Salt stress-induced FERROCHELATASE 1 improves resistance to salt stress by limiting sodium accumulation in Arabidopsis thaliana

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Ferrochelatase-1 as a terminal enzyme of heme biosynthesis regulates many essential metabolic and physiological processes. Whether FC1 is involved in plant response to salt stress has not been described. This study shows that Arabidopsis overexpressing AtFC1 displays resistance to high salinity, whereas a T-DNA insertion knock-down mutant fc1 was more sensitive to salt stress than wild-type plants. AtFC1 conferred plant salt resistance by reducing Na⁺ concentration, enhancing K⁺ accumulation and preventing lysis of the cell membrane. Such observations were associated with the upregulation of SOS1, which encodes a plasma membrane Na⁺/H⁺ antiporter. AtFC1 overexpression led to a reduced expression of several well known salt stress-responsive genes such as NHX1 and AVP1, suggesting that AtFC1-regulated low concentration of Na⁺ in plants might not be through the mechanism for Na⁺ sequestration. To investigate the mechanism leading to the role of AtFC1 in mediating salt stress response in plants, a transcriptome of fc1 mutant plants under salt stress was profiled. Our data show that mutation of AtFC1 led to 490 specific genes up-regulated and 380 specific genes down-regulated in fc1 mutants under salt stress. Some of the genes are involved in salt-induced oxidative stress response, monovalent cation-proton (Na⁺/H⁺) exchange, and Na⁺ detoxification.

Soil salinity is one of the major environmental problems that seriously cause osmotic stress and ionic toxicity, thus limiting the productivity of crops. Plants have to develop remarkable capabilities for adapting themselves to adverse environments⁵. Plants also evolve various strategies to cope with the environmental challenge via perceiving stressful signals and transmitting them through diverse metabolic pathways; and upon receipt of the signal, a number of molecular and cellular responses are initiated⁷.

Plant growth responds to salt stress in three phases: Phase 0: initial response to NaCl; Phase I: osmotic phase that physiologically inhibits growth of young leaves; and Phase II: ionic phase that facilitates senescence of mature leaves⁶. Phase 0 occurs during the first minutes to hours after salt exposure. There are transient changes in turgor, growth, membrane potential⁴. During the first phase, ion accumulation in root medium may reduce the water potential and water availability to plants⁸. The second phase occurs when vacuoles no longer sequester incoming salts, but the concentration of toxic ions rises rapidly in leaves⁹. Na⁺ is the main toxic ion. Accumulation of excessive Na⁺ in cytosol is detrimental to many metabolic and physiological processes. Thus, maintaining a low concentration of cytoplasmic Na⁺ under salt stress is critical for plant growth and development⁷. Plants prevent Na⁺ accumulation in symplast through multiple ways such as extrusion, influx restriction and vacuolar sequestration⁷. Na⁺ efflux is catalyzed by a plasma membrane Na⁺/H⁺ antiporter encoded by Salt Overly Sensitive 1.

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Ferrochelatase 1 (FC1, EC4.99.1.1) is the terminal enzyme of heme biosynthesis, catalyzing the insertion of ferrous iron into protoporphyrin IX\(^{11}\). Protoporphyrin IX is the branch point of the tetrapyrrole biosynthesis pathway of heme and chlorophyll. While chlorophyll is well known for its functions associated with solar energy absorption, transfer and photosynthesis in plants, heme is one of the important tetrapyroles with functions associated with fundamental cellular aspects\(^{12}\). It is apparent that FC1 is a critical enzyme in the tetrapyrrole biosynthesis pathway for coordination of heme and apoprotein production\(^{13}\). FCs are conserved in organisms. In Arabidopsis and several other plants such as barley and cucumber, only two isoform genes of FC are available\(^{11,14-18}\). While \textit{AtFC1} (At5g26030) is expressed in both mitochondria and chloroplasts, \textit{AtFC2} (At2g30390) is only present in chloroplasts\(^{11,16}\). Although \textit{AtFC1} and \textit{AtFC2} share high identity of nucleotides (83\%) and amino acids (69\%), both did originate from a segmental duplication event in the Arabidopsis genome and thus belong to two distinct groups of plant ferrochelatases\(^{11,12,18}\). \textit{AtFC1} and \textit{AtFC2} have different expression patterns\(^{11,13,15,16,19}\). Compared to \textit{AtFC1} that ubiquitously expresses in whole plants, \textit{AtFC2} tends to express in stems, flowers and leaves, but not in roots\(^{15,16}\). Identifying promoters in response to biotic and abiotic stresses shows that the leaf \textit{AtFC1} promoter activity increased in response to nonfriuran (an inhibitor of carotenoid biosynthesis), wounding and viral infection, whereas \textit{AtFC2} promoter activity was in most instances repressed\(^{18}\). Furthermore, expression of \textit{AtFC1} could be triggered by reagents generating reactive oxygen species (ROS), inhibitors of cytoplasmic protein synthesis and other environmental stresses\(^{33,38}\). These results suggest that \textit{FC1} is mainly involved in stress responses, whereas \textit{FC2} is thought of generating heme for photosynthetic cytochromes\(^{15,16,18,20}\). In this paper, we showed that \textit{AtFC1} could positively regulate Arabidopsis resistance to salinity stress. \textit{AtFC1} overexpression improved seed germination and primary root elongation and reduced Na\(^+\) accumulation under salt stress, whereas the loss of function \textit{fc1} mutant had adverse phenotypes. \textit{AtFC1} overexpression altered expression of a set of genes responsible for Na\(^+\) uptake, efflux and detoxification in plants.

