Genome-wide transcriptome analysis of the orphan crop tef (Eragrostis tef (Zucc.) Trotter) under long-term low calcium stress

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Calcium (Ca2+) is one of the essential mineral nutrients for plant growth and development. However, the effects of long-term Ca2+ deficiency in orphan crops such as Tef [(Eragrostis tef) (Zucc.) Trotter], which accumulate high levels of Ca in the grains, remained unknown. Tef is a staple crop for nearly 70 million people in East Africa, particularly in Ethiopia and Eritrea. It is one of the most nutrient-dense grains, and is also more resistant to marginal soils and climatic conditions than main cereals like corn, wheat, and rice. In this study, tef plants were grown in a hydroponic solution containing optimum (1 mM) or low (0.01 mM) Ca2+, and plant growth parameters and whole-genome transcriptome were analyzed. Ca2+-deficient plants exhibited leaf necrosis, leaf curling, and growth stunting symptoms. Ca2+ deficiency significantly decreased root and shoot Ca, potassium (K), and copper content in both root and shoots. At the same time, it greatly increased root iron (Fe) content, suggesting the role of Ca2+ in the uptake and/or translocation of these minerals. Transcriptomic analysis using RNA-seq revealed that members of Ca2+ channels, including the cyclic nucleotide-gated channels and glutamate receptor-like channels, Ca2+-transporters, Ca2+-binding proteins and Ca2+-dependent protein kinases were differentially regulated by Ca2+ treatment. Moreover, several Fe/metal transporters, including members of vacuolar Fe transporters, yellow stripe-like, natural resistance-associated macrophage protein, and oligo-peptide transporters, were differentially regulated between shoot and root in response to Ca2+ treatment. Taken together, our findings suggest that Ca2+ deficiency affects plant growth and mineral accumulation by regulating the transcriptomes of several transporters and signaling genes.

Calcium (Ca2+) is an essential plant nutrient with two crucial roles. It is a structural component of the cell wall and membranes where it binds to an acidic group of the membrane lipids and crosslinks pectin. Ca2+ also acts as a ubiquitous second messenger in environmental stress signaling. Several environmental stimuli lead to the release of stored Ca2+, which binds to calmodulin. The Ca2+-calmodulin complex recruits several kinases and phosphatases that regulate transcription-dependent cellular processes. Plant cells also possess other Ca2+-regulated proteins, including Ca2+-dependent protein kinase (CDPKs), calcineurin B-like (CBL) proteins, CIPK, interacting protein kinases (CIPKs), CDPK-related kinases (CRKs), etc., which have been well characterized.

In plants, Ca2+ is taken up from the soil solution by roots and translocated to the shoot via the xylem. Ca2+ enters plant cells through Ca2+ channels. Plant glutamate receptor-like (GLR) channels, which are analogs to the mammalian ionotropic glutamate receptors (iGLuRs), are implicated in Ca2+ sensing, signaling, and...
long-distance root to shoot Ca²⁺ wave propagation during wounding. Several members of the AtGLRs have been characterized. The role of AtGLR3.1 in Ca²⁺ sensing in Arabidopsis shoot has been reported. Another class of Ca²⁺ channels known as cyclic nucleotide-gated channels (CNGCs) has also been implicated in Ca²⁺ transport. Cellular Ca²⁺ concentration is tightly regulated to allow signaling role, control Ca²⁺ translocation to the shoot and prevent cation toxicity. Antiporters, uniporters and Ca²⁺-ATPases localized in all major plant cellular membranes and rapidly expel Ca²⁺ from the cytosol. The Arabidopsis genome encodes five different Ca²⁺ efflux systems, called autoinhibited Ca²⁺-ATPases (ACAs), ER-type Ca²⁺-ATPases (ECAs), and P1-ATPases (HMA1), the mitochondrial calcium uniporter complex (MCUC) and Ca²⁺ exchangers (CAX). Similarly, Ca²⁺/Cation-exchangers have also been reported to play a role in cation homeostasis in plants.

Ca²⁺ deficiency is not commonly observed in most soils due to its relative abundance and regular liming practice. However, developing tissues may experience Ca²⁺ deficiency due to lack of Ca²⁺ remobilization from older conditions, under conditions of low transpiration, in sandy soils untreated with lime (CaCO₃ or Ca(OH)₂), and inhibition of Ca²⁺ uptake due to competition from other cations. Plants exposed to Ca²⁺ deficiency may experience necrosis of the meristems, shoot and root growth stunting and leaf curling. Ca²⁺-deficiency increases the level of reactive oxygen species, causing oxidative stress in high Ca-demanding plant species such as cabbage and broccoli. Ca²⁺ treatment has been shown to enhance the antioxidant system and decrease oxidative stress in plants grown under high temperature, drought, salinity and heavy metals.

Calcium-sensing networks, including the calcineurin B-like proteins (CBL)-CBL-interacting protein kinase (CIPK), has been implicated in plant abiotic stresses, including K⁺ deficiency. The CBL1/9—CIPK11 interaction has been involved in regulating the bHLH transcription factor FIT; a crucial regulator of Arabidopsis Fe acquisition. Fe deprivation has been linked to alteration of Ca²⁺ signatures evoked by exogenous ATP, suggesting crosstalk between Ca²⁺ and Fe homeostasis. However, detailed studies on the interconnection between Ca²⁺ and Fe nutrition are missing. Recently, studies of factors modulating Ca²⁺-iron homeostasis are emerging.

