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Lucas Roani Ponte

Papel dos genes *OsASR* na homeostase de ferro em arroz (*Oryza sativa* L.) e estudos sobre o mecanismo de importe vacuolar de ferro mediado pelos transportadores OsVIT1 e OsVIT2

Porto Alegre
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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES 8
RESUMO 11
ABSTRACT 12
1. INTRODUÇÃO 13
1.1. Importância científica, socioeconômica e nutricional do arroz 13
1.2. Importância biológica geral do ferro (Fe) e impacto na saúde humana 15
1.3. Importância biológica do Fe em plantas 17
1.4. Estratégias de absorção de Fe em plantas 18
1.5. Toxidez por Fe em arroz 19
1.6. Regulação da homeostase de Fe em arroz 21
1.7. Genes *ABSCISIC ACID*, *STRESS*, *RIPENING* (*ASR*) de arroz 22
1.8. Genes *VACUOLAR IRON TRANSPORTER* (*VIT*) de arroz 27
2. OBJETIVOS 29
2.1. Objetivo geral 29
2.2. Objetivos específicos 29
CAPÍTULO 1 31
CAPÍTULO 2 46
CAPÍTULO 3 69
DISCUSSÃO GERAL 90
REFERÊNCIAS BIBLIOGRÁFICAS GERAIS 92
***CURRICULUM VITAE* resumido** 116

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

%	Por cento
=	Equivalente a
ABA	Ácido abscísico
AIDS	Síndrome da Imunodeficiência Adquirida
Al	Alumínio
Al^{3+}	Íon alumínio trivalente
amiRNA	microRNA artificial
APX	Ascorbato peroxidase
As	Arsênio
ASR	ABSCISIC ACID, STRESS, RIPENING
AtFRO2	FERRIC-CHELATE REDUCTASE OXIDASE 2 de Arabidopsis
AtIRT1	IRON-REGULATED TRANSPORTER 1 de Arabidopsis
AtVIT1	VACUOLAR IRON TRANSPORTER 1 de Arabidopsis
bHLH	BASIC HELIX-LOOP-HELIX
Ca	Cálcio
CAT	Catalase
Cd	Cádmio
Co^{2+}	Cobalto catiônico
Cr	Crômio
Cu	Cobre
DMA	Ácido deoximugineico
DNA	Ácido desoxirribonucleico
e^{-}	Elétron
EMS	Etil-metano-sulfonato
EROs	Espécies reativas de oxigênio
FBN	Fixação biológica de nitrogênio
Fe	Ferro
$Fe(OH)_3$	Hidróxido de ferro
Fe^{2+}	Íon ferroso
Fe^{3+}	Íon férrico
Fe^{3+}-DMA	Complexo de íon férrico quelado a ácido deoximugineico
Fe-Mo	Cofator ferro-molibdênio
Fe-S	*Cluster* ferro-enxofre
Fe-SOD	Ferrosuperóxido dismutase
GA_3	Ácido giberélico
H^{+}	Próton (hídron)
H+-ATPase	Bomba de prótons
H_2O_2	Peróxido de hidrogênio
JA	Ácido jasmônico
JIF	*Journal Impact Factor*
K	Potássio
Kg	Quilograma
MDA	Malondialdeído
Mg	Magnésio
Mn	Manganês

N	Nitrogênio
N_2	Gás nitrogênio
NA	Nicotianamina
NH_3	Amônia
O	Oxigênio
O_2	Gás oxigênio
OH^-	Íon hidroxila
OH•	Radical hidroxila
OsASR1	ABSCISIC ACID, STRESS, RIPENING 1 de arroz
OsASR2	ABSCISIC ACID, STRESS, RIPENING 2 de arroz
OsASR3	ABSCISIC ACID, STRESS, RIPENING 3 de arroz
OsASR5	ABSCISIC ACID, STRESS, RIPENING 5 de arroz
OsASR6	ABSCISIC ACID, STRESS, RIPENING 6 de arroz
OsbHLH133	BASIC HELIX-LOOP-HELIX 133 de arroz
OsCIPK15	CALCINEURIN B-LIKE (CBL)-INTERACTING PROTEIN KINASE 15 de arroz
OsFIT/OsbHLH156	FER-LIKE FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR de arroz
OsHRZ1	HEMERYTHRIN MOTIF-CONTAINING RING- AND ZINC-FINGER PROTEIN 1 de arroz
OsHRZ2	HEMERYTHRIN MOTIF-CONTAINING RING- AND ZINC-FINGER PROTEIN 2 de arroz
OsIDEF1	IRON DEFICIENCY-RESPONSIVE ELEMENT-BINDING FACTOR 1 de arroz
OsIDEF2	IRON DEFICIENCY-RESPONSIVE ELEMENT-BINDING FACTOR 2 de arroz
OsIRO2/OsbHLH056	IRON-RELATED BHLH TRANSCRIPTION FACTOR 2 de arroz
OsIRO3/OsbHLH063	IRON-RELATED BHLH TRANSCRIPTION FACTOR 3 de arroz
OsIRT1	IRON-REGULATED TRANSPORTER 1 de arroz
OsIRT2	IRON-REGULATED TRANSPORTER 2 de arroz
OsNCED1	9-CIS-EPOXYCAROTENOID DIOXYGENASE 1 de arroz
OsPRI1/OsbHLH060	POSITIVE REGULATOR OF IRON DEFICIENCY RESPONSE 1 de arroz
OsPRI2/OsbHLH058	POSITIVE REGULATOR OF IRON DEFICIENCY RESPONSE 2 de arroz
OsPRI3/OsbHLH059	POSITIVE REGULATOR OF IRON DEFICIENCY RESPONSE 3 de arroz
OsSTAR1/OsENA2	SENSITIVE TO ALUMINUM RHIZOTOXICITY 1/NICOTIANAMINE EXPORTER 2 de arroz
OsSTAR2	SENSITIVE TO ALUMINUM RHIZOTOXICITY 2 de arroz
OsTOM1/OsZIFL4	TRANSPORTER OF MUGINEIC ACID 1/ZINC-INDUCED FACILITATOR-LIKE 4 de arroz
OsVIT1	VACUOLAR IRON TRANSPORTER 1 de arroz
OsVIT2	VACUOLAR IRON TRANSPORTER 2 de arroz
OsYSL15	YELLOW STRIPE LIKE 15 de arroz
OsYSL2	YELLOW STRIPE LIKE 2 de arroz

P	Fósforo
P_i	Fósforo/fosfato inorgânico
S	Enxofre
Si	Silício
SOD	Super óxido dismutase
TRA	Teor relativo de água
UDP	Uridina-difosfato
VIT	VACUOLAR IRON TRANSPORTER
ZIP	ZINC-REGULATED, IRON-REGULATED TRANSPORTER-LIKE PROTEIN
ZmYS1	IRON-PHYTOSIDEROPHORE TRANSPORTER YELLOW STRIPE 1 de milho
Zn	Zinco
Zn^{2+}	Zinco catiônico

RESUMO

A homeostase de ferro (Fe) em plantas é regulada por uma complexa rede formada por fatores de transcrição e transportadores. Dada a sua importância socioeconômica e científica, o arroz (*Oryza sativa* L.) é considerado uma espécie-modelo ideal para o estudo dos mecanismos regulatórios envolvidos no transporte e distribuição de Fe. Nesta tese, foram abordadas duas hipóteses distintas: (1) os genes da família *ASR* (*ABSCISIC ACID*, *STRESS*, *RIPENING*) tem papel na homeostase de Fe em arroz; e (2) as proteínas da família VIT (VACUOLAR IRON TRANSPORTER) interagem, formando um heterodímero. No Capítulo 1, foram analisados os efeitos da supressão dos genes *OsASR* em plantas OsASR5-RNAi, que apresentaram maior sensibilidade à deficiência de Fe. Estas plantas apresentaram clorose foliar precoce, redução na altura, biomassa e fotossíntese. Embora não tenham sido observadas alterações significativas na expressão de genes-chave relacionados à rota de absorção de Fe, o gene *OsYSL2*, que codifica um importante transportador envolvido no carregamento de Fe no floema, apresentou aumento de expressão. Isso indica, pela primeira vez, a atuação das proteínas OsASR na homeostase de Fe, possivelmente numa via regulatória de transporte de longa distância de Fe. No Capítulo 2, as plantas OsASR5-RNAi foram submetidas à condição de excesso de Fe. Em condição controle, através de uma análise detalhada, foi observado que o sistema de raízes foi menos desenvolvido. Isso demonstra que a supressão dos genes *OsASR* é prejudicial para o crescimento e desenvolvimento geral das plantas. No Capítulo 3, foi investigada a potencial heterodimerização entre os transportadores vacuolares OsVIT1 e OsVIT2. Ensaios de duplo-híbrido (Y2H) em levedura (*Saccharomyces cerevisiae*) não evidenciaram interação direta, possivelmente devido a limitações técnicas intrínsecas a este sistema de expressão heteróloga. Além disso, uma versão truncada de OsVIT2, sem o seu domínio N-terminal (OsVIT2mod), foi expressa na linhagem mutante de levedura Δ*ccc1* para avaliar o seu potencial papel no transporte de Fe^{2+}. Não foram detectadas diferenças fenotípicas entre as linhagens expressando OsVIT2 nativa e OsVIT2mod. Nossos resultados ampliam o entendimento sobre a homeostase de Fe em arroz, colocando as proteínas ASR como parte da regulação das concentrações de Fe.

Palavras-chave: arroz, ASR, deficiência de ferro, excesso de ferro, vacúolo, transportador, ionômica.

ABSTRACT

Iron (Fe) homeostasis in plants is regulated by a complex network formed by transcription factors and transporters. Given its socioeconomic and scientific importance, rice (*Oryza sativa* L.) is considered an ideal model species for studying the regulatory mechanisms involved in Fe transport and distribution. This thesis addressed two distinct hypotheses: (1) genes from the *ASR* (*ABSCISIC ACID*, *STRESS*, *RIPENING*) family play a role in Fe homeostasis in rice; and (2) proteins from the VIT (VACUOLAR IRON TRANSPORTER) family interact, forming a heterodimer. In Chapter 1, the effects of suppression of the *OsASR* genes in OsASR5-RNAi plants, which showed greater sensitivity to Fe deficiency, were analyzed. These plants showed early leaf chlorosis, reduced height, biomass, and photosynthesis. Although no significant changes were observed in the expression of key genes related to the Fe uptake pathway, the *OsYSL2* gene, which encodes an important transporter involved in Fe phloem loading, showed increased expression. This indicates, for the first time, the role of OsASR proteins in Fe homeostasis, possibly in a regulatory pathway for long-distance Fe transport. In Chapter 2, OsASR5-RNAi plants were exposed to Fe excess treatment. Under control condition, through a detailed analysis, it was observed that the root system was less developed. This demonstrates that the suppression of *OsASR* genes is detrimental to overall plant growth and development. In Chapter 3, the potential heterodimerization between the vacuolar transporters OsVIT1 and OsVIT2 was investigated. Two-hybrid (Y2H) assays in yeast (*Saccharomyces cerevisiae*) did not show direct interaction, possibly due to technical limitations intrinsic to this heterologous expression system. In addition, a truncated version of OsVIT2, lacking its N-terminal domain (OsVIT2mod), was expressed in the mutant yeast strain Δ*ccc1* to assess its potential role in Fe^{2+} transport. No phenotypic differences were detected between the transformants expressing native OsVIT2 and OsVIT2mod. Our results broaden the understanding of Fe homeostasis in rice, placing ASR proteins as components of Fe concentration regulation.

Keywords: rice, ASR, iron deficiency, iron excess, vacuole, transporter, ionomic.

1. INRODUÇÃO

1.1. Importância científica, socioeconômica e nutricional do arroz

O gênero *Oryza* pertence à família das gramíneas (*Poaceae*) e clado das monocotiledôneas, sendo composto por 27 espécies catalogadas (Fornasiero *et al.*, 2025). Dentre estas, duas espécies de arroz são cultivadas: *O. sativa* e *O. glaberrima*. Enquanto o cultivo de *O. glaberrima* está concentrado em países da África Ocidental, o cultivo de *O. sativa* está difundido em todos os continentes, com exceção da Antártida (Coclanis, 2024; Kehinde *et al.*, 2024). Semelhante às demais espécies do seu gênero, *O. sativa* é uma planta semiaquática, de aparato fotossintético C_3 e pode sobreviver como planta perene, embora seja cultivada como cultura agrícola de ciclo anual (Li *et al.*, 2021).

Na literatura científica, *O. sativa* é considerada uma planta-modelo para estudos fisiológicos e genéticos, servindo como referência para as demais monocotiledôneas (Lohithaswa *et al.*, 2007; Kim *et al.*, 2014). Isso se deve ao fato de o arroz ter sido a primeira cultura agrícola e a segunda espécie vegetal a ter o genoma completamente sequenciado, após *Arabidopsis thaliana* (Hamilton *et al.*, 2025). O genoma de arroz é constituído por 12 cromossomos (2n = 24), sendo um dos menores dentre as gramíneas (Tkach *et al.*, 2025). Além disso, apresenta um alto grau de sintenia com os genomas de outros cereais de importância agronômica, como milho (*Zea mays*), trigo (*Triticum aestivum*), cevada (*Hordeum vulgare*), etc. (Salse *et al.*, 2004, 2008; Thiel *et al.*, 2009).

A domesticação de *O. sativa* para cultivo e consumo humano provavelmente ocorreu entre 8.000 a 10.000 anos atrás no vale do Rio Yangtzé, China (Gross & Zhao, 2014). Ao longo de milhares de anos, esta cultura foi distribuída pelos continentes através de migrações e rotas de comércio (Gutaker *et al.*, 2020; Spengler *et al.*, 2021). Isso resultou na sua subespeciação: *indica*, *japonica* e *japonica tropical*, esta última também identificada como *javanica*. Tais subespécies são diferenciadas por características morfológicas e bioquímicas (Singh *et al.*, 2022). Destaca-se que a pressão de seleção, tanto ambiental quanto antropogênica, imposta a plantas de *O. sativa* em diferentes ecossistemas proporcionou que esta espécie se tornasse um dos principais cereais cultivados em escala global e consequentemente um alimento básico na dieta de mais de metade da população mundial (Zhou *et al.*, 2025).

Em torno de 90% do arroz produzido globalmente origina-se da Ásia, sendo Índia, Tailândia e Vietnã os maiores mercados exportadores. Em paralelo, a China é o principal

produtor, consumidor e importador (FAO, 2024). A produção mundial de grãos de arroz na safra 2024/25 resultou em mais de 530 milhões de toneladas métricas, sendo somente China e Índia responsáveis por cerca de 54% deste montante (USDA, 2025). Fora da Ásia, o maior produtor mundial é o Brasil (FAO, 2024). Historicamente concentrada na Região Sul, a produção orizícola brasileira na safra 2024/25 gerou mais de 12 milhões de toneladas métricas, das quais cerca de 70% foram provenientes do Estado do Rio Grande do Sul (CONAB, 2025).

O consumo mundial de arroz está associado a tradições culturais e pela sua acessibilidade econômica, o que o torna uma fonte de sustento energético crucial para populações de baixa renda (Fitrawaty *et al*., 2023). Estima-se que 19% do total de calorias consumidas diariamente pelos seres humanos advém do arroz (Elert, 2014). Esta estimativa ultrapassa 50% quando analisada a dieta de populações asiáticas de baixa renda (Muthayya *et al.*, 2014). Para efeito de comparação, enquanto o consumo *per capita* anual de arroz no Brasil é por volta de 30 kg, este é superior a 100 kg em países do Sudeste Asiático, como Filipinas, Mianmar, Laos, etc. (OECD/FAO, 2023).

O arroz branco é a forma predominante pela qual os grãos deste cereal são destinados para o consumo humano, sendo um produto industrialmente processado. Neste processo, os grãos são descascados e posteriormente polidos, havendo a eliminação do farelo e gérmen (embrião) (Müller *et al.*, 2022). Resta assim somente o endosperma, o qual é composto majoritariamente por carboidratos, como o amido. Isso justifica o fato de a demanda calórica humana ser saciada em dietas nas quais o arroz branco está dentre os principais constituintes (Zaffer *et al.*, 2025). No entanto, o consumo generalizado de arroz branco está associado com a continuidade da desnutrição, também chamada de fome oculta (Yilmaz & Yilmaz, 2025). Dado que os grãos de arroz apresentam baixas concentrações de minerais essenciais para a saúde humana, estas são ainda mais reduzidas através do seu polimento, tornando o arroz branco uma fonte nutricionalmente pobre (Hansen *et al.*, 2012; Tuncel *et al.*, 2025). Grande porção de proteínas, vitaminas e minerais estão presentes na camada de aleurona do grão, um dos constituintes do farelo. Este após ser descartado durante o processamento dos grãos, é geralmente utilizado como matéria-prima na composição de rações destinadas à alimentação animal (Bodie *et al.*, 2019; Manlapig & Matsui, 2025). Dentre os minerais, estima-se que cerca de 70% do ferro (Fe) presente nos grãos é perdido em função do seu polimento (Prom-u-thai *et al.*, 2007).

Diferentes métodos de processamento do arroz levam a alterações nas concentrações de nutrientes. O arroz integral, por exemplo, sofre apenas o descasque, apresentando maiores teores de compostos antioxidantes, assim como de minerais em relação ao arroz branco, como cálcio (Ca), magnésio (Mg), potássio (K) e zinco (Zn), além do Fe (Mir *et al.*, 2020; Wu *et al.*, 2023; Tuncel *et al*., 2025). Também há a parboilização, que ocorre antes do descasque e é estruturada nas seguintes etapas: exposição do grão inteiro a altas temperaturas, gelatinização parcial ou total do endosperma e secagem do grão, podendo o mesmo ser polido ou não (Muchlisyiyah *et al*., 2023). Diferentes estudos relataram a maior retenção de minerais em grãos parboilizados, principalmente de Zn (Balbinoti *et al*., 2018; Taleon *et al*., 2020, 2022). No entanto, já foi constatado que a substituição em grande escala do arroz branco para outros tipos de arroz comercialmente disponíveis trata-se de um processo gradual e laborioso. Isso é decorrente da preferência dos consumidores ao arroz branco, em função das suas propriedades organolépticas, aliada ao pouco conhecimento do público acerca das características nutricionais dos diferentes tipos de arroz beneficiado (Kumar *et al*., 2011; Milani *et al*., 2016; Chowdhury *et al*., 2022). Além disso, o maior teor de lipídios presente no arroz integral acelera a sua deterioração, o que consequentemente diminui o seu tempo de prateleira (Mir *et al.*, 2020; Tuncel *et al*., 2025).

1.2. Importância biológica geral do ferro (Fe) e impacto na saúde humana

O Fe é um metal pesado indispensável para a vida terrestre, exceto para algumas bactérias, como o patógeno *Borrelia burgdorferi*, agente causador da doença de *Lyme*, e possivelmente algumas espécies do gênero *Lactobacillus* (Aguirre *et al*., 2013; Seyoum *et al*., 2021). Isso é decorrente dos átomos de Fe serem capazes de doarem ou receberem elétrons (e^-), sendo tipicamente encontrados em dois estados de oxidação sob pH celular: íon ferroso (Fe^{2+}) ou férrico (Fe^{3+}). A capacidade de formar 6 ligações coordenadas com átomos doadores de e^- permite que o Fe se ligue a proteínas, agindo como grupo prostético destas na forma de grupos heme, não-heme e *clusters* ferro-enxofre (Fe-S) (Sánchez *et al*., 2017). Desta forma, o Fe é vital para a estabilização estrutural de diversas proteínas e enzimas, assim como para a catálise de reações de oxirredução, nas quais pode haver o uso ou a produção de gás oxigênio (O_2), como a respiração celular e fotossíntese. Em seres humanos e demais mamíferos, o Fe faz parte da estrutura de diversas proteínas, como a

hemoglobina e mioglobina, necessárias para o transporte de O_2 na corrente sanguínea e armazenamento de O_2 nos músculos, respectivamente (Dev & Babitt, 2017).

A deficiência de ferro (Fe) é a deficiência nutricional que mais comumente afeta os seres humanos, podendo ter origem em fatores fisiológicos ou patológicos (Pasricha *et al*., 2021). Sua principal consequência é a anemia, a qual acomete aproximadamente 2 bilhões de indivíduos (Gardner *et al*., 2023). Em países subdesenvolvidos e/ou em desenvolvimento, a ocorrência de doenças infecciosas, como a síndrome da imunodeficiência adquirida (AIDS), tuberculose, malária, etc., contribui para o agravamento da anemia (Chaparro & Suchdev, 2019). Comprometimento no desenvolvimento motor e cognitivo de recém-nascidos e crianças, ocorrência de alterações comportamentais e maiores fadiga e suscetibilidade a infecções, principalmente das vias aéreas superiores, em especial em mulheres em idade fértil e/ou gestantes, estão dentre os principais efeitos da anemia (Costa & Ayres-Silva, 2023). No Brasil, foi estimado que uma em cada três crianças com menos de cinco anos sofre de anemia por falta de Fe na dieta, cenário potencialmente agravado durante a pandemia do vírus SARS-CoV-2 (COVID-19) (Nogueira-de-Almeida *et al*., 2021).

A biofortificação vegetal destaca-se como solução para este problema. Trata-se do conjunto de diferentes estratégias que visam a geração de plantas com maiores concentrações de micronutrientes nas suas porções comestíveis (Ofori *et al*., 2022). Diferentes estudos já enfatizaram o melhor custo-benefício da biofortificação quando comparada com medidas paralelas no combate à anemia/desnutrição, como a suplementação e fortificação minerais (Meenakshi *et al*., 2010; De Steur *et al*., 2012; Zhang *et al*., 2018). Visto o baixo poder aquisitivo das populações carentes, a biofortificação torna-se uma medida economicamente viável a longo prazo para combater a anemia/desnutrição em países subdesenvolvidos e/ou em desenvolvimento (Osendarp *et al*., 2018; Kiran *et al*., 2022). Para tornar possível a geração de plantas capazes de acumularem maiores concentrações de Fe, é indispensável a compreensão dos mecanismos de homeostase de Fe em arroz, a qual engloba desde a sua absorção na rizosfera para as raízes, transporte via tecidos vasculares, remobilização de tecidos vegetativos e acúmulo no endosperma dos grãos (Sperotto *et al*., 2012; Wairich *et al*., 2022).

1.3. Importância biológica do Fe em plantas

O Fe é o quarto elemento mais abundante na crosta terrestre, sendo oxigênio (O), silício (Si) e alumínio (Al) respectivamente os três principais constituintes em massa (Yaroshevsky, 2006; Wu *et al*., 2019). Entretanto, o Fe tende a ser encontrado predominantemente como óxidos e hidróxidos de Fe^{3+} [$Fe(OH)_3$], moléculas com solubilidade extremamente baixa em solos aerados, principalmente os que apresentam pH neutro a alcalino (Jones, 2020). Isso facilita a ocorrência da deficiência de Fe em diferentes espécies vegetais, visto que a maioria das culturas agrícolas são cultivadas em solos aerados e manejados com práticas agrícolas visando a correção do pH, como as calagens (Vélez-Bermúdez & Schmidt, 2023). Em decorrência da baixa mobilidade do Fe no interior das plantas, um sintoma típico da sua deficiência é a clorose internerval em folhas jovens, que em casos mais severos, pode tornar-se generalizada nos tecidos foliares (Jones, 2020). Em conjunto, redução da atividade fotossintética, estagnação do crescimento vegetativo e desenvolvimento de órgãos reprodutivos, o que pode levar à eventual morte das plantas, também são causadas pela deficiência de Fe (Krohling *et al*., 2016). A deficiência de Fe também interfere na homeostase de outros elementais vitais para as plantas, como cobre (Cu), enxofre (S), fósforo (P), K, nitrogênio (N) e Zn (Khan *et al*., 2022; Therby-Vale *et al*., 2022; Yang *et al*., 2024; Wairich *et al*., 2025).

A relevância do Fe no ciclo de vida das plantas é destacada pela sua presença na estrutura de enzimas atuantes na biossíntese das clorofilas (Hu *et al*., 2017). Em uma única cadeia transportadora de e^- no aparato fotossintético são encontrados mais de 20 átomos de Fe. Deste modo, estima-se que cerca de 90% do Fe presente em tecidos foliares está situado nos cloroplastos (Kroh & Pilon, 2020). O Fe também é essencial para a fixação biológica de nitrogênio (FBN) que ocorre nas raízes de espécies da família *Fabaceae*, através da sua relação simbiótica com bactérias dos gêneros *Rhizobium*, *Bradyrhizobium*, etc. (Liu *et al*., 2023). Isso é decorrente da presença de *clusters* Fe-S e um cofator ferro-molibdênio (Fe-Mo) na nitrogenase, enzima que catalisa a conversão do nitrogênio atmosférico (N_2) em amônia (NH_3) (Schmidt *et al*., 2024). O Fe também está presente na estrutura da leghemoglobina como grupo heme. Semelhante à hemoglobina, a leghemoglobina é uma proteína que quela O_2, auxiliando na manutenção de baixas concentrações deste gás no interior dos nódulos, o que é essencial para a atividade da nitrogenase, visto que esta enzima é sensível à presença de O_2 (Day & Smith, 2021).

Uma adequada homeostase de Fe em plantas também é crítica para a atividade de enzimas antioxidantes. Pelo fato de serem organismos sésseis, as plantas estão continuamente expostas a diferentes tipos de estresses abióticos, como seca, altas temperaturas e alta intensidade luminosa (Dixit *et al*., 2024). A ocorrência destes fenômenos deve ser agravada pelas mudanças climáticas em escala global nas próximas décadas, o que impõe riscos para a produção de alimentos e consequentemente para a segurança alimentar global (Yuan *et al*., 2024; Nóia Júnior *et al*., 2025). Como resultado da exposição das plantas a estas flutuações ambientais, espécies reativas de oxigênio (EROs) tendem a ser produzidas de forma excessiva (Wang *et al*., 2024). Assim, para mitigar a ocorrência de danos no nível celular e tecidual, as EROs necessitam ser metabolizadas por enzimas de ação antioxidante, como a ascorbato peroxidase (APX), catalase (CAT) e superóxido dismutase (SOD) (Mishra *et al*., 2023). A atividade destas enzimas é dependente da presença de Fe, visto que enquanto CAT e APX possuem grupos heme, existem isoformas de SOD que apresentam Fe na sua estrutura, conhecidas como Fe-SOD (Santos *et al*., 2019; da Fonseca-Pereira *et al*., 2023).

1.4. Estratégias de absorção de Fe em plantas

Na literatura científica, Marschner *et al*. (1986) foram os primeiros a propor dois mecanismos de absorção de Fe pelas plantas, denominados Estratégia I e Estratégia II. A Estratégia I ocorre em eudicotiledôneas e monocotiledôneas, exceto as gramíneas (*Poaceae*). Esta estratégia baseia-se na redução do Fe^{3+} para Fe^{2+}, íon com maior solubilidade, facilitando assim a sua absorção pelas raízes. Localizadas na epiderme das raízes, bombas de prótons (bombas H^+-ATPase) realizam a extrusão de prótons/hídrons (H^+). O aumento do gradiente de cargas elétricas positivas promove a redução do pH na rizosfera (Santi & Schmidt, 2009). Esta acidificação local aumenta a solubilidade dos íons Fe^{3+}, os quais são posteriormente convertidos em íons Fe^{2+} por ação de ferro-quelato redutases situadas na membrana plasmática das células epidérmicas, como a AtFRO2 (FERRIC-CHELATE REDUCTASE OXIDASE 2) (Yi & Guerinot, 1996; Robinson *et al*., 1999; Connolly *et al*., 2003). Por fim, os íons Fe^{2+} são absorvidos por transportadores transmembrana específicos, como o AtIRT1 (IRON-REGULATED TRANSPORTER 1), pertencente à família gênica ZIP (ZINC-REGULATED, IRON-REGULATED TRANSPORTER-LIKE PROTEIN) (Eide *et al*., 1996; Korshunova *et al*., 1999; Vert *et al*., 2002). Recentemente, Rodrigues *et al*. (2023) constataram que a estratégia de absorção de Fe^{2+} via proteínas ZIP provavelmente

evoluiu de forma independente pelo menos duas vezes ao decorrer da evolução das plantas terrestres.

Em paralelo, a Estratégia II ocorre em gramíneas. Neste caso, há a extrusão de fitossideróforos, compostos derivados da metionina (Mori & Nishizawa, 1987; Nozoye *et al*., 2007). Os fitossideróforos, com destaque para o ácido deoximugineico (DMA) e seu precursor nicotianamina (NA), são compostos de baixo peso molecular que apresentam a capacidade de quelarem diferentes íons metálicos (Seregin & Kozhevnikova, 2021, 2023). Em arroz, a extrusão de DMA para a rizosfera ocorre através do transportador OsTOM1/OsZIFL4 (TRANSPORTER OF MUGINEIC ACID 1/ZINC-INDUCED FACILITATOR-LIKE 4) (Nozoye *et al*., 2011; Ricachenevsky *et al*., 2011). Os complexos Fe^{+3}-DMA são absorvidos pelo transportador ZmYS1 (IRON-PHYTOSIDEROPHORE TRANSPORTER YELLOW STRIPE 1) em milho e por seus ortólogos, como o OsYSL15 (YELLOW STRIPE LIKE 15) em arroz (von Wiren *et al*., 1994; Curie *et al*., 2001; Schaaf *et al*., 2004; Inoue *et al*., 2009; Lee *et al*., 2009).

