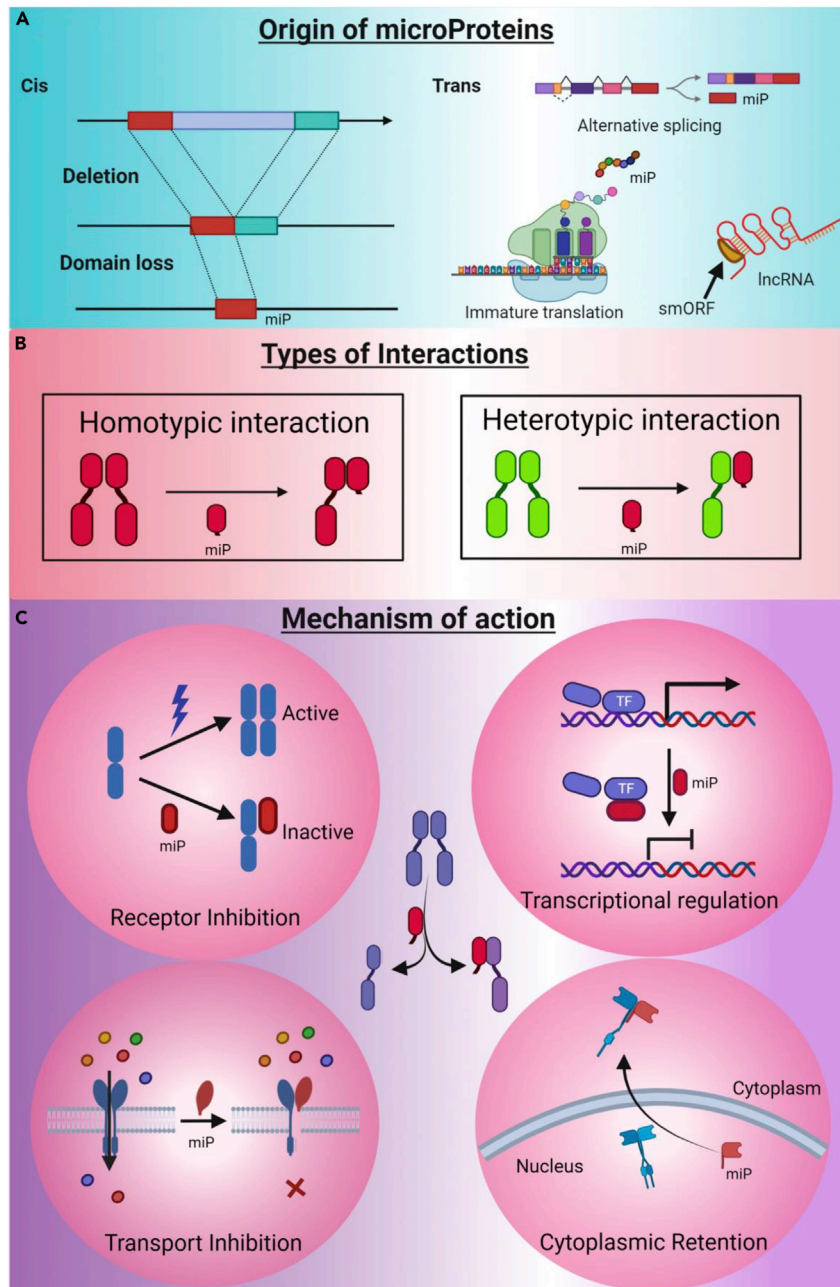


Figure 1. Origin and mode of action of microProteins

(A) MicroProteins originate via *cis* pathway through deletion and subsequent domain loss as well as via *trans* pathway involving alternative splicing, immature translation of multidomain proteins, and translation of lncRNA.

(B) MicroProteins undergo homotypic interaction with identical domain proteins or heterotypic interaction with non-identical but similar or homologous domain-containing proteins.

(C) Mechanism of action of microProteins include inhibiting receptors by binding them and making them inactive, transcriptional regulation by binding to transcription factors and inhibiting them from binding to their promoters, inhibiting transport across the membrane by binding to membrane transporters and promoting cytoplasmic retention of proteins.

(Figure 1A) (Bhati et al., 2018). Many of the miPs are synthesized by the translation of smORFs (small Open Reading Frames) located on lncRNAs (Figure 1A). A large number of smORFs have been found in organisms across kingdoms including bacteria, plants, and animals; and may constitute about 5–10% of the total

genome of an organism (Fesenko et al., 2021; Khitun and Slavoff, 2019; Saghatelian and Couso, 2015). smORFs translated products were assumed to have no relevant function. However, the discovery of functional translated products of less than 100 amino acids such as miPs, opened a new chapter in understanding the genome of an organism more deeply (Khitun and Slavoff, 2019).

MicroProteins interact with domains of the proteins in two ways (Figure 1B). In homotypic interaction, miP interacts with a protein having a similar domain. One example of such an interaction is miP1a/b or BBX31/BBX30 interacting with CONSTANS and TOPLESS via B-box motif to regulate flowering (Graeff et al., 2016). When an miP interacts with a non-identical but compatible domain, the interaction is known as heterotypic interaction (Figure 1B). For example, B-box domain of miP1a/b interacts with bHLH domain of PHYTOCHROME INTERACTING FACTOR (PIFs) and both coiled-coil and C-terminal DNA binding domain of ETHYLENE INSENSITIVE 3 (EIN3) to promote photomorphogenic development (Wu et al., 2020). This heterotypic interaction of miPs might have allowed the control over targets that are not evolutionarily related and broadened the scope of microProtein functions.

MicroProteins are functionally inactive until they interact with other homologous proteins of larger size and physiological importance. Hence, in a typical signaling pathway, miPs are involved in fine-tuning the activity of proteins at post-translational level (Bhati et al., 2018). Most of the commonly known miPs interact with and regulate transcription factors through shared homologous domains (Figure 1C) (Wu et al., 2022). However, miPs are also known to interact and regulate proteins other than transcription factors in certain organisms. For example, Vpu (Viral Protein U), an accessory protein of HIV, sequesters the mammalian K^+ -channel TASK-1 and renders it dysfunctional (Hsu et al., 2004). The skeletal muscle-specific lncRNA MYOREGULIN (MLN) encodes for an miP that interferes with SERCA, a calcium ATPase type P-ATPase, and impedes the Ca^{2+} transport in the sarcoplasmic reticulum (Anderson et al., 2015). MicroProteins also regulate proteins by promoting their retention in the cytoplasm (Figure 1C) (Bhati et al., 2018). Taken together, microProteins are highly variable in their form and mechanism of action.

