Molecular and Biochemical Characterization of the Fe(III) Chelate Reductase Gene Family in *Arabidopsis thaliana*

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Iron chelate reductase is required for iron acquisition from soil and for metabolism in plants. In the genome of *Arabidopsis thaliana* there are eight genes classified into the iron chelate reductase gene family (*AtFROs*) based on sequence homology with *AtFRO2* (a ferric chelate reductase in Arabidopsis). They are localized on chromosome 1 (three *AtFROs*) and chromosome 5 (five *AtFROs*) of Arabidopsis and show a high level of amino acid sequence similarity to each other. An assay for ferric chelate reductase activity revealed that *AtFRO2*, *AtFRO3*, *AtFRO4*, *AtFRO5*, *AtFRO7* and *AtFRO8* conferred significantly increased iron reduction activity compared with the control when expressed in yeast cells, indicating that the six *AtFROs* encode iron chelate reductases functioning in iron homeostasis in Arabidopsis. *AtFRO2* displayed the highest iron reduction activity among the *AtFROs* investigated, further demonstrating that *AtFRO2* is a major iron reductase gene in Arabidopsis. *AtFRO2* and *AtFRO3* were mainly expressed in roots of Arabidopsis, *AtFRO5* and *AtFRO6* in shoots and flowers, and *AtFRO7* in cotyledons and trichomes, whereas the transcription of *AtFRO8* was specific for leaf veins. Considering the tissue-specific expression profiles of *AFRO* genes, we suggest that *AtFRO2* and *AtFRO3* are two Fe(III) chelate reductases mainly functioning in iron acquisition and metabolism in Arabidopsis roots, while *AtFRO5*, *AtFRO6*, *AtFRO7* and *AtFRO8* are required for iron homeostasis in different tissues of shoots.

**Keywords:** Arabidopsis — *AtFROs* — Fe(III) chelate reductase — Gene family — Iron homeostasis.

Abbreviations: FRO, ferric reduction oxidase; GUS, β-glucuronidase; RT–PCR, reverse transcription–PCR.

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**Introduction**

Iron is an essential element for all organisms. Coordinated at metalloprotein active sites, iron participates in the redox reaction and also in many vital enzymatic reactions required for fundamental biological processes such as DNA synthesis, photosynthesis and respiration, as well as hormone synthesis. Although abundant in soil, iron is one of the most common nutrients limiting plant growth and development due to its extremely low solubility in aerobic environments at neutral and alkaline pH (Guerinot and Yi 1994). Iron deficiency results in a decrease of Fe-containing pigment proteins and chlorophylls, causing chlorosis in young leaves. Plants need effective mechanisms to acquire iron from the soil to meet the demand for growth and development on the one hand and to avoid toxicity by iron excess on the other hand because excessive iron in cells catalyzes the generation of hydroxyl radicals that can damage cellular constituents (Halliwell and Gutteridge 1992).

For effective acquisition of iron, two mechanisms, known as strategy I and strategy II (Römheld and Marschner 1986), have been well described in higher plants. All plants, with the exception of the grasses, employ the strategy I mechanism to acquire iron effectively from soil under iron stress. This mechanism includes acidification of the rhizosphere by enhanced extrusion of protons and organic acids to drive more ferric iron into solution, reduction of Fe(III) to Fe(II) on the root surface and transport of Fe(II) across the root epidermal cell membrane. In iron-efficient plants, these iron deficiency responses [proton extrusion, Fe(III) chelate reduction and the Fe(II) transporter activities] are obviously enhanced under iron-limiting stress (Bienfait et al. 1983, Fox et al. 1996).

*Arabidopsis thaliana* is a model plant for molecular biological study in plants based on its small genome size and the availability of its complete genome sequence, as well as a short generation time (Arabidopsis Genome Initiative 2000). Arabidopsis exhibits typical iron deficiency responses of strategy I under iron limitation. Two major discoveries in understanding the molecular mechanism of iron uptake in strategy I plants have been made with Arabidopsis in the past decade. First, expression of an Arabidopsis cDNA library in the *Saccharomyces cerevisiae* fet3fet4 double mutant strain, impaired in both low and high affinity iron transport, enabled cloning of a cDNA encoding a putative Arabidopsis Fe(II) transporter named IRT1 (Eide et al. 1996). Loss of function of IRT1 in its knock-out mutant revealed typical iron deficiency symptoms causing severe leaf chlorosis and lethality in soil.

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indicating that IRT1 is an essential transporter for iron uptake from soil and for plant growth (Henriques et al. 2002, Varotto et al. 2002, Vert et al. 2002). IRT1 expression was controlled at the transcriptional and post-transcriptional levels responding to iron deficiency (Connolly et al. 2002). Secondly, a PCR-based cloning approach using degenerated oligonucleotides derived from the yeast ferric reductase (FRE) sequences enabled isolation of ferric reduction oxidase 2 (FRO2) in Arabidopsis. This gene is allelic to FRD1-1 (ferric chelate reductase-defective) mutant plants and its expression was induced in roots under iron deficiency conditions (Robinson et al. 1999). Connolly et al. (2003) observed that FRO2 was regulated at both the transcriptional and post-transcriptional levels coordinately with IRT1 by iron and zinc.

