OsACL-A2 is involved in iron transport and positively regulates iron dependent disease resistance

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Abstract

Iron is an essential nutrient required for growth, development and also impacts disease resistance in plants. Plant absorb iron through their roots and citrate plays a vital role in transport of Fe³⁺ through the xylem. In this study, we found that the rice cytoplasmic ATP-citrate lyase subunit A2, OsACL-A2 (Os12g0566300) serves as a crucial factor in iron uptake and translocation. Leaf iron levels were depressed in the osacl-a2 mutant which caused iron deficiency induced chlorosis, activated defense signaling and eventually necrosis in mature leaves. Furthermore, loss of OsACL-A2 activity reduced disease resistance in leaves that had not reached development of necrosis. Citrate levels are increased in the osacl-a2 mutant, while ACL activity in the wild type is upregulated in response to both iron starvation and pathogen attack. We hypothesize that OsACL-A2 activity generates source-sink gradients through targeted lysis activity thus directing the movement of citrate-Fe³⁺.

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ABSTRACT

Iron is an essential nutrient required for growth, development and also impacts disease resistance in plants. Plant absorb iron through their roots and citrate plays a vital role in transport of Fe$^{3+}$ through the xylem. In this study, we found that the rice cytoplasmic ATP-citrate lyase subunit A2, OsACL-A2 (Os12g0566300) serves as a crucial factor in iron uptake and translocation. Leaf iron levels were depressed in the osacl-a2 mutant which caused iron deficiency induced chlorosis, activated defense signaling and eventually necrosis in mature leaves. Furthermore, loss of OsACL-A2 activity reduced disease resistance in leaves that had not reached development of necrosis. Citrate levels are increased in the osacl-a2 mutant, while ACL activity in the wild type is upregulated in response to both iron starvation and pathogen attack. We hypothesize that OsACL-A2 activity generates source-sink gradients through targeted lysis activity thus directing the movement of citrate-Fe$^{3+}$.

Keywords: Rice, Iron translocation, OsACL-A2, Disease resistance.

INTRODUCTION

Iron is an essential element that plays crucial roles in key processes including photosynthesis, respiration, DNA synthesis, cell division, and nitrogen metabolism by acting as a co-factor to cell pigments, hemoglobin, ferritin, and various enzymes (Camprubi et al., 2017). During photosynthesis, iron is indispensable in the electron transport chain and mutations in iron homeostasis genes such as OsPRI2/3, OsLFNR1 or OsFIT impair photosynthesis causing to iron deficiency induced chlorosis (Zhang et al., 2020; Da et al., 2023; Liang et al., 2020). Under iron-deficient conditions, both the roots and aboveground parts of pri2 and pri3 mutants exhibit significant shortening, with the aboveground parts showing chlorosis. (Zhang et al., 2020). The last step of the photosynthetic electron transfer chain involves an important iron-containing protein called NADP$^+$ oxidoreductase, specifically OsLFNR1 (Da et al., 2023; Yang et al., 2016). OsFIT is an iron-deficiency-induced bHLH transcription factor. Under normal iron supply conditions, theosbhhlh156 mutant does not significantly differ from the wild type. However, under iron deficiency, the mutant manifests obvious chlorosis symptoms, decreased SPAD values in leaves, decreased iron content in the aboveground parts, and inhibited plant growth (Liang et al., 2020).

Interestingly, while iron deficiency restricts photosynthesis and thus plant growth, iron deficiency also activates plant defense responses (Herlihy et al., 2020). Localized iron deficiency may result from invading pathogens siphoning iron. In response plants sequester iron from the pathogens. During the battle against pathogen invasion, Arabidopsis thaliana identifies the effector protein avrRps4 via EDS1 and RPS4, suppressing plant iron uptake by inhibiting its interaction with the BTS protein. This leads to a decrease in extracellular iron accumulation (Xing et al., 2021). Plants secrete the protein PDF1.1 into the extracellular matrix, where it can bind iron in an unusable form. Consequently, intracellular iron deficiency signals are triggered, prompting plant immune responses (Hsiao et al., 2017). Ironically, iron accumulation near the infection site is part of the defense response, which involves generation of reactive oxygen species (ROS) through processes such as the Fenton reaction (Ye et al., 2014; Liu et al., 2007). For example, in the rice-blast disease system, trivalent iron accumulates at the site of pathogen attack. The excessive iron accumulation inhibits pathogen growth through the burst of ROS generated by Fenton reactions (Dangol et al., 2019; Lianget al., 2021). Conversely, in the Arabidopsis-D. dadantii pathogen system, strong iron accumulation occurs in healthy areas surrounding infected tissues. Low iron content is closely associated with the localization of bacterial cells in infected tissues, indicating that Arabidopsis adopts an iron-restriction strategy to resist D. dadantii infection (Aznar et al., 2014). How iron is transported to or from sites of infection remains unknown.