Tef is an important orphan crop in the Horn of Africa and some other countries including the United States where it is grown primarily for animal feed. Recently, tef has been getting increased attention in many laboratories due to its health benefits and potential roles in sustainable agriculture. Its mineral nutrient composition has been studied. Tef is one of the orphan crops reported to have higher mineral contents; mainly, the Ca²⁺ content of tef is higher than other crops such as sorghum, barley, wheat, and rice. The Ca²⁺ content in the plant varies from 0.1 to 5% of the shoot dry weight and the average seed Ca²⁺ content may vary between 981 and 1811 mg/kg in tef as compared to 33 mg/kg in maize, 153 mg/kg in rice and 533 mg/kg in wheat.

Similarly, in millets such as finger millet, the Ca²⁺ contents were higher than in other major crops such as wheat, rice, and maize. Low Ca²⁺ consumption in the diet has been related to several disorders in humans, many of which can have significant health repercussions over time. Because most staple food grains are low in Ca²⁺, tef, which is an orphan crop with high Ca²⁺ content, has enormous promise as a nutritional security crop. However, orphan crops’ physiological and transcriptomic response to Ca²⁺ nutrition has not been studied before.

This study exposed tef plants to long-term Ca²⁺ deficiency and analyzed the physiological and transcriptomic responses. Our findings revealed that prolonged low Ca²⁺ treatment decreased shoot and root biomass, Ca, K, and copper (Cu) content while significantly suppressing Fe translocation from the roots to the shoot. Transcriptomic analysis using RNA-seq revealed that some members of Ca²⁺ transporters, including CNGC, GLR and CaUP, signaling genes such as CBP and CDPK and genes implicated in Fe homeostasis such as YSL, OPT, VIT and NRAMP are differentially regulated by Ca²⁺ treatment or between roots and shoots.

**Results**

**Low Ca²⁺ treatment inhibits plants growth.** To understand the effect of long-term Ca²⁺ deficiency in tef, plants were grown in a modified Hoagland’s hydroponic solution supplemented with predetermined low (0.01 mM) or optimum (1 mM) Ca²⁺ for one month, and the plants were analyzed. As shown in Fig. 1, plants grown in low Ca²⁺ exhibited typical Ca²⁺ deficiency symptoms, including leaf tip necrosis and growth stunting, compared to plants grown at optimum Ca²⁺ level (Fig. 1A). Plants were then dried at 65 °C overnight, and root and shoot dry matters were determined. As shown in Fig. 1B, Ca²⁺ deficiency decreases shoot and root biomass by 45% and 35%, respectively. The results also showed that prolonged low Ca²⁺ treatment decreased shoot and root elongation and plant height significantly from 66 to 38 cm, while root length was affected only slightly from about 15 cm to 12 cm (Fig. 1C).

**Ca²⁺ deficiency disturbs minerals homeostasis.** Fourteen-day-old seedlings were cultured in hydroponic solutions supplemented with 0.01 mM or 1 mM Ca²⁺ for one month. Mineral content from the dried shoot and root samples was determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES). Plants grown in low Ca²⁺ media accumulated 0.5 g/Kg Ca²⁺ in the shoot (LCS) as compared to plants grown at optimum Ca²⁺ level (ConS), which accumulated 3 g/Kg Ca²⁺ (Fig. 2A). However, there was no statistically significant difference in root Ca²⁺ content. Similarly, there was no significant difference in root P content in plants grown at low (LCR) and optimum Ca²⁺ (ConR). At the same time, there was a small but statistically significant difference between LCS and ConR (Fig. 2B). S contents in root were slightly lower in plants grown at low Ca²⁺ while shoot S content was significantly lower in LCS than LCR (Fig. 2C). Ca²⁺ deficiency significantly suppressed root and shoot K content (Fig. 2D). In roots, K content decreased from 24.2 g/Kg in control plants to 10.3 g/Kg in Ca²⁺ deficient plants. Similarly, shoot K content decreased from 32.9 g/Kg in control plants to 23.2 g/Kg in Ca²⁺ deficient plants, suggesting the role of Ca²⁺ in K⁺ uptake and homeostasis. Ca²⁺ deficiency also decreased Mg content in the roots, but it did not affect shoot Mg content (Fig. 2E). Interestingly, Fe content in LCR was 2.5-fold higher than ConR, 0.95 g/Kg and 0.36 g/Kg, respectively (Fig. 2F). However, Fe content in...
LCS (0.06 g/Kg) was slightly lower than in control plants (0.08 g/Kg), suggesting that Ca$^{2+}$ deficiency impairs root to shoot translocation of Fe. The Zn content was slightly higher in LCR as compared to ConR, while in shoots, Zn content was slightly lower in LCS than ConS (Fig. 2G). We also observed that Cu accumulation was significantly affected by Ca$^{2+}$ treatment. Ca$^{2+}$ deficiency decreased root Cu content by three-folds (from 0.04 g/kg in ConR to 0.013 g/Kg in LCS), while in shoots, a small but significant decrease in Cu content was observed (0.01 g/Kg in ConS to 0.01 g/kg in LCS) (Fig. 2H). Both zinc (Zn) and copper (Cu) contents were lower in shoots than in shoots.

Figure 1. The phenotypic response of tef (Eragrostis tef) plants to long-term Ca treatment. (A) Phenotypes of tef plants treated with low (10 µM) or optimum or control (1 mM) calcium in modified Hogland’s hydroponics media for four weeks. The picture was taken using an Apple iPhone camera by AL-O. (B) Root and shoot biomass, and root length of tef plants grown in Hoagland solution containing 10 µM or 1 mM Ca. LCS, low calcium grown shoots; ConS, control/optimum Ca grown shoots. LCR, low calcium grown roots; ConR, optimum calcium treated roots. (C) Shoot and root length of tef plants grown under control (ConS and ConR) and low Ca (LCS and LCS) conditions. Bars represent mean and SD of at least 4 independent biological replicates, and different letters indicate statistically significant difference ($p < 0.05$).