## Results

### Expression of \textit{AtFC1} is upregulated by salt stress.

The transcriptional expression pattern of \textit{AtFC1} was analyzed during plant growth and development. RT-PCR analysis showed that \textit{AtFC1} was ubiquitously expressed, including early or late developing cotyledons, roots, stems, shoots, leaves and flowers, but the abundance of their transcripts varied considerably (Fig. 1A). Roots showed a high abundance of \textit{AtFC1} transcripts. Compared to young seedlings, mature stems and flowers also had a higher \textit{AtFC1} expression level.

Several studies have indicated that expression of \textit{AtFC1} was induced by wounding, ozone and oxidative stress\(^{15,19,20,22}\). To investigate the expression pattern of \textit{AtFC1} under salt stress, transcript levels of \textit{AtFC1} were assessed by RT-PCR. As shown in Fig. 1B, exposure of two-week old Arabidopsis seedlings to 100–400 mM NaCl induced \textit{AtFC1} expression by 1.8–3.5 fold. The time-kinetics study, in which seedlings were treated with 200 mM NaCl for 12 hours, also showed 2.3–4.3 fold enhanced expression of \textit{AtFC1} under salt stress (Fig. 1C). These results were well confirmed by qRT-PCR (Fig. 1D,E).

### Overexpression of \textit{AtFC1} in Arabidopsis enhances salt resistance.

To test the biological function of \textit{AtFC1} in Arabidopsis in response to salt stress, a T-DNA insertion mutant of \textit{AtFC1} (SALK-150001.42.45x, \textit{fc1}) was identified. The mutant \textit{fc1} was verified by diagnostic PCR using gene-specific primers and found with a T-DNA insertion in the 5′-untranslated region (Fig. 2A). The RT-PCR analysis proved the right T-DNA insertion in the homozygous mutant (Fig. 2B). \textit{AtFC1} transcripts in \textit{fc1} were assessed by RT-PCR, showing substantial decrease compared to the wild-type (Fig. 2C,D), which was in a good agreement with the recent report\(^{18}\). We further generated transgenic lines overexpressing \textit{AtFC1} driven by the cauliflower mosaic virus (CaMV) 35S promoter. The homozygous transgenic lines were filtered in. The \textit{AtFC1} mRNA level of the 35S::\textit{AtFC1} transgenic lines was confirmed by qRT-PCR. The transgenic plants carrying 35S::\textit{AtFC1} showed 13.5 to 22.8-fold higher transcripts of \textit{AtFC1} than the wild-type (WT) plants (Fig. 2E).

Seed germination in response to salt stress was determined in wild-type, \textit{fc1} mutants and \textit{AtFC1}-overexpression plants. Seeds were placed on the solid 1/2 MS medium supplemented with 0, 100 and 200 mM NaCl. First, the response of wild-type and \textit{fc1} mutants to salt stress was compared. The \textit{fc1} mutants always had a lower germination rate under salt stress (Fig. 3A,B). Further, a salt acclimation experiment was made with WT and \textit{fc1} mutant seeds before exposure to 100 mM NaCl. Seeds were pretreated with 10 mM NaCl for 48 h. The WT seeds had a rapid germination after the 48 h salt acclimation, whereas \textit{fc1} seeds had no significant increase in germination after that (Fig. 3C), indicating that loss of \textit{AtFC1} function impaired seed germination. We then studied the effect of \textit{AtFC1} overexpression on seed germination under salt stress. The seed germination responded differently to NaCl stress. The 35S::\textit{AtFC1} lines always had higher germination rates than WT under salt stress (Fig. 3D,E).

Root growth is sensitive to NaCl and is often used as a biomarker of salt stress\(^{23}\). Under 5–100 mM NaCl exposure for 7 d, the primary root elongation of \textit{fc1} mutants was relatively weak compared to WT (Fig. 4A). In contrast, 35S::\textit{FC1} plants displayed strong root growth compared to the wild-type under salt stress (Fig. 4B). Under 150 mM NaCl stress, elongation of 35S::\textit{AtFC1} roots was 1.4–1.5 fold higher than that of wild-type. The \textit{AtFC1}-improved plant growth was also found in shoots. Three-week-old WT, \textit{fc1} and 35S::\textit{AtFC1} seedlings were treated with 50 mM NaCl for 7 and 14 d. Thereafter, the fresh weight was measured. Compared to WT, the total fresh weight of \textit{fc1}...
mutants was lower, whereas the fresh weight of 35S::AtFC1 plants was higher (Fig. 4C–E). The fresh weight of 35S:FC1-1 and 35S:FC1-3 seedlings with 150 mM NaCl was increased 27–34% compared to WT (Fig. 4F).

Overexpression of AtFC1 attenuates electrolyte leakage and proline accumulation. The electrolyte leakage represents the damage of plasma membrane in plant cells and the degree towards abiotic stress response. Compared to WT, 100–300 mM NaCl induced a higher level of electrolyte leakage in fc1 mutants, whereas a lower level was observed in 35S::AtFC1 plants, (Fig. 5A).