Reduction of Fe(III) to Fe(II) on the root surface is an obligatory process for iron acquisition in strategy I plants (Yi and Guerinot 1996). Loss of the inducible reduction function of Fe(III) in roots of the Arabidopsis mutant frd1 led to growth impairment under iron-limiting conditions. The reduction of Fe(III) to Fe(II) is carried out by a plasma membrane-bound Fe(III) chelate reductase (Buckhout et al. 1989, Holden et al. 1991). Three iron chelate reductase genes (FRO2 from Arabidopsis and FRO1 from tomato) responsible for iron reduction on the root surface have been isolated and characterized (Robinson et al. 1999, Waters et al. 2002, Li et al. 2004). Recent studies demonstrated that LeFRO1 in tomato and FRO2 in Arabidopsis are controlled by the regulatory genes FER (Ling et al. 2002, Li et al. 2004) and FIT1 (Colangelo and Guerinot 2004, Jakoby et al. 2004), respectively, at the transcriptional level. In addition, CHLN which encodes a nicotianamine synthase LeNAS in tomato (Higuchi et al. 1999, Ling et al. 1999) is required in the transcriptional down-regulation of LeFRO1 under iron sufficiency condition in tomato (Li et al. 2004).

In dicots and non-graminaceous monocots, iron is first reduced on the root surface from Fe(III) to Fe(II) by Fe(III) chelate reductase and then transferred across the rhizodermal plasma membrane barrier by a high affinity Fe(II) transporter such as IRT1 into roots. It is then oxidized and transported as an Fe(III) citrate complex for long-distance transportation in xylem to the shoot (Hell and Stephan 2003). For assimilation in leaves and other tissues, iron is again reduced. It is still unknown which genes are involved in the Fe(III) reduction process in different tissues. In the genome of Arabidopsis, seven additional genes, encoding protein sequences highly similar to the ferric reduction oxidase 2 (FRO2), were predicted and together classified into the FRO gene family. Except for FRO2, the biological functions of the other seven genes in this family are not known. In this paper, we report on the molecular and biochemical characterization of the AtFRO gene family in Arabidopsis, describing the molecular characteristics of the eight AtFRO genes, their Fe(III) chelate reductase activity and their expression profiles.

Results

FRO gene family in Arabidopsis

The genome sequence of Arabidopsis was blasted against the amino acid sequence of the ferric chelate reductase AtFRO2 (Robinson et al. 1999). Eight predicted genes (At1g01580, At1g01590, At1g23020, At5g23980, At5g23990, At5g49730, At5g49740 and At5g50160) were found and they showed high sequence similarity to each other at the protein level (Table 1). They all contain the conserved features of Fe(III) chelate reductases (Table 2) described by Waters et al. (2002) and are classified into the FRO gene family of Arabidopsis. AtFRO2, AtFRO1 and AtFRO3 described previously by Robinson et al. (1999) correspond to the predicted genes At1g01580, At1g01590 and At1g23020, respectively, and are localized in the terminal region of Arabidopsis chromosome 1. The remaining five predicted genes are found at two loci on chromosome 5. We designated them AtFRO4 (At5g23980), AtFRO5 (At5g23990), AtFRO6 (At5g49730), AtFRO7 (At5g49740) and AtFRO8 (At5g50160). Sequence analysis with the TMMTOP program indicates that five AtFROs (AtFRO1, AtFRO2, AtFRO3, AtFRO4 and AtFRO5) contain nine and the remaining three (AtFRO6, AtFRO7 and AtFRO8) contain 10 integral transmembrane domains. An additional peripheral membrane domain was predicted in AtFRO1, AtFRO2, AtFRO3, AtFRO4, AtFRO5 and AtFRO8 (Table 2).

On the basis of the predicted open reading frame sequences in the database, we designed 5′ and 3′ primers (see the Supplementary material) for the eight AtFRO genes and the cDNAs of these genes were amplified with the primers from total RNA isolated from Fe deficiency-induced seedlings by reverse transcription–PCR (RT–PCR) with the exception of AtFRO1 because of its extremely low expression. The cDNAs were sequenced and their sequences were compared with the predicted sequences in the database. With the exception of AtFRO7, they were all identical. AtFRO7 showed differential splicing and has three different mRNA products. The coding sequence of AtFRO7 (GenBank accession number AY912280) is 2244 bp and was 24 bp longer than the computer-predicted sequence due to an incorrect splice at the second intron. The other two mRNAs of AtFRO7 were 2354 bp (containing the fourth intron) and 2435 bp (containing the sequences of the fourth and seventh introns) in length.