In this review, we highlight how microProteins modulate the activity of different types of proteins including transcription factors, membrane receptors, and channels. We summarize the different techniques used in the identification and characterization of miPs. We further discuss the functional roles of miPs in different developmental stages of plants. Although natural miPs are being discovered in plants, novel approaches to design synthetic miPs are also being explored (Dolde et al., 2018). Synthetic miPs may be engineered to inhibit the function of proteins post-translationally and to fine-tune different physiological processes in plants. We conclude by discussing the prospects of the role of microProteins in regulating crop improvement and food security.

TECHNIQUES TO MAP THE MICROPROTEINS AND SHORT PEPTIDES IN PLANTS

The versatility and significance of microProteins emphasize the demand for methods to identify and characterize novel microProteins. MicroProteins are small-sized proteins, but not all tiny proteins are microProteins (Bhati et al., 2021; Eguen et al., 2015). Therefore, there is a need to differentiate potential miPs from other small proteins. Here, we attempt to bring together various approaches that may be adapted to identify putative miPs and their potential targets. Because of the small size of miP genes, these may not be annotated in complex genomes. Hence, it was difficult to identify and characterize the miPs earlier. The advancement of computational tools, however, allows the identification of miP genes based on adjustable sets of criteria discussed in later sections. Multiomics approaches are generally used to discover potential miPs, but these approaches have their limitations and need further validation (Bhati et al., 2018). Presently, high throughput mass spectrometry techniques, computational algorithms, and predictive protein tools are anticipated to simplify the finding of new microProteins.

Mass spectrometry-based proteomics approach to forecast microProteins

Chromatography techniques along with mass spectrometry have tremendous potential to characterize small proteins. High throughput proteomics using liquid chromatography-tandem mass spectrometry (LC-MS/MS) is generally adopted to identify small candidate peptides (Figure 2A) (Chick et al., 2015). In MS methodology, generally protein-lysates are used to detect proteins using shotgun and top-down proteomics approaches (Kaulich et al., 2020). To detect small proteins, sample preparation is a crucial step that focuses on effective denaturation, reduction, and digestion of protein samples (Figure 2A) (Donnelly et al., 2019). Sometimes, protein precipitation is performed with suitable organic solvents, such as

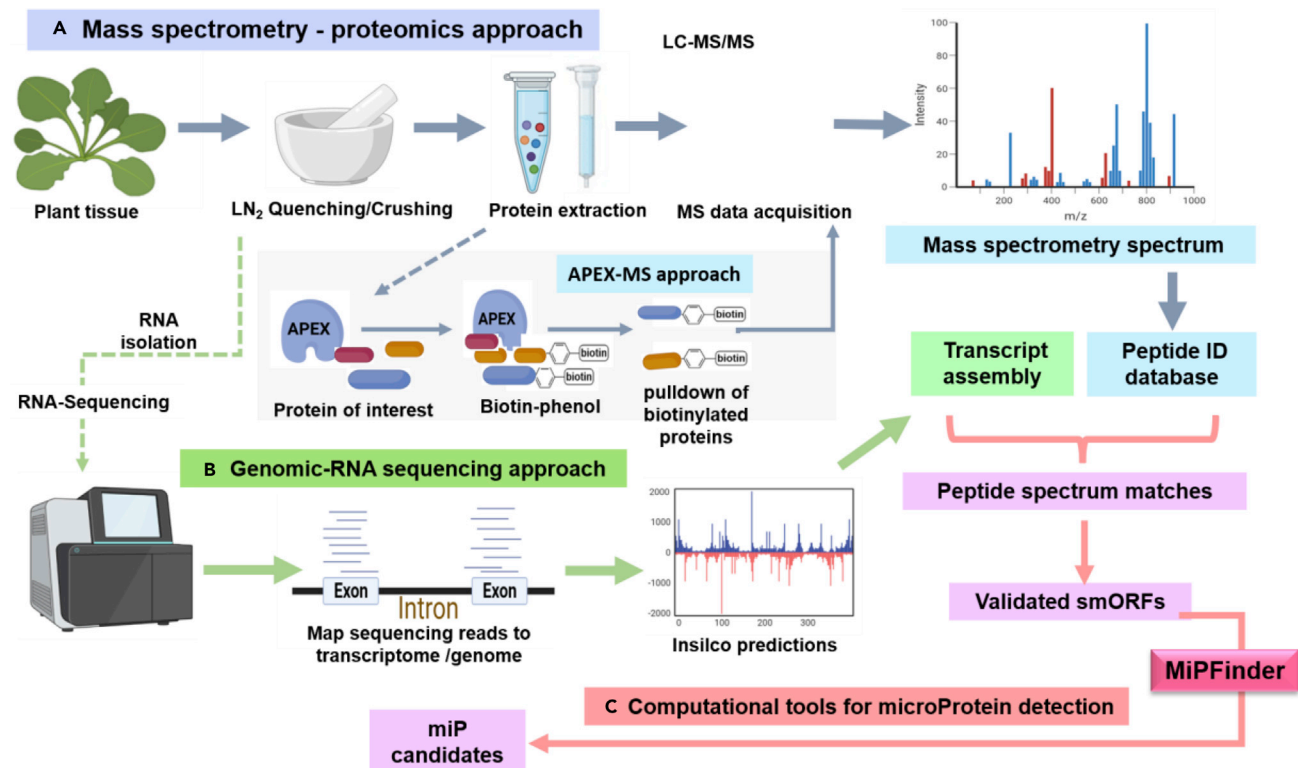


Figure 2. Workflow for identification of microProteins in plants using biochemical, genomic, and computational tools

(A) Liquid chromatography integrating mass spectrometry-based proteomics approaches to characterize small proteins. The APEX-MS tagging method can be used to elucidate interactions of the microProtein of interest.

(B) Next-generation sequencing techniques integrating with various transcript and peptide databases to identify novel microProteins.

(C) Computational tools like miPFinder are often used to validate and filter microProteins from other small proteins. In the figure gray, green and pink lines represent MS, sequencing, and computational approaches respectively. Dotted lines indicate multiple steps might be involved in the workflow separately.

methanol and acetone, to concentrate and purify target proteins. Separation of small proteins can also be performed by SDS-PAGE, although it demands some adjustment in steps to avoid the loss of small proteins from the sample (Kaulich et al., 2020). This can be adapted to extract the fragmentation pattern of peptides (Bupp and Wirth, 2020). Additionally, in the top-down approach, size exclusion and ion-exchange chromatography also enrich small proteins. However, these approaches have their constraint in the classification of miPs from small proteins (Leong et al., 2022). This is due to less standard workflow, challenges in specific protein extraction, protein digestion techniques, number of optimisation steps in liquid chromatography, data acquisition and the need for complex data analysis (Ahrens et al., 2022). MS methods can be incredibly beneficial for detecting small proteins, m/z fragments patterns, and constructing structures from protein complexes. MS can also be utilized to discover the function of miPs by integrating with various high-quality computational tools. However, Information about small proteins and their molecular weight can also be analyzed directly using matrix-assisted laser desorption ionization (MALDI) integrated with mass spectrometry (Zabret et al., 2021).

