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Mysterious microproteins as a novel tool for crop improvement: A review

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Abstract
Microproteins (miPs) are minute regulatory proteins that range in size from 5 to 20 kilodaltons. They are known to be produced as a result of the translation of small open reading frames consisting of 150 to 200 nucleotides. They are classified into two categories based on their origin: cis and trans microproteins. Microproteins are involved in various roles throughout the development of plants and animals. Generally, proteins function by forming a complex with similar or dissimilar peptides through protein-protein interactions. The microproteins are known to form a complex with such multi-domain proteins and alter their activity. It is known that miPs control the post-translational regulation of gene expression. Microproteins are essential for the growth and development of plants, including seed germination, seedling development, stomatal opening, root growth, plant pigment production, flowering, and floral development. Synthetic microproteins can be created artificially by delivering genes governing their production through technologies like CRISPR-CAS to target a particular multi-domain protein and regulate its expression. A key step in turning microproteins into practical biotechnological tools for crop bioengineering comprehends the regulatory mechanisms of the microproteins.

Keywords: Microproteins, multidomain, gene regulation, post translation, proteomics

Introduction
Microproteins (miPs) are the small regulatory proteins of size 5–20 kilodaltons known to regulate gene expression at the post-translational level through protein-protein interactions (Straub and Wenkel, 2017) [21]. These proteins contain a single domain encoded by small open reading frames containing up to 150 codons (Thambu *et al*., 2021) [22]. Although few of these proteins are not small, we refer to them as microproteins because the effects of their actions are analogous to those of micro RNAs (miRNAs), which regulate mRNAs negatively. The miPs do not regulate gene expression directly because they lack any DNA-binding or activation domain. However, they are known to regulate indirectly by forming heterodimers by binding with similar proteins. miPs operate as post-translational regulators by building homotypic dimers with their targets and suppressing protein complex function in a dominant-negative manner (Eguen *et al*., 2015) [6].

Deletions and duplications in the genes coding multidomain proteins led to the evolution of single-domain proteins, and these interact with their ancestor protein from where they originated (Straub and Wenkel, 2017) [21]. It is hypothesized that microproteins and the proteins with which they interact have evolutionary relations. Microproteins have been proven to have a significant impact on biological processes in both animals and plants. Through in silico analysis, many putative and functional microproteins in plants have been discovered, but only a few have been structurally and functionally annotated (Bhati *et al*., 2018) [2]. Microproteins can be categorized as cis-miPs or trans-miPs, depending on their origin. Trans microproteins originated through alternate splicing or alternate 5' translation initiation and 3' translation termination site. In contrast, cis-micro proteins evolved through the duplication of genomic regions and subsequent loss of the domains (Kushwaha *et al*., 2022) [13].

DNA binding Inhibitor (Id) protein was the first miP discovered in living organisms. It has a helix-loop-helix domain of 16 kDa size and binds with various transcription regulators such as E2A/TCF3, HEB/TCF12, and E2-2/TCF4 (Ke *et al*., 2018) [9]. E2A/TCF3 in normal and cancerous cells regulates cell proliferation negatively and promotes cell differentiation (Patel and Chaudhary 2012) [16]. HEB/TCF12 is a basic helix-loop-Helix transcription factor that mediates the osteogenic differentiation of bone marrow mesenchymal stem cells (Yi *et al*.,

2012) [35]. The LITTLE ZIPPER (ZPR) protein was the first miP family reported in plants. ZPR contains a leucine zipper domain and modulates plant development by interacting with

other zipper-containing proteins. The ZPR proteins were identified to promote abaxial leaf development in Arabidopsis (Wenkel *et al*., 2007) and shoot apical meristem development (Kim *et al*., 2008) [11].

Recently few microproteins have been identified, and their role in growth and development is delineated. This review deals with the origin of microproteins, techniques to map microproteins, their function in plant growth and development, and strategies for crop improvement through microproteins.

Origin of microproteins

Microproteins are produced due to the translation of small open-reading frames. miPs are produced from long non-coding RNAs (lncRNAs). Generally, the length of lncRNAs contains more than 200 nucleotides (Ma *et al*., 2013) [14]. The trans microproteins originated from alternative splicing, where exons from the same gene are spliced in different combinations (Wang *et al*., 2015) [28]. The alternative initiation and termination of translation of the same pre-miRNA produce them. The other mechanism of origin includes the immature termination of the translation of proteins having multiple domains (Kushwaha *et al*., 2022) [13]. The cis miPs are produced through the deletion of genomic regions coding polypeptide and subsequent loss of the domain (Bhati *et al*., 2022) [2]. The mechanism of origin is shown in Figure 1.