The sequence analysis of the eight FRO genes together with LeFRO1 from tomato (Li et al. 2004) and PsFRO1 from pea (Waters et al. 2002) showed that the phylogenetic relationship of the eight FROs can be classified into four groups (Fig. 1). The three FRO genes located on chromosome 1 were grouped together whereas the five genes on chromosome 5 were clustered into three groups (AtFRO4 with AtFRO5, AtFRO6 with AtFRO7, and AtFRO8). AtFRO8 is the most divergent from the other seven FRO genes and is clustered alone in a group. The well characterized Fe(III) chelate reductase genes LeFRO1 from tomato and PsFRO1 from pea are
Iron chelate reductase gene family in Arabidopsis

Comparative analysis of the structures among the eight AtFRO genes showed that eight exons and seven introns are present in AtFRO1, AtFRO2, AtFRO3, AtFRO4, AtFRO5 and AtFRO8, whereas nine exons and eight introns were identified in AtFRO6 and AtFRO7. The locations of splice sites vary widely among some genes (e.g. between AtFRO8 and the other seven AtFROs) while they are identical in others (e.g. between AtFRO4 and AtFRO5, AtFRO6 and AtFRO7). The relationship between the exon number and location of splice sites of each gene closely reflects the phylogenetic relationships shown in Fig. 1.

**Fe(III) chelate reductase activity assay of AtFROs in yeast cells**

It was shown earlier that AtFRO2 was an essential protein for Fe(III) chelate reduction in Arabidopsis (Robinson et al. 1999). The other seven AtFROs have high homology to AtFRO2 at the protein level, suggesting that they all encode Fe(III) chelate reductases involved in iron homeostasis in *A. thaliana*. To test this hypothesis, the iron reduction activity of the seven AtFROs was measured and the results are presented in Table 1.

Table 1 Percentage amino acid residue identity or similarity (italics) between the products of the AtFRO genes, as calculated by the BESTFIT algorithm of the GCG package software

<table>
<thead>
<tr>
<th>AtFRO</th>
<th>AtFRO2</th>
<th>AtFRO3</th>
<th>AtFRO4</th>
<th>AtFRO5</th>
<th>AtFRO6</th>
<th>AtFRO7</th>
<th>AtFRO8</th>
</tr>
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<tbody>
<tr>
<td>AtFRO1</td>
<td>73.02</td>
<td>84.41</td>
<td>60.12</td>
<td>59.76</td>
<td>45.97</td>
<td>48.22</td>
<td>47.79</td>
</tr>
<tr>
<td>AtFRO2</td>
<td>65.80</td>
<td>71.66</td>
<td>63.68</td>
<td>62.36</td>
<td>46.94</td>
<td>45.51</td>
<td>48.85</td>
</tr>
<tr>
<td>AtFRO3</td>
<td>80.08</td>
<td>64.39</td>
<td>58.57</td>
<td>57.73</td>
<td>46.52</td>
<td>47.40</td>
<td>51.06</td>
</tr>
<tr>
<td>AtFRO4</td>
<td>48.96</td>
<td>51.66</td>
<td>47.09</td>
<td>93.13</td>
<td>45.78</td>
<td>45.16</td>
<td>44.38</td>
</tr>
<tr>
<td>AtFRO5</td>
<td>48.24</td>
<td>50.86</td>
<td>45.66</td>
<td>91.27</td>
<td>43.99</td>
<td>44.33</td>
<td>47.22</td>
</tr>
<tr>
<td>AtFRO6</td>
<td>33.24</td>
<td>34.40</td>
<td>34.39</td>
<td>33.48</td>
<td>32.40</td>
<td>94.44</td>
<td>46.09</td>
</tr>
<tr>
<td>AtFRO7</td>
<td>35.76</td>
<td>33.38</td>
<td>34.71</td>
<td>32.70</td>
<td>32.56</td>
<td>92.54</td>
<td>47.03</td>
</tr>
<tr>
<td>AtFRO8</td>
<td>35.77</td>
<td>35.79</td>
<td>38.60</td>
<td>32.98</td>
<td>35.49</td>
<td>34.34</td>
<td>35.16</td>
</tr>
</tbody>
</table>

Table 2 Summarized features of the AtFRO family open reading frames (ORFs)

<table>
<thead>
<tr>
<th>ORF name</th>
<th>Other name</th>
<th>ORF size</th>
<th>Transmembrane regions</th>
<th>FAD-binding motif</th>
<th>NADPH-binding motif</th>
<th>Bis-heme-binding motif</th>
<th>Oxidoreductase signature motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g01580</td>
<td>AtFRO2</td>
<td>725</td>
<td>9+1</td>
<td>HPFT</td>
<td>GPYG</td>
<td>+</td>
<td>LVMVCQGSITPFPIS</td>
</tr>
<tr>
<td>At1g01590</td>
<td>AtFRO1</td>
<td>704</td>
<td>9+1</td>
<td>HPFT</td>
<td>GPYG</td>
<td>+</td>
<td>LVMVSQGSITPFPIS</td>
</tr>
<tr>
<td>At1g23020</td>
<td>AtFRO3</td>
<td>693</td>
<td>9+1</td>
<td>HPFT</td>
<td>GPYG</td>
<td>+</td>
<td>LVMVSQGSITPFPIS</td>
</tr>
<tr>
<td>At5g23980</td>
<td>AtFRO4</td>
<td>699</td>
<td>9+1</td>
<td>HPFT</td>
<td>GPYG</td>
<td>+</td>
<td>LILVQQGQITPFPIS</td>
</tr>
<tr>
<td>At5g23990</td>
<td>AtFRO5</td>
<td>707</td>
<td>9+1</td>
<td>HPFT</td>
<td>GPYG</td>
<td>+</td>
<td>LILVQQGQITPFPIS</td>
</tr>
<tr>
<td>At5g49730</td>
<td>AtFRO6</td>
<td>738</td>
<td>10</td>
<td>HPFS</td>
<td>GPYG</td>
<td>+</td>
<td>LFLVAGGIGTPPLS</td>
</tr>
<tr>
<td>At5g49740</td>
<td>AtFRO7</td>
<td>747</td>
<td>10</td>
<td>HPFS</td>
<td>GPYG</td>
<td>+</td>
<td>LFLVAGGIGTPPLS</td>
</tr>
<tr>
<td>At5g50160</td>
<td>AtFRO8</td>
<td>728</td>
<td>10+1</td>
<td>HPFS</td>
<td>GPYG</td>
<td>+</td>
<td>LFLVAGGIGTPPLS</td>
</tr>
</tbody>
</table>