Next-generation sequencing and computational tools for microProtein detection

Modern algorithms and cutting-edge advances in high throughput next-generation sequencing have given us chance to analyze comprehensive genomic data to discover novel microProteins (Figure 2B). However, precise *in silico* classification of miPs remains a challenge. miPFinder is one of the standard tools to filter miP from small proteins (Figure 2C). This computational tool requires some information like annotated genome, size of proteins, protein domain, evolutionary origin, protein organization of potential targets, and protein-protein interaction evidence from older databases to predict the potential function of small proteins as miP (Straub and Wenkel, 2017). For acquiring this information, miPFinder tool is dependent on python, BLAST, ClustalW2, hmmbuild, Pfam, and iPfam computational tools for multiple sequence

alignment and annotation. Specifically in plants, Gene Ontology (GO) functions were derived from Plant GO, agriGO and agriGO v2.0, GOSlimViewer, GOSlim Set, AgBase v2.0, and PANTHER v11. GO helps in the functional annotation of protein classes through evolutionary relationships and putative ancestors of miPs (Du et al., 2010; McCarthy et al., 2006; Mi et al., 2017; Tian et al., 2017a, 2017b). Next-generation ribosomal sequencing (Ribo-seq) can detect short open reading frames (smORFs). Studies reported that smORFs with ribosomal activity may encode miPs (Olexiuk et al., 2016). The novel database and tools like sORF finder, Coding Region Identification Tool Invoking Comparative Analysis (CRITICA), Coding Potential Calculator (CPC), PhastCons, PhyloCSF and Micropeptide detection pipeline (micPDP), ARA-PEPs and PsORF can provide detailed information to predict functional small open reading frames (smORFs). Most of these databases are demonstrated for animal models like human, mouse, and fruit fly. However, these computational tools can also be extended to detect plant miPs (Olexiuk et al., 2016; Pueyo et al., 2016). Additionally in Arabidopsis, paired-end analysis of transcription start sites (PEAT) is a bioinformatics tool that provides information about transcription start site (TSS) and might also be used to predict microProteins in plants (Morton et al., 2014).

Techniques to decipher regulatory functions of microProteins

Computational and MS approaches can predict small open reading frames (smORFs) and potential microProteins. In most cases, the regulatory function of miPs needs additional insights and comprehensive experimental validations to predict their functions (Bhati et al., 2018; Dolde et al., 2018; Straub and Wenkel, 2017). The elucidation of miP-target interactions by observing phenotypes of -gain-of-function and -loss-of-function mutants is an effective approach to characterize the physiological functions. So, critical observations of different mutants of miP-coding genes can provide strong leads in their phenotypic, physiological, and regulatory characterization (Graeff et al., 2016; Wenkel et al., 2007). In biochemical methods, immunoprecipitation of FLAG-tagged miPs provides a general approach for revealing interacting partners of miPs leading to the elucidation of their physiological functions. However, sometimes these methods also show nonspecific interactions of miPs due to their very small size. The new *in situ* tagging method, which relies on engineered ASCORBATE PEROXIDASE 2 (APEX) to elucidate microProtein-protein interactions, is also being used to check the interactions (Figure 2A). It has been found that APEX tagging is superior to traditional immunoprecipitation methods for miPs, as less nonspecific interactors were observed in the APEX tagging experiments by employing suitable controls. Altogether, extensive genetic, molecular, and biochemical experiments like structural analysis, immunoprecipitation methods, imaging, and yeast two-hybrid screenings can also help in predicting potential targets and functions of miPs (Chu et al., 2017; Dolde et al., 2018). In the future, synthetically derived miPs may also interpret the regulatory functions of microProteins (Dolde et al., 2018).

FUNCTIONAL ROLE OF MICROPROTEINS IN PLANTS

Several microProteins belonging to different families have been identified in plants primarily in Arabidopsis (Figure 3). Advancement of genomic and molecular tools has also led to the identification and characterization of miPs in plants of economic importance (Table 1). These miPs have been characterized for a wide range of physiological functions including epidermal cell patterning during root hair and trichome development, light-mediated responses, leaf development, pigment biosynthesis, and floral development (Figure 3). Here, we provide an account of functionally characterized plant-miPs, classified according to their size, origin, and mode of action typical of miPs.

Epidermal cell patterning in trichome and root hair development

Trichomes are specialized hairy structures on aerial surfaces of plants that are differentiated from epidermal cells. The functional role of trichomes may be enlisted as protection from UV light, pests, and excess transpiration. The functional characterization of the first microProtein from plants may be traced back to the screening of trichome mutants from a population of EMS mutants (Hülkamp et al., 1994). Out of the 21 genes identified one was characterized as TRYPTYCHON (TRY) which coded for a 106 aa long (~13 kDa) protein consisting of an R3 single-repeat MYB domain and no transcriptional activation domain, typical hallmarks of an miP. In the *try* mutants, nests of up to four enlarged, abnormally branched trichomes were reported in place of a single trichome. These phenotypes may be attributed to defective epidermal cell patterning. Further analysis identified that TRY was downstream to GL1 and TTG, while upstream to STI (STICHEL), DIS1 (DISTORTED TRICHOMES 1), DIS2, and GL3, the key genes for epidermal cell patterning. A series of studies soon after suggested that TRY and GL1/TTG antagonistically control the

