Genome-wide investigation of ZINC-IRON PERMEASE (ZIP) genes in Areca catechu and potential roles of ZIPs in Fe and Zn uptake and transport

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ABSTRACT

Iron (Fe) and Zinc (Zn) are essential nutrient elements for plant growth and development. Here, we observed the effects of Fe and Zn deficiency in seedlings of Areca catechu L. (areca palm), one of the most cultured palm trees in tropic regions. Results revealed that Fe deficiency causes strong chlorosis with the significantly decreased chlorophyll biosynthesis level and photosynthetic activities in the top third young leaf (L3) of seedlings. Zn deficiency caused light chlorosis in all three young leaves which slightly decreased chlorophyll biosynthesis and photosynthetic activities. Analysis of the Fe and Zn concentration in leaves and roots indicated that absorption and distribution of these two ions share cooperative pathways, since Zn deficiency caused Fe increasing, and vice versa. Therefore, we focused on the ZINC-IRON PERMEASE (ZIP) genes in areca trees. From the whole-genome data set we obtained, 6 ZIP genes were classified, and a phylogenetic tree was constructed with other 38 ZIP genes from model plants to find their potential functions. We also analyzed the expression pattern of AcZIP1-6 genes under Zn and Fe deficiency by transcriptomic approaches. With these results, we constructed an expression atlas of AcZIP1-6 genes in leaves and roots of areca seedlings with the dynamic expression levels under Fe and Zn deficient conditions. In conclusion, we provide evidence to understand the absorption and transport of nutrient elements, Fe and Zn, in the tropical agricultural plant A. catechu.

INTRODUCTION

Iron (Fe) and zinc (Zn) are essential micro-nutrients, both for plants and animals. Zn ions exist primarily as complexes with proteins and play various roles as activators of many proteins, participate in protein, nucleic acid, carbohydrates, and lipid metabolism, maintaining the integrity of cellular membranes, regulation of auxin synthesis, and pollen formation.1-3 It is also serving as a cofactor for more than 300 enzymes i.e. RNA polymerase, alkaline phosphatase, alcohol dehydrogenase, Cu/Zn superoxide dismutase, and carbolic anhydrase.4 It plays an associative role with gene transcriptional regulatory proteins and coordination of other biological processes.5 Zn also plays a key role in the regulation and expression of the gene required for the tolerance of environmental stresses in plants, such as light intensity and temperature.6-8

Fe is another essential micro-nutrients available in oxidation-reduction states (Fe2+ and Fe3+). It is an auxiliary group of many enzymes in plants, such as cytochrome oxidase, peroxidase, and catalase, and also plays an important role in electron transfer in photosynthesis and respiration.7 Besides being essentials, Zn and Fe could be potentially toxic at an excessive level for biological systems. A high concentration of free Fe (Fe2+/Fe3+) can generate oxygen and hydroxyl-free radicals through the Fenton reaction that can lead redox reaction and cause intra-cellular damages of DNA and lipids. Similarly, excess Zn binds to sulfur, nitrogen, and oxygen-containing functional groups in biological molecules because of its unregulated high affinity.8,9

Therefore, to balance the absorption, utilization, and storage of these metal ions, plants have established decisively multiform control transport systems.10 Besides other essential metal ions, Fe and Zn are present in the soil in soluble form and need to be transported from the soil solution into the root and then distributed throughout the plant, crossing both cellular and organelles membranes. Since trace elements are often present in the soil solution in exceedingly low amounts thus difficult to diffuse freely across the cell membrane.11 Therefore, plants use high-affinity transport systems to accumulate these ions and transport them into the cytoplasm.