O. sativa apresenta a Estratégia Combinada para a absorção de Fe. Este mecanismo é constituído pela Estratégia II somada à Estratégia I parcialmente (Ishimaru *et al*., 2006). A presença parcial da Estratégia I é decorrente da absorção de Fe^{2+} via transportadores OsIRT1 e OsIRT2 (Bughio *et al*., 2002; Nakanishi *et al*., 2006; Soviguidi *et al*., 2025). Inicialmente presumido como exclusivo de *O. sativa*, Wairich *et al*. (2019) demonstraram que este mecanismo ocorre em espécies não-domesticadas do gênero *Oryza* próximas ao arroz cultivado.

1.5. Toxidez por Fe em arroz

A produção mundial de arroz é historicamente baseada no seu cultivo em solos alagados, isto é, solos que são submetidos à presença contínua de uma lâmina hídrica sobre a superfície (Bindraban *et al*., 2006). Ao contrário da maioria das culturas agrícolas, o arroz é uma planta semiaquática adaptada a desenvolver-se em solos alagados, devido à existência de aerênquimas no colmo (caule) e raízes, tecidos parenquimáticos modificados, cuja função é a distribuição de O_2 (Yamauchi *et al*., 2013). A tolerância à submersão prolongada durante os estágios iniciais de crescimento, incrementada em algumas cultivares via edição gênica, é outra particularidade existente no arroz (Ashikari *et al*., 2025). No entanto, o cultivo de

arroz pode ser afetado por distúrbios nutricionais que tendem a ocorrer em solos alagados, como a toxicidade por Fe^{2+} (Wairich *et al*., 2024).

O alagamento do solo a longo prazo leva à depleção do O_2 através da sua rápida metabolização por populações bacterianas aeróbicas (Gschwend *et al*., 2020). A condição de anaerobiose torna a solução do solo um ambiente redutor, isto é, com excesso de cargas elétricas negativas (Pezeshki & DeLaune, 2012). Em adição, há o estabelecimento e multiplicação de populações microbianas anaeróbicas, que utilizam o Fe^{3+} como receptor de e^- no processo respiratório (Kosman, 2019; Gschwend *et al*., 2020). Havendo a conversão para Fe^{2+}, este íon tem a sua concentração aumentada na solução do solo (Wu *et al*., 2019). Como comentado previamente, os íons Fe^{2+} apresentam uma alta solubilidade, sendo assim facilmente absorvidos pelas raízes das plantas de arroz (Soviguidi *et al*., 2025). Um sintoma visual típico em plantas de arroz cultivadas em solos com altas concentrações de Fe^{2+} é o bronzeamento dos tecidos foliares, o que também já é um indicativo da ocorrência de danos gerados por este distúrbio nutricional (Engel *et al*., 2012; Santana *et al*., 2014).

O bronzeamento das raízes também é um sintoma visual típico do cultivo de arroz em solos com altas concentrações de Fe^{2+}. Neste caso, é decorrente da formação de placas férricas sobre a superfície das raízes (Zandi *et al*., 2021, 2023). Vale ressaltar que se trata de um processo natural desencadeado pela respiração das raízes, ou seja, pela liberação de O_2 na rizosfera através dos aerênquimas (Ejiri *et al*., 2021). As moléculas de O_2 promovem a oxidação dos íons Fe^{2+} em Fe^{3+} (Maisch *et al*., 2019). Os íons Fe^{3+} gerados tendem a interagir com íons hidroxila (OH^-) presentes na solução do solo, formando moléculas de $Fe(OH)_3$, que por sua vez acabam por precipitar sobre a epiderme (Zandi *et al*., 2021, 2023). O acúmulo gradual das moléculas de $Fe(OH)_3$ leva à formação das placas férricas (Zandi *et al*., 2021, 2023). É sabido que estas placas são benéficas para as plantas de arroz, pois atuam como uma barreira apoplástica para a absorção de elementos tóxicos, como arsênio (As), cádmio (Cd) e crômio (Cr) (Sebastian & Prasad, 2016; Xiao *et al*., 2021; Zandi *et al*., 2023; Yu *et al*., 2024). Entretanto, é a formação excessiva das mesmas que acaba por interferir na absorção de água e de elementos vitais para as plantas, como P, manganês (Mn) e Zn, acarretando no crescimento reduzido das raízes (Zhang *et al*., 2022a; Li *et al*., 2024).

A absorção excessiva de Fe^{2+} resulta no aumento das suas concentrações intracelulares, tanto nas raízes quanto em folhas. Isso propicia a interação entre Fe^{2+} e moléculas de peróxido de hidrogênio (H_2O_2) através da ocorrência da chamada Reação de Fenton, a qual

tem como produto radicais hidroxila (OH•) (Becana *et al*., 1998). Dada a natureza altamente reativa destes radicais, as células vegetais tornam-se propensas à peroxidação lipídica, à oxidação de proteínas e a danos nas fitas de ácido desoxirribonucleico (DNA), o que inevitavelmente leva à redução da performance fotossintética e necrose nos tecidos (Pereira *et al*., 2013; Müller *et al*., 2015, 2017; Pinto *et al*., 2016). Em casos severos de toxicidade por Fe, são observados o atrofiamento do crescimento e desenvolvimento vegetal e consequentemente perdas de produtividade (Frei *et al*., 2016).

1.6. Regulação da homeostase de Fe em arroz

A homeostase de Fe em arroz é rigorosamente regulada para garantir a sua absorção e distribuição ao longo da planta e, paralelamente, evitar toxicidade (Gao & Dubos, 2021). Na via de resposta à deficiência de Fe em arroz é sabido que os fatores de transcrição OsIDEF1 (IRON DEFICIENCY-RESPONSIVE ELEMENT-BINDING FACTOR 1) e OsIDEF2 ligam-se a elementos cis-regulatórios para promover a expressão de genes-alvo (Kobayashi *et al*., 2007, 2009, 2010; Ogo *et al*., 2008). Dentre os genes-alvo destaca-se *OsYSL2*, sendo indireta e diretamente regulado por OsIDEF1 e OsIDEF2, respectivamente (Ogo *et al*., 2008). OsYLS2 desempenha papel fundamental no transporte de Fe^{2+}-NA, sendo altamente expresso nas células companheiras dos vasos de floema, situados no colmo e bainhas foliares. OsYSL2 também atua no importe de Fe para o endosperma e embrião dos grãos (Koike *et al*., 2004; Nozoye *et al*., 2007; Ishimaru *et al*., 2010). Outro gene-alvo que se destaca é *OsIRO2* (IRON-RELATED BHLH TRANSCRIPTION FACTOR 2), cuja proteína atua como regulador positivo central de genes constituintes da Estratégia II (Ogo *et al*., 2006, 2007; Wang *et al*., 2020a). OsIRO2 regula diretamente genes envolvidos na biossíntese de NA e DMA, na extrusão de DMA para a rizosfera, como *OsTOM1*/*OsZIFL4* e na absorção de Fe^{+3}-DMA, como *OsYSL15* (Ogo *et al*., 2007, 2011).

Ressalta-se que *OsIRO2* pertence à família de fatores de transcrição bHLH (BASIC HELIX-LOOP-HELIX), sendo também identificado como *OsbHLH056* (Zhang *et al*., 2023). *OsIRO2* é regulado por OsPRI1/OsbHLH060 (POSITIVE REGULATOR OF IRON DEFICIENCY RESPONSE 1) (Zhang *et al*., 2017). *OsPRI1* possui dois parálogos: *OsPRI2*/*OsbHLH058* e *OsPRI3*/*OsbHLH059*, cujas proteínas também atuam como reguladores positivos da resposta à deficiência de Fe em arroz. OsPRI2 regula diretamente OsIRO2, tendo sobreposição funcional parcial a OsPRI1 (Kobayashi *et al*., 2019). Além

disso, outro fator de transcrição bHLH, OsFIT/OsbHLH156 (FER-LIKE FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR) facilita o transporte e acúmulo de OsIRO2 no núcleo (Liang *et al*., 2020; Wang *et al*., 2020a). Interessantemente, OsIRO2 é regulado por OsIRO3/OsbHLH063, o qual também é induzido por baixas concentrações de Fe (Zheng *et al*., 2010). Porém, OsIRO3 reprime a expressão de IRO2, assim como genes atuantes na biossíntese de NA, atuando *in tandem* com os reguladores positivos OsPRI1 e OsPRI2 para manter o equilíbrio entre a deficiência e toxicidade de Fe (Wang *et al*., 2020b; Carey-Fung *et al*., 2022; Li *et al*., 2022). Por fim, OsbHLH133 também foi observado como regulador positivo da via de resposta à deficiência de Fe, promovendo a distribuição de Fe entre raízes e parte aérea (Wang *et al*., 2013).

Quando encontradas concentrações ideais de Fe nos tecidos vegetais, os fatores de transcrição OsPRIs são regulados negativamente pelas proteínas OsHRZ1 (HEMERYTHRIN MOTIF-CONTAINING RING- AND ZINC-FINGER PROTEIN) e OsHRZ2. OsHRZ1 e OsHRZ2 são classificadas como E3 ubiquitina ligases, isto é, promovem a degradação de proteínas-alvo através da via de ubiquitina-proteossoma 26S (Kobayashi *et al*., 2013). Recentemente, Shinkawa *et al*. (2025) reportaram que as homeostases de Fe e Zn são cruciais para a atividade de OsHRZ1 e OsHRZ2, visto a existência de sítios de ligação a átomos de Fe e Zn na estrutura de ambas as duas proteínas (Kobayashi *et al*., 2013).

1.7. Genes *ABSCISIC ACID*, *STRESS*, *RIPENING* (*ASR*) de arroz

Os genes *ASR* (*ABSCISIC ACID*, *STRESS*, *AND RIPENING*) estão associados principalmente a respostas a estresses abióticos, à sinalização de ácido abscísico (ABA) e ao amadurecimento de frutos (Zhang *et al*., 2024). Na literatura científica, estes genes foram primeiramente identificados em tomateiro (*Solanum lycopersicum*) (Golan *et al*., 2014). Desta forma, é conhecido o papel das proteínas ASR como fatores de transcrição de genes-chave atuantes no importe e acúmulo de açúcares simples (hexoses) durante o amadurecimento de frutos de tomateiro e também de videira (*Vitis vinifera*) (Saumonneau *et al*., 2012; Golan *et al*., 2014; Parrilla *et al*., 2022). Vale ressaltar que não há genes *ASR* em *A*. *thaliana*, o que indica uma possível deleção desta família gênica em algum ancestral comum pertencente à família *Brassicaceae* (Barros *et al*., 2025a). Em paralelo, seis genes *ASR* são encontrados no genoma de arroz (Frankel *et al*., 2006; Philippe *et al*., 2010).

Recentemente, a presença de genes *ASR* foi detectada em 115 genomas em *Archaeplastida*, havendo variação no número de genes encontrados (Barros *et al*., 2025a). Eventos de duplicação *in tandem* são provavelmente o principal mecanismo que impulsionou a expansão da família gênica *ASR* dentre as espécies do conjunto estudado (Barros *et al*., 2025a). Infelizmente a nomenclatura dos genes *OsASR* é inconsistente na literatura científica (Frankel *et al*., 2006; Philippe *et al*., 2010; Joo *et al*. 2013a). Portanto, neste trabalho seguimos a nomenclatura proposta por Philippe *et al*. (2010), a qual foi previamente utilizada pelos nossos colaboradores (Arenhart *et al*., 2013). A Tabela 1 sintetiza a relação da nomenclatura dos genes *OsASR* com seus respectivos identificadores únicos no *Michigan State University Rice Genome Annotation Project* (MSU-RGAP) e no *Rice Annotation Project Database* (RAP-DB).

Tabela 1. Nomenclatura e identificadores únicos (IDs) dos genes *ASR* de arroz.

Nome do gene	ID MSU	ID RAP
OsASR1	LOC_Os01g72900	Os01g0959100
OsASR2	LOC_Os01g72910	Os01g0959200
OsASR3	LOC_Os02g33820	Os02g0543000
OsASR4	LOC_Os04g34600	Os04g0423400
OsASR5	LOC_Os11g06720	Os11g0167800
OsASR6	LOC_Os01g73250	Os01g0963600

Inicialmente, destaca-se a análise efetuada por Pérez-Díaz *et al*. (2014), na qual a expressão dos seis genes *OsASR* foi verificada sob condições de estresse por ABA, desidratação e baixa temperatura. Os autores observaram que os genes *OsASR* são diferencialmente expressos de maneira específica conforme o estresse e tecido selecionados (Pérez-Díaz *et al*., 2014). *OsASR1* e *OsASR5* apresentaram alta expressão em tecidos vasculares sob estresse, bem como expressão basal em raízes, anteras e escutelo (Pérez-Díaz *et al*., 2014).

Joo *et al*. (2013a) investigaram funcionalmente *OsASR3* e *OsASR5* (denominado no trabalho como *OsASR1*). *OsASR3* apresentou maior expressão nas raízes e é mais sensível ao ácido giberélico (GA_3) e à glicose, enquanto *OsASR5* é expresso predominantemente nas folhas, sendo fortemente induzido por ABA e tratamento com sacarose (Joo *et al*., 2013a). Plantas de arroz superexpressando *OsASR3* ou *OsASR5* apresentaram maior tolerância à seca e baixa temperatura (Joo *et al*., 2013a). A superexpressão de *OsASR5* também acarretou em

menor sensibilidade ao ABA e incremento na tolerância de plântulas de arroz à salinidade e estresse osmótico (Joo *et al*., 2013b). Ademais, maiores quantidades de glicose e frutose foram encontradas nas raízes das plantas superexpressando *OsASR5*, indicando que OsASR5 através do ABA integra vias de resposta a estresses com o metabolismo de açúcares na raízes (Joo & Song, 2015). Nota-se que maiores níveis proteicos de OsASR5 foram observados nas bainhas foliares de plântulas de arroz tratadas com GA_3 exógeno. Porém não foram observadas alterações nos níveis de transcritos, o que indica uma possível regulação de OsASR5 por GA_3 em nível pós-transcricional (Takasaki *et al*., 2008).

Plantas superexpressando *OsASR5* também foram geradas por Li *et al*. (2017), os quais também verificaram uma maior tolerância à seca. Em contraste a Joo *et al*. (2013b), os autores observaram uma hipersensibilidade a ABA na germinação e estágios iniciais de desenvolvimento nas plantas superexpressando *OsASR5*, assim como maiores concentrações endógenas do fitohormônio (Li *et al*., 2017). A superexpressão de *OsASR5* também levou ao aumento da expressão de genes atuantes na biossíntese de ABA e, paralelamente, a repressão de genes envolvidos em vias de resposta antioxidante, levando ao acúmulo de H_2O_2 (Li *et al*., 2017). Maiores concentrações desta ERO estimularam um maior nível de fechamento estomático, colaborando para a maior tolerância à seca observada, visto que maiores teores relativos de água (TRAs) foram encontrados nos tecidos das plantas superexpressando *OsASR5*. Resultados contrários foram observados nas plantas da linhagem de perda de função *osasr5*. Desta forma, os autores propuseram que OsASR5 modula o fechamento estomático através do acúmulo de H_2O_2 de forma dependente a ABA na condição de seca (Li *et al*., 2017). Ressalta-se que a potencial atividade chaperona-*like* de OsASR5 também foi proposta, já que foi observada a sua interação com duas proteínas responsivas à seca (Li *et al*., 2017).

A atividade funcional de OsASR5 (denominado no trabalho como OsASR1) também foi investigada através da sua expressão heteróloga em células de levedura (*Saccharomyces cerevisiae*) por Kim *et al*. (2012). Células transformadas apresentaram melhor crescimento sob diferentes tratamentos de estresse abiótico em comparação com células não transformadas. Sob tratamento de H_2O_2, as células transformadas apresentarem menores concentrações de EROs, indicando uma possível atividade antioxidante de OsASR5. Além disso, OsASR5 mitigou a inativação térmica de catalase de forma dose-dependente, indicando uma possível atividade chaperona-*like* da proteína (Kim *et al*., 2012).

Park *et al*. (2020) geraram plantas de arroz superexpressando *OsASR3* (denominado no trabalho como *OsASR1*). A linhagem apresentou aproximadamente o dobro da taxa de sobrevivência em comparação com plantas do tipo selvagem após o tratamento de estresse por salinidade ou seca, em concordância com resultados anteriores (Joo *et al*., 2013a). Semelhante às plantas superexpressando *OsASR5* (Li *et al*., 2017), plantas superexpressando *OsASR3* apresentaram maiores concentrações endógenas de ABA e hipersensibilidade ao fitohormônio durante a germinação e estágios iniciais de desenvolvimento (Park *et al*., 2020). Sob seca, uma maior nível de fechamento estomático, reduções significativas da condutância estomática e transpiração foram observadas, aliadas a maiores TRAs nos tecidos (Park *et al*., 2020). A superexpressão de *OsASR3* também induziu o aumento da expressão de genes envolvidos na biossíntese de ABA, indicando que OsASR3, assim como OsASR5, é um regulador positivo de ABA em condições de estresse (Li *et al*., 2017; Park *et al*., 2020).

Similar ao observado para *OsASR3* e *OsASR5* (Joo *et al*., 2013a; Li *et al*., 2017), a superexpressão de *OsASR6* levou a um melhor desempenho das plantas sob seca, apresentando maiores comprimento de raízes e densidade de raízes laterais, assim como maior produtividade em comparação com o tipo selvagem após aplicação do tratamento de seca durante a fase reprodutiva (Srivastava *et al*., 2022). Maiores atividades de enzimas antioxidantes, como APX, CAT e SOD também foram observadas, em paralelo com menores concentrações de malondialdeído (MDA), composto marcador da ocorrência de peroxidação lipídica (Srivastava *et al*., 2022). Vale destacar que vários genes atuantes na biossíntese e sinalização de ácido jasmônico (JA) foram induzidos nas plantas superexpressando *OsASR6*, indicando a participação da respectiva proteína em vias de resposta à seca através da modulação da sinalização de JA (Srivastava *et al*., 2022).

Plantas de arroz superexpressando *OsASR4* (denominado no trabalho como *OsASR6*) foram geradas por Zhang *et al*. (2022b), que observaram melhores taxas de germinação e sobrevivência de plântulas submetidas a tratamento de salinidade. Os autores também observaram maiores atividades de enzimas antioxidantes, aliadas a menores concentrações de H_2O_2 e MDA (Zhang *et al*., 2022b). Em adição, foi observada a interação direta, tanto *in vitro* quanto *in vivo*, de OsASR4 com OsNCED1 (9-CIS-EPOXYCAROTENOID DIOXYGENASE 1), enzima-chave atuante na biossíntese de ABA (Wan & Li, 2006; Changan *et al*., 2018). Isso indica que, semelhante a OsASR3 e OsASR5, OsASR4 é um

regulador positivo de vias de resposta a estresses abióticos dependentes de ABA (Zhang *et al*., 2022b).

Em relação à homeostase de metais, a caracterização funcional de OsASR1 e OsASR5 na resposta de tolerância ao Al foi progressivamente detalhada (Arenhart *et al*., 2012, 2013, 2014, 2016). Primeiramente, Arenhart *et al*. (2013) demonstraram que *OsASR5* é induzido em plantas expostas ao Al. Além disso, foi gerada a linhagem OsASR5-RNAi, cujas plantas apresentaram maior sensibilidade ao Al, principalmente no crescimento de raízes. É crucial destacar que os autores observaram reduções nos níveis de transcritos de todos os genes *OsASR* nas plantas OsASR5-RNAi. Também foi verificada a localização subcelular de OsASR5, sendo este detectado no citosol e núcleo, o que sugere a atuação desta proteína como um fator de transcrição (Arenhart *et al*., 2013).

Arenhart *et al*. (2012) também observaram a presença de OsASR5 nos cloroplastos, propondo um possível transporte da respectiva proteína, dos cloroplastos para o núcleo celular, promovendo assim a ativação de vias de defesa ao Al. Subsequentemente, genes-alvo de OsASR5 foram identificados (Arenhart *et al*., 2014). Dentre estes, estão *OsSTAR1* (*SENSITIVE TO ALUMINUM RHIZOTOXICITY 1*) e *OsSTAR2*. Ambas as proteínas codificadas por cada gene formam um complexo funcional. Este complexo medeia o transporte de uridina-difosfato de glicose (UDP-glicose), colaborando para um possível mecanismo de defesa na parede celular aos íons de Al trivalente (Al^{3+}) (Huang *et al*., 2009). Curiosamente, *OsSTAR1* também foi identificado na literatura científica como *OsENA2* (*NICOTIANAMINE EXPORTER 2*), o qual é induzido pela deficiência de Fe, mediando o transporte de NA (Nozoye *et al*., 2011). Até o momento, não está clara a função destas proteínas na resposta a Al ou Fe.

Plantas de arroz apresentando o silenciamento específico de *OsASR5* via microRNA artificial (amiRNA) foram geradas por Arenhart *et al*. (2016). Diferentemente das plantas OsASR5-RNAi, as plantas amiRNA apresentaram crescimento de raízes comparável ao tipo selvagem quando expostas ao Al (Arenhart *et al*., 2016) Além disso, os autores notaram um incremento na expressão de *OsASR1*, cuja proteína também foi detectada no citosol e núcleo. Desta forma, foi verificado que OsASR1 regula parcialmente um conjunto de genes-alvo de OsASR5, incluindo *OsSTAR1*. Estes dados levaram os autores a concluírem que OsASR1 e OsASR5 são fatores de transcrição com redundância funcional parcial entre si na resposta ao estresse por Al (Arenhart *et al*., 2016).

Plantas da linhagem OsASR5-RNAi também foram utilizadas por Barros *et al*. (2025b). Neste caso, foi observado que o silenciamento dos genes *OsASR* atrasa o desenvolvimento de raízes laterais e adventícias em plântulas. Isso gerou a hipótese de que as plantas OsASR5-RNAi poderiam não estar respondendo à deficiência de fosfato (P_i), visto a estrutura do sistema de raízes similar a um fenótipo insensível a P_i observado previamente na literatura (Péret *et al*., 2014). Deste modo, foi observada uma arquitetura do sistema de raízes das plantas OsASR5-RNAi semelhante à do tipo selvagem sob deficiência de P_i (Barros *et al*., 2025b). Por meio de análise transcriptômica, genes envolvidos no desenvolvimento de raízes e resposta à deficiência de P_i apresentaram menores níveis de expressão em plantas OsASR5-RNAi quando comparadas ao tipo selvagem (Barros *et al*., 2025). Além disso, em plantas do tipo selvagem submetidas à deficiência de P_i, foram observados incrementos nas expressões de *OsASR6* e principalmente *OsASR5*, indicando que OsASR5 possa ser um regulador positivo de genes-chave responsivos à deficiência de P_i (Barros *et al*., 2025).

1.8. Genes *VACUOLAR IRON TRANSPORTER* (*VIT*) de arroz

Primeiramente, o gene *AtVIT1* de *A. thaliana* foi caracterizado como ortólogo do gene *CCC1* de levedura, o qual codifica um transportador de Fe^{+2} do citosol para o vacúolo (Li *et al*., 2001; Kim *et al*., 2006). Enquanto a linhagem Δ*ccc1* apresenta crescimento comprometido em meio de cultura contendo excesso de Fe, a complementação com o gene *AtVIT1* restaurou o seu crescimento. Em sementes da linhagem de perda de função *atvit1-1*, as concentrações de Fe^{+2} não foram afetadas, porém a sua distribuição espacial sim, indicando a participação de AtVIT1 na homeostase de Fe (Kim *et al*., 2006; Roschzttardtz *et al*., 2009). Além disso, mutantes *atvit1-1* apresentaram germinação prejudicada em solo alcalino, no qual o Fe externo é pouco disponível, sugerindo que a correta localização de Fe na semente é importante durante a germinação (Kim *et al*., 2006).

Em arroz, foi verificado que os genes homólogos a *AtVIT1*, *OsVIT1* e *OsVIT2*, são expressos principalmente na folha-bandeira (Zhang *et al*., 2012). A complementação da linhagem Δ*ccc1* com cada gene restaurou parcialmente seu crescimento sob o tratamento de excesso de Fe, indicando que estes dois transportadores também medeiam o importe vacuolar de Fe^{+2} (Zhang *et al*., 2012). Os autores observaram que as linhagens mutantes *osvit1* e *osvit2* apresentaram aumento moderado nas concentrações não somente de Fe, mas também de Zn, em tecidos embrionários e com destaque em grãos polidos, o que também

foi observado para *osvit2* por Bashir *et al*. (2013). Posteriormente, foi observado que OsVIT2 é expresso, além da bainha da folha-bandeira, em nós do colmo e na camada de aleurona do grão. Assim, a perda de função deste gene acarreta em maiores deposições de Fe no endosperma (Che *et al*., 2021). Em adição, Kandwal *et al*. (2022) identificaram uma mutação induzida por etil-metano-sulfonato (EMS) em *OsVIT2* na linhagem 1095_k. Quando cultivadas a campo, as plantas desta linhagem não apresentaram diferenças em parâmetros de produtividade com o seu respectivo tipo selvagem. Além disso, os grãos de 1095_k apresentaram maiores concentrações de Fe e Zn. Plantas F_2 do cruzamento entre 1095_k e o tipo selvagem apresentaram resultados similares, ressaltando o potencial desta linhagem para a geração de cultivares com grãos biofortificados.

Aliada ao entendimento sobre a função molecular e fisiológica das proteínas OsVIT1 e OsVIT2 na homeostase de Fe, há a busca por elucidar as características estruturais e o mecanismo de transporte efetuado por estas proteínas. Através da modelagem estrutural das proteínas VIT1 do patógeno *Plasmodium falciparum*, principal agente causador da malária, e de *Eucalyptus grandis*, é sabido que as proteínas VIT são estruturadas como homodímeros, possuindo um domínio transmembrana e um domínio citoplasmático de ligação a metais de transição, como Fe^{+2}, Zn^{2+} e cobalto (Co^{2+}) (Labarbuta *et al*., 2017; Kato *et al*., 2019). Foi proposto que as proteínas VIT atuam como transportadores antiporte, isto é, acoplam a entrada de Fe^{2+} e de outros íons divalentes para o vacúolo com a saída de H^+ para o citosol (Labarbuta *et al*., 2017; Kato *et al*., 2019). Recentemente, em concordância com a indicação de que as proteínas da família VIT apresentam um dobramento (*folding*) exclusivo e conservado, análises *in silico* realizadas por Arend *et al*. (2025) identificaram as mesmas características ao modelar estruturalmente OsVIT2. Simulações de dinâmica molecular revelaram uma mobilidade extensa da porção citoplasmática do primeiro segmento transmembrana, a qual é correspondente ao domínio N-terminal de OsVIT2. Portanto se supõe que esta porção possa atuar como um "braço flexível" e que poderia funcionar para prospectar o citosol próximo à proteína, contribuindo na captura de íons metálicos (Arend *et al*., 2025).

Levando em conta o potencial da edição dos genes *OsVIT* para a biofortificação de Fe em grãos de arroz, Benato *et al*. (2025) geraram plantas duplo-mutantes *osvit1osvit2* via CRISPR/Cas-9. Tais plantas apresentaram incrementos significativos nas concentrações de Fe nos grãos, semelhante ao que foi observado previamente com as linhagens singulares

osvit1 e *osvit2* (Zhang *et al*., 2012; Bashir *et al*., 2013). Isso indica que as proteínas OsVIT1 e OsVIT2 possam atuar em conjunto no transporte de Fe^{2+}, ou seja, formem heterodímeros, algo ainda não evidenciado dentre as proteínas VIT. Benato *et al*. (2025) também observaram uma maior suscetibilidade das plantas *osvit1osvit2* ao estresse por excesso de Fe, apresentando crescimento e produtividade reduzidos. Este fenótipo é provavelmente decorrente da menor capacidade de compartimentalização de Fe nos vacúolos, demonstrando uma desvantagem causada pelo silenciamento simultâneo dos dois genes *OsVIT* (Benato *et al*., 2025).

2. OBJETIVOS

2.1. Objetivo geral

Analisar a contribuição funcional das proteínas da família *OsASR* para a homeostase de ferro (Fe) em arroz (*Oryza sativa* L.), assim como a possibilidade de heterodimerização entre os transportadores vacuolares OsVIT1 e OsVIT2, bem como o possível papel do domínio N-terminal de OsVIT2 no transporte de Fe^{2+}.

