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Spotlight

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MicroProteins: Expanding functions and novel modes of regulation

MicroProteins are small, 5–15-kDa single-domain proteins that are evolutionarily related to multi-domain proteins with sequence homology (Eguen et al., 2015). The single domain of microProteins is often a protein–protein interaction (PPI) domain, through which they can interact with their multi-domain protein targets (Figure 1). The first experimental insight that microProteins exist and how they act came from the identification of the regulatory feedback mechanism of class III homeodomain-leucine zipper (HD-ZIPIII) transcription factors by LITTLE ZIPPER (ZPR) microProteins (Wenkel et al., 2007; Kim et al., 2008). In *Arabidopsis*, the LITTLE ZIPPER microProtein family consists of four members (ZPR1-4) containing only a leucine zipper domain. The HD-ZIPIII transcription factor REVOLUTA directly transcriptionally upregulates multiple ZPR genes. ZPR proteins physically interact with their HD-ZIPIII targets and suppress their DNA binding ability. Thus, ZPRs establish a direct negative feedback module that controls the activity of the shoot apical meristem.

Soon after the identification of ZPR-type microProteins, the zinc finger B-box-containing miP1a/b -type microProteins were identified by *in silico* screening of the *Arabidopsis* genome (Graeff et al., 2016). MiP1a/b interact with B-box-containing CONSTANS (CO)/CONSTANS-like (COL) proteins. CO is a prominent flowering activator in *Arabidopsis*, and the interaction of CO with miP1a/b renders transgenic plants overexpressing miP1a/b late flowering. However, instead of inactivating CO through heterodimerization, miP1a/b recruit the TOPLESS co-repressor protein through a short C-terminal peptide motif. Removal of this motif from miP1a/b proteins strongly reduces the efficacy of miP1a/b to delay flowering. Thus, it seems that miP1a/b-type microProteins do not act by simply sequestering transcription factors but by engaging transcription factors in novel transcriptional repressor-type complexes. Surprisingly, a recent report suggests that the function of miP1a/b-type microProteins extends beyond the regulation of B-box-containing transcription factors. Wu et al. (2020) showed that miP1a/b microProteins physically interact with the basic-helix-loop-helix transcription factors PHYTOCHROME-INTERACTING FACTORs (PIFs) and ETHYLENE-INSENSITIVE 3 (EIN3). PIFs hetero-oligomerize with EIN3 to negatively regulate light responses during germination. It was then shown that the *miP1a/b* transcript was rapidly induced by exposing etiolated seedlings to a brief 30-min light pulse. Subsequently, miP1a/b sequester the PIF–EIN3 hetero-oligomer from binding to target promoters and thereby control photomorphogenesis. This finding is particularly intriguing as all microProtein/target interactions identified in the past were homotypic, meaning that the PPI domains involved were the same or highly similar. The finding that microProteins can engage in heterotypic interactions to control targets to which they are not evolutionarily related broadens the scope for microProtein functions.

All so-far identified and experimentally validated microProteins are related to transcription factors. Computational analysis geared to identify small proteins that are evolutionary related to much larger proteins revealed the existence of many potential microProtein candidates belonging to diverse protein classes (Dolde et al., 2018). A synthetic microProtein approach in which potential regions mediating protein interactions of candidate proteins were overexpressed revealed that microProteins could potentially regulate diverse protein classes. It was, for example, shown that overexpression of potential PPI domains of CRYPTOCHROME 1 (CRY1) and BRASSINOSTEROID INSENSITIVE1 (BRI1) resulted in transgenic plants phenocopying the *cry1* and *bri1* mutant phenotypes, respectively. The finding that overexpression of microProteins results in transgenic plants phenocopying the mutant phenotype of the targets they regulate has often been observed. Surprisingly, overexpression of the PAZ domain of DICER LIKE1 (DCL1) as a synthetic microProtein did not inactivate, but rather enhanced, DCL1 activity. These findings suggest that the synthetic microProtein might sequester a potential negative regulator of DCL1 (Dolde et al., 2018; Choi et al., 2020).

The identification of microProteins in complex genomes is not trivial. MicroProtein genes are small and hence might not be annotated in larger crop genomes. Besides, the degree of sequence similarity to the ancestral protein is sometimes low, especially when microProtein genes evolved early during genome evolution. Computational tools allow the identification of microProtein genes based on adjustable sets of criteria (Straub and Wenkel, 2017; Bhati et al., 2020). But, are microProteins important for crop performance, and can they be used as biotechnological targets to improve traits of interest? The first question was recently answered when the *defective tomato meristem* (*dtm*) mutant was cloned. The *dtm* mutant carries a loss-of-function mutation in a LITTLE ZIPPER gene (Xu et al., 2019). Loss of *DTM* function results in smaller tomato plants with enlarged meristems that give rise to fasciated stems, leaves, and flowers.