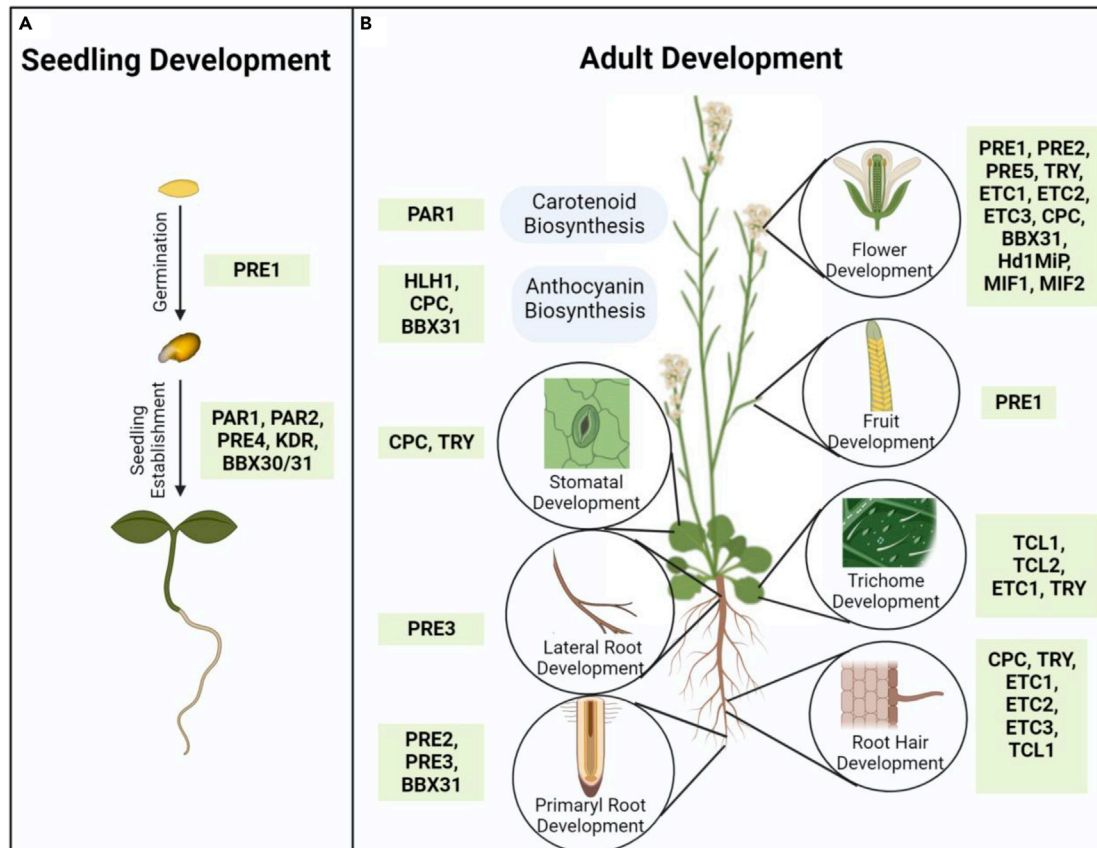


Figure 3. Functional role of microProteins during different developmental processes in plants

(A) MicroProteins involved during seed germination and seedling establishment.

(B) MicroProteins involved in different developmental processes during the adult stage of plants like root development, trichome and stomatal development in leaves, carotenoid, and anthocyanin biosynthesis as well as fruit and flower development. MicroProteins involved in a particular process are grouped and written in a green box beside the circle showing the particular development.

endoreduplication of epidermal and trichome cells (Schnittger et al., 1998, 1999; Szymanski and Marks, 1998). It was discovered later that TRY interferes with the GL1 binding to GL3 and disturb the formation of MBW (MYB-bHLH-WDR) complex. TTG1, GL1, GL3, and EGL3 participate in the formation of MBW activator complex that activates the expression of *GL2* and *TTG2*. MBW complex along with NTL8 (NTM1-LIKE 8) also promote expression of *TRY* (Tian et al., 2017a, 2017b).

Later, CAPRICE (CPC), an MYB microProtein of ~11 kDa was also identified during the screening of transfer DNA tagged lines of Arabidopsis (Wada et al., 1997). Mutation in *CPC* resulted in sparsely distributed root hairs of no identifiable difference in size and morphology when compared to the wild-type. Ectopic expression of *CPC* increased the root hairs along with a negative effect on trichome development in aerial parts. *CPC* which promotes root-hair cell differentiation is thought to be evolved from another epidermal cell fate regulator WEREWOLF (WER) after the truncation of the activation domain and loss of DNA binding capacity. WER-GL3/TTG1-EGL3 transcriptional complex promotes *GL2* expression which prevents root hair formation in non-hair cells. *CPC*-mediated root hair cell specification is achieved due to the movement from non-hair cells to hair cells. *CPC* moves from non-hair cells to hair cells where it competes with WER for binding to this transcriptional complex which leads to the inhibition of *GL2* expression, resulting in root hair formation (Wada et al., 2002).

WER was also found to act negatively on the *CPC* (Lee and Schiefelbein, 1999). Later, WER was reported to negatively regulate the *CPC* by binding directly to its promoter (Ryu et al., 2005). Another MYB miP, ENHANCER OF TRY AND CPC 1 (ETC1) (~9 kDa), was characterized to have functions partially redundant with TRY and CPC (Kirik et al., 2004). Analysis of 5 R3-MYB miP homologs revealed that CPC,

Table 1. List of microProteins characterized in economically important plants

MicroProtein Family	Species	Known miPs	Targets	Function	References
Cis-microProteins	<i>Brachypodium distachion</i>	LNJ	NINJA	Shoot architecture	(Hong et al., 2020)
NAC	<i>Populus tomentosa</i>	PtRD26 ^{IR}	PtRD26	Leaf Senescence	(Wang et al., 2021)
HLH	<i>Oryza sativa</i>	ILI1/PRE1	IBH1	Leaf inclination, cell elongation	(Zhang et al., 2009a)
Synthetic BBX	<i>Oryza sativa</i>	Hd1miP	HD1	Flowering	(Eguen et al., 2020)
ZPR	<i>Solanum lycopersicum</i>	SIDTM	HD-ZIPIII/REV	Stem cell homeostasis	(Xu et al., 2019)
MIF	<i>Solanum lycopersicum</i>	SIMIA	SIKNU, TPL, HDA19	Floral development, stem cell homeostasis	(Bollier et al., 2018)
KNATM	<i>Solanum lycopersicum</i>	SIPTS	BIP	Leaf Development	(Kimura et al., 2008)
R3-MYB	<i>Solanum lycopersicum</i>	SITRY	SIGL3	Trichome and root hair development, Anthocyanin biosynthesis	(Tominaga-Wada et al., 2013)
R3-MYB	<i>Gossypium spp.</i>	GhCPC	MYC1, TTG1/4	Cotton fiber elongation	(Liu et al., 2015)