Despite the importance of Zn and Fe both in plant and animal nutrition, the knowledge of how plants acquire, transport, store, and utilize these metals at the molecular level is quite limited. In recent years, several metal transporters have been identified in plants, including the copper transporter (CTR), natural resistance-associated macrophage protein (NRAMP) family, Heavy Metal ATPase (HM-ATPase) proteins, and cation diffusion facilitator (CDF) family.12,13 Recently, much progress has been made toward understanding the molecular and transport mechanisms of the ZRT, IRT-like protein (ZIP) family of plants, animals, fungi, bacteria, and Archaea. ZIP proteins generally contribute to metal ion home-
ostasis by transporting cations into the cytoplasm. Functional complementation in yeast indicated that ZIP proteins can import, transport various divalent cations: Fe\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), and Ni\(^{2+}\), from outside the cell or within an intracellular compartment. This family was first identified in plants and yeast (Arabidopsis and yeast), taking its name from the two founding members as “ZRT, IRT-like protein”. ZRT1 and ZRT2 are the high- and low-affinity zinc-regulated plasma membrane transporters of yeast, respectively. IRT1 (iron-regulated transporter) is an Arabidopsis thaliana plasma membrane transporter expressed in the roots of iron-deficient plants. The expression of these genes was detected in various tissues, including roots, shoots, nodules, and flowers, and it seems likely that these genes were transcriptionally regulated by metal levels. Most ZIP proteins are predicted to have eight potential transmembrane domains and a similar membrane topology in which the amino- and carboxyl-terminal ends of the protein are located on the outside surface of the plasma membrane. ZIP proteins range from 309 to 476 amino acids in length; this difference is largely due to the length between transmembrane domains III and IV, designated the ‘variable region’. In conclusion, ZIPs are the key transporters for Zn and Fe uptake and translocation in plants, considerable progress has been achieved in cloning and characterizing its functions in various plants to understand the distribution and usage of elemental nutrients.

Areca catechu L. (areca palm), a monocotyledon plant, belongs to the Arecaceae family, originated from the tropical rain forests of Malaysia. As one of the most commercially and economically important crops, it is widely distributed in India, Pakistan, the Philippines, Thailand, and other South and Southeast Asian countries. Researches on the elemental nutrient supply in areca trees are still limited. In this study, we observed the chlorosis phenomenon and physiological effect of Fe and Zn deficiency in areca seedlings. From the whole genomic dataset, we cloned and sequenced 6 ZIP genes, AcZIP1-6, in A. catechu. Their expression atlas was constructed and the dynamic changes of AcZIP1-6 under Fe and Zn deficiency were analyzed.

Materials and methods

Plant material and growth conditions

The seedlings of an A. catechu cultivar "Reyan NO.1," used in this study were provided from the Coconut Research Institute of the Chinese Academy of Tropical Agricultural Sciences by Dr Liu Liyun and Dr Huang Liyun. Research work was conducted in the experimental base of Hainan University. The areca seedlings selected in this experiment are 6-month-old seedlings, that having one opened leaf and the top leaf is still closed. The seedlings were planted and cultured in hydroponic pots with a whole vegetable nutrient solution for adaptive cultivation (Hoagland) (Table S1). After 2 weeks, the seed bulbs were removed from the stem base (to eliminate the interference of nutrients in the endosperm of the seed bulbs) and incubate for a week. The seedlings were cultured in whole nutrient solution (CK), Fe deficient medium without Fe-EDTA (Na)\(_2\), and Zn deficient medium without Zn\(^{2+}\), respectively. Areca seedlings in the treated group were sampled for subsequent experiments when there were obvious phenotypes. Keep the air pump inflated twice a day for half an hour each time. The modified Hoagland solution was used for the formulation of the whole nutrient solution.

Measurement of fresh and dry weight and chlorophyll content

The seedlings, above and below ground, were collected and measured their fresh weight. The dry weights were measured after dried at 40°C for 12 h in the oven. For chlorophyll (Chl) measurement, 0.1 g fresh leaves were extracted in 80% acetone. The absorbance was measured at 645,663 nm wavelength using a UV721 spectrophotometer and repeated three times for each sample.

Chl fluorescence parameters analyses

The leaves of areca seedlings treated with -Fe and -Zn deficiency were measured. Chl fluorescence parameters were measured using a DUAL-PAM/F100 instrument (Walz, Germany) after 15 min of dark adaption. After fully dark adaption, the leaves were irradiated with a measuring light of less than 0.1 to obtain initial fluorescence (F0). Turning on a saturation pulse [8000 µmol/(m\(^2\)*s)], the maximum fluorescence (Fm) under dark adaptation was obtained. When the fluorescence drops to a stable value, the photochemical light [450 µmol/(m\(^2\)*s)] is turned on. When it reaches a stable value again, the saturation pulse is turned on to obtain Fm'. The maximum quantum yield (Fv/Fm), the quantum yield of regulated energy dissipation of PSII (Y(NPQ)), and photochemical quenching coefficient (qP) were obtained. Each sample will be repeated three times.

