Commentary

Shall we talk? New details in crosstalk between copper and iron homeostasis uncovered in *Arabidopsis thaliana*

Micronutrients copper (Cu) and iron (Fe) are essential for the growth and development of all organisms. These elements have similar physiochemical properties; thus, it is not surprising that their metabolism is intertwined. Crosstalk between Cu and Fe homeostasis has been documented in many organisms, including plants, but the molecular mechanisms of the homeostatic effect of one element on the other are not well understood. The essentiality of the tight regulation of Cu/Fe crosstalk stems from the fact that while both elements are nutritious in small amounts, they become toxic when they overaccumulate in cells. To account for this, Cu and Fe uptake, and the ratio of Cu : Fe in plant tissues, is tightly controlled in response to local Cu and Fe availability in the rhizosphere and the physiological demands of the developing shoots. In an article published in this issue of *New Phytologist*, Cai *et al.* (2024, 1206–1217) identified a novel aspect of Cu/Fe interplay in model plant *Arabidopsis thaliana* by studying the regulation of Cu homeostasis. Findings by Cai *et al.* bring us a step closer to untangling the complexity of Cu/Fe crosstalk used by plants to ensure balanced Cu and Fe nutrition.

'Understanding the basic principles that plants use to fine-tune their Cu and Fe demands to these elements' uptake, transport and utilization will help in devising targeted biofortification strategies and improving crop yield on Cu- and Fe-deficient soils.'

Plants are self-sufficient autotrophs that, in addition to converting solar energy to the synthesis of organic compounds during photosynthesis, mine the rhizosphere for inorganic compounds such as essential micronutrients Cu and Fe, assimilating, and utilizing them to sustain growth and development. Similar physiochemical properties of Cu and Fe, such as their ability to accept and donate electrons, are key to these elements' essentiality in important biological processes, including photosynthesis and respiration, but this property also acts as a double-edged sword as excess Cu and Fe can cause oxidative stress (Broadley *et al.*, 2012; Ravet & Pilon, 2013). Plants can experience Cu and Fe deficiency in alkaline and organic soils that occupy > 30% of the world's arable land. By contrast, 50% of the world's soils are acidic and can cause Fe toxicity (Broadley *et al.*, 2012; Ravet & Pilon, 2013). Cu, on the other hand, is a common additive in pesticides and insecticides, so uncontrolled use of these substances could cause a buildup of toxic Cu levels in soils. To control internal Cu and Fe concentrations in response to the availability of these elements in the rhizosphere and the physiological demands, plants developed sophisticated transcriptional and posttranscriptional mechanisms to regulate Cu and Fe uptake, internal transport, sequestration, and release from internal stores (reviewed in Riaz & Guerinot, 2021; Rahmati Ishka *et al.*, 2022). Transcriptional regulation of Cu uptake rely on two transcription factors (TFs), SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE7 (SPL7), a homolog of the algal Cu sensor, COPPER RESPONSE REGULATOR1 (CRR1) and COPPER DEFICIENCY-INDUCED TRANSCRIPTION FACTOR1 (CITF1) (Kropat *et al.*, 2005; Yamasaki *et al.*, 2009; Rahmati Ishka *et al.*, 2022; Fig. 1). SPL7, in part, controls the expression of *CITF1*, and both regulate the expression of Cu deficiency-responsive genes (Yan *et al.*, 2017; Schulten *et al.*, 2022). The increased expression of SPL7- and CITF1-regulated genes, including a high-affinity Cu(I) transporter gene, *COPT2*, and *FERRIC REDUCTASE OXIDASES 4* and *5* (*FRO4* and *FRO5*) that reduce Cu(II) to Cu(I) along with the increased expression of *CITF1*, constitute a signature of the Cu deficiency response in *A. thaliana* (Fig. 1). Posttranscriptional regulation of Cu deficiency was reported in other species (van den Berghe & Klomp, 2010) but not yet in plants. In addition to local regulation of Cu uptake, orchestrated by SPL7, a phloem companion cell-localized, plasma membrane Cu/Fe transporter, OLIGOPEPTIDE TRANSPORTER3 (OPT3) participates in systemic shoot-to-root Cu status signaling and crosstalk with systemic shoot-to-root Fe signaling (Araki *et al.*, 2018; Chia *et al.*, 2023).

