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Molecular Mechanisms Underlying the Impacts of Available-Iron Levels on the Accumulation and Translocation of 6:2 Chlorinated Polyfluoroalkyl Ether Sulfonate in Soybean (*Glycine max* L. Merrill)

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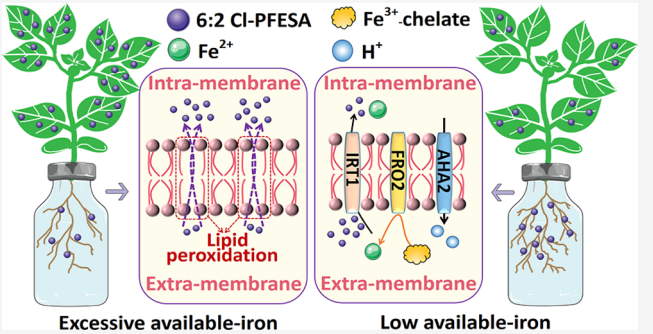
ABSTRACT: Soil available-iron (Fe) is crucial for various physiological properties and processes in plants, particularly those related to the accumulation and translocation of per- and polyfluoroalkyl substances (PFAS). However, the mechanisms underlying the impact of available-Fe levels on PFAS accumulation and translocation in plants remain unclear. In this study, we investigated the impacts of available-Fe levels on the accumulation of an emerging PFAS, 6:2 chlorinated polyfluoroalkyl ether sulfonate (6:2 Cl-PFESA), in soybean, a typical dicot, through hydroponic experiments. Interestingly, Fe deficiency significantly enhanced soybean root volume, surface area, root tip count, and lipid content, thus favoring 6:2 Cl-PFESA adsorption on the root epidermis. However, its absorption and translocation to aboveground tissues were markedly suppressed due to significantly reduced transpiration rate and soluble protein content induced by Fe deficiency. Conversely, although excessive available-Fe also inhibited transpiration, it notably increased root membrane permeability and soluble protein content in aboveground tissues, thus greatly facilitating the absorption and translocation of 6:2 Cl-PFESA within soybeans. These findings demonstrate that appropriate Fe application in agricultural soils is essential to promote the growth of dicot crops and mitigate the potential ecological risks associated with the accumulation of 6:2 Cl-PFESA in the aboveground tissues of soybeans.



KEYWORDS: *6:2 chlorinated polyfluoroalkyl ether sulfonate (6:2 Cl-PFESA), available-iron, absorption, translocation*

INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are widely used in various industries and commercial products owing to their excellent stability and surface properties.[1] As a fluorinated alternative to perfluorooctane sulfonate (PFOS), 6:2 polyfluoroalkyl ether sulfonate (6:2 Cl-PFESA) is frequently detected in various environmental matrices,[2,3] with concentrations in agricultural soils comparable to or even higher than those of PFOS,[4,5] thus posing a great potential for root accumulation and translocation to aboveground plant tissues.[6] PFAS absorption in plants occurs through diffusion from soil to interstitial water, followed by root adsorption. A fraction of the adsorbed PFAS may enter the xylem, translocate to aboveground tissues, and ultimately enter the food chain.[7,8] It is well accepted that absorption and translocation of PFAS within plants are related to plasma membrane (PM) H^+-ATPase,[9] transpiration stream,[10] cell membrane permeability[11] and the protein content of plant tissues.[12] Notably, exogenous substances can alter these physiological processes. Our previous studies found that fulvic acid facilitated the absorption of 6:2 Cl-PFESA in wheat roots by stimulating the activity of PM H^+-ATPase,[9] while humic acid increased 6:2 Cl-PFESA transmembrane potential by regulating slow-type anion channels mediated by Ca^{2+}-dependent protein kinases.[13]

Iron (Fe) is a trace but essential nutrient for plant growth,[14] which plays a vital role in photosynthesis, respiration, and cellular redox processes.[15] Although the total Fe content in agricultural soil is high, it tends to form insoluble ferric hydroxide complexes and becomes unavailable to plants,[16] especially in alkaline soil, where Fe deficiency is common. It is reported that roughly one-third of the world's soils are calcareous, with approximately 40% being Fe-deficient.[16] Fe deficiency could lead to plant chlorosis and reduced crop yield and quality.[17] To cope with Fe deficiency, plants evolve two primary strategies to solubilize and absorb Fe from the soil: the

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reduction strategy (Strategy I) and the chelation strategy (Strategy II).[18,19] Dicot plants, such as soybean, tomato, and pea, as well as nongraminaceous monocots employ a reduction-based strategy (Strategy I), where root acidification increases the solubility of Fe^{3+} through H^+-ATPase-mediated proton extrusion in PM, followed by reduction of Fe^{3+} to Fe^{2+} and subsequent absorption of Fe^{2+} in the roots.[20] Strategy II is used by graminaceous monocots, such as rice and maize. These plants use a chelation strategy in which they exude phytosiderophores to chelate with Fe^{3+}, and the resulting complexes are imported by the roots.[21,22] As a typical dicot, soybean develops a reduction strategy, particularly in alkaline soils in arid and semiarid areas.[23] Fe deficiency affects root morphology, such as root length and lateral root development, as well as various plant physiological processes, including PM H^+-ATPase and protein synthesis.[19,24] Iron-regulated transporter 1 (IRT1) is the main transporter of Fe^{2+},[25] which generally exhibits low selectivity for substrates. It is overproduced under Fe-deficient conditions and enters cells through endocytosis.[25] Yang et al. found that the amino acid transporter bound with substituted polycyclic aromatic hydrocarbons (PAHs) and facilitated their absorption in legume plants.[26] Similarly, PFAS tend to bind to multiple proteins due to their proteophilic properties.[27] However, it remains unclear whether IRT1 can bind to PFAS and indirectly participate in their absorption or if PFAS absorption is, instead, facilitated by endocytosis under Fe-deficient conditions. In agricultural practices, an appropriate amount of Fe fertilizer is beneficial for plant growth.[28] However, excessive application, which is common due to improper fertilization,[29] can damage root cell membranes by triggering the production of reactive oxygen species (ROS). This, in turn, increases the potential for facilitating the absorption and translocation of 6:2 Cl-PFESA in plants.[30] Thus, it is hypothesized that the available-Fe levels in soil may significantly affect the accumulation and translocation of PFAS by altering multiple physiological properties and processes in soybeans, though the underlying mechanisms need further investigation.