TRY, ETC1, ETC2, and ETC3 (~9–13 kDa) have similar and redundant functions in root epidermal cell patterning. Moreover, TRY and ETCs may substitute for the function of CPC (Simon et al., 2007; Tominaga et al., 2008). Identification of TRICHOMELESS 1 (TCL1) (~10 kDa), another R3-MYB microProtein as a negative regulator of trichome formation in inflorescence epidermis added additional information in organ-specific epidermal cell fate program. Loss-of-function mutant *tcl1-1* had unique, ectopic trichomes along the inflorescence and pedicel epidermis, while gain-of-function *tcl1-1D* had trichomeless phenotype (Wang et al., 2007). Genetic analyses revealed that TCL1 suppressed the expression of *GL1* and acted synergistically with CPC. It is interesting to mention while overexpression of other single repeat R3-MYBs such as CPC, TRY, ETC1, and ETC2 form ectopic root hair cells, overexpression of *TCL1* does not affect root hair formation and patterning. This indicates that *TCL1* protein is functionally different from other R3-MYBs (Wang et al., 2007). Another close homolog of *TCL1* was soon identified as *TCL2* (~12 kDa) with 80% of sequence similarity. Genetic analyses revealed that these two had redundant, but not equivalent functions (Gan et al., 2011). Arabidopsis and tomato both have trichomes, but their trichomes differ in morphology and functions. However, the identification of *SITRY* (~11 kDa) (Table 1) and its functional characterization in Arabidopsis concluded that both species might share a common pathway to regulate epidermal cell patterning in trichome and root hair development (Tominaga-Wada et al., 2013). Cotton fibers are derived from trichomes developed from epidermal cell patterning of seeds. *GhCPC* (~9 kDa) (Table 1) is a homolog of *AtCPC* whose overexpression delayed the initiation of the cotton fiber and reduced its length. Moreover, transgenic plants overexpressing *GhCPC* have reduced expression *GhHOX3/GL2* (Liu et al., 2015).

Phosphate (Pi) is one of the major elements found in soil, which is essentially required for optimal plant growth and development. The induction of root hairs is one of the many strategies plants adapt to counter limited phosphate conditions. Comparative RNAseq analysis identified *ETC1* as one of the upregulated genes under Pi-deficiency, while expression of other key genes for epidermal cell fate determination (*WER*, *SCM*, *CPC*, *GL3*, *EGL3*, *GL2*) remained constant (Savage et al., 2013). CPC and ETC1, both are known regulators of root hair development. Analysis of *etc1* and *cpc etc1* double mutants concluded that ETC1 is required for increased root hair density typically during Pi-deficient conditions. RNAseq data of *cpc*, *try*, and *etc1* further confirmed that in addition to the root hair, the three MYBs also regulate several of the Pi-deficiency-related processes (Chen and Schmidt, 2015).

Light-mediated responses in plants

Light is a crucial environmental cue for the growth and development of plants. In the dark, seedlings exhibit skotomorphogenesis which is characterized by the formation of long hypocotyl and closed cotyledons forming a hook-like structure called the apical hook. Once exposed to light seedlings undergo photomorphogenesis characterized by the suppression of hypocotyl elongation and opening and greening of cotyledons. ELONGATED HYPOCOTYL 5 (HY5) is one of the central players in light signaling and photomorphogenesis during seedling development (Yadukrishnan and Datta, 2021). HY5 directly regulates the transcription of *MICROPROTEIN 1A (miP1a)/B-BOX DOMAIN PROTEIN 31 (BBX31)* (~13 kDa) under white as well as UV light. However, BBX31 regulates photomorphogenesis independent of HY5 (Yadav et al., 2019a, 2019b, 2019b). miP1a and miP1b are BBX proteins consisting of a single B-box domain for protein-protein interaction but lack the CCT domain responsible for DNA binding. During dark to light transition rapidly accumulated miP1a/b (~13 kDa) interact with PIF3 and EIN3 and disrupt their oligomerization, thereby directing the inhibition of their transcriptional activities (Wu et al., 2020). Screening of activation-tagged mutants identified a mutant showing long hypocotyl under continuous blue light. The gene responsible for the mutation was characterized as *KIDARI (KDR)*, a member of the HLH family of miPs (Hyun and Lee, 2006). The KDR (~10 kDa) specifically interacted with (LONG HYPOCOTYL IN FAR-RED) HFR1 to form heterodimer affecting the sequestration of HFR1 and release of PIFs from HFR1-PIF heterodimer complex (Hong et al., 2013). Overexpression of a MINI ZINC FINGER miP, MIF1 (~11 kDa), resulted in multiple developmental defects affecting chlorophyll levels, flowering, fertility, apical dominance, and longevity and altered sensitivity to phytohormones (Hu and Ma, 2006). However, transgenics did not show any skotomorphogenic phenotype in dark and were equivalent to the light grown seedlings compared to the wild type. This suggested that MIF1 promotes photomorphogenesis along with affecting some adult developmental stages.

Under the shade, seedlings exhibit shade avoidance responses characterized by hypocotyl elongation and changes in photosynthetic pigment accumulation. Two shade-responsive HLH domain containing miPs, PHY RAPIDLY REGULATED 1 (PAR1) and PAR2 (~13 kDa both) were identified from the existing genomic data. Ectopic expression of these genes led to the formation of short hypocotyl, reduced longitudinal cotyledon expansion, increased levels of chlorophylls and carotenoids, dwarf and dark-green plants (Roig-Vilanova et al., 2006, 2007). The impaired shade avoidance response was attributed to negative transcriptional regulation of a set of hormonally inducible *SAUR* genes. Later it was shown that the light signal stabilizes the PAR1 protein and PAR1 interacts through its HLH domain with PIF4, a key protein in the light signaling module (Hao et al., 2012). This interaction inhibited PIF4-mediated transcriptional activation of downstream genes such as *PIL1*, *HFR1*, and *INDOLE-3-ACETIC ACID INDUCIBLE 29 (IAA29)* resulting in reduced hypocotyl length (Hao et al., 2012).

Leaf development

Leaves are the major photosynthetic organs of plants. Leaves develop from the pluripotent cells of shoot apical meristems with a dorsoventral asymmetry. That means the adaxial surface of the developing leaves remains adjacent to the meristem, while the abaxial surface develops toward the opposite side (Canales et al., 2005). The polarity of the adaxial/abaxial surfaces of the leaves is critical for the proper leaf development, cellular specialization of the leaf functions, and development of axillary meristem. HD-ZIPIII proteins are the potent regulator of this development. Transcriptome profiling of the seedlings under inducible HD-ZIPIII activity leads to the identification of LITTLE ZIPPER 1/2/3/4 (ZPR1/2/3/4) (~7–16 kDa), the four members of a LITTLE ZIPPER family of miPs (Wenkel et al., 2007). These miPs directly interact with HD-ZIPIII/REV and abolish its DNA binding activity. Overexpression of the ZPRs result in the inhibition of the HD-ZIPIII/REV activity. Furthermore, transcriptional regulation of ZPRs by HD-ZIPIII/REV creates a negative feedback loop (Brandt et al., 2013). In the transition from a primordium on the flank of shoot apical meristem to a fully functional organ, a developing leaf follows indeterminate to determinate growth. This transition is mediated and regulated by intrinsic developmental programs and environmental cues. Mini Zinc Finger (MIF) is a 101 aa long miP that acts in the leaf developmental process. Pleiotropic developmental phenotype produced by the 35S:MIF1 plants suggested that MIFs are involved in multiple hormonal responses (Hu and Ma, 2006). Overexpression of *MIF2* and *MIF3* (coding ~9–10 kDa miPs) leads to phenotypes like smaller leaf size and abnormal expansion similar to 35S:MIF1 mutants suggesting their redundant functional role (Hu et al., 2011). MIFs have zinc finger domain but lack other protein domains proteins that are normally present in transcription factors making these dependent on other transcription factors for activity. MIF1 interacts with Zinc Finger HomeoDomain 5 (ZHD5) to regulate floral architecture and leaf development. In