2.2. Objetivos específicos

2.2.1. Analisar os efeitos morfofisiológicos da supressão dos genes *OsASR* em plantas OsASR5-RNAi sob condição de deficiência de ferro, com foco em parâmetros de crescimento e fotossíntese;

2.2.2. Analisar os efeitos da supressão dos genes *OsASR* em plantas OsASR5-RNAi no ionoma de raízes, folhas e grãos;

2.2.3. Analisar os efeitos moleculares da supressão dos genes *OsASR* em plantas OsASR5-RNAi sob condição de deficiência de ferro, com foco em genes relacionados à absorção e distribuição de ferro nas raízes;

2.2.4. Analisar a resposta das plantas OsASR5-RNAi ao excesso de ferro, com ênfase em alterações morfológicas nas raízes e no desenvolvimento vegetal geral;

2.2.5. Avaliar a interação física entre os transportadores vacuolares OsVIT1 e OsVIT2, por meio de ensaios de duplo-híbrido em levedura (Y2H);

2.2.6. Caracterizar funcionalmente uma versão truncada de OsVIT2 em linhagem mutante de levedura Δ*ccc1*, com o objetivo de verificar a função do domínio N-terminal de OsVIT2 no transporte de ferro.

CAPÍTULO 1

Abscisic acid, stress, ripening (ASR) proteins play a role in iron homeostasis in rice (*Oryza sativa* L.)

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Abscisic acid, stress and ripening (ASR) proteins play a role in iron homeostasis in rice (*Oryza sativa* L.)

Lucas Roani Ponte [a], Jover da Silva Alves [a], Yugo Lima-Melo [a], Paloma Koprovski Menguer [a], Hadrien Georges Boulanger [b], Ricardo Fabiano Hettwer Giehl [c], Cristiane Paula Gomes Calixto [b], Márcia Margis-Pinheiro [a,d], Felipe Klein Ricachenevsky [a,e,*]

[a] Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil
[c] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) OT Gatersleben, Seeland, Germany
[d] Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[e] Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

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ABSTRACT

Iron (Fe) is essential for plant growth, playing a key role in photosynthesis, respiration, and nitrogen fixation. Despite its abundance, Fe is not easily available for uptake by roots, making Fe deficiency a common issue that reduces crop yields. The ASR (Abscisic acid/Stress/Ripening) proteins are known to be responsive to different abiotic stresses. Two ASR proteins from rice (*Oryza sativa* L.), OsASR1 and OsASR5, were described as transcription factors involved in aluminum (Al) toxicity response, and were shown to be partially redundant in their function. Here we explored a possible role of ASR proteins in Fe deficiency in rice plants. We showed that rice plants silenced for ASR genes (named OsASR5-RNAi) had increased sensitivity to Fe deficiency, with early and more severe chlorosis, as well as reduced photosynthesis, stunted growth, reduced seed set and altered ionome in roots, leaves and seeds. Transcriptomic analysis indicated that roots of OsASR5-RNAi plants had similar expression of Fe uptake genes, such as *OsIRT1* and *OsYSL15*. However, long distance phloem transporter *OsYSL2* was up-regulated in OsASR5-RNAi roots to a larger extent compared to WT, suggesting ASR proteins negatively regulate *OsYSL2* expression. We also identified other interesting candidate genes, such as *OsZIFL2* and *Thionins*, that are dependent on ASR proteins for regulation under Fe deficiency. Our work demonstrated that OsASR proteins are important for proper Fe deficiency response in rice.

1. Introduction

Iron (Fe) is essential for plants. It is present in the structure of several proteins and complexes that participate in electron transfer reactions during photosynthesis and respiration, including photosystems I and II, ferredoxins and cytochromes (Przybyla-Toscano et al., 2021). Fe is also a critical cofactor for nitrogenase used in symbiotic nitrogen fixation by rhizobia (Liu et al., 2023; Sankari et al., 2022). However, despite being abundant in the Earth's lithosphere, Fe is not readily available for uptake by plant roots. Consequently, crops tend to be susceptible to Fe deficiency (Trofimov et al., 2024). Often manifested as leaf chlorosis, Fe deficiency impairs photosynthesis, resulting in stunted growth, delayed flowering and yield losses (Huang and Suen, 2021; Krohling et al., 2016). On other hand, Fe excess can be harmful, since Fe can produce reactive oxygen species (ROS) by Fenton's reactions, leading to toxicity (Becana et al., 1998; Wairich et al., 2024). Therefore, Fe homeostasis must be finely regulated to ensure adequate Fe concentrations in different plant tissues.

Plants have two strategies for Fe uptake, known as Strategy I and Strategy II (Rodrigues et al., 2023; Wairich et al., 2022). Strategy I is performed by non-grass plants and is based on the solubilization of ferric ion (Fe^{3+}) by the release of H^+ protons into the rhizosphere; reduction of Fe^{3+} to ferrous ion (Fe^{2+}) by ferric chelate reductases, such as FRO2 (Robinson et al., 1999; Connolly et al., 2003); and Fe^{2+} uptake by high

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* Corresponding author. Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.
E-mail address: felipecruzalta@gmail.com (F.K. Ricachenevsky).

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affinity plasma membrane transporters, such as IRT1 (Iron-Regulated Transporter 1) (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002). On the other hand, Strategy II is performed by grasses and is based on the release of Fe^{3+} chelators, named phytosiderophores (PSs), into the rhizosphere (Carrillo and Borthakur, 2021; Nozoye et al., 2011). The intact Fe^{3+}-PS complexes are then taken up by YS1/YSL15 (Yellow-Stripe Like) transporters (Curie et al., 2001; Inoue et al., 2009; Lee et al., 2009). Plants within the *Oryza* genus, including rice (*Oryza sativa* L.), use the Combined Strategy, which consists of Strategy II coupled with the up-regulation of an IRT-like transporter, allowing these species to uptake both Fe^{3+}-PS complexes and Fe^{2+} ions (Ishimaru et al., 2006; Wairich et al., 2019).

Aluminum (Al^{3+}) toxicity is another common disorder for plants, resulting in significant yield losses. Al toxicity is typically observed in acidic soils, particularly in pH lower than 5.5. Under these conditions, Al is highly soluble and readily available for uptake by roots (Bojórquez-Quintal et al., 2017). Because of that, plants have developed mechanisms to tolerate Al toxicity. Al-tolerant plant species are known to release organic acids into the rhizosphere, such as citrate, malate and oxalate, which chelate Al^{3+} ions and prevent their entry into roots (Yang et al., 2019). In parallel, to minimize Al toxic effects on vital cellular processes, some Al-tolerant plants also show enhanced antioxidant defense systems, including the production of enzymes, such as superoxide dismutase, catalase, and peroxidases, to scavenge ROS (Ranjan et al., 2021). Furthermore, uptake by plasma membrane and subsequent vacuolar detoxification of Al^{3+} in root and/or shoot cells is also described (Yan et al., 2022).

Rice has been used as a model plant for studying Fe homeostasis due to its genetic and physiological similarities with other important cereal crops such as bread wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) (Kim et al., 2014; Song et al., 2018). The presence of the combined strategy in rice allows comparisons with Strategy I plants, with several homologous proteins playing similar roles in upstream regulatory networks and uptake mechanisms (Grillet and Schmidt, 2019, Wairich et al., 2025), as well as with Strategy II plants, for which rice is a good model given its small genome and availability of tools for molecular genetics. Since rice is usually cultivated in acidic soils, it provides an ideal system to investigate the adaptive responses and tolerance mechanisms of plants under Al toxicity (Arenhart et al., 2015; Zhang et al., 2019). Moreover, rice is considered the most Al-tolerant cereal (Famoso et al., 2010). Interestingly, Al has been shown to affect Fe homeostasis in rice as the supply of Al alleviated the chlorotic phenotype of Fe-deficient plants (Alves et al., 2025). It was suggested that Al could be causing P deficiency, which is known to alleviate Fe deficiency-induced chlorosis (Nam et al. 2021; Therby-Vale et al., 2022). However, little is known about the underlying mechanisms, and which proteins could be involved in regulating both Al toxicity and Fe deficiency responses.

The *ASR* (*Abscisic acid/Stress/Ripening*) genes are plant-specific proteins known to be involved in fruit ripening, sugar metabolism and to be responsive to different abiotic stresses, such as drought, salinity and cold (Jia et al., 2016; Li et al., 2018; Parrilla et al., 2022; Yacoubi et al., 2022). There are six *ASR* genes in the rice genome, and since *ASR* genes are not present in the *Brassicaceae* family, rice is the model of choice to understand the function of these proteins in plants (Parrilla et al., 2022; Philippe et al., 2010). Two members of the *OsASR* family, namely *OsASR1* and *OsASR5,* were linked to Al stress response in rice (Arenhart et al., 2013, 2014, 2016). Both genes are responsive to Al stress (Arenhart et al., 2013). OsASR5 was shown to control several Al-responsive genes by directly binding to their promoters (Arenhart et al., 2014). It was shown that OsASR1 and OsASR5 can functionally compensate for each other at Al responsive target gene promoters, suggesting that these genes have at least partial functional redundancy (Arenhart et al., 2016). Among other Al-responsive genes, OsASR5 is able to directly bind to *Sensitive To Al Rhizotoxicity 1* (*OsSTAR1*) promoter and is involved in *OsSTAR1* up-regulation in Al toxicity (Arenhart et al., 2014; Huang et al., 2009). *OsSTAR1* is also named *Efflux transporter of NA 2* (*OsENA2*), which has a role in Fe homeostasis (Nozoye et al., 2011). Moreover, *OsASR* transcripts have been found to be responsive to Fe in transcriptomic studies (Stein et al., 2019; Wairich et al., 2019, 2021). However, whether these proteins play a role in Fe homeostasis has hitherto remained unexplored.

Here we evaluated how knockdown rice plants of *ASR* genes respond to Fe deficiency. We found that down-regulation of the *OsASR* genes increases sensitivity to low Fe, with early onset and more pronounced leaf chlorosis, and decreases photosynthesis, growth and seed set. We also found that the mineral element composition of seeds, root and leaf tissues are altered. Transcriptomic analyses in roots indicate that OsASR5-RNAi phenotype is not associated with the main Fe deficiency pathway, i.e., regulating OsYSL15 and OsIRT1 transporters. However, *OsYSL2* is strongly up-regulated in OsASR5-RNAi plants, suggesting that Fe distribution might be involved in the observed phenotype. Our work demonstrates that ASR proteins play a role in Fe homeostasis.

2. Material and methods

2.1. Plant materials and growth

We used *Oryza sativa* L. cv Nipponbare and the previously described OsASR5-RNAi line (Arenhart et al., 2013). OsASR5-RNAi line have been characterized in detail in several other papers of our group (Arenhart et al., 2013, 2014, 2016). The construct decreases expression of several *OsASR* transcripts (see data below; Fig. 8).

Seeds of both genotypes were germinated in Petri dishes with filter paper and distilled water for 7 days and transferred to vermiculite. After two weeks, plants were transferred to a hydroponic system. The nutrient solution consisted of 700 μM K_2SO_4, 100 μM KCl, 100 μM KH_2PO_4, 2 mM $Ca(NO_3)_2$, 500 μM $MgSO_4$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 100 μM Fe^{3+}-EDTA. The pH of the nutrient solution was adjusted to 5.4 and the solution was renewed twice a week. Plants were exposed to treatments after two weeks of acclimation. For Fe deficiency treatment (−Fe), Fe was omitted from nutrient solution, while control plants were cultivated in complete nutrient solution. Plants were kept under treatment or under control condition (CC) for up to 24 days. Growth conditions were 27 °C ± 1 °C under photoperiod of 16h/8h light/dark and irradiance of 150 μmol photons m^{-2} s^{-1}. Plants grown until maturity were cultivated in greenroom conditions with photoperiod of 12h/12h light/dark and irradiance of 200 μmol photons m^{-2} s^{-1}.

2.2. Growth measurements and agronomic traits

Measurements of shoot height and root length of individual plants (n = 5) were performed using a graduated ruler. Shoot and root samples were dried in a forced ventilation oven at 60 °C for one week. The dry mass values were obtained using an analytical precision balance. At maturity, plants were evaluated for total number of panicles and number of filled and empty seeds (n = 12 to 24).

2.3. SPAD, gas exchange and fluorescence analyses

The relative chlorophyll content were measured with the Soil Plant Analysis Development (SPAD) chlorophyll meter SPAD 502 (Konica Minolta, Inc., Tokyo, Japan) from the 11th to the 21st day of treatment every two days. Measurements were performed in the central region of the fifth fully expanded leaf of the same plant. Data was obtained from five different plants (n = 5).

Gas exchange and chlorophyll *a* fluorescence was measured in the youngest fully expanded leaves from both WT and OsASR5-RNAi lines under control condition or −Fe. The measurements were performed at the 7th, 14th, and 21st days of treatment. CO_2 assimilation rate (A; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}), internal CO_2

concentration (C_i; μmol CO_2 mol air^{-1}) and transpiration (E; mmol H_2O m^{-2} s^{-1}) were measured with a LCpro-SD portable InfraRed Gas Analyzer system (IRGA; ADC Bioscientific, Hoddesdon, England). The internal parameters in the IRGA chamber during gas exchange measurements were: 27 °C, 1000 μmol photons m^{-2} s^{-1}, 420 ppm CO_2 concentration, ambient humidity (80 % ± 5 %). Each sample corresponded to the normalized value of two leaves from different plants and the measurement recordings were performed 15 min after placing the samples inside the measuring chamber with the internal parameters described previously. The water use efficiency (μmol CO_2 mmol H_2O^{-1}) was calculated as A/E and the carboxylation efficiency was calculated as A/Ci. Normalized data is used for figures presentation.

In vivo chlorophyll *a* fluorescence was measured using an OS-30p chlorophyll fluorometer (Opti-Sciences, Hudson, NH, USA). The fluorescence parameters were measured by the saturation pulse method (Schreiber et al., 1995). The maximum and the minimum fluorescence (Fm and Fo, respectively) were measured in 30-min dark-adapted plants and the maximum quantum yield of photosystem II (PSII) [Fv/Fm; (Fm–Fo)/Fm] was calculated.

2.4. Mineral element analyses

Whole root or leaf samples were dried at 65 °C and weighed in polytetrafluoroethylene tubes. Samples were digested with concentrated HNO_3 (67–69 %; Bernd Kraft) and pressurized in a high-performance microwave reactor (UltraCLAVE IV, MLS GmbH). The digested samples were diluted with deionized water (Milli-Q Reference A+, Merck Millipore). Elemental analysis was carried out by high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS) (ELEMENT 2, Thermo Fisher Scientific, Germany).

Elemental concentration of seeds was determined using an inductively couple plasma mass spectrometer (ICP-MS; Elan DRCe, PerkinElmer; or NexION 300D, PerkinElmer) as described previously (Zhang et al., 2014). The radar charts were plotted using the online tool available at https://www.bioinformatics.com.cn/en (Tang et al., 2023).

2.5. RNA extraction, library preparation and RNA-seq analyses

For the RNA-seq analyses, plants were cultivated as described above, and roots from 4 plants per sample were collected after five days of treatment. RNA extraction was performed with the Direct-zol™ RNA MiniPrep Plus Kit (Zymo Research). For each treatment and genotype, three libraries (n = 3) were sequenced using the Illumina® NovaSeq6000 system, generating 100 bp paired-end reads.

Pre-processing and analysis of RNA-seq datasets were carried out according to Vitoriano and Calixto (2021). Briefly, residual adaptor sequences were removed from raw paired-end reads using Trimmomatic version 0.39 (Bolger et al., 2014). To generate quantitative transcript-specific expression data, the trimmed reads were pseudo-aligned to the rice reference transcriptome (all cDNAs) available at the MSU Rice Genome Annotation Project Database, Osa1 Release 7 (Kawahara et al., 2013) using Salmon version 1.4.0 (Patro et al., 2017). Full differential gene expression and differential alternative splicing analysis were carried out with the 3D RNA-seq app (Guo et al., 2021).

For this, we firstly generated read counts and Transcript per Million reads (TPM) information from each Salmon output file using tximport R package version 1.10.0 and the lengthScaledTPM method. Next, transcripts with less than 1 count per million (CPM) in ≥11 out of 12 samples were removed. Subsequently, the normalization of read counts was carried out across the samples using the weighted Trimmed Mean of M-values (TMM) method. Lastly, the voomWeights pipeline of limma R package was used for differential expression analysis, setting the following thresholds: absolute $\log_2$-fold change (L2FC) ≥ 1 and adjusted P-value (FDR) ≤ 0.01. In these analyses, the contrast groups were set as OsASR5-RNAi CC x WT CC, WT −Fe x WT CC, OsASR5-RNAi −Fe x OsASR5-RNAi CC, and OsASR5-RNAi −Fe x WT −Fe, in which "CC" means control condition and "−Fe" as the Fe deficiency treatment. Raw data can be found at Sequence Read Archive (SRA) database at NCBI (National Center for Biotechnology Information), accession number PRJNA1210584.

Hierarchical clustering was used to partition the differentially expressed genes (DEGs) into 10 clusters with euclidean distance and ward.D clustering algorithm (Saraçli et al., 2013). ComplexHeatmap R package version 2.16.0 was used to make the heatmap (Gu, 2022).

For the gene ontology (GO) analysis, DEGs were separated into up- and down-regulated genes, taking into account the Fe deficiency treatment in the same genotype [i.e. WT (−Fe x CC) and OsASR5-RNAi (−Fe x CC)]. To identify enriched gene ontologies (Fisher's test, $P \leq 0.05$), we used the web-based platform Comprehensive Annotation of Rice Multi-Omics (CARMO) (Wang et al., 2015). The Venn diagrams and GO enrichment bubble graphics were plotted using the online tool available at https://www.bioinformatics.com.cn/en (Tang et al., 2023).

2.6. Statistical analyses

Our data were tested for normality and homogeneity of variance using the Shapiro-Wilk test and Levene test, respectively. Afterwards, we used Student's -test or One-Way ANOVA, followed by Tukey's test, if both assumptions were met. If the data were not normal, we followed with the Mann-Whitney U test (for independent samples) or the Kruskal-Wallis test, followed by Dunn's test for pairwise comparisons (for multiple comparisons). If the data were normal but did not meet the assumption of homogeneity of variance, we performed Welch's ANOVA, followed by the Games-Howell post-hoc test.

3. Results

3.1. Plants silenced for ASR genes are more sensitive to Fe deficiency

Transgenic rice plants expressing a construct to silence *OsASR5* (hereafter OsASR5-RNAi) were previously generated in *Oryza sativa* cv. Nipponbare (hereafter WT) background. The data presented in the original paper and here (see below) show that the expressed construct leads to knockdown of ASR proteins (Arenhart et al., 2013; see below). Since these genes can substitute one another at the promoter binding sites of their target genes and compensate for each other's loss of function (Arenhart et al., 2016), this transgenic line is excellent for exploring the role of ASR proteins in rice mineral nutrition.

We exposed 30-day-old plants of WT and OsASR5-RNAi genotypes to either control (CC) or Fe deficiency (−Fe) treatments for up to 21 days. Fe deficiency-induced chlorosis was observed in leaves of WT plants but was clearly more pronounced in OsASR5-RNAi (Fig. 1A). To better quantify the differences in Fe deficiency-induced changes in leaf color in WT and OsASR5-RNAi plants, we used SPAD to measure chlorosis in the fifth leaf of both genotypes in a time-course −Fe experiment (Fig. 1B). Measurements started after 11 days after the onset of treatment, since the fifth leaf was the last to fully expand during treatment in both genotypes. While SPAD values were slightly lower in OsASR5-RNAi plants under control condition compared to WT, OsASR5-RNAi plants showed considerably lower values compared to WT control under Fe deficiency plants (Fig. 1B). These data further demonstrate that the knockdown of the *OsASR* genes lead to more pronounced leaf chlorosis under −Fe.

We also measured shoot height, root length and their respective dry masses of WT and OsASR5-RNAi plants under control condition and −Fe. The shoot height from the two genotypes were similar in control condition, while both shoot height and shoot dry mass were lower in OsASR5-RNAi under −Fe (Fig. 1C and D). While the WT roots were shorter in −Fe than in control, OsASR5-RNAi had roots longer than WT regardless of treatment (Fig. 1E and G). Despite longer roots, OsASR5-RNAi plants showed lower root dry mass compared to WT under −Fe, while being similar in control condition (Fig. 1F and G), suggesting that OsASR5-RNAi plants partition less carbon to roots. Taken together, these

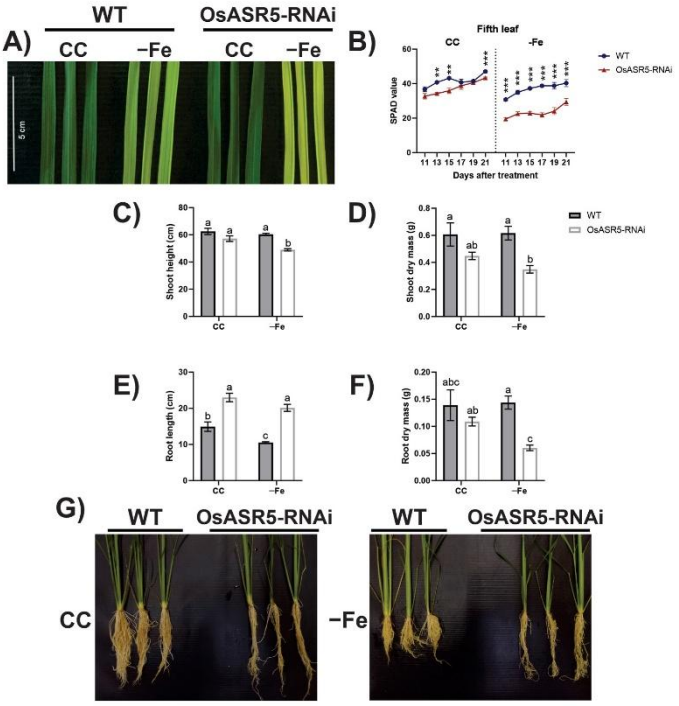


Fig. 1. The phenotype of OsASR5-RNAi plants under Fe deficiency treatment (−Fe). (A) Representative photograph of the fifth leaf of WT and OsASR5-RNAi plants after 21 days of treatment. (B) SPAD values on the fifth leaf of both genotypes during the treatment period. The asterisks denote statistically significant differences between the two genotypes within the same treatment. (C) Shoot height, (D) shoot dry mass, (E) root length and (F) root dry mass of WT and OsASR5-RNAi plants after 23 days of treatment. (G) Representative photographs of the root systems of WT and OsASR5-RNAi plants after 21 days of treatment. Error bars indicate standard error. Statistically significant differences are denoted by asterisks (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$) or by distinct letters. CC = control condition.

results suggest that OsASR5-RNAi plants are more sensitive to −Fe, showing earlier chlorosis and decreased shoot and root biomass accumulation when Fe is low.

3.2. *OsASR5-RNAi plants photosynthesis is reduced under Fe deficiency*

To quantify possible differences in photosynthesis associated with increased chlorosis in OsASR5-RNAi under −Fe, we quantified photosynthetic parameters at 7, 14 and 21 days of treatment (Fig. 2). −Fe clearly decreased the CO_2 assimilation rate in WT plants at 7 and 21 days, and in OsASR5-RNAi plants at 7 and 14 days (Fig. 2A). Interestingly, the overall values for OsASR5-RNAi under control condition was similar to WT plants exposed to −Fe, while OsASR5-RNAi under −Fe showed the lowest values in all three time points. There were no differences comparing time points within each genotype/treatment (Fig. 2A).

Stomatal conductance was similar to all genotypes at 7 days of treatment (Fig. 2B). At 14 days, stomatal conductance showed to be lower in OsASR5-RNAi under −Fe compared to both OsASR5-RNAi under control condition and WT under −Fe. At 21 days, stomatal conductance of OsASR5-RNAi under −Fe slightly increased but it was still lower compared to WT plants under −Fe (Fig. 2B). Consistently, OsASR5-RNAi plants under −Fe showed the highest internal CO_2 concentration (Fig. 2C). Transpiration rate was similar when comparing most genotype-treatment interactions, but OsASR5-RNAi plants exposed to −Fe showed reduction at 14 and 21 days, both compared to 7 days after treatment (Fig. 2D). Water use efficiency (Fig. 2E) and carboxylation efficiency (Fig. 2F) showed similar patterns as found for the CO_2 assimilation rate (Fig. 2A), with OsASR5-RNAi plants with the lowest values when exposed to −Fe.

We also measured chlorophyll *a* fluorescence. Interestingly, Fv/Fm values decreased at 14 days of treatment in OsASR5-RNAi plants under −Fe and recovered at the 21st day (Fig. 2G). WT plants under −Fe, on the other hand, only showed a slight decrease at the 21st day (Fig. 2G). Few differences were observed for Fm values (Fig. 2H), while Fo was clearly increased in OsASR5-RNAi plants under −Fe at 14 days of treatment (Fig. 2I). Taken together, these data strongly suggest that while OsASR5-RNAi plants have defects in photosynthesis, −Fe exacerbates these phenotypes.

3.3. *Elemental profiling of leaves and roots of OsASR5-RNAi plants*

To understand whether down-regulation of *OsASR* genes affected the ionome, we quantified the concentrations of elements in leaves and roots of WT and OsASR5-RNAi plants under control condition and −Fe (Fig. 3). In both treatments, we observed increases in concentrations of some elements in leaves of OsASR5-RNAi plants compared to WT, such as: calcium (Ca; 72 %), copper (Cu; 81 %), manganese (Mn; 67 %), phosphorus (P; 28 %) and Fe (46 %) under control condition; and Cu (32 %), P (55 %), potassium (K; 16 %), sulphur (S; 20 %) and zinc (Zn; 31 %)

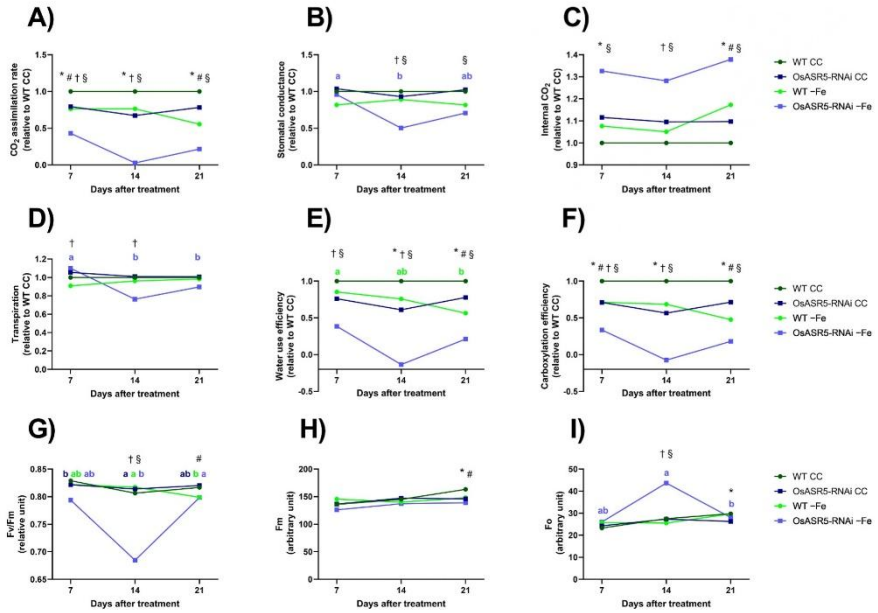


Fig. 2. The impact of *OsASR* genes knockdown and Fe deficiency treatment on photosynthesis. Changes in (A) CO_2 assimilation rate, (B) stomatal conductance, (C) internal CO_2 concentration, (D) transpiration rate, (E) water use efficiency, (F) carboxylation efficiency, (G) maximum quantum efficiency of PSII photochemistry (Fv/Fm), and (H; Fm) maximal and (I; Fo) minimal fluorescence of WT and OsASR5-RNAi rice plants under control condition (CC) or Fe deficiency treatment (−Fe) for 7, 14, and 21 days. All values were normalized relative to WT CC. The symbols indicate statistically significant differences determined by Student's t-test ($P \leq 0.05$) between WT CC and OsASR5-RNAi CC (*), WT CC and WT −Fe (#), OsASR5-RNAi CC and OsASR5-RNAi −Fe (†), or WT −Fe and OsASR5-RNAi −Fe (§) in each time of treatment. Different letters with the same color mean statistically significant difference comparing treatment times within the genotype-treatment group represented with the corresponding color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

under −Fe (Fig. 3A and B). Increases were also observed in roots of OsASR5-RNAi plants in both treatments: Ca (11 %), Cu (163 %), Mn (40 %) and Zn (19 %) under control condition; and Cu (94 %), Fe (94 %), Mn (48 %), P (29 %) and Zn (57 %) under −Fe (Fig. 3C and D). K was the only element to show reduced concentrations in OsASR5-RNAi roots in both treatments: 15 % in control condition and 28 % in −Fe (Fig. 3C and D). Furthermore, S showed a 13 % decrease only in the control condition in OsASR5-RNAi roots (Fig. 3C).

To understand how −Fe affect both genotypes, we also compared the ionomes of plants from the same genotype in control condition and −Fe in both leaves and roots (Fig. S1). There is an obvious decrease in Fe concentration in leaves and roots of both genotypes, as expected (Fig. S1). Interestingly, we found that P concentration is increased in OsASR5-RNAi leaves, but not in WT leaves, when plants are under −Fe (Figs. S1A and S1B). Zn, S, Ca, Mn and Mg decreased in WT leaves, whereas Cu and K increased (Fig. S1A). In OsASR5-RNAi leaves, Ca, Mn and Mg were decreased, whereas K increased by Fe deficiency (Fig. S1B). In roots, P and Mn is decreased in both genotypes, whereas Cu increased (Figs. S1C and S1D). Taken together, these results suggest that OsASR5-RNAi ionome changes differ from that of WT, which could be related to the −Fe chlorotic phenotype.

