Unraveling the iron deficiency responsive proteome in *Arabidopsis* shoot by iTRAQ-OFFGEL approach

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**Keywords:** iTRAQ-CEX, iTRAQ-OFFGEL, *Arabidopsis* shoots, Fe deficiency, excess Zn, *irt1-1* mutant

**Abbreviations:** CEX, Cation exchange column; GO, Gene ontology; iTRAQ, Isobaric tags for relative and absolute quantification

Iron (Fe) is required by plants for basic redox reactions in photosynthesis and respiration, and for many other key enzymatic reactions in biological processes. Fe homeostatic mechanisms have evolved in plants to enable the uptake and sequestration of Fe in cells. To elucidate the network of proteins that regulate Fe homeostasis and transport, we optimized the iTRAQ-OFFGEL method to identify and quantify the number of proteins that respond to Fe deficiency in the model plant *Arabidopsis*. In this study, Fe deficiency was created using Fe-deficient growth conditions, excess zinc (Zn), and use of the *irt1-1* mutant in which the IRT1 Fe transporter is disrupted. Using the iTRAQ-OFFGEL approach, we identified 1139 proteins, including novel Fe deficiency-responsive proteins, in microsomal fractions isolated from 3 different types of Fe-deficient shoots compared with just 233 proteins identified using conventional iTRAQ-CEX. Further analysis showed that greater numbers of low-abundance proteins could be identified using the iTRAQ-OFFGEL method and that proteins could be identified from numerous cellular compartments. The improved iTRAQ-OFFGEL method used in this study provided an efficient means for identifying greater numbers of proteins from microsomal fractions of *Arabidopsis* shoots. The proteome identified in this study provides new insight into the regulatory cross talk between Fe-deficient and excess Zn conditions.

**Introduction**

Iron (Fe) is an essential element for life on earth and is required by both plants and animals for a range of essential enzymatic and metabolic reactions. In plants, Fe is required for basic redox reactions in photosynthesis and respiration, and for many key enzymatic reactions involved in important biological processes such as DNA replication, lipid metabolism, and nitrogen fixation. According to the World Health Organization, 30% of the world’s population is affected by Fe deficiency.

To better understand the mechanisms that regulate Fe transport and homeostasis in plants, omics-based approaches have been used to examine shifts in pI due to iTRAQ labeling; however, physiological parameters were not considered in that study.5 To identify and quantify a greater number of proteins, Chenau et al. (2008) established a method that coupled iTRAQ with OFFGEL electrophoresis (iTRAQ-OFFGEL).4 The iTRAQ-OFFGEL approach is important for quantitative proteomics studies because greater amounts of sample can be loaded and separated allowing subsequent analytical steps to be optimized. In the plant proteomics field, the iTRAQ-OFFGEL method has been used to examine shifts in pI due to iTRAQ labeling; however, physiological parameters were not considered in that study.

In this study, we used the iTRAQ-OFFGEL method to analyze microsomal fractions isolated from shoots of wild-type *Arabidopsis* (Col-0) under conditions of Fe deficiency and excess zinc (Zn) and the *irt1-1* mutant, which lacks a functional IRT1 Fe transporter, and showed that this method is an effective approach for understanding plant physiological phenomena. We identified 1139 proteins using the iTRAQ-OFFGEL method compared to only 233 proteins using the conventional iTRAQ-CEX method, which couples the iTRAQ method with peptide fractionation on a cationic exchange column. Moreover, the iTRAQ-OFFGEL method enabled the efficient identification of a large number of membrane proteins, including low-abundance proteins. We focused on the highly upregulated and downregulated microsomal proteins in *Arabidopsis* shoots.
that were identified by iTRAQ-OFFGEL to provide insight into regulatory cross talk between the conditions of Fe deficiency and excess Zn.

**Results**

**iTRAQ-CEX analysis of Fe deficiency-responsive proteins**

An analysis using iTRAQ-CEX was recently performed on microsomal fractions isolated from Arabidopsis Col-0 plants grown on normal MGRL medium (Normal), medium without Fe (0-Fe), and medium containing 300 µM ZnSO₄ (300-Zn) and from shoots of the *irt1-1* mutant grown on Normal medium to identify changes in protein expression due to Fe deficiency. To ensure that the Fe content in each type of Fe-deficient sample (0-Fe, 300-Zn, and *irt1-1*) was less than in Col-0 grown on Normal medium, the concentrations of 11 elements in Arabidopsis shoots were examined by inductive coupled plasma mass spectroscopy (ICP-MS) (Table S1). The Fe content was lower in all 3 types of Fe-deficient shoots than in shoots from Col-0 grown on Normal medium (Fig. 1A). The experimental scheme followed in this study is depicted in Figure 2. A total of 233 proteins were identified and quantified with a false discovery rate (FDR) of less than 1% in 3 biological replicates (Table S2). Among the identified proteins, reproducible increases in protein amounts of more than 2.0-fold were observed for only 3 proteins in the 0-Fe samples and proteins in the 300-Zn samples, while no increases in protein amounts greater than 2.0-fold were observed for the *irt1-1* mutant (Table 1). Only 16 proteins decreased in amount to less than 0.50-fold in the 0-Fe samples; no proteins from the 300-Zn or *irt1-1* mutant samples decreased in amount to less than 0.50-fold (Table 1). The identification of small numbers of highly responsive proteins indicates a need to adopt alternate methodologies to

![Figure 1](image-url). Effect of Fe deficiency on Fe and Zn levels. Fe (A) and Zn (B) contents in shoots from Arabidopsis Col-0 plants grown on Normal, 0-Fe, or 300-Zn media and in shoots from *irt1-1* mutant plants grown on Normal medium for 10 days. The Fe levels are presented as mean values from 3 biological replicates analyzed in triplicate. Error bars indicate the SEs of the biological replicates.