Metal concentration analyses

The aboveground and underground parts of areca nut were sampled, respectively. The collected aboveground samples were washed and dried until the samples were completely dry. Roots need to be washed on ice with 5 mM PbNO\(_3\) solution for 30 minutes to remove ions attached to the root surface, then washed with distilled water three times, ultra-pure water for one time, put in a clean filter paper to dry, put in an oven at 80°C to bake for 2-3 d until the sample is completely dried. After being completely digested at room temperature with concentrated nitric acid, the contents of Zn and Fe were determined using ICP-MS. Each experiment was repeated three times.

Whole genomic sequencing

The genomic data of Areca catechu (cultivar information) were obtained by utilizing PacBio RSII single-molecule real-time (SMRT) sequencing technology. Incorporated Hi-C technology was further used for scaffolding. The yielded 284 G PacBio clean data of the genome from was resulted into 127 scaffolds,
with a scaffold N50 of 67.3 Mb and a total length of ~2.73 Gb. The data were uploaded JAHSCV000000000 (biosample SAMN19591864).

**Database searches and bioinformatics analysis for ZIP family genes**

To identify the members of the ZIP gene family, the hidden Markov model (HMM) profile of the ZIP domain (PF02535) provided by the Pfam database (http://pfam.xfam.org/) was used to search the ZIPs of *A. catechu* by using HMMER 3.0 software. The E-value of the HMMER search results was less than 0.001 as mentioned in previous studies (File S1). An evolutionary tree was constructed for the ZIP protein sequences of *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana*, and *A. catechu*. The protein sequences of ZIP transporter family members were collected from Phytozome (www.phytozome.net) website (File S2 and S3). The protein sequences and phylogenetic tree of ZIP genes were constructed by using MEGA 6 software with the maximum likelihood method based on the Jones-Taylor-Thornton matrix-based model. The bootstrap values were from 1000 replicates. MEME (https://meme-suite.org/meme/tools/meme), online programs, was used to analyze the conserved motif of ZIPs of four species. TBtools software was used to visualize the results.17,26

**RNA sequence and quantitative real-time PCR**

The first top leaf (L1), the third top leaf (L3), and the roots (R) of areca seedlings were sampled. Total RNA was extracted by using Trizol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and purity were analyzed by Agilent Bioanalyzer 2100 with RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, Redwood, CA). The samples were sent to Biomarker Technologies Co., Ltd. Sequencing libraries were generated using NEBNext“Ultra” RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) following the manufacturer’s instructions. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina, San Diego, CA) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated at Biomarker Technologies Corporation (Beijing, China). After passing the library inspection, the pooling of different libraries is conducted according to the target offline data volume, and the sequencing is conducted with the Illumina platform. RNA concentration and purity were measured using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). Each sample was mixed from three biological replicates.25

Quantitative real-time PCR (qPCR) was used to compare and verify the expression of the ZIP family genes in the presence of Fe and Zn deficiency treatments with stably expressed endogenous reference genes, i.e., Housekeeping genes (*AaActin*). Primer sequences of these genes are listed in Table S2. Total RNA were extracted from the sample (Same as the transcriptome sequencing sample) using RNAprep Pure Plant Plus Kit (Polysaccharide & Polyphenolics-Rich) and cDNA were reversed from the RNA by the Fast King RT Kit (With gDNase) respectively according to the manufacturer’s instructions (TIANGEN, Beijing, China). ChamQ Universal SYBR qPCR Master Mix was used for qPCR according to the manufacturer’s instruction (Vazyme, Nanjing, China). The relative gene expressions were calculated using the 2^-ΔΔCT^ method and normalized by the geometric mean of the three endogenous reference genes. Each biological replicate was technically replicated three times.

**Results**

**Chlorosis phenomenon and photosynthetic activities in Areca seedling leaves under -Fe and -Zn treatment**

We observed the chlorosis phenomenon of areca seedlings with three opened leaves grown under Fe (-Fe) and Zn (-Zn) deficient conditions. Comparing to the seedlings grown in whole nutrient solutions (Figure 1a), -Fe treatment caused significant leaf yellowing in all three young leaves, the top one has the most significant chlorosis feature (Figure 1b). Meanwhile, the -Zn treatment caused light yellowing in all three young leaves (Figure 1c). Fe and Zn deficiency also affected biomass accumulation of areca seedlings (Figure 1d).

