Functional characterization of the putative Theobroma cacao Iron Regulated Transporter 1 (IRT1) and its role in cadmium uptake from the soil

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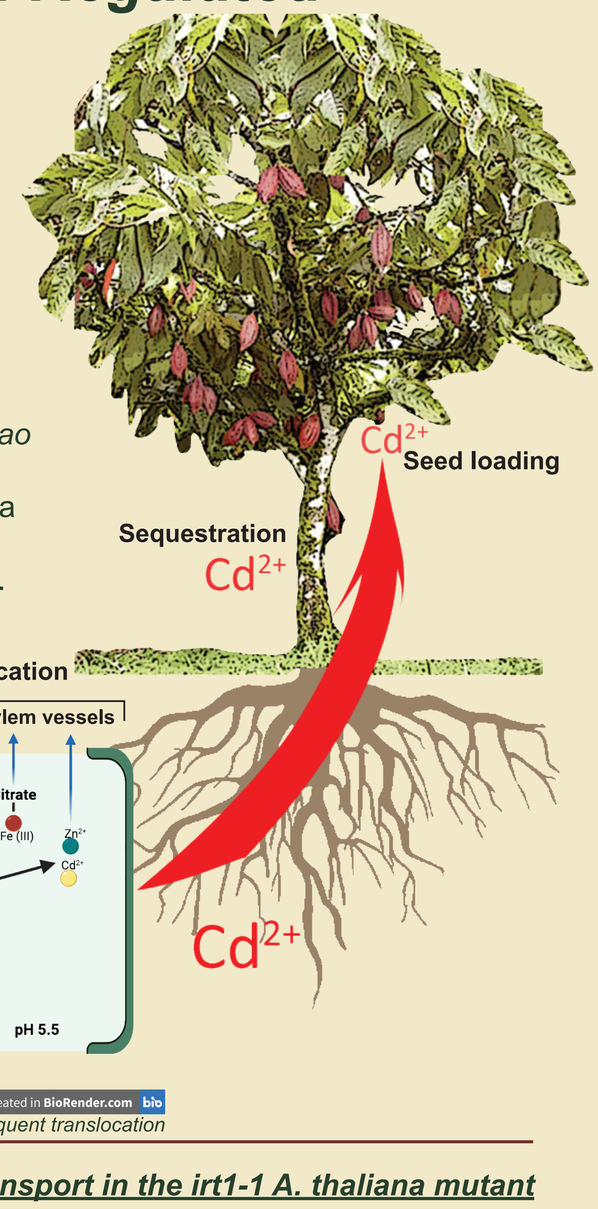

Figure 1. A diagram of the strategy I Fe uptake pathway and subsequent translocation

Cadmium limits cocoa farmers' ability to export

Problem: Cadmium (Cd) is a toxic heavy metal that accumulates in the edible portion of some plants including the seeds of *Theobroma cacao* (*T. cacao*). Cd exposure in humans predominately occurs from dietary sources. Extended exposure to Cd can elevate a person's health risk. To mitigate this, the European Union and other government bodies have enacted maximum permissible levels of Cd in foods, including cocoa products. Cocoa farmers' in Latin America and Caribbean countries have been most adversely affected by these regulations as cocoa from this region tends to have higher Cd concentrations and can exceed limits importers use to avoid violating regulations like (EU) NO 488/2014.

Cadmium enters via the iron uptake pathway

Background: Iron (Fe) is an essential plant micronutrient involved in biological processes like photosynthesis. Despite the abundance of Fe in soils, it is generally unavailable as ferric iron (Fe^{3+}). To overcome this, dicots rely on a reduction strategy (Strategy I), which is induced in Fe deficient conditions and is well characterized in the model species, *Arabidopsis thaliana* (Fig. 1). This process involves the Plasma Membrane Proton Atpase 2 - (AHA2) which effluxes protons, acidifying the soil; Ferric reduction oxidase 2 (FRO2) which reduces Fe^{3+} to Fe^{2+}, the form which the high-affinity Iron-Regulated Transporter 1 (IRT1) readily transports. IRT1 and functional homologs characterized in other plant species are not entirely specific to Fe and can mediate transport of Cd and other divalent cations. In several cases, Fe deficiency was observed to increase the accumulation of Cd, indicating that transport of Cd via the Fe pathway may be feature shared among dicots.