Transcriptional and posttranscriptional regulation of Fe homeostasis in *A. thaliana* and other nongrass species involves members of the IVb, IVc, and Ib subgroups and FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) of the basic loop–helix (bHLH) TF family (Riaz & Guerinot, 2021; Fig. 1). This TF network regulates the expression of genes encoding the Fe uptake system, including the H^+-ATPase 2 (AHA2), FERRIC REDUCTASE OXIDASE2 (FRO2) and IRON-REGULATED TRANSPORTER 1 (IRT1) (Fig. 1). The upregulated expression of *AHA2*, *FRO2* and *IRT1* in *A. thaliana* and their orthologous in nongrass species is a hallmark of Fe deficiency response in the root (Riaz & Guerinot, 2021). Posttranscriptional regulation of Fe deficiency responses includes phosphorylation events of UPSTREAM REGULATOR OF IRT1

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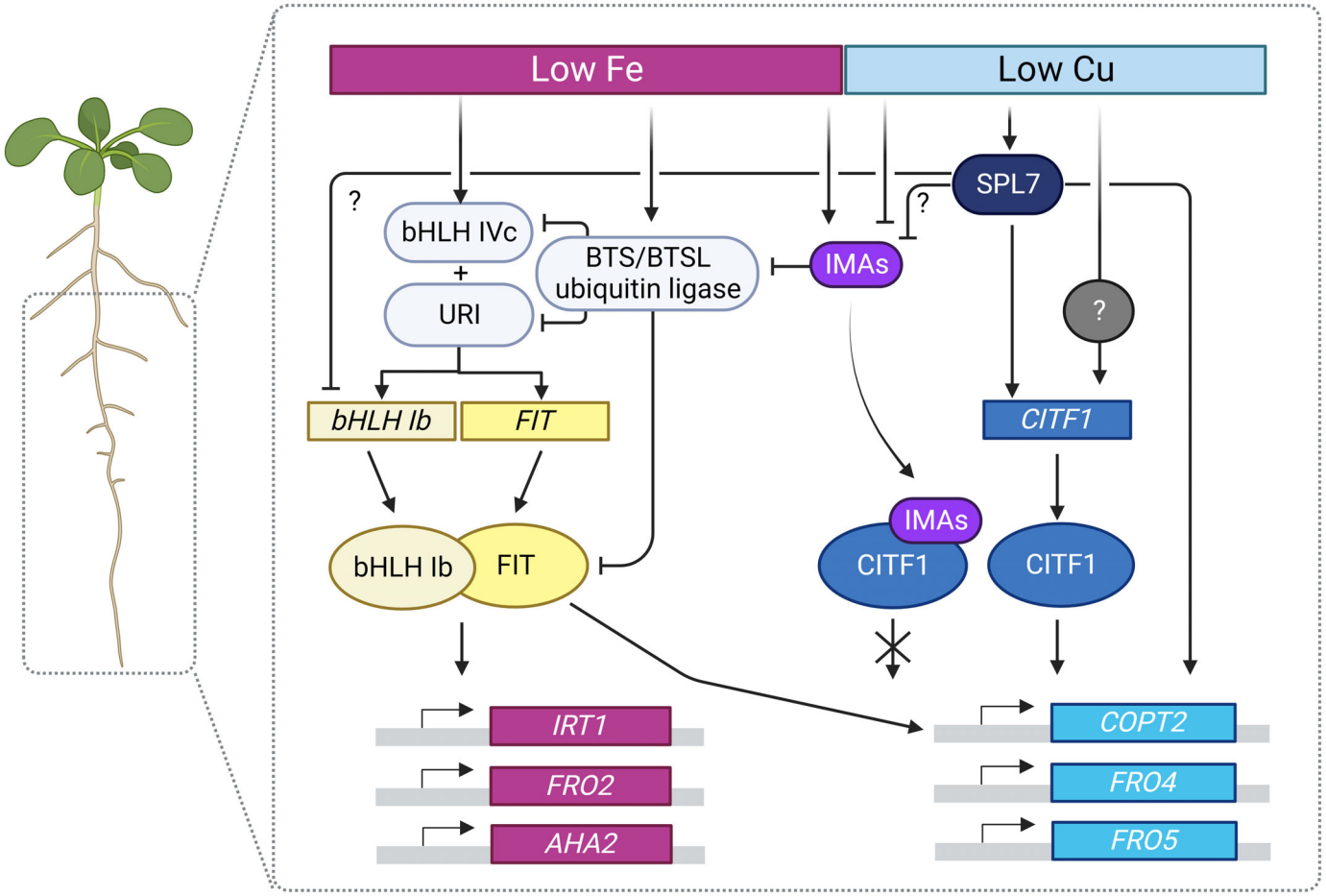

