Small-Molecules Selectively Modulate Iron-Deficiency Signaling Networks in Arabidopsis

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Plant growth requires optimal levels of iron (Fe). Fe is used for energy production, numerous enzymatic processes, and is indispensable for cellular metabolism. Recent studies have established the mechanism involved in Fe uptake and transport. However, our knowledge of Fe sensing and signaling is limited. Dissecting Fe signaling may be useful for crop improvement by Fe fortification. Here, we report two small-molecules, R3 and R6 [where R denotes repressor of IRON-REGULATED TRANSPORTER 1 (IRT1)], identified through a chemical screening, whose use blocked activation of the Fe-deficiency response in Arabidopsis thaliana. Physiological analysis of plants treated with R3 and R6 showed that these small molecules drastically attenuated the plant response to Fe starvation. Small-molecule treatment caused severe chlorosis and strongly reduced chlorophyll levels in plants. Fe content in shoots was decreased considerably by small-molecule treatments especially in Fe deficiency. Small-molecule treatments attenuated the Fe-deficiency-induced expression of the Fe uptake gene IRT1. Analysis of FER-LIKE IRON-DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) and subgroup Ib basic helix-loop-helix (bHLH) gene (bHLH38/39/100/101) expression showed that R3 affects the FIT-network, whereas R6 affects both the FIT and Ib bHLH networks. An assessment of the effects of the structural analogs of R3 and R6 on the induction of Fe-dependent chlorosis revealed the functional motif of the investigated chemicals. Our findings suggest that small-molecules selectively modulate the distinct signaling routes that operate in response to Fe-deficiency. R3 and R6 likely interrupt the activity of key upstream signaling regulators whose activities are required for the activation of the Fe-starvation transcriptional cascade in Arabidopsis roots.

Keywords: Arabidopsis thaliana, chemical biology, iron deficiency signaling, iron homeostasis, small-molecules

INTRODUCTION

Many cellular functions occurring during plant growth and development depend on iron (Fe) availability; therefore plants regulate Fe homeostasis by tightly controlling its uptake and allocation. Fe, although abundant in soil, is not so readily available to plants in soils with high pH due to poor solubility (Colombo et al., 2014). Hence, plants employ different mechanisms for efficient acquisition of Fe from soil. To date, two mechanisms have been identified in higher plants, namely Strategy I or the reduction strategy and Strategy II or the chelation strategy (Kobayashi and Nishizawa, 2012; Connorton et al., 2017) for Fe acquisition.
protons, mediated by H9 (PDR9) (Fourcroy et al., 2014; Clemens and Weber, 2016) and of coumarins, facilitated by PLEIOTROPIC DRUG RESISTANCE (Kobayashi and Nishizawa, 2012). For Fe uptake, large amounts of Fe(II)-EDTA2+ are pumped into the rhizosphere. These processes help to solubilize and mobilize the insoluble ferric Fe (Fe3+) in the rhizosphere (Chen et al., 2017; Jeong et al., 2017). Arabidopsis then reduces the soluble Fe3+ into ferrous Fe (Fe2+) by the action of FERRIC REDUCTASE OXIDASE 2 (FRO2) (Robinson et al., 1999) at the cell surface. And the IRON-REGULATED TRANSPORTER 1 (IRT1), a plasma membrane localized divalent cation transporter, then imports ferrous Fe from the extracellular space (Connolly et al., 2002; Vert et al., 2002).

Iron uptake and transport is coordinated by the actions of transcription factors. Several basic helix-loop-helix (bHLH) transcription factors are involved in orchestrating Fe transport and utilization. A subgroup of IIA bHLH member, FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) is involved in controlling the Fe uptake via regulating the expression of IRT1 and FRO2 (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). Members of subgroup Ib of the bHLH proteins (bHLH38/39/100/101) redundantly interact with FIT and control the Fe uptake-associated genes (Wang et al., 2007, 2013; Yuan et al., 2008). Recent studies have revealed the upstream transcriptional regulation under Fe-starvation. The subgroup IVc bHLH factors (bHLH34/104/105/115), form heterodimers among themselves, directly regulate the expression of Ib bHLH genes and indirectly regulate the expression of the FIT (Zhang et al., 2015; Li et al., 2016; Liang et al., 2017). The Ivc bHLH protein levels are post-translationally controlled by BRUTUS (BTS), a hemerythrin E3 ligase, via proteosomal degradation (Selote et al., 2015). BTS has been proposed to be involved in Fe sensing (Kobayashi and Nishizawa, 2015). BTS negatively regulates the Fe-starvation responses. Hindt et al. showed that the BTS paralogs, BTS LIKE1 (BTSL1) and BTS LIKE2 (BTSL2) act redundantly as negative regulators of the Fe starvation response (Hindt et al., 2017). Therefore, both positive and negative regulators coordinately fine tune the plant responses under the Fe starvation response.