Figure 2. Mineral content profiling of tef plants grown under low or optimum Ca conditions. Shoot and root Ca (A), P (B), S (C), K (D), Mg (E), Fe (F), Zn (G), Cu (H), Mn (I) and B (J) were analyzed using ICP-OES as described in the “Materials and methods” section. Bars represent mean and SD of at least 4 independent biological replicates, and different letters indicate statistically significant difference ($p < 0.05$).
Note that values are averages of reads from four biological replicates.

**Characteristics of RNA sequencing data.** Since Ca^{2+} deficiency-induced visual symptoms in tef plants, including growth stunting and leaf necrosis in tef (Fig. 1A), we aimed at analyzing genome-wide transcriptomic changes in response to Ca^{2+} treatment. Total RNA was isolated from control and low Ca^{2+}-grown shoots and roots (ConR, ConS, LCR, and LCS). Complementary DNA libraries were generated from four independent biological replicates and sequenced using the paired-end sequenced using Illumina Hiseq 4000. A total of 476.5 million raw reads with an average of 30 million reads were obtained for each sample. The total clean reads of the 16 libraries were aligned to the tef draft genome sequence. On average 85.7% of the reads were uniquely aligned to the reference genome (Tables 1, S1).

**Analysis of differentially expressed genes (DEGs).** The quality of sequence reads was assessed using fastqc application, and 15 poor-quality bases were trimmed from the 5'-end. The remaining good-quality reads were aligned to the tef reference genome using hisat2 aligner. For each replicate, per-gene counts of uniquely mapped reads were calculated using the htseq-count script from the HTSeq python package. The count matrix was imported to R statistical computing environment for further analysis. Initially, genes that have no count in the replicate samples were discarded. The remaining count data were normalized and differentially expressed genes were identified using the DESeq2 R package.

To determine the integrity of RNA samples, we performed principal component analysis (PCA). The PCA shows no mark variation among most RNA samples from the same Ca^{2+} treatment or tissue (Supplementary Fig. 1A,B). Our analysis identified many DEGs in response to Ca^{2+} treatment suggesting the crucial role of Ca^{2+} in plant physiological processes, including overall plant performance (Fig. 1) and mineral homeostasis (Fig. 2).

The Venn diagram in Fig. 3A summarizes the pairwise comparisons of different groups (LCR, LCS, ConR, and ConS) based on the number of DEGs regulated in roots and shoots of tef plants grown under low and optimum Ca^{2+} conditions. As compared to ConR, 824 DEGs were detected in LCR, of which 85 are unique to LCR. A total of 5,420 DEGs out of 28,796 genes were detected in LCS as compared to ConS of which 531 DEGs were uniquely detected in LCS. Relative to LCS, 10,752 DEGs were seen in LCR of which 532 DEGs are unique to LCR. A total of 13,504 DEGs were detected in ConR as compared to LCR, of which 1,886 DEGs were uniquely detected in ConR. A total of 249 DEGs were common in all the pairwise comparisons (LCR vs. ConR, LCS vs. ConS, LCR vs. LCS, and ConR vs. ConS).

Figure 3B illustrates the transcriptomic response of tef plants grown in optimum and low Ca^{2+} conditions. A large number of genes (4,654) were upregulated in roots and shoots of low Ca^{2+} relative to control, of which 366 and 4009 genes were uniquely upregulated in LCR and LCS relative to ConR and ConS, respectively. A total of 279 genes were commonly upregulated in LCR and LCS. We also detected 1279 DEGs that were downregulated in LCR (179) and LCS (1132) compared to ConR and ConS, respectively. A total of 32 genes were commonly downregulated in LCR and LCS as compared to ConR and ConS, respectively. Ca^{2+} deficiency also induced significant transcriptomic changes between roots and shoots. A total of 7045 DEGs were upregulated in roots, of which 4833 DEGs were upregulated in LCR relative to LCS, 6670 DEGs were upregulated in ConR relative to ConS, and 4458 DEGs were commonly upregulated in both LCR and ConR. Similarly, 7430 DEGs were downregulated in roots, of which 6,192 DEGs were downregulated in LCR relative to LCS, 6834 DEGs were downregulated in ConR relative to ConS, and 5396 DEGs were commonly downregulated in both LCR and ConR relative to LCS and ConS, respectively. Taken together, the number of transcripts that were upregulated in response to Ca^{2+} deficiency in both roots and shoots were higher than those whose transcript abundance was downregulated. Moreover, the number of DEGs that were upregulated in ConR versus ConS were higher than those DEGs that were upregulated in LCR versus LCS (Fig. 3B).

**GO annotation of DEGs.** We performed Gene Ontology (GO) enrichment analysis using the AgriGo database to determine the potential function of DEGs identified in our analysis. In the GO analysis, the DEGs were grouped into molecular function, cellular components, and biological process categories (Fig. 4). Of these categories, DEGs with catalytic activity (> 1300 DEGs) were the most highly enriched group (Fig. 4A) for the

| Treatments | LCR | LCS | ConR | ConS |
|------------|-----|-----|------|------|
| Number of input reads | 22,769,930 | 25,179,862.3 | 36,184,172.5 | 34,989,385.3 |
| Number of uniquely mapped reads | 18,361,838.8 | 21,930,270.8 | 28,709,324.8 | 31,726,862.3 |
| Percentage of uniquely mapped reads | 80.7 | 86.9 | 84.5 | 90.7 |
| Number of reads mapped to multiple loci | 1,956,523.75 | 1,285,027 | 2,371,067.75 | 1,791,236.5 |
| Unmapped reads (too short) percent | 8.45 | 5.115 | 6.555 | 5.175 |

Table 1. RNA sequencing profile of tef samples under two different Ca^{2+} levels in root and shoots. Samples were LCR (low calcium roots), LCS (low calcium shoots), ConR (control roots) and ConS (control shoots). Note that values are averages of reads from four biological replicates.
LCR vs ConR comparison, followed by those predicted to have transport activity (200 DEGs). In the cellular component category, all the DEGs that were enriched are associated with membranes. In contrast, in the biological process category, about 300 DEGs are predicted to be involved in establishing localization, transport, and transmembrane transport. We also noted that DEGs with metal ion transporter activity, ATPase activity, primary active transporter activity, inorganic cation transporter activity, and substrate-specific transporter activity were on top of the list (Fig. 4A). When LCS versus ConS comparison was used for GO analysis, we observed that DEGs with hydrolase activity, ATP binding, and kinase activity were most enriched in the molecular function category. In contrast, in the biological process category, genes with cellular process followed by the primary metabolic process is the most enriched group (Fig. 4B).