3.4. The knockdown of the OsASR genes reduces seed set and changes the seed ionome

We quantified seed set and panicle number in WT and OsASR5-RNAi plants. Panicle number did not differ between WT and OsASR5-RNAi (Fig. 4A). The total number of seeds produced by both genotypes was similar. However, OsASR5-RNAi plants showed higher number of empty seeds and consequently lower number of filled seeds (Fig. 4B). We next analyzed the ionome of seeds from OsASR5-RNAi plants compared to WT. We found that concentrations of several elements are increased in seeds of OsASR5-RNAi plants other than Fe (14 %), such as K (31 %), P (25 %), magnesium (Mg; 16 %), Zn (47 %), Mn (56 %), Cu (58 %) and cadmium (Cd; 254 %) (Fig. 4C). Therefore, these data suggest that the knockdown of *OsASR* genes affects seed set in rice plants, which may also result in increased concentration of several elements in filled seeds.

3.5. Transcriptomic analyses of Fe deficiency response in OsASR5-RNAi plants

To gain insight into possible changes in the transcriptome of OsASR5-RNAi plants that can be associated with increased −Fe sensitivity, we performed RNA-seq of root tissues of both WT and OsASR5-RNAi plants under control condition and −Fe (n = 3). An average of 24.2 million pairs of reads per sample were generated and used to obtain transcript-specific expression data using Salmon (Patro et al., 2017). Approximately 87 % of the 200-bp read pairs mapped to the rice reference transcriptome Osa1 Release 7 (Kawahara et al., 2013) (Supplementary Table S1), allowing for expression quantification of 55, 683 genes (Supplementary Table S2).

The R package 3D RNA-seq was used to perform analyses of differential gene expression (Guo et al., 2021). We identified 1412 differentially expressed genes (DEGs; Supplementary Table S3 and Fig. 5A). From these, 948 genes were regulated by −Fe in WT (406 up-regulated and 542 down-regulated), while 797 genes were regulated in

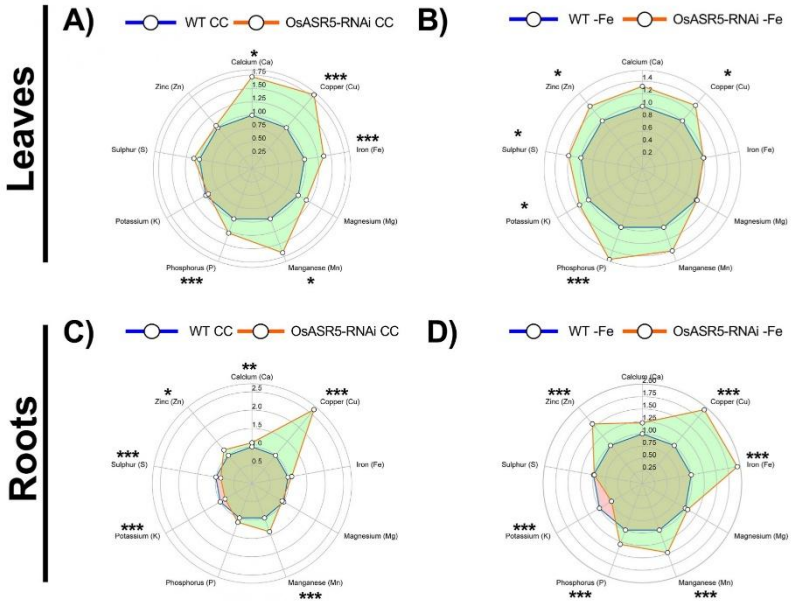


Fig. 3. Radar charts representing the average concentration (n = 6; μg g^{-1} dry mass) of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), sulphur (S) and zinc (Zn) in the leaves and roots of the OsASR5-RNAi line relative to the wild-type (WT) under control condition (CC) or iron deficiency (−Fe) at 21 days of treatment. Statistically significant differences were determined by Student's t-test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

OsASR5-RNAi plants (355 up-regulated and 442 down-regulated). Comparing the up-regulated gene sets, we found 288 genes commonly up-regulated in both genotypes, whereas WT plants showed 118 specific up-regulated genes, and OsASR5-RNAi plants showed 67 genes (Fig. 5B). Among the down-regulated genes, 247 were commonly down-regulated in both genotypes, whereas 295 and 195 were specific to WT and OsASR5-RNAi, respectively (Fig. 5C). These results confirm widespread transcriptomic changes in response to not only Fe deficiency but also knockdown of *ASR* genes in rice.

To gain insight into the pathways and functions potentially associated with the role of OsASR proteins under −Fe, we performed functional enrichment analysis of the DEG groups (Fig. 5B and C) using CARMO (Wang et al., 2015). A total of 22 statistically significant molecular function (MF) categories were detected for the group of genes up-regulated by Fe deficiency in both WT and OsASR5-RNAi genotypes (Fig. 6A). Among these, we found "Transporter activity", "Metal ion transmembrane transporter activity", "Nicotianamine synthase activity", "Metal ion binding", "Zinc ion transmembrane transporter activity", "Iron ion binding" and "Iron ion transmembrane transporter activity" (Fig. 6A and Supplementary Table S4). Interestingly, eight categories were specifically enriched among up-regulated genes in WT, including "Iron ion binding", "Oxidoreductase activity", "ATPase activity", "Heme binding", "Nucleoside-triphosphatase activity" and "Transporter activity" (Fig. 6B and Supplementary Table S4). The genes exclusively up-regulated in OsASR5-RNAi plants were only enriched for "Heme binding" and "Oxidoreductase activity" (Supplementary Table S4). These results confirm that −Fe significantly upregulated genes related to Fe metabolism and metals transport.

We found 27 statistically significant categories commonly enriched in down-regulated genes between WT and OsASR5-RNAi (Fig. 6C and Supplementary Table S4). Among these, we found "Oxidoreductase activity", "Heme binding", "Iron ion binding", "Calcium ion binding", "Metal ion binding", "Nutrient reservoir activity" and "Transporter activity". In WT, 15 categories were specific considering the down-regulated genes, including categories such as "Iron ion binding" and "Metal ion binding" (Fig. 6D and Supplementary Table S4). In OsASR5-RNAi, 19 categories were enriched considering the down-regulated genes, including "ATP binding", "ADP binding", "Kinase activity", "Nucleotide binding", "Protein kinase activity" and "Protein serine/threonine kinase activity" (Fig. 6E and Supplementary Table S4). Taken together, these results demonstrate that, despite similarities, the two genotypes have some differences in how they respond to −Fe.

3.6. Differentially expressed genes under Fe deficiency in OsASR5-RNAi plants

To better understand how OsASR5-RNAi plants differ in their response to Fe deficiency compared to WT plants, we performed hierarchical clustering of all 1412 DEGs based on expression patterns, and separated them into 10 clusters (Fig. 5A and Supplementary Table S5).

Among these, we identified two clusters that contained mostly genes up-regulated by −Fe, regardless of the genotype: clusters 4 and 7. Cluster 4 has 196 genes, including the transcription factors *OsIRO2/OsbHLH056* (Ogo et al., 2006, 2011), *OsIRO3/OsbHLH063* (Li et al., 2022a; Zheng et al., 2010), *OsbHLH133* (Wang et al., 2013) and *OsFIT/OsbHLH156* (Wang et al., 2020); E3 ubiquitin ligase *OsHRZ2* (Guo et al., 2022; Kobayashi et al., 2013); *OsOPT7* (Bashir et al., 2015); *OsIMA1* (*IRONMAN* (Kobayashi et al., 2021; Peng et al., 2022)); *OsMIR* (de Oliveira et al., 2020; Ishimaru et al., 2009); the nicotianamine (NA)/deoxymugineic acid (DMA) transporter *OsZIFL4/OsTOM1* (Nozoye et al., 2011; Ricachenevsky et al., 2011); the two efflux transporters of NA *OsENA1* and *OsENA2/OsSTAR1* (Huang et al., 2009; Nozoye et al., 2019); and several other transporters such as *OsNRAMP1* (Curie et al., 2000), *OsIRT2* (Nakanishi et al., 2006), *OsYSL13* (Ponte

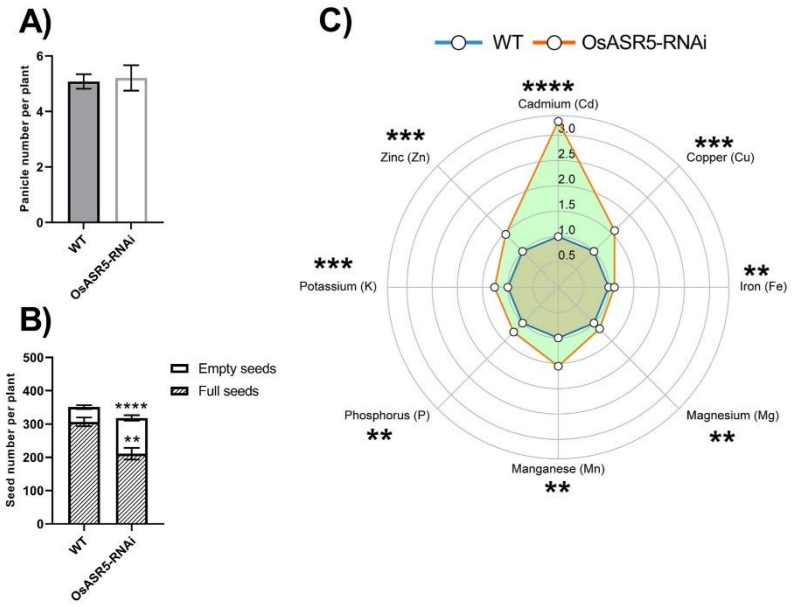


Fig. 4. The impact of *OsASR* genes knockdown on seed set and ionome composition. (A) Panicle number per plant and (B) number of filled and empty seeds per plant between WT and OsASR5-RNAi genotypes. (C) Radar chart representing the average concentration (n = 5; μg g^{-1} dry mass) of cadmium (Cd), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P) and potassium (K) in seeds of the OsASR5-RNAi line relative to the wild-type (WT). Statistically significant differences were determined by Student's t-test (**$P \leq 0.01$; ***$P \leq 0.001$).

et al., 2023; Zhang et al., 2018) and *OsZIFL12/OsVMT* (Che et al., 2019; Ricachenevsky et al., 2011) (Fig. S2 and Supplementary Table S5). Genes encoding transporters for other nutrients were also identified, such as the Cu transporter *OsCOPT7* (Guan et al., 2024; Tang et al., 2024), the sulphate transporter *OsSULTR3;3* (Zhao et al., 2016) and the Zn transporter *OsZIP4* (Ishimaru et al., 2005). In addition, the arsenic (As)-responsive transporters *OsABCB11* (LOC_Os01g50100) (Wang et al., 2017) and *OsMATE2* (Das et al., 2018) were also present (Fig. S2 and Supplementary Table S5). Cluster 7 has 191 genes, including other well-known genes responsive to −Fe such as the E3 ubiquitin ligase *OsHRZ1* (Kobayashi et al., 2013), and important transporters, such as *OsIRT1* (Bughio et al., 2002; Lee and An, 2009), *OsYSL15* (Inoue et al., 2009; Lee et al., 2009), *OsYSL9* (Senoura et al., 2017) and *OsPEZ2* (Bashir et al., 2011) (Fig. S3 and Supplementary Table S5).

While all genes in Cluster 4 are similarly regulated in WT and OsASR5-RNAi plants, 15 genes in Cluster 7 show differences in expression levels comparing the two genotypes, being either not up-regulated by −Fe in OsASR5-RNAi plants or up-regulated to a lower extent compared to WT (Supplementary Table S5). Among these genes, we found the pleiotropic drug resistance (PDR) ABC transporter *OsPDR16/OsABCG32* (*LOC_Os01g24010*) (Crouzet et al., 2006; Verrier et al., 2008); a hypothetical protein encoding a small peptide (*LOC_Os02g37260*), a terpene synthase (*LOC_Os08g07120*) and the laccase gene *OsLAC23* (*LOC_Os11g42200* (Liu et al., 2017);) (Fig. 7A−7D). Another eight genes are located in chromosome 7, with five located within ~200 kb. These include one transposon gene (*LOC_Os07g25030*) and four thionin-like genes, organized in two in tandem pairs (*LOC_Os07g24820* and *LOC_Os07g24830*; *LOC_Os07g25050* and *LOC_Os07g25060*) (Fig. 7E−7I). Moreover, *OsZIFL2* was up-regulated by −Fe in WT but not in OsASR5-RNAi (Fig. 7J). These genes seem to be directly or indirectly dependent on OsASR proteins for up-regulation under −Fe.

In Cluster 10, we found genes that are negatively regulated by OsASR proteins, since their expression was up-regulated under −Fe only in OsASR5-RNAi plants (Fig. 5A and Supplementary Table S5). Among them, we found *OsYSL2*, a known Fe-NA transporter located in the phloem that is important for long distance Fe translocation. *OsYSL2* is induced by −Fe in roots of WT plants, in agreement with data from the literature (Ishimaru et al., 2010; Koike et al., 2004). Interestingly, OsASR5-RNAi plants show expression levels under control condition comparable to WT under −Fe, and much more pronounced up-regulation under −Fe (Fig. 8A). The result suggests that OsASR5-RNAi might not be able to adequately regulate long-distance Fe transport.

Cluster 5 contained genes down-regulated in response to −Fe in both genotypes (Fig. 5A and Supplementary Table S5), including *OsFER1* and *OsFER2* (Stein et al., 2009), which are known to be positively regulated by Fe excess (Wairich et al., 2021) and negatively by −Fe (Wairich et al., 2019) (Fig. 8B and C). We also observed that *OsASR* genes are grouped in Cluster 3 and Cluster 9 (Supplementary Table S5 and Supplementary Table S6). *OsASR1* and *OsASR2* are in Cluster 3. They were both down-regulated by −Fe in WT, alongside *OsASR3* (Fig. 8D−8F). Intriguingly, *OsASR1* was the only *OsASR* gene to be not down-regulated in OsASR5-RNAi under the control condition (Fig. 8D). *OsASR3* and the three remaining *OsASR* genes, located in Cluster 9, were down-regulated in OsASR5-RNAi in both treatments (Fig. 8F−8I), which demonstrates that the construct is decreasing the expression of transcripts from this gene family. Altogether, these results demonstrate that *OsASR1*, *OsASR2* and *OsASR3* transcripts tend to be down-regulated in roots of WT plants under −Fe, and that the OsASR5-RNAi plants have decreased expression of *OsASR* genes, except for *OsASR1*.

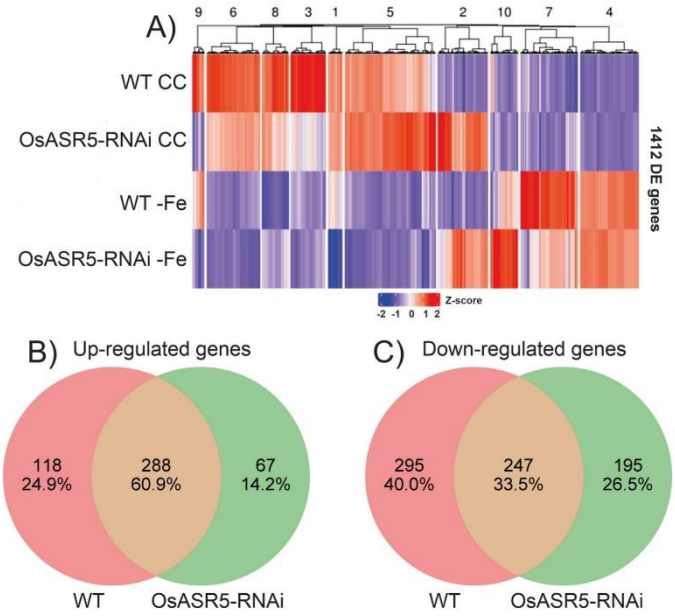


Fig. 5. Differentially expressed genes (DEGs) under Fe deficiency (−Fe) in both WT and OsASR5-RNAi genotypes. (A) Heatmap of 1412 DE genes between WT and OsASR5-RNAi plants under control condition (CC) or Fe deficiency (−Fe). (B and C) Venn diagrams showing the specific and common up- or down-regulated genes between the two genotypes under −Fe.

4. Discussion

ASR proteins are known to play a role in plant responses to abiotic stresses, such as drought, salinity and cold, as well as in fruit ripening regulation (Jia et al., 2016; Li et al., 2018; Parrilla et al., 2022; Yacoubi et al., 2022). Two ASR proteins from rice, OsASR1 and OsASR5, are well characterized as transcription factors and are positive regulators of genes involved in aluminum (Al) tolerance (Arenhart et al., 2013, 2014, 2016). In these studies, OsASR5-RNAi plants were more sensitive than WT to Al toxicity, as well as showing decreased transcript levels for *OsASR* genes (Arenhart et al., 2013). The latter is in accordance with our RNA-seq data (Fig. 8D–I; Supplementary Table S2). *OsASR3* to *OsASR6* showed lower expression levels in OsASR5-RNAi plants regardless of the treatment (Fig. 8F–8I; Supplementary Table S6). *OsASR2* showed lower expression levels in OsASR5-RNAi only under the control condition (Fig. 8E; Supplementary Table S6). In addition, *OsASR1*, *OsASR2* and *OsASR3* showed to be down-regulated by −Fe in WT plants (Fig. 8D–8F; Supplementary Table S6). Thus, our data indicate that the expression of some *OsASR* genes is altered regarding the Fe status of the plant.

We observed that OsASR5-RNAi plants are more sensitive to −Fe, showing decreased shoot growth, more pronounced chlorosis and decreased photosynthetic activity (Figs. 1 and 2). Under control condition, OsASR5-RNAi plants have increased Fe concentration in leaves, whereas under −Fe, OsASR5-RNAi plants leaf Fe concentration was similar to WT (Fig. 3A and B). Roots of OsASR5-RNAi plants have higher Fe concentration under −Fe compared to WT (Fig. 3D). Therefore, it seems that Fe distribution in OsASR5-RNAi is disturbed, with roots retaining Fe under −Fe, which may be linked to lower shoot growth. It is interesting to note that P concentration is increased in OsASR5-RNAi leaves in both treatments (Fig. 3A and B), and in roots under −Fe (Fig. 3D). Fe deficiency is known to increase P concentration in roots and shoots of rice plants (Alves et al., 2025), and antagonistic interaction of Fe and P homeostasis has been extensively studied (Guo et al., 2022; Lay-Pruitt et al., 2022; Nam et al., 2021; Therby-Vale et al., 2022; Yang et al., 2024; Wairich et al., 2025). In *Arabidopsis thaliana*, −Fe leads to the accumulation of ROS in the chloroplast, which triggers the down-regulation of *AtbZIP58*, a basic leucine zipper transcription factor that controls the expression of several photosynthesis-related genes (Nam et al., 2021). This results in the occurrence of −Fe-induced chlorosis in leaves, which is dependent on P bioavailability. On the other hand, under combined Fe and P deficiency (−Fe −P), ascorbic acid accumulates in the chloroplast via the inorganic phosphate (Pi) transporter AtPHT4; 4, preventing the accumulation of ROS and consequently blocking the down-regulation of *AtbZIP58*. Thus, −Fe-induced chlorosis is not observed in −Fe −P (Nam et al., 2021). In addition, a reciprocal inhibitory module was also described between the negative regulators of Fe homeostasis, OsHRZ1 and OsHRZ2, and the central regulators of Pi signaling, OsPHR1 and OsPHR2 (PHOSPHATE STARVATION RESPONSE). Under −Fe, the HRZs are up-regulated both transcriptionally and translationally, which triggers the degradation of PHRs. This is supposedly a mechanism to prevent Pi accumulation from interfering with Fe root-to-shoot translocation (Guo et al., 2022). Furthermore, *phr2* and *phr1 phr2* plants were more chlorotic and had lower Fe shoot/root rates under −Fe and also in −Fe −P. Opposite results were observed in *phr2 hrz2* plants, indicating that OsPHRs positively promote the −Fe response by indirectly promoting the degradation of OsHRZs (Guo et al., 2022). Our results indicate that OsASR5-RNAi plants have higher P concentrations, which could be involved in the increased susceptibility to −Fe-induced chlorosis. Further work will be necessary to explore if the mechanism involving OsPHR2/OsHRZs are linked to ASR proteins.

It is also important to mention the increases observed in the

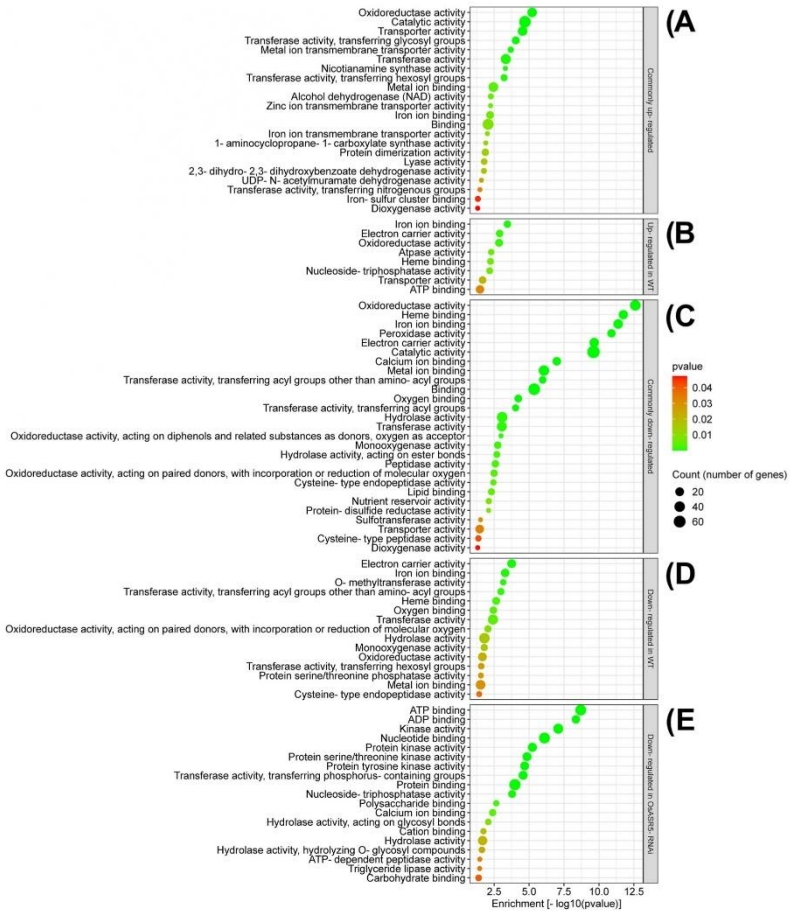


Fig. 6. Enrichment analysis of molecular functions (MF) categories of: (A) commonly up-regulated genes, (B) up-regulated genes in WT, (C) commonly down-regulated genes, (D) down-regulated genes in WT and (E) down-regulated genes in OsASR5-RNAi, comparing -Fe and control data for each genotype.

concentrations of other elements. Elemental homeostases are known to cross-talk with that of Fe by common regulatory pathways or simply by non-specific transport performed by induced divalent metal transporters (Rai et al., 2021; Wu et al., 2022; Wairich et al., 2025). Cu showed higher concentrations in the roots and leaves of OsASR5-RNAi plants under both treatments, when compared with the WT (Fig. 3). It was demonstrated that −Fe stimulates greater uptake and accumulation of Cu in different plant species (Garcia-Molina et al., 2020; Kastoori Ramamurthy et al., 2018; Waters et al., 2012; Waters and Armbrust, 2013), supposedly as a strategy for Cu/Zn superoxide dismustases (SODs) to compensate for the low levels of Fe-containing SODs in ROS scavenging (Waters et al., 2012). Regarding Zn, we observed higher concentrations in OsASR5-RNAi leaves and roots under −Fe compared to WT in the same conditions (Fig. 3B and D). Kastoori Ramamurthy et al. (2018) also observed higher Zn concentrations in Arabidopsis roots and rosettes under −Fe, but lower in comparison with the increases in Cu concentrations. It is worth noting that the Fe(II) transporter OsIRT1, which is up-regulated under −Fe, is also capable of transporting Zn, which may explain the increases in Zn concentrations in plants subjected to −Fe (Korshunova et al., 1999; Wu et al., 2022). However, *OsIRT1* was not differentially expressed between the two genotypes in our RNA-Seq data (Fig. S3B). It will be an interesting avenue to explore how other micronutrients might be involved in OsASR5-RNAi phenotype under Fe deficiency.

Our data suggest that the mechanism by which OsASR proteins might be affecting Fe homeostasis does not involve altered Fe uptake (Kobayashi and Nishizawa, 2012). Besides *OsIRT1*, we found that −Fe markers such as *OsYSL15*, *OsIRO2*, *OsHRZ1* and *OsHRZ2*, among others (Bughio et al., 2002; Inoue et al., 2009; Kobayashi et al., 2013; Lee et al., 2009; Lee and An, 2009), are similarly up-regulated in roots of both genotypes (Fig. S2 and Fig. S3). These data suggest that OsASR5-RNAi plants are able to properly induce the main regulatory network controlling Fe uptake, which is regulated by the transcription factor OsIDEF1 (Kobayashi et al., 2007; Kobayashi and Nishizawa, 2012). Therefore, this suggests that Fe homeostasis in OsASR5-RNAi plants is misregulated in other aspects, such as long-distance Fe transport.

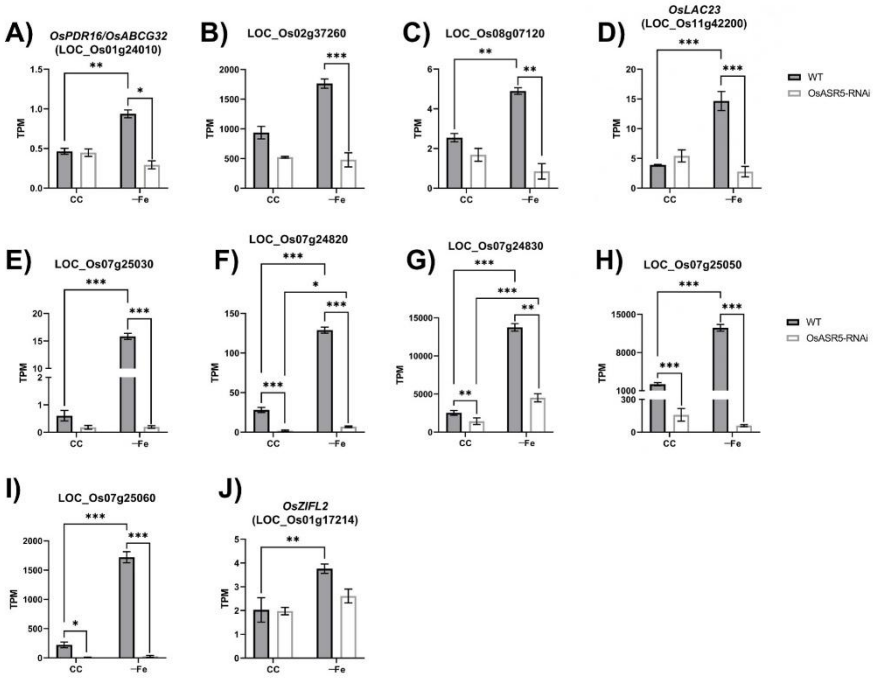


Fig. 7. Normalized expression levels from RNA-Seq of key genes located into cluster 7 under control condition (CC) or Fe deficiency (−Fe), such as (A) *OsABCG32/OsPDR16*, (B) a hypothetical protein encoding a small peptide (LOC_Os02g37260), (C) a terpene synthase (LOC_Os08g07120), (D) a laccase (LOC_Os11g42200), (E) a transposon gene (LOC_Os07g25030), (F to I) four thionin-like genes and (J) *OsZIFL2*. Statistically significant differences were carried out with the 3D RNA-seq app (* adjusted $P \leq 0.01$; ** adjusted $P \leq 0.001$; *** adjusted $P \leq 0.0001$). TPM, transcripts per million.

In agreement with that, *OsYSL2* expression was up-regulated in OsASR5-RNAi plants compared to WT, either in control condition or −Fe (Fig. 8A). OsYSL2 is a Fe(II)/Mn(II)-NA transporter that is involved in Fe phloem long-distance transport within the plant and Fe translocation into grains (Ishimaru et al., 2010; Koike et al., 2004; Nozoye et al., 2007). Its expression is directly regulated by the NAC transcription factor IDEF2, which is constitutively expressed regardless of the Fe status of the plant (Ogo et al., 2008). Since IDEF2-OsYSL2 regulatory module is distinct from OsIDEF1-OsIRO2-OsYSL15 module (Kobayashi et al., 2010; Kobayashi and Nishizawa, 2012), our data suggest that OsASR proteins might act as a regulator of this branch of Fe deficiency response in roots.