To understand the optimal balance between positive and negative regulation, it is important to shed light on the signaling that is specific to each regulator (positive or negative). By modulating selective signaling branches we might be able to dissect the Fe starvation transcriptional network and the related complicated transcriptional machinery. Many molecules/metabolites such as sucrose, putrescine, nitric oxide (NO) and S-nitrosoglutathione (GSNO), and the hormones auxin and ethylene participate in the signaling process and positively regulate Fe-deficiency transcription (Chen et al., 2010; Lin et al., 2015; Lucena et al., 2015; Liu et al., 2016; Zhu et al., 2016; Kailasam et al., 2018); whereas the hormones, cytokinin, abscisic acid (ABA) and jasmonic acid (JA) act negatively on the network (Liu et al., 2016; Cui et al., 2018). A recent study by Garcia et al. (2018) discussed the different signaling modes, in the form LODIS (Long Distance Iron Signal) or LODIS-derived and also via NO/GSNO, to the transcription factors. We previously undertook a chemical screening and dissected the Fe-signaling pathway using a small-molecule named R7 (Kailasam et al., 2018). R7 blocked the transfer of the Fe-deficiency generated signal from NO to the FIT by inhibiting the cellular levels of GSNO, a carrier of NO bio-activity, whose levels are critical for the activation of FIT expression. By using the small-molecule R7, we clarified the signaling pathway from NO (Kailasam et al., 2018).

Despite these findings, the identity of the signal that is transferred to transcription factors from NO is still unclear. Moreover, it is not clearly known whether the Fe-dependent signal is conveyed to the transcription factors through only one route or through many routes. With this focus, we used a chemical biology approach to further dissect the signaling routes of Fe starvation response. The chemical screening undertaken yielded two small-molecules named R3 and R6 (R denotes Repressor of IRON-REGULATED TRANSPORTER 1), whose actions during Fe-starvation are uncovered in this report. Small-molecule treatment resulted in severe Fe-dependent chlorosis and decreased Fe levels in shoots. R6 inhibited the expression of both FIT and Ib bHLH genes whereas R3 only inhibited FIT expression. Our finding clearly reveals that these small-molecules modulate Fe-deficiency by targeting specific signaling branches to central transcription factors, further suggesting that multiple routes are used for transferring the Fe-deficiency born signals to the central transcription factors in roots. Our work also highlights that small-molecules can be used to decode novel signaling pathways that modulate the transcription factors responsible for Fe-deficiency.

**MATERIALS AND METHODS**

**Plant Growth Conditions**

Arabidopsis thaliana Col-0 and the reporter line ProIRT1::LUC (Kailasam et al., 2018) were used. Seeds were surface-sterilized for 4 min in 70% ethanol and treated for 8 min with 1.2% sodium hypochlorite containing 0.02% SDS, finally washed several times in double-distilled H2O. Two-day-stratified seeds were grown on half-strength Murashige and Skoog (½MS) (Duchefa Biochemie) medium supplemented with 2.3 mM MES, 1% sucrose and 0.7% type A agar (Sigma-Aldrich) (pH 5.8). For Fe-sufficiency treatments [50 µM Fe(II)-EDTA], ½MS was used. For the Fe0 condition, Fe was omitted [0 µM Fe(II)-EDTA], whereas for the –Fe condition, 100 µM FerroZine was added to the Fe0 medium. For small molecule treatment, the indicated concentration was added in the medium, whereas in mock treatments dimethyl sulfoxide (DMSO) was added. All plants in this study were grown under a 16-h light/8-h dark photoperiod at 23°C.

**Small Molecule Screening**

The small molecules R3 and R6 were isolated by screening DIVERSet library (ChemBridge, United States) for inhibition of ProIRT1::LUC expression (Kailasam et al., 2018). Briefly, the DIVERSet library compounds were dissolved in DMSO and added a final concentration of 100 µM to 48-well plates containing –Fe medium. Two to three ½MS-grown-seedlings of...
5 day old were transferred to the wells. Two days after treatment, plants were subjected to luminescence analysis. For luminescence assay, plants were submerged in 0.5 mM luciferin solution that contain 0.01% Triton X-100 and kept for 10 min in the dark. The luminescence was then captured by using the IVIS Lumina imaging system (Xenogen Corp., United States) with 1-min exposure times.