*The first number indicates integral membranes and the second number refers to peripheral membranes (TMMTOP).*

Fe(III) chelate reductase activity assay of AtFROs in yeast cells

It was shown earlier that AtFRO2 was an essential protein for Fe(III) chelate reduction in Arabidopsis (Robinson et al. 1999). The other seven AtFROs have high homology to AtFRO2 at the protein level, suggesting that they all encode Fe(III) chelate reductases involved in iron homeostasis in *A. thaliana*. To test this hypothesis, the iron reduction activity of the seven AtFROs was measured and the results are presented in Table 1.
Iron chelate reductase gene family in Arabidopsis

Fig. 2 Fe(III) chelate reductase activity assay of AtFROs in yeast cells. The wild-type yeast strain BJ2168 transformed with the pYES2.0 (vector) or the pYES2.0-AtFRO plasmid was grown in iron-limiting medium (supplemented with 1 mM EDTA) and assayed for Fe(III) chelate reductase activity as described by Eide et al. (1992). The data shown are average values of five individual experiments. * and ** indicate significant differences at P < 0.05 and P < 0.01, respectively, compared with the control.

Fig. 3 Expression profiles of AtFRO genes in Arabidopsis. The gene transcription intensities among different tissues were analyzed by RT–PCR analysis using gene-specific oligonucleotides described in the Supplementary data. (a) Expression patterns of the AtFRO gene family in roots and shoots under iron-limiting stress and iron-sufficient conditions. (b) Transcription profiles of the eight AtFRO genes in flowers and cotyledons.

Expression profiles of AtFROs in Arabidopsis plants

Among the eight FRO genes in Arabidopsis, AtFRO2 is mainly expressed in roots and enhanced under iron deficiency (Robinson et al. 1999). Its mRNA was also detected in leaves and flowers at a low level (Connolly et al. 2003). To study the expression patterns of the other AtFRO genes, total RNA was extracted from roots, shoots, cotyledons and flowers and analyzed by semi-quantitative RT–PCR with gene-specific primers (Supplementary material). The results are shown in Fig. 3. Consistent with previous reports, AtFRO2 was mainly expressed in roots and the expression intensity was strongly up-regulated under iron deficiency conditions (Fig. 3a). AtFRO3 showed a similar expression pattern to that of AtFRO2. It was mainly expressed in roots and the expression intensity was strongly enhanced under iron-limiting conditions (Fig. 3a). Compared with AtFRO2 and AtFRO3, transcription of AtFRO6 and AtFRO7 was mainly detected in shoots. Their transcription levels were high, regardless of the iron status. AtFRO8 transcription was clearly induced in roots under iron starvation, whereas its expression was constitutive in shoots at a relatively high level (Fig. 3a). Transcripts of AtFRO4 and AtFRO5 were found at low level in roots and shoots, especially AtFRO4. Among the eight AtFRO genes, AtFRO1 showed the lowest expression intensity, its transcript was detected only by RT–PCR with a large number of PCR cycles (>40, data not shown). Bauer et al. (2004) investigated the expression of AtFRO1, AtFRO2, AtFRO3, At5g23980 (AtFRO4) and At5g23990 (AtFRO5) in roots and leaves under iron-deficient and -sufficient conditions by RT–PCR combined with Southern hybridization, and generally showed similar expression patterns to those described above. The high transcription levels of AtFRO2 in roots under the culture condition with iron and in leaves without iron differ from the results reported in this work, and those of Robinson et al. (1999) and Connolly et al. (2003). This might be due to different culture conditions and analysis method used.

In flowers, the transcripts of AtFRO2, AtFRO3, AtFRO5, AtFRO6, AtFRO7 and AtFRO8 were clearly observed, while the transcription of AtFRO1 and AtFRO4 was not detected. AtFRO5 mRNA was most abundant among the six AtFRO genes expressed in flowers (Fig. 3b). Four of the eight AtFRO transcripts (AtFRO3, AtFRO4, AtFRO6 and AtFRO7) were detected in cotyledons. AtFRO7 showed the highest transcrip-
Histochemical analysis of GUS activity driven by AtFRO gene promoters in transgenic Arabidopsis lines