In general, PFAS are absorbed by plant roots through the apoplast (intercellular spaces) and symplast (passing from cell to cell through the plasmodesmata) pathways. Since the apoplast pathway is generally blocked by the Casparian strip in the endodermis, PFAS must cross at least one lipid bilayer to reach the endodermal symplast and enter the xylem before being subjected to translocation.[6] Thus, the transmembrane transport of PFAS into the symplast pathway is critical in their translocation to aboveground tissues. The transmembrane transport of PFAS has typically been assessed using subcellular fractionation of plant roots in the previous studies.[13] However, PFAS in the apoplast pathway cannot be effectively separated using this approach, potentially leading to misleading results. Therefore, it is essential to accurately evaluate the impact of available-Fe levels on the transmembrane transport of PFAS in soybean roots and their translocation potential by characterizing the distribution of PFAS in the apoplast and symplast of soybean roots.

This study aimed to investigate the impact mechanisms of available-Fe levels on the accumulation and translocation of 6:2 Cl-PFESA in soybeans through hydroponic experiments. The extraction method was optimized to effectively separate apoplast and symplast fractions in soybean roots, enabling direct investigation of the transmembrane transport of 6:2 Cl-PFESA. Molecular docking and pharmacological inhibition experiments were performed to indirectly elucidate the role of IRT1 in 6:2 Cl-PFESA absorption in soybean roots. Multiple plant physiological properties were monitored to identify the major factors influencing the accumulation and translocation of 6:2 Cl-PFESA in soybeans regulated by available-Fe levels. This study provides valuable insights for optimizing the application of Fe fertilizers in agricultural soils to reduce the ecological risks associated with PFAS in soil-plant systems.

MATERIALS AND METHODS

Chemicals and Reagents. Details of chemicals and reagents used are provided in Text S1 of the Supporting Information.

Exposure Experiments. Soybean (*Glycine max* L. Merrill, variety: Dongsheng-1, which is widely cultivated in China due to its strong adaptability) is a globally cultivated crop that exhibits high sensitivity to soil available-Fe and serves as a representative dicot plant that employs Strategy I to cope with Fe deficiency.[31] A hydroponic exposure experiment was conducted to minimize interference from soil microorganisms and components.[32] The design of the hydroponic experiment followed the methods from our previous studies.[9] In brief, intact soybean seeds of similar size were disinfected with an 8% H_2O_2 solution for 15 min, rinsed with deionized water, and then soaked at room temperature for 12 h. The seeds were placed in a dark germination tank for 5 days. Germinated seedlings with similar growth were transferred to five sterile nutrient solutions (1/4 strength Hoagland's solution) containing varying levels of available-Fe. The selected levels of available-Fe (Fe^{2+}) were according to the results previously reported in field soil, which ranged from 5.49 to 163.34 mg kg^{-1}, with a mean value of 28.16 mg kg^{-1} (25.2 μM).[33] Soils with Fe concentration exceeding 50 mg kg^{-1} (44.8 μM) were considered as having excessive available-Fe.[34] The corresponding concentrations were converted to Fe^{2+} under hydroponic conditions based on the soil solution ratios in the field. Fe^{3+} is generally the predominant form of Fe in Fe-deficient soils.[35] Generally, Fe^{2+} is directly absorbed and utilized by soybeans due to its high bioavailability, whereas Fe^{3+} is not readily absorbed and utilized by soybeans due to its low bioavailability. Although soybeans are capable of reducing Fe^{3+} to Fe^{2+} for absorption, the transformation remains limited.[25] Thus, Fe^{3+} was chosen to more realistically simulate Fe-deficient conditions (low available-Fe). One solution without any Fe and two solutions containing 25 μM Fe^{3+} and 75 μM Fe^{3+} ($Fe_2(SO_4)_3$), designated as LAF1, LAF2, and LAF3, respectively, were prepared. Two other solutions with 25 μM Fe^{2+} and 75 μM Fe^{2+} ($FeSO_4{\cdot}7H_2O$) were prepared and labeled as AAF (appropriate available-Fe) and EAF (excessive available-Fe, simulating overfertilization), respectively. Soybean seedlings were cultured in a light incubator with a cycle of 14 h at 27 °C (day) and 10 h at 22 °C (night). After 8 days, three uniform-sized seedlings were transferred into a 125 mL brown polypropylene (PP) bottle containing 100 mL nutrient solution with 6:2 Cl-PFESA.[9] The selected exposure concentration of 6:2 Cl-PFESA was determined based on its environmental concentrations in agricultural soils, its bioavailability following water irrigation and sewage sludge application, and the detection limit of the analytical instrument.[1,5] Its initial concentration was 50 μg L^{-1}, as determined by LC−MS/MS. After exposure periods of 12, 24, 48, 72, 96, 144, and 192 h, the three-soybean seedlings (including root, shoot, and leaf) cultured in a single bottle (n = 3) were harvested and pooled as