Other regulators of meristem development are mini zinc finger (MIF)-type microProteins. MIF-type microProteins are related to zinc finger homeodomain (ZHD) proteins that act as chromatin regulators. MIFs physically interact with ZHDs, and this interaction prevents the nuclear import of the heterodimeric complex and abolishes DNA binding (Hong et al., 2011). A recent study in tomato showed that one of tomato MIFs interacted with the ZHD protein KNUCKLES that functions as a meristem regulator. The loss of the tomato MIF microProtein SlIMA (INHIBITOR OF

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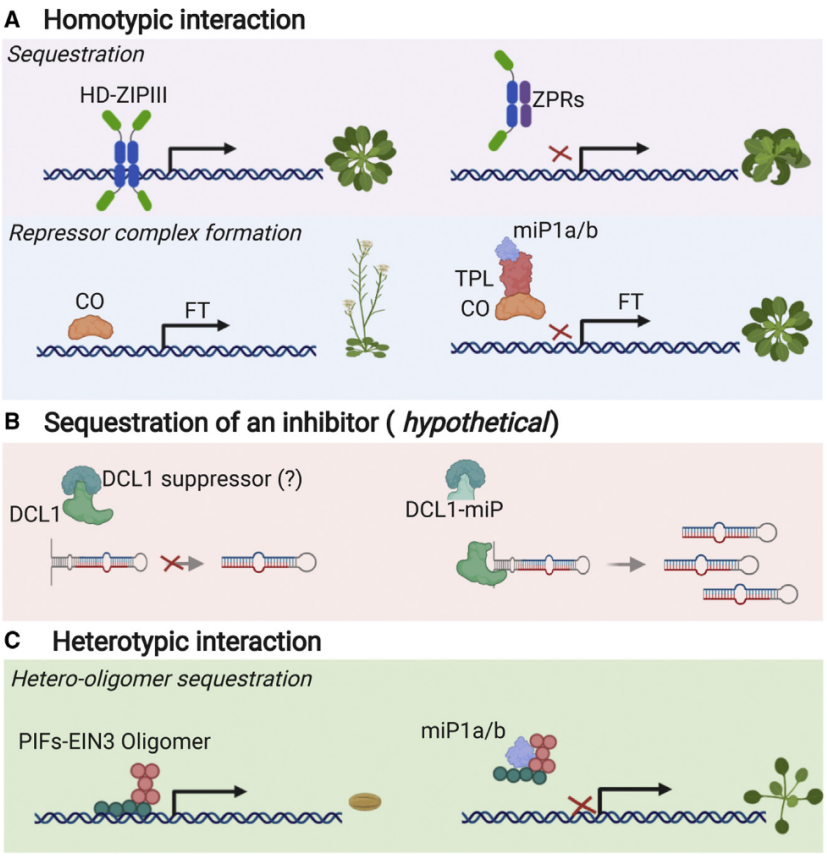

Figure 1. Mechanisms of microProtein-mediated target regulation.

(A) MicroProteins participate in homotypic interactions with the target protein carrying a similar protein–protein interaction domain. Homotypic interactions can lead to the sequestration of the target (here HD-ZIPIII and ZPRs) and the formation of a repressor complex (miP1a/b and CONSTANS-TOPLESS).

(B) The hypothetical model for potential target inhibitor (DCL1 activation by sequestration of a potential inhibitor).

(C) Heterotypic interaction can also lead to the sequestration of protein partners (here miP1a/b and the PIF-EIN3 hetero-oligomer). The schematic illustration was created on the Biorender (biorender.com) platform.

MERISTEM ACTIVITY) caused severe growth defects, including misshapen fruits (Bollier et al., 2018). Interestingly, like miP1-type microProteins, SIIMA also functions as adapter for the TOPLESS co-repressor complex. These examples highlight that microProteins have functional importance beyond *Arabidopsis* and might serve as biotechnological tools to alter traits of interest.

An analysis of microProteins that are present in *Brachypodium* plants has identified a microProtein related to NINJA proteins, called LITTLE NINJA (LNJ) (Hong et al., 2020). LNJ is a potential splice isoform of a gene encoding a NINJA-like protein. Interestingly, LNJ affected NINJA proteins and caused, when overexpressed, the de-repression of jasmonic acid response genes. Overexpression of *LNJ* in *Brachypodium*, rice, and barley resulted in transgenic plants that were stunted but produced a larger number of tillers. Finally, the authors showed in *Arabidopsis* that CRISPR-based genome engineering could be used to convert genes encoding multi-domain proteins into microProteins.

In summary, these few examples highlight the versatility and importance of microProteins to control biological processes. The abundance of small proteins is manifold, and it is safe to assume that many microProteins have not yet been identified. However, recent progress in predicting three-dimensional protein structures and the proximal environment around protein complexes with high accuracy can boost microProtein discovery. In conjunction with improvements of effective genome-engineering strategies, novel functions of microProteins will be uncovered.

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Kaushal Kumar Bhati[1,], Ulla Dolde[2,*] and Stephan Wenkel[3,*]*

[1]Louvain Institute of Biomolecular Sciences, Catholic University of Louvain, Louvain la Neuve 1348, Belgium
[2]Plant Science Research Laboratory (LRSV), UMR5546 CNRS/Université Toulouse 3, 31320 Castanet-Tolosan, France
[3]Copenhagen Plant Science Centre, Department of Plant & Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

***Correspondence: Kaushal Kumar Bhati (kaushal.bhati@uclouvain.be), Ulla Dolde (ulla.dolde@lrsv.ups-tlse.fr), Stephan Wenkel (wenkel@plen.ku.dk)**

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