The current knowledge of iron transport mechanisms in plants has developed through the study of root to shoot iron translocation. In rice, two uptake models have been proposed for uptake of soluble Fe$^{2+}$ and insoluble Fe$^{3+}$ respectively (Palmgren et al., 2001; Marschner et al., 1994; Nozoye et al., 2011). The first strategy involves root tip epidermal cells secreting hydrogen ions, which acidify the surrounding soil.
This acidification reduces Fe$^{3+}$ to Fe$^{2+}$, facilitating iron absorption and subsequent transport (Santi and Schmidt., 2009; Brumbarova., 2015). Another strategy is the synthesis of plant iron carriers (PS) within rice plants. These carriers are then released into the roots to form Fe$^{3+}$-PS chelates, which are transported into the cells. Insoluble Fe$^{3+}$ is the most abundant form and uptake is dependent on carrier molecules (Nozoye et al., 2011). Various organic chelators are released from the root hair zone and scavenge mineral ions in the neighboring soil bringing them before the endodermal barrier (Takagi et al., 1984; Mori and Nishizawa et al., 1987). At the rice endodermis, iron is loaded into the xylem primarily as a citrate-Fe$^{3+}$ complex by the citrate membrane transporter OsFRDL1 (Wu et al., 2018; Yokosho et al., 2009, 2016). Citrate has been demonstrated to be an important iron carrier in many crop species for example tomato, peanut, rapeseed and sugar beet (Müller et al., 2019; Solti et al., 2012). Studies on rapeseed chloroplasts have shown that the chloroplasts more effectively utilize citrate-Fe$^{3+}$ salts rather than Fe-nicotinamide complexes (Müller et al., 2019). In support of this, mutants of OsFRDL1 in rice or the citrate efflux protein AtFRD3 in Arabidopsis result in impaired iron trafficking to the shoot and overaccumulation in the roots (Scheepers et al., 2019; Green et al., 2004; Rogers., 2002). The mechanism of how citrate-Fe$^{3+}$ is directed towards target locations has yet to be elucidated.

Existing research has demonstrated that OsACL-A2 can interact with histone acetyltransferase HAG704, contributing to the establishment and maintenance of acetyl coenzyme A concentrations in specific genomic regions. Consequently, this interaction regulates histone acetylation and cellular processes (Xu et al., 2023). We characterized a loss of function osacl-a2 mutant and found a clear decrease in iron content despite overall increased citrate levels. Furthermore the iron deficiency was sufficient to both activate defense responses and cause spontaneous cell death. We hypothesize that OsACL-A2 may drive iron movement through generation of citrate source-sink gradients by differential lysis of citrate at sink sites.

**METHODS**

**Plant Materials and Growth Conditions**

The osacl-a2 mutant was identified by map based cloning from an EMS library of the Indica rice variety ZH8015 (Figure S1). All field plants were grown under standard field conditions at the Fuyang Experimental Base of China National Rice Research Institute in Hangzhou, China and the Lingshui Experimental Base in Hainan, China. Hydroponically grown plants were germinated by soaking at room temperature for two days, 37°C for one day, and then sown in 96-well hydroponic trays. For the first week plants were grown in double-distilled water, followed by 2 weeks in modified Yoshida media (1.25mM NH$_4$NO$_3$, 0.32mM NaH$_2$PO$_4$, 0.3mM K$_2$SO$_4$, 1mM CaCl$_2$, 1mM MgSO$_4$, 9μM MnCl$_2$-4H$_2$O, 0.52μM (NH$_4$)$_6$MoO$_{24}$·4H$_2$O, 18μM H$_2$BO$_3$, 0.77μM ZnSO$_4$·7H$_2$O, 0.32μM CuSO$_4$·5H$_2$O, 50μM FeCl$_3$·6H$_2$O, pH 5.8-6) changed every three days (Sánchez-Saumy et al., 2022). Plants were grown in growth chambers with a 14h 30°C/10h 25°C day/night cycle. For iron treatments, the Fe concentration was adjusted to 0 or 500μM for deficient and excessive iron treatments respectively.

**Map based cloning**

To investigate the molecular mechanism controlling the phenotype of the osacl-a2 mutation, we conducted a cross between the mutant osacl-a2 and the japonica rice variety 02428. In the F1 generation, all individuals displayed a phenotype similar to that of the wild type. Selfing of the F1 generation resulted in the F2 generation, where the phenotypes of both the wild type and the mutant followed the expected Mendelian segregation ratio of 3:1 (Table S2). To map the mutation gene, we performed linkage analysis using polymorphic simple sequence repeat (SSR) markers distributed on the 12 chromosomes. Genotyping of 126 recessive plants allowed us to initially locate the mutation locus between two markers, RM519 and RM6306, on the long arm of chromosome 12. For fine mapping, we developed six InDel markers and expanded the population to 763 recessive plants for genotyping. This allowed us to narrow down the mutation locus between markers InDel1296 and 12105.

We further investigated the region and identified eleven predicted open reading frames (ORFs) according to the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.edu). Through further analysis,
we discovered a single base substitution from G to A at the ninth exon of the third ORF, \textit{LOC}\textsubscript{Os012g37870}. This substitution resulted in an amino acid change from glycine to aspartic acid. (Primer sequences are listed in Table S1). To confirm whether the mutation in the gene \textit{LOC}\textsubscript{Os012g37870} is responsible for the spotted phenotype, we constructed complementation and overexpression plasmids. The complementation plasmid contained an 8357-bp sequence which included the entire genomic DNA sequence of the gene, along with 2547-bp upstream and 1282-bp downstream sequences. The overexpression plasmid contained the full-length coding DNA sequence (CDS) of \textit{OsACL-A2}. We introduced these two plasmids into both the wild type and \textit{osacl-a2} mutant to generate two transgenic lines for functional validation.