To confirm and localize the expression of the AtFRO genes in plant tissues, putative promoters for AtFRO gene family members were used to construct AtFRO promoter:β-glucuronidase (AtFRO-GUS) fusions for transformation of Arabidopsis. Each construct contained approximately 1.2–2.5 kb of genomic DNA sequence upstream of the transcription start site. The eight constructs were introduced into the Arabidopsis genome by floral transformation with Agrobacterium tumefaciens. Stable transformants were selected for further analysis. The GUS transcription profiles in the transgenic plants were evaluated by RT–PCR analysis before histochemical assay (Fig. 4). The GUS transcripts appeared in roots of the transgenic plants with AtFRO2-GUS and AtFRO3-GUS and in shoots of the seedlings with AtFRO5-GUS, AtFRO6-GUS, AtFRO7-GUS and AtFRO8-GUS. The enhanced GUS transcription under iron deficiency was observed in the transgenic plants with AtFRO2-GUS, AtFRO3-GUS and AtFRO5-GUS (Fig. 4). The GUS expression in seedlings of AtFRO1-GUS and AtFRO4-GUS was low and it was detected only by RT–PCR with more PCR cycles (from 28 to 40). This is fully consistent with the expression patterns of AtFRO genes described in Fig. 3.

Histochemical assays of GUS activity with the seedlings of transgenic plants were performed and the results are presented in Fig. 5. Four different GUS staining patterns were observed among the transgenic Arabidopsis seedlings with AtFRO-GUS: (i) clear GUS staining in roots of transgenic plants with AtFRO2-GUS and AtFRO3-GUS (Fig. 5a–d); (ii) in shoots with AtFRO5-GUS and AtFRO6-GUS (Fig. 5e–h); (iii) in leaves and trichomes (Fig. 5i–k) with AtFRO7-GUS; and (iv) in leaf veins with AtFRO8-GUS (Fig. 5l–n). No GUS staining was found in the seedlings of the transgenic plants with AtFRO1-GUS and AtFRO4-GUS. The GUS staining intensity was clearly stronger under iron-limiting than under iron-sufficient conditions in AtFRO2-GUS, AtFRO3-GUS and AtFRO5-GUS plants (Fig. 5a–d, g, h). The histochemical assay results of the GUS activity in seedlings are consistent with the RT–PCR analysis results presented above.

GUS activities in cotyledons were determined with 4-day-old seedlings of the transgenic Arabidopsis lines germinated on MS solid medium with iron. Consistent with RT–PCR results of the AtFRO genes (Fig. 3b), GUS activity was detected in cotyledons of AtFRO3-GUS, AtFRO4-GUS, AtFRO6-GUS and AtFRO7-GUS lines (Fig. 6a–e). The cotyledons of AtFRO7-GUS plants revealed a strong GUS staining (Fig. 6e), whereas a relatively weak GUS activity appeared in cotyledons of AtFRO3-GUS and AtFRO6-GUS lines (Fig. 6a, d). Interestingly, the GUS activity in cotyledons of the AtFRO4-GUS line was mostly localized in cotyledon veins (Fig. 6b, c).

We also studied the expression of AtFRO genes in flowers by histochemical analysis of GUS activity with the transgenic AtFRO-GUS plants. In developing flowers of the AtFRO5-GUS plants, GUS activity was observed in floral buds and localized mainly in the sepals (Fig. 6f, g). In open flowers, the GUS activity was detected in stigma and anther filament (Fig. 6h). A strong AtFRO5-GUS expression was also displayed in pedicel and inflorescence stem (Fig. 6i). AtFRO6-GUS showed intense expression in floral buds and open flowers throughout sepals, anther filaments and stigma (Fig. 6j–l). In the transgenic plants with AtFRO8-GUS, the GUS activity was detected only in the pedicel, and prominently in the transition zones between pedicel and inflorescence stem (Fig. 6m). Consistent with data from Connolly et al. (2003), GUS activity was determined in anthers and anther filaments in flowers of AtFRO2-GUS plants (data not shown). No GUS staining in flowers was observed in transgenic plants with the rest of the constructs (AtFRO1-GUS, AtFRO3-GUS, AtFRO4-GUS and AtFRO7-GUS).

Effect of FIT1 on AtFRO gene expression

FIT1 (Colangelo and Guerinot 2004) or FRU (Jakoby et al. 2004) as a homolog of tomato FER (Ling et al. 2002) was recently identified as an essential protein involved in iron homeostasis in Arabidopsis. It regulates AtFRO2 at the transcriptional level and AtIRT1 at the protein level (Colangelo and Guerinot 2004). To check whether FIT1 regulates other AtFRO genes in Arabidopsis, we isolated total RNA from roots of fit1 mutant plants, which grew under iron starvation conditions for 14 d. The expression patterns of the eight AtFRO genes in the roots of the wild type and the fit1 mutant were compared (Fig. 6n).
Consistent with the report by Colangelo and Guerinot (2004), \textit{AtFRO2} was regulated by \textit{FIT1}. The loss-of-function mutant of Arabidopsis \textit{fit1} led to abolished transcription of \textit{AtFRO2} under iron starvation. In addition to \textit{AtFRO2}, the mRNA abundance of \textit{AtFRO3} and \textit{AtFRO4} was also significantly decreased in the \textit{fit1} mutant plants compared with the wild type, suggesting that transcription of \textit{AtFRO3} and \textit{AtFRO4} is partially controlled by \textit{FIT1} directly or indirectly. No obvious changes in root gene expression were observed among the other five \textit{AtFROs} between the \textit{fit1} mutant and wild type (Fig. 7).