Fig. 1 IRON MAN/FE UPTAKE-INDUCING PEPTIDES (IMA/FEP) play antagonistic roles in Fe and Cu deficiency response. When Fe availability in the rhizosphere is low, the IVc subgroup transcription factors (TFs) from the basic loop–helix (bHLH) family interact with UPSTREAM REGULATOR OF IRT1 (URI), a IVb subgroup bHLH TF, and initiate Fe deficiency responses, including the transcriptional activation of Ib subgroup bHLH TFs and *FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR* (*FIT*). This leads to the activation of genes encoding Fe(II) uptake system that includes IRON-REGULATED TRANSPORTER 1 (IRT1), H^+-ATPase 2 (AHA2), FERRIC REDUCTASE OXIDASE 2 (FRO2). These events lead Fe uptake into roots (reviewed in Riaz & Guerinot, 2021). BRUTUS (BTS) and BTS-LIKE (BTSL) ubiquitin E3 ligases act as negative regulators of Fe deficiency response by facilitating proteasome degradation of IVc subgroup bHLH TFs, URI, and FIT (Riaz & Guerinot, 2021). When Cu deficiency occurs, a master regulator of Cu homeostasis SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE7 (SPL7) activates the expression of *COPPER DEFICIENCY-INDUCED TRANSCRIPTION FACTOR1* (*CITF1*) and genes encoding a Cu transporter COPT2, and cupric reductases FRO4 and FRO5 (reviewed in Rahmati Ishka *et al*., 2022). CITF1 also regulates the expression of *COPT2*, *FRO4*, and *FRO5* directly (Rahmati Ishka *et al*., 2022). In addition, SPL7 acts as a key regulatory hub of Cu and Fe homeostasis by directly or indirectly repressing IRON MAN/FE UPTAKE-INDUCING PEPTIDE (IMA/FEP) peptides and Ib bHLH TFs expression under Cu deficiency (Kastoori Ramamurthy *et al*., 2018). In the posttranslational regulation of Fe and Cu deficiency response, IMA/FEP peptides play important, yet antagonistic roles. IMA/FEP peptides positively regulate Fe deficiency response by physically interacting with BTS/BTSL and inhibiting proteasome degradation of bHLH TFs (Li *et al*., 2021; Riaz & Guerinot, 2021). By contrast, *Cai et al*. (2024; pp. 1206–1217, in this issue of *New Phytologist*) have shown that IMA/FEP peptides negatively regulate Cu deficiency response by interacting with CITF1 and preventing CITF1 from activating *COPT2*, *FRO4* and *FRO5* expression.

(URI) that is a member of IVb subgroup bHLH family; URI initiates bHLH-mediated transcriptional cascades leading to the upregulation of *AHA2*, *FRO2* and *IRT1* and other Fe deficiency-regulated genes (Riaz & Guerinot, 2021). Proteasomal degradation of URI, IVc subgroup bHLH TFs and FIT by the BRUTUS (BTS) and BTS-LIKE (BTSL) ubiquitin E3 ligases (BTS/BTSL) fine-tunes Fe deficiency responses to ensure that an adequate (nontoxic) amount of Fe is absorbed by plant roots (Fig. 1). Long-distance, shoot-to-root, systemic Fe deficiency signaling involves OPT3 that loads Fe into the phloem companion cells in leaves and recirculates it via the phloem to sink tissues including roots, thereby conveying Fe status of the shoot to the root; OPT3 is also involved in Fe delivery to seeds (Stacey *et al*., 2008; Mendoza-Cózatl *et al*., 2014; Zhai *et al*., 2014). As noted above OPT3 also transports Cu and participates in crosstalk between Cu and Fe in shoot-to-root signaling (Chia *et al*., 2023). The recently discovered IRON MAN/FE UPTAKE-INDUCING PEPTIDE (IMA/FEP) peptides are also implicated in shoot-to-root communication of Fe status (Grillet *et al*., 2018; Hirayama *et al*., 2018). The genome of *A. thaliana* encodes eight *IMA/FEP*s, and all are transcriptionally upregulated under Fe deficiency (Grillet *et al*., 2018; Hirayama *et al*., 2018). IMA/FEPs are positive regulators of Fe deficiency

signaling: they compete for binding to BTS/BTSL with IVc subgroup bHLHs, thus relieving these TFs degradation and contributing to the upregulation of *AHA2/FRO2/IRT1* expression (Grillet *et al.*, 2018; Hirayama *et al.*, 2018; Li *et al.*, 2021; Fig. 1).