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one sample, and the solution was also collected. During the exposure period, the PP bottles were randomly placed in a light incubator, and 2 mL/day of nutrient solution was added to each bottle to replenish the solution lost through evaporation and plant absorption. After pretreatment, the concentrations of 6:2 Cl-PFESA in soybean tissues and solution were determined, and the remaining samples were stored in liquid nitrogen at −80 °C for soybean physiological analysis. Blank groups, including 6:2 Cl-PFESA-spiked nutrient solution without plants (Blank A) and plants in nutrition solution without 6:2 Cl-PFESA (Blank B), were set up to determine the sorption of 6:2 Cl-PFESA on the bottle's inner surface and possible volatilization of 6:2 Cl-PFESA from the exposure system. Additionally, *in vivo* and *in vitro* transformation experiments of 6:2 Cl-PFESA in soybeans, along with crude enzyme extraction, were conducted based on the method described in a previous study,[9] with details provided in Text S2.

Sample Pretreatment and Extraction. The pretreatment and extraction procedures for 6:2 Cl-PFESA from soybean tissues and nutrient solutions were adapted from our previous studies with minor modifications.[32,36] Detailed information is provided in the Text S3.

Measurement of Enzymatic Activities and Soluble Protein, Lipid, and ROS Concentrations. The level of malondialdehyde (MDA), the activity of PM H^+-ATPase, and the concentration of ROS were measured using ELISA kits and a multimode microplate reader. ELISA kits were purchased from Jiangsu Meimian Industrial Co., Ltd. The methods for determining soluble protein and lipid content in soybean tissues were adapted from a previous study with some minor modifications,[37] as detailed in Texts S4 and S5.

Determination of Membrane Permeability of Soybean Roots. The membrane permeability of soybean roots was assessed by measuring electrolytic leakage from the cell membrane following the method of Wei et al. with minor modifications.[38] Detailed procedures are provided in Text S6.

Extraction of Apoplast and Symplast Saps in Soybean Roots. Apoplast sap extraction from soybean roots was based on the vacuum-infiltration−centrifugation method by Zhang et al.[39] and Wu et al.[40] with modifications to the centrifugal force for effective separation. Since the root structure of soybean is different from that of wheat, an appropriate centrifugal force is essential to keep the PM intact. Glucose-6-phosphate dehydrogenase (G6PDH), a key intracellular enzyme in the plant pentose phosphate pathway, was used to determine whether apoplast sap was contaminated by symplast sap.[41] If the ratio of G6PDH activity in the apoplast extract to that in the total protein extract was less than 1%, contamination by symplast sap was considered negligible.[42] Five centrifugal speeds (1000, 2000, 3000, 4000, and 5000 g) were selected to determine the activity of G6PDH in the apoplast extract. As shown in Table S1, the activity ratio of G6PDH was less than 1% at 1000 and 2000 g. When the centrifugal speed reached 3000 g, the G6PDH activity ratio exceeded 1%, suggesting cell membrane rupture and leakage of symplast sap. Therefore, 2000 g was selected as the optimal centrifugal speed for apoplast sap extraction. Further details on the extraction process are provided in Text S7.

Molecular Docking Analysis. The soybean IRT1 protein structure was obtained through online homology modeling (https://swissmodel.expasy.org).[43−45] The molecular docking mode between 6:2 Cl-PFESA and IRT1 was determined using AutoDock (v.1.5.7).[46] The conformation with the lowest binding energy was selected as the bioactive conformation, and the interactions between IRT1 and 6:2 Cl-PFESA were visualized using Pymol and Ligplot. Additional details are available in Text S8.

Effect of IRT1 on the Absorption of 6:2 Cl-PFESA in Soybean Roots. Two inhibition tests were conducted. Since there are currently no specific inhibitors targeting clathrin-mediated endocytosis of IRT1 in plants, chlorpromazine hydrochloride (30 μM), which has been previously reported to inhibit clathrin-mediated endocytosis in wheat,[47] was selected to explore whether clathrin-mediated endocytosis of IRT1 affects the absorption of 6:2 Cl-PFESA in soybean roots. Brefeldin A (10 μM), as an exocytosis inhibitor, was added to the solutions to inhibit the transport of IRT1 between the endoplasmic reticulum and Golgi apparatus. All the exposure conditions were consistent with the above experiments, and all treatments were harvested after 24 h. To minimize interference caused by 6:2 Cl-PFESA adsorbed on the root epidermis, the roots were rinsed with a methanol−water solution after sample collection to remove the adsorbed fraction. All the treatments were performed in triplicate.

Instrumental Analysis and Data Processing. 6:2 Cl-PFESA concentrations were analyzed using ultraperformance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS, Xevo TQ-S, Waters, Milford, MA), operated in negative electrospray ionization (ESI^-) and multiple reaction mode (MRM). The sample injection volume was set to 10 μL, and separation was conducted on an ACQUITY C18 column (Waters, 1.7 μm, 2.1 mm × 50 mm). Mass spectrometry operating parameters are listed in Table S2 of the Supporting Information.

The root concentration factor (RCF) and leaf translocation factor (LTF) were calculated according to eqs 1 and 2.

$$\mathrm{RCF} = C_{\mathrm{root}}/C_{\mathrm{solution}} \tag{1}$$

$$\mathrm{LTF} = C_{\mathrm{leaf}}/C_{\mathrm{root}} \tag{2}$$

where C_{root}, C_{leaf}, and C_{solution} represent the concentrations of 6:2 Cl-PFESA in soybean root (ng g^{-1}), leaf (ng g^{-1}), and solution (ng L^{-1}), respectively.