**Calcium transporter genes are differentially regulated in response to Ca²⁺ treatment.** Several families of genes have been implicated in Ca²⁺ transport in plants. This study identified transcriptomic changes in some families of Ca²⁺ transporters, including GLRs, CNGCs, Ca²⁺ uniporters, cations/Ca²⁺ exchangers, and Ca²⁺-transporting ATPases. Our analysis identified Ca²⁺ transporters including Ca²⁺ uniporters, cation/Ca²⁺ exchangers and Ca²⁺-transporting ATPase (Fig. 5A) etc. Expression of putative Ca²⁺ uniporters CaUP2 and CaUP4 were higher in roots than shoots in both low and optimum Ca²⁺ conditions. Transcripts of several transporters, including CaE1-1, CaTA5-1, CaTA9-1, CaUP6, CaUP3, NaCaE1 were more abundant in ConS relative to ConR while transcripts of CaE1-2, CaUP3, CaTA3, and CaUP4 were more abundant in LCR than LCS. Transcripts of CaE1 was more abundant in LCS than ConS, while CaTA9, CaTA5-1, CaTA9-1, CaUP6, and CaTA10 were more abundant in ConS than LCS. CaE1, CaE1-1, and CaUP4 are more abundant in LCR than ConR.
whereas CaTA5 is more abundant in ConR than LCR. Transcripts of several members of Ca²⁺ transporting proteins are not markedly affected by Ca²⁺ treatment and between roots and shoots (Fig. 5A).

In response to Ca²⁺ treatment, several Ca²⁺-binding, Ca²⁺ signaling, and other transporters, including Ca²⁺ transporters and Ca²⁺-gated channels, are differentially expressed in both roots and shoots. We analyzed the expression of the CNGCs, which are implicated in the transport of Ca²⁺ and other cations (Fig. 5B). Transcripts of CNGC2, CNGC4, CNGC4.1, and CNGC4.2 were more abundant in shoots than roots under low and optimum

Figure 4. Gene Ontology (GO) enrichment terms of DEGs. Bars represent the number of genes enriched in LCR versus ConR (A), and the number of genes enriched in LCS versus LCS (B). GO annotation was performed as described in the “Materials and methods” section.
Figure 5. Heatmaps of differentially expressed genes. (A) Calcium transporters, (B) CNGCs, (C) GLRs and (D) Fe/metal transporters. Fe, iron; GLR, glutamate receptor; CNGC, cyclic nucleotide-gated channel; OPT, oligopeptide transporter; VIT, vascular iron transporter; NRAMP, metal transporter Nramp; YSL, yellow stripe-like; CaC1, calcium load-activated calcium channel; CaCCI, calcium permeable stress-gated cation channel 1; CaUP, calcium unipporter protein; CaMC, calcium-binding mitochondrial carrier protein; CaTA, calcium-transporting ATPase; CaE, cation/calcium exchanger; CaME, mitochondrial protons/calcium exchanger protein; NaCaE, sodium/calcium exchanger; TPCaC, two pore calcium channel protein. *p<0.05; **p<0.01; ***p<0.001. (A) Et_4A_032234, CaC1; Et_3B_028475, CaCCI; Et_1A_007032, CaUP2; Et_4A_035795, CaUP4; Et_3B_029644, CaUP6; Et_10A_001249, CaUP6-1; Et_6A_048187, CaUP6-2; Et_2A_008615, CaUPS; Et_7B_055244, CaUP4; Et_2B_021219, CaMC-3; Et_4B_039870, CaTA10-1; Et_4A_033710, CaTA3; Et_4B_037342, CaTA3-1; Et_4A_033168, CaTA3-2; Et_4A_034993, CaTA4; Et_1A_007426, CaTA5; Et_8A_057406, CaTA5-1; Et_8A_057411, CaTA5-2; Et_7B_054771, CaTA6; Et_1A_006195, CaTA7; Et_2A_016261, CaTA8. (B) Et_1B_013097, OPT4; Et_1A_003655, OPT3; Et_2B_020954, OPT1; Et_3B_028366, OPT2; Et_4A_033828, OPT1; Et_4A_034655, Opt2; Et_5B_045010, OPT3. (C) Et_1A_006195, GLR2.7; Et_2A_016261, GLR2.4; Et_2A_018314, GLR2.8; Et_2B_043951, GLR3.4-1; Et_7A_052266, GLR3.4-2; Et_1A_018316, GLR3.4-3; Et_7B_054916, GLR3.4-4; Et_7B_054916, GLR3.4-5. (D) Et_1A_007771, CNGC1-1; Et_2A_015772, CNGC17-1; Et_2A_01786, CNGC17-2; Et_7B_054569, CNGC6. The graphs were generated using R Software42(URL https://www.R-project.org/).
Ca²⁺ conditions (Fig. 5B). The transcripts of these genes were more abundant in LCS than ConS, although not statistically significant. Expression of CNGC1 and CNGC20 are significantly downregulated in ConR and LCR as compared to ConS and LCS, respectively. There was no difference in the expression of CNGCs between LCR and ConS.