Proline acts as an osmoprotectant which plays an important role in osmotic balancing and increasing the turgor necessary for cell expansion under salt stress; in addition, it serves as a redox potential regulator.
Figure 2. Identification of fc1 mutant and 35S::AtFC1 transgenic lines in Arabidopsis. (A) Schematic diagram of AtFC1 structure and T-DNA diagnostic PCR and RT-PCR. The coding region and 5′ and 3′ untranslated regions are illustrated by the black and white boxes, respectively; introns are indicated by lines. The position of the T-DNA insertion is indicated by a triangle. The locations of the primer pairs used to analyze the mutation by RT-PCR are indicated by arrows. (B) RT-PCR analysis of the fc1 insertion mutant. The reverse transcription products were PCR-amplified using primer pairs LP-1 + RP-1, LP-1 + LBb1.3. (C) RT-PCR analysis of AtFC1 transcript levels in fc1 mutant. (D) qRT-PCR analysis of AtFC1 transcript levels in fc1 mutant. E: qRT-PCR analysis of AtFC1 transcript levels in 35S::AtFC1 lines. Fourteen day-old seedlings were used for RT-PCR analysis. The number below the band indicates the relative abundance of the corresponding proteins with respect to the loading control. Vertical bars represent mean values ± SE. Asterisk indicates the significant difference in expression between the fc1 mutant/35S::AtFC1 lines and wild type (p < 0.05). The color of image in C was inversed. The uncropped images are shown in Supplementary Data 9.

protecting macromolecules (e.g. proteins or enzymes) from damage or denaturation caused by heat, NaCl, and other stresses2. Similarly, a higher level of proline was found in the fc1 mutants than in WT under NaCl stress; in contrast, the concentration of proline in 35S::AtFC1 plants was lower than WT (Fig. 5B).

AtFC1 overexpression maintains Na+ and K+ homeostasis under salt stress. Excessive Na+ is toxic for plants, whereas K+ is an opponent against Na+ under salt stress24. To figure out the physiological role of AtFC1 in resistance to salt stress, three week-old WT, fc1 mutant and 35S::AtFC1 plants were treated with 200 mM NaCl for 3 d, and the concentrations of Na+ and K+ in roots and shoots were determined by coupled plasma-atomic emission spectrometry. There was no difference of Na+ concentrations in WT, fc1 mutant and 35S::AtFC1 plants grown in absence of NaCl (Fig. 6A,C). However, when exposed to 200 mM NaCl, the fc1 mutants accumulated more Na+ in shoots and roots, whereas the Na+ concentration in the tissues was relatively lower in 35S::AtFC1 plants compared to wild-type. In absence of NaCl, no difference of shoot K+ concentrations was observed between WT and fc1 mutants (Fig. 6B); although fc1 mutants accumulated less K+ in roots, there was no significant difference (Fig. 6D). Under control conditions, 35S::AtFC1 plants accumulated a litter higher level of K+ in plants. However, the 35S::AtFC1 plants accumulated more K+ in their shoots and roots under salt stress, whereas the K+ concentration was significantly lower in fc1 mutants than in WT. We further analyzed the K+/Na+ ratio which represents the balance of the two ions under salt stress26. It was unclear for K+/Na+ ratio between fc1, 35S::AtFC1 and WT plants under normal condition. But the imbalance of Na+ and K+ uptake led to an increase in K+/Na+ ratio in 35S::AtFC1 plants and a decrease in K+/Na+ ratio in fc1 mutants (Fig. 6E,F).

To get an insight into the Na+ location, we analyzed the Na+ uptake on root surface and Na+ translocation from roots to shoots based on the method described previously27. Under normal condition, there was no significant difference of the Na+ uptake on root surface between WT and 35S::AtFC1 or fc1 mutant plants; however under salt stress (200 mM NaCl), the concentration of Na+ on the root surface was lower in 35S::AtFC1 plants but higher in fc1 mutant compared to WT (Fig. 7). Also, the AtFC1 overexpressing plants showed less Na+ transfer from roots to shoots. For fc1 mutants, no much difference was found.

The altered Na+ accumulation due to the AtFC1 expression prompted us to analyze genes responsible for Na+ and K+ acquisition and homeostasis. The SOS (Salt Overly Sensitive) pathway plays an important role in Arabidopsis to regulate Na+ concentration and maintain ionic homeostasis2. SOS1 encodes a plasma membrane-localized Na+/H+ antiporter, functioning in extrusion of Na+ from cells and contributes to salt resistance in Arabidopsis8. SOS2 encodes a member of the CBL-interacting protein kinase family, which combines with SOS3. SOS3 encodes a protein that shares significant sequence similarity with the calcineurin B subunit from yeast and neuronal calcium sensors from animals; this kind of intracellular calcium signaling through a calcineurin-like pathway mediates the beneficial effect of calcium on plant salt tolerance2. qRT-PCR analyses
Figure 3. Germination responses of fc1 mutants and 35 S::AtFC1 plants to salt stress. (A) Phenotypes of wild-type (WT) and fc1 mutant seeds exposed to 0, 100 and 200 mM NaCl for 7 d. (B) Quantification of germination rates of WT and fc1 seeds with 0–100 mM NaCl for 2 d. C: Quantification of germination rates of WT and fc1 seeds in an acclimation way of 10 mM NaCl pre-treatment for 48 h (circles) and then 100 mM NaCl to the end of treatment (108 h) (triangles). Both 0 (square) and 100 (circles) mM NaCl treatments were set as control. D: Phenotypes of wild-type (WT) and 35 S::AtFC1 lines exposed the indicated concentrations of NaCl for 7 d. E: Quantification of germination rates of WT and fc1 seeds with 0, 100 and 200 mM NaCl for 3 or 4 d. Vertical bars represent mean values ± SE. Asterisk indicates the significant difference in germination rate between fc1 mutants/35 S::AtFC1 lines and wild type (p < 0.05).
showed that compared to the controls, expression of SOS1 and SOS3 was lower in fc1 mutants, whereas it was higher in 35S::AtFC1 plants under salt stress (Fig. 8A,C). SOS2 showed a similar expression pattern, but the SOS2 transcription was very weak in 35S::FC1-3 plants under salt stress (Fig. 8B).
the number of germinating seeds (Fig. 9A–C). Addition of hematin led to a 43% recovery in germination rate compared to the control in which no hematin was added. The exogenous hematin could also recover 47% fresh weight of seed compared to the control in which no hematin was added. The exogenous hematin could also recover 47% fresh weight of seed compared to the control in which no hematin was added.