**ROS related measurements**

ROS related measurements was done using commercial kits following the provided instructions (Nanjing Jiancheng Bioengineering Institute). Briefly, 100mg of leaves were ground in 1ml of PBS. After centrifugation at 3500 rpm for 10 minutes the supernatant was collected. The samples were then measured for the following parameters following respective kit instructions; total soluble protein (A045-2-2), H\textsubscript{2}O\textsubscript{2} (A064-1-1), catalase activity (A007-1-1), peroxidase activity (A084-3-1), superoxide dismutase activity (A001-1-2) and malondialdehyde (A003-1-2). Three biological reps were used for each measurement.

**Evans Blue and DAB staining**

Evans Blue and 3,30-diaminobenzidine (DAB) staining was done as previously described (Cui et al., 2019). Briefly, submerge the leaves in a 10% Sodium Dodecyl Sulfate (SDS) solution for 10 minutes to eliminate the waxy layer. Thoroughly rinse the leaves with water thereafter. Next, immerse the leaves in a solution containing 1mg/mL Diaminobenzidine (DAB) and Evans Blue, ensuring complete coverage, and allow them to incubate in darkness for 6-12 hours. Discard the staining solution and eliminate chlorophyll by placing the leaves in a boiling water bath with 95\text% ethanol.

**RNA extraction and qRT-PCR**

RNA extraction was done with commercial kits following the provided instructions (DP432, Tiangen). RNA samples were reverse-transcribed using the ImProm-II Reverse Transcription System (Promega). qRT-PCR was performed using the GoTaq\textsuperscript{®}qPCR Master Mix (Promega) on a Light-Cycler 480 instrument (Roche Applied Science). qPCR used the following cycle: 95 for 5 min, followed by 45 cycles of 95 for 10s, 58 for 10s, and 72 for 15s. Values were normalized using UBQ10 as reference. The 2\textsuperscript{-}\textit{t} method was used to determine relative expression level. All primers used for qRT-PCR were listed in Table S1.

**Subcellular localization assay**

Transient expression in tobacco and rice protoplasts was done as previously described (Yan et al., 2012). Briefly, OsACL-A2-GFP was generated by insertion of the full length CDS into the EcoRI site of pYBA-1132. For transient expression in tobacco the plasmid was introduced into agrobacterium strain GV3101. Fluorescence signals of the rice protoplasts at 48 hours post-transformation and tobacco leaf infiltrations at 72 hours post-infection were observed using a LSM 700 confocal laser scanning microscope (ZEISS). The primers used for vector construction are listed in Table S1.

**ACL activity measurement**

ACL activity was measured using commercial kits following the provided instructions (G0817W, Suzhou Grace Biotechnology). In brief, 0.1g of leaves was ground in 1ml of ice cold PBS and then centrifuged at 12,000rpm for 10 minutes at 4. The supernatant was then collected for measurement. Three biological reps were used for each measurement.

**Citric acid content measurement**

Citric acid was measured using commercial kits following the provided instructions (QYS-233004 Shanghai QiYI Biotechnology). In brief, 0.1g of leaves was ground in 1ml of ice cold PBS and then centrifuged at
12,000rpm for 10 minutes at 4. The supernatant was then collected for measurement. Three biological reps were used for each measurement.

**Blast resistance assay**

Rice blast resistance assays were done as previously described using *Magnaporthe oryzae* strains 14-1 (Liu et al., 2017). *M. oryzae* was grown on oatmeal media at 25°C for 10 days in the dark. The mycelium was then washed off with a 0.01% concentration of Tween20, and impurities were filtered out using gauze. The spore count was adjusted to approximately 20-30 spores under a 10x magnification microscope. For spray method, when the rice seedlings reached the three-leaf stage, the washed spore suspension was evenly sprayed onto the leaves using a spray bottle, ensuring that no water droplets formed. The treatment was conducted in a dark environment at 28°C for 24 hours, followed by a return to normal light conditions (12 hours light, 12 hours dark) while maintaining humidity at 80%. Disease development could be observed after approximately 7 days. Samples were taken at different time intervals (0h, 6h, 12h, 24h, 36h, 48h, 72h, and 96h) during this period for RNA extraction. For punch method, Rice plants cultivated in the greenhouse for approximately 60 days were selected. The leaves were injured using a hole puncher, creating a circular wound. Then, 10μL of the washed rice blast fungus suspension was carefully pipetted onto the wound, which was sealed with transparent tape to prevent the liquid from drying out. Disease development was promoted by keeping the plants in the greenhouse. After around 8-14 days, the area of the lesions was measured. At least 40 plants were used per test.

**Blight resistance assay**

Bacterial blight resistance assays were done as previously described using *Xanthomonas oryzae* strains CR4, PXO86 and PXO96 (Liu et al., 2017). *X. oryzae* was grown on Potato Dextrose Agar (PDA) 7 days. Wash the bacterial suspension with PBS for inoculation. We performed leaf clip inoculation with 10 fully expanded leaves during the heading stage of the plants. After 14 days of inoculation, we measured the length of the lesions. The average lesion length on the leaves was compared and analyzed, and the disease phenotype of the leaves was scanned and recorded.

**Brown leaf spot resistance assay**

Brown leaf spot resistance assays were done as previously described using *Xanthomonas oryzae pv Oryzicola* (Xiao et al., 2017). *X. oryzae* was grown on Nutrient Agar (NA) 7-10 days. Wash the bacterial suspension with PBS for inoculation. Inject the bacterial suspension into the leaves while avoiding the main veins using a syringe. After 14 days of inoculation, we measured the length of the lesions. The average lesion length on the leaves was compared and analyzed, and the disease phenotype of the leaves was scanned and recorded.