| Table 1. Number of proteins identified as per the expression levels using iTRAQ-CEX and iTRAQ-OFFGEL methods |
|--------------------------------------------------|----------------------|----------------------|
| Expression levels                                | iTRAQ-CEX            | iTRAQ-OFFGEL         |
| Col-0 0-Fe/Col-0 Normal                          |                      |                      |
| More than 2.0-fold                               | 3                    | 66                   |
| More than 1.5-fold                               | 5                    | 159                  |
| More than 1.2-fold                               | 19                   | 330                  |
| Less than 0.833-fold                             | 110                  | 355                  |
| Less than 0.667-fold                             | 36                   | 184                  |
| Less than 0.5-fold                               | 16                   | 64                   |
| Col-0 300-Zn/Col-0 Normal                        |                      |                      |
| More than 2.0-fold                               | 0                    | 31                   |
| More than 1.5-fold                               | 2                    | 99                   |
| More than 1.2-fold                               | 8                    | 284                  |
| Less than 0.833-fold                             | 38                   | 245                  |
| Less than 0.667-fold                             | 4                    | 81                   |
| Less than 0.5-fold                               | 0                    | 13                   |
| *irt1-1* Normal/Col-0 Normal                     |                      |                      |
| More than 2.0-fold                               | 2                    | 7                    |
| More than 1.5-fold                               | 3                    | 20                   |
| More than 1.2-fold                               | 11                   | 186                  |
| Less than 0.833-fold                             | 45                   | 44                   |
| Less than 0.667-fold                             | 6                    | 3                    |
| Less than 0.5-fold                               | 0                    | 0                    |
better understand the mechanisms of Fe homeostasis in plant cells.

Efficiency of iTRAQ-OFFGEL analysis over iTRAQ-CEX

The compatibility of OFFGEL fractionation with iTRAQ labeling was reported previously. The iTRAQ-OFFGEL method has been adopted in several studies, mostly in animal systems. In this study, a large number of proteins were identified from Arabidopsis microsomal fractions using the iTRAQ-OFFGEL method (Fig. 2). A total of 1139 proteins were identified and quantified in 3 biological replicates with an FDR of less than 1% (Table S3). The number of proteins identified using iTRAQ-OFFGEL was 4.9-fold greater than with iTRAQ-CEX, indicating a higher recovery rate of fractionated peptides.

Based on GO annotations, the proteins identified using the iTRAQ-OFFGEL method corresponded to a larger number of cellular compartments than the proteins identified using the iTRAQ-CEX method (Tables S4 and S5). Additionally, higher proportions of proteins belonging to “membrane and membrane bound proteins” were identified by iTRAQ-OFFGEL than by iTRAQ-CEX (Tables S4 and S5). Moreover, 245 proteins identified by iTRAQ-OFFGEL were predicted to have 2 or more transmembrane (TM) regions compared to only 31 proteins identified by iTRAQ-CEX based on an analysis using TMHMM Server v. 2.0 software (Fig. 3, http://www.cbs.dtu.dk/services/TMHMM/). The fractionation of iTRAQ-labeled peptides was an effective method for increasing the numbers of detectable peptides, including low-abundance peptide. Peptides derived from low-abundance proteins are often not detected by MS due to ionization suppression.

Cross talk between Fe deficiency and excess Zn conditions

From the iTRAQ-OFFGEL analysis, growth on 0-Fe and 300-Zn caused 66 and 31...
Table 2. iTRAQ-OFFGEL analysis based identified proteins with more than 2.0-fold expression