All experimental data were presented as means and standard deviations (SD) of three replicates. Statistical significance was assessed using SPSS 22.0 (Chicago, IL), with significance set at $p < 0.05$.

Quality Control and Quality Assurance. Quality control was conducted by regularly analyzing procedural and reagent blanks after every 10 samples to monitor instrumental and background contamination. Quantification of 6:2 Cl-PFESA was performed using mass-labeled internal standard calibration curves with six points ($R^2 > 0.997$). A recovery experiment was conducted using the soybean tissues that had not been exposed to 6:2 Cl-PFESA. Recoveries of 6:2 Cl-PFESA were determined by spiking soybean tissues with 5.0, 10.0, and 15.0 ng of the target compound. The pretreatment and extraction procedures are detailed in the Text S3. The mean recoveries ($n = 3$) of the 6:2 Cl-PFESA ranged from 86.9 to 97.4% for soybean roots, shoots, and leaves. The method detection limit (MDL) and limit of quantification (LOQ) were determined based on signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. Detailed information on mean recoveries, MDL, and LOQ of 6:2 Cl-PFESA are provided in Table S3.

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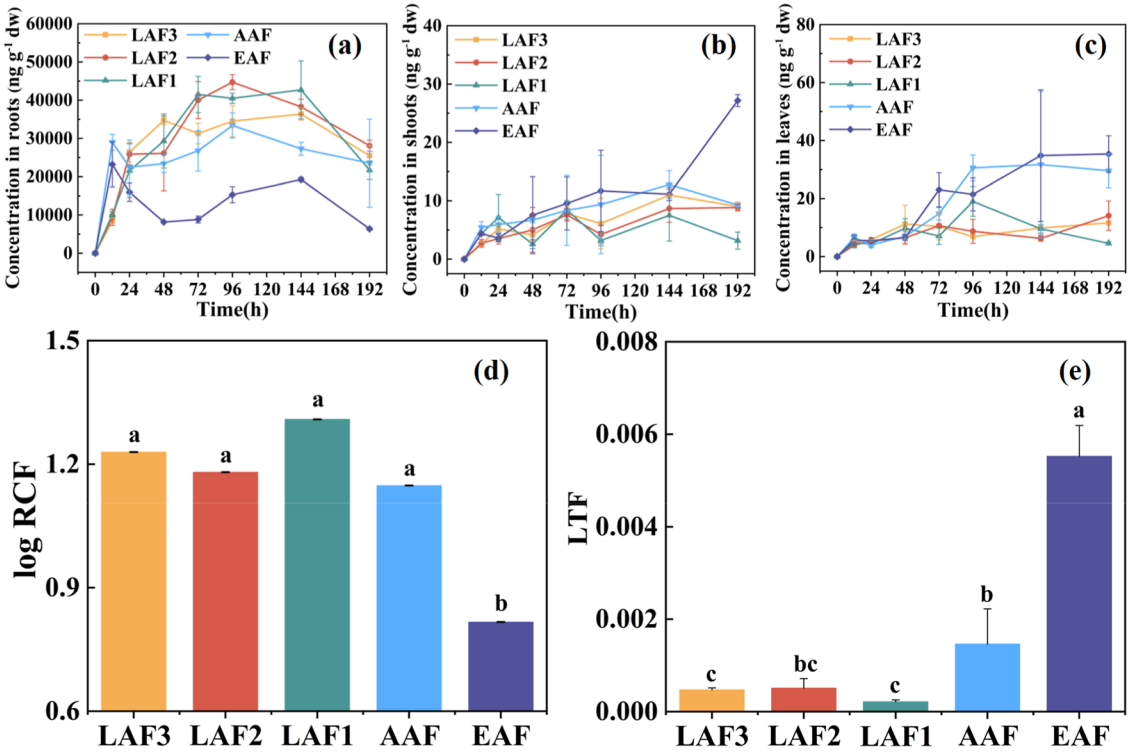

Figure 1. Time-dependent concentrations of 6:2 Cl-PFESA in the soybean roots (a), shoots (b), and leaves (c) under different available-iron levels. Mean values of root concentration factor (log RCF) (d) and leaf translocation factor (LTF) of 6:2 Cl-PFESA (e). Error bars represent the mean ± standard deviation (n = 3). Different letters indicate significant differences (p < 0.05).