**Leaf shading experiment**

Shading experiments were conducted as reported (Ruan et al.). Aluminum foil was wrapped around mature leaves of field grown plants at the tillering stage. After one week, the samples were collected in replicates of five.

**Chlorophyll content measurement**

Chlorophyll measurement was done as reported by (Cui et al). Briefly, 100 mg of leaves were extracted in 10ml of 80% methanol under darkness for 24 hours. The extracts were measured at wavelengths of 470, 645, and 663nm using a DU800 UV-Visible spectrophotometer (Beckman, California, USA). All experiments were performed in triplicate, and the average values of the three replicates were reported.

**Transmission electron microscopy**

Leaf segments were fixed with 2.5% glutaraldehyde in sodium phosphate buffer (pH 7.2) for 4 h at 4°C. Ultrathin samples were made as previously described (Zhou et al., 2013), and viewed using a transmission electron microscope.

**Statistical analysis**
Data was compared using Student’s *t*-test or one-way analysis of variance (ANOVA). Calculations and graphs were done using GraphPad Prism 8.0. Variance and p-values used are stated in individual figure legends.

RESULTS

ATP-citrate lyase mutant *osacl-a2* is a lesion mimic in *Indica* rice

Lesion mimic mutants spontaneously undergo cell death producing a phenotype reminiscent of the hypersensitive response induced by disease resistance (Cui et al., 2021). To identify potential resistance genes, we screened an EMS library constructed in the *Indica* rice variety ZH8015 for lesion mimic mutants. We mapped a lesion mimic mutant to the cytoplasmic ATP-citrate lyase subunit A2 (*OsACL-A2*, LOC:Os012g37870) we named *osacl-a2* which harbored a point mutation changing glycine to aspartic acid at position 378 (Figure 1A). Lesion formation in *osacl-a2* was suppressed by complementation or overexpression with the wild type allele, indicating that *osacl-a2* was both casual and recessive (Figure 1B-C, Figure S1). Lesions in *osacl-a2* first appeared in older leaves at the seedling stage and continued to accumulate as the plant matured (Figure 1D-G). Compared to the wild type, the mutant *osacl-a2* exhibited reduced ATP-citrate lyase activity and increased citrate content in mature plants, indicating that *osacl-a2* was a loss-of-function allele (Figure 1H-I).

Previously, Ruan *et al.* (2019) had reported an *OsACL-A2* lesion mimic mutant in the *Japonica* rice variety Nipponbare that displayed red-brown lesions, heightened ROS content, activated defense responses, and augmented resistance to bacterial leaf blight; we found similar results in mature *osacl-a2* plants (Figure S2-S4). Additionally, *osacl-a2* was also more resistant to rice blast at the heading stage (Figure S4). Thus, loss of *OsACL-A2* ATP-citrate lyase activity resulted in a lesion mimic phenotype in rice.

Lesion formation in *osacl-a2* is due to iron deficiency

We considered possible causative reasons behind *osacl-a2* lesion formation. Based on previous literature, we initially believed that the lesions resembled iron toxicity induced necrosis (Dangol *et al.*, 2019; Conrad *et al.*, 2018; Distéfano *et al.*, 2021). Cytoplasmic ATP-citrate lyase catalyzes lysis of cytoplasmic citrate, unrelated to the mitochondrial TCA cycle, and cytoplasmic citrate has been shown to chelate insoluble Fe$^{3+}$ which functions in both uptake and intercellular transport of iron (Yokosho *et al.*, 2009, 2016). We hypothesized that decreased citrate lyase activity led to increased citrate levels and would heighten cellular iron to toxic levels. Measurement of plant iron content immediately refuted this as iron levels in *osacl-a2* were lower than the wild type (Figure 2A-B). Instead it appeared that iron deficiency could be responsible for *osacl-a2* lesions as necrotic leaf spots formed under severe iron deficiency have been reported before (Kobayashi *et al.*, 2021; Li *et al.*, 2022).

To test whether *osacl-a2* lesions was due to iron deficiency, we tested the effect of growing rice seedlings in hydroponic solutions with different iron content: deficient (0 uM), sufficient (50 uM), and excessive (500 uM). Compared to plants grown under normal iron, *osacl-a2* lesion formation in mature leaves was strongly enhanced by transfer to iron deficient media while transfer to excess iron media suppressed lesion development (Figure 2C). Iron deficiency in rice is often accompanied by characteristic transcriptome changes as the plant reconfigures its iron strategy (Zhang *et al.*, 2017). The bHLH transcription factor *OsIRO2* is a key iron uptake regulator that is transcribed in response to iron starvation in grasses (Liang *et al.*, 2020). Iron starvation upregulates transcription of iron transporters such as *OsYSL2* (Ishimaru *et al.*, 2010). Ferritin proteins store cellular free iron in large complexes which also mitigates potential iron toxicity; correspondingly transcription of *OsFER2* positively correlates with iron status (Nguyen *et al.*, 2022). Complementing previous studies, *OsIRO2* and *OsYSL2* were strongly upregulated in the wild type in response to iron starvation, and both transcripts were high in *osacl-a2* under normal conditions (Figure 2D). *OsFER2* transcript levels in the wild type decreased in response to iron starvation, whereas transcript levels in *osacl-a2* did not significantly change. Other iron status marker genes were also tested but some responded erratically in ZH8015 (Figure S5). Insoluble Fe$^{3+}$ uptake by citrate chelation is highly dependent on the citrate transporter *OsFDRL1* which is transcribed in response to iron deficiency (Wu *et al.*, 2018). Interestingly while *OsFDRL1* transcript
levels in the wild type behaved as expected, OsFDRL1 levels were lower in osacl-a2 and furthermore did not increase in response to iron starvation (Figure 2D). Perhaps the higher citrate content in osacl-a2 negatively regulates OsFDRL1 transcription. Taken together, iron status marker genes suggest osacl-a2 is normally akin to the wild type under iron starvation. Furthermore, osacl-a2 displayed stronger iron deficiency induced chlorosis accompanied by reduced chlorophyll pigments when grown in iron deficient medium (Figure 3A-B). Excess iron on the other hand also suppressed the defence gene expression observed in osacl-a2 grown under normal iron conditions (Figure 3C). These results suggest that osacl-a2 suffered from iron deficiency under normal growth conditions which in turn induced lesion formation in mature leaves.