**Protein Isolation and Immunoblot**

Total protein isolation and western blot analysis were conducted according to (Shin et al., 2013). Ten-day-old seedlings underwent a small-molecule treatment for 3 day before analysis. Small molecules were used at a final concentration of 50 µM. Total protein from roots was extracted by using protein extraction buffer: 125 mM Tris-HCl (pH 6.8), 15% glycerol, 5.5% SDS, 0.05% 2-mercaptoethanol, and Protease Inhibitor extraction buffer contained 0.01% Triton X-100 and kept for 10 min in the dark. Blots were probed with an anti-IRT1 antibody (Shanmugam et al., 2011).

**Chlorophyll Estimation**

Nine-day-old seedlings that have been grown on ½/MS media were transferred onto ½/MS (Fe50) or Fe0 media with 0 or 50 µM small molecules. After a 9-day treatment, the leaves were harvested and their fresh weight was measured. Total chlorophyll was extracted in 1.0 ml of 80% acetone at 4°C in the dark for 12–16 h until the leaves became white. The clear supernatant was then analyzed in a spectrophotometer (Power Wave XS; BioTek) at 470, 646, and 663 nm spectra. The total chlorophyll content was calculated according to (Wellburn, 1994).

**Determination of Elemental Contents**

Tissue elements were estimated by inductively coupled plasma-optical emission spectrometry (ICP-OES; OPTIMA 5300; PerkinElmer) as described (Shanmugam et al., 2011). Ten-day-old seedlings from ½/MS plates were transferred to ½/MS or Fe0 media containing 0 or 50 µM small molecules and grown for 10 days. Shoots were harvested and rinsed with 10 mM CaCl₂ for 20 min. After washing with de-ionized water, shoots were dried at 70°C for 3 day, then digested with 1 ml 65% HNO₃ (Merck, Tracepur) and 0.5 ml H₂O₂ (Merck, Suprapur). Digested samples were analyzed in ICP-OES for quantification.

**Quantitative Reverse Transcription-PCR**

For gene expression analysis, 9-day-old seedlings from ½/MS plates were transferred to ½/MS or –Fe ½/MS without Fe²⁺-EDTA and with 100 µM FerroZine) containing 0 or 25 µM small molecules. After 3 days of treatment, the roots were harvested. RNA isolation, complementary DNA (cDNA) synthesis and quantitative reverse transcription-PCR (qPCR) analysis were conducted according to manufactures protocol. In short, the total RNA was extracted by using Total RNA isolation kit (GeneDireX). The RNA samples were treated with gDNA wipeout RNase-free DNase (Qiagen) at 42°C for 2 min for genomic DNA contamination elimination. Approximately 1 µg of total RNA was used for first-strand cDNA synthesis by using a QuantiTect Reverse Transcription kit (Qiagen). 25 ng of RNA was subjected for quantitative PCR (qPCR) using Fast SYBR Green Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR instrument (Applied Biosystems). Three biological replicates were used for the quantification of expression of each gene. Each biological replicate was analyzed in triplicate. Relative transcript levels were calculated by normalizing to UBC21. Expression was calculated by using the formula 2⁻ΔΔCT (Schmittgen and Livak, 2008). The primers described (Zhang et al., 2015) were used for bHLH100 and bHLH101. The primers described in (Shin et al., 2013) were used for IRT1 and UBC21. The primers described in (Shanmugam et al., 2015) were used for FRO2 and FIT. The primers described in (Kailasam et al., 2018) were used for bHLH38 and bHLH39.

**RESULTS**

**Small Molecules R3 and R6 Block Fe-Deficiency-Induced IRT1 Expression**

We previously employed a chemical screen on ProIRT1:LUC reporter lines and isolated a small-molecule named R7 (R denotes repressor of IRON-REGULATED TRANSPORTER I) that represses the Fe-deficiency response in Arabidopsis (Kailasam et al., 2018). This screen yielded two more small molecules named R3 and R6. In this report, we analyzed the physiological and molecular responses of plants to understand the role of R3 and R6 in detail. R3 or R6 treatment inhibited the Fe-deficiency-inducible ProIRT1:LUC expression (Figure 1A). Small-molecule treatment caused no luminescence in roots under Fe-deficient medium as compared to mock-treated that showed stronger luminescence. These results suggest that R3 and R6 may modulate endogenous IRT1 expression.