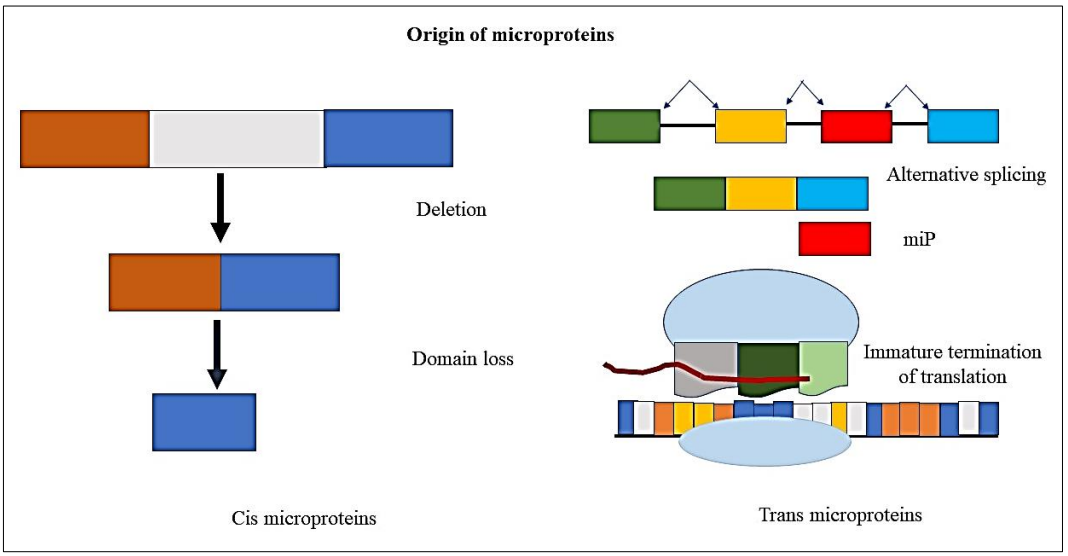

Fig 1: Shows The mechanism of origin

Mode of action

The immediate product produced after the translation of small open reading frames does not have any function on its own (Khitun and Slavoff, 2019) [10]. Generally, they work through protein-protein interactions. It is of two types, *viz*., homotypic and heterotypic interactions (Figure 2). Homotypic interactions occur by binding two identical proteins with similar protein-protein interacting domains. In comparison, heterotypic interactions occur through binding two non-identical proteins but having compatible protein-protein interacting domains (Staudt and Wenkel 2011) [20]. miPs BBX19, BBX30, and BBX31 interact through a homotypic mode (Wang *et al*., 2014) [27], while miP1a and miP1b interact through a heterotypic mode (Graeff *et al*., 2016) [7].