RESULTS AND DISCUSSION

Effects of Available-Fe Levels on the Accumulation and Translocation of 6:2 Cl-PFESA in Soybean. After 192 h of exposure to 6:2 Cl-PFESA, the soybean seedlings grew well without any signs of wilting or mortality in all treatment groups. The concentration of 6:2 Cl-PFESA in the Blank A solution remained between 81.8 and 93.5% of the nominal dose, indicating that the loss due to evaporation or adsorption on bottle walls was negligible. The root biomass was lowest in the EAF treatment, followed by the AAF treatment, while the biomass in the LAF treatments was significantly higher (p < 0.05) than in the AAF and EAF treatments (Table S4). However, the biomass of shoots and leaves showed no significant differences among treatments (p > 0.05). Figure 1a–c illustrate the temporal trends of 6:2 Cl-PFESA concentrations in soybean roots, shoots, and leaves across all treatments. The concentrations of 6:2 Cl-PFESA in roots initially increased rapidly, reaching the maximum levels of 40,498, 44,722, 34,525, 28,986, and 23,204 ng g^{-1} in LAF1, LAF2, LAF3, AAF, and EAF, respectively. This was followed by a gradual decrease, likely due to the translocation of 6:2 Cl-PFESA to the aboveground tissues, the transformation of 6:2 Cl-PFESA within the soybean, or/and the growth dilution effect. After 192 h of exposure, the mass composition ratio of 6:2 Cl-PFESA in the aboveground tissues across treatments accounted for approximately 0.024–0.203% of the initial mass in the whole system, suggesting that the amount of 6:2 Cl-PFESA transported from roots to aboveground tissues was relatively low (Figure S1). One of our previous studies found that the transformation rate of 6:2 Cl-PFESA to form 6:2 H-PFESA in wheat was very low (0.46‰).[9] In this study, 6:2 H-PFESA was not detected in either *in vivo* or *in vitro* experiments (Table S5). Furthermore, the mass of 6:2 Cl-PFESA did not significantly decrease over the 192 h exposure period, suggesting that the transformation of 6:2 Cl-PFESA within soybean tissues was marginal. Notably, the mass of 6:2 Cl-PFESA in soybean roots stabilized after 96 h of exposure (Figure S2), while the root biomass significantly increased (p < 0.05). These findings suggest that the observed decrease in root 6:2 Cl-PFESA concentrations was primarily attributed to the growth dilution effect. The log RCF values were calculated to assess the accumulation potential of 6:2 Cl-PFESA in soybean roots. As shown in Figure 1d, the average log RCF values of 6:2 Cl-PFESA were higher in the three LAF treatments [LAF1 (1.31), LAF3 (1.23), LAF2 (1.18)] than in the AAF and EAF treatments [AAF (1.15), EAF (0.816)]. Previous studies suggest that root accumulation of 6:2 Cl-PFESA involves both adsorption on the root epidermis and absorption into the internal root compartments, with adsorption on the root epidermis being predominant.[9] These results suggest that the adsorption of 6:2 Cl-PFESA on soybean roots was significantly promoted (p < 0.05) in the LAF treatments, possibly due to the increased root specific surface areas, thus providing more adsorption sites for 6:2 Cl-PFESA.

In Blank B, 6:2 Cl-PFESA was not detected in the shoots and leaves of soybean, suggesting that absorption from the air was negligible. The concentrations of 6:2 Cl-PFESA in shoots gradually increased throughout the exposure period, reaching 9.04, 8.86, 3.18, 9.29, and 27.2 ng g^{-1} after 192 h in LAF3, LAF2, LAF1, AAF and EAF, respectively. Similar trends were observed in leaves, with concentrations of 11.6, 14.1, 4.58, 29.6, and 35.4 ng g^{-1}, respectively. Considering that leaves serve as the final site of 6:2 Cl-PFESA accumulation in aboveground tissues, LTF values were calculated to evaluate

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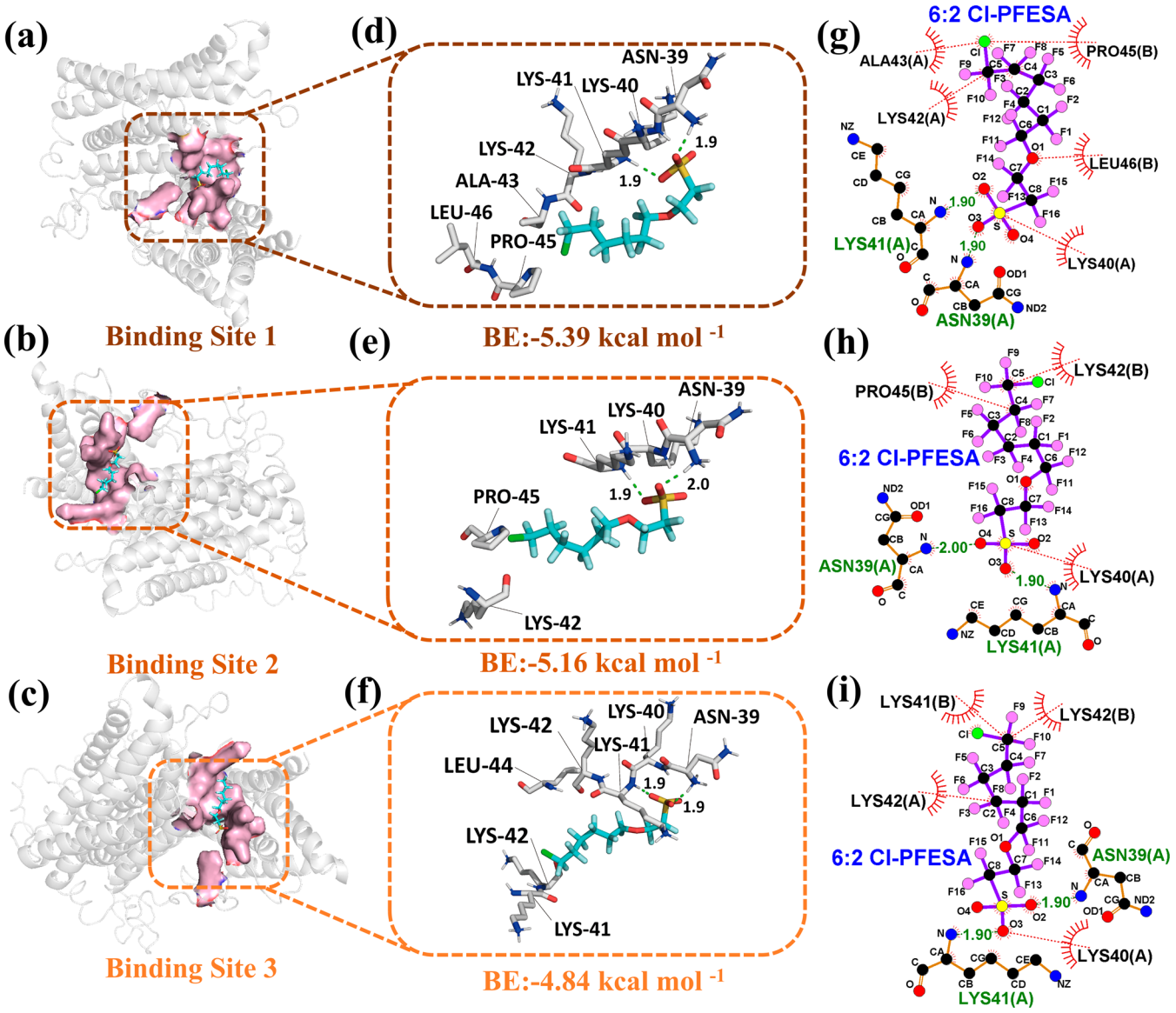