The chlorophyll biosynthesis in the L1 and L3 was differently affected by -Fe and -Zn treatment. In the L1 leaf, both the Chl a and Chl b contents were significantly decreased under both -Fe and -Zn treatment, while the -Fe treatment decreased the Chl a contents more than 3 times. Meanwhile, the Chl a/Chl b ratio was significantly increased in L1 under both -Fe and -Zn treatment, the Chl a/Chl b ratio under -Fe treatment was slightly greater than that under -Zn treatment (Figure 1e). -Fe and -Zn treatment decreased the Chl a and Chl b contents in the L3 leaf too. However, the -Fe treatment only decreased the Chl a content for about twice than that in the control condition. The -Zn treatment did not affect the Chl a/Chl b ratio, while -Fe treatment slightly decreased it (Figure 1f).

Photosynthetic activities and Chl fluorescence parameters of both PSI and PSII were recorded by Dual-PAM-100. Fv/Fm decreased significantly in L1, but not in L3 under both -Fe and -Zn treatment (Figure 1g). The NPQ increased significantly in the L1 leaf under -Zn treatment, while there was no significant change in the L3 leaf under -Fe treatment. Meanwhile, in L3 leaf both -Fe and -Zn treatment, the NPQ was not significantly changed. The qP was decreased significantly in both L1 and L3 leaf under -Fe treatment. Under the -Zn treatment, the qP was decreased significantly in L1 leaf, while there was no significant change in the L3 leaf (Figure 1h).

**The accumulation and translocation of Fe and Zn in plants**

As can be seen from Figure 2, under the -Fe treatment, the Fe ion content in the L1 leaf and root of areca seedlings decreased significantly, while the Fe ion content in L3 leaf did not change significantly (Figure 2a). Zn ion contents increased in all tissues under Fe treatment, but increased significantly in L3 leaf and...
roots (Figure 2b). Under -Zn treatment, the Fe ion content of the L1 leaf and root had no significant change, while the Fe content in the L3 leaf increased significantly (Figure 2c). Meanwhile, the Zn content decreased significantly in the L1 leaf and root, while the zinc ion content of the L3 even increased to some extent (Figure 2d).

Identification and characterization of AcZIPs from A. catechu

Six ZIP genes in the A. catechu genome were identified using the HMM profile of the ZIP domain (PF02535) and BLASTP search tools. The main ZIP characteristics are listed in Table 1, including gene name, gene ID, full-length of cDNA (642–1230
In order to further verify the expression of ZIP transporters in Fe and Zn deficiency of A. catechu, RNA-seq and qPCR were performed for 6 ZIP transporters (Figure 4). AcZIP1 was significantly decreased in L3 leaf under -Fe treatment and increased in roots under -Zn treatment. AcZIP2 was decreased significantly in L1 leaf under -Fe treatment and increased significantly in all three -Zn treatment tissues. AcZIP3 was downregulated in all tissues treated with Fe-deficiency. The expression was down-regulated in L3 leaf under -Zn treatment, but up-regulated in the root. The expression of AcZIP4 was down-regulated in the L3 leaf under both -Fe and -Zn treatment, while the opposite trend was observed in the root. AcZIP5 was upregulated in all tissues treated with Zn-deficiency and also in roots treated with Fe-deficiency. AcZIP6 was down-regulated in all tissues treated with Fe-deficiency but up-regulated in both L1 leaf and L3 leaf treated with Zn-deficiency.

**Expression profiles of ZIP membrane transporters**

In order to further verify the expression of ZIP transporters in Fe and Zn deficiency of A. catechu, RNA-seq and qPCR were performed for 6 ZIP transporters (Figure 4). AcZIP1 was significantly decreased in L3 leaf under -Fe treatment and increased in roots under -Zn treatment. AcZIP2 was decreased significantly in L1 leaf under -Fe treatment and increased significantly in all three -Zn treatment tissues. AcZIP3 was downregulated in all tissues treated with Fe-deficiency. The expression was down-regulated in L3 leaf under -Zn treatment, but up-regulated in the root. The expression of AcZIP4 was down-regulated in the L3 leaf under both -Fe and -Zn treatment, while the opposite trend was observed in the root. AcZIP5 was upregulated in all tissues treated with Zn-deficiency and also in roots treated with Fe-deficiency. AcZIP6 was down-regulated in all tissues treated with Fe-deficiency but up-regulated in both L1 leaf and L3 leaf treated with Zn-deficiency.