Citrate can also chelate other metal ions and may directly or indirectly affect their uptake (Rauser et al., 1999). We noticed a reduction in Mn, Zn, Cu and Mg levels in osacl-a2 plants, but neither starvation or excess tests had an obvious effect on lesion formation suggesting that this phenotype is specific to iron deficiency (Figure S6). In conclusion, osacl-a2 plants normally suffers from iron deficiency at a level sufficient to spontaneously form lesions in mature rice leaves.

OsACL-A2 contributes to iron uptake through source-sink dynamics

Since osacl-a2 caused iron deficiency under normal conditions we asked, how does OsACL-A2 affect iron uptake? As stated before Fe\(^{3+}\) chelation by citrate is a known iron uptake pathway (Yokosho et al., 2009, 2016). Thus it is sensible that iron starvation would increase citrate levels and vice versa with excess iron; indeed this was the case in wild type mature seedling leaves (Figure 4A). Yet despite possessing higher levels of citrate than the wild type, the iron content of osacl-a2 was lower (Figure 2A-B). Since citrate quantity could not explain the lower iron content of osacl-a2, we next measured whether ACL activity changed in response to iron treatment. Surprisingly, ACL activity increases in response to iron starvation in the wild type and slightly decreases with excess iron (Figure 4B). The iron starvation induced ACL activity appears to be primarily due to OsACL-A2 as there is no activity change in the mutant. Taken together, this suggests that increased OsACL-A2 activity rather than steady-state citrate levels may be important for iron uptake.

To explain the mechanism of OsACL-A2 in iron uptake we hypothesized that ACL activity may act through source-sink dynamics. Dynamic removal of molecules in sink cells maintains the concentration gradient necessary to drive solute movement from source cells (Jeong et al., 2017). Previous studies have shown that the vast majority of iron in the shoots is sent to the chloroplasts, where iron is a necessary cofactor for several pathways, most notably photosynthesis (Wang et al., 2020). Thus photosynthetic cells in shoot tissue are the major iron sink, while the primary source of iron comes from root absorption (Jeong et al., 2017). Iron absorption mainly occurs in the root hair zone and travel upwards by citrate-Fe\(^{3+}\) shuttles in the xylem before unloading into shoot phloem cells for redistribution. Our source-sink hypothesis predicts that OsACL-A2 should be relatively more active in sink locations. In agreement, transcript measurements show a greater abundance of OsACL-A2 in shoots relative to roots (Figure 4C), providing some support that OsACL-A2 is at least differentially expressed in sink vs source cells.

OsACL-A2 positively regulates rice resistance through free iron

Our experiments linked the biological role of OsACL-A2 to iron uptake. Free iron plays a pivotal role in plant disease immunity as such mutants defective in iron level or movement impair disease resistance (Nguyen et al., 2022). The enhanced disease resistance observed in heading stage osacl-a2 mutants seemingly contradicts the positive role OsACL-A2 plays in seedling iron uptake. Therefore we wondered whether OsACL-A2 functions differently between disease resistance and iron uptake.

We retested the blast resistance of hydroponically grown osacl-a2 seedlings after transfer to different iron solutions (Figure 5A-D). Wild type young leaves were almost disease free yet osacl-a2 developed obvious infection spots (Figure 5A,C). Disease resistance in young leaves positively correlated with iron supplementation in both wild type and mutant seedlings. In contrast, disease development in mature leaves progressed rapidly (Figure 5A,D). As with young leaves, iron starvation decreased wild type resistance while excess iron enhanced it. However osacl-a2 responded differently; resistance increased under iron starvation while excess had little effect. The bioassay results for biomass determination of the rice blast fungus also demonstrated...
the same trend. (Figure 5E-F). They key difference between young and mature *osacl-a2* leaves is that spontaneous lesions had already formed in the latter, and as noted before was more severe with iron deficiency. Our interpretation of these results is that older leaves however develop lesions due to iron deficiency and the enhanced “disease resistance” of *osacl-a2* is a side-effect of lesion formation rather a negative resistance regulator function of OsACL-A2. In young leaves that have yet to develop lesions, *osacl-a2* resistance is likely impaired due to iron deficiency. Therefore we suggest that OsACL-A2 plays a positive role in iron dependent disease resistance.

The role of OsACL-A2 in iron dependent disease resistance could simply be transporting sufficient levels of iron to the leaves but it might also play an active part in gathering iron at sites of incursion. In wild type leaves transcription of *OsACL-A2* increases 72h post inoculation with blast (Figure 6A). Complementing this the ACL activity in challenged leaves sharply increases between 72 and 96h post inoculation (Figure 6B). Once again, steady-state citrate content showed no particular correlation with ACL activity (Figure 6C) suggesting an alternative purpose for heightened ACL activity.