Figure 2. Possible binding conformations of 6:2 Cl-PFESA with IRT1 (a−c). Interactions in the lowest binding energy state, with binding energies shown below each graph (d−f). Green lines represent hydrogen bonds, with corresponding residues and bond lengths labeled. Molecular representation of the top docking complex from the molecular docking of 6:2 Cl-PFESA with IRT1 (g−i). Green dashed lines indicate hydrogen bonds, dark red semicircular residues represent hydrophobic interactions, and green-labeled residues denote potential hydrogen bond formation sites.

the translocation ability of 6:2 Cl-PFESA from roots to aboveground tissues. As shown in Figure 1e, the LTF values followed the order of EAF > AAF > LAF2 > LAF3 > LAF1, with significantly higher ($p < 0.05$) values in EAF compared to other treatments, in contrast to the trend for log RCF observed. Notably, the LTF values in the EAF treatment were approximately 10−28 times higher than those in the AAF and LAF treatments, respectively. Additionally, the mass composition ratio of 6:2 Cl-PFESA translocated to the aboveground tissues of soybean followed the order: EAF (0.203%) > AAF (0.103%) > LAF3 (0.079%) > LAF2 (0.074%) > LAF1 (0.024%), with the EAF treatment being significantly higher ($p < 0.05$) than the other treatments (Figure S1). Generally, most of the 6:2 Cl-PFESA accumulated in soybean roots was adsorbed on the root epidermis, and only a small fraction was absorbed and translocated to the aboveground tissues.[6] Therefore, the translocation of 6:2 Cl-PFESA from soybean roots to shoots is distinctly related to its absorption by soybean roots. These results indicate that excessive available-Fe greatly enhanced the absorption and translocation of 6:2 Cl-PFESA to aboveground tissues, even though root adsorption was significantly lower. This further strengthens our previous finding that not all PFAS adsorbed on roots are absorbed and translocated to aboveground tissues.[6,48]

As aforementioned, the higher log RCF values observed in the LAF treatments may be attributed to the increased root specific surface areas, which provide more adsorption sites for 6:2 Cl-PFESA under Fe-deficient conditions. Consequently, root phenotypic parameters likely play a crucial role in this process. The results from root scanning indicated that the root length, surface area, and tip count in the LAF treatments were significantly higher ($p < 0.05$) than those in the AAF and EAF treatments (Figures S3 and S4), suggesting an adaptive strategy developed by soybeans to counter Fe deficiency

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stress. This enhanced lateral root growth, root volume, and specific surface area, increasing adsorption sites for 6:2 Cl-PFESA. Plant lipids, primarily neutral lipids, phospholipids, and fatty acids, also play essential roles in the adsorption of PFAS.[7] Previous studies reported that phospholipids promote the sorption capacity for PFAS due to their zwitterionic properties.[27] Liu et al. demonstrated that hydrophobic compounds tend to localize on root surface membranes because of lipid partitioning, thereby reducing their translocation to aboveground tissues.[48] In this study, the lipid content of soybean roots followed the order of LAF1 (155 mg g^{-1}) > LAF2 (150 mg g^{-1}) > LAF3 (127 mg g^{-1}) > AAF (117 mg g^{-1}) > EAF (109 mg g^{-1}). The significantly elevated ($p < 0.05$) lipid content in the LAF treatments could further contribute to the adsorption of 6:2 Cl-PFESA on the root epidermis (Table S6).

Mechanisms of Fe Deficiency on the Absorption and Translocation of 6:2 Cl-PFESA in Soybean. Under Fe-deficient conditions, protons are released into the rhizosphere by PM H^+-ATPase, resulting in local acidification.[49] This acidification reduces Fe^{3+} to Fe^{2+} via the membrane-bound ferric-chelate reduction oxidase (FRO),[25] enabling Fe^{2+} absorption by root epidermal cells through IRT1,[50] a primary Fe^{2+} transporter and multitransmembrane protein.[25] During this process, PM H^+-ATPase and IRT1 are upregulated. In this study, PM H^+-ATPase activity in the LAF1, LAF2, and LAF3 treatments was significantly higher ($p < 0.05$) than in the AAF treatment (Figure S5). Our previous study demonstrated that PM H^+-ATPase regulated the absorption of PFAS by plant roots.[9] However, the lower STF of 6:2 Cl-PFESA in these treatments suggests that additional factors may influence its absorption and translocation. While it remains unclear if IRT1 mediates 6:2 Cl-PFESA absorption in soybean, molecular docking shows that 6:2 Cl-PFESA binds to IRT1 mainly through three sites, with binding energies ranging from −5.39 to −4.84 kcal mol^{-1} (Figure 2a–f). These interactions involve hydrogen bonding with lysine 41 (LYS41) and aspartic acid 39 (ASN39), as well as hydrophobic interactions with other amino acid residues (Figure 2g–i). Similar hydrogen bonding interactions have been observed between 6:2 Cl-PFESA and lysine 15 in the thyroid hormone transporter.[51]