**Discussion**

The \textit{FRO} gene family comprising eight members in Arabidopsis was characterized at the molecular and biochemical levels. The predicted proteins of AtFROs all contain conserved features of Fe(III) chelate reductase, and six of them showed significantly increased iron reduction activity when expressed in yeast cells, suggesting that they encode Fe(III) chelate reductases functioning in iron homeostasis in Arabidopsis. RT–PCR analysis and monitoring with the promoter–GUS method revealed the tissue specificity of gene expression among the eight \textit{AtFRO} genes. These indicate that the \textit{AtFROs} individually function in different tissues of Arabidopsis for iron homeostasis.

Iron is a transition metal involved in electron transfer reactions of many fundamental biological processes. Reduction of this metal is necessary prior to its transport through the plasma membrane (Georgatsou and Alexandraki 1999). In strategy I plants, iron is first reduced from Fe(III) to Fe(II) on the root surface and then taken up in root cells. There, it is oxidized to Fe(III) and is then transported as an Fe(III) citrate complex for long-distance transport in the xylem from root to shoot.
Iron chelate reductase gene family in Arabidopsis

For assimilation in cells of leaves and other tissues, iron again needs to be reduced. Reasonably, the iron reduction in different tissues or cells of Arabidopsis is performed by different AtFROs. AtFRO2 and AtFRO3 were mainly expressed in the roots, especially under iron starvation conditions. These results were also supported by the histochemical assays of GUS activity in transgenic plants, suggesting that AtFRO2 and AtFRO3 principally function in roots of Arabidopsis. Previous studies demonstrated that AtFRO2 is essential and a major Fe(III) chelate reductase of Arabidopsis involved in iron reduction in roots (Robinson et al. 1999). In situ hybridization experiments showed that AtFRO2 is detected at high levels in the epidermal cells but not in the vascular cylinder of the Arabidopsis roots (Connolly et al. 2003), suggesting that AtFRO2 functions mainly on the root surface for reducing Fe(III) to Fe(II). AtFRO3 revealed the same expression pattern as AtFRO2 under iron-limiting stress and was expressed at a relatively high level even under iron sufficiency conditions. AtFRO3 might possibly function both in iron metabolism in roots and in iron reduction on the root surface for uptake. Based on the fact that GUS expression driven by AtFRO3 and AtFRO6 promoters was delimited in shoots (leaves and stems) and AtFRO8 only in the tissue of leaf vein, the three AtFROs possibly function in iron reduction in shoots, where AtFRO8 might specifically reduce Fe(III) in leaf shoot (Hell and Stephan 2003). For assimilation in cells of leaves and other tissues, iron again needs to be reduced. Reasonably, the iron reduction in different tissues or cells of Arabidopsis is performed by different AtFROs. AtFRO2 and AtFRO3 were mainly expressed in the roots, especially under iron starvation conditions. These results were also supported by the histochemical assays of GUS activity in transgenic plants, suggesting that AtFRO2 and AtFRO3 principally function in roots of Arabidopsis. Previous studies demonstrated that AtFRO2 is essential and a major Fe(III) chelate reductase of Arabidopsis involved in iron reduction in roots (Robinson et al. 1999). In situ hybridization experiments showed that AtFRO2 is detected at high levels in the epidermal cells but not in the vascular cylinder of the Arabidopsis roots (Connolly et al. 2003), suggesting that AtFRO2 functions mainly on the root surface for reducing Fe(III) to Fe(II). AtFRO3 revealed the same expression pattern as AtFRO2 under iron-limiting stress and was expressed at a relatively high level even under iron sufficiency conditions. AtFRO3 might possibly function both in iron metabolism in roots and in iron reduction on the root surface for uptake. Based on the fact that GUS expression driven by AtFRO3 and AtFRO6 promoters was delimited in shoots (leaves and stems) and AtFRO8 only in the tissue of leaf vein, the three AtFROs possibly function in iron reduction in shoots, where AtFRO8 might specifically reduce Fe(III) in leaf

Fig. 6 GUS activity in cotyledons and flowers of transgenic Arabidopsis lines. (a–e) Histochemical assays of GUS activity with 4-day-old seedlings. AtFRO3-GUS (a), AtFRO4-GUS (b), AtFRO6-GUS (d) and AtFRO7-GUS (e) are four transgenic lines revealing GUS activity in cotyledons. (c) A partially enlarged picture of (b) marked by a square. (f–i) GUS activity assays with flowers. (f–i) GUS staining in sepals of floral buds (i) and in opening flowers (g); in anther filament and stigma (h) and in pedicel and inflorescence stem (i) of the flowers of AtFRO5-GUS plants; (j–l) are GUS staining in sepals (j and k), anther filament and stigma (l) of flowers of the AtFRO6-GUS line; (m) is GUS staining in pedicel and transition zones between the pedicel and inflorescence stem of AtFRO8-GUS plants.