Further functional analysis of hematin was performed by assessing NaCl accumulation in wild-type and fc1 mutant plants (Fig. 8A–C). Three week-old WT, fc1 and 35 S::AtFC1 plants were exposed 0 and 100 mm NaCl for 4 h before determination of free proline concentration. Vertical bars represent mean values ± SE. Asterisk indicates the significant difference between fc1/35 S::AtFC1 and WT plants (p < 0.05).

HK1 encodes a member of the histidine kinase family, and is considered as an osmosensor. Analysis of HK1 transcripts showed that expression of HK1 in salt-treated fc1 mutants was lower than in WT, accounting for about 70% of the WT (Fig. 8D). We further assessed the expression of HK1, NHX1 and AVP1. HK1 encodes a sodium transporter expressed in xylem parenchyma cells, but SOS3 and other salt-related protein in the SOS pathway can inhibit the expression of HK1 to reduce the intracellular concentration of Na+. NHX1 encodes a vacuolar Na+/H+ antiporter involved in salt resistance and transports Na+ into the vacuole using the electrochemical gradient of protons generated by the vacuolar H+-translocating enzymes, H+-adenosine triphosphatase and H+-inorganic pyrophosphatase. AVP1 encodes an H+-translocating (pyrophosphate-energized) inorganic pyrophosphatase located in the vacuolar membrane. Under salt stress, expression of HK1, NHX1 and AVP1 was higher in fc1 mutants, while their expression in 35 S::AtFC1 plants was lower (Fig. 8E–G). AVP1 was an exception because it had a similar expression pattern under both –NaCl and + NaCl conditions (Fig. 8G). P5CS1 (Delta-1-pyrroline-5-carboxylate synthase 1) is a rate-limiting enzyme in proline biosynthesis, whose mRNA is induced by drought and salinity. RD22 belongs to the DRE/CRT (drought responsive/C-repeat) elements-containing class of stress-responsive genes. qRT-PCR analysis showed that both genes had an expression pattern similar to NHX1 in fc1 mutants and 35 S::AtFC1 plants under salt stress (Fig. 8H,I).

Exogenous hematin alleviates tissue damage of fc1 mutant under salt stress. Knowing that AtFC1 was involved in salt plant stress response, we asked whether feeding fc1 with heme, the product of AtFC1, could restore the impaired seed germination caused by NaCl stress. To validate the assumption, the fc1 mutant plants were treated with hematin (protoheme), a highly stable heme substitute. Under salt stress (100 mM NaCl), the fc1 seeds showed a reduced germination rate, but adding 2 μM hematin to the growth media increased the number of germinating seeds (Fig. 9A–C). Addition of hematin led to a 43% recovery in germination rate compared to the control in which no hematin was added. The exogenous hematin could also recover 47% fresh weight of fc1 mutant plants (Fig. 9D). These results indicate that the heme produced by FC1 is partially responsible for resistance of plant to salt stress. Further functional analysis of hematin was performed by assessing Na+ accumulation in fc1 mutant plants. Three week-old WT and fc1 plants were exposed to 100 mM NaCl with or without 2 μM hematin for 3 d. Compared to NaCl treatment alone, concomitant supply of hematin resulted in a significant decrease of Na+ accumulation in fc1 mutants (Fig. 9E). Furthermore, qRT-PCR analysis showed that expression of SOS1 with NaCl was enhanced by hematin (Fig. 9F).

Regulation of FC1 involves extensive transcriptome remodeling. To identify putative FC1 downstream genes associated with the salt stress response in Arabidopsis, we comparatively analyzed the global transcripts of fc1 mutant and wild-type plants by RNA-sequencing. In total, 30.0–31.6 million clean reads were generated in four libraries using illumina sequencing technology (Supplementary Data 1). Mapping the reads to Arabidopsis genome led to identification of 6460 (3086 up and 3374 down) and 5556 (3114 up and 2442 down) genes in wild-type and fc1 mutant plants (> two fold change, p < 0.05) under salt stress, respectively (Supplementary Data 2–5). Figure 9A shows the distribution of the differentially expressed genes (DEGs) represented by color dots (blue, down, and red, up). Compared to the wild-type Col-0, fc1 mutants under salt stress showed more red dots (more DEGs up-regulated) and less blue dots (less DEGs down-regulated). Under the control (–NaCl) and NaCl treatment, more red dots were detected than blue dots for fc1 / Col-0 ratio, indicating that in the fc1 mutant plants more genes were induced relative to wide-type (Col-0) (Fig. 10A). This observation was reflected by the heat-map graph (Fig. 10B), from which the same results could be figured out. To detail the gene expression, Venn diagrams were plotted and showed that 1010 genes were specifically induced and 1717 were repressed in Col-0, while 1038 genes were specifically induced and 785 genes were repressed in fc1 mutant plants under saline stress.