First, to confirm that the small-molecule effect is not due to dysfunctional ProIRT1:LUC under the treatment, we analyzed the endogenous IRT1 level under both Fe sufficient and deficient conditions (Figure 1B). As expected, the IRT1 protein was accumulated under Fe deficiency in mock treatment, whereas R3 or R6 treatment abolished the IRT1 accumulation. This indicates that R3 and R6 (Figure 1C) block the accumulation of Fe-deficiency-induced IRT1 protein.

**R3 and R6 Cause Severe Fe-Deficiency Chlorosis**

Iron deficiency in the environment causes chlorosis and affects the chlorophyll level in plants. To investigate the effect of small-molecule treatment on plant photosynthetic capacity under Fe starvation, phenotypic analysis was conducted (Figure 2). Compared to mock plants whose leaves were pale-green when grown under Fe-limited conditions, small molecule-treated plants were highly chlorotic (Figure 2A). We further measured the chlorophyll level in both Fe sufficient and deficient
conditions. The small molecule treatment caused a decrease in levels of chlorophyll even under Fe-sufficiency and the levels were drastically reduced under Fe-limited conditions (Figure 2B). These data indicate that R3 and R6 perturb the physiological responses to Fe starvation.

**Small-Molecule Treatment Affects Metal Content**

Perturbation in cellular levels of metals often results in chlorosis (Vert et al., 2002). R3 and R6 caused chlorosis; therefore, we next analyzed the cellular level of Fe in response to small molecule treatment (Figure 3). Under Fe sufficiency, R6 treatment did not alter the shoot Fe level, whereas R3 treatment led to a decrease in shoot Fe level (Figure 3A). In Fe-limited medium, the mock treatment showed reduced Fe levels, as expected. The small-molecule treatment caused a drastic reduction in the levels of shoot Fe under Fe-limited conditions. Since, IRT1 also transports manganese (Mn) and zinc (Zn), we then measured Mn and Zn levels. The Mn levels were significantly decreased in response to small molecule treatment under both Fe-sufficiency and -deficiency (Figure 3B). R3 or R6 treatment did not alter the Zn levels in shoots (Figure 3C). These data indicate that R3 and R6 treatment affect cellular metal contents, particularly Fe.

**Fe-Acquisition Genes Are Down Regulated in Small-Molecule Treatments**

The above findings suggested that small-molecule treatment might impair the transcription of genes involved in Fe uptake. To test this, we measured the expression levels of Fe-uptake-associated genes, IRT1, FRO2 and FIT, in response to small molecule treatment in roots. Loss-of-function mutants of these genes display a decrease in cellular Fe levels and chlorosis. We found that IRT1, FRO2, and FIT expression was induced 51.4-, 60.7- and 5.4-fold, respectively by Fe-deficiency in mock-treated plants (Figure 4). The small-molecule treatment strongly inhibited the transcripts of these genes under Fe-deficiency. These results indicate that R3 and R6 inhibit the molecular response to Fe-deficiency by affecting the central transcription factor.

**R3 and R6 Are Involved in Different Signaling Branches of Fe-Deficiency**

FIT forms a dimeric complex with members of the lb bHLH factors (bHLH38/39/100/101) to regulate the expression of Fe-uptake genes, IRT1 and FRO2. Fe-deficiency also induces the transcripts of lb bHLH genes. Hence, we wondered whether small
molecule treatment deregulates the expression of these genes as well or not. Expression analysis of \( bHLH38/39/100/101 \) revealed that their transcripts are indeed induced in mock-treated plants under Fe-deficiency (Figure 5). Interestingly the R3 treatment did not influence the transcript levels of \( Ib bHLH \) genes under Fe-deficiency whereas R6 treatment inhibited the expression. The R6 inhibition level was 29.4, 17.6, 41.8, and 58.0% of the mock treatment for \( bHLH38, bHLH39, bHLH100, \) and \( bHLH101 \), respectively. These data indicate that R3 is not involved in the pathway for \( Ib bHLH \) gene expression whereas R6 is and further implies that small molecules R3 and R6 modulate the Fe-deficiency transcriptional networks selectively.