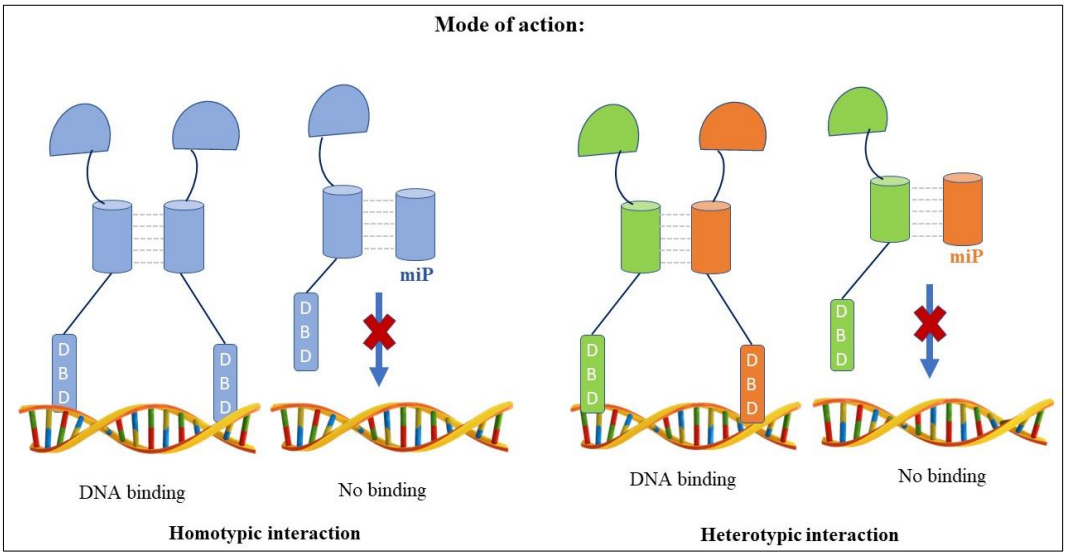

Fig 2: Shows homotypic and heterotypic interactions

Plant microproteins generally regulate gene expression in various ways. Includes

1) Transcription sequestration, where microproteins interact and bind to transcription factors to form a non-functional complex making them incapable of binding to the promoter region. miPs ZPR, KNATM, and HLH act through this mechanism (Magnani *et al*, 2014) [15].
2) Competitive protein interaction: in this case, miP competes with another protein that binds to the target protein; binding of miP to a target protein prevents it from interacting with other associated proteins, allowing it to bind to the promoter region. miPs HLH and R3-MYB act through this mechanism (Andrea *et al*., 2020) [1].
3) Repressor recruitment, miPs recruit repressors and co-repressors to target proteins forming transcription repressor complex. The miPs MIF2 and miP1a/b regulate by forming a repressor complex (Wu *et al*., 2022) [32]. 4) Allosteric regulation, binding of microproteins to transcription factors and receptors causes a change in their native form, making them functionally inactive. The miPs miP1a, miP1b, and BICs cause allosteric changes in their target proteins (Wu *et al*, 2020) [31].

Techniques to map microproteins

Computational approach

The basic principle includes the separation of protein components based on size. The microproteins are separated from small proteins of similar size. After filtration, tools like MiPFinder can identify novel and true microproteins (Straub and Wenkel, 2017) [21]. The MiPFinder utilizes protein information like its size, protein-protein interacting domains present, and evolutionary history. The identified miPs can be evaluated in biological experiments to confirm their function (Kushwaha *et al*., 2022) [13].

Proteomics approach based on mass spectrometry:

Mass spectroscopy and chromatographic techniques were used to separate and identify small proteins. It starts with the extraction of proteins present in the cell and the precipitation of the proteins using organic solvents like acetone and methanol. SDS-PAGE does the separation of proteins with some modifications or ion exchange chromatography. For the effective separation and identification of small proteins, matrix-assisted laser desorption ionization (MALDI) time of flight (TOF) and mass spectroscopy are used. However, the disadvantage of this method is incapable of differentiating small proteins from true microproteins (Kushwaha *et al*., 2022) [13]. The separated proteins are characterized using a protein ID database or transcript assembly to identify true microproteins (Kushwaha *et al*., 2022) [13].

Next-generation sequencing

With the advances in sequencing technologies, a comprehensive analysis can be made to detect microproteins. This approach starts with isolating all protein-coding RNA and sequencing RNAs in a paired-end method. The sequenced reads are mapped on a transcriptome or genome. In-silico predictions are made to identify the putative smORFs. The identified smORFs are validated using a protein ID database or transcriptome assembly to identify true miP coding smORFs (Kushwaha *et al*., 2022) [13].

The computational analysis with MiP Finder can be used along with next-generation sequencing and mass spectroscopy-based approaches to validate and filter true microproteins from other small proteins (Kushwaha *et al*., 2022) [13].

Significance of microproteins (miP) in plant growth and development

Small, single-domain proteins called microproteins bind to target protein complexes directly, preventing them from assembling. There is mounting evidence that microproteins are effective post-translational regulators of protein activity (Wu *et al*., 2022) [32]. Microproteins play a significant role in plant developmental processes such as seed germination, seedling growth, stomatal opening, root development, pigment biosynthesis in plants, flowering, and floral development.