| Arabidopsis Genome Initiative Code | Protein | Peptide$^a$ | Coverage$^b$ | Ratio (Col-0 0-Fe/Col-0 Normal) | Ratio (Col-0 300-Zn/Col-0 Normal) | Ratio (irt1-1 Normal/Col-0 Normal) |
|-----------------------------------|---------|------------|-------------|-------------------------------|---------------------------------|----------------------------------|
| AT3G57260                         | beta-1,3-glucanase 2 | 1.0 | 3.0 | 58.833 ± 35.422 | 7.621 ± 5.062 | 1.285 ± 0.602 |
| AT2G18193                         | P-loop containing nucleoside triphosphate hydrolases superfamily protein | 1.3 | 2.8 | 14.417 ± 9.381 | 20.651 ± 16.525 | 2.792 ± 2.269 |
| AT4G39909                         | Papain family cysteine protease | 2.3 | 12.8 | 12.889 ± 1.938 | 3.892 ± 1.034 | 1.000 ± 0.192 |
| AT5G26340                         | Major facilitator superfamily protein | 1.7 | 2.2 | 8.895 ± 4.114 | 4.125 ± 1.113 | 1.034 ± 0.139 |
| AT5G10760                         | Eukaryotic aspartyl protease family protein | 1.3 | 2.5 | 7.770 ± 9.065 | 3.901 ± 4.076 | 1.007 ± 0.174 |
| AT3G52400                         | syntaxin of plants 122 | 3.7 | 13.0 | 7.158 ± 2.860 | 2.843 ± 0.373 | 1.245 ± 0.192 |
| AT5G20230                         | blue-copper-binding protein | 3.0 | 21.4 | 7.026 ± 1.667 | 4.625 ± 1.218 | 1.370 ± 0.387 |
| AT3G26000                         | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein | 1.0 | 6.5 | 6.949 ± 4.416 | 2.561 ± 0.940 | 0.950 ± 0.239 |
| AT2G18690                         | unknown protein | 3.0 | 7.8 | 6.173 ± 0.992 | 3.610 ± 0.888 | 1.447 ± 0.253 |
| AT3G25610                         | ATPase E1-E2 type family protein/haloid-c-dehalogenase-like hydrolase family protein  | 1.0 | 1.2 | 5.788 ± 3.425 | 3.793 ± 2.025 | 0.913 ± 0.258 |
| AT4G16370                         | oligopeptide transporter | 12.0 | 16.7 | 5.574 ± 0.565 | 4.625 ± 1.218 | 1.370 ± 0.387 |
| AT2G47000                         | ATP binding cassette subfamily B4 | 11.0 | 15.7 | 4.858 ± 2.582 | 6.587 ± 4.535 | 1.258 ± 0.166 |
| AT5G06320                         | NDR1/HIN1-like 3 | 6.3 | 25.8 | 4.779 ± 0.978 | 2.087 ± 0.368 | 1.233 ± 0.033 |
| AT1G1910                          | aspartic proteinase A1 | 10.7 | 26.2 | 4.549 ± 0.295 | 1.905 ± 0.343 | 0.917 ± 0.076 |
| AT3G26080                         | plastid-lipid associated protein PAP/fibrillin family protein | 1.0 | 3.9 | 4.546 ± 0.679 | 1.436 ± 0.639 | N.D. |
| AT2G38290                         | ammonium transporter 2 | 1.7 | 4.6 | 4.348 ± 0.791 | 2.388 ± 0.422 | 1.167 ± 0.059 |
| AT3G13080                         | multidrug resistance-associated protein 3 | 12.3 | 8.8 | 4.305 ± 1.467 | 7.100 ± 2.457 | 1.408 ± 0.298 |
| AT1G02920                         | glutathione S-transferase 7 | 1.7 | 33.5 | 4.116 ± 0.382 | 2.573 ± 0.134 | 1.015 ± 0.105 |
| AT2G3810                          | tetratranin | 5.7 | 17.8 | 3.955 ± 0.866 | 2.079 ± 0.323 | 1.250 ± 0.159 |
| AT4G27170                         | seed storage albumin 4 | 1.7 | 11.6 | 3.890 ± 2.794 | 0.803 ± 0.254 | 3.058 ± 2.330 |
| AT3G19930                         | sugar transporter 4 | 3.3 | 6.7 | 3.722 ± 0.351 | 1.674 ± 0.115 | 1.301 ± 0.146 |
| AT1G02930                         | glutathione S-transferase 6 | 1.7 | 33.2 | 3.693 ± 0.317 | 2.091 ± 0.203 | 0.958 ± 0.121 |
| AT4G15610                         | Uncharacterised protein family (UPD0497) | 3.0 | 14.5 | 3.470 ± 1.610 | 2.406 ± 0.911 | 0.906 ± 0.102 |
| AT4G35840                         | FAD/NAD(P) binding oxidoreductase family protein | 2.3 | 4.8 | 3.291 ± 0.443 | 2.702 ± 0.507 | 1.156 ± 0.177 |
| AT5G67330                         | natural resistance associated macrophage protein 4 | 4.0 | 8.7 | 3.053 ± 0.195 | 3.395 ± 0.023 | 2.203 ± 0.871 |
| AT1G71880                         | sucrose-proton symporter 1 | 6.0 | 12.0 | 2.902 ± 0.528 | 2.121 ± 0.042 | 1.105 ± 0.042 |
| AT4G21960                         | Peroxidase superfamily protein | 3.7 | 14.6 | 2.899 ± 1.637 | 2.534 ± 1.009 | 3.693 ± 2.543 |
| AT3G5410                          | lipoygenase 2 | 5.0 | 5.6 | 2.866 ± 1.617 | 2.698 ± 1.668 | 1.105 ± 0.372 |
| AT4G20830                         | FAD-binding Berberine family protein | 2.0 | 3.0 | 2.684 ± 1.655 | 2.106 ± 1.136 | 1.116 ± 0.366 |
| AT4G27160                         | seed storage albumin 3 | 2.3 | 15.0 | 2.679 ± 0.203 | 0.988 ± 0.191 | 2.716 ± 0.204 |
| AT5G25260                         | SPFH/Band 7/PHB domain-containing membrane-associated protein family  | 3.7 | 21.1 | 2.605 ± 0.507 | 1.513 ± 0.403 | 1.118 ± 0.214 |
| AT1G44575                         | Chlorophyll A-B binding family protein | 6.0 | 28.2 | 2.556 ± 0.151 | 1.362 ± 0.164 | 1.653 ± 0.744 |
| AT2G31880                         | Leucine-rich repeat protein kinase family protein | 5.0 | 11.7 | 2.548 ± 0.420 | 2.196 ± 0.628 | 1.026 ± 0.054 |
| AT1G08450                         | calreticulin 3 | 4.3 | 9.5 | 2.538 ± 0.893 | 1.514 ± 0.144 | 1.119 ± 0.110 |

$^a$Peptide indicates average number of assigned peptides. $^b$Coverage indicates average percentage of assigned peptides to the predicted protein. The values were calculated as the ratio of 115 (Col-0 0-Fe) to 114 (Col-0 Normal), 116 (Col-0 300-Zn) to 114 (Col-0 Normal), or 117 (irt1-1 Normal) to 114 (Col-0 Normal). Microsomal proteins from the Col-0 grown on Normal, 0-Fe or 300-Zn media and from the irt1-1 mutant grown on Normal medium were labeled with iTRAQ-114, 115, 116 and 117 reagents, respectively. Proteins that increased more than 2.0-fold were selected and ordered based on fold change values in Col-0 0-Fe/Col-0 Normal. However, the response of proteins in the Col-0 300-Zn/Col-0 Normal and irt1-1 Normal/Col-0 Normal were ordered as per their position in fold change values in Col-0 0-Fe/Col-0 Normal. Data were mean ± SD of 3 independent experiments. N.D. means protein and/or iTRAQ reporter ions were not detected.
Table 2. iTRAQ-OFFGEL analysis based identified proteins with more than 2.0-fold expression (continued)