this process, MIF1 blocks DNA binding activity as well as prevent nuclear localization of ZHD5 by forming a MIF1-ZHD5 complex (Hong et al., 2011). Moreover, 35S:MIF1 and 35S:MIF3 plants developed ectopic meristems along the margins of late rosette leaves due to *ERECTA* dependant reduced responses to auxin and GA. Cell elongation, expansion, and cell division are the key attributes of plant morphogenesis and development. Leaf laminal inclination in rice involves cell elongation and is a key response to Brassinosteroids (BRs). Screening of a large collection of transfer DNA insertional mutants in rice resulted in the identification of a line with increased leaf inclination which had a mutation in *ILI1*, which encodes an ortholog of PRE1, an Arabidopsis bHLH-type miP (Zhang et al., 2009a). The *ili1-D* mutant also showed hypersensitivity to BR treatment showing the role of ILI1 in BR signaling. Molecular and genetic analyses showed that ILI1/PRE1 (~11 kDa) interacts with ILI1 Binding bHLH (IBH1) via bHLH domain and inhibits its activity. Both proteins act downstream of BRASSINAZOLE RESISTANT 1 (BZR1), a key intermediate in BR signaling, and work as a key intermediate in BR-mediated cell expansion as well as leaf inclination.

Leaf senescence is a complex late stage in the leaf development program which helps in the translocation of nutrients from dying leaves to the growing organs of plants. Therefore, tight regulation of the senescence process is critical to plant fitness. Exact mechanism of microProtein mediated-regulation of senescence is not known in Arabidopsis. However, transcript profiling of senescing leaves in *Populus* identified *PtRD26/PtNAC013* and its miP encoding alternative splice variant *PtRD26^{IR}* (*Populus tremula* RESPONSIVE TO DESICCATION 26^{intron retention}) (Table 1). *PtRD26* was found to directly regulate multiple SAGs (Senescence-Associated Genes) including *ORESARA 1* (*ORE1*), *ETHYLENE-INSENSITIVE3* (*EIN3*), and *STAY-GREEN 1* (*SGR1*). *PtRD26^{IR}* retains the first intron during the splicing event and was detected only in senescing leaves suggesting that this splicing event is senescence-associated (Table 1). The translated product of *PtRD26^{IR}* was only 62aa (~7 kDa) long compared to the original 343 aa of *PtRD26*, and had lost DNA binding domain. *PtRD26^{IR}* interacted with *PtRD26* through its common domain retained after the splicing event and inhibited its interaction with its target SAG genes. Additionally, it also interfered with the binding of *PtNAC055*, *PtNAC086/PtNAP*, and *PtNAC109/PtORE1* to their target. Identification of *PtRD26^{IR}* introduces alternative splicing as another aspect of the regulatory program for senescence (Wang et al., 2021).

Pigment biosynthesis

Since its identification in 1997 (Wada et al., 1997), the R3-MYB type miP CAPRICE (CPC) has been established as a key regulator of the cell fate determination of the hair-forming-special cells following a series of investigations mentioned in an earlier section. In an attempt to explore other aspects of CPC's functional role, *AtCPC* was ectopically expressed in *Nicotiana* (Zhang et al., 2009b). Transgenic tobacco lines showed affected trichome/root hair distribution and variegated flowering pattern. The variegated flowering pattern was correlated with differences in anthocyanin levels, *AtCPC* mRNA levels, and differential expression of late flavonoid pathway genes. Moreover, Y2H analysis identified that *AtCPC* could bind to the bHLH proteins ANTHOCYANIN 1 (AN1) and JAF13 of *Petunia*, which are known regulators of anthocyanin biosynthesis. These data suggested that *AtCPC* might negatively regulate anthocyanin biosynthesis via inhibiting the transcriptional activity of AN1 and JAF13 (Zhang et al., 2009b). Similar observations were made when Arabidopsis *cpc* mutants were grown under different stresses (Zhu et al., 2009). The levels of anthocyanin were proportional to the expression levels of *CPC* in mutants. Microarray and qPCR analyses suggested that late flavonoid biosynthesis genes were repressed under ectopic expression of the *CPC*. Further molecular studies like Y2H suggested that *CPC* competitively inhibits the interaction of the R2R3-MYB protein PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) with ENHANCER OF GLABRA 3 (EGL3) by binding to bHLH protein EGL3. This interaction negatively modulates PAP1-mediated transcriptional regulation of anthocyanin biosynthesis genes in Arabidopsis (Zhu et al., 2009).

When *TRY* isolated from tomato was expressed in Arabidopsis under *CPC* promoter, transgenic *CPC:SITRY* exhibited repressed accumulation of anthocyanins. Moreover, transgenic *GL3:SIGL3* also showed a similar reduction in anthocyanin levels, suggesting that *TRY* and *GL3* also influence anthocyanin biosynthesis (Tomimaga-Wada et al., 2013). Similar results were obtained when *AtCPC* was overexpressed in tomatoes. 35S:*CPC* tomato plants accumulated less pigment compared to the wild type. The anthocyanin level was further correlated with the mRNA levels of key pathway genes, *PHE AMMONIA LYASE 1* (*PAL1*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), *CHALCONE SYNTHASE* (*CHS*), and *ANTHOCYANIDIN SYNTHASE* (*ANS*) (Wada et al., 2014). High doses of UV light induce DNA damage and increase the accumulation of ROS leading to cellular damage. To combat such stresses, plants tend to accumulate

photoprotective compounds such as anthocyanins. Overexpression of *miP1a/BBX31*, a gene encoding BBX-domain containing miP, showed reduced loss of chlorophyll and accumulation of anthocyanins under high UV light (Yadav et al., 2019a). BBX31 is known to regulate flowering by interacting with BBX domain of CONSTANS and inhibit its activity. Moreover, ectopic expression of *miP1a/BBX31* elevated the levels of UV-protective compounds such as coumaric acid, hydroxybenzoic acid, and vanillic acid. BBX31 enhances mRNA levels of *EARLY LIGHT INDUCED PROTEIN1 (ELIP1)* and *ELIP2*, the key genes involved in DNA repair. However, BBX31 being an miP does not contain any DNA binding domain, the mechanism behind its transcriptional regulation is not known as it cannot interact with HY5 (Yadav et al., 2019a). Similarly, overexpression of *HLH4* gene coding for a ~17 kDa bHLH type miP resulted in the downregulation of many flavonoid biosynthesis genes. When *HLH4* mutants were exposed to high light stress, anthocyanins were accumulated in WT and *hlh3*, *hlh4*, while the levels in overexpression lines remain unchanged (Hou et al., 2022). HLH4 interacts with several bHLH proteins including CIB5 and PRE1 but the mechanism for regulating anthocyanin biosynthesis genes is still not known. Carotenoids are photoprotective compounds that accumulate in chloroplasts in response to high light stress. They protect plants against ROS produced under stress. The first rate-limiting enzyme of carotenoid biosynthesis is *PHYTOENE SYNTHASE (PSY)*. The miP PAR1 interacts with bHLH domain-containing protein PHY-INTERACTING FACTOR 1 (PIF1) to modulate PIF1-mediated regulation of *PSY* (Bou-Torrent et al., 2015).