Fig. 7 The expression profiles of the eight AtFRO genes in roots of the FIT1 knock-out mutant fit1 under iron deficiency conditions. The expression patterns and intensities of the AtFRO genes were analyzed by RT–PCR using gene-specific primers (see the Supplementary material). Wt, wild type (ecotype Columbia); fit1, FIT1 knock-out mutant fit1.
veins for iron transport. Interestingly, the transcription of *AtFRO7* was detected only in shoots (Fig. 3a) and it was most abundant in cotyledons (Fig. 3b). Histochemical assays showed a strong GUS activity in cotyledons and trichomes of *AtFRO7-GUS* seedlings. These indicated that AtFRO7 might be mainly involved in iron mobilization from cotyledons during germination and iron homeostasis in trichomes. Fe(III) chelate reductase activity has been reported in leaves (de la Guardia and Alcantara 1996). *LeFRO1* in tomato and *PsFRO1* in pea were proposed to be Fe(III) chelate reductases participating in iron reduction in leaves based on their abundant transcripts in leaves (Waters et al. 2002, Li et al. 2004).

Monitoring the expression profile of *AtFRO* genes in flowers by histochemical assays displayed a strong expression of the GUS reporter gene in sepals, anther filaments, stigmas and pedicels, as well as inflorescence stems of *AtFRO5-GUS* transgenic plants (Fig. 6f–i). Similar transcription patterns were also observed in flowers of the *AtFRO6-GUS* plants, but the GUS staining intensity was significantly lower compared with the transgenic plants of *AtFRO5-GUS* (Fig. 6j–l). Obviously, *AtFRO5* and *AtFRO6* are two genes also functioning in iron metabolism in reproductive organs. Together with results of RT–PCR analysis (highest transcriptional intensity of *AtFRO5* in flowers), we suggest that AtFRO5 is a dominant contributor to iron reduction in flowers. A strong GUS activity in anthers was detected in flowers of transgenic *AtFRO2-GUS* plants, indicating that AtFRO2 also plays an important role in iron homeostasis in anthers in addition to its function in roots. The hypothesis that expression patterns correlate with relative contributions to Fe(III) reduction in plant tissues will be tested by evaluating a comprehensive set of knock-out mutants of these genes.

The Fe(III) chelate reductase activity assay showed that the AtFROs of Arabidopsis exhibited different activities of Fe(III) reduction when expressed in yeast cells. Six of the eight AtFROs showed more or fewer transcripts in roots under iron-limiting conditions (Fig. 3a). Considering the diversity of iron resources in soil, the redundancy of the AtFROs in roots might confer the capacity to utilize iron from various sources effectively. The differences of iron reductase activity among AtFROs might possibly be due to different substrate specificities. The substrate specificity of iron chelate reductase has been observed in yeast (Yun et al. 2001). The plasma membrane reductases FRE1 and FRE2 are required for reduction and uptake of free ferric iron in yeast, whereas FRE3 and FRE4 are siderophore-iron reductases reducing specifically siderophore-iron. Besides iron, some AtFROs are possibly involved in homeostasis of other metals. Increased copper chelate reductase activity was observed in AtFRO2-overexpressing plants when they grew in iron-deficient conditions (Connolly et al. 2003). In yeast, FRE1 and FRE7 are regulated by copper through the transcriptional regulator Mac1 (Martins et al. 1998) and function in the reduction of copper (Georgatsou et al. 1997).

The eight AtFROs are localized on five loci on chromosome 1 and chromosome 5 of Arabidopsis. A large duplication in the Arabidopsis genome did not occur in the regions where AtFRO genes are located (Arabidopsis Genome Initiative 2000, Blanc et al. 2000, Vision et al. 2000, Simillion et al. 2002), indicating that the AtFRO genes possibly originate from one ancestor via local duplications. Based on the high sequence similarity and gene structure identity, it would appear that local tandem duplications occurred more recently between AtFRO4 and AtFRO5 and between AtFRO6 and AtFRO7.

In conclusion, we have studied the expression patterns of the *AtFRO* gene family in Arabidopsis and functionally characterized seven of the eight members of this family. Our data firstly provide evidence that the *AtFRO* genes encode active iron chelate reductase and exhibit tissue-specific expression, indicating that they possess tissue-specific functions involving iron homeostasis in Arabidopsis plants. The further characterization of this gene family with single, double and multiple knock-out mutants will give an insight into understanding the biological functions of each *AtFRO* gene and form the possible basis for the manipulation of plants to generate iron-efficient and iron-rich crops for improvement of human iron nutrition.

**Materials and Methods**

Cloning cDNAs of AtFROs and sequence analysis

For identification of all *FRO* genes in Arabidopsis, the complete Arabidopsis genome sequence (Arabidopsis Genome Initiative 2000) was blasted against the amino acid sequence of AtFRO2 (GenBank accession No. Y09581, Robinson et al. 1999) and AGI gene codes for the AtFRO family members were obtained from (http://www.mips. biochem.mpg.de/proj/thal/db/index.html). Based on predicted DNA sequences of *AtFRO* genes in the database, 3′ and 5′ primers for each *AtFRO* gene were designed (Supplementary material) and the coding sequences were amplified from total RNA of Arabidopsis with the primers by RT–PCR. They were purified with a GFX gel purification kit (Pharmacia, Sweden), subsequently cloned into pGEM T-easy vector (Promega, Madison, WI, USA) and sequenced. Multiple alignments of sequences were performed using the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html) (Thompson et al. 1994). The transmembrane domains of putative AtFRO proteins were predicted using the TMTOP program (http://www.enzim.hu/tmmtop/server/tmtop.cgi). Gene positions on chromosomes were determined using SeqViewer (http://arabidopsis.org/servlets/sv).