| AT3G01290 | SPRF/Band 7/PHB domain-containing membrane-associated protein family | 9.7 | 51.0 | 2.513 ± 0.868 | 1.766 ± 0.574 | 1.085 ± 0.224 |
| AT5G11040 | TRS120 | 5.0 | 4.1 | 2.486 ± 1.351 | 2.297 ± 0.945 | 0.870 ± 0.163 |
| AT4G09010 | ascorbate peroxidase 4 | 4.3 | 12.6 | 2.464 ± 0.397 | 1.177 ± 0.197 | 1.313 ± 0.443 |
| AT5G35735 | Auxin-responsive family protein | 2.0 | 6.4 | 2.418 ± 1.106 | 1.451 ± 0.683 | 0.870 ± 0.380 |
| AT2G05380 | glycine-rich protein 3 short isoform | 2.0 | 25.9 | 2.392 ± 0.665 | 3.418 ± 0.693 | 1.359 ± 0.303 |
| AT4G13510 | ammonium transporter 1;1 | 3.0 | 9.8 | 2.390 ± 1.063 | 1.911 ± 0.771 | 0.956 ± 0.084 |
| AT1G77510 | PDI-like 1-2 | 12.3 | 34.7 | 2.389 ± 0.266 | 1.603 ± 0.215 | 1.063 ± 0.108 |
| AT3G26210 | cytochrome P450, family 71, subfamily B, polypeptide | 1.3 | 4.5 | 2.383 ± 1.080 | 1.567 ± 0.440 | 1.158 ± 0.195 |
| AT1G03860 | prohibitin 2 | 2.0 | 12.9 | 2.345 ± 0.388 | 1.771 ± 0.177 | 1.292 ± 0.254 |
| AT4G02520 | glutathione S-transferase PHI 2 | 6.0 | 43.9 | 2.337 ± 0.521 | 1.536 ± 0.533 | 1.006 ± 0.164 |
| AT1G65820 | microsomal glutathione s-transferase, putative | 3.7 | 25.6 | 2.336 ± 0.165 | 1.872 ± 0.204 | 1.159 ± 0.096 |
| AT3G11820 | syntaxin of plants 121 | 5.0 | 18.8 | 2.315 ± 0.119 | 1.607 ± 0.145 | 1.114 ± 0.072 |
| AT3G51670 | SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein | 3.3 | 8.7 | 2.312 ± 0.027 | 2.130 ± 0.256 | 1.620 ± 0.465 |
| AT3G62600 | DNAJ heat shock family protein | 5.0 | 14.8 | 2.310 ± 0.375 | 1.727 ± 0.323 | 1.326 ± 0.137 |
| AT3G12740 | aLa-interacting subunit 1 | 3.0 | 11.4 | 2.307 ± 0.729 | 1.725 ± 0.334 | 1.186 ± 0.187 |
| AT3G48890 | membrane-associated progestosterone binding protein 3 | 8.3 | 32.3 | 2.263 ± 0.159 | 1.554 ± 0.085 | 1.242 ± 0.154 |
| AT1G27770 | autoinhibited Ca2++-ATPase 1 | 3.0 | 4.2 | 2.259 ± 0.542 | 1.725 ± 0.334 | 1.186 ± 0.187 |
| AT4G08850 | Leucine-rich repeat receptor-like protein kinase family protein | 18.3 | 18.7 | 2.216 ± 0.442 | 1.937 ± 0.641 | 1.154 ± 0.126 |
| AT3G23120 | photosystem II stability/assembly factor, chloroplast (HCIF136) | 3.3 | 9.2 | 2.215 ± 0.271 | 1.122 ± 0.134 | 1.446 ± 0.422 |
| AT5G42020 | Heat shock protein 70 (Hsp 70) family protein | 2.7 | 49.9 | 2.160 ± 0.311 | 1.365 ± 0.255 | 1.069 ± 0.106 |
| AT4G22890 | PGRS-LIKE A | 1.0 | 3.7 | 2.159 ± 0.294 | 1.244 ± 0.160 | 1.451 ± 0.604 |
| AT4G34150 | calcium-dependent lipid-binding (CaLB domain) family protein | 1.7 | 7.0 | 2.157 ± 0.461 | 1.604 ± 0.221 | 1.274 ± 0.205 |
| AT5G40770 | prohibitin 3 | 2.3 | 8.3 | 2.138 ± 0.418 | 1.635 ± 0.495 | 1.198 ± 0.162 |
| AT1G21750 | PDI-like 1-1 | 20.7 | 48.8 | 2.114 ± 0.493 | 1.569 ± 0.059 | 1.200 ± 0.079 |
| AT4G16660 | heat shock protein 70 (Hsp 70) family protein | 8.7 | 9.2 | 2.105 ± 0.623 | 1.372 ± 0.107 | 1.108 ± 0.112 |
| AT1G80530 | Major facilitator superfamily protein | 1.3 | 2.1 | 2.088 ± 0.332 | 2.648 ± 0.323 | 1.217 ± 0.440 |
| AT2G43350 | glutathione peroxidase 3 | 1.0 | 6.6 | 2.086 ± 0.321 | 1.861 ± 0.658 | 1.375 ± 0.185 |
| AT2G03510 | SPRF/Band 7/PHB domain-containing membrane-associated protein family | 3.3 | 10.4 | 2.007 ± 0.580 | 1.996 ± 0.508 | 1.362 ± 0.451 |