The reduced expression of Fe-deficiency response transcription factors under small molecule treatment could be the result of defective signaling from plant hormones/metabolites. Auxin, ethylene, NO and GSNO act as positive regulators and exogenous applications of these substances are able to improve plant molecular response and fitness under Fe-starvation. Hence, we were interested in investigating whether providing these substances could alleviate the inhibitory effects caused by R3 or R6. We monitored the \( \text{Pro}^{\text{IRT1}}:\text{LUC} \) expression under R3, R6, or R7, a small-molecule that blocks the signal from NO to FIT (Kailasam et al., 2018). \( \text{LUC} \) expression was not rescued under R3 or R6 treatment by providing any of the positive regulators [naphthaleneacetic acid (NAA) or 1-Aminocyclopropane-1-carboxylic acid (ACC) or GSNO] (Figure 6A). Under R7 treatment, supplying NAA or ACC did not rescue the \( \text{LUC} \) expression either, but supplying GSNO alleviated the R7 inhibition as demonstrated previously (Kailasam et al., 2018). Further we also measured the NO levels under these small-molecule treatments (Figure 6B). There was sufficient NO level, in fact higher, in roots under Fe deficiency upon treatment with any of the small-molecules. These data suggest that R3 and R6 act independently or downstream of these positive regulators.

**DISCUSSION**

Crop improvement toward fortification of Fe has great significance for human health as large populations depend on plant-feeds for dietary Fe. Enhancing Fe levels in plants is therefore useful. In order to achieve this, however; an adequate knowledge of Fe homeostasis is needed. Fe homeostasis in plants is controlled through at least five cellular processes: uptake systems, internal transport and distribution, utilization, storage, and finally the regulation (Connorton et al., 2017). Of these coordinated processes, uptake is the most critical, that depends on soil pH, redox environment and interactions with other minerals (Colombo et al., 2014). To overcome this kind of environment and for efficient uptake, plants have evolved sophisticated mechanisms. Until now two systems for Fe-uptake, Strategy I, and Strategy II have been identified (Kobayashi and Nishizawa, 2012). Much meticulous work has helped to establish the Fe uptake and transport and the regulation process in Arabidopsis (Brumbarova et al., 2015; Curie and Mari, 2017). However, despite this knowledge, the precise sensing, both external and internal, and the associated signaling for Fe availability is still a poorly understood process.

Small-molecule-based chemical biology is an effective approach to dissect the nutrient-starvation response, especially signaling (Bonnot et al., 2016; Kailasam et al., 2018). In the current study, we investigated the role of two small-molecules,
R3 and R6 in Fe deficiency response (Table 1). R3 and R6 blocked Fe-deficiency induced IRT1 expression (Figure 1). The induction of IRT1 is important for coping with Fe-deficiency (Connolly et al., 2002; Vert et al., 2002). Physiological analysis revealed that R3 and R6 severely affected the chlorophyll levels in plants and imporated stronger chlorosis under Fe-limited conditions (Figure 2). This might be the result of reduced Fe levels under small molecule treatment (Figure 3A). Interestingly, under Fe sufficiency, R3-treated plants had a low Fe level with apparent chlorosis as well. By contrast, R6 treatment reduced the chlorophyll content, with no decrease in shoot Fe level under Fe sufficiency (Figures 2, 3A). Hence, total Fe level is not the only reason for chlorosis. The missing link between Fe content and chlorosis was also observed in the triple mutant bhlh39bhlh100bhlh101, which showed no defective total Fe level in shoots but strong chlorosis (Maurer et al., 2014). Treatment with R3 or R6 affected the Mn level (Figure 3B). Surprisingly the Zn level, whose level is subjected to increase under Fe-deficiency (Korshunova et al., 1999; Vert et al., 2002), is not affected under both Fe-sufficient and -deficient conditions (Figure 3C). One possibility for the unchanged Zn levels in shoots is higher translocation rate for Zn. Translocation of Fe, Mn and Zn is depends on the metal-chelating nicotiamine (NA)/citrate levels in the vasculature (Durrett et al., 2007; Schuler et al., 2012). Formation of NA-Zn complexes over Fe and Mn could be favored under...
conditions of limited Fe and the limiting levels of vasculature NA (Palmer et al., 2013). If R3 or R6 treatment brings down the levels of NA, then Zn would be the favorable substrate for the translocation. Other possibility might account for is that small-molecules may block the transport of metals in a selective manner.