It is also known that there is natural variation of *OsYSL2* in rice genotypes. A minor *OsYSL2* allele, named as *CF1*, was linked to Cd accumulation in grains indirectly by affecting Fe homeostasis (Li et al., 2022b). The authors observed that near isogenic lines with the high-expression, high Fe affinity *CF1*-allele, compared to the major allele, had higher concentrations of Fe in shoots and grains, while having lower concentrations of Cd. They suggested that since CF1 has no affinity to Cd-NA, Fe status is higher in plants with the *CF1* allele, which results in decreased expression of Fe and Cd transporter *OsNRAMP5* and *OsNRAMP1*, consequently decreasing Cd translocation to aerial tissues (Ishimaru et al., 2012; Takahashi et al., 2011, 2014; Zhang et al., 2024). Although we observed higher expression of *OsYSL2* in OsASR5-RNAi and increased chlorosis, but no change in *OsNRAMP5* or *OsNRAMP1*, it is possible that OsASR5-RNAi is interfering with the regulation of OsYSL2, changing Fe distribution and resulting in increased chlorosis. Moreover, we found that *OsASR* down-regulation changed the seed ionome, comparable to the phenotype observed in CF1 minor allele-harboring plants (Li et al., 2022b). For example, we observed a substantial increase in the concentration of Cd in the grains of *OsYSL2*-up-regulated OsASR5-RNAi plants, when compared to WT (Fig. 4C). Higher concentration of Cd was also observed in the grains of plants carrying the high Fe affinity CF1-allele, suggesting that OsASR5-RNAi plants misregulating of OsYSL2 might be linked to the observed seed phenotype. Taken together, these data suggest that OsASR proteins might be involved in the regulation of long-distance Fe transport through OsYSL2.

Another possible downstream candidate for OsASR proteins regulation is OsZIFL2 (ZINC-INDUCED FACILITATOR LIKE) (Ricachenevsky et al., 2011). *OsZIFL2* was up-regulated by −Fe in roots of WT plants, but not in OsASR5-RNAi plants (Fig. 7J). The data suggest that OsASR proteins might be positive regulators of *OsZIFL2*. AtZIF1 from *Arabidopsis thaliana* was the first ZIFL protein functionally characterized (Haydon et al., 2012; Haydon and Cobbett, 2007). It was shown to be a vacuolar transporter, affecting Fe and Zn homeostasis by changing NA distribution. Two paralogs, *AtZIFL1* and *AtZIFL2*, were also characterized, and it was shown that AtZIFL1 is at least partially redundant with AtZIF1, whereas AtZIFL2, albeit also affecting Fe and Zn homeostasis, does not functionally overlap with AtZIF1 (Lee et al., 2021). In rice and other monocots, the *ZIFL* gene family is extensively expanded (Ricachenevsky et al., 2011). This might be related to the diversification of substrates, since *Poaceae* species synthesize and use phytosiderophores (PSs) for metal uptake and movement within the plant. For

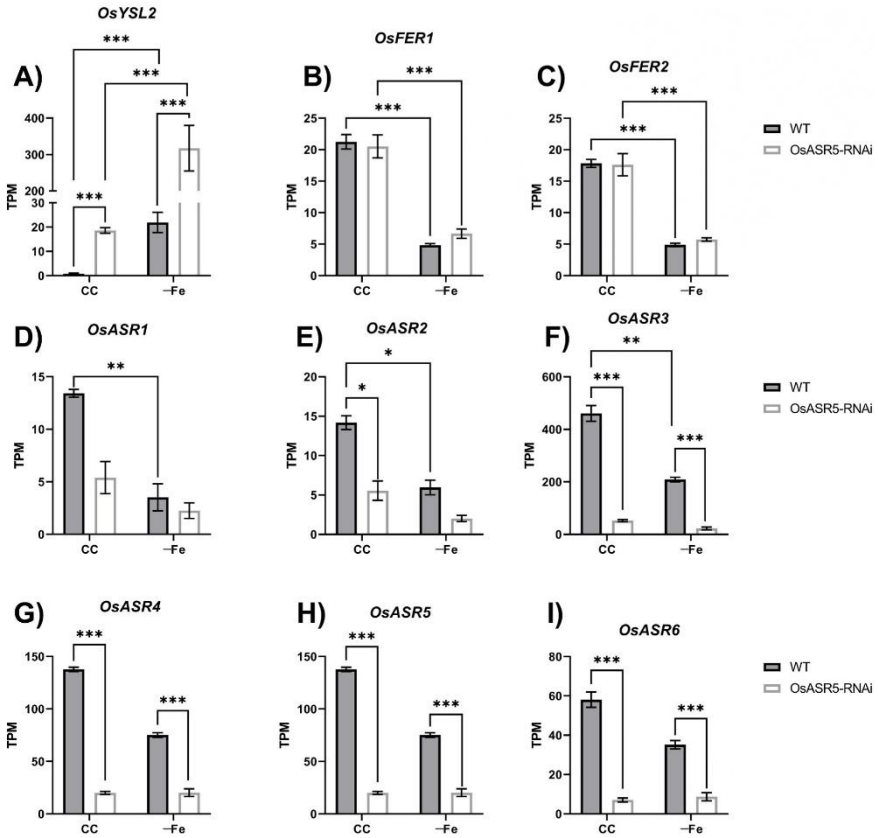


Fig. 8. Normalized expression levels from RNA-Seq of (A) *OsYSL2*, (B) *OsFER1*, (C) *OsFER2* and (D to I) the six rice *OsASR* genes under control condition (CC) or Fe deficiency (−Fe). Statistically significant differences were determined using the 3D RNA-seq app (* adjusted $P \leq 0.01$; ** adjusted $P \leq 0.001$; *** adjusted $P \leq 0.0001$). TPM, transcripts per million.

example, OsZIFL4/TOM1 is a plasma-membrane-localized transporter that secretes PSs to the rhizosphere (Nozoye et al., 2011), whereas OsZIFL12/OsVMT is a vacuolar NA/DMA transporter (Che et al., 2019). Knockdown or knockout of either gene affects Fe homeostasis. Yet, OsZIFL2 has no characterized function, but it is plausible that it may play a role in NA and/or PS transport within plant cells, and therefore might be involved in regulating Fe transport.

The four genes annotated as *thionins-like* (LOC_Os07g24820, LOC_Os07g24830, LOC_Os07g25050 and LOC_Os07g25060) also represent potential downstream targets of the OsASR proteins, since all were significantly less expressed in OsASR5-RNAi plants compared to the WT in −Fe (Fig. 7F–7I). To a lesser extent, the same was observed in the control condition for LOC_Os07g24820 and LOC_Os07g25050. Interestingly, LOC_Os07g24820 and LOC_Os07g24830 were expressed in OsASR5-RNAi under −Fe, when compared to the control condition. Thionins are small peptides that contain around 45–48 amino acid residues and have a characteristic cysteine-rich structure (Stec, 2006). They are typically known to have a broad-spectrum antimicrobial activity, which makes them important components of the plant's innate immune system against different pathogens, such as bacteria, fungi and nematodes (Höng et al., 2021). In this sense, it is known that leguminous plants secret small cysteine-rich peptides from roots to promote Fe sequestration. This triggers an increase in Fe uptake by bacteria, which stimulates nitrogenase, thus improving the nitrogen symbiotic fixation process (Sankari et al., 2022). In the rice genome, 44 genes coding for canonical thionins (OsTHIONs) were identified (Silverstein et al., 2007). To our knowledge, few rice thionin genes have been studied (Boonpa et al., 2019; Ji et al., 2015; Kitanaga et al., 2006). It was observed that the overexpression of *OsTHION9* resulted in Cd-sensitive yeast growth in media containing 10 μM or 20 μM Cd (Liu et al., 2023). *OsTHION9*-overexpressing rice plants showed higher yield per plant and reduced Cd levels in shoots and grains compared to the WT when grown in Cd-contaminated soil. The opposite results were observed in OsTHION9-knockout plants. Taken together, thionins and thionins-like seem to be interesting candidate genes for a possible role in Fe homeostasis and other metals.

5. Conclusions

Our work demonstrates that ASR proteins have a role in Fe

homeostasis. We observed decreased growth and photosynthesis, increase leaf chlorosis, and altered ionome in plants silenced for ASR genes. Transcriptomic data suggests that *OsYSL2* is misregulated in OsASR5-RNAi plants, suggesting that the phenotype might result from altered Fe long-distance transport. We also identified other candidate genes that might be involved in Fe deficiency sensitivity, such as putative thionin and thionin-like peptides.

Declaration of competing interest

The authors declare that there are no potential competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.109882.

Data availability

Data will be made available on request.

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CAPÍTULO 2

Impacts of the down-regulation of *ABSCISIC ACID*, *STRESS*, *RIPENING* (*ASR*) genes in rice (*Oryza sativa* L.) plants exposed to iron toxicity stress

Impacts of the down-regulation of *Abscisic acid*, *Stress*, *Ripening* (*ASR*) genes in rice (*Oryza sativa* L.) plants exposed to iron toxicity stress

Lucas Roani Ponte[1], Yugo de Lima de Melo[2], Márcia Margis-Pinheiro[3], Felipe Klein Ricachenevsky[1,2]

[1] Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

[3] Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

ABSTRACT

ASR (*Abscisic acid*, *Stress*, *Ripening*) genes play key roles in plant responses to abiotic stresses, sugar metabolism, and phytohormone signaling, especially abscisic acid (ABA). The simultaneous down-regulation of all six *ASR* genes from rice (*Oryza sativa* L.), exhibited in the OsASR5-RNAi line, showed to be associated with a stunted root system under optimal nutrient availability and also insensitive to phosphate starvation. In addition, it was shown the involvement of OsASR proteins in the regulation of iron (Fe) deficiency response. This study focused on the morphophysiological characterization of OsASR5-RNAi plants under Fe toxicity, a stress condition frequently encountered in paddy fields. Our data demonstrated that OsASR5-RNAi plants showed impaired photosynthetic activity and specially reduced root growth and development compared to wild-type plants. Collectively, our findings underscore the multifaceted roles of *OsASR* genes in coordinating stress responses, root development, and metabolic balance.

Keywords: rice, ASR, iron excess, roots.

1. Introduction

Rice (*Oryza sativa* L.) plays a crucial role in human nutrition due to its cultural significance and economic affordability for low-income populations (Mohidem *et al*., 2022). Unlike most crops, which are cultivated on aerated soils, rice is mostly grown on waterlogged soils around the world. Waterlogging makes soil anaerobic and highly reductive, a condition that promotes reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), which becomes the predominant form of iron (Fe) found in the soil solution of paddy fields (Kirk *et al*., 2022).

Since rice root cells release oxygen gas (O_2), creating a localized O_2-rich zone around themselves, Fe^{2+} can be oxidized back to Fe^{3+}, which in turn can react with hydroxide ions (OH^-), producing Fe hydroxide [$Fe(OH)_3$] molecules (Ejiri *et al*., 2021; Lin *et al*., 2024). The $Fe(OH)_3$ molecules tend to precipitate as solid particles on root surfaces due to their low solubility in water. Their accumulation leads to the formation of Fe plaques, which end up acting as a natural defense mechanism of rice roots, mitigating the entry of heavy metals (Tripathi *et al*., 2014; Zandi *et al*., 2023). Nevertheless, high concentrations of Fe^{2+} tend to be harmful to rice plants because of the excessive formation of Fe plaques on roots, physically interfering with the uptake of water and other essential minerals, therefore restricting root growth (Kirk *et al*., 2022; Zhang *et al*., 2022a; Li *et al*., 2024; Sonu *et al*., 2024).

Excessive amounts of Fe^{2+} can be uptaken by roots due to its high solubility in the soil solution. High concentrations of Fe^{2+} within plant cells leads to the production of reactive oxygen species (ROS) as signaling molecules for defense pathways (Wang *et al*., 2024). However, the coexistence of Fe^{2+} ions and hydrogen peroxide (H_2O_2) molecules can trigger the generation of hydroxyl radicals (•OH), enhancing the occurrence of oxidative damage to various cell components due to their high reactivity (Becana *et al*., 1998). Furthermore, excessive upload and accumulation of Fe^{2+} into shoots leads to the occurrence of leaf bronzing, a hallmark symptom observed in plants under this stressful condition (Engel *et al*., 2012; Santana *et al*., 2014). In leaves, reduced photosynthesis and chlorosis, which may progress to necrosis, are also likely to occur (Pereira *et al*., 2013; Müller *et al*., 2015, 2017; Pinto *et al*., 2016). Taken together, the damage caused by Fe^{+2} toxicity results in stunted growth, tissue damage and ultimately significant yields losses (Frei *et al*., 2016). Therefore, it is vital to elucidate the defense mechanisms to Fe^{2+} toxicity in rice (Wairich *et al*., 2024).

The six *ASR* (*Abscisic acid*, *Stress*, *Ripening*) genes of rice are known to be responsive to different types of abiotic stresses, such as drought and salinity (Joo *et al*., 2013; Pérez-Díaz *et al*., 2014; Srivastava *et al*., 2022; Zhang *et al*., 2022b). In addition, OsASR1 and OsASR5 play a role as transcription factors in the response to aluminum (Al) toxicity in rice (Arenhart *et al*., 2012, 2013, 2014, 2016). OsASR1 regulates the target genes of OsASR5 in a non-preferential way, indicating that both transcription factors are partially functionally redundant. *OsSTAR1* was found among the genes responsive to Al toxicity and regulated by both OsASR proteins (Huang *et al*., 2009; Arenhart *et al*., 2014, 2016). Heterodimers composed by OsSTAR1 and OsSTAR2 proteins transport UDP-glucose that supposedly competes with Al^{3+} ions for binding sites in the cell wall, preventing its Al-mediated disruption (Huang *et al*., 2009). Curiously, *OsSTAR1* has also been described as *OsENA2* and shown to transport nicotianamine (NA), a molecule that binds to Fe^{+2}. The Fe^{+2}-NA complex is found mainly in the phloem sap and is essential for the long-distance transport of Fe within rice plants (Nozoye *et al*., 2011; Carrillo & Borthakur, 2021).

In order to functionally characterize OsASR5, Arenhart *et al*. (2013) generated an RNA interference (RNAi) rice line, named OsASR5-RNAi. Besides the plants showing greater sensitivity to Al stress, the RNAi construct designed to specifically silence *OsASR5* was able to target the other five *OsASR* genes, due to their high level of similarity. As such, apart from its potential for studying Al stress tolerance mechanisms, the OsASR5-RNAi line can also be used to study the pathways involved in Fe homeostasis in rice. In this regard, a previous work of our group demonstrated that OsASR proteins are involved in the Fe deficiency response (Ponte *et al*., 2025). Therefore, in order to further evaluate the role of OsASR proteins in Fe homeostasis, we evaluated the morphophysiological responses of OsASR5-RNAi plants under Fe excess. Our data demonstrate that the down-regulation of *OsASR* genes leads to diminished plant growth, biomass accumulation and photosynthetic performance, even under favorable conditions, highlighting the critical role of these genes in overall plant growth and development.

2. Materials and methods

2.1. Plant materials and growth conditions

The nomenclature of rice *ASR* genes is not consistent in the literature. Here, we followed the nomenclature proposed by Philippe *et al*. (2010), which has been used in our previous

works (Arenhart *et al*., 2013; Barros *et al*., 2025; Ponte *et al*., 2025). Seeds of the OsASR5-RNAi line, described in detail by previous works (Arenhart *et al*., 2013, 2014, 2016), and its parental background, *O*. *sativa* cv. Nipponbare (hereafter WT – wild-type), were germinated in recipients containing sheets of filter paper moistened with distilled water in a BOD incubator set to 28°C with long-day photoperiod (16h/8h light/dark). After one week, the seedlings were transferred to pots containing vermiculite and watered with standard nutrient solution (700 µM K_2SO_4, 100 µM KCl, 100 µM KH_2PO_4, 2mM $Ca(NO_3)_2$, 500 µM $MgSO_4$, 10µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2µM $CuSO_4$, 0.01µM $(NH_4)_6Mo_7O_{24}$, and 100 µM Fe^{3+}-EDTA). After two weeks, the plants were transferred to a hydroponics system, which consisted of containers filled with 4.5 liters of nutrient solution. After a week of acclimation, the treatments were applied for 15 days: control condition (CC) or iron excess (+Fe), in which 5 mM $FeSO_4 \cdot 7H_2O$ was added as the only Fe source during the preparation of the solution. The nutrient solution was renewed twice a week and its pH was adjusted to 5.4. Growth conditions were 25°C under a long-day photoperiod.

2.2. Growth parameters and dry mass of shoots and roots

After 15 days of treatment, the shoot height and root length of five plants per genotype per treatment were determined using a graduated ruler, measuring from the base of the culm to the leaf tip and from the base of the culm to the root apex, respectively. Subsequently, these same samples were dried in a forced ventilation oven at 60°C for one week and weighted.

2.3. Root measurements

After 14 days of treatment, roots of five plants per genotype per treatment were collected for root biometric evaluations. Initially, the roots were enveloped in filter paper, moistened with distilled water and then frozen at −20°C. The roots were then thawed, soaked in a 30% ethyl alcohol solution, and stored at 4°C. A photo scanner (Regent Instruments Inc. STD4800) was used to digitalize the root images at 600 dpi resolution. The WinRHIZO™ Pro 2020a (Regent Instruments Inc., Canada) software was used to analyze the images and determine the following parameters: total root length (cm), surface area (cm^2), volume (cm^3) and mean diameter (mm).

2.4. IRGA and fluorometer analyses

Gas exchange and chlorophyll α fluorescence was measured in the youngest fully expanded leaves from both WT and OsASR5-RNAi under both treatments. The measurements were performed at the 7th and 14th days of treatment. CO_2 assimilation rate (A), stomatal conductance, internal CO_2 concentration (Ci) and transpiration (E) were measured with a LCpro-SD portable InfraRed Gas Analyzer system (IRGA; ADC Bioscientific, Hoddesdon, England). The internal parameters in the IRGA chamber during gas exchange measurements were: 27 °C, 1000 µmol photons m^{-2} s^{-1}, 420 ppm CO_2 concentration, ambient humidity. Each sample corresponded to the normalized value of two leaves from different plants and the measurement recordings were performed during 15 min after placing the leaves inside the measuring chamber with the internal parameters described previously. The water use efficiency was calculated as A/E and the carboxylation efficiency was calculated as A/Ci.

In vivo chlorophyll α fluorescence was measured using an OS-30p chlorophyll fluorometer (Opti-Sciences, Hudson, NH, USA). The fluorescence parameters were measured by the saturation pulse method in leaves of plants exposed to 30-min dark-adapted conditions (Schreiber *et al*., 1995). The maximum and the minimum fluorescence (Fm and Fo, respectively) were measured in 30-min dark-adapted plants and the maximum quantum yield of photosystem II (PSII) [Fv/Fm; (Fm – Fo)/Fm] was calculated.

2.5. Malondialdehyde and hydrogen peroxide concentration quantifications

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations were quantified according to De Palma & Fett-Neto (2024). After 7 days of treatment, leaf samples of four plants per genotype x treatment were collected, frozen in liquid nitrogen and stored at −80°C. The frozen material was then pulverized. Around 100 mg were weighed for each sample using an analytical precision balance. For the crude extraction, 667 µl of 0.1% trichloroacetic acid (TCA; w/v) solution was added to each sample. The material was homogenized using a vortex. The samples were centrifuged at 16,000 g for 20 min at 4°C.

For the MDA quantification, 200 µl of the supernatant formed from the crude extract was collected. Next, 400 µl of the 0.5% thiobarbituric acid (TBA; w/v) in 20% TCA (w/v) solution was added. The solutions were homogenized using a vortex. Then, the samples were incubated in a water bath at 95°C for 30 min. To stop the reaction, the samples were put on

ice for 5 min. For the absorbance readings, the samples were centrifuged at 16,000 g for 5 min a 4°C. A total of 150 µl of each sample was pipetted into a 96-well plate in triplicate. The absorbance readings were obtained at 532 nm and 600 nm in a SpectraMax® M2 reader (Molecular Devices LLC, USA).

For the H_2O_2 quantification, a total of 100 µl of the same supernatant was collected and mixed with 100 µl of 10 mM potassium phosphate buffer (pH 7) and 400 µl of 1M potassium iodide (KI). The solutions were homogenized using a vortex. The samples were incubated for 60 min at room temperature under darkness. Following homogenization, a volume of 150 µl of each sample was pipetted into a 96-well plate in triplicate. The absorbance readings were obtained at 392 nm.

2.6. Data analysis

Our data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene test, respectively. If both assumptions were met, we performed One-Way ANOVA, followed by Tukey's test. If the data were normal but did not meet the assumption of homogeneity of variance, we performed Welch's ANOVA, followed by the Dunnett´s T3 post-hoc test. If the data were not normally distributed, we performed the Kruskal Wallis test, followed by Dunn's test for multiple comparisons. We also verified the data for any outliers using the two-side Grubbs' test with $P < 0.05$.

3. Results

3.1. Down-regulation of *OsASR* genes is detrimental to growth and shoot biomass accumulation

We exposed four-week-old plants of wild-type (WT) and OsASR5-RNAi genotypes to either control or Fe excess (+Fe) conditions for up to 15 days. At the end of the treatment period, shoot height and dry mass of OsASR5-RNAi plants were respectively 19.4% and 55.5% lower than those of the WT in control condition (Fig. 1A and 1B). No such differences were observed between the two genotypes under Fe excess, in which only a 44.1% decrease was observed in the shoot dry mass of WT, when compared with control condition (Fig. 1B). Regarding root length, no differences were observed between the genotypes on the same treatment or between the two treatments (Fig. 1C). However, the dry mass of OsASR5-RNAi roots was 59.9% and 38.4% lower compared to the WT in control and +Fe, respectively (Fig.

1D). Taken together, these results indicate that the down-regulation of *OsASR* genes affects both growth and biomass accumulation in the shoot of plants. Furthermore, exposure to Fe excess does not seem to have affected these two parameters in OsASR5-RNAi plants, while both were reduced in the WT.

3.2. OsASR5-RNAi plants develop a reduced root system regardless of treatment

As we observed no differences in the root length between the WT and OsASR5-RNAi plants (Fig. 1C), but reduced root dry masses of OsASR5-RNAi in both treatments (Fig. 1D), we decided to further evaluate the root architecture of both genotypes. By scanning the roots, we were able to observe reductions of 33.4%, 39.8% and 51.6% respectively for the total root length, surface area and volume of OsASR5-RNAi plants under control condition, in comparison with the WT (Fig. 2A–C). Comparing the genotypes under +Fe, we also observed reductions in OsASR5-RNAi: 60.8% for total root length, 62.3% for root surface area and 65.6% for root volume (Fig. 2A–C). Exposure to Fe excess also led to reductions in total root length (50% and 70.6% for WT and OsASR5-RNAi, respectively), root surface area (48.5% and 67.8% for WT and OsASR5-RNAi, respectively) and volume (43.8% for the WT) (Fig. 2A–C). No differences were observed in the average root diameter between the genotypes and/or treatments (Fig. 2D). Altogether, these results indicate that the down-regulation of *OsASR* genes is detrimental to root growth and development, resulting in a stunted root system (Fig. 2E).

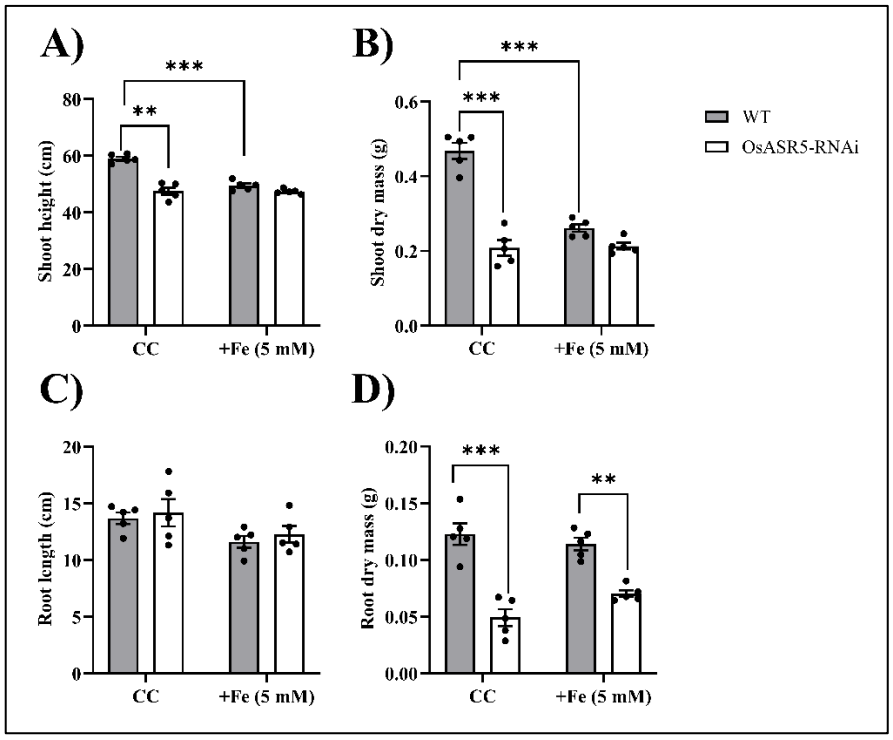


Figure 1. Down-regulation of *OsASR* genes impacts dry masses of shoot and roots. Shoot height (A), shoot dry mass (B), root length (C) and root dry mass (D) from wild-type (WT) and OsASR5-RNAi plants under control condition (CC) or Fe excess treatment (+Fe; 5 mM) at 14 days. Error bars indicate standard error. Asterisks denote statistically significant differences (** $P < 0.01$; *** $P < 0.001$). Each black dot represents an individual measurement (n = 4 or 5).

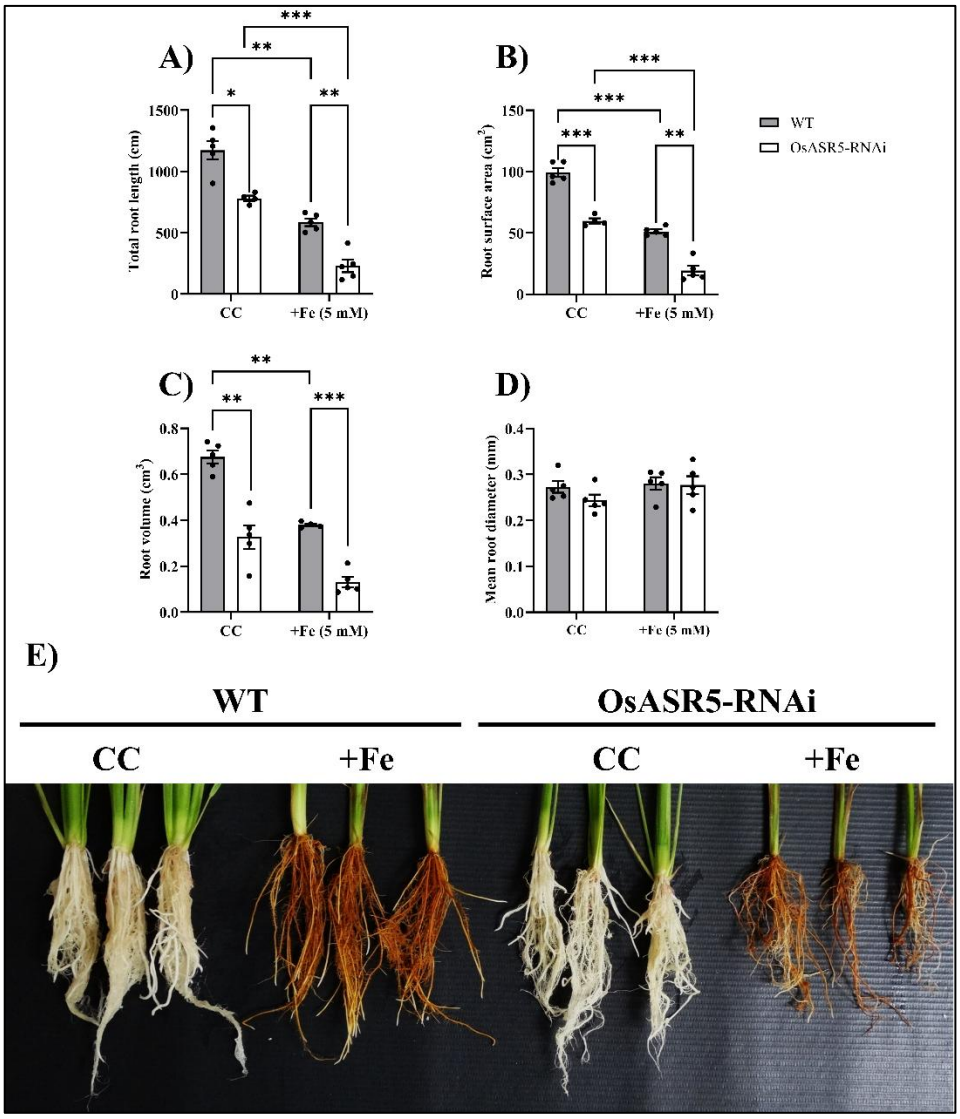


Figure 2. Down-regulation of *OsASR* genes impacts root development and increases sensitivity to Fe excess. Total length (A), surface area (B), volume (C) and mean diameter (D) from roots of wild-type (WT) and OsASR5-RNAi plants under control condition (CC) or Fe excess treatment (+Fe; 5 mM) at 14 days. (E) Representative photograph of roots at 14 days of treatment. Error bars indicate standard error. Asterisks denote statistically significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Each black dot represents an individual measurement (n = 4 or 5).