*Peptide indicates average number of assigned peptides. Coverage indicates average percentage of assigned peptides to the predicted protein. The values were calculated as the ratio of 115 (Col-0 0-Fe) to 114 (Col-0 Normal), 116 (Col-0 300-Zn) to 114 (Col-0 Normal), or 117 (irt1-1 Normal) to 114 (Col-0 Normal). Microsomal proteins from the Col-0 grown on Normal, 0-Fe or 300-Zn media and from the irt1-1 mutant grown on Normal medium were labeled with iTRAQ-114, 115, 116 and 117 reagents, respectively. Proteins that increased more than 2.0-fold were selected and ordered based on fold change values in Col-0 0-Fe/Col-0 Normal. However, the response of proteins in the Col-0 300-Zn/Col-0 Normal and irt1-1 Normal/Col-0 Normal were ordered as per their position in fold change values in Col-0 0-Fe/Col-0 Normal. Data were mean ± SD of 3 independent experiments. N.D. means protein and/or iTRAQ reporter ions were not detected.
proteins, respectively, to be upregulated more than 2.0-fold (Tables 1 and 2). All of the 31 proteins that were upregulated in response to excess Zn were also upregulated more than 2.0-fold by growth on 0-Fe. In contrast, 64 and 13 proteins were downregulated to less than 0.5-fold by growth on 0-Fe and 300-Zn, respectively. Seven of these downregulated proteins were in common between growth on 0-Fe and 300-Zn (Tables 1 and 3). The functions of most of the downregulated proteins were related to photosynthesis as evidenced by the visual effects of Fe deficiency and excess Zn on physiological parameters such as the development of chlorosis. The common regulation of most of the proteins in response to Fe deficiency and excess Zn indicates regulatory cross talk activity between the 2 conditions.

### Regulation of protein expression in the irtl-1 mutant

Of the 7 proteins identified by the ITREAX-OFFGEL method that were induced more than 2.0-fold in the irtl-1 mutant, 6 proteins were also expressed more than 2.0-fold in Col-0 plants grown on 0-Fe medium (Table S3). However, no proteins in the irtl-1 mutant were reduced in amount to less than 0.5-fold. The number of highly responsive proteins was less in the irtl-1 mutant than in Col-0 grown on 0-Fe or 300-Zn media or in Col-0 grown on Normal medium (Table 1). Moreover, photosystem I was affected less in the irtl-1 mutant than in Col-0 grown on 0-Fe medium (Table 3). These results indicate a broad effect of mineral stress on protein expression levels rather than a limited effect caused by the disruption of just one Fe transporter.

### Discussion

#### Overview of proteins upregulated more than 2.0-fold in all 3 Fe-deficient conditions

Two transporter proteins were identified among the proteins that were upregulated by more than 2.0-fold in all 3 Fe-deficient conditions; that is, growth on 0-Fe and 300-Zn media and in the irtl-1 mutant. Oligopeptide transporter (OPT3, AT4G16370) was upregulated by 5.574-, 6.045-, and 3,341-fold by growth on 0-Fe medium and 300-Zn medium and in the irtl-1 mutant, respectively (Table 2). OPT3 is involved in the transport of small peptides that may have roles in nutrition.\(^{12,13}\) OPT3 plays a critical role in the maintenance of whole-plant Fe homeostasis and Fe nutrition in developing seeds, and it plays an important role in shoot-to-root signaling for the regulation of Fe deficiency responses in roots.\(^{14}\) Stacey et al. (2006) showed that OPT3 expression was enhanced by Fe limitation.\(^{15}\) The high level of OPT3 expression under Fe-deficient conditions shown in this study provides further support for the role of this protein in defense against Fe deficiency stress. The expression of another important transporter protein, natural resistance-associated macrophage protein 4 (NRAMP4, AT5G67330), was increased by 3.053-, 3.395-, and 2.203-fold by growth on 0-Fe medium and 300-Zn medium and in the irtl-1 mutant, respectively (Table 2). NRAMP4 is also upregulated under Fe-deficient conditions in both shoots and roots.\(^{16}\) OPT3 and NRAMP4 may serve as backup systems for Fe homeostasis because they were also upregulated in the irtl-1 mutant on Normal medium.

P-loop containing nucleoside triphosphate hydrolases superfamily protein (AT2G18193) showed the strongest upregulation, with increases of 14.417-, 20.651-, and 2,792-fold in response to growth on 0-Fe medium and 300-Zn medium and in the irtl-1 mutant, respectively (Table 2). The superfamily, which includes AT2G18193, includes 4 of the 6 primary classes of enzymes as defined by their Enzyme Commission number. Among them, the S1 family functions in signal transduction, the S2 family consists of DNA-binding proteins or transporter proteins, and the S3 family includes transferases, ATP-dependent kinases, and sulfotransferases.\(^{17}\) Fe deficiency may trigger a signal transduction pathway that upregulates the expression of transporters and other transferases to increase the efficiency of Fe transport. Moreover, this may help in the stabilization of DNA molecules.

Peroxidase superfamily protein (AT4G21960) expression was increased by 2.899-, 2.534-, and 3.693-fold in response to growth on 0-Fe medium and 300-Zn medium and in the irtl-1 mutant, respectively (Table 2). In addition to the known defense-related induction pathway, peroxidase superfamily genes are induced via other signal transduction pathways.\(^{18}\) Fe deficiency is also known to affect different peroxidase isoenzymes to varying degrees. For example, Fe induces the generation of \(\text{H}_2\text{O}_2\), leading to oxidative stress in sunflower.\(^{19}\) Thus, Fe deficiency, as well as excess Zn, may play a role in inducing a signaling pathway that directs defense against Fe deficiency stress.

#### Cross talk between Fe-deficient and excess Zn conditions

We also showed that the expression of some of the identified proteins was higher with growth on 0-Fe and 300-Zn media, but was not induced to the same level as in the irtl-1 mutant. Major facilitator superfamily protein (AT5G26340) was highly upregulated in response to growth on 0-Fe and 300-Zn media. ZINC-INDUCED FACILITATOR1 (ZIF1), which encodes a member of the major facilitator superfamily of membrane proteins, is involved in a novel mechanism of Zn sequestration possibly by the transport of a Zn ligand or Zn ligand complex into vacuoles.\(^{20}\) The Zn content in shoots grown under Fe-deficient or excess Zn conditions increased to a level greater than in Col-0 shoots grown on Normal medium (Fig. 1B). The stronger expression of AT5G26340 under Fe-deficient and excess Zn conditions suggests a role in the sequestration of Zn into vacuoles. Multidrug resistance-associated protein 3 (MRP3, AT3G13080), a transporter protein, was highly upregulated by 4.305 – and 7.100-fold by growth on 0-Fe and 300-Zn media, respectively. Cadmium, nickel, arsenic, cobalt, and lead exposure induced MRP3 expression in Arabidopsis roots and shoots. Previously, a slight difference in MRP3 expression was observed in response to treatment with higher levels of Zn and Fe.\(^{21}\) MRP3 is also thought to have separate binding sites for different substrates.\(^{22,23}\) Therefore, MRP3 might be able to transport both metal conjugates and non-metal-containing toxic compounds. The increased expression of MRP3 observed in this study suggests a role in assisting the transport of Fe under Fe-deficient conditions. However, further evidence is needed to confirm the mechanism underlying increased MRP3 expression under Fe-deficient conditions.