Many genes are strongly induced in response to Fe-deficiency (Buckhout et al., 2009). Both R3 and R6 treatments inhibited the expression of Fe-uptake-associated genes IRT1 and FRO2 (Figures 4A,B). This inhibition is due to low transcript levels of central transcription factor FIT under Fe-deficiency (Figure 4C) upon small molecule treatment. FIT is the central modulator and is responsible for the activation of many Fe-deficiency-associated genes in root epidermal cells (Mai et al., 2016). We found that R6 downregulated the expression of I b bHLH genes, FIT-partners under Fe-deficiency, whereas R3 did not (Figure 5). Our findings thus reveal that R3 and R6 may target the transcriptional response through distinct branches under Fe starvation.

The expression of the transcription factors responsible for Fe-deficiency is regulated by the upstream signaling molecules. Any defect in the levels or activity of these signaling-molecules causes decreased expression of transcription factors, and exogenous supply increases the transcription factor expression (Chen et al., 2010; Liu et al., 2016; Kailasam et al., 2018). Based on our data, none of the signaling-molecules (auxin/ethylene/GSNO) alleviated the inhibitory effects caused by R3 or R6 when they were supplied externally (Figure 6). NO levels were higher under R3 or R6 treatment than in the mock (Figure 6C). This supports the notion that R3 and R6 work downstream of auxin/ethylene/NO/GSNO, or alternatively that a novel pathway to the transcription factor exists independent of these hormones (Figures 4–6).

The observed decrease in expression of FIT and I b bHLH transcription factors under R6 treatment (Figures 4, 5) might be due to a blocked signal passage from NO. It has been demonstrated that NO acts immediately upstream to these transcription factors but downstream of auxin (Chen et al., 2010; Garcia et al., 2010; Kailasam et al., 2018). Recently it has
also been shown that IVc bHLH factors (bHLH34/104/105/115) directly control the \( \text{Ib} \) bHLH gene expression and indirectly control \( \text{FIT} \) (Zhang et al., 2015; Li et al., 2016; Liang et al., 2017). However, it is not clear whether or not IVc bHLH transcription factors work under NO. Our previous study showed that NO did not regulate the transcripts of IVc bHLH genes suggesting that control could be post-translational (Kailasam et al., 2018). One possibility that may account for the effect of R6 is that R6 may target these IVc bHLH proteins thereby reducing the expression of \( \text{Ib} \) bHLH and \( \text{FIT} \) genes (Figure 9).

If this is so, it will be worth investigating how R6 regulates IVc factors.

Given that R3 treatment only affected the expression of \( \text{FIT} \) and not the \( \text{Ib} \) bHLH transcription factors (Figures 4, 5) and together with the data in Figure 6, it is highly likely that R3 targets the signaling route that is specific to \( \text{FIT} \) alone (Figure 9). Similar inhibition of the expression of Fe-homeostatic genes was also found with R7 treatment (Kailasam et al., 2018); however, R7 possibly interrupts the signaling pathway from NO to \( \text{FIT} \). GSNO has been shown to be involved in mediating the signal from NO specifically to the \( \text{FIT} \) (Kailasam et al., 2018); however, the precise mechanism and the signal identity is unknown. As the external supply of GSNO did not rescue the R3 inhibition of \( \text{Pro}_{\text{IRT1}}:\text{LUC} \) expression (Figure 6), R3 may interrupt the signal downstream of GSNO or target an independent unknown signaling pathway to \( \text{FIT} \). We did not find any structural similarity between R3 and R7. This suggests the presence of multiple signal inputs for \( \text{FIT} \), whose routes are selectively and independently targeted by the structurally less-related R3 and R7 compounds. Under Fe starvation, a wide range of chemical signals coordinate and trigger the transcriptional response (Liu et al., 2016). Some studies have suggested that cellular Fe, especially the levels in leaf vasculature itself act as a sensing/signaling component (Kumar et al., 2017; Garcia et al., 2018; Khan et al., 2018). Based on these findings, together with action of R3 and R7, it is clear that multilayered signaling networks exist. Importantly, there is lot of interconnection and feed-/forward-back between these signaling molecules, that