Generally, Fe^{2+} is taken up into root epidermal cell layers by the specialized Fe^{2+} transporter IRT1. Under Fe-deficient conditions, the expression of IRT1, located on the PM, is elevated to facilitate Fe absorption.[25] When Fe^{2+} content meets the plant's growth requirements, IRT1 is ubiquitinated and subsequently enters the cells through clathrin-mediated endocytosis to prevent Fe overload.[50] Thus, this study hypothesizes that 6:2 Cl-PFESA may enter cells by binding to IRT1 during its endocytosis under Fe-deficient conditions. To verify this, brefeldin A, an exocytosis inhibitor, was used to block the transport of IRT1 between the endoplasmic reticulum and Golgi apparatus, thereby inhibiting its function in transporting Fe^{2+} on the PM. Chlorpromazine hydrochloride was applied to inhibit clathrin-mediated endocytosis to investigate whether this pathway contributes to the absorption of 6:2 Cl-PFESA.[52] As shown in Figure 3, the concentrations of 6:2 Cl-PFESA in brefeldin A-treated LAF1 and LAF2 were slightly lower but not significantly different from the control ($p > 0.05$), suggesting that IRT1 does not directly mediate the absorption of 6:2 Cl-PFESA in soybean roots. In contrast, the concentrations of 6:2 Cl-PFESA decreased significantly in chlorpromazine hydrochloride-treated LAF treatments ($p < 0.05$), possibly due to the overexpression of IRT1 under Fe-deficient conditions. Once sufficient Fe is absorbed by soybean, IRT1 is internalized into cells via endocytosis, facilitating the absorption of 6:2 Cl-PFESA in the roots. However, chlorpromazine hydrochloride is not exclusively limited to inhibiting clathrin-mediated endocytosis of IRT1 in plants. Therefore, these results only suggest that clathrin-mediated endocytosis of IRT1 may contribute to 6:2 Cl-PFESA absorption, while other pathways cannot be excluded.

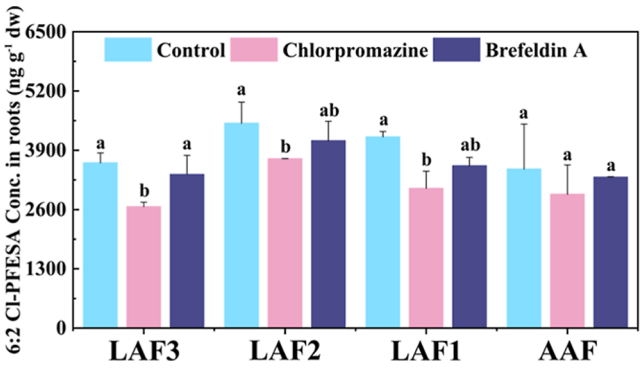

Figure 3. Impact of endocytosis and exocytosis inhibitors on the absorption of 6:2 Cl-PFESA after 24 h exposure. Different letters indicate significant differences ($p < 0.05$).

The movement of compounds in the apoplast is typically blocked by the endodermal Casparian strip. Therefore, 6:2 Cl-PFESA in the apoplast must cross at least one phospholipid bilayer to enter the symplast before it can be transported to aboveground tissues.[6] The proportions of 6:2 Cl-PFESA in the symplast pathway were measured to assess the impact of PM H^+-ATPase and IRT1 upregulation on its transmembrane transport in roots. As illustrated in Figure 4a, the proportions of 6:2 Cl-PFESA in the symplast sap in the LAF3 (18%), LAF2 (26%), and LAF1 (45%) treatments were lower compared to the AAF (49%) treatment, consistent with the trend in TF values. These results suggest that although PM H^+-ATPase activity and IRT1 were significantly upregulated under Fe deficiency, the transmembrane transport of 6:2 Cl-PFESA in soybean roots was not enhanced. Other factors may, therefore, influence its absorption and translocation to aboveground tissues. It is known that transpiration stream and protein content are important factors regulating the translocation of PFAS in plants.[10] Thus, stomatal conductance (G_s) and transpiration rate (E) were measured to evaluate plant transpiration intensity. The G_s values in LAF1 (0.287 mol m^2 s^{-1}), LAF2 (0.178 mol m^2 s^{-1}), and LAF3 (0.150 mol m^2 s^{-1}) were significantly lower ($p < 0.05$) than in AAF (0.370 mol m^2 s^{-1}). A similar trend was observed for the E values (Figure 4b), suggesting that appropriate Fe supplementation, as in the AAF treatment, is essential for maintaining healthy transpiration in soybeans. However, Fe deficiency markedly inhibited transpiration in soybeans ($p < 0.05$). Additionally, the soluble protein content, which plays an important role in PFAS translocation, was lower in the three Fe-deficient treatments than in AAF ($p > 0.05$, Table S6). These results underscore that reduced transpiration and soluble protein content under Fe deficiency contribute to the decreased absorption and translocation of 6:2 Cl-PFESA in soybeans.