Syntaxin of plants 122 (SYP122, AT3G52400) was also highly expressed under both 0-Fe and 300-Zn growth conditions.
SYP122 was upregulated 7.158 – and 2.843-fold by growth on 0-Fe and 300-Zn media, respectively. SYP121 (AT3G11820), which has functional redundancy with SYP122, showed 2.315 – and 1.607-fold increases in expression with growth on 0-Fe and 300-Zn media, respectively. SYP122 and SYP121 are key element of the soluble N-ethylmaleimide-sensitive factor protein attachment receptor (SNARE) complex on target membranes and can bind the SNARE complex to a vesicle to establish docking. SYP121 is involved in localization of the K⁺ channel KAT1 and aquaporin PIP2;5 transporter to the plasma membrane. Therefore, SYP122 might be involved in the localization of Fe transporters to the plasma membrane, although SYP122-interacting proteins that might assist the transport of Fe, or other metals whose accumulation is enhanced under Fe-deficient conditions, have not been reported.

Blue copper (Cu) binding protein (AT5G20230) was also highly expressed with 7.026 – and 4.625-fold increases in expression with growth on 0-Fe and 300-Zn media, respectively. This protein may function to sequester Cu, a potentially toxic element that is also an essential cellular catalyst for redox reactions. Elemental analysis showed that the accumulation of Cu was higher in shoots grown on 0-Fe and 300-Zn media (Table S1), indicating that AT5G20230 might also play a role in the sequestration of excessive Cu caused by Fe deficiency and excess Zn. This potential role was further supported by the lack of an increase in blue Cu binding protein expression in the *irt1-1* mutant corresponding to the levels of Cu in the *irt1-1* mutant.

Conclusions

We showed that iTRAQ-OFFGEL is an efficient method to identify and quantify a large number of proteins from microsomal fractions of *Arabidopsis* shoots. The number of proteins identified in this study using conventional iTRAQ-CEX was less than half the 521 proteins identified in an iTRAQ-CEX analysis of roots in our previous study. In contrast, we identified a 4.9-fold greater number of proteins using the iTRAQ-OFFGEL method. Moreover, many cellular compartments were targeted by our iTRAQ-OFFGEL analysis. To determine the impact of the iTRAQ-OFFGEL method on protein recovery, we also applied the method to a microsomal fraction from *Arabidopsis* roots. Overall, the number of proteins identified in roots was increased but it was not as large as that observed using the iTRAQ-CEX method (Y. Fukao, personal communication). The identification of a slightly smaller number of proteins in shoots may be due the abundance of chloroplast proteins in addition to storage, ribosomal, and cytoskeletal proteins. Peptides from chloroplast proteins might be preferentially detected by MS over low-abundance proteins due to ionization suppression, thereby decreasing the total number of proteins identified. We confirmed that the higher-resolution peptide separation of the iTRAQ-OFFGEL method is more effective for protein samples that include high-abundance proteins. In this study, higher expression levels of metal ion transporters such as NRAMP4, OPT3, MRP3, and other membrane proteins such as SYP122 under both Fe-deficient and excess Zn conditions could be detected using the iTRAQ-OFFGEL method. Our study provides new insight into the regulatory cross talk that occurs in response to Fe deficiency and excess Zn, although a detailed analysis is needed to fully understand the regulatory network involved in this cross talk.

Materials and Methods

Plant material and sample preparation

*Arabidopsis (Arabidopsis thaliana)* ecotype wild type Col-0 and *irt1-1* mutant seeds were germinated on sterile plates of MGLR medium containing 2.3 mM MES-KOH, pH 5.7, 1.0% (w/v) sucrose, and 1.2% agar. The Col-0 and *irt1-1* mutant were given different treatments i.e., normal MGLR medium (Normal), without Fe (0-Fe) and with 300 µM ZnSO₄ (300-Zn). The seedlings were grown for 10 d at 22 °C under 16h light/ 8h dark conditions. Shoots (approximately 0.2 g fresh weight) were harvested and homogenized with buffer A (50 mM HEPES-KOH, pH 7.5, 5 mM EDTA, 400 mM sucrose, and protease inhibitor cocktail). The homogenates were centrifuged at 1,000g at 4 °C for 20 min, and the supernatants were centrifuged at 8,000g at 4 °C for 20 min. The supernatants were centrifuged at 100,000g at 4 °C for 60 min to prepare the microsomal fraction. The pellets were washed by buffer A twice under the same condition and then dissolved in iTRAQ buffer (AB SCIEX). The protein concentration was determined using Nano drop (Thermo Scientific) by taking the OD. Three replicates were prepared from 3 independent experiments of plants grown at different times.

Determination of metal content

Ten-day-old shoots were harvested from Col-0 and *irt1-1* mutant, that were given different treatments i.e. Normal, 0-Fe and 300-Zn for Col-0 and *irt1-1* on Normal. Harvested shoots were dried at 60 °C for 2 d. Dried samples weighing more than 10 mg were digested with ultrapure HNO₃, using a Microwave Digestion System (Milestone General). Elemental content in the digests was determined by ICP-MS (Agilent technology). Measurement was performed with 3 independent biological replicates.