Flowering and floral development

MicroProteins have been characterized to play important roles both in flower development as well as the regulation of flowering time. The gynoecium is the last structure to be developed from the floral meristem. The floral development processes are mediated by the *WUSCHEL (WUS)*, *AGAMOUS (AG)*, and *KNUCKLES (KNU)*. AG induces the expression of *MIF1* and *SIIMA (INHIBITOR OF MERISTEM ACTIVITY)*. The MIF1 miP and its tomato ortholog SIIMA (~10 kDa) interact with KNU to recruit TPL and HISTONE DEACETYLASE19 (HDA19) to form a repressor complex and transcriptionally regulate *WUS* (Bollier et al., 2018). *ab initio* analysis of 44 putative miPs in Arabidopsis identified two BBX proteins: miP1a/BBX31 and miP1b/BBX30. These miPs were shown to interact with BBX domain of CO. Ectopic expression of either *miP1a/BBX31* or *miP1b/BBX30* in transgenic plants reduced the *FT* leading to severe delay in flowering. Moreover, miP1a/b also interacted with carboxy-terminal PF(V/L)FL motif of *TOPLESS (TPL)/TOPLESS-RELATED (TPR)*, another corepressor of the flowering (Graeff et al., 2016). This CO-miP1a/b-TPL trimeric complex represses flowering by inhibiting *FLOWERING LOCUS T (FT)* expression. *ENHANCER OF TRY AND CPC3 (ETC3)/CAPRICE LIKE 3 (CPL3)* is one of the R3-MYB miPs which regulates flowering. *cp3* mutants flower early while *35S:CPL3* plants show delayed flowering. qPCR analysis showed that key flowering genes *FLOWERING LOCUS T (FT)*, *CONSTANS (CO)*, and *SUPPRESSOR OF OVEREXPRESSION OF C O 1 (SOC1)* were differentially expressed in mutant plants (Tominaga et al., 2008; Wada and Tominaga-Wada, 2015). CPL3 interacts with GL3, EGL3 and AtMYC1 but none of them regulates flowering, so the mechanism behind the regulation of flowering is not known. A bHLH miP *PACLOBUTRAZOL RESISTANCE 1 (PRE1)* is a Gibberlin (GA) responsive microProtein (Lee et al., 2006). Ectopic expression of *PRE1* affects the transition from vegetative growth to flowering (Lee et al., 2006).

SYNTHETIC MICROPROTEINS IN PLANTS

Since microProteins interact and fine-tune the target proteins employing compatible PPI domains, it may be speculated that synthetic miPs may be designed by targeting protein-protein interaction domains of key proteins of physiological pathways. Designing synthetic miPs with single or multiple functional domains might lead to more specific or diversified effects. Following this approach, Dolde et al. generated transgenic Arabidopsis plant ectopically expressing PPI domains of *DICER-LIKE1 (DCL1)*, *BRASSINOSTEROID INSENSITIVE1 (BRI1)*, and *CRYPTOCHROME1 (CRY1)*, which are involved in miRNA biogenesis, BR-response and light signaling (Dolde et al., 2018). In all three cases, normal physiological processes regulated by DCL1, BRI1 and CRY1 were disrupted. Transcending this proof of concept to the commercially important monocot model rice, Eguen et al. engineered an miP from *HEADING DATE 1 (Hd1)*. Hd1 is the rice ortholog of CO, which regulate the flowering by controlling the expression of *HEADING DATE 3A (Hd3a)* and *RICE FLOWERING LOCUS T 1 (RFT1)*. A synthetic miP was designed by truncating the CCT domain of Hd1 (Eguen et al., 2020). The resulting protein Hd1miP was found to interact with Hd1. Plants expressing *Hd1miP* exhibited early flowering phenotype in a dose-dependent manner. The early flowering phenotype was independent of the photoperiod. Moreover, the presence of Hd1miP also influenced yield parameters such as grain length and width. It also inhibits the Hd1-mediated suppression of *Hd3a* and *RFT1* expression levels.

Putative miPs annotated in the genomes of organisms may also be used as templates to design synthetic miPs to be used in other species. In one such case, computational screening resulted in the identification of several putative monocot-specific miP sequences from reference genomes. From a subset of such sequences from *Brachypodium* genome, five synthetic miPs were designed and expressed in Arabidopsis. One of these proteins was identified as LITTLE NINJA/LNJ due to the presence of NINJA domain related to the ABI5-BINDING PROTEIN2 (AFP2) protein of Arabidopsis (Hong et al., 2020). *LNJ-OX* plants showed stunted growth and lost apical dominance in contrast to *afp2* plants. The phenotype of *LNJ-OX* plants resembles *jaz-D*, a decuple mutant of JA signaling (Hong et al., 2020). Comparative transcriptomic analysis indeed found differential expression of multiple genes related to JA signaling. Moreover, LNJ was found to be functional in *Brachypodium*, barley, and rice, suggesting that it can be a potential target for crop engineering.

FUTURE OUTLOOK

MicroProteins are small in size and consist of a single functional domain. Its mode of action suggests that miPs act in a dominant-negative manner on the related target proteins. In due course of evolution, miPs have lost their DNA binding domain and retained only the PPI domain to interact with their target and modulate their role in diverse developmental and physiological processes as discussed earlier. It is also evident from the published work that a single miP such as CPC may be involved in multiple regulatory pathways and physiological functions. The advances in analytical, molecular, and computational methods have helped to expand the pool of miPs characterized across species. However, there is a lot to know about the cellular dynamics and specificities concerning the miPs. In this section, we highlight the studies which have been explored in other organisms and may be investigated in plants to answer many fundamental questions about microProteins.