**Discussion**

Fe and Zn deficiency caused leaf yellowing in areca seedlings. The dry weight of areca seedlings decreased significantly due to Fe and Zn deficiency. The chlorophyll content in the L1 and L3 also decreased significantly. As the components of Chl synthase, the Chl synthesis was blocked by Fe and Zn deficiency treatment.1–3 Chl fluorescence parameters also indicated that Fe and Zn deficiency led to the decline of the photosynthetic capacity of areca seedlings. Correspondingly, the ion content in plants also decreased, and the absorption of Fe and Zn ions appeared complementary phenomenon. At the same time, the Fe content in the L3 decreased not significantly or even increased. It was consistent with yellowing phenomenon in the L3. Both excessive and deficient of iron decreases fresh weight and photosynthetic rate in A. catechu seedlings, demonstrating that A. catechu is an iron-sensitive species. Moreover, A. catechu has a fine regulatory network to adapt to the influence of ion content changes in the external environment.47

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**Table 1.** List of ZIP genes in the A. catechu whole-genome dataset, which has been deposited at GenBank under the accession JAHSVC000000000.

| Gene name | Gene ID   | Full length of cDNA | protein length |
|-----------|-----------|----------------------|----------------|
| AcZIP 1   | AC03G045000 | 1080                | 359            |
| AcZIP 2   | AC05G102430 | 642                 | 213            |
| AcZIP 3   | AC06G108490 | 1230                | 409            |
| AcZIP 4   | AC08G023500 | 894                 | 267            |
| AcZIP 5   | AC09G005640 | 885                 | 294            |
| AcZIP 6   | AC13G102360 | 1047                | 348            |

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**Figure 2.** Fe and Zn concentration in leaves and roots of areca seedlings under Fe and Zn deficiency. (a) Fe concentration under Fe deficiency; (b) Zn concentration under Fe deficiency; (c) Fe concentration under Zn deficiency; (d) Zn concentration under Zn deficiency. L1: the top leaf. L3: the top third leaf. R: root. Different letters above error bars indicate values (mean ± SE, n = 3) are significantly different at the 0.05 level by Duncan multiple range test.

bp, protein length (213–409 aa) (Table 1). A phylogenetic tree was constructed from A. catechu and other 3 plant species, i.e., O. sativa, Z. mays, and A. thaliana (Figure 3a). According to the evolutionary tree, a total of 40 ZIP transporters of the four species were divided into 8 clusters, among which the ZIP family of A. catechu was in 6 clusters. The evolutionary tree shows that ZIPs of A. catechu are closer to maize and rice. Motif structural analysis showed that all ZIP genes contained conserved ZIP superfamily domains (Figure 3b). Motif analysis shows that AcZIP1, AcZIP2, AcZIP3, AcZIP5, and AcZIP6 have similar motif compositions which are widespread in other species, while the AcZIP4 has a unique motif composition that is similar to a few ZIP transporters in other species.
Across the entire genome, we found 6 ZIP genes. The motifs of six ZIP genes were analyzed which were similar to those of arabidopsis, rice, and maize. In order to further understand the evolutionary relationship between ZIP gene family members, a phylogenetic tree was constructed among 6 A. catechu ZIPs (AcZIP), 12 O. sativa ZIPs (OsZIP), 10 Z. mays ZIPs (ZmZIP), and 12 A. thaliana ZIPs (AtZIP). Evolutionary relationship indicated that the amino acid sequences of AcZIPs were closely related to ZIPs from other plants and existed as homologous (AcZIP1 and OsZIP3, OsZIP4, ZmZIP4, ZmZIP5; AcZIP3 and ZmZIP7, OsZIP10; AcZIP4 and ZmZIP6; AcZIP5 and OsZIP6, ZmZIP11; AcZIP6 and OsZIP8, ZmZIP10; AcZIP2 is in a separate branch), suggesting that the ZIPs of these species may have a common ancestor. ZmZIP4 and ZmZIP5 are sensitive to environmental Fe conditions in shoots and roots. ZmZIP7 may play a role in absorbing Fe and Zn in the roots and stems of maize. Yeast complimentary analysis of ZmZIP6 may play a role in absorbing Fe and Zn. OsZIP6 can be induced in the absence of Fe and Zn and plays a role in the absorption of Zn from the roots to the shoots. OsZIP8 was