In-solution trypsin digestion and iTRAQ labeling for iTRAQ-CEX

Each 20 µL of microsomal protein fraction (2.5 mg mL⁻¹) was reduced by tris-(2-carboxyethyl) phosphine at 60 °C for 60 min and then alkylated by methyl methanethiosulfonate at room temperature for 10 min. Samples were digested using 10mL of trypsin (1 mg mL⁻¹) at 37 °C for 16 h. The peptides from Col-0 grown on Normal medium, 0-Fe, 300-Zn medium and *irt1-1* mutant grown on Normal medium, were labeled with iTRAQ-114, – 115, – 116, and – 117 reagents, respectively, at room temperature for 60 min. For iTRAQ-CEX analysis, the mixed peptides were manually separated by 25, 50, 75, 100, 200, 350, and 1000 mM KCl using strong cation exchange (AB SCIEX) and then desalted on Sep-Pak C18 cartridges (Waters). The labeled peptides were concentrated by a vacuum concentrator. However, for iTRAQ labeling coupled with OFFGEL fractionation, the mixed peptide mixture was directly desalted
Table 3. iTRAQ-OFFGEL analysis based identified proteins with less than 0.5-fold expression

| Protein Description | Peptide Coverage a | Ratio (Col-0 0-Fe/Col-0 Normal) | Ratio (Col-0 300-Zn/Col-0 Normal) | Ratio (irt1-1 Normal/Col-0 Normal) |
|---------------------|-------------------|---------------------------------|-----------------------------------|---------------------------------|
| delta tonoplast integral protein | 2.3 | 0.171 ± 0.058 | 0.378 ± 0.053 | 1.116 ± 0.386 |
| Aldolase superfamily protein | 3.3 | 0.182 ± 0.009 | 0.371 ± 0.058 | 0.811 ± 0.324 |
| Auxin-responsive family protein | 2.3 | 0.245 ± 0.070 | 0.415 ± 0.027 | 0.777 ± 0.179 |
| Major facilitator superfamily protein | 4.0 | 0.247 ± 0.031 | 0.451 ± 0.070 | 0.883 ± 0.204 |
| ribulose-bisphosphate carboxylases | 20.7 | 0.283 ± 0.034 | 0.622 ± 0.050 | 0.849 ± 0.125 |
| 5-adenosyl-l-homocysteine (SAH) hydrolase | 1.3 | 0.301 ± 0.044 | 0.505 ± 0.071 | 0.966 ± 0.196 |
| Unknown protein | 2.3 | 0.311 ± 0.271 | N.D. | N.D. |
| pyruvate dehydrogenase E1 beta | 2.7 | 0.314 ± 0.033 | 0.492 ± 0.043 | 0.793 ± 0.316 |
| Plasma membrane intrinsic protein 1C | 4.0 | 0.316 ± 0.056 | 0.753 ± 0.152 | 1.167 ± 0.070 |
| Photosystem I reaction centre subunit IV / Psae protein | 2.0 | 0.319 ± 0.066 | 0.528 ± 0.058 | 0.844 ± 0.051 |
| chloroplast RNA binding | 6.3 | 0.328 ± 0.088 | 0.759 ± 0.121 | 1.439 ± 0.302 |
| Photosystem I reaction center subunit PSI-N, chloroplastic, putative / PSI-N, putative (PSAN) | 3.7 | 0.335 ± 0.051 | 0.532 ± 0.075 | 0.791 ± 0.120 |
| Iron-sulfur cluster binding/electron carriers;4 iron, 4 sulfur cluster binding | 1.3 | 0.340 ± 0.090 | 0.563 ± 0.127 | 0.939 ± 0.160 |
| cytochrome P450, family 83, subfamily A, polypeptide 1 | 3.3 | 0.341 ± 0.030 | 0.427 ± 0.109 | 0.746 ± 0.232 |
| photosystem I subunit K | 1.0 | 0.348 ± 0.109 | 0.515 ± 0.059 | 0.800 ± 0.082 |
| ribulosebisphosphate carboxylase small chain 1A | 3.3 | 0.348 ± 0.011 | 0.728 ± 0.105 | 0.867 ± 0.158 |
| long chain acyl-CoA synthetase 9 | 3.7 | 0.348 ± 0.062 | 0.601 ± 0.063 | 0.796 ± 0.238 |
| Protein of unknown function, DUF538 | 8.3 | 0.351 ± 0.074 | 0.564 ± 0.066 | 1.941 ± 0.566 |
| Photosystem I, Psaa/PsaB protein | 7.7 | 0.356 ± 0.096 | 0.552 ± 0.080 | 0.891 ± 0.070 |
| HAD superfamily, subfamily IIIb acid phosphatase | 9.0 | 0.358 ± 0.087 | 0.781 ± 0.156 | 1.194 ± 0.071 |
| Neurofilament protein-related | 17.0 | 0.362 ± 0.090 | 0.556 ± 0.104 | 1.091 ± 0.147 |
| fatty acid desaturase 6 | 2.0 | 0.369 ± 0.047 | 0.506 ± 0.032 | 0.659 ± 0.167 |
| K+ efflux antiporter 1 | 9.7 | 0.375 ± 0.005 | 0.815 ± 0.148 | 0.865 ± 0.132 |
| sedoheptulose-bisphosphatase | 2.0 | 0.378 ± 0.196 | 0.675 ± 0.081 | 0.869 ± 0.034 |
| Involved in response to salt stress. Knockout mutants are hypersensitive to salt stress. | 4.3 | 0.379 ± 0.035 | 0.502 ± 0.061 | 1.073 ± 0.073 |
| Dihydrolipoyl dehydrogenases | 3.0 | 0.381 ± 0.124 | 0.507 ± 0.095 | 0.808 ± 0.351 |
| Uncharacterised protein family (UPF0497) | 2.0 | 0.387 ± 0.082 | 0.544 ± 0.009 | 0.900 ± 0.063 |
| 3-ketoacyl-CoA synthase 6 | 1.7 | 0.394 ± 0.104 | 0.600 ± 0.207 | 1.080 ± 0.396 |
| Lipoamide dehydrogenase 1 | 2.0 | 0.404 ± 0.059 | 0.551 ± 0.076 | 0.785 ± 0.266 |
| Unknown protein | 4.3 | 0.406 ± 0.015 | 0.611 ± 0.094 | 0.832 ± 0.162 |
| monogalactosyldiacylglycerol synthase 1 | 2.0 | 0.409 ± 0.138 | 0.565 ± 0.157 | 0.905 ± 0.131 |
| Photosynthetic electron transfer D | 1.3 | 0.410 ± 0.061 | 0.492 ± 0.072 | 0.874 ± 0.094 |
| Photosystem I subunit G | 2.3 | 0.410 ± 0.112 | 0.581 ± 0.056 | 0.887 ± 0.068 |
| Photosystem I, Psaa/PsaB protein | 10.7 | 0.411 ± 0.067 | 0.609 ± 0.117 | 1.067 ± 0.115 |
| Remorin family protein | 13.3 | 0.412 ± 0.043 | 0.628 ± 0.056 | 0.814 ± 0.222 |