Results

T. cacao IRT1 (TcIRT1) is orthologous to other characterized IRT1s

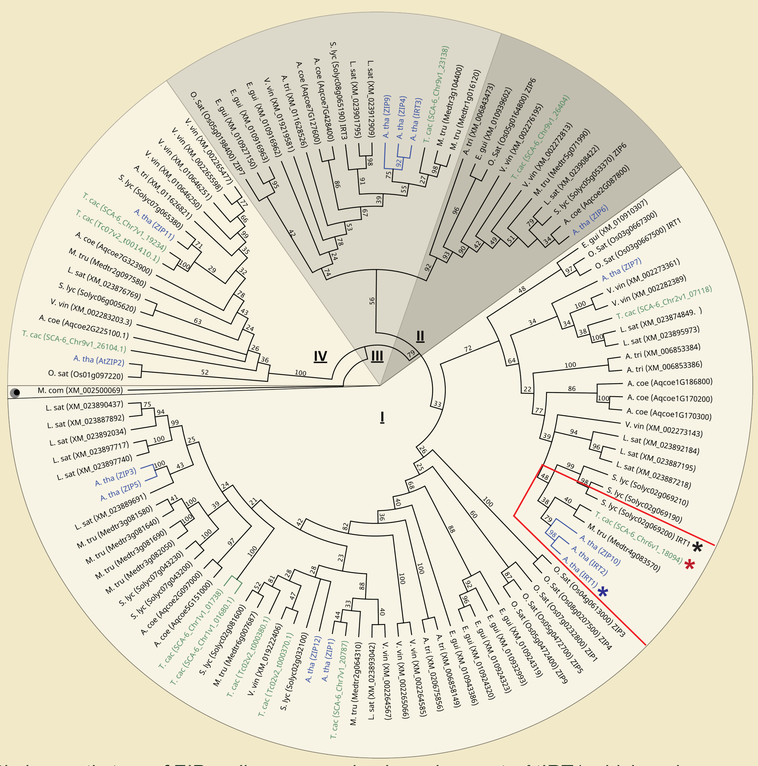

Figure 2. Phylogenetic tree of ZIP coding sequencing homologous to AtIRT1 which make up a representative reduced evolutionary phylogeny of plants including T. cacao. Alignments were made with Geneious MUSCLE codon alignment software and the tree was constructed using RAXML bootstrap (N=1,000). Asterisk represent TcIRT1 from T. cacao (red), A. thaliana IRT1 (blue), Solanum lycopersicum IRT1 (black).

TcIRT1 tissue-specific expression indicate functional homology to AtIRT1

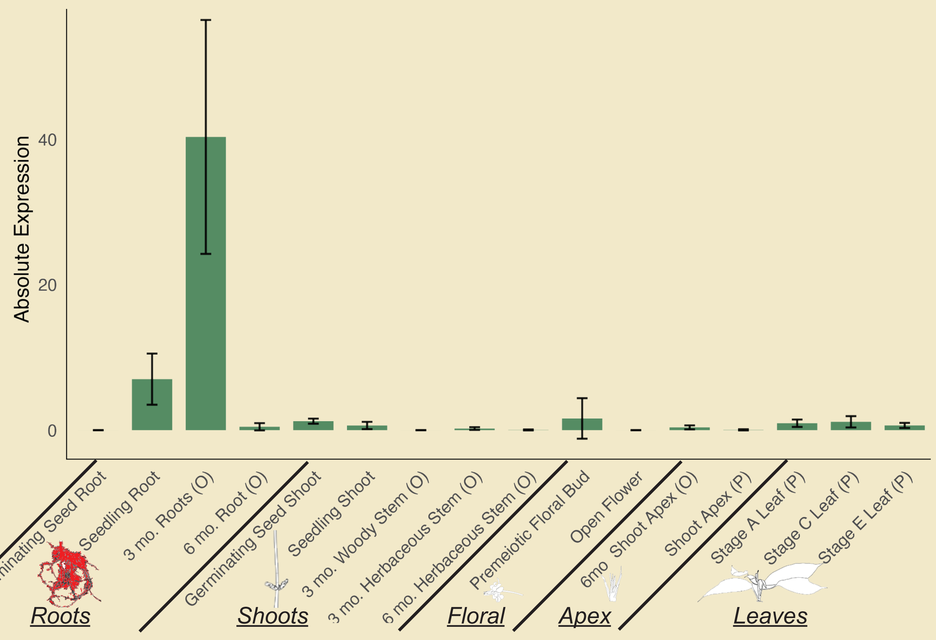

Figure 3. Expression levels of the putative TcIRT1 candidate gene grouped by tissue types. Tissue specific expression was collected from the Cacao Developmental Atlas found in the eFP Browser (University of Toronto). The highest absolute gene expression value was found in 3-month-old orthotropic roots with little to no detectable levels in any of aerial tissues. Orthotropic (O) and plagiotropic (P).

TcIRT1 mediates Fe, Zn, Mn transport in yeast and Fe transport in the irt1-1 A. thaliana mutant

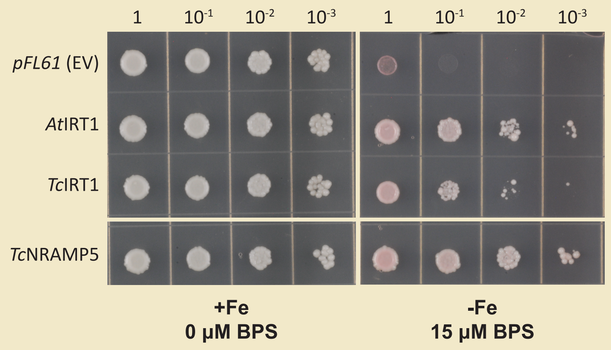

Figure 4. Fe-transport-deficient yeast (Δfet3/fet4) transformed with the pFL61 vector containing AtIRT1, TcIRT1, TcNRAMP5 or nothing (empty vector (EV)). Ten-fold dilutions (left to right) are made to visualize individual colonies. All constructs grow equally as well in the lowest dilution (rightmost) when on Fe sufficient media (left). Growth is severely inhibited in the yeast mutant with pFL61 (EV), whereas yeast expressing Fe transporters grow similar to Fe sufficient conditions.