**Figure 5.** Physiological responses of fc1 and 35 S::AtFC1 plants to salt stress. (A) Leaf tissues from three week-old WT, fc1 and 35 S::AtFC1 plants were carefully excised 4 h after 0–300 mM NaCl treatments and used for electrolyte leakage (see Materials and Methods). (B) Three week-old WT, fc1 and 35 S::AtFC1 plants were exposed 0 and 100 mm NaCl for 4 h before determination of free proline concentration. Vertical bars represent mean values ± SE. Asterisk indicates the significant difference between fc1/35 S::AtFC1 and WT plants (p < 0.05).
under salt stress (Fig. 10C). By comparative analysis of transcripts between fc1 mutant and wild-type plants, 1159 genes were found to be specifically induced and 924 genes were repressed under control condition, whereas 490 genes were specifically induced and 380 genes were repressed under salt stress (Fig. 10D). The expression patterns of some randomly selected genes were well validated by qRT-PCR (Supplementary Data 6).

By BGI WEGO (Web Gene Ontology Annotation Plotting), the DEGs from salt-exposed fc1/Col-0 samples were functionally classified. Based on their functional specificity, these DEGs were subdivided into three major groups including biological process, cellular component and molecular function (Fig. 11A). Several categories such as metabolic process, response to stimulus, transcription regulation activity and transporter activity were presented. We further specified some DEGs based on the GO categories. The first group contains genes encoding Na efflux transporters (Fig. 11B), which are responsible for Na exclusion in plants when exposed to excess salt. The second group comprised genes encoding Na uptake transporters and Na tolerant-related proteins, such as sodium transporter HKT1 and sodium/metabolite co-transporter (Fig. 11C). Compared to WT, these genes in fc1 mutants were induced under salt stress. Potassium is an important ion balancing excessive sodium during plants subjected to salt stress. Examination of K⁺ uptake transporter genes revealed that five were transcriptionally

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**Figure 6.** Effects of AtFC1 overexpression on Na and K accumulation. Three week-old wild type, fc1 mutant and over-expression transgenic plants were treated with 200 mM NaCl for 3 d. Sodium (A,C) and potassium (B,D) contents in shoots and roots were quantified. K⁺/Na⁺ ratio in shoots and roots of the 35S::AtFC1 transgenic lines and wild type after treatment with 0 (E) or 200 mM NaCl (F) for 3 d. Vertical bars represent mean values ± SE. Asterisk indicates the significant difference between fc1/35S::AtFC1 and WT plants (p < 0.05).
repressed in fc1 mutants (Fig. 11D). Finally, expression of a group of ROS (reactive oxygen species)-responsive genes was also found to be lower in fc1 mutants than in wild-type (Fig. 11E). These results indicate that disruption of FC1 expression could modify transcription of genes responsible for salt exclusion and detoxification.

**Discussion**

Although several biological and defense functions of FC1 in Arabidopsis and other plant species have been identified in recent years\(^{11,12,18,19,38,39}\), the functional regulation of plant response to salt stress by FC1 has not been described. A recent study using ATH1 microarray chip demonstrated that some genes (e.g. SALT-INDUCIBLE ZINC FINGER 2 and WRKY33) associated with salt stress could be co-regulated by FC1\(^{18}\), suggesting that FC1 would be involved in the salt stress response. The present study provided genetic and physiological evidence that AtFC1 was able to regulate plant resistance to salt stress. AtFC1 was transcriptionally induced by NaCl. AtFC1 overexpression resisted salt stress by promoting seed germination (Fig. 3E), root and shoot growth (Fig. 4), accumulating less Na\(^+\) and more K\(^+\) (Fig. 6) in plants. By contrast, AtFC1 loss of function led to adverse phenotypes. Importantly, several genes responsible for Na\(^+\)/H\(^+\) antiporter were up-regulated in 35 S::AtFC1 plants. These results indicate that expression of AtFC1 is required for plant resistance to salt stress.