Figure 5 also shows that several putative tef glutamate receptors such as GLR3.1, GLR2.8, GLR4-1, and GLR3.4-1 are differentially expressed in response to Ca²⁺ deficiency, as shown in Fig. 5C. In ConS, transcripts of GLR2.4, GLR2.7, GLR3.1, GLR3.4, and GLR3.4-1 were more abundant than ConR, while GLR2.4, GLR3.4, and GLR3.4-1 were more abundant in LCS than ConR. However, the transcripts of GLR2.8 were more abundant in LCR than ConR.

**Low calcium regulates the expression of Fe and metal transporter genes.** Our analysis also revealed that several members of the putative iron transporters are differentially regulated in response to Ca²⁺ treatment and between tissue types (Fig. 5D). Transcripts of several tef homologs of Arabidopsis and rice iron transporter, including VIT5, VIT1, OPT1, NRAMP5, YSL16, NRAMP4, YSL9, and YSL2 were more abundant in LCS and ConS than LCR and ConR, respectively. YSL2 is a critical enzyme in long-distance Fe transport through the xylem. The VITs transport Fe across the vacuolar membrane, and Nramps and OPTs are involved in Fe other metal transport. The expression of YSL14, NRAMP2, and NRAMP3 was downregulated under low Ca²⁺ conditions in both roots and shoots. Transcripts of OPT1, OPT4, and NRAMP5 were upregulated in LCR relative to LCS, while NRAMP1 and NRAMP2 were more abundant in LCS than LCR. Transcripts of YSL16 is upregulated in ConS than ConR, while YSL12 is upregulated in ConR than ConS (Fig. 5B).

**Calcium signaling genes are differentially regulated by long-term Ca²⁺ treatment.** Because Ca²⁺ plays a key role in various abiotic stress signaling, we analyzed whether transcripts of signaling genes respond to long-term Ca²⁺ deficiency. As shown in Fig. 6, many signaling genes, including CDPK1, CDPK2, CDPK3, CDPK4, CDPK5, CDPK6, CDPK17, CBP1, CBP2, CBP3, CBP6, CBP10, CBP12, CBP15, CBP16, CBP17, CCDSTK1, CNGC17, and CNGC17 were upregulated in shoots than roots under both Ca²⁺ levels. At the same time, transcripts of several CDPK and CBP, including CDPK1, CDPK2, CDPK12, CDPK3, and CBP15, were more abundant in the roots of both control and low Ca²⁺ conditions. In roots, the expression of CDPK1, CDPK2, CDPK3, CCDS1T1, and CBP15, is upregulated by Ca²⁺ deficiency. Overall, our findings highlight the differential expression of some signaling genes in response to long-term Ca²⁺ deficiency.

**Validation of RNA-seq DEGs.** To the RNA-seq data, we performed quantitative RT-PCR (qPCR) to analyze the expression of a few DEGs, including CaE1, CGC2, GLR3.1, and CNGC17. As shown in Fig. 7, we performed Pearson correlation of Log² fold changes in gene expression by RNA-seq and qPCR analysis. A strong positive correlation was observed between the two assays for CaE1 (R² = 0.9167), CNGC2 (R² = 9626) and CaE1 (R² = 9626), while a positive but weak correlation was observed for CNGC17 (R² = 0.1689). These findings validate the RNA-seq data.

**Discussion**

The Chloridoideae subfamily of Poaceae, including tef and finger millet, accumulate higher levels of Ca²⁺ in grains than other cereals. However, the effect of prolonged Ca²⁺ deficiency on their physiology and transcriptome remained unknown. In the present study, we examined the response of tef to prolonged Ca²⁺ deficiency based on agronomic, physiological, and transcriptome changes in plants grown in hydroponics solution under optimum and Ca²⁺ deficient conditions.

Tef plants grown under Ca²⁺ deficient conditions showed typical deficiency symptoms in shoots, including leaf curling, tip necrosis, and growth stunting (Fig. 1A), which is in line with previous reports. We performed the elemental analysis of the shoots and roots to validate our phenotypic analysis. Our findings revealed that prolonged Ca²⁺ deficiency significantly decreased Ca²⁺, K, and Cu content in both the roots and the shoots, and S, Zn, and Mn in the shoots. Interestingly, Fe translocation from roots to shoots is significantly suppressed by Ca²⁺ deficiency. Root Fe content was 2.5-fold higher in roots than in shoots. These suggest that Ca²⁺ deficiency suppresses plant growth by perturbing homeostasis of essential minerals, including macro and micronutrients. Ca²⁺ deficiency is rare in nature but may happen when plants are grown in acidic soil. Although there is no report on the effect of Ca²⁺ deficiency on plant Fe translocation, some studies have reported on the interaction between Ca²⁺ and Fe. A high level of Ca²⁺ has been reported to cause Fe chlorosis by suppressing Fe acquisition in field crops and decreased Fe uptake in dry bean plants.

To understand the transcriptomic change in response to Ca²⁺ deficiency, we performed RNA-seq using the Illumina HiSeq platform. Our analysis revealed that many transcripts are differentially regulated between control and Ca²⁺ deficient conditions, and between roots and shoots of plants grown under both Ca²⁺ treatments (Figs. 3 and 5). Moreover, our GO analysis highlighted significant enrichment of several Ca²⁺ transporters, signaling genes, those genes with transport activities, ATPase activity, catalytic activities etc. (Fig. 5). Such transcriptomic changes are expected since Ca²⁺ is an integral part of plant cell walls, mineral homeostasis, and signaling.