*Peptide indicates average number of assigned peptides. Coverage indicates average percentage of assigned peptides to the predicted protein. The values were calculated as the ratio of 115 (Col-0 0-Fe) to 114 (Col-0 Normal), 116 (Col-0 300-Zn) to 114 (Col-0 Normal), or 117 (irt1-1 Normal) to 114 (Col-0 Normal). Microsomal proteins from the Col-0 grown on Normal or 0-Fe or 300-Zn media and from the irt1-1 mutant grown on Normal medium were labeled with iTRAQ-114, 115, 116, and 117 reagents, respectively. Proteins that decreased less than 0.5-fold were selected and ordered based on fold change values in Col-0 0-Fe/Col-0 Normal. However, the response of proteins in the Col-0 300-Zn/Col-0 Normal and irt1-1 Normal/Col-0 Normal were ordered as per their position in fold change values in Col-0 0-Fe/Col-0 Normal. Data were mean ± SD of 3 independent experiments. N.D. means protein and/or iTRAQ reporter ions were not detected.
on Sep-Pak C18 cartridges (Waters) followed by lyophilization to concentrate the peptides and then subjected to OFFGEL fractionation.

**iTRAQ-OFFGEL fractionation analysis**

The trypsin digested and iTRAQ labeled peptides were mixed with the supplied OFFGEL buffer to obtain a 3.60 mL sample solution, which was then subjected to isoelectric focusing using immobilized pH gradient strips in the liquid phase. The peptides were separated into 24 fractions with a 3100 OFFGEL fractionator (Agilent Technologies) using a 24 cm IPG gel, pH 3-10 (GE Healthcare) at 4500 V for 50,000 Vh at 50 µA, according to the manufacturer's instructions. The fractionated peptides were automatically purified using C-TIP (AMR); the details are described in Fukao et al. (2013).

**LC-MS/MS analysis**

iTRAQ analysis was performed on the LTQ Orbitrap XL-HTC-PAL-Paradigm MS4 system. The iTRAQ-labeled peptides were loaded on the column (75mm internal diameter,
15 cm; L-Column; CERI) using a Paradigm MS4 HPLC pump (Michrom Bioresources) and an HTC-PAL autosampler (CTC Analytics). Buffers were 0.1% (v/v) acetic acid and 2% (v/v) acetonitrile in water (A) and 0.1% (v/v) acetic acid and 90% (v/v) acetonitrile in water (B). A linear gradient from 5% to 45% B for 70 min was used, and peptides eluted from the column were introduced directly into an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with a flow rate of 200 nL min−1 and a spray voltage of 2.0 kV. The range of the mass spectrometric scan was mass-to-charge ratio 450 to 1500, and the top 3 peaks were subjected to tandem mass spectrometry analysis. The obtained spectra were compared against data in The Arabidopsis Information Resource 10 (TAIR10; http://www.arabidopsis.org/) using the MASCOT server (version 2.4; Matrix Science) via Proteome Discover software (version 1.3; Thermo Scientific). The following search parameters were used for database searching: threshold set-off at 0.05 in the ion-score cutoff; protein identification cutoff set to 2 assigned spectra per predicted protein; peptide charge of 2+ or 3+; tandem mass spectrometric scan was mass-to-charge ratio 450 to 1500, and peptide tolerance at 10 ppm; predicted protein; protein identification cutoff set to 2 assigned spectra per predicted protein; peptide tolerance at ±0.2 Da; peptide charge of 2+ or 3+; trypsin as the enzyme and allowing up to one missed cleavage; iTRAQ label and methyl methanethiosulfonate on Cys as a fixed modification; and oxidation on Met as a variable modification. iTRAQ data for 3 biological replicates were analyzed by MASCOT, and the data only with FDR of less than 1% were used for subsequent analysis. Only proteins that were identified in all 3 independent experiments were considered. Statistical analysis was conducted using the Student t test.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
The authors thank Dr Izumi Mori and Ms Sanae Rikishi (Institute of Plant Science and Resources, Okayama University) for technical assistance to ICP-MS analysis. This work was supported by a Grant-in-Aid for Organelle Differentiation as the Strategy for Environmental Adaptation in Plants for Scientific Research of Priority Areas (No. 19039022 to Fukao Y) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Grant-in-Aid for Scientific Research from Nara Institute of Science and Technology supported by The Ministry of Education, Culture, Sports, Science and Technology, Japan. Zargar SM acknowledges the DBT, New Delhi, India for award of CREST, Overseas fellowship. This research was supported by Japan Advanced Plant Science Network.

Supplementary Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/psb/article/26892

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