Minimizing the concentration of cytoplasmic Na\(^+\) is critical for plant growth and development under salt stress. Strategies for fighting against excess Na\(^+\) in plant cells were proposed\(^{1,2}\). Na\(^+\) exclusion is one of the most efficiently resistant mechanisms\(^{7}\). The SOS pathway is one of the most extensively studied mechanisms for controlling Na\(^+\) accumulation in plants\(^{2}\). During salt stress, a myristoylated calcium-binding protein encoded by SOS3 presumably senses the salt-elicited calcium signal and translates it to downstream responses\(^{40}\). SOS3 interacts with and activates SOS2, a serine/threonine protein kinase\(^{28}\). Both SOS2 and SOS3 regulate the expression of SOS1, a plasma membrane Na\(^+\)/H\(^+\) antiporter which mediates Na\(^+\) efflux\(^{8}\). SOS1 can act as a Na\(^+\) sensor of adjusting Na\(^+\) long-distance transport from roots to shoots and protecting cells from Na\(^+\) toxicity\(^{8,41}\). AtFC1-improved resistance to salt stress was associated with activation of SOSs because these genes, particularly for SOS1 were induced in 35 S::AtFC1 plants. The upregulation of SOS1 might be responsible for the low level of Na\(^+\) in the 35 S::AtFC1 plants. We examined cellular and tissue Na\(^+\) distribution in transgenic plants and found that AtFC1 overexpression led to a lower concentration of Na\(^+\) on the root cellular surface and less Na\(^+\) translocated from roots to shoots (Fig. 7). This is consistent with the result that 35 S::AtFC1 plants accumulated less Na in roots and shoots. In addition, RNA-seq profiled many Na efflux transporter genes repressed, while some Na uptake transporter genes were enhanced in fc1 mutant plants (Fig. 11). Several other salt stress-responsive genes, such as NHX1 and AVP1 were examined. Both genes are responsible for sequestration of Na\(^+\) into vacuoles\(^{35,36}\). However, expression of NHX1 and AVP1 was down-regulated in 35 S::AtFC1 plants, suggesting that sequestration of Na\(^+\) into vacuoles by NHX1 and AVP1 was not be involved in AtFC1-mediated salt tolerance mechanism.
Heme is one of the most important tetrapyrroles in plants as it serves as a cofactor for many enzymes, transporters and proteins involved in the essential biological processes\textsuperscript{36,39,42}. Furthermore, FC1 was shown to be induced with demand for heme for respiratory cytochromes and heme-proteins as part of general defense responses\textsuperscript{13,18,19}. To address the question whether heme is also involved in salt stress response, its regulatory function in this regard was tested by supplying hematin to fc1 mutants and WT plants. Exogenous hematin could partially improve seed germination rate and fresh weight of fc1 mutant plants under salt stress, and addition of hematin could also reduce the Na content in fc1 mutants. These results suggest that AtFC1-improved plant resistance to salt stress not only depended on AtFC1 itself but on heme as well. Although AtFC1 generates heme in plastids, AtFC1 could co-induced with heme-proteins outside plastids, which consequently coordinate the defense response to wounding and ozone-induced oxidative stresses\textsuperscript{13}. One of the striking heme-containing enzymes is heme oxygenase-1 (HO1 or HY1) that regulates biosynthesis of phytochrome by taking advantage of heme as substrate to yields biliverdin IX\textsubscript{α}, carbon monoxide (CO) and iron\textsuperscript{43}. HO1 was induced by abiotic stresses including salinity and heavy metals\textsuperscript{36,42}. In Arabidopsis, transgenic ho1 mutants overexpressing AtHO1 resisted salt stress by limiting K\textsuperscript{+} efflux and facilitating H\textsuperscript{+} efflux\textsuperscript{44}. The enhanced H\textsuperscript{+} efflux was associated with activation of plasma membrane H\textsuperscript{+}-ATPases (AHA1/2/3) in root epidermis and Na\textsuperscript{+}/H\textsuperscript{+} antiporter (SOS1) in the plasma membrane of AtHO overexpressors\textsuperscript{44}. In this way, AtHO1 acted as an essential component of the salt acclimation signaling pathway\textsuperscript{36}. In addition to HO1, many other proteins such as ascorbate peroxidases and cytochrome P450 reductases with heme as co-enzyme are also involved in plant response to abiotic stress\textsuperscript{42,43}. Thus, AtFC1-improved salt stress resistance likely depends on heme through interacting with heme-proteins.

It is mentionable that AtFC1-regulated salt stress resistance involved potassium homeostasis. Our data showed that the 35S::AtFC1 plants had a higher concentration of tissue K\textsuperscript{+}, whereas the fc1 mutant plants had a lower concentration of K\textsuperscript{+} than WT under salt stress. Overload of Na\textsuperscript{+} can dramatically depolarize the plasma membrane, leading to K\textsuperscript{+} efflux via depolarization-activated outward rectifying K\textsuperscript{+} (KOR) channels\textsuperscript{45}. AtFC1

**Figure 8.** qRT-PCR analysis of salt stress-responsive genes in fc1 mutant and 35S::AtFC1 plants. Two week-old wild type, fc1 mutant and 35S::AtFC1 plants were treated with 0 and 200 mM NaCl for 4h. Total RNA was isolated from the plants and analyzed by qRT-PCR. The graphs (y-axis) indicate the induction fold of the genes with 200 mM NaCl as compared with the control (0 mM NaCl) (x-axis). Vertical bars represent mean values ± SE. Asterisks indicate that mean values are significantly different between the fc1/35S::AtFC1 and WT (p < 0.05).
overexpression prevented the loss of K\(^+\) in salt-stressed roots. This was reinforced by our RNA-seq datasets, from which a group of K\(^+\) uptake transporter genes in response to salt stress was profiled. For example, the cation/calcium exchanger 3 (AtCCX3) identified here has been well characterized as an endomembrane H\(^+\)-dependent K\(^+\) transporter\(^{46}\). ABC transporter C family member 5 (AtABCC5 or AtMRP5) mediating guard cell opening was characterized involving ABA signaling\(^{47}\). Both AtCCX3 and AtABCC5 are involved in abiotic stress responses, but both genes showed significantly lower abundance in fc1 mutants than in WT under salt stress (Fig. 11D).