Our results suggest that OsACL-A2 positively regulates iron dependent disease resistance.

**DISCUSSION**

Iron is an essential element for nearly all living organisms on earth. Iron serves as a cofactor in several key reactions of vital processes such as photosynthesis and respiration (Camprubi et al., 2017). As such plants need to continuously absorb iron as they grow while avoiding dangerous free iron levels (Awasthi et al., 2019). Accordingly, iron uptake is adjusted in response to both internal and environmental availability (Jeong et al., 2017). We too observed that citrate levels negatively correlated with iron availability, but unexpectedly so did ATP-citrate lyase activity. Considering that the *osacl-a2* mutant is impaired in iron uptake, we suggest that ACL plays a role in iron movement, hypothetically through source sink gradients (Figure 5). OsACL-A2 mediated lysis of citrate-Fe$^{3+}$ complexes at sink sites could release iron while helping to maintain a citrate concentration gradient. Supporting this concept, *OsACL-A2* transcripts were more abundant in leaves than roots. This would suggest that ACL or at least OsACL-A2 activity would be relatively lower in source cells, primarily in the root hair zone, compared to sink cells in the shoot, primarily photosynthetic tissue (Jeong et al., 2017). Exploring the impact of changes in ACL enzyme activity in different tissue sites on the flow of iron from source to sink may be of future interest.

Regardless of the precise mechanism the outcome of loss of *OsACL-A2* is iron deficiency induced cell death. Normally, iron deficiency causes gradually worsening from chlorosis towards cell death (Zhang et al., 2020). However, mature leaves of the *osacl-a2* mutant rapidly develops necrotic lesions in response to iron starvation prior to visible symptoms appearing in the wild type. This indicates that photosynthetic leaves are highly vulnerable to the disruption of iron influx. Recent reports have shown that iron deficiency induced chlorosis is the result of an active plant response involving downregulation of photosynthesis and is cross-regulated by the status of other mineral nutrients; i.e. low Mn, S, or P inhibits iron deficiency induced chlorosis (Therby-Vale et al., 2021). Taken together, we can deduce the possibility that plants need to dial down the photosynthesis reactors in the face of temporary iron influx shortages or suffer a meltdown leading to cell death. This could be due to over generation of chloroplastic ROS from photosystem electron stress to a level sufficient to activate damage/stress response pathways, which if true would conveniently explain the defense response related phenotypes seen in the *osacl-a2* mutant (Figure S3, S7). Iron deficiency has been demonstrated to activate plant defense responses, which may reflect the circumstance of cellular iron being siphoned away by invading pathogens (Herlihy et al., 2020). Indeed, the spontaneous defense response of *osacl-a2* is dependent on leaf iron level. However, simply having lower iron levels alone was insufficient to activate defense responses in *osacl-a2* as it only occurred in leaves beyond a certain stage of maturity. Furthermore, shading reduces
lesion severity in the field (Figure S7A) and in growth chambers lesions do not develop when light intensity is low (data not shown). Hence lesion development is likely dependent on active photosynthesis. Therefore we suggest that iron deficiency induced defense response originates from chloroplastic stress only produced in photosynthetic leaves.

We have proven that OsACL-A2 is not a negative regulator of defense responses as previously proposed (Ruan et al., 2019). Instead, OsACL-A2 as a positive regulator of iron transport by extension also positively regulates iron dependent plant defense. Iron dependent defenses have been proposed to be due to restriction of iron nutrition available to invading pathogens as well as concentrating iron at infection sites for use in key defensive redox reactions (Dangol et al., 2019). While the overall lower iron status of osacl-a2 mutants may explain their impaired defense response, OsACL-A2 may play a more active role. In support of this both OsACL-A2 transcripts and overall ACL activity increase after infection. It is tempting to consider differential ACL activity as a general mechanism to direct intercellular iron movement. If so, OsACL-A2 could be used to both draw iron away from pathogens and concentrate iron for defensive reactions. We currently lack the means to precisely observe and measure OsACL-A2 in individual cells, but this could be an interesting area to test in the future.

In conclusion, we identified a lesion mimic mutant in Indica rice ZH8015 that was due to the loss of function of the cytoplasmic ATP-citrate lyase subunit A, OsACL-A2. We demonstrated that cell death in osacl-a2 arose from iron deficiency triggered plant stress and defense responses in photosynthetic cells. Our results suggest that OsACL-A2 activity is a key factor in iron transport, which we hypothesize to occur by lysis of citrate-Fe$^{3+}$ at sink sites thereby creating a concentration gradient against source sites. The same mechanism may also occur during plant defense responses to sequester and concentrate iron at infection sites. Figure 6 depicts the normal functioning of the ACL enzyme in wild type (WT), which is crucial for transporting iron from roots to leaves—a process vital for photosynthesis. In contrast, the mutant osacl-a2 exhibits decreased ACL enzyme activity, resulting in impaired iron translocation to aerial parts, diminished iron content in foliage, citric acid buildup, and compromised leaf photosynthesis, ultimately culminating in the development of disease spots. Following pathogen invasion, the sufficient iron levels in WT confer enhanced resistance, whereas the compromised ACL enzyme activity in osacl-a2 leads to a reduction in disease resistance.