3.3. The down-regulation of *OsASR* genes substantially affects the photosynthetic performance of plants

To verify whether Fe excess would affect the photosynthetic performance of OsASR5-RNAi plants, photosynthetic parameters were evaluated at 7 days of treatment. The CO_2 assimilation rate of OsASR5-RNAi plants was 65.8% and 54.5% lower in control condition and +Fe compared to WT, respectively. Meanwhile, the CO_2 assimilation rate of WT showed

a reduction of 42.3% in +Fe (Fig. 3A). Although the internal CO_2 concentrations in WT leaves in both treatments were close to 350 μmol CO_2 mol air^{-1} (Fig. 3E), the carboxylation efficiency showed a reduction of 53% in WT under +Fe (Fig. 3B). This was probably a consequence of the lower CO_2 assimilation rate observed in WT under this condition (Fig. 3A). Notably, the carboxylation efficiency of OsASR5-RNAi plants was 81.6% and 78.6% lower than that of the WT under control condition and +Fe, respectively (Fig. 3B). Despite the higher transpiration rate in OsASR5-RNAi under control condition (Fig. 3D), the water use efficiency of the genotype did not differ between treatments (Fig. 3C). This was probably due to the lower CO_2 assimilation rates observed in both treatments (Fig. 3A). Compared to WT, water use efficiency of OsASR5-RNAi was 78.8% lower in control condition (Fig. 3C). Concerning the chlorophyll α fluorescence, a reduction of 16% was observed in the maximum fluorescence (Fm) of OsASR5-RNAi leaves compared with the WT under control condition (Fig. 3I). We observed that the fifth leaf of the OsASR5-RNAi plants, which was the last to be fully expanded during the course of the experiment, was smaller than those of the WT under control condition, which is in line with the lower dry mass observed (Fig. 1B and 3J). In addition, the leaves were chlorotic and rolled under +Fe in both genotypes, appearing to be more yellowish in OsASR5-RNAi (Fig. 3J). Taken together, these results demonstrate that the down-regulation of *OsASR* genes drastically affects the photosynthetic performance. The effect is so drastic that no differences in CO_2 assimilation rate, carboxylation efficiency and water use efficiency were observed in OsASR5-RNAi plants when comparing the two treatments. Furthermore, the differences in these parameters between WT and OsASR5-RNAi were reduced under +Fe, indicating that the treatment also affected the photosynthetic performance of WT plants, as expected.

We also evaluated the photosynthetic performance of both genotypes over 14 days of treatment (Fig. S1). The differences in CO_2 assimilation rate, carboxylation efficiency and water use efficiency remained between WT and OsASR5-RNAi under control condition. However, these differences were no longer observed under +Fe, indicating the detrimental effect of long-term exposure to Fe excess on WT plants (Fig. S1A−C).

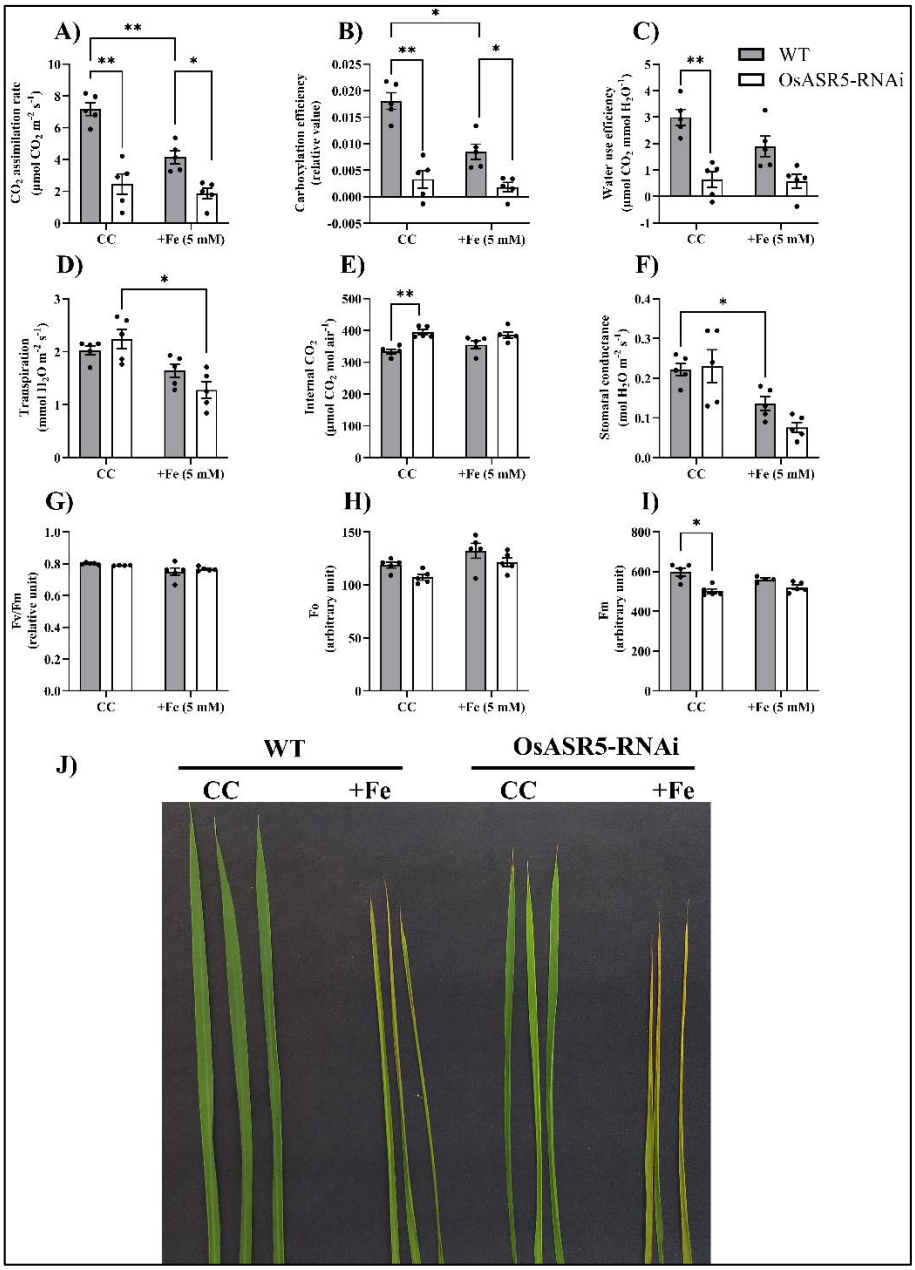


Figure 3. Impacts of down-regulation of *OsASR* genes on photosynthetic parameters. (A) CO_2 assimilation rate, (B) carboxylation efficiency, (C) water use efficiency, (D) transpiration rate, (E) internal CO_2 concentration, (F) stomatal conductance, (G) maximum quantum yield of photosystem II (Fv/Fm), (H) minimum fluorescence (Fo) and (I) maximum fluorescence (Fm) from leaves of wild-type (WT) and OsASR5-RNAi plants under control condition (CC) or Fe excess treatment (+Fe; 5 mM) at 7 days. (J) Representative photograph of leaves at 14 days of treatment. Error bars indicate standard error. Asterisks denote statistically significant differences (* $P < 0.05$; ** $P < 0.01$). Each black dot represents an individual measurement (n = 4 or 5).

3.4. WT and OsASR5-RNAi plants under Fe excess seem to respond similarly regarding oxidative stress

Given the chlorotic phenotype observed in the fifth leaf of OsASR5-RNAi plants under +Fe at the end of the experiment, we decided to quantify the concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in leaf tissues, hallmark components of oxidative stress. Leaf samples collected 7 days prior to the observed phenotype were used. A 38.6% increase in H_2O_2 concentration was observed in WT leaves under +Fe compared to control condition (Fig. 4A). Interestingly, no significant differences in H_2O_2 concentration were observed in OsASR5-RNAi leaf tissues compared with the WT, and between treatments (Fig. 4A). In addition, the MDA concentration increased 96% in OsASR5-RNAi under +Fe compared to control condition (Fig. 4B).

At 7 days of treatment, these results indicate that H_2O_2 metabolism does not seem to have been altered in OsASR5-RNAi plants when exposed to Fe excess. Furthermore, based on the MDA concentrations, both genotypes showed similar levels of lipid peroxidation, although we did not observe a statistically significative increase in MDA concentration in WT under +Fe (Fig. 4B). Thus, we assume that compared to the WT, the more sensitive phenotype observed in OsASR5-RNAi leaves cannot be explained solely by the level of lipid peroxidation at 7 days of +Fe treatment.

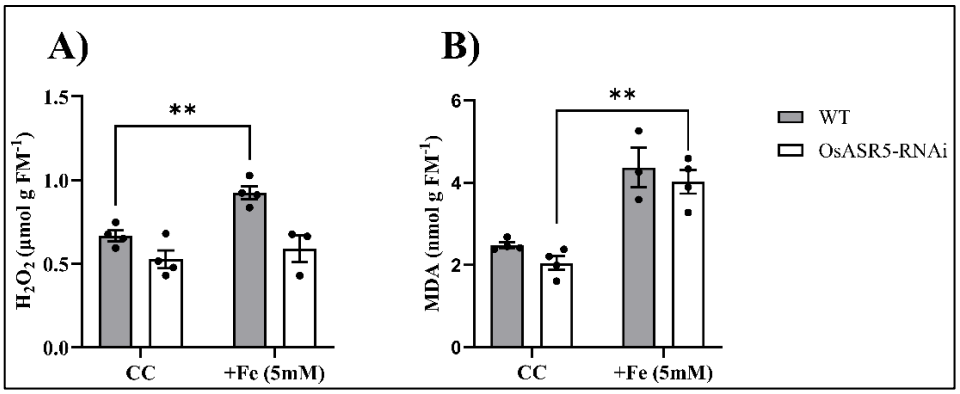


Figure 4. Down-regulation of *OsASR* genes does not seem to significantly alter the oxidative response in plants. (A) Hydrogen peroxide (H_2O_2) and (B) malondialdehyde (MDA) concentrations in wild-type (WT) and OsASR5-RNAi plants under control condition (CC) or Fe excess treatment (+Fe; 5 mM) at 7 days. Error bars indicate standard error. Asterisks denote statistically significant (* $P < 0.05$; ** $P \leq 0.01$). Each black dot represents an individual measurement (n = 3 or 4). FM, fresh mass.

4. Discussion

It is known that *OsASR* genes are responsive to different stresses, as it was observed the presence of several cis-elements in their 5' regulatory regions associated with responses to abiotic stresses, sugar metabolism and phytohormones, especially abscisic acid (ABA) (Joo *et al*., 2013; Pérez-Díaz *et al*., 2014). In addition, the overexpression of different *OsASR* genes led to greater tolerance to drought or salinity stresses (Li *et al*., 2017; Srivastava *et al*., 2022; Zhang *et al*., 2022b). In relation to metal homeostasis, it has been detailed shown that OsASR1 and OsASR5 act as transcription factors that regulate genes responsive to Al stress (Arenhart *et al*., 2013, 2014, 2016). OsASR5-RNAi plants, which in fact are knockdown for the six *OsASR* genes (Arenhart *et al*., 2013; Ponte *et al*., 2025), are more sensitive to Al stress (Arenhart *et al*., 2013). One of the genes regulated by OsASR1 and OsASR5 is *OsSTAR1*, also known as *OsENA2* (Huang *et al*., 2009; Nozoye *et al*., 2011; Arenhart *et al*., 2014, 2016). OsENA2 is a NA efflux transporter, which is a crucial phytosiderophore for long-distance Fe^{2+} transport (Nozoye *et al*., 2011; Carrillo & Borthakur, 2021). Considering that our previous work demonstrated that OsASR proteins play a role in the regulation of Fe deficiency response (Ponte *et al*., 2025), we decided to evaluate the morphophysiological responses of OsASR5-RNAi plants to Fe toxicity, a nutritional disorder that tends to occur in waterlogged soils and it has been a recurring problem in paddy fields around the world (Wairich *et al*., 2024).

Our present data demonstrated that the down-regulation of *OsASR* genes affects plant growth and development, especially in terms of root development. Arenhart *et al*. (2013) observed no differences between the OsASR5-RNAi line and its parental background in terms of shoot height and root development. Intriguingly, we observed greater root length in OsASR5-RNAi plants both in control and Fe deficiency conditions compared to the WT in our previous work (Ponte *et al*., 2025). However, similar to what we currently observed, Barros *et al*. (2025) reported the occurrence of a stunted root system in OsASR5-RNAi plants under control condition. Remarkably, this root phenotype showed to be insensitive to P starvation, which prompted the authors to investigate the role of OsASR proteins in P homeostasis. It was observed that the down-regulation of *OsASR* genes dramatically alters the regulation of early root response to P starvation (Barros *et al*., 2025). In addition, the gene *OsWAK60* (LOC_Os04g30240) in the OsASR5-RNAi line under control condition showed to be down-regulated (Barros *et al*., 2025). *OsWAK60* is a member of the *Wall-*

Associated Kinase (*WAK*) gene family, a subfamily of receptor-like kinases (RLKs) involved in various cellular processes, including cell expansion (Wagner & Kohorn, 2001; de Oliveira *et al*., 2014). In this regard, reduced growth and root development due to the disruption of *WAK* genes have already been observed in *Arabidopsis thaliana* and rice (Lally *et al*., 2001; Kanneganti & Gupta, 2011). Altogether, these results suggest that ASR proteins may be positive regulators of genes involved in cell growth and expansion.

Based on their tissue-specific expressions, *OsASR* genes showed to be involved in sugar transport and signaling (Pérez-Díaz *et al*., 2014). In fact, Joo & Song (2015) observed that the overexpression of *OsASR5* (named in their work as *OsASR1*) increased glucose and fructose levels in roots, mainly under ABA treatment. The authors also observed that the roots of *OsASR5*-overxpressing plants showed to be hypersensitive to glucose treatment, and the transcript levels of genes involved in sugar metabolism and allocation were altered. The role of *ASR* genes from other plants species in sugar metabolism have also been described, such as the *ASR1* of tobacco (*Nicotiana tabacum*) and its ortholog, the *MSA* (*Maturation, Stress, Abscisic acid-induced*) of grape (*Vitis vinifera*) (Dominguez *et al*., 2013; Parrilla *et al*., 2022). *Asr1*-silenced tobacco plants showed higher levels of glucose in leaf tissues, since its assimilation appeared to be impaired by altered expression of key genes involved in its transport, such as *Hexose Transporter 1* (*HT1*), and its signaling, such as *Hexokinase 1* (*HXK1*) and *SnRK1* [*Sucrose Non-Fermenting1* (*SNF1*)*-Related Kinase 1*] (Çakir *et al*., 2003; Dominguez *et al*., 2013; Peixoto & Baena-González, 2022; Vanderwall & Gendron, 2023). Therefore, further research is needed to determine whether the stunted phenotype, particularly in OsASR5-RNAi roots, is also caused by impaired sugar metabolism resulting from the down-regulation of *OsASR* genes, as previously observed with the silencing of *NtASR1* (Dominguez *et al*., 2013).

Similar to what we observed in OsASR5-RNAi plants, *Asr1*-silenced tobacco plants showed dwarfism, lower biomass accumulation and reduced CO_2 assimilation rate. However, higher leaf H_2O_2 concentrations in these plants were detected (Dominguez *et al*., 2013). The authors also observed higher and lower transcript levels of *HXK1* and *SnRK1*, which are respectively positive and negative regulators of leaf senescence (Cho *et al*., 2012; Zheng *et al*., 2021). Intriguingly, Li *et al*. (2017) observed increased leaf H_2O_2 concentrations in *OsASR5*-overexpressing plants. The authors proposed that OsASR5 contributes to the ABA-dependent stomatal closure pathway, as OsASR5 induces the

expression of ABA biosynthesis genes and, in parallel, represses some H_2O_2-scavenging genes. The resulting H_2O_2 accumulation, which biosynthesis is also stimulated by ABA under drought conditions, ends up promoting stomatal closure (Li *et al*., 2017). Likewise, the overexpression of *OsASR3* (named as *OsASR1*) also led to greater drought tolerance due to higher ABA contents in leaf tissues, resulting in lower transpiration rate and stomatal conductance (Park *et al*., 2020). As we did not observe remarkable differences regarding transpiration rate and stomatal conductance between OsASR5-RNAi and WT plants, it is worth mentioning that phenotypic divergences among overexpression and silenced lines of *ASR* genes are to be expected, given their responsiveness to different stimuli and partial functional redundancy between each other (Joo & Song, 2015; Arenhart *et al*., 2016; Barros *et al*., 2025; Ponte *et al*., 2025). Future work would be of great value in investigating ABA biosynthesis and signaling in the OsASR5-RNAi genotype.

Regarding exposure to Fe excess, both WT and OsASR5-RNAi plants showed reduced biometric parameters related to roots, which was expected (Sonu *et al*., 2024). Along with the potential adverse impact caused by impaired root development and sugar metabolism, a disrupted chaperone-like activity of OsASR5 may be another contributing factor to the occurrence of this phenotype. The promotion of ROS scavenging and the chaperone-like activity of OsASR5 (named as OsASR1) increased abiotic stress tolerance in yeast (*Saccharomyces cerevisiae*) cells (Kim *et al*., 2012). However, we did not detect significant differences between genotypes for H_2O_2 or MDA concentrations under Fe excess. One possible explanation is that tissue sampling may have been performed before an initial oxidative burst, or even after it. In the latter case, the results may have been masked due to the activation of antioxidant systems. Another possibility is the compensation for the loss of ASR-mediated ROS scavenging through the induction of alternative antioxidant pathways, thus preventing measurable differences in bulk H_2O_2 and MDA concentrations. In this case, the lack of substantial changes in oxidative stress markers suggests that the sensitivity of OsASR5-RNAi plants to Fe excess may be driven more by structural and metabolic limitations than by exacerbated ROS accumulation.

5. Conclusions

In summary, our study shows that the down-regulation of *OsASR* genes leads to reduced plant growth, biomass accumulation, and photosynthetic performance under control condition. Accordingly, this demonstrates that OsASR proteins play a significant role in overall plant growth and development, beyond their regulation of abiotic stress responses and metal homeostasis. Future work regarding the effects caused by the down-regulation of *OsASR* genes in the OsASR5-RNAi genotype would be of great interest to: (1) verify the occurrence of morphological changes in root tissues caused by suppressed expressions of genes involved in cell growth and expansion; (2) identify changes in sugar and ABA biosynthesis, transport and signaling pathways; (3) further explore the chaperone-like activity of the OsASR proteins under stress conditions, especially OsASR5.

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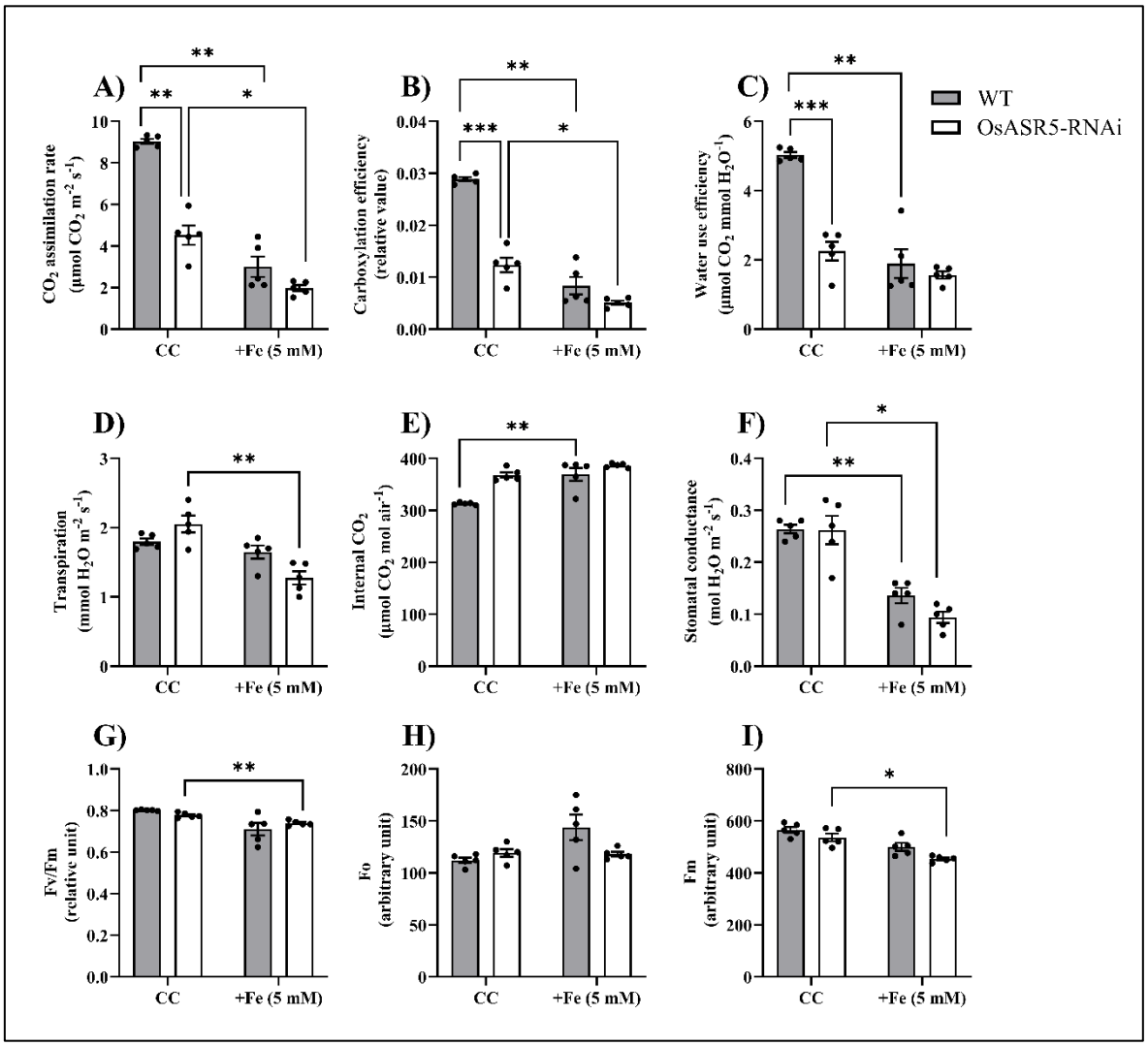
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Supplemental material



Supplemental Figure 1. Impacts of down-regulation of *OsASR* genes on photosynthetic parameters. (A) CO_2 assimilation rate, (B) carboxylation efficiency, (C) water use efficiency, (D) transpiration rate, (E) internal CO_2 concentration, (F) stomatal conductance, (G) maximum quantum yield of photosystem II (Fv/Fm), (H) minimum fluorescence (Fo) and (I) maximum fluorescence (Fm) from leaves of wild-type (WT) and OsASR5-RNAi plants under control condition (CC) or Fe excess treatment (+Fe; 5 mM) at 14 days. Error bars indicate standard error. Asterisks denote statistically significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Each black dot represents an individual measurement (n = 4 or 5).

CAPÍTULO 3

Investigating OsVIT1–OsVIT2 interaction and the functional role of the N-terminal domain of OsVIT2 in iron transport in rice (*Oryza sativa* L.)

Investigating OsVIT1–OsVIT2 interaction and the functional role of the N-terminal domain of OsVIT2 in iron transport in rice (*Oryza sativa* L.)

Lucas Roani Ponte[1], Abdullah Shalmani[2], Jingyi Guo[2], Ricardo Fabiano Hettwer Giehl[2], Felipe Klein Ricachenevsky[1,3]

[1] Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Leibniz Institute of Plant Genetics & Crop Plant Research (IPK), Gatersleben, Germany

[3] Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

ABSTRACT

Iron (Fe) is essential for both human health and plant development. However, its bioavailability tends to be often limited, contributing to widespread Fe-deficiency anemia and reduced crop productivity. In rice (*Oryza sativa* L.), the vacuolar iron transporters OsVIT1 and OsVIT2 are key regulators of Fe storage and distribution, especially into grain tissues. While previous studies have described the mechanism of Fe^{2+} transport carried out by plant VIT proteins, the potential heterodimerization between OsVIT1 and OsVIT2, as well as the functional role of OsVIT2 N-terminal domain in Fe^{2+} transport, remain unexplored. Here, we employed yeast two-hybrid (Y2H) assays to assess the possible occurrence of OsVIT1–OsVIT2 interaction. We observed no evidence of interaction. Additionally, a truncated OsVIT2 lacking its N-terminal domain (OsVIT2mod) was expressed in the yeast mutant strain Δ*ccc1* to evaluate its Fe^{2+} transport capacity. No phenotypic differences were detected between OsVIT2 and OsVIT2mod-expressing lines. Overall, our negative results thus far highlight the technical and biological challenges of characterizing plant membrane transporters in heterologous yeast systems, underlining the usefulness of more sensitive and *in planta* assays to further investigate the function and regulation of OsVITs.

Keywords: iron, yeast-two hybrid, yeast complementation, VIT transporter, vacuole.

1. Introduction

Iron (Fe) is indispensable for human life, serving as a critical cofactor in cellular processes such as DNA synthesis, mitochondrial electron transfer, respiration and oxygen gas (O_2) transport (Sánchez *et al*., 2017). In the human body, up to 70% of Fe is bound to hemoglobin proteins, while the remainder can be found bound to ferritins, myoglobin and various enzymes (Dev & Babitt, 2017). The imbalance of Fe bioavailability in food diets leads to widespread human health issues, especially Fe-deficiency anemia, which affects over two billion people around the world (Gardner *et al*., 2023). Likewise, plants depend on tightly controlled Fe uptake and distribution, due to its role in chlorophyll biosynthesis, photosynthetic electron transfer, nitrogen fixation, etc. (Hu *et al*., 2017; Kroh & Pilon, 2020; Liu *et al*., 2023). Although Fe is abundant in soils, it is frequently found in insoluble forms at neutral to alkaline pH, resulting in low availability to crops in aerated soils (Vélez-Bermúdez & Schmidt, 2023). Fe deficiency causes reduced photosynthetic capacity, growth delay and chlorosis, mainly in younger leaves (Krohling *et al*., 2016).

Rice (*Oryza sativa* L.) is a staple food for half the world's population but fails to supply adequate dietary Fe (Trinidad *et al*., 2009; Gibson *et al*., 2010). In waterlogged soils, rice roots uptake readily the highly soluble ferrous ions (Fe^{2+}) (Soviguidi *et al*., 2025). In rice grains, Fe accumulates mostly in the aleurone layer, pericarp and embryo, which represent the parts of the grains that are removed during industrial processing to produce white rice, the main form of rice consumption worldwide (Prom-u-thai *et al*., 2007; Tuncel *et al*., 2025). Consequently, rice biofortification efforts seek to redirect Fe into the grains' starchy endosperm (Wang *et al*., 2021; Wairich *et al*., 2022).

The rice genome has two genes coding for vacuolar iron transporters (VIT), *OsVIT1* and *OsVIT2*. They function as key regulators of Fe storage and partitioning within plants (Krishna *et al*., 2023; Arend *et al*., 2025; Benato *et al*., 2025). Previous works have shown that OsVIT1 and OsVIT2 mediate Fe sequestration into vacuoles, and mutation in either genes have altered Fe distribution within vegetative tissues and grains (Zhang *et al*., 2012; Bashir *et al*., 2013; Che *et al*., 2021; Kandwal *et al*., 2022). Recently, it was reported that the double knockout of *OsVIT1* and *OsVIT2* leads to increased Fe accumulation in the embryo of the grains (Benato *et al*., 2025). On the other hand, it reduces tolerance to Fe excess, demonstrating a trade-off between biofortification and stress resilience (Benato *et al*., 2025). Interestingly, both single and double mutants have altered Fe distribution within

grains, suggesting that OsVIT1 and OsVIT2 may work redundantly (Zhang *et al*., 2012; Benato *et al*., 2025).

Despite these advances, the molecular mechanisms by which VIT plant proteins transport and sequester Fe have not been extensively studied. The structure of VIT plant transporters has been elucidated using VIT1 from *Eucalyptus grandis* (Kato *et al*., 2021). EgVIT1 functions as a homodimer, and uses a proton (H^+) gradient across the tonoplast as a driving force to transport Fe^{2+} ions from the cytosol into the vacuolar lumen (Kato *et al*., 2021). Thus, plant VIT proteins function as Fe^{2+}/H^+ antiporters, as also described in protozoa and fungi (Labarbuta *et al*., 2017; Sorribes-Dauden *et al*., 2020; Aghabi *et al*., 2023). Recently, Arend *et al*. (2025) performed *in silico* structural analyses of OsVIT2. The authors modelled OsVIT2 homodimer, simulating how it operates by a dynamic and H^+-coupled transport mechanism. Furthermore, the study highlighted the presence of flexible loop regions located in the N-terminal domain of OsVIT2, referred to as cytoplasmic arms, which have been hypothesized to recognize and guide cytosolic Fe^{2+} ions toward the transmembrane pore, contributing to an efficient substrate delivery (Arend *et al*., 2025). However, there is no biological evidence for that.