Figure 9. Effect of exogenous hematin on growth, Na\(^+\) concentration and SOS1 expression in fc1 mutants under salt stress. (A, B) Wild-type and fc1 mutant plants after germination were grown in MS medium containing 0 or 2 \(\mu\)M hematin with or without 100 mM NaCl for 14 d. Exogenous hematin effect was assayed as the whole plants fresh weight when treated with or without hematin. C: Germination rate of WT and fc1 seeds with 100 mM NaCl and/or 2 \(\mu\)M hematin for 7 d. D: Fresh weight of WT and fc1 seeds with 100 mM NaCl and/or 2 \(\mu\)M hematin for 7 d. E: Three-week-old wild type and fc1 mutant plants were treated with 100 mM NaCl with and/or 2 \(\mu\)M hematin for 3 days. Then sodium ion contents in whole plants were detected. F: Two-week-old WT and fc1 mutant plants were treated with 0 and 200 mM NaCl with and/or 2 \(\mu\)M hematin for 4 h. Total RNA was isolated from the plants and analyzed by qRT-PCR. SOS1 transcript level was analyzed by qRT-PCR. Vertical bars represent Values represent mean values \(\pm\) SE. Asterisk indicates the significant difference between fc1 mutant and wild type (p < 0.05).
mediated by a mechanism for K⁺ influx and Na⁺ efflux. Further investigation will be required to highlight the essential role of AtFC1 in mediating K⁺ and Na⁺ homeostasis in plants.

To figure out the impact of AtFC1 on its downstream genes and the mechanism for AtFC1 regulation, we profiled transcriptomes of fc1 mutant plants exposed to −NaCl and +NaCl. Our data show that mutation of
AtFC1 led to 1038 specific genes up-regulated in fc1 mutants under salt stress; by contrast there were 785 specific genes down-regulated in fc1 mutants. These results indicate that mutation of AtFC1 was able to modify the transcriptional pattern of more genes under salt stress. By profiling the specifically up- or down-regulated genes in fc1 mutants, we show that many genes were involved in biological pathways including cellular components and molecular functions. A fairly number of genes that are responding to or mediate plant response to salt stress under FC1 pathway have been identified. For example, the NaCl-specific responsive genes such as cytochrome P450, was repressed in fc1 mutants under salt stress, and many other genes related to salt stress response were also found to be altered. The vacuolar-type H⁺-ATPase (V-ATPase) is a multi-subunit endomembrane proton pump involved in ion vesicle trafficking and adaptation to salt stress. Overexpression of AtFC1 conferred plant resistance to NaCl stress. The strong expression of AtFC1, which ensures the supply of the cofactor for a variety of heme-proteins and other putative metabolism, is required in plant resistance to salt stress. The salt stress-induced AtFC1 expression in the tetrapyrrole pathway was associated with activation or suppression of many genes responsible for Na⁺ exclusion or sequestration, antioxidative stress,
phytohormones regulation and other defense components, suggesting a cross-talk between the FCI pathway and salt-responsive resistance pathway. Thus, our data broaden our understanding of a new role of FCI in mediating plant resistance to salt stress.

Materials and Methods

Plant materials and growth condition. Arabidopsis thaliana (ecotype Col-0) was used throughout the study. The FCI (At5g26530) T-DNA insertion mutant fci (SALK_15000.142.45.X, Col background) were obtained from the Arabidopsis Biological Resource Center. Seeds were surface sterilized and germinated on half-strength MS medium containing 1 to 3% sucrose and 0.8% phytagar (pH 5.7) in a growth chamber at 22 °C with 100 μE m⁻² s⁻¹ photosynthetically active radiation and a 16 h light/8 h dark cycle. Two week-old seedlings were used for NaCl (100–400 mM) treatments for 0–12 h depending on the experiment conducted.

Mutant analysis. The T-DNA insertion position and homozygous lines were verified according to the instructions on the Salk Signal website (http://signal.salk.edu/isects.html). Primers were designed using the SALK T-DNA verification primer design program (http://signal.salk.edu/tdnaprimers2.html). For qRT-PCR analysis of the insertion mutants, total RNA was isolated and reverse-transcribed. The reverse transcription products were then PCR-amplified. The internal control was normalized with Actin.

RT-PCR analysis. Total RNA was extracted using column plant RNAout Kit (Tiandze). A 1% agarose gel, stained by ethidium bromide, was run to check the integrity of the RNA. All RNA samples were quantified and examined for protein contamination (A260 nm/A280 nm ratios) and reagent contamination (A260 nm/A230 nm ratios) by a Nanodrop ND 1000 spectrophotometer.

The first strand cDNA was synthesized from 1.0 μg total RNA by Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) using oligo (dT) primers. Transcription of genes was analyzed by quantitative real-time RT-PCR (qRT-PCR) using the fluorescent intercalating dye SYBR-Green in a detection system (MJ Research, Opticon 2) in a final volume of 20 μL containing 2 μL of a 1/10 dilution of cDNA in water, 10 μL of the 2 × SYBR Premix Ex Taq (TaKaRa) and 200 nM of forward and reverse primers (Supplementary Data 7). The thermal cycling conditions were 40 cycles of 95 °C for 5 s for denaturation and 60 °C for 30 s for annealing and extension. All reactions were run in triplicate by monitoring the dissociation curve to control the dimers. PCR efficiency was determined by a series of 2-fold dilutions of cDNAs. The calculated efficiency of all primer pairs was 0.9 to 1.0. Gene Actin 2 was used as a reference and relative expression levels of genes were presented by 2^{-ΔΔCT}. For semi-quantitative RT-PCR (sqRT-PCR), the gene specific primer (Supplementary Data 7) were used for PCR reactions under the following conditions: pre-denaturation at 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at a specific annealing temperature (57 °C), and 30 s at 72 °C. As an internal control and to exclude genomic contamination, Actin was amplified (same cycling conditions as above for 28 cycles) from the same cDNA samples.