When plants undergo certain environmental stresses, Ca²⁺ signals are generated, which trigger a rapid rise in cytosolic Ca²⁺ levels. Several protein families are implicated in plants’ Ca²⁺ transport processes, including Ca²⁺-permeable ion channels, Ca²⁺/H⁺ exchangers, and pumps (Ca²⁺-ATPases). Several Ca²⁺-permeable channels have been implicated in the uptake of Ca²⁺ from the soil solution to plant roots. These include the GLRs, CNGCs, two-pore channels (TPCs), mechanosensitive-like channels (MSLs) etc. Ca²⁺ pumps are involved in maintaining homeostasis by controlling Ca²⁺ efflux from the cytosol to organelles and/or to the apoplast.
In our dataset, we identified several DEGs including Ca²⁺ uniporters (CaUPs), cation/Ca²⁺ exchangers (CaE), Ca²⁺-transporting ATPases (CaTA) etc. (Fig. 5). Our findings revealed that the CaE1, CaE1-2, CaUP6, CaUP3, NaCaE1, CaTA10, CaUP4 and CaTA3 were downregulated in ConR as compared to ConS (Fig. 5A). Whereas CaE1, CaE1-2, CaUP3, and CaTA3 were downregulated in LCR compared to LCS (Fig. 5A). When the LCS was compared to ConS, the CaE1 was upregulated, and CaTA9, CaTA5-1, CaTA9-1, CaUP6, and CaTA10 were downregulated. Similarly, when LCR was compared with ConR, the CaUP4 and CaE1 genes were upregulated, and CaTA5 was downregulated (Fig. 5A). It was previously reported that Ca²⁺ uniporters play an important role in stress signaling in Arabidopsis⁵¹. In another study, Ca²⁺ uniporter proteins were implicated in pollen tube germination⁵². As reported in previous studies, Ca²⁺ uniporters play an important role in Ca²⁺ signaling and homeostasis in Arabidopsis⁵¹ and homologs in mammals play role in Ca²⁺ uptake into the mitochondrial matrix and regulate calcium signaling, aerobic metabolism, and apoptosis⁵³. None of these transporters have been isolated and characterized from tef before. However, several homologs have been characterized in other plant species such as Arabidopsis⁵⁴, rice⁵⁵ and apple (Malus domestica)⁵⁶.

The CaCA superfamily has been reported to facilitate Ca²⁺ transport against its concentration gradient and enhance the influx of monovalent cations (K⁺, Na⁺, and H⁺)⁵⁷. For example, overexpression of the apple cation/ Ca²⁺ exchanger MdCCX1 in transgenic Arabidopsis and apple root cells has been shown to decrease Na⁺ accumulation and ROS levels and increase salt tolerance in transgenic cells⁵⁸. In this study, Ca²⁺ deficiency decreased...
Ca and K content in roots. Therefore, upregulation of CaE1 in LCR and LCS could be a mechanism by which roots enhance K⁺ or Ca²⁺ acquisition from the external solution or facilitate their distribution between tissues. Transcripts of CaTA9-1, CaTA5-1, CATA9, and CaUP6 are downregulated by Ca²⁺ deficiency in the shoots. The CaTA5 was the only gene among the transporters that was downregulated by Ca²⁺ deficiency in roots. CaTA5 is a homolog of the plasma membrane Ca²⁺-transporting ATPases (PMCA), which are high-affinity Ca²⁺-pumps that export Ca²⁺ ions from the cytosol into the extracellular solution. CaTA5 may be a functional Ca²⁺-pump, and its downregulation under Ca²⁺ deficiency could be a strategy to suppress the cytosolic Ca²⁺ export to the extracellular solution. The autoinhibited Ca²⁺-ATPase ACA13 has been implicated in successful pollination in the Brassicaceae by exporting pollen to compatible pollen tubes.

This study also identified various Fe/metal transporters, including OPTs, VITs, NRAMPs, and YSLs. All these transporters proteins were differentially expressed in the roots and shoots of low Ca²⁺ and control plants. In Arabidopsis, Oligopeptide Transporter 1 (AtOPT1) is a major nitrate/oligopeptide transporter family. The Arabidopsis OPT3 is a phloem-specific iron transporter and its rice counterpart, OsOPT7, has been implicated in Fe loading into the phloem, the long-distance Fe transport, and loading to seed. OPT4 is involved in oligopeptide transport. Furthermore, Arabidopsis Oligopeptide Transporter 6 (AtOPT6) was reported to transport glutathione and a possible connection between prolonged Ca²⁺ deficiency, redox status, and increased iron transport is a likely scenario as suggested in a recent review. In this study, transcript levels of several transporters such as OPT1, EtNRAMP5, EtVIT5, and EtYSL2 were upregulated by several folds in the shoot during Ca²⁺ deficiency. Similarly, the VIT1, YSL16, NRAMP4, YSL9 and OPT4 were upregulated in both LCR vs. LCS and ConR vs. ConS. The transcripts of OPT1, NRAMP5, and OPT4 were also upregulated, while that of NRAMP1 and NRAMP2 were downregulated in the LCR compared to ConR. The maize ZmYS1 and rice OsYSL1 import Fe³⁺-phytosiderophore (PS) across the root cell membrane, while the rice OsYSY2 is implicated in the long-distance transport of Fe associated with a PS precursor, nicotianamine (NA). The role of YSL2 in Fe uptake and distribution has been studied previously in maize and Arabidopsis. The NRAMPs such as AtNRAMP3 and AtNRAMP4 function in transporting Fe out of vacuoles under Fe-limited conditions, while members of the VIT family mediate Fe transport into vacuoles. For example, overexpression of the wheat VIT2 under the control of an endosperm-specific promoter increases Fe content in white flour fractions of wheat. Some of these transporters are not specific to Fe but also transport other minerals, including Zn, Cd, Cu, and Mn. Taken together, differential regulation of several mineral transporters in response to Ca nutrition suggests the involvement of Ca²⁺-signaling in the homeostasis of these minerals. The decrease in root to shoot Fe translocation in this study due to Ca²⁺ deficiency (Fig. 2F) suggests the involvement of Ca²⁺ signaling in Fe homeostasis. Ca²⁺-signaling has been implicated in the homeostasis of minerals such as K⁺. In this regard, Gratz et al. reported that CIPK11-dependent phosphorylation modulates the activity of FIT (a transcription factor that regulates Fe acquisition), promoting Fe uptake in Arabidopsis. Ca²⁺ promotes the interaction of C2-Domain Protein Enhanced Bending1 (EBI) and the root Fe-regulated transporter IRT1, inhibiting Fe acquisition in Arabidopsis. However, further studies are needed to understand the crosstalk between Fe and Ca nutrition in Ca and Fe-rich cereals such as tef and finger millet.