Given that single *osvit1* and *osvit2* mutant lines showed similar phenotypic differences in embryo Fe localization as the double mutant (Zhang *et al*., 2012; Benato *et al*.; 2025), we hypothesized that OsVIT1 and OsVIT2 may function as heterodimers. Therefore, the goals of this study were: (1) to investigate the possibility of interaction between OsVIT1 and OsVIT2 proteins; and (2) to investigate whether Fe^{2+} transport by OsVIT2 is impaired by the absence of its N-terminal domain.

2. Materials and methods

2.1. Plant materials and growth conditions

Rice (*O. sativa* ssp. *japonica* cv. Nipponbare) seeds were disinfected with 20% (w/v) sodium hypochlorite for 20 min, washed with distilled water three times and soaked in distilled water for 24 hours. After germination, seedlings were planted in autoclaved soil and grown into a phytochamber (Percival E-41L2X), with long day-photoperiod (16/8h light/dark) and adjusted temperature (28°C) and relative humidity (50%). The plants were irrigated with tap water twice a week.

2.2. DNA constructs

Total RNA from homogenized rice leaf material was extracted using the Z6 extraction buffer (containing 8 M guanidine hydrochloride) method (Logemann *et al*., 1987). After extraction, RNA quantification was performed using a Nanodrop® (Thermo Fisher Scientific) spectrophotometer. Total RNA was treated with DNAse I (Invitrogen®), and first-strand cDNA synthesis was performed with OligodT and reverse transcriptase M-MLV (Invitrogen®) using 1 µg of DNAse-treated RNA, according to the manufacturer's instructions. The coding sequence (CDS) of the cDNA fragments of *OsVIT1*, *OsVIT2* and *OsVIT2* modified (without the nucleotide sequence corresponding to the protein N-terminal domain), hereafter *OsVIT2mod*, were amplified by RT-PCR, using high-fidelity Phusion DNA polymerase (New England Biolabs) and primers OsVIT1-FWD-TOPO, OsVIT1-RVS, OsVIT2-FWD-TOPO, OsVIT2-RVS and OsVIT2mod-FWD-TOPO. The gene-specific primer pairs used in this study are listed in Supplemental Table S1. The amplified DNA fragments were introduced into the pENTR/D-TOPO plasmid, using the pENTR™/D-TOPO™ Cloning Kit (Thermo Fisher Scientific). The OsVIT1-pENTR/D-TOPO, OsVIT2-pENTR/D-TOPO and derived constructs were all confirmed via restriction digestion and further sequencing.

For the yeast complementation assays, the Gateway® LR Clonase™ Enzyme Mix (Thermo Fisher Scientific) was used to subclone *OsVIT2* and *OsVIT2mod* (OsVIT2 without its N-terminal domain, which corresponds to the first 81 nucleotides of its coding region) fragments into the yeast expression pFL61-GW plasmid (Minet *et al*., 1992; Sinclair *et al*., 2018) to generate the constructs OsVIT2-pFL61-GW and OsVIT2mod-pFL61-GW. *OsVIT2* and *OsVIT2mod* fragments were also introduced into another yeast expression plasmid, the pYES260 (Melcher, 2000). In this case, the fragments were amplified by RT-PCR, using high-fidelity Phusion DNA polymerase (New England Biolabs) and primers OsVIT2-BamHI-FWD, OsVIT2-HindIII-RVS and OsVIT2mod-HindIII-RVS to generate the OsVIT2-pYES260 and VIT2mod-pYES260 constructs.

For the yeast double-hybrid (Y2H) assay, we used the Gateway® LR Clonase™ Enzyme Mix (Thermo Fisher Scientific) to subclone *OsVIT1* and *OsVIT2* fragments into the yeast expression plasmids of the ProQuest™ Two-Hybrid System (Thermo Fisher Scientific). To verify the possible interaction between OsVIT1 and OsVIT2 by another Y2H screening system, we subcloned *OsVIT1* and *OsVIT2* fragments into the pBT3-SUC and pPR3-N

plasmids [Yeast Two-Hybrid (Membrane System) Vector Kit; ProNet Biotech], respectively. In this case, the fragments anchored with SfiI restriction sites were amplified by RT-PCR, using high-fidelity Phusion DNA polymerase (New England Biolabs) and primers OsVIT1-pBT3-FWD, OsVIT1-pBT3-RVS, OsVIT2-pPR3-FWD and OsVIT2-pPR3-RVS to generate the OsVIT1-pBT3 and OsVIT2-pPR3 constructs.

2.3. Yeast complementation assay

Saccharomyces cerevisiae mutant strain Δ*ccc1* (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR220w::kanMX4*) was transformed with the pYES260, OsVIT2-pYES260 and OsVIT2mod-pYES260 plasmids or pFL61-GW, OsVIT2-pFL61-GW and VIT2mod-pFL61-GW plasmids via the lithium acetate/single-stranded/PEG method (Li *et al*., 2001; Gietz & Schiestl, 2007). The respective wild-type strain, BY4741 (*MATa; his3D1; leu2D0; met15D0; ura3D0*), was transformed with the empty pYES260 or pFL61-GW plasmids. Transformed yeast cells were incubated for 16 hours in liquid Synthetic Defined (SD) medium without uracil (SD-Ura). The optical density (OD) at 600 nm of the resulting suspensions were measured. After the values were adjusted to 0.4, serial dilutions (1:10, 1:100 and 1:1,000) of the suspensions were prepared. A volume of 7 µl of each dilution was spotted onto SD-Ura plus glucose (for pFL61-GW constructs) or galactose (for pYES260 constructs) plates. This difference was attributed to the presence of the GAL1 promoter upstream of the open reading frame (ORF) site in the pYES260 plasmid. Fe was supplied to the SD medium by adding $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich) from a fresh stock solution. The plates were incubated at 28°C for two days before being scanned. Another mutant strain Δ*ccc1* (*MATa/MATα; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; LYS2/lys2Δ0; met15Δ0/MET15; ura3Δ0/ura3Δ0; YLR220w/YLR220w::kanMX4*), and its respective wild-type, BY4743 (*MATa/alpha; his3D1/his3D1; leu2D0/leu2D0; met15D0/MET15; LYS2/lys2D0; ura3D0/ura3D0*), were also transformed and spotted as previously described. This second mutant strain was identified as Δ*ccc1/43*. The described yeast strains were purchased from the EUROSCARF repository.

2.4. Yeast double-hybrid (Y2H) assays

S. cerevisiae mutant strain MaV203 (*MATα; leu2-3,112; trp1-901; his3Δ200; ade2-101; gal4Δ; gal80Δ; SPAL10::URA3; GAL1::lacZ; HIS3*$_{UAS\ GAL1}$*::HIS3@LYS2; can1*^{R}*; cyh2*^{R})

was transformed via the lithium acetate/single-stranded/PEG method (Gietz & Schiestl, 2007). Transformed yeast cells were plated on Synthetic Complete (SC) medium without tryptophan (SD-Trp) for the pDEST22-derived clones and SC-Leu medium (without leucine) for the pDEST32-derived clones. In turn, cotransformed yeast cells were plated on SC-Leu-Trp medium. To perform the Y2H assay, cotransformed yeast cells were incubated for 16 hours in liquid SC-Leu-Trp medium. The OD at 600 nm of the resulting suspensions were measured. After the values were adjusted to 0.4, serial dilutions (1:10, 1:100 and 1:1,000) of the suspensions were prepared. A volume of 7 µl of each dilution was spotted onto different selective media. The plates were incubated at 28°C for two days before being scanned. The MaV203 line cotransformed with Krev1-pEXP32 + RalGDS-pEXP22 served as the strong positive interaction control, according to the manufacturer's instructions.

S. cerevisiae mutant strain NMY5 (*MATa his3Δ200 trp1-901 leu2-3,112 ade2, LYS2::(lexAop)$_4$-HIS3, ura3::(lexAop)$_8$-lacZ, ade2::(lexAop)$_8$-ADE2 GAL4*) was directly cotransformed via the lithium acetate/single-stranded/PEG method (Gietz & Schiestl, 2007). Cotransformed yeast cells were plated in SC-Leu-Trp medium. The Y2H assay was carried out as previously described. The NMY5 line cotransformed with the empty pBT3 and pPR3 plasmids served as the double negative activation control.

3. Results

3.1. Investigating OsVIT1/OsVIT2 interaction

To investigate the potential interaction between OsVIT1 and OsVIT2 proteins, we performed a yeast two-hybrid (Y2H) assay (Fig. 1). The coding sequences (CDS) of *OsVIT1* and *OsVIT2* were cloned into both pDEST22 (prey) and pDEST32 (bait) plasmids in reciprocal combinations. All cotransformed yeast lines grew on control plates lacking leucine and tryptophan (SC-Leu-Trp), confirming successful transformation (Fig. 1). Since the MaV203 yeast strain can exhibit basal HIS3 reporter activity, interaction between OsVIT1 and OsVIT2 was assessed by monitoring growth on SD-Leu-Trp-His medium supplemented with varying concentrations of the HIS3 inhibitor 3-amino-1,2,4-triazole (3AT). No growth was observed in any of the cotransformed lines expressing OsVIT1 and OsVIT2 (Fig. 1A). As expected, the negative control lines showed no growth, while the positive interaction control showed growth even at 100 mM 3AT (Fig. 1A). To further validate these results, we assessed URA3 reporter activity by testing growth on SD-Leu-Trp-

Ura medium. No growth of any cotransformed lines was observed under these conditions (Fig. 1B). Correspondingly, all cotransformed lines grew on medium supplemented with 0.2% 5-fluoroorotic acid (5FOA), indicating a lack of URA3 activation and therefore absence of interaction (Fig. 1B). Because both OsVIT1 and OsVIT2 have been reported to form homodimers (Kato *et al*., 2019; Arend *et al*., 2025), we also tested self-interactions, but observed no growth under the same selective conditions (Fig. 1).

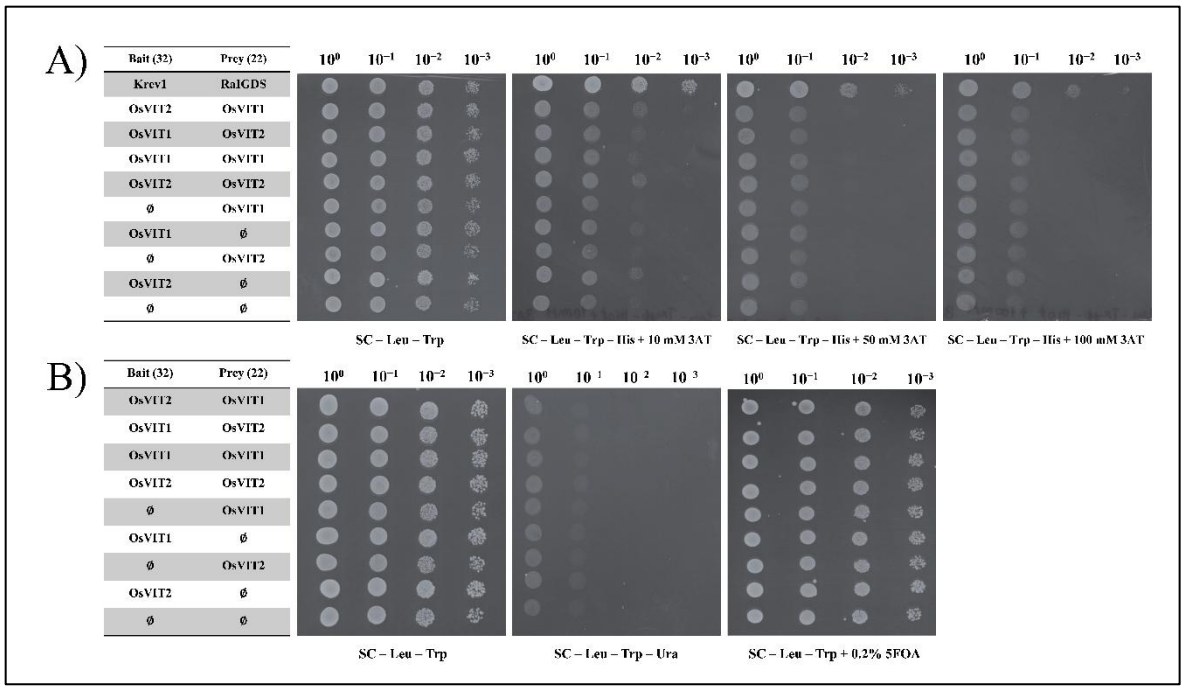


Figure 1. Evaluation of the interaction between OsVIT1 and OsVIT2 through a Y2H assay. (A) Yeast cell growth on standard SD-Leu-Trp medium or supplied with different concentrations of 3-amino-1,2,4-triazole (3AT). (B) Yeast cell growth on SD-Leu-Trp-Ura medium or SD-Leu-Trp medium supplied with 0.2% 5-fluoroorotic acid (5FOA). Ø, empty plasmid (negative activation control).

To investigate the possibility of a weak interaction, the Y2H assay was repeated using lower 3AT concentrations (Fig. 2A). Additionally, we tested whether the presence of exogenous Fe could trigger this interaction (Fig. 2B). Although weak growth was observed at lower 3AT concentrations, this growth was comparable to that of negative controls, and much lower than the positive control, suggesting it was nonspecific (Fig. 2A). Similarly, Fe supplementation did not promote interaction, indicating that exogenous Fe does not significantly affect OsVIT1–OsVIT2 interaction in this system (Fig. 2B). Given that OsVIT

proteins are localized to the tonoplast, we also employed a membrane protein-specific Y2H system based on the split-ubiquitin mechanism, which enables the detection of interactions of full-length membrane proteins in their native cellular environment, thereby overcoming the nuclear localization requirement of the classic Y2H. However, no interaction between OsVIT1 and OsVIT2 was detected in this system either (Fig. S1).

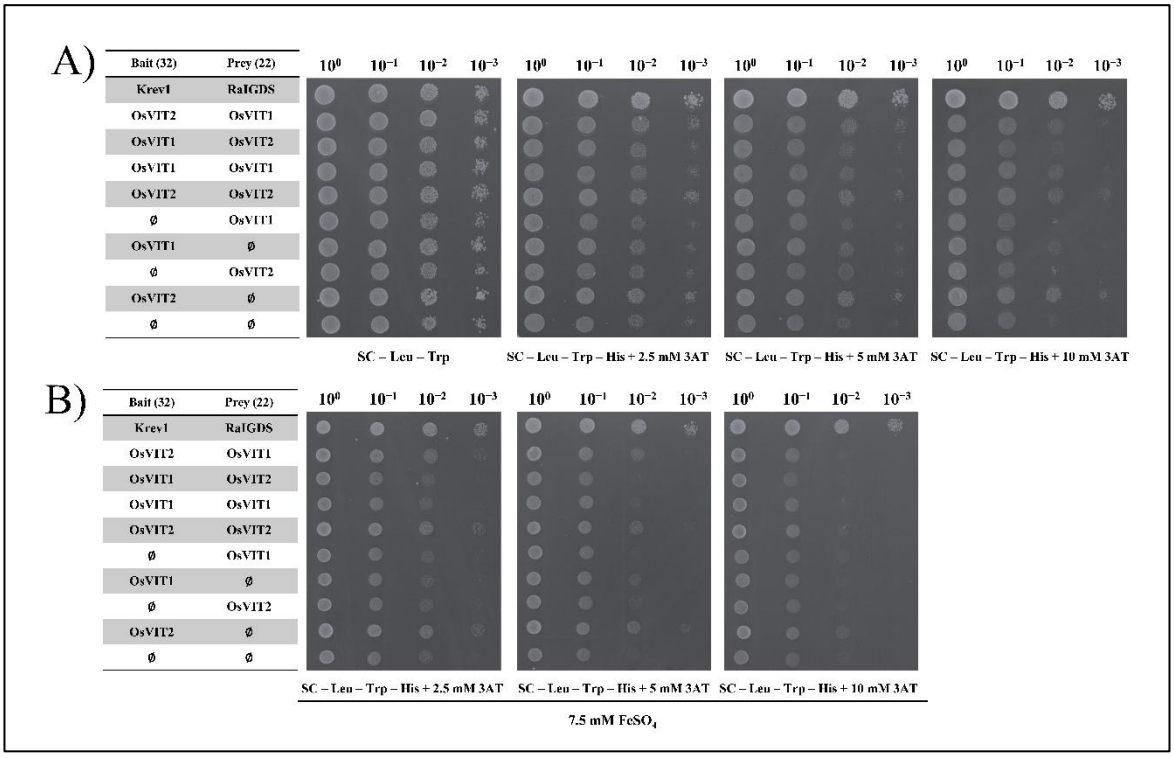


Figure 2. Evaluation of the interaction between OsVIT1 and OsVIT2 through a Y2H assay. (A) Yeast cell growth on standard SD-Leu-Trp medium or supplied with different concentrations of 3-amino-1,2,4-triazole (3AT). (B) Yeast cell growth on SD-Leu-Trp medium supplied with different 3AT concentrations and 7.5 mM $FeSO_4$. Ø, empty plasmid (negative activation control).

3.2. Investigating the functional role of the N-terminal domain of OsVIT2 in Fe^{2+} transport

To investigate the potential functional role of the N-terminal domain of OsVIT2 in Fe^{2+} transport, we generated a modified version of OsVIT2 lacking the N-terminal domain coding sequence (27 amino acids), referred to as OsVIT2mod. This construct, along with the native OsVIT2, was expressed in the yeast mutant strain Δ*ccc1* using the pYES260 plasmid. In

yeast, CCC1 functions as a Fe^{2+}/H^{+} antiporter, localizing to the tonoplast where it sequesters excess cytosolic Fe^{2+} into the vacuole (Li *et al*., 2001). While all lines grew under control conditions, the transformant expressing the native OsVIT2 failed to grow at or above 5 mM Fe (Fig. 3A). This was an unexpected outcome, as it was previously shown that OsVIT1 and OsVIT2 can partially complement the Δ*ccc1* phenotype in yeast (Zhang *et al*., 2012). To determine whether this was linked to potential issues regarding the GAL1-mediated expression from the pYES260 plasmid, both native and OsVIT2mod sequences were additionally cloned into the pFL61-GW plasmid. However, these transformants also failed to grow under Fe treatment (Fig. 3B). One possibility to explain the failed complementation of Δ*ccc1* was that the strain used had some inherent issues.

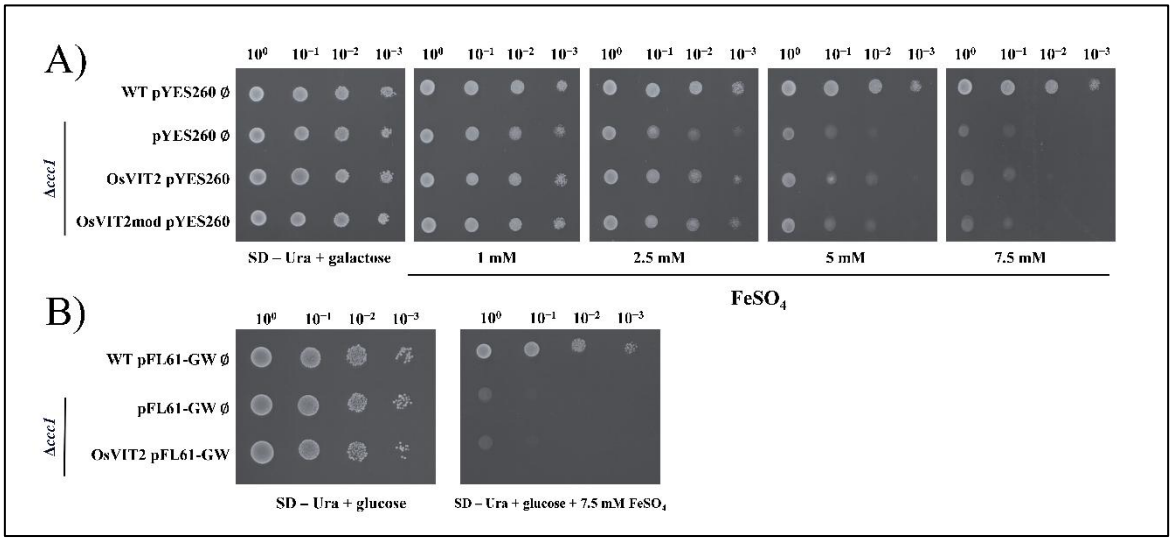


Figure 3. Functional analysis of the OsVIT2 N-terminal in Fe^{2+} transport. (A) Spot assay of yeast mutant strain Δ*ccc1* transformed with OsVIT2 pYES260 or VIT2mod pYES260 on SD-URA medium + galactose supplied with different concentrations of $FeSO_4$. (B) Spot assay of Δ*ccc1* transformed with OsVIT2 pFL61-GW on standard SD-Ura medium or supplied with 7.5 mM $FeSO_4$. WT, wild-type (BY4741 strain). Ø, empty plasmid.

To address this possibility, a new Δ*ccc1* strain along with a second, independent Δ*ccc1* mutant in the BY4743 background (designated hereafter as Δ*ccc1/43*) were acquired. Both strains were transformed with the native and modified OsVIT2 sequences in the pFL61-GW plasmid. Surprisingly, all transformants in the original Δ*ccc1* background again showed no growth under Fe treatment (Fig. S2). Therefore, subsequent analyses were carried out

exclusively using the Δ*ccc1/43* strain. Although the Δ*ccc1/43* transformant expressing OsVIT2 showed a slightly better growth at 7.5 mM and 10mM Fe, this was only apparent with pFL61-GW (Fig. 4A), but not with the pYES260 plasmid (Fig. 4B). It is important to note that the negative control lines exhibited similar growth to those expressing OsVIT2 or OsVIT2mod (Fig. 4).

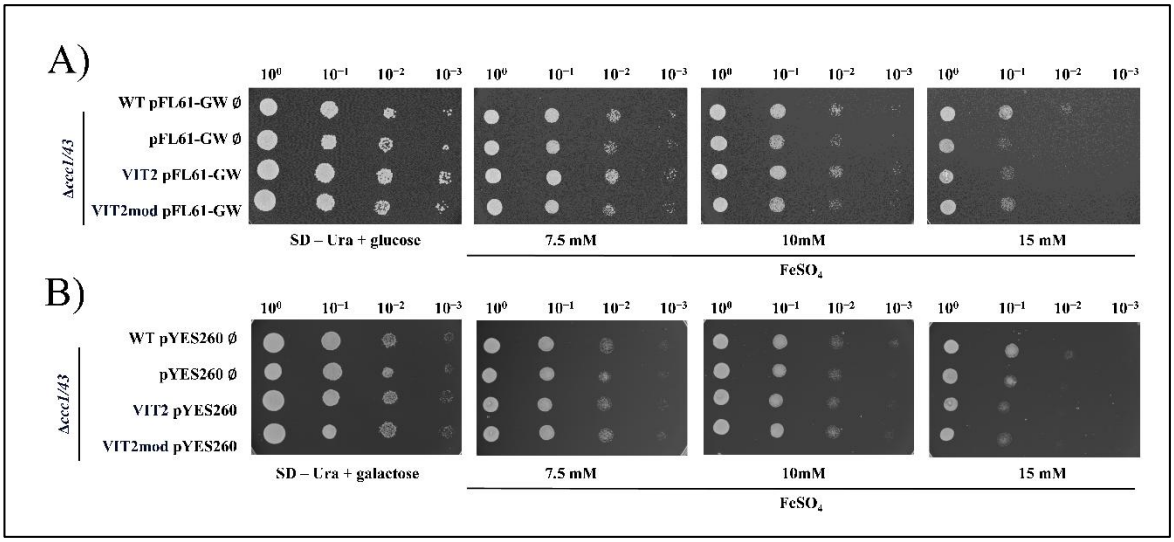


Figure 4. Functional analysis of the OsVIT2 N-terminal in Fe^{2+} transport. (A) Spot assay of yeast mutant strain Δ*ccc1/43* transformed with OsVIT2 pFL61-GW or VIT2mod pFL61-GW on SD-URA medium supplied with different concentrations of $FeSO_4$. (B) Spot assay of Δ*ccc1/43* transformed with OsVIT2-pYES260 or VIT2mod-pYES260 on SD-URA medium + galactose supplied with different concentrations of $FeSO_4$. WT, wild-type (BY4743 strain). Ø, empty plasmid.

4. Discussion

In rice, OsVIT1 and OsVIT2 play a crucial role in mediating Fe^{2+} sequestration into vacuoles, thus maintaining cytosolic Fe balance and contributing to both stress responses and grain Fe concentrations (Krishna *et al*., 2023; Benato *et al*., 2025). Recent structural analysis of OsVIT2 has further explored the mechanistic basis of its function, showing that OsVIT2 contains an extended cytoplasmic region, at its N-terminal domain, which was hypothesized to transiently bind to Fe^{2+} ions and deliver them to the entrance of the protein channel (Arend *et al*., 2025). Exploring whether this region is required for Fe^{2+} transport could not only elucidate the functional modularity of OsVIT2, but also provide deeper

insights into the evolution and engineering of metal transporters for biofortification applications. Furthermore, given that VIT plant transporters act as homodimers (Kato *et al*., 2019; Arend *et al*., 2025), the possibility of OsVIT1 and OsVIT2 interacting with each other is compelling, as the disruption of each gene did not result in distinct phenotypes between *osvit1*, *osvit2* and *osvit1osvit2* plants (Zhang *et al*., 2012; Benato *et al*., 2025). The uncovering of a physical interaction between these two proteins would not only provide information on the structural mechanisms of vacuolar Fe^{2+} transport, but could also reveal new regulatory pathways essential for Fe partitioning and storage in plant cells.

4.1. No evidence for physical interaction between OsVIT1 and OsVIT2 according to independent Yeast Two-Hybrid (Y2H) systems

In this study, we employed Y2H assays to investigate the potential interaction between OsVIT1 and OsVIT2 (Fig. 1, 2 and S1). Despite reciprocal cloning of both proteins into bait and prey plasmids and testing under different conditions, no evidence of protein–protein interaction was observed. Although our results may suggest the absence of interaction between OsVIT1 and OsVIT2, they must be interpreted cautiously, as the Y2H system has intrinsic limitations when applied to membrane-bound proteins. Conventional Y2H assays rely on the reconstitution of a transcriptional activator in the nucleus, which typically requires soluble protein domains capable of interacting in the cytosolic space (Brückner *et al*., 2009). *S. cerevisiae* is also widely used for expressing plant membrane proteins due to its eukaryotic folding mechanism and ability to perform certain post-translational modifications (Kesidis *et al*., 2020). However, membrane proteins such OsVIT1 and OsVIT2, which are naturally embedded in the tonoplast and contain multiple transmembrane domains, may be misfolded, mislocalized, or incapable of proper interaction in this system (Bao *et al*., 2009; Popova *et al*., 2023). This is further supported by the absence of interaction for both VIT proteins as homodimers, which are presumably occurring.

Considering this limitation of conventional Y2H, the putative interaction was further investigated using a membrane protein-specific Y2H system. Likewise, no interaction between OsVIT1 and OsVIT2 was detected in this system either. Although membrane-optimized Y2H systems are designed to accommodate transmembrane proteins (Lentze & Auerbach, 2008; Snider *et al*., 2010), these assays may not accurately mimic the lipid composition, redox environment, or ion concentrations of the plant vacuole (Zhang *et al*.,

2015). Furthermore, weak or transient interactions may fall below the detection threshold of optimized Y2H systems. This is particularly relevant for transport proteins, which may require specific metal-binding states, cofactors, or pH-dependent conformational changes to be able to interact properly (Brückner *et al*., 2009). Thus, the two Y2H systems used in the present study were not able to provide experimental evidence for the formation of OsVIT homo- and/or heterodimers previously demonstrated *in silico* (Kato *et al*., 2019; Arend *et al*., 2025). Given the limitations of Y2H, these interactions should be investigated directly *in planta*, e.g., employing co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation (BiFC). These methods would allow to investigate a possible interaction of OsVITs in a context closer to native conditions and may provide evidence of the heterodimerization between the two OsVIT proteins.

4.2. Potential functional role of the N-terminal domain of OsVIT2 remains unresolved in yeast complementation assays

In this study, we also investigated the potential functional role of the N-terminal domain of OsVIT2 in Fe^{2+} transport by expressing a truncated form, OsVIT2mod, in the yeast mutant Δ*ccc1* strain (Li *et al*., 2001). The initial assumption was that Δ*ccc1* would provide a sensitive and reliable model to reveal functional differences in vacuolar Fe transport activity, as partial complementation of the phenotype was observed with OsVIT1 and OsVIT2 under 7.5 mM Fe (Zhang *et al*., 2012). Unexpectedly, expression of the native OsVIT2 in Δ*ccc1* yielded no growth at or above 5 mM Fe, despite successful expression under both pYES260 (GAL1 promoter) and pFL61-GW plasmids (Fig. 3 and S2). While the use of the Δ*ccc1/43* strain appeared to improve experimental consistency, no remarkable visual differences in growth were observed between the transformants expressing native OsVIT2 and OsVIT2mod under different Fe concentrations (Fig. 4 and S2). One possibility to explain these results is that vacuolar Fe loading is not completely abolished in the Δ*ccc1* strains used in this study. Amaral *et al*. (2021) identified an internal promoter within the yeast CCC1 coding region that promotes the expression of a N-terminally truncated isoform, referred to as s-Ccc1, indicating evolutionary pressure to maintain minimal transporter function. Although s-Ccc1 is less efficient than full-length CCC1, it is still capable of sequestering Fe into vacuoles, partially rescuing Δ*ccc1* survival under Fe^{2+} excess (Amaral *et al*., 2021). Thus, it is possible that the unchecked expression of s-Ccc1 could have resulted in residual

vacuolar Fe^{2+} transport, attenuating the selective pressure required to distinguish phenotypic differences between OsVIT2 and OsVIT2mod-expressing transformants, especially if the constructs partially complement the Δ*ccc1* phenotype, as previously observed for the native OsVIT2 (Zhang *et al*., 2012).