Transformation of AtFC1 in Arabidopsis. The AtFC1 genomic sequence was inserted into the downstream of the CaMV35s promoter in the binary vector pBI121. The constructs were then transferred into Agrobacterium tumefaciens for Arabidopsis transformation by the floral dip method. Positive transgenic lines were selected on the 1/2 MS medium with 50 mg/L kanamycin. More than fifteen independent transgenic lines were obtained, and four of them were presented. In this study, the homozygous lines (T4) were used.

Germination assay, root growth and fresh weight measurement. Seeds of wild-type, mutant and transgenic plants were grown on the same plate containing MS medium with or without different concentrations of NaCl or hematin. Plants were grown under conditions of 22 °C with 100 μE m⁻² s⁻¹ photosynthetically active radiation and a 16 h light/8 h dark cycle. The germination (fully emerged radicle) was recorded. The root length was measured with a ruler, and the fresh mass was weighted at the indicated times.

Analysis of plasma membrane permeability and proline. Plasma membrane permeability of tissues was determined according to the method described previously. Leaf and root segments were immersed in tubes with deionized water for 30 min, followed by measurement of conductivity of bathing medium (EC1) with a conductivity meter (METTLER TOLEDO FE30-FiveEasy). Samples were boiled for 20 min and the conductivity of bathing medium (EC2) was measured. The percent leakage of electrolytes was calculated as the ratio of EC1/EC2. For proline analysis, seedlings were harvested, frozen in liquid nitrogen and dried by lyophilization. Approximately 50 mg of dried seedling tissue was ground in 3% sulfosalicylic acid to extract free proline. Proline concentration was determined as described previously.

Quantification of ion concentration. Fresh seedlings were harvested and dried at 80 °C, and digested with the mixture of nitric acid and hydrogen peroxide using microwave system (MARS, CEM). The digested samples were used to quantify Na⁺ and K⁺ in roots and shoots using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Optimal 2100DV, Perkin Elmer Instruments). The Na⁺ uptake (1) and Na⁺ translocation (2) was calculated as follows: Na⁺ uptake = total Na⁺ content / root dry weight (1); and Na⁺ translocation = shoot Na⁺ content / root Na⁺ content (2).

Preparation of total RNA libraries and mRNA sequencing. Two week-old Arabidopsis seedlings were treated with 0 and 200 mM NaCl and sampled at 1, 2 and 4 h, respectively. Total RNA from NaCl-exposed and NaCl free seedlings was isolated using the TRIzol Reagent (Invitrogen, USA) and pooled for RNA sequencing. The extracted RNA was treated with DNaseI (Qiagen, USA) at 25 °C for 30 min and confirm in quality. mRNA was purified with oligo (dT)-rich magnetic beads and broken into short fragments. The first and second
strand cDNAs were synthesized. The cDNAs were end-repaired and phosphorylated using T4 DNA polymerase and Klenow DNA polymerase. The Illumina paired-end solexa adaptors were ligated to these cDNA fragments. The ligated products were purified on a 2% agarose gel. Four libraries (Col-0-NaCl, fci-1NaCl, Col-0 + NaCl and fci-1 + NaCl) were sequenced using an Illumina hiseq. 2500 with paired-end of Solexa RNA.

The original image data generated by the sequence providers were transferred into nucleotide sequences data by base calling, defined as raw reads and saved as ‘fastq’ files. All subsequent analyses were performed on the high-quality clean read datasets according to the bioinformatics analysis approach summarized in Supplementary Data 1. A rigorous algorithm was used to identify differentially expressed genes (DEGs) between the samples. The expression level for each transcript was calculated as FPKM (fragments per kilobase of exon per million fragments mapped)-derived read counts based on the number of uniquely mapped reads that overlapped with exonic regions. FDR was used to determine the threshold of the p-value in multiple tests, which corresponded to the differential gene expression test. In this study, FDR < 0.001 and the absolute value of Log Ratio > 1 were used as a threshold to judge the significant differences of gene expression.

**Gene ontology analysis.** The Gene Ontology (GO) category of the DEGs with functional significance was subject to the ultra-geometric test with Benjamini-Hochberg correction (http://www.geneontology.org/). GO terms with corrected p-value < 0.05 were regarded as significant enrichment for the DEGs compared to the genome background.

**Statistical analysis.** Experiments in the study were independently performed in triplicate. Each result shown in the figures was the mean of three replicated treatments, and each treatment contained at least 15–20 seedlings. Samples for analysis were randomly selected from all transgenic lines. The significant differences between treatments were statistically evaluated by standard deviation and ANOVA methods (p < 0.05).
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Author Contributions

Z.M.Y. conceived, designed and wrote the manuscript; F.F. and T.K. revised the manuscript; W.Z., H.L. and N.L.L. carried out the molecular and physiological experiments; S.J.F. analyzed the transcriptome data. All authors have read and approved the manuscript.

Additional Information

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