Our study also revealed that several Ca²⁺-transporting channels were differentially expressed by Ca treatment. Our findings showed that transcripts of the CNGC, including CNGC2, CNGC4, and CNGC4-2, were more abundant in the shoots regardless of the Ca treatment (Fig. 5B). This may suggest that the CNGC may be involved.
in Ca²⁺ distributions in the shoots. The CNGC has lower ion selectivity permeating K⁺, Na⁺ and Ca²⁺ across the plasma membrane. The Arabidopsis genome consists of 20 members of the CNGC, and all the CNGC tested so far transport K⁺, while some members of the CNGC also translocate Ca²⁺. The Arabidopsis CNGC2 and CNGC4 are implicated in pathogen-induced Ca²⁺-signaling. The CNGC2 and CNGC4 have also been implicated in plasma membrane external ATP-activated Ca²⁺ influx in pollen tubes of Arabidopsis. As shown in Fig. 5C, GLR2.8 and GLR2.9 were significantly upregulated in ConR compared to ConS while GLR3.4-2, GLR2.7, GLR3.4-1 GLR2.4 and GLR3.1 were downregulated. Similarly, GLR2.8 and GLR2.9 were upregulated in LCR compared to LCS, while GLR3.4-1, GLR2.4, and GLR3.1 were downregulated. In Arabidopsis, the GLRs such as GLR 3.1, GLR 3.2, and GLR 3.3 GLR 3.6 have been reported to mediate long-distance Ca²⁺ signaling during wound responses. Heterologous expression of the rice OsGLR2.1 in mammalian cells resulted in Glu-triggered Ca²⁺ increase. Similarly, in the basal land plant mosses (Physcomitrella patens), it has been shown that GLR1 encodes a non-selective ion channel that is permeable to Ca²⁺. Upregulation of GLR2.8 and GLR2.9 in roots compared to shoots in this study may suggest their involvement in Ca²⁺ uptake in the roots. Upregulation of GLR2.8 in LCR compared to ConR may suggest its role in Ca²⁺ acquisition under Ca²⁺-deficient conditions. However, the GLRs which were upregulated in the shoots under both high and low Ca²⁺, such as GLR3.4-1, GLR2.4, and GLR3.1, may play a role in Ca²⁺ distribution in the shoots, which remains to be studied in the future.

In conclusion, we found that Ca²⁺ deficiency severely inhibits the growth of tef plants by disturbing the homeostasis of some minerals, including Ca, K, Fe, Mn, and Cu. Furthermore, our transcriptomic analysis revealed various genes, including those implicated in Ca²⁺-signaling, Ca²⁺-transporters, and Ca²⁺-permeable channels; Fe and metal transports which were differentially regulated by Ca²⁺ deficiency and/or between root and shoot tissues, suggesting their involvement in Ca²⁺ homeostasis. Heterologous expression and loss-of-function mutations studies of the DEGs identified in this study would elucidate their physiological functions in tef.

**Materials and methods**

**Plant materials and growth condition.** We recently performed seed mineral profiling of diverse tef (Eragrostis tef) accessions obtained from the USDA-GRIN germplasm collection. We used the accession with the highest seed Ca (1811 mg/kg) content in this study. Tef seeds were sterilized according to a method previously published with slight modifications. Briefly, seeds were soaked in 70% ethanol for one minute, followed by soaking in 1% NaOCl solution containing 0.1% Tween-20 for 10 min with vortexing every 1 min. Subsequently, the seeds were washed with autoclaved high purity water (18.2 MΩ (megohms)) five times. The seeds were then transferred to a petri-dish with a sterile wet filter paper disc. The plates were incubated in a growth chamber (Conviron, Winnipeg, Canada) for six days under 16 h/8 h day-night cycles at 28 °C. The seedlings were then transferred to a modified Hoagland hydroponic solution in which the Ca(NO₃)₂ was replaced with NH₄NO₃ to investigate the effect of Ca²⁺. Plants were grown in the media containing either 1 mM Ca²⁺ (optimum or control) or 10 μM (low Ca or Ca-deficient). The nutrient solution was continuously aerated and was renewed every 4 days.

**Plant phenotyping/photography.** Images were taken from the plants grown in a hydroponics system after ~28 days. Root and shoot dry weight biomass were measured at the end of the prolonged Ca²⁺ deficiency treatment. The measurements were taken for all replicates and mean values were calculated.