Figure 3. Evolutionary relations and structural characteristics of the ZIP membrane transporters. (a) Phylogenetic tree constructed by 40 ZIP transporters. (b) Motif analysis of 17 ZIP transporters selected by the closed genes with the AcZIPs. The ZIP phylogenetic tree was constructed from 44 ZIP transporter protein sequences collected from 4 plant species. The 6 ZIP transporters of A. catechu were shown in red background. The protein sequences of ZIP transporter family members were collected from Phytozome (www.phytozome.net) website. Os: Oryza sativa. Zm: Zea mays. At: Arabidopsis thaliana. Ac: Areca catechu.
Figure 4. (a) Heat map analysis of the expression patterns of ZIP membrane transporters in Fe-deficient (-Fe) and Zn-deficient (-Zn) in A. catechu. The numbers in the heat map reflect the real numbers, and the colors of the heat map are normalized. L1: the top leaf, L3: the top third leaf. –Fe: Fe deficient. –Zn: Zn deficient. (b) The qRT-PCR analysis of ZIP genes in L1, L3, and root of areca seedlings under no treatment group (CK), Fe-deficient (-Fe), and Zn-deficient (-Zn) conditions. The relative expression of each gene was calculated as the $2^{-\Delta\Delta CT}$ value and normalized to the endogenous reference genes. Different letters above error bars indicate values (mean ± SE, n = 3) are significantly different at the 0.05 level by Duncan multiple range test. Linear correlation between RT-qPCR and transcriptome results is $R^2 = 0.98$. 
induced to be expressed under Zn deficiency treatment and played a role in the absorption and enrichment of zinc in roots.\textsuperscript{39} The function of these homologs may provide clues to the functional prediction of \textit{AcZIP}s.

The expression levels of \textit{AcZIP}1 and \textit{AcZIP}2 were significantly up-regulated in roots treated with Zn deficiency, indicating that they may play a role in the absorption of Zn ions in the roots of areca seedlings. At the same time, \textit{AcZIP}2 was up-regulated in the L1 leaf and L3 leaf, suggesting that \textit{AcZIP}2 also plays a role in Zn transport. However, the expression of \textit{AcZIP}2 was down-regulated in the L1 leaf of Fe-deficient areca seedlings, but not significantly changed in L3 leaf and roots, indicating that \textit{AcZIP}2 plays a role in Fe transport in new leaves. The downregulation of \textit{AcZIP}3 in all tissues treated with Fe deficiency suggests that \textit{AcZIP}3 may play a role in Fe absorption and transport in areca seedlings. However, the up-regulated expression level of \textit{AcZIP}3 in roots under the Zn deficiency condition indicated that it plays a role in the absorption of Zn in roots. \textit{AcZIP}4 is upregulated in both Fe and Zn deficient roots and down-regulated in L3 leaf, suggesting that it plays a role in ion absorption of Fe and Zn. \textit{AcZIP}5 was significantly upregulated in all three tissues with Zn deficiency, indicating that it plays a role in the absorption and transport of Zn. It was also up-regulated in Fe-deficient, suggesting that plays a role in Fe absorption. The expression of \textit{AcZIP}6 was down-regulated in all three tissues under the condition of Fe deficient, indicating that \textit{AcZIP}6 plays a role in the absorption and transport of Fe. At the same time, it was down-regulated in the L3 leaf and up-regulated in the L1 leaf, indicating playing a role in leaf ion transport (Figure 5).