Cai *et al.* show that *A. thaliana* IMA/FEPs, act as negative regulators of Cu homeostasis, unlike their function in Fe deficiency (Fig. 1). Specifically, the authors showed that, as observed previously (Kastoori Ramamurthy *et al.*, 2018), the expression of all eight IMA/FEP genes was downregulated in roots of Cu-deficient *A. thaliana* seedlings. To evaluate the biological significance of this finding, the authors used octuple mutants (*ima8x*) generated by Grillet *et al.* (2018). Unlike the *ima8x* mutant seedling lethality under Fe deficiency (Grillet *et al.*, 2018), Cai *et al.* found that the *ima8x* mutant was somewhat more tolerant to Cu deficiency as evidenced by the longer root length of *ima8x* vs wild-type. The increased tolerance to Cu deficiency of the *ima8x* mutant observed in Cai *et al.* was accompanied by significantly increased expression of the Cu uptake system, *COPT2*, *FRO4* and *FRO5*. By contrast, ectopic overexpression of *IMA1* or *IMA3* in wild-type plants decreased the root length of seedlings grown under Cu-limited conditions, and these changes were accompanied by decreased levels of *COPT2*, *FRO4* and *FRO5* transcripts.

The IMA/FEP peptides negatively regulate Cu deficiency responses in *A. thaliana* seedlings. But how is this achieved considering that IMA/FEPs are positive regulators of Fe homeostasis? Under Fe deficiency, IMA/FEPs bind to BTS/BTSL and prevent it from degrading IVc subgroup bHLHs, and in doing so, upregulate the expression of the Fe uptake system (Li *et al.*, 2021; Fig. 1). Perhaps, in regulating Cu deficiency responses, IMA/FEPs act on a positive regulator of Cu homeostasis and inactivate it? To test this, Cai *et al.* selected CITF1 as a putative IMA/FEPs interacting partner as the authors showed that the expression of CITF1 targets, *COPT2*, *FRO4* and *FRO5*, but not other Cu deficiency-responsive genes that are controlled mainly by SPL7, and modulated by IMA/FEPs. Using a variety of assays, the authors showed that indeed all eight IMA/FEPs physically interact with CITF1, and these interactions prevent CITF1 from binding to the promoters of its targets and CITF1's ability to activate gene expression (Fig. 1). Finally, the authors generated *A. thaliana* plants lacking functional CITF1 and all eight IMA/FEPs (*ima8x citf1*) and found that the effect of IMA/FEPs on root growth and the expression of *COPT2*, *FRO4* and *FRO5* disappeared. Taken together, these results show that IMAs function in Cu homeostasis is dependent on CITF1.

How is the antagonistic regulation of Cu and Fe homeostasis achieved by IMA/FEPs? How does it relate to Cu/Fe crosstalk? Is this crosstalk biologically relevant? IMA/FEPs are known to bind Fe^{2+} and Cu^{2+} as well as other transition metals such as zinc (Zn^{2+}) and manganese (Mn^{2+}) (Grillet *et al.*, 2018). However, whether metal binding or IMA/FEPs mismetallation affects their function and contributes to the interplay between Cu and Fe homeostasis is unknown. In this regard, it is noteworthy that the hallmark of Cu/Fe crosstalk is the overaccumulation of Cu under Fe deficiency and the overaccumulation of Fe under Cu deficiency in *A. thaliana* roots and shoots (Bernal *et al.*, 2012; Waters & Armbrust, 2013; Kastoori Ramamurthy *et al.*, 2018; Chia *et al.*, 2023). In addition, recent studies showed that Cu and Fe can partially substitute each other in long-distance signaling (Chia *et al.*, 2023). Thus, the relationship of the metal bound to IMA/FEPs to their repressive or promotive function in Cu and Fe deficiency responses, respectively, is a potential area for exploration. Given the nutritive yet potentially toxic nature of Cu and Fe, these interactions are essential for fine-tuning Cu and Fe uptake. This adaptation allows plants to respond effectively to fluctuations in the availability of Cu and Fe in the rhizosphere and aligns with plant physiological demands while avoiding toxicity. Although many unknowns in Cu/Fe interactions remain, the work presented by Cai *et al.* brings us a step closer to untangling molecular components of Cu/Fe interactions and stimulates future studies. Understanding the basic principles that plants use to fine-tune their Cu and Fe demands to these elements' uptake, transport and utilization will help in devising targeted biofortification strategies and improving crop yield on Cu- and Fe-deficient soils.

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