**Mineral element analysis.** One-month-old tef plants grown in 1/4-strength Hoagland’s hydroponics solution was analyzed. Roots and shoots were separated and rinsed three times with Milli-Q (18.2 MΩ) water. Before weight measurements, the tissues were oven dried at 65 °C overnight and ground to fine powder using a Waring Laboratory spice blender. Briefly, 500 mg of ground tissue was digested using concentrated HNO₃ for 30 min in a microwave at 200 °C, and mineral content was analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

**Library construction and RNA sequencing.** For RNA extraction, root and shoot samples were ground into powder under liquid nitrogen using a mortar and pestle. Total RNA was isolated using the GeneJET RNA purification kit following manufacturer’s procedure (Thermo Fisher Sci., Waltham, MA). The quality and quantity of the RNA were determined using a Nanodrop (Thermo Fisher Sci, Waltham, MA). mRNA-seq libraries were prepared using NEBNext® Ultra II RNA Library Prep Kit for Illumina (New England Biolab EB, Ipswich, MA) according to the manufacturer’s instructions. For each library, 500 ng total RNA was used. The mRNA-seq libraries were sequenced on an Illumina HiSeq platform using the 150 bp paired-end sequencing strategy.

**RNA-seq data analysis.** Data analysis for RNA-Seq was performed by the Bioinformatics Core associated with the Center for Human Health and Environment at North Carolina State University. Quality control of read data was briefly evaluated with FastQC, and alignment trimming was performed using sickle with the options –q 12 and –i 15 (version 1.33). Alignment was performed using hisat2 short read aligner to Tef reference genome downloaded from the CoGe Comparative Genomics website (V3.1, id59854). All default parameters were used except --very-sensitive and --summary-file options passed to the hisat2 aligner. The number of reads mapped to a genome feature was determined using htseq-count command line script from the HTSeq python package (-s reverse -a 10 -t exon -I Parent -m union) and count data was imported to the R statistical computing environment for further analysis. Initially, genes that have no count in at least 4 of the replicates samples were excluded from the analysis, and the remaining 28,796 features were used for downstream analysis. Data normalization based on dispersion and differential analysis were conducted using the DESeq2 package. We fitted a generalized linear model (~ Treatment) between the expression count. Finally, differential expression analysis was performed.
between ConR versus LCR, ConS versus LCS, Con R versus ConS, and LCR versus LCS, and differential expressions were identified by applying multiple testing correction using Benjamini-Hochberg procedure.

**Quantitative reverse transcription PCR (qRT-PCR).** To validate the RNA-seq data for representative genes, quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using a QuantStudio 3 real-time PCR system and the PowerUP™ SYBR Green pre-formulated 2 × master mix (Applied Biosystems, Foster City, CA). Total RNA was treated with DNase 1 (Invitrogen, Carlsbad, CA) to remove contaminating genomic DNA before first-strand cDNA synthesis. First-strand cDNA was synthesized using 5 μg of total RNA and the SuperScript™ III First-Strand Synthesis System (Thermo Fisher, Waltham, MA) following the manufacturer’s instructions. First-strand cDNA was used in the real-time qRT-PCR reaction containing 2xSYBR Green Master Mix, 500 nM forward, and reverse gene-specific primers in a 20 μL reaction volume. Gene-specific sense and antisense primers 5′-tttgctctggtgactctta-3′ and 5′-gcgactcgaggtgtgaatgaagag-3′ for GLR3.1, 5′-tcatctctagctgccaaggg-3′ and 5′-tctacgttgccgctttggt-3′ for GLC2, 5′-gcgaccaaggtttgacagag-3′ and 5′-atgtaaggccggaaacaaag-3′ for CNGC17, and 5′-tctgtaacaggcttg-3′ and 5′-tcatctctagctgccaaggg-3′ were used to amplify CaE1. The internal control, eukaryotic elongation factor (EF1A) was used as a housekeeping gene and amplified using sense 5′-cataaactctctgtgatacctcag and antisense 5′-gatgcctcagcctgtgatt-3′ primers. Gene expression in the untreated samples were used as a calibrator, and blank (without template) was used as a reference control. The qPCR parameters were as follows: 95°C for 30 s, 40 cycles of 95°C for 10 s, 60°C for 30 s, and a final incubation at 72°C for 5 min followed by melting curve analysis. Relative expression levels based on fold changes were calculated using the ΔACT method available with the QuantStudio3 software (Applied Biosystems, Foster City, CA, USA) and in MS-Excel (Microsoft Corp, Seattle, WA).

**Gene annotation.** Coding sequences (CDS) of *Eragrostis tef* were annotated by comparing it to the genome sequences of *Setaria italica* (GCF_000263155.2), *Oryza sativa* (Oryza sativa b, and *Sorghum bicolor* (GCF_00003195.3). The CDS of *E. tef* were compared to *S. italica*, *O. sativa*, and *S. bicolor* using Blastx. A CDS was considered significant with e-value <0.0001. Significant hits were first annotated by comparison to *S. italica* (25,876 significant CDS), then to *O. sativa* (873 additional CDS) and *S. bicolor* (483 additional CDS). A total of 94.6% of the CDS of *E. tef* had a significant hit to a CDS of these three genomes, and the corresponding gene annotations were used.

**GO analysis.** Go analysis was performed using the agriGO. Signature genes from the RNAseq analysis were used as an INPUT, and significantly enriched pathways and/or families were selected based on molecular function, cellular structure, and biological process category.

**Data analysis.** Treatments were replicated four times, and two independent experiments were conducted. Data were analyzed by the one-way ANOVA using the PROC GLM procedure in SAS. After the significant F-tests, the Tukey’s multiple comparisons were used to separate the means (P ≤ 0.05). Values represent mean ± SD of six (Figs. 1B,C) and four (Fig. 2) biological replicates.

**Statement on ethics and approvals to use tef.** Tef is primarily cultivated in East Africa and is becoming popular in several countries including South Africa, Australia, and the U.S. The tef accessions used in this study was obtained from Dr. John Cushman at the University of Nevada Reno. All methods are performed in accordance with relevant guidelines and regulations.

**Data availability** Sequence data files have been stored at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201043.

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Competing interests

The authors declare no competing interests.