According to the ion content and gene expression, \textit{AcZIP}3, \textit{AcZIP}4, \textit{AcZIP}5, and \textit{AcZIP}6 played a role in Fe ion transport and absorption in the roots under Fe-deficient conditions. \textit{AcZIP}1, \textit{AcZIP}2, \textit{AcZIP}3, \textit{AcZIP}4, and \textit{AcZIP}6 are associated with iron ion transport in areca nut leaves. \textit{AcZIP}1, \textit{AcZIP}2, \textit{AcZIP}3, \textit{AcZIP}4, and \textit{AcZIP}5 play an important role in the absorption and transport of zinc in the root of zinc deficiency. \textit{AcZIP}2, \textit{AcZIP}3, \textit{AcZIP}4, \textit{AcZIP}5, and \textit{AcZIP}6 are associated with zinc transport in areca nut leaves under zinc deficiency treatment. Further analysis showed that \textit{AcZIP}2, \textit{AcZIP}3, \textit{AcZIP}4, and \textit{AcZIP}5 are associated with the increase of Zn ions in L3 and the roots of areca nut seedlings under iron deficiency conditions, while \textit{AcZIP}3, \textit{AcZIP}4, and \textit{AcZIP}6 are associated with the increase of Fe ions in L3 leaf and roots under Zn deficiency condition.

Meanwhile, ZIP transporters also affect plant ion homeostasis by interacting with other proteins in mutant and overexpressed plants, including CTR, CDF, CAX, NRAMP, and HM-ATPase.\textsuperscript{12,13} The members of the ZIP family also interact with each other to regulate plant ion homeostasis.\textsuperscript{30,31} The complexity of the function of ZIP transporter is also related to the wide distribution of homology of its sequence structure. The high and low affinity of ZIP transporters to ions in plants and the difference of ion content in different parts of plant development indicate that the complexity of ZIP transporters in maintaining ion homeostasis in plants.\textsuperscript{32-34} The function of the tissue-specific ZIP gene may require further studies to validate its function. Our findings recommend that ZIP genes play a role in the uptake and translocation of Zn and Fe and involving in detoxification and storage of metals in plant cells. The results could be useful to better understand the transport mechanism of Fe and Zn in the areca tree and provide convenience for the future study of gene function. Therefore, more accurate and specific functional studies are needed to verify the function of the relevant genes.

\textbf{Conclusion}

Fe and Zn play an important role in growth and development of plants. No previous studies have explored the physiological and biochemical functions of Fe and Zn in Palms. In this experiment, areca seedlings were treated with Fe and Zn deficiency, and it was found that Fe and Zn deficiency caused the chlorosis...
of areca seedlings, as well as the decrease of dry weight and photosynthetic capacity. The content of Fe and Zn ions in L1, L3, and roots was determined. It was found that the absorption of Fe and Zn by areca seedlings was complementary. The ZIP family plays an important role in the uptake and transport of Fe and Zn in plants, and six ZIP genes have been identified from the whole genome of *A. catechu*. Through gene structure and phylogenetic tree analysis, we found that the ZIP gene of *A. catechu* has a similar structure to Arabidopsis, rice, and maize, and is closer to maize in an evolutionary relationship. Through specific expression of the ZIP family in top first leaf the L1, the top third leaf L3, and root, we found that ZIP transporters play an important role in the absorption and transport of Fe and Zn in areca seedlings (Table 2). We reveal that physiological, biochemical, and molecular responses of areca seedlings to Fe and Zn deficiency treatments, providing a clue for palm to cope with Fe and Zn deficiency treatments. The preliminary study on iron deficiency and zinc deficiency of areca catechu will provide basis for more accurate and complex understanding of iron deficiency and zinc deficiency in plants.

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### Disclosure statement

No potential conflict of interest was reported by the authors.

### Author contributions

QA did most experimental works and wrote the manuscript; CC did the elemental deficient treatment, NMK helped in preparing the manuscript, GZ did the evolutionary analysis, YW supervised this study.

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### Table 2. The predicted function of the ZIP transporters.

| Gene name | Predicted function                        |
|-----------|------------------------------------------|
| AcZIP1    | Zinc absorption                          |
| AcZIP2    | Zinc absorption and transport            |
| AcZIP3    | Iron absorption and transport, zinc absorption |
| AcZIP4    | Iron and zinc absorption                 |
| AcZIP5    | Zinc absorption and transport, iron absorption |
| AcZIP6    | Iron absorption and transport, zinc transport |

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