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**FIGURE 8** | Effect of R6 structural derivatives. (A) The phenotype of plants under structural derivatives treatment. 9-days 1/2 MS-grown plants were treated in Fe50 or Fe0 for 9 days in the presence or absence of 50 µM small-molecules. (B) The 2D structure of R6 structural derivatives. (C) IRT1 protein accumulation in roots under R6SD1 treatment. 10-days 1/2 MS-grown plants were transferred to +Fe or −Fe with or without R6SD1 for 3 days. IRT1 protein was detected using an anti-IRT1 antibody. CB, coomassie blue stain.
FIGURE 9 | Proposed model for the possible action of R3 and R6 in Fe-deficiency transcriptional-network of Arabidopsis. Fe-status determines the BTS/BTSL stability/function. Under Fe deficiency, BTS is likely degraded, thereby allowing the accumulation of IVC bHLH proteins, which activates the transcription of IVC bHLH genes (directly) and FIT (indirectly). R6 inhibits the Fe-deficiency-stimulated expression of these IVC bHLH targets. R6 may block the IVC bHLH function by directly affecting the stability or indirectly promoting the degradation through BTS action. In addition to these signals, an unknown Fe-deficiency born signal directly activates the FIT transcription. FIT forms a heterodimer with each member of IVC bHLH transcription factors and regulate the Fe-uptake gene IRT1. R3 most likely intercepts this unknown signal, thus repressing the FIT expression. Solid lines indicate data-supported information from previous studies. Open arrow indicates translation. Proteins are represented by colored-ovals and the genes are indicated by colored-boxes.

influence each other, levels and activity under Fe-starvation (Garcia et al., 2011, 2018; Brumbarova et al., 2015; Liu et al., 2016). Therefore, further study of R3 may reveal the identity of a hidden unknown novel component that regulates the central transcription factor FIT.

Assaying the structural analogs of R3 did not help us to narrow down the active region of R3 and indicated that modifying the R3 parent compound will lead to loss of activity (Figure 7). R3 belongs to the benzothiazole class of compounds (Figure 1C and Table 1). Benzothiazole derived compounds are used in clinical studies and the benzothiazole moiety has been widely used as a template structure for the development of therapeutic agents (Ali and Siddiqui, 2013). However, the core benzothiazole (R3SD3) structure alone did not mimic the R3 effect and neither did the other R3 derivatives (Figure 7). Therefore, it seems that the parent structure of R3 itself is necessary for its activity. On the other hand, the structural derivatives of R6 provided some clues about the core motif required for the action of R6 (Figure 8). R6 is a benzofuran class compound. Benzofuran is an important pharmacophore and its derivatives are employed in medicinal chemistry for a wide range of drugs (Khanam and Shamsuzzaman, 2015). The benzofuran core (R6SD4) itself did not produce any observable phenotype under Fe-limited conditions. However, one structural derivative R6SD1 mimicked the R6 effect, in fact the phenotype caused more effect than R6 (Figure 8). The main difference between R6 and R6SD1 is that presence of a carboxylic moiety (-COOH) at the fifth position of the benzofuran unit. R6SD1 does not have a –COOH moiety. It should therefore be worthwhile to study the effect of the –COOH moiety in R6 on Fe-deficiency response. Further, characterizing many structural analogs of R3 and R6 might help us to better understand the core motif required for chemical activity, which will in turn benefit identification of its cellular targets, an important study.

CONCLUSION

The data presented here strongly support the view that small molecules target signaling pathways in the Fe starvation response network and specifically modulate a particular pathway. This work also shows the usefulness of small-molecules in dissecting known signal transduction pathway(s). Furthermore, the selective inhibition of signaling pathways suggests the usefulness of R3 and R6 and chemical genetics per se to interpret networks and to identify new components in Fe-signaling. Based on our observations, the small-molecule R3 targets a novel unknown signaling pathway to the transcription factor FIT, whereas R6 may influence the IVC bHLH transcription factors under Fe-starvation (Figure 9). In summary, this study unraveled a new unknown Fe-signaling route and increases our understanding of plant Fe starvation signaling.

AUTHOR CONTRIBUTIONS

K-CY conceived the research. SK designed and performed the experiments. SK and K-CY wrote the manuscript. W-FC performed the chemical library screening and western-blot. All authors read and approved the manuscript.

FUNDING

This research was supported by grants (to K-CY) from the Ministry of Science and Technology (MOST 107-2321-B-001-018) of Taiwan and Academia Sinica of Taiwan and a postdoc fellowship (to SK) from Ministry of Science and Technology (MOST 107-2811-B-001-578) of Taiwan.

ACKNOWLEDGMENTS

We thank Jing-Chi Lo and Chia-Lin Wu for their assistance.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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