Mechanisms of Excessive Available-Fe on the Absorption and Translocation of 6:2 Cl-PFESA in

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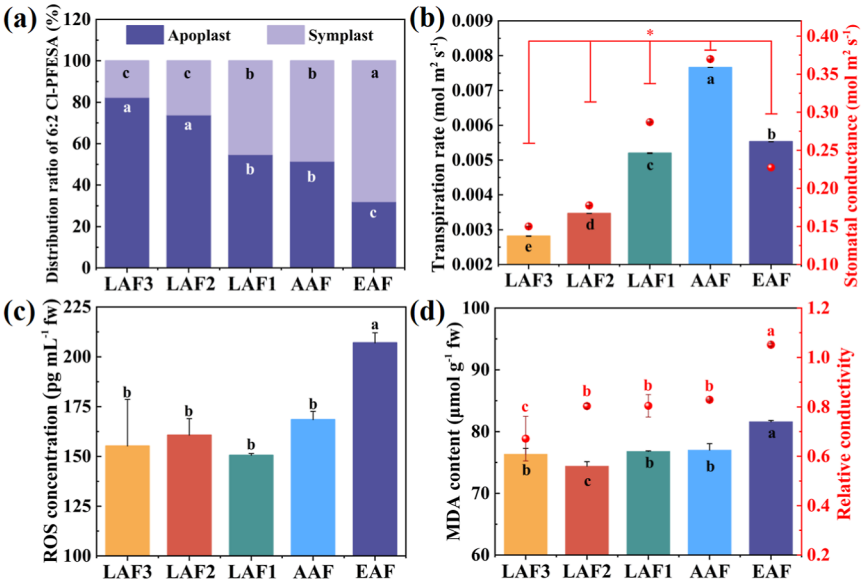

Figure 4. Distribution ratio of 6:2 Cl-PFESA concentrations in the apoplast and symplast following 192 h exposure (a). Effects of different available-iron levels on transpiration rate and stomatal conductance (b). ROS concentration in soybean roots (c), and changes in MDA content and conductivity (d). Error bars represent the mean ± standard deviation (n = 3). Different letters and asterisks indicate significant differences (p < 0.05).

Soybean. Notably, the G_s and E values in the EAF treatment were significantly lower (p < 0.05) than those in the AAF treatment (Figure 4b), indicating that excessive Fe supplementation greatly inhibited the transpiration rate in soybeans. This finding is consistent with previously reported results.[53] Additionally, the PM H^+-ATPase activity in the EAF treatment was similar to that in the AAF treatment (p > 0.05, Figure S5). These results contrast with the observation that the LTF of 6:2 Cl-PFESA was significantly higher (p < 0.05) in the EAF treatment compared to the AAF treatment, implying that other factors may enhance 6:2 Cl-PFESA absorption and translocation in soybean under excessive available-Fe conditions. As shown in Figure 4a, the proportion of 6:2 Cl-PFESA in the symplast sap (68%) was significantly higher (p < 0.05) in EAF than in other treatments, suggesting that excessive Fe greatly facilitates the transmembrane transport of 6:2 Cl-PFESA in soybean roots. It was hypothesized that Fe^{2+} induces the production of free radicals, leading to peroxidation of cell membrane lipids and consequently increasing the permeability of the root cell membrane.[11] Indeed, the production of ROS in soybean roots under the EAF treatment was significantly higher (p < 0.05) than in the AAF and LAF treatments, implying that excessive Fe triggered an oxidative response (Figure 4c). Correspondingly, the MDA content, a key indicator of lipid peroxidation,[54] was significantly higher in the EAF treatment (p < 0.05) compared to the AAF and LAF treatments (Figure 4d), suggesting that excessive available-Fe substantially induced lipid peroxidation in the soybean root membrane. Lipid peroxidation may compromise membrane integrity, resulting in membrane instability and increased permeability.[11] Significant positive correlations (p < 0.05) were observed between the relative proportion of 6:2 Cl-PFESA in the symplast, LTF values, and electrolyte leakage, as available-Fe levels increased (Figure S6a,b). This indicates that excessive available-Fe induces oxidative stress and lipid peroxidation in soybean roots, leading to increased membrane permeability, which significantly facilitates the transmembrane transport of 6:2 Cl-PFESA in soybean roots. Furthermore, the soluble protein content in soybean tissues, particularly in leaves, was significantly higher (p < 0.05) in the EAF treatment than in AAF (Table S6). These findings collectively demonstrate that excessive available-Fe boosts protein synthesis in soybean tissues, especially in leaves, thus enhancing the translocation of 6:2 Cl-PFESA from roots to aboveground tissues.

Environmental Implications. Fe is essential for photosynthesis, respiration, and other biological processes in plants. The available-Fe levels in soil affect multiple physiological processes in plants related to the accumulation and translocation of 6:2 Cl-PFESA. This study reveals that soybean adapts to Fe deficiency by altering its root morphology, including root specific surface area, root tips, root volume, and total root length, thereby promoting 6:2 Cl-PFESA adsorption on the root epidermis. IRT1 can strongly bind to 6:2 Cl-PFESA and may indirectly participate in its absorption via the clathrin-mediated endocytosis pathway. Although PM H^+-ATPase and IRT1 were upregulated under Fe-deficient conditions, the significantly reduced transpiration and soluble protein content in soybeans suppressed the transmembrane transport of 6:2 Cl-PFESA in roots and its subsequent translocation to aboveground tissues. In contrast, excessive available-Fe enhanced root membrane permeability and soluble protein content in soybean aboveground tissues, which facilitated 6:2 Cl-PFESA absorption in roots and its translocation to aboveground tissues. Therefore, the appropriate application of Fe fertilizer is essential in agricultural soils with significant PFAS contamination to mitigate potential ecological risks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.4c11993.

Chemicals and reagents; sample pretreatment and extraction; transformation experiments and extraction of crude enzymes and results; determination of enzyme

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activity; mass composition; analysis of phenotypic parameters of soybean roots; electrolyte leakage analysis and its correlation; IRT1 structural conformation; differences in biomass of soybean tissues under different available-Fe levels conditions; determination of soluble protein and lipid content; phenotypic parameters of soybean roots; molecular docking; methods of data analysis (PDF)

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Notes

The authors declare no competing financial interest.

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