**Plant materials and growth conditions:**

The Columbia ecotype of *A. thaliana* and the knock-out mutant *fit1* (Salk_126020, NASC) was used in this work. Unless otherwise stated, MS medium (Sigma, St. Louis, MO, USA), supplemented with 3% sucrose and 1% phytoagar at pH 5.8, was applied for growing wild-type *A. thaliana* and transgenic lines under sterile conditions at 23°C with a 16 h light period. On the fourth day, cotyledons of some seedlings were harvested for analysis. According to the purposes of the experiments, 4- to 6-week old leaf stage seedlings were then transferred to plates with either iron-sufficient [by supplementing with 50 μM Fe(II)-EDTA] or iron-deficient [by adding 300 μM Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate] replacing Fe(III)-EDTA] medium for treatments. Four days later, roots and shoots were harvested separately for further analysis.
RT–PCR analysis

To investigate the expression patterns of AtFRO genes among tissues and in different environments, total RNA was extracted from the desired tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA contamination in RNAs was eliminated following the description of Li et al. (2004). After quantification with an UV spectrophotometer, 10 µg of total RNAs for each sample were converted to cDNAs by reverse transcription in a 20 µl reaction volume according to the manufacturer’s instructions (GeneAmp RNA PCR; Perkin Elmer, Foster City, CA, USA). The reverse transcription reactions were diluted 6-fold, and the contents of cDNAs in the reverse transcription mixtures were standardized by amplifying ACTIN transcripts using primers described in the Supplementary material. Transcription levels of each AtFRO in different tissues samples were evaluated using the standardized cDNA mixtures and gene-specific primers. The oligonucleotide sequences of the gene-specific primers for the eight AtFRO genes and their expected fragment lengths amplified from genomic DNA and from cDNA are shown in the Supplementary material. The RT–PCRs were carried out in 30 µl of reaction solution. The reaction conditions were 45 s at 94°C, 45 s at 55°C and 90 s at 72°C for 28 cycles and adding a 6 min incubation at 72°C for extension. Subsequently, the PCR products were separated by electrophoresis on a 1.5%(w/v) agarose gel with ethidium bromide and documented by a CCD camera.

Plasmid construction of AtFRO promoter–GUS fusions and plant transformation

In order to investigate the location of expression of each AtFRO in Arabidopsis tissues, an approximately 1.2–2.5 kb fragment of genomic DNA upstream of the transcriptional start site of each AtFRO gene was amplified by PCR using primers (Supplementary material) and cloned into PMD18-T vector (TAKARA BIOTECHNOLOGY, Dalian, China). They were sequenced for confirmation of sequence correction. BLAST analysis of the putative promoter regions confirmed the absence of sequences from neighboring genes. Subsequently, the promoter fragment was excised from PMD18-T vector by corresponding restriction endonucleases, purified with the GFX gel purification kit and subcloned into binary vector pCAMBIA1381 upstream of the ATG of the GUS gene. The clones containing different AtFRO promoter–GUS constructs were transformed by the standard method into A. tumefaciens strain GV3101. Plasmids were isolated from Agrobacterium and verified by restriction digests. Arabidopsis Columbia ecotype was then transformed with A. tumefaciens carrying the FRO promoter–GUS by the floral dip method (Clough and Bent 1998). T1 seeds were plated on the germination medium containing 60 µg ml−1 hygromycin and 100 µg ml−1 ampin to select for transformants. Hygromycin-resistant T1 seedlings were transferred to soil and grown to maturity. Three dependent homozygous T2 lines of each construct were investigated for analysis of promoter activity.

GUS activity assay

The T2 transgenic lines were germinated and grown on MS medium as described above. Four-day-old and 5-leaf-stage seedlings were collected for histochemical assays of GUS activity using the method of Jefferson et al. (1987). For determination of GUS activity in generative tissues, flowers were harvested from transgenic plants growing in a growth chamber.

Expression of AtFROs in yeast cells

For assaying Fe(III) reduction activity of AtFRO proteins, the coding sequences of AtFRO2–AtFRO8 were amplified from the total RNA by RT–PCR using the primers shown in the Supplementary material and cloned into pGEM T-easy vector after purification with the GFX gel purification kit. After verification of sequence correction by sequencing, the coding sequence of each gene was excised by NcoI and subcloned into a yeast expression vector pYES2.0 (Invitrogen, Carlsbad, CA, USA) to generate the pYES2.0–AtFRO plasmid. They were then introduced into the Saccharomyces cerevisiae wild-type strain BJ2168 with the lithium acetate method according to the manufacturer’s manual. BJ2168 yeast cells containing the plasmid pYES2.0–AtFRO or containing pYES2.0 as a control were grown following the protocol described by Li et al. (2004). Cells were harvested in the mid-log phase, and Fe(III) chelate reductase activity was determined as described by Eide et al. (1992).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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