MicroProteins as a potential player in liquid-liquid phase separation

Spontaneous demixing of two distinct phases, a dense phase, and a dilute phase, mediate the formation of membrane-less cytoplasmic and nuclear compartments. These non-stoichiometric phase-separated assemblies hold liquid-like properties and are termed “biomolecular condensates” (Emenecker et al., 2020). These assemblies are mainly composed of proteins and nucleic acids (Emenecker et al., 2020). Plants are exposed to various environmental stresses during their growth and development which cause the evolution of plant-specific condensates formed via liquid-liquid phase separation (LLPS) (Emenecker et al., 2020). It has been observed that sORF-produced protein candidates are enriched in the intrinsically disordered region. It contributes to the flexibility and adaptability of the miPs and enhances their participation in protein-protein interaction networks (Schlesinger and Elsässer, 2022). It has been reported that intrinsically disordered human miP NBDY (NoBody), a 68-amino acid component of membrane-less organelles, also known as P-bodies can undergo liquid-liquid phase separation (LLPS), in the presence of RNA *in vitro* (Na et al., 2021). Phosphorylation of NBDY promotes the dissociation of condensates (Na et al., 2021). It has been reported that an RNA-binding protein FLOWERING CONTROL LOCUS A (FCA), which regulates flowering in Arabidopsis, forms subnuclear condensates having liquid-like properties. The predicted intrinsically disordered prion-like domain is necessary and sufficient for phase separation under experimental conditions (Fang et al., 2019). We have already discussed the roles of miPs CPL3, miP1a, and miP1b in floral transition by interacting with other floral regulators. Example from other organisms might evoke the idea to explore plant miPs functioning as biomolecular condensates which form via LLPS, a biophysical process thought to underlie the formation of membrane-less organelles.

MicroProteins in cell-cell communication

While plant-pathogen interaction studies have been going on for a long time, the role of extracellular vesicles (EVs) mediated intercellular as well as cross-kingdom communication during plant-pathogen interaction has been an open field to explore (H et al., 2022; He et al., 2021; U Stotz et al., 2022). Studies on cell lines in animals and some of the examples from plants suggest that extracellular vesicles mediate the trafficking of proteins, lipids, and short and long non-coding RNAs in a cell-cell manner which results in the reprogramming of various cellular functions (H et al., 2022; He et al., 2021). Cai et al. first reported the EV-mediated transfer of miPs as a novel mechanism in cell-cell communication (Cai et al., 2021). They reported the identified miPs from the EVs as a potential biomarker for glioma diagnosis (Cai et al., 2021). In a similar direction, it's tempting to speculate trafficking of miPs via EVs during pathogen invasion in plants might be leading to the silencing of the pathogen virulence-related genes and proteins. Using the synthetic biology approach,

extracellular vesicles (exosomal vesicles) can be synthesized to target miPs to enhance the plant defense system and improve the yield.

Limitations of the study

One possible future question would be the determination of the size limitation of microProteins. What determines a protein be categorized as microProtein? A B-box domain protein BBX32 which is consisting of a single B-box domain shows PPI with its target proteins. The studies published so far on BBX32 showed that it doesn't have DNA binding capability. BBX32 shows diverse activity where it interacts with BZR1 and PIF3 and modulates cotyledon opening (Ravindran et al., 2021). On the other hand, it interacts with BBX21, a positive regulator of photomorphogenesis, to prevent its binding with HY5 and inhibit photomorphogenesis (Holtan et al., 2011). BBX32 also regulates floral transition by interacting with BBX4 (Tripathi et al., 2017). These studies indicate the diverse functionality and presence of BBX32 in different tissues. But based on its size, it is not considered an miP. While it's paralogs, two B-box proteins BBX30/miP1b and BBX31/miP1a, are considered miPs based on the sequence length. In a similar case, RESPONSE TO ABSCISIC ACID AND SALT1 (RAS1) was reported as miP that negatively regulates salt tolerance and promotes immunity against the bacterial pathogen (Magnani et al., 2014). However, in later reports, it is not mentioned as a miP due to its size of 26 kDa.

There are other questions that need to be investigated further, whether microProteins control the formation of non-functional protein dimers and determine their physiological fate. However, the homodimerization of miPs is still not identified. Still, there is no evidence that miPs could act also non-cell autonomously. To date, the studies on miPs have been limited to their dominant negative effects on their targets. Besides four modes of action, namely, sequestration of multimeric complexes, cytoplasmic retention, repressor formations, and ion-channel inhibitions, whether miPs positively regulate the multimeric complex stability and functionality, is yet to be explored. Also, what are the criteria that will distinguish microProtein from micropeptides and microproteins, is still being consolidated. For example "cyclotide," a group of small proteins, that are often categorized as "microproteins" and made up of 30–40aa residues (Craik et al., 1999). Cyclotides are translated as precursor proteins consisting of multiple domains and undergo post-translational processing including enzymatic cleavage through its cyclic cysteine knot (CCK) motif and subsequent cyclization (Burman et al., 2014). According to Bhati et al. (2018) because of the absence of recognizable protein domains (cyclotides retain only the cyclotide domain after the cleavage and cyclization) and absence of the relationship with larger multidomain proteins these cyclic peptides are not considered microProteins (Bhati et al., 2018). However, their circular topology and cysteine knot, cyclotides are the emerging molecules to be used as drug development and bioimaging tools (Camarero, 2017; Camarero and Campbell, 2019). A few studies on cell lines suggest that some of the cyclotides can also cross cell membranes via endocytic pathways and have the potential to modulate intracellular protein-protein interactions both *in vitro* and *in vivo* (Contreras et al., 2011; Henriques et al., 2015; Ji et al., 2013). Although transfer DNA insertional mutants are available in Arabidopsis stock centers, it's difficult to obtain transfer DNA insertional mutants for the genes coding for microProteins. Because of their short sequence length, microRNA-induced gene silencing procedure is favored to obtain mutants of these genes. Recently CRISPR-Cas editing technology has been employed as a targeted approach for getting knock-out mutants that can enhance microProtein characterization in the future.

Conclusions

Increased anthropogenic activity globally is resulting in stressful environments for plant growth. The cumulative effects of climate change and unsustainable agricultural practices are challenging food security, augmenting the need for finding sustainable and novel solutions. MicroProteins, as discussed above, play a substantial role in regulating various physiological functions in plants. Their small size makes them good candidates for designing synthetic miPs that can be used to promote plant stress resilience leading to enhanced productivity. Modulating the functioning of these small but powerful miPs, and utilizing gene editing tools like CRISPR-cas9 can contribute to crop engineering.

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AUTHOR CONTRIBUTIONS

All authors contributed to the planning of the manuscript. A.K.K., S.D., A.M., and M.L. contributed equally to the writing and figure preparation. All authors helped in finalizing the draft.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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