1). Role of microproteins in light signalling responses: Light is one of the leading environmental elements affecting a plant's development. It aids in photosynthesis, detects seasonal and diurnal changes, and serves as a cue for changing a plant's developmental pattern (Chory *et al*., 1996) [5]. Recent research has highlighted the critical roles of microproteins (miPs) in regulating light signalling, which enables plants to thrive in different environmental conditions. The HLH miP type in light signalling is the most researched miP type. Six of the eight Arabidopsis HLH miPs, including PAR1, PAR2, KDR, PRE1/BNQ1, BNQ2, and PRE4/BNQ3, have been demonstrated to be light signalling regulators. Due to the fact that simulated shade quickly and precisely increased PAR1 gene expression, PAR1 was initially discovered as the primary target of plant photoreceptor phytochrome signalling during shade avoidance (Roig-Villanova., 2006) [18]. One of the key participants in light signalling and photomorphogenesis during seedling development is ELONGATED HYPOCOTYL 5(HY5). Microprotein 1A (miP1a)/B-Box Domain Protein 31 (BBX31) (13 kDa) transcription is directly regulated by HY5 under both white and UV light. BBX31 controls photomorphogenesis independently of HY5 (Yadav *et al*., 2019) [34]. microproteins are crucial for plant growth and development since it plays a significant role in light signalling pathways.

2). Role in the development of the photosynthetic organ, The leaves: The leaves are an essential organ in plants since it plays a vital role in photosynthesis, transpiration, guttation, exchange of gases during respiration, storage (as a source of food), and defence. Multiple tissue layers are organized along the dorsoventral (adaxial/abaxial) axis in the leaves. Plants' ability to establish leaf polarity is greatly influenced by transcription factors known as class III homeodomain leucine zipper (HD-ZIPIII). Microproteins of the LITTLE ZIPPER (ZPR) type can bind with HD-ZIPIII proteins to produce attenuated protein complexes. The interaction of these microRNA/microProtein feedback loops controls plant stem cell activity and polarity establishment (Brandt *et al*.,2013) [4]. In addition to their known function in fundamental pattern formation, class III homeodomain leucine zipper (HD-ZIPIII) transcription factors also regulate the beginning of leaf senescence in Arabidopsis (Xie *et al*., 2014) [33].

3). Role in regulating floral development and flowering time: microproteins were identified to regulate developmental activities in plants such as floral organ development and time of flowering. CONSTANS (CO), a potent regulator of

flowering time, physically interacts with two Arabidopsis thaliana microproteins, miP1a and miP1b. The extra carboxy-terminal PF (V/L) FL motif is found in dicotyledonous plant miP1a/b-type microproteins. This motif enables the interaction of miP1a/b microproteins with TOPLESS/TOPLESS-RELATED (TPL/TPR) proteins leading to late flowering under long-day conditions (Graeff *et al*., 2016) [7]. Genetic suppression studies revealed the function of suppressors of mip1a (sum) in suppressing the function of mip1a which is responsible for late flowering induction in plants (Rodrigues *et al*., 2021) [17]. The prominent role of microproteins in regulating floral development is preventing premature floral transitions in shoot apical meristem (SAM) by interacting with proteins responsible for flowering time and floral development in plants.

4). Role in regulating root hair and hair-like epidermal structures (trichomes): Trichomes are the epidermal appendages found mainly on leaves and stems of plants. Trichomes are important during plant development since they act as a barrier to plants from natural dangers like herbivores, UV radiation, disease and pest attacks, excessive transpiration, and during the germination of seeds. Plant trichome initiation, growth, and development are regulated by various transcription factors (Wang *et al*., 2021) [29]. Functional characterization of microproteins was done during the genetic dissection studies of trichome development in Arabidopsis. Out of the 21 genes discovered, one was named TRYPTYCHON (TRY), which encoded for a 106 amino acid (aa) long (13 kDa) protein without a transcriptional activation domain but with the R3 single-repeat MYB domain, indicating the nature of a miP (Hülskamp *et al*., 1994) [8]. Further, through advanced studies, it became known that TRY interferes with GL1, stops its binding to GL3 and successfully disturbs the MBW (MYB-bHLH-WDR) complex, which is the crucial player for trichome development (Tian *et al*., 2017) [23]. CAPRICE (CPC), an MYB microprotein of around 11 kDa, was found during the screening of transfer DNA-tagged lines of Arabidopsis. Compared to the wild type, the CPC mutation produced sparsely dispersed root hairs with no discernible differences in size or shape (Wada *et al*.,1997) [26]. Kirik*et al*. (2004) [12] reported that an MYB miP that enhances TRY and CPC function was functionally characterized.