Figure 5. The A. thaliana irt1-1 mutant is unable to grow in soil without copious Fe fertilizer. Transgenic irt1-1 mutants expressing AtIRT1 driven by the AtIRT1 promoter restores growth to the same levels as the mutant background (Ws). TcIRT1 is also able to rescue the mutant phenotype indicating its functionally homologous to AtIRT1.

TcIRT1 mediates Cd transport resulting in increased Cd sensitivity in yeast and A. thaliana

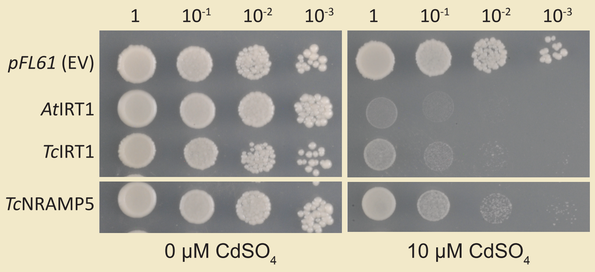

Figure 6. Wild type yeast are insensitive to Cd below 20 µM as observed by the lack of change in growth between the pFL61 empty vector (EV) yeast grown on the 0 µM Cd control (left) and with 10 µM Cd (right). Expression of AtIRT1, TcIRT1, and TcNRAMP5 increase the sensitivity to Cd at this concentration due to their ability to mediate Cd transport.

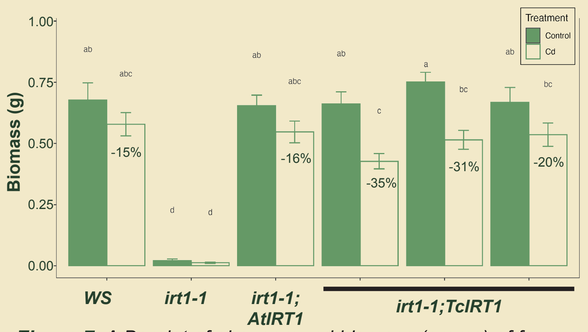

Figure 7. A Barplot of aboveground biomass (grams) of four-week-old A. thaliana plants grown in soil (control) or 10mg Cd/kg soil (Cd). Bars with different letters are significantly different from one another ($P<0.05$) as determined using Tukey's HSD test.

Conclusions and future directions

TcIRT1 contributes to the uptake and accumulation of Cd

Based on our cumulative results, we believe that TcIRT1 is involved in the root uptake of divalent cations including Cd from the soil. This conclusion is supported by our findings, which show that TcIRT1 expression is root specific and that when TcIRT1 is expressed in heterologous systems it can mediate Fe, Mn, Zn, and Cd transport.

Clarification about Cd uptake in T. cacao and TcIRT1's involvement in that process is an important first step in understanding ways to manage and reduce it. However, some outstanding questions remain; **1)** Is TcIRT1 regulated by Fe like it is in A. thaliana; **2)** If it is, could managing soil levels of Fe or competitive divalent cations potentially at toxic concentrations relieve Cd accumulation? **3)** Does modifying expression of TcIRT1 influence the metal profile of T. cacao?

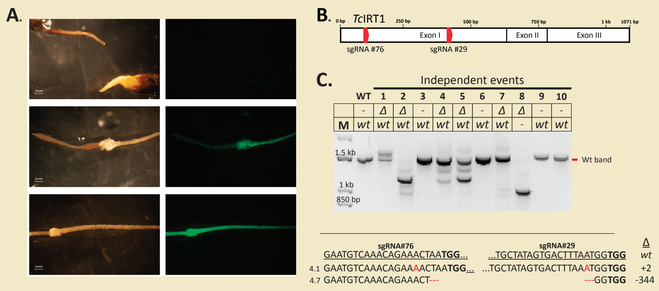

Figure 8. Panel A: Bright field (left) and eGFP filter (right) root images from a non-transformed T. cacao embryo (top), R. rhizogenes transformed hairy root with the empty vector containing an eGFP marker (middle), TcIRT1-CRISPR construct in the same vector (bottom). Panel B: coding sequence model of TcIRT1 with sgRNA sites (red). Panel C: Cleaved amplified polymorphic sequence screening in hairy roots transformed with the TcIRT1-CRISPR construct.

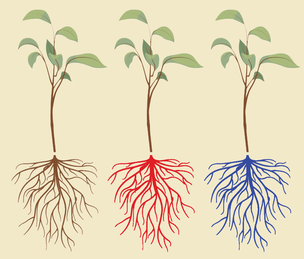

Figure 9. Diagram of composite plants with root systems comprised of R. rhizogenes transgenic roots co-transformed with the empty vector control, 35S::TcIRT1 for overexpression, or RNAi-TcIRT1 for silencing.

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