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CONFLICTS OF INTEREST

W.D., P.X, and Q.L. designed experiments and analyzed data. Y.Z., and L.S. created material. A, provided crucial guidance and suggestions. B.W., Y.C., Y.Z., M.J., and D.L. performed the experiments. W.D. and Aron wrote the manuscript. W.W., X.Z., S.C., and L.C. supervised the project. All authors read and approved the final manuscript.

REFERENCE


Aznar, A., Chen, N.W, Rigault, M., Riache, N., Joseph, D., Desmaele, D., Mouille, G., Boutet,


Zhang, H., Li, Y., Pu, M., Xu, P., Liang, G. (2020). Oryza sativa POSITIVE REGULATOR OF IRON DEFICIENCY RESPONSE 2 (OsPRI2) and OsPRI3 are involved in the maintenance of Fe homeostasis. Plant, cell & environment. 43: 261-274.


SUPPLEMENTAL DATA

Supplemental Figure 1. Gene expression analysis about OsACL-A2.

Supplemental Figure 2. Comparison of agronomic traits across WT and osacl-a2 plants.

Supplemental Figure 3. H2O2 content and the activity of ROS scavenging enzymes.

Supplemental Figure 4. Identification of resistance in osacl-a2 during tillering stage.

Supplemental Figure 5. Transcriptome analysis of iron-related transport genes.

Supplemental Figure 6. ACL affects the expression of steady-state related genes and content.

FIGURE LEGENDS

Figure 1. ATP-citrate lyase mutant osacl-a2 is a lesion mimic in Indica rice
(A) The amino acid sequences of the WT and osacl-a2.

(B-C) Phenotype of WT, osacl-a2, OE and COM plants at the tillering stage, Bar = 20 cm.

(C-E) Lesion mimic phenotype in osacl-a2 at the seedling stage (26 days after seed sowing in the paddy field), Bar = 10 cm.

(F-G) Lesion mimic phenotype in osacl-a2 at the tillering stage (60 days after seed sowing in the paddy field), Bar = 10 cm.

(H) Enzyme activity of ACL in WT and osacl-a2 at the heading stage. FW, fresh weight.

(I) Leaf contents of citric acid at the heading stage in WT and osacl-a2. FW, fresh weight.

Data are means ± SE. H-I, n = 12. ** indicates significance at P<0.01 and * indicates significance at P<0.05 (Student’s t test).

**Figure 2.** Lesion formation in osacl-a2 is due to iron deficiency

(A) Fe content in leaves in WT and osacl-a2 at the tillering stage in nature environment.

(B) Fe content in roots and leaves in WT and osacl-a2 in nutrient solution two weeks after germination.

(C) Symptoms of leaves in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination, Bar = 5 cm.

(D) Gene expression analysis related to iron absorption and transport in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination.

Data are means ± SE. A - B, E - G, n = 3. ** indicates significance at P<0.01 and * indicates significance at P<0.05 (Student’s t test). Different letters above the bars indicates significant differences (P<0.05). P-value was determined and analyzed using the GraphPad Prism 8.0 by one-way ANOVA followed by Tukey’s multiple comparisons. (C - G) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe.

**Figure 3.** Excess iron restores osacl-a2 photosynthetic capacity and disease gene expression.

(A) WT and osacl-a2 plants treated by the indicated exogenous iron concentrations two weeks after germination, Bar = 20 cm.

(B) Chla, Chlb, Car and Chl content of leaves in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination.

(C) Analysis of gene expression levels related to defence-related markers in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination.

Data are means ± SE. A - B, E - G, n = 3. ** indicates significance at P<0.01 and * indicates significance at P<0.05 (Student’s t test). Different letters above the bars indicates significant differences (P<0.05). P-value was determined and analyzed using the GraphPad Prism 8.0 by one-way ANOVA followed by Tukey’s multiple comparisons. (C - G) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe.

**Figure 4.** OsACL-A2 contributes to iron uptake through source-sink dynamics

(A) Leaf contents of citric acid in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination. FW, fresh weight.

(B) Leaf enzyme activity of ACL in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination. FW, fresh weight.

(C) Gene expression analysis of OsACL-A2 at root and leaf in WT treated by the indicated exogenous iron concentrations two weeks after germination.
Data are means ± SE. A-F, n = 3. ** indicates significance at P≤ 0.01 and * indicates significance at P≤ 0.05 (Student’s t test). (A - F) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe.

**Figure 5.** OsACL-A2 positively regulates rice resistance through free iron

(A) Spray inoculation of WT and osacl-a2 (treated by the indicated exogenous iron concentrations two weeks after germination) with *M. Oryzae* (blast isolate 14-1 was used for inoculation), leaves were photographed after 7 days, Bar = 2 cm.

(B) Two types of lesions caused by rice blast disease, Bar = 1 mm.

(C-D) Number of different lesion types in the first leaf (C) and the third leaf (D).

(E-F) Fungal growth in the first leaf (E) and the third leaf (F).

(G) qRT-PCR analysis of transcript levels of OsACL-A2 in WT during *M. oryzae* infections.

(H-I) Leaf enzyme activity of ACL and contents of citric acid in WT during *M. oryzae* infections.

Data are means ± SE. E-I, n = 3. Different letters above the bars indicates significant differences (P <0.05). P-value was determined and analyzed using the GraphPad Prism 8.0 by one-way ANOVA followed by Tukey’s multiple comparisons. (A-F) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe. The first leaf means the absence of disease spots phenotype. The third leaf means the presence of disease spot phenotype.