5). Role in the biosynthesis of plant pigments: Pigments are collections of various natural chemical compounds produced in plants and serve various vital biological purposes. Chlorophylls (Chl), carotenoids, flavonoids, and betalains are the four main categories of pigments (Solovchenko *et al*., 2019) [19]. Differences in anthocyanin concentrations, AtCPC mRNA levels, and differential expression of late flavonoid pathway genes were all associated with the variegated flowering pattern in tobacco. Additionally, Y2H research revealed that AtCPC might bind to the recognized regulators of anthocyanin biosynthesis, Petunia's ANTHOCYANIN 1 (AN1), and JAF13 bHLH proteins. These findings suggested that AtCPC might decrease the transcriptional activity of AN1 and JAF13 to control anthocyanin production adversely (Zhang *et al*., 2009) [36]. Further studies show that Under intense UV radiation, miP1a/BBX31, a gene expressing a miP with a BBX-domain, showed reduced chlorophyll loss and an increase in anthocyanins. However, UV-protective substances such as coumaric acid, hydroxybenzoic acid, and vanillic acid levels were increased by ectopic expression of miP1a/BBX31. The important genes involved in DNA repair, EARLY LIGHT INDUCED PROTEIN1 (ELIP1) and ELIP2, have higher amounts of mRNA when BBX31 is present (Yadava *et al*., 2019) [34]. Thus, microproteins play a regulatory role in pigment biosynthesis. Tetraterpene molecules known as carotenoids generate the yellow, orange, and red colours and build up in chloroplasts in response to severe light stress. They defend plants from ROS generated during stress. PHYTOENE SYNTHASE is the first enzyme that restricts the rate of carotenoid production (PSY). To modify the PIF1-mediated regulation of PSY, the miP PAR1 interacts with the bHLH domain-containing protein PHY-INTERACTING FACTOR 1 (PIF1) (Bou-Torrent.,2015) [3].

List of some microproteins characterized in plants

Microproteins	Species	Function	Reference
TRYPTYCHON (TRY)	Arabidopsis	Trichome architecture	(Hülskamp *et al*., 1994) [8]
CAPRICE (CPC)	Arabidopsis	Root hair development	(Wada *et al*., 1997) [26]
MIP1A AND. MIP1B	Arabidopsis	Light responsive signalling	(Wu *et al*., 2020) [31]
LITTLE ZIPPER(LZ)	Arabidopsis	Leaf architecture	(Wenkel *et al*., 2007) [30]
SlTRY	SOLANUM SPECIES	Trichome and root architecture	(Tominga-wada *et al*., 2013) [24]
3-MYB type miP CAPRICE (CPC)	Tobacco	Pigment biosynthesis	(Zhang *et al*., 2009) [36]
miP1a/ BBX31 and miP1b/BBX30	Arabidopsis	Flower and floral development	(Graeff *et al*., 2016) [7]
Hd1miP	Rice	Flower development	(Eguen *et al*., 2020) [6]

Microproteins – versatile tool for crop improvement

Microproteins are considered a novel tool for post-translational regulation of target proteins since they interact and modify their targets using compatible Protein-protein interaction (PPI) domains. It is possible to hypothesize that synthetic miPs may be created by selecting important proteins involved in physiological pathways for their PPI domains. Hence it can be used to influence different physiological processes that are crucial for plant growth and development. Critical biochemical and physiological processes, such as leaf development, seedling development, flowering time, and floral development stages, can be controlled using synthetic microproteins.

Synthetic microproteins are a valuable tool in targeting and inhibiting target proteins involved in the plant developmental process more precisely and reducing the likelihood of off-target effects. It has a significant potential for biotechnological usage because of its capacity to function as a dominant regulator in a focused manner. (Bhati *et al*., 2018) [2]. Synthetic miPs, because of their small size, can be exploited for plant stress resilience and increased crop productivity. Recent studies show that microproteins are involved in cell-cell communication (H *et al*., 2022); hence, they can be used in plant-pathogen interaction studies. Microproteins are a novel tool in designing crop plants' growth and development process; hence, they can be used in

future crop improvement programs.

Conclusion

Food security is being challenged by the cumulative consequences of climate change and unsustainable agricultural methods, which increases the need for sustainable and innovative solutions since microproteins are essential regulators of several physiological processes in plants. They are excellent candidates for creating synthetic miPs that can be employed to support plant stress resilience leading to increased productivity. Understanding the microproteins' regulatory mechanisms is a crucial step in developing microproteins into useful biotechnological tools for crop bioengineering.

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