**Figure 6.** OsACL-A2 regulates the iron transport in rice and proposes a working model for positively regulating disease resistance.

We depicted the normal functioning of the ACL enzyme in wild type (WT), which is crucial for transporting iron from roots to leaves—a process vital for photosynthesis. In contrast, the mutant osacl-a2 exhibits decreased ACL enzyme activity, resulting in impaired iron translocation to aerial parts, diminished iron content in foliage, citric acid buildup, and compromised leaf photosynthesis, ultimately culminating in the development of disease spots. Following pathogen invasion, the sufficient iron levels in WT confer enhanced resistance, whereas the compromised ACL enzyme activity in osacl-a2 leads to a reduction in disease resistance. The yellow circle represents citric acid, the green triangle arrow represents ATP citric acid lyase, the green long arrow represents promotion, and the red represents inhibition.

**Hosted file**

Supporting Information TableS1-2.docx available at https://authorea.com/users/757964/articles/731079-osacl-a2-is-involved-in-iron-transport-and-positively-regulates-iron-dependent-disease-resistance
**Fig1.** ATP-citrate lyase mutant *osacl-a2* is a lesion mimic in *Indica* rice

(A) The amino acid sequences of the WT and *osacl-a2*.

(B-C) Phenotype of WT, *osacl-a2*, OE and COM plants at the tillering stage, Bar = 20 cm.

(C-E) Lesion mimic phenotype in *osacl-a2* at the seedling stage (26 days after seed sowing in the paddy field), Bar = 10 cm.

(F-G) Lesion mimic phenotype in *osacl-a2* at the tillering stage (60 days after seed sowing in the paddy field), Bar = 10 cm.

(H) Enzyme activity of ACL in WT and *osacl-a2* at the heading stage. FW, fresh weight.

(I) Leaf contents of citric acid at the heading stage in WT and *osacl-a2*. FW, fresh weight.

Data are means ± SE. H-I, n = 12. ** indicates significance at P≤ 0.01 and * indicates significance at P≤0.05 (Student’s t test).
Fig2. Lesion formation in *osacl-a2* is due to iron deficiency

(A) Fe content in leaves in WT and *osacl-a2* at the tillering stage in nature environment.
(B) Fe content in roots and leaves in WT and *osacl-a2* in nutrient solution two weeks after germination.
(C) Symptoms of leaves in WT and *osacl-a2* treated by the indicated exogenous iron concentrations two weeks after germination, Bar = 5 cm.
(D) Gene expression analysis related to iron absorption and transport in WT and *osacl-a2* treated by the indicated exogenous iron concentrations two weeks after germination.

Data are means ± SE. A - B, E - G, n = 3. ** indicates significance at P≤ 0.01 and * indicates significance at P≤0.05 (Student’s t test). Different letters above the bars indicates significant differences (P<0.05). P-value was determined and analyzed using the GraphPad Prism 8.0 by one-way ANOVA followed by Tukey’s multiple comparisons. (C - G) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe.
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(C) Analysis of gene expression levels related to defence-related markers in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination.

Data are means ± SE. A - B, E - G, n = 3. ** indicates significance at P ≤ 0.01 and * indicates significance at P ≤ 0.05 (Student’s t test). Different letters above the bars indicates significant differences (P < 0.05). P-value was determined and analyzed using the GraphPad Prism 8.0 by one-way ANOVA followed by Tukey's multiple comparisons. (C - G) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe.
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(A) Leaf contents of citric acid in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination. FW, fresh weight.

(B) Leaf enzyme activity of ACL in WT and osacl-a2 treated by the indicated exogenous iron concentrations two weeks after germination. FW, fresh weight.

(C) Gene expression analysis of OsACL-A2 at root and leaf in WT treated by the indicated exogenous iron concentrations two weeks after germination.

Data are means ± SE. A-F, n = 3. ** indicates significance at P≤ 0.01 and * indicates significance at P≤0.05 (Student’s t test). (A - F) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe.
Fig5. OsACL-A2 positively regulates rice resistance through free iron

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(C-D) Number of different lesion types in the first leaf (C) and the third leaf (D).

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(G) qRT-PCR analysis of transcript levels of OsACL-A2 in WT during M. oryzae infections.

(H-I) Leaf enzyme activity of ACL and contents of citric acid in WT during M. oryzae infections. Data are means ± SE. E-I, n = 3. Different letters above the bars indicates significant differences (P<0.05). P-value was determined and analyzed using the GraphPad Prism 8.0 by one-way ANOVA followed by Tukey's multiple comparisons. (A-F) D, 0 μM Fe; S, 50 μM Fe; E, 500 μM Fe. The first leaf means the absence of disease spots phenotype. The third leaf means the presence of disease spot phenotype.
Fig6. OsACL-A2 regulates the iron transport in rice and proposes a working model for positively regulating disease resistance.

We have outlined the fundamental function of the ACL enzyme in the wild type (WT), which plays a pivotal role in iron translocation from roots to leaves. This process is essential for the growth and development of plants, and it also exerts a positive regulatory influence on the plant's disease resistance response. When the plant roots combine citric acid with iron ions and store them in the reservoir, ACL catalyzes the hydrolysis of the citric acid-iron complex, releasing free iron ions for growth. Upon encountering pathogen invasion, the plant elevates the activity of ACL to enhance the delivery of free iron ions to the infected site, generating ROS to counteract the pathogen. The yellow circular icons represent citric acid, the yellow triangle icons symbolize concentration gradient potential, and the blue arrows indicate the stored reservoir.