Strikingly, Sorribes-Dauden *et al*. (2021) reported that the deletion of the N-terminal domain of CCC1 mitigated the toxicity observed upon constitutive overexpression of full-length CCC1 in Fe-sufficient media, without compromising the transporter's ability to accumulate Fe in the vacuole. The authors also observed that the N-terminal domain of CCC1 may influence membrane localization patterns. While the full-length CCC1 tended to cluster in specific tonoplast regions, the N-terminally truncated protein, in this case designated as "NtΔCcc1", showed a more uniform distribution (Sorribes-Dauden *et al*., 2021). Although we did not assess OsVIT2 subcellular localization in yeast, these findings collectively suggest that the N-terminal domain of OsVIT2, similar to that of CCC1, may regulate protein activity and/or localization. Overall, these findings suggest a possible limitation of using Δ*ccc1* strains with potentially incomplete deletions.

It is worth mentioning that the regulation of CCC1 is part of a complex transcriptional network involving multiple stress-responsive transcription factors, which modulate CCC1 expression in response to Fe availability and oxidative stress (Li *et al*., 2008, 2012, 2017; Pimentel *et al*., 2012; Rietzschel *et al*., 2015). Variability in these pathways between yeast strains, especially between genetically engineered backgrounds, may influence the cellular response to Fe stress and potentially explain the differential sensitivity of Δ*ccc1* and Δ*ccc1/43* mutants observed in our experiments (Fig. 3, 4 and S2), suggesting strain-specific traits unrelated to promoter-driven expression or plasmid systems. This underscores the need for careful strain validation, and the use of more quantitative assays to detect subtle differences in vacuolar Fe^{2+} transport capacity. Therefore, based on the internal promoter activity in CCC1, it would be valuable to test whether OsVIT2 has cryptic internal promoters producing truncated isoforms *in planta*, and whether such forms are functional or redundant. Direct Fe^{2+} uptake or vacuolar accumulation assays to quantify any subtle functional losses should also be considered (Holmes-Hampton *et al*., 2013). It may also be beneficial to generate yeast strains with strictly null CCC1 function via CRISPR/Cas9 to prevent expression of truncated endogenous proteins, thereby reducing potential background activity (Wu *et al*., 2025).

5. Conclusions

In summary, our Y2H assays did not detect interactions between OsVIT1 and OsVIT2, nor self-interactions, likely due to the inherent limitations of the system for studying tonoplast-localized, multi-transmembrane proteins. Similarly, functional complementation assays of the yeast mutant strain Δ*ccc1* using the native and a truncated sequence of OsVIT2 failed to reveal phenotypic differences regarding the potential role of N-terminal domain of OsVIT2 in Fe^{2+} transport. Altogether, these findings highlight technical and biological challenges of studying plant membrane transporters in yeast systems.

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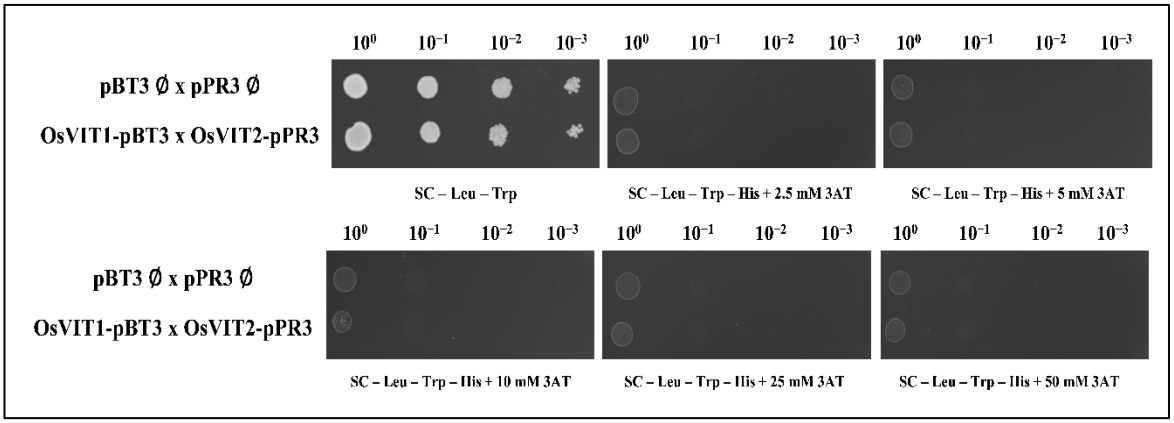
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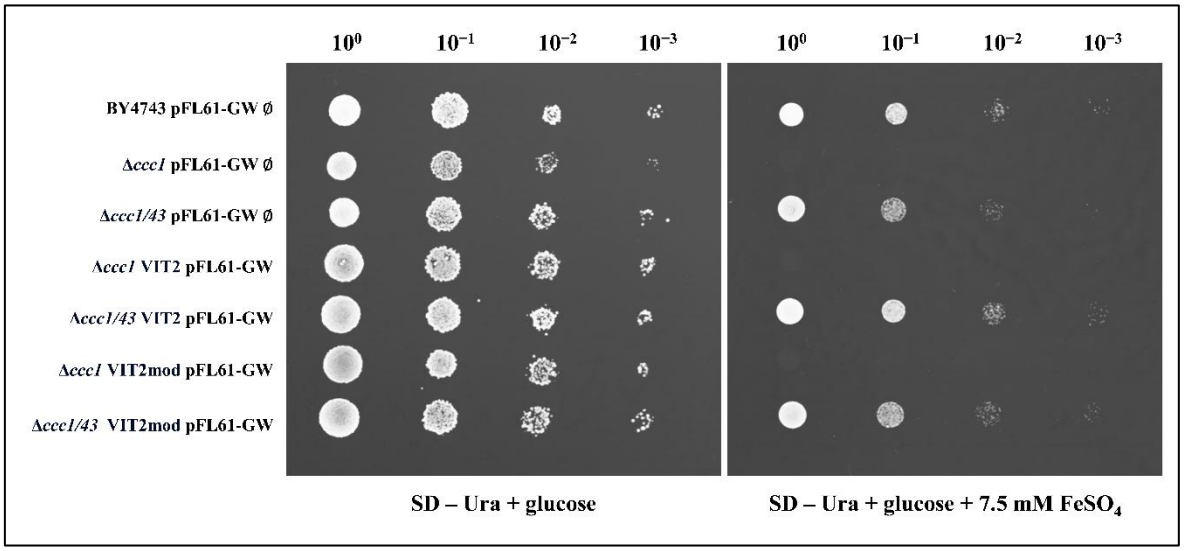
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Supplemental material



Supplemental Figure 1. Evaluation of the interaction between OsVIT1 and OsVIT2 through the growth analysis of yeast mutant strain NMY51 cotransformed with pBT3 and pPR3 constructs (plasmids specific for membrane-associated proteins) on SD-Leu-Trp solid selection media with different concentrations of 3-amino-1,2,4-triazole (3AT). Ø, empty plasmid (negative activation control).



Supplemental Figure 2. Growth analysis of Δ*ccc1* (background BY4741) and Δ*ccc1/43* (background BY4743) mutant strains transformed with OsVIT2-pFL61-GW and OsVIT2mod-pFL61-GW constructs on SD-URA medium supplied with 7.5 mM $FeSO_4 \cdot 7H_2O$. Ø, empty plasmid.

Supplemental Table 1. List of primers used in this work.

Primer identification	Sequence (5'→3')
OsVIT1-FWD-TOPO	CACCATGGCGGCGGCGACGGACGGC
OsVIT1-RVS	TCATCTAGTTTGCACGGCCTTGG
OsVIT2-FWD-TOPO	CACCATGGTGAAGGAGTTCGTGCAGGAC
OsVIT2-RVS	TTAGATGGACTGCACGGCCTTGGC
OsVIT2mod-FWD-TOPO	CACCATGGAGGTGGTCCGCGACATCATCAT
OsVIT2-BamHI-FWD	GGATCCTTAGATGGACTGCACGGC
OsVIT2-HindIII-RVS	AAGCTTATGGTGAAGGAGTTCGTGC
OsVIT2mod-HindIII-RVS	AAGCTTATGGAGGTGGTCCGCGAC
OsVIT1-pBT3-FWD	ATTAACAAGGCCATTACGGCCATGGCGGCGGCGACGGACGGC
OsVIT1-pBT3-RVS	AACTGATTGGCCGAGGCGGCCCCTCTAGTTTGCACGGCCTTGG
OsVIT2-pPR3-FWD	ATTAACAAGGCCATTACGGCCATGGTGAAGGAGTTCGTGCAGGAC
OsVIT2-pPR3-RVS	AACTGATTGGCCGAGGCGGCCTTAGATGGACTGCACGGCCTTGGC

DISCUSSÃO GERAL

A homeostase de Fe em plantas é mantida por um complexo sistema regulatório composto por fatores de transcrição e transportadores (Gao & Dubos, 2021). Estes componentes atuam em conjunto para assegurar a adequada percepção, distribuição e armazenamento de Fe, especialmente em resposta a flutuações ambientais (Krohling *et al*., 2016). A compreensão dessas vias regulatórias fundamenta o desenvolvimento de estratégias de biofortificação voltadas ao incremento da qualidade nutricional de órgãos vegetais destinados à alimentação humana, com destaque para os grãos de arroz (Wairich *et al*., 2022). Neste contexto, investigamos: (1) a regulação transcricional mediada pelas proteínas OsASR, com foco em alterações morfofisiológicas e moleculares observadas em plantas OsASR5-RNAi; e (2) o transporte vacuolar de Fe^{2+} mediado pelas duas proteínas OsVIT, enfatizando uma possível dinâmica funcional entre ambas e nas características estruturais de OsVIT2.

É sabido que as proteínas OsASR estão associadas a respostas a diferentes estresses abióticos, como seca (Li *et al*., 2017; Park *et al*., 2020; Srivastava *et al*., 2022), salinidade (Zhang *et al*., 2022), toxidez por Al (Arenhart *et al*., 2013, 2014, 2016) e deficiência de P (Barros *et al*., 2025b). Nossos resultados expandem este panorama ao demonstrar que a supressão dos genes *OsASR* na linhagem OsASR5-RNAi resulta em plantas mais vulneráveis ao estresse por Fe, tanto em condição de deficiência quanto de excesso. Sob deficiência de Fe, esta maior sensibilidade foi evidenciada por reduções na altura da parte aérea, acúmulo de biomassa e performance fotossintética, além de clorose foliar antecipada. É interessante notar que não foram observadas alterações significativas na indução de genes-chave relacionados à absorção de Fe. Contudo, a expressão alterada de *OsYSL2*, cuja proteína desempenha papel importante no transporte de Fe a longa distância via floema (Koike *et al*., 2004; Nozoye *et al*., 2007; Ishimaru *et al*., 2010), sugere que as proteínas OsASR podem atuar em vias regulatórias alternativas.

Curiosamente, as plantas OsASR5-RNAi apresentaram um sistema de raízes severamente atrofiado e menos ramificado em condição controle e em excesso de ferro. Este fenótipo foi similarmente observado em deficiência de fosfato (Barros *et al*., 2025b). Além disso, foi relatado o envolvimento de OsASR5 no metabolismo de açúcares (Joo & Song, 2015), bem como na biossíntese e sinalização de ABA (Li *et al*., 2017). Em conjunto, esses resultados indicam que as proteínas OsASR podem atuar integrando a regulação da

homeostase de metais, níveis de açúcares e respostas a estresses, o que é crucial para manter o crescimento de raízes e o equilíbrio energético, principalmente sob condições adversas.

Os transportadores vacuolares OsVIT1 e OsVIT2 foram associados à regulação do acúmulo de Fe nos grãos de arroz (Zhang *et al*., 2012; Bashir *et al*., 2013), o que os torna alvos relevantes para estratégias de biofortificação (Benato *et al*., 2025). Visto que não foram observadas diferenças nas concentrações de Fe no embrião de grãos entre plantas *osvit1*, *osvit2* e *osvit1osvit2* (Zhang *et al*., 2012; Benato *et al*., 2025), e considerando modelos *in silico* que indicam que estas duas proteínas atuam como homodímeros (Kato *et al*., 2019; Arend *et al*., 2025), investigamos a possível heterodimerização entre OsVIT1 e OsVIT2. Para isso, realizamos ensaios de duplo híbrido em levedura (Y2H), nos quais não foram observadas interações entre as duas proteínas, nem entre os monômeros de cada uma delas. Embora isso possa indicar ausência de interação, é provável que limitações técnicas do sistema Y2H, especialmente com proteínas transmembrana, tenham inibido uma detecção precisa (Bao *et al*., 2009; Popova *et al*., 2023). Interações transitórias ou específicas de proteínas embebidas em membranas tendem a ser mal reproduzidas nesse sistema heterólogo (Brückner *et al*., 2009). Abordagens futuras, como co-imunoprecipitação (Co-IP) ou ensaios de interação baseados em microscopia confocal de fluorescência (BiFC) em amostras vegetais, poderão fornecer resultados mais conclusivos.

Análises estruturais recentes demonstraram que OsVIT2 contém um domínio citosólico N-terminal capaz de se ligar transitoriamente a íons Fe^{2+}, facilitando sua entrega ao canal proteico (Arend *et al*., 2025). Para elucidar a relevância funcional desta região, expressamos uma versão truncada de OsVIT2 (OsVIT2mod) na linhagem mutante de levedura Δ*ccc1* (Li *et al*., 2001). Para nossa surpresa, tanto a versão *full-length* quanto a truncada de OsVIT2 apresentaram comportamento semelhante em termos de crescimento da levedura em alto teor de Fe. Estudos demonstraram que uma isoforma truncada de CCC1 pode ser expressa por meio de promotores internos, resultando em um resgate parcial do fenótipo mutante mesmo na ausência de CCC1 canônica (Amaral *et al*., 2021; Sorribes-Dauden *et al*., 2021). Assim, este fenômeno pode ter mascarado diferenças funcionais entre as duas versões de OsVIT2. Além disso, permanece a possibilidade de que o domínio N-terminal de OsVIT2 exerça funções regulatórias ou de localização da proteína, à semelhança do que foi observado em levedura (Sorribes-Dauden *et al*., 2021).

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APÊNDICES

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Research Article
Plant Molecular Genetics - Special Issue

Enhanced expression of *OsNAC5* leads to up-regulation of *OsNAC6* and changes rice (*Oryza sativa* L.) ionome

Andriele Wairich[1†], Ariane Vitali[2&], Janete Mariza Adamski[2], Karina Letícia Lopes[1Σ], Guilherme Leitão Duarte[2], Lucas Roani Ponte[1], Henrique Keller Costa[3], Paloma Koprovski Menguer[1], Rinaldo Pires dos Santos[2], Janette Palma Fett[1,2], Raul Antonio Sperotto[4,5] and Felipe Klein Ricachenevsky[1,2]

[1]*Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia, Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Porto Alegre, RS, Brazil.*
[2]*Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Departamento de Botânica, Porto Alegre, RS, Brazil.*
[3]*Universidade Federal de Santa Maria, Instituto de Ciências Naturais e Exatas, Departamento de Biologia, Porto Alegre, RS, Brazil.*
[4]*Universidade do Vale do Taquari (Univates), Programa de Pós-Graduação em Biotecnologia (PPGBiotec), Lajeado, RS, Brazil.*
[5]*Universidade Federal de Pelotas, Programa de Pós-Graduação em Fisiologia Vegetal (PPGFV), Pelotas, RS, Brazil.*

Abstract

NAC transcription factors are plant-specific proteins involved in many processes during the plant life cycle and responses to biotic and abiotic stresses. Previous studies have shown that stress-induced *OsNAC5* from rice (*Oryza sativa* L.) is up-regulated by senescence and might be involved in control of iron (Fe) and zinc (Zn) concentrations in rice seeds. Aiming a better understanding of the role of *OsNAC5* in rice plants, we investigated a mutant line carrying a T-DNA insertion in the promoter of *OsNAC5*, which resulted in enhanced expression of the transcription factor. Plants with OsNAC5 enhanced expression were shorter at the seedling stage and had reduced yield at maturity. In addition, we evaluated the expression level of *OsNAC6*, which is co-expressed with *OsNAC5*, and found that enhanced expression of *OsNAC5* leads to increased expression of *OsNAC6*, suggesting that *OsNAC5* might regulate *OsNAC6* expression. Ionomic analysis of leaves and seeds from the *OsNAC5* enhanced expression line revealed lower Fe and Zn concentrations in leaves and higher Fe concentrations in seeds than in WT plants, further suggesting that OsNAC5 may be involved in regulating the ionome in rice plants. Our work shows that fine-tuning of transcription factors is key when aiming at crop improvement.

Keywords: Oryza sativa, NAC, iron, zinc, stress.

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Introduction

Rice (*Oryza sativa* L.) is one of the three most important crops in the world, being the staple food for over three billion people (Ali and Wani 2021). However, rice plants often suffer from a variety of biotic and abiotic stresses, such as mineral imbalance, salt, drought, cold, high temperature, pathogens, and phytophagous pests. These biotic and abiotic stresses directly or indirectly affect plant growth and development and may decrease crop yield (Ali *et al*., 2021). To cope with stress, plants have evolved a series of defence mechanisms, which commonly include transcription factors (TFs) controlling downstream genes that code for proteins involved in stress response, acclimation, and tolerance.

The NAC protein family comprises plant-specific TFs that are characterized by the presence of a highly conserved N-terminal DNA binding domain (NAC domain), as well as non-conserved C-terminal domains (Ernst *et al*., 2004; Olsen *et al*., 2005; Fang *et al*., 2008). NAC is an acronym derived from three proteins containing the domain: NAM (*no apical meristem*), ATAF1,2 (*Arabidopsis transcription activator factor*) and CUC2 (*cup-shaped cotyledon*) (Souer *et al*., 1996; Aida *et al*., 1997). The NAC proteins have been implicated in transcriptional control of a variety of plant processes, including responses to phytohormones and to stresses (Meng *et al*., 2007; Sperotto *et al*., 2009; Jeong *et al*., 2010, 2013; Kim *et al*., 2013; Ricachenevsky *et al*., 2013; Lee *et al*., 2017; Liu *et al*., 2020; Li *et al*., 2021). The rice genome has 151

Send correspondence to Felipe Klein Ricachenevsky. Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia, Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Av. Bento Gonçalves, 9500, prédio 43423, sala 224, Bairro Agronomia, 90650-001, Porto Alegre, RS, Brazil. E-mail: felipecruzalta@gmail.com.

† Present address: Justus-Liebig University Giessen, Institute for Agronomy and Plant Breeding I, Department of Agronomy and Crop Physiology, Giessen, Germany.
& Present address: PSC Biotech Ltd, Dublin, Ireland.
Σ Present address: Dartmouth College, Geisel School of Medicine, Department of Biochemistry and Cell Biology, Hanover, NH.

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OsYSL13 transporter may play a role in Mn homeostasis in rice (*Oryza sativa* L.)

Lucas Roani Ponte · Júlia Gomes Farias · Bianca Knebel Del Frari · Henrique Keller Costa · Luís Henrique Ferreira Prigol · Milena Caye · Janette Palma Fett · Flávia Brito Hanzel · Valderi Luiz Dressler · Raul Antonio Sperotto · Gustavo Brunetto · Felipe Klein Ricachenevsky

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Abstract Iron (Fe) is an essential element for almost all living organisms. In plants, it is a key player in electron transport in both photosynthesis and respiration. Rice (*Oryza sativa* L.) is a crop that feeds half of the world's population. However, rice grains have low Fe concentrations, making populations that base their diet on this crop prone to Fe deficiency. Nutritional Fe deficiency can be addressed by biofortification. To achieve this goal, however, it is necessary to understand plants' Fe homeostasis mechanisms. Here we evaluate the physiological consequences of loss of *OsYSL13* gene in Fe homeostasis and possible alterations in manganese (Mn) homeostasis. We found that leaves of *osysl13* mutant plants have lower concentrations of Fe and Mn when extra Mn is added to the soil, compared to wild-type (WT) plants. Mutant *osysl13* plants less compared to WT not only in control, but also under Mn deficiency, with decreased shoot biomass accumulation and root length, as well as increased root diameter, suggesting that OsYSL13 has a function in Mn homeostasis. Interestingly, we observed that *osysl13* mutant plants accumulate higher H_2O_2 levels in their leaves, causing visible damages. Mn concentration was also higher in *osysl13* leaves compared to WT. Our data suggest that OsYSL13 might be relevant in Mn

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L. R. Ponte · J. P. Fett · F. K. Ricachenevsky (✉)
Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
e-mail: felipecruzalta@gmail.com

J. G. Farias · B. K. Del Frari · H. K. Costa · L. H. F. Prigol · M. Caye
Departamento de Biologia, Instituto de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

J. P. Fett · F. K. Ricachenevsky
Departamento de Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

F. B. Hanzel · V. L. Dressler
Departamento de Química, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

R. A. Sperotto
Programa de Pós-Graduação em Biotecnologia (PPGBiotec), Universidade do Vale do Taquari - Univates, Lajeado, RS, Brazil

R. A. Sperotto
Programa de Pós-Graduação em Fisiologia Vegetal (PPGFV), Universidade Federal de Pelotas, Pelotas, RS, Brazil

G. Brunetto
Departamento de Solos, Programa de Pós-Graduação em Ciência do Solo, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

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Aluminum alleviates iron deficiency chlorosis by interfering with phosphorus homeostasis in rice (*Oryza sativa* L.)

Jover da Silva Alves [a], Paloma Koprovski Menguer [a], Yugo Lima-Melo [a], Victor Hugo Rolla Fiorentini [a], Lucas Roani Ponte [a], Raquel Vargas Olsson [a], Vic Martini Sasso [b], Nicolás De Palma [c], Luciane Almeri Tabaldi [b], Gustavo Brunetto [b], Ricardo F.H. Giehl [d], Márcia Margis-Pinheiro [a,e], Felipe Klein Ricachenevsky [a,c,*]

[a] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Brazil
[b] Departamento de Biologia, Universidade Federal de Santa Maria, Brazil
[c] Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Brazil
[d] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) OT Gatersleben, Corrensstr 3, 06466, Seeland, Germany
[e] Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil

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Iron and aluminum interaction
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ABSTRACT

Rice (*Oryza sativa* L.) is a staple food for more than half of the human population. Rice plants are cultivated in several different environments, and face various abiotic stresses, including nutritional imbalance in soils. The ionome, the inorganic composition of an organism, is known to be tightly regulated, as changes in concentration of one element affect concentrations of others. Iron (Fe) is an essential element that is involved in redox reactions, nitrogen metabolism and chlorophyll synthesis. The hallmark of Fe deficiency in plants is leaf chlorosis, a phenotype known to be alleviated by deficiencies of other elements, such as phosphorus (P). Aluminum (Al) is abundant in soils and limits plant growth in acidic soils. Despite its well-established detrimental effects, Al has been proposed to have a positive effect on growth for some species, but little is known about this phenomenon. Here we aim to understand whether Al affects Fe homeostasis in rice. We found that Al alleviated Fe deficiency-induced chlorosis. +Al–Fe treatment decreased expression of Fe deficiency marker genes and partially recovered photosynthesis. We also observed that Al induced expression of a P deficiency marker gene, and addition of excess P to nutrient solution reversed effects of Al on chlorosis. Our data show that Al alleviates Fe deficiency-induced chlorosis, and suggests that this occurs indirectly by inducing P deficiency in leaves.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most cultivated and consumed cereals in the world and is considered a staple food for more than half of the world's population, providing 21% of the calories consumed by humans (Elert, 2014). Rice is cultivated on all continents, with rice genotypes and cultivars adapted to different environmental, climatic, and agronomic conditions. Due to the large cultivation area, rice fields are often exposed to abiotic stresses, such as flooding, drought, salinity, heat, cold, deficiency of essential nutrients, and toxicity of hazardous elements, which can have detrimental impacts in yield and quality (Ganie et al., 2024; Ray et al., 2015). These stresses can occur simultaneously, forcing plants to respond to combinations of stresses (Priya et al., 2023). Diverse edaphic conditions also result in combined mineral element-related stresses (DeLoose et al., 2024), since changes in the concentration of one element can affect the homeostasis of others (Huang and Salt, 2016; Pita-Barbosa et al., 2019; Ricachenevsky et al., 2018). Therefore, it is crucial to understand how accumulation of one element might affect the ionome balance.

Aluminum (Al) is one of the most abundant elements in the Earth's crust and a major limiting factor for plant growth worldwide (Imadi et al., 2016; Ofoe et al., 2023). This is because Al^{3+} becomes highly soluble and causes phytotoxicity under acidic conditions, which are prevalent in approximately 50% of arable soils (Kochian et al., 2015). Most plants are sensitive to Al, which inhibits root growth even when present at micromolar concentrations, thereby decreasing water and

* Corresponding author. Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Brazil.
E-mail address: felipecruzalta@gmail.com (F.K. Ricachenevsky).

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The role of *ASR* (ABA, Stress, and Ripening) genes in responses to phosphate starvation in rice roots

Nicolle Louise Ferreira Barros [a,#], Breno Xavier Gonçalves [a,#], Thomaz Stumpf Trenz [a], Paloma Koprovski Menguer [a,b], Lucas Roani Ponte [b], Cristiane P.G. Calixto [c], Felipe Klein Ricachenevsky [b,d], Marcia Margis-Pinheiro [a,b,*]

[a] Programa de Pós-Graduação em Genética e Biologia Molecular (PPGBM), Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91540-000, Brazil
[b] Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91540-000, Brazil
[c] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP 05508-090, Brazil
[d] Programa de Pós-Graduação em Botânica, Departamento de Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91540-000, Brazil

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ABSTRACT

Phosphorus (P) is a crucial macronutrient for plant growth and development, absorbed by plant roots as inorganic phosphate, which is frequently limited in soil. Plants use only 30 % of the total phosphate fertilizers applied to increase yield. Compared to other nutrients, the understanding of the molecular mechanisms involved in phosphate homeostasis in crops, particularly in the early transcriptional responses to change the root system architecture remain underexplored. Addressing these knowledge gaps requires studies that offer insights into the role of transcription factors in response to endogenous and exogenous signals associated with the nutritional status of crops. ASR (ABA, Stress and Ripening) proteins function as molecular chaperones, transcription factors, and homeostasis sensors. They also regulate the development and response to stress in plants. Our results show that *ASR* genes play an important role in phosphate homeostasis in rice (*Oryza sativa* L.) roots. Silencing of *OsASR* genes (OsASR-RNAi plants) delays development of adventitious and lateral roots, and alters the expression of genes associated with root development and the response to phosphate starvation. These findings suggest that *OsASR* play a role in regulating root system architecture, nutrient perception and signal transduction in rice plants.

Introduction

Large amounts of phosphate (Pi) are applied to the soil for the cultivation of economically important plants such as rice, soybeans, and maize. However, only one-third of this nutrient present in the soil is absorbed by the root system (Johnston et al. 2014; Khan et al. 2023). In addition to the high cost, the unabsorbed mineral contaminates water bodies, causing eutrophication of lakes and blooms of toxic cyanobacteria (Plaxton and Lambers, 2015), which are harmful to human health. Furthermore, the geological source of this nutrient is limited (Johnston et al. 2014; Khan et al. 2023). Therefore, strategies targeting the biological components regulating plant-phosphate relationship, such as studies emphasizing molecular resources activated by plants to manage nutritional status properly may contribute, in the long term, to mitigate environmental issues associated with phosphate uses in agriculture.

To increase phosphate uptake, plants develop adaptive strategies, including changes in root system architecture, increased transporter activity, phosphatase induction, and internal P remobilization (Smith et al. 2011; Jain et al. 2012; Jia et al. 2017). The change in the root system architecture is among the main physiological mechanisms related to an increase in the root surface area that allows a more efficient absorption of phosphate, enabling greater growth and development of the plants (Péret et al. 2011; Gu et al. 2016; Jia et al. 2017). This change in the architecture of the root system mainly consists of new root development, including adventitious roots, lateral roots, and root hairs. For the emergence of lateral roots, a well-orchestrated process begins with the formation of lateral root primordia (Torres-Martínez et al. 2019), which is then followed by a highly organized sequence of cell

* Corresponding author.
E-mail address: marcia.margis@ufrgs.br (M. Margis-Pinheiro).
# These authors contributed equally to this work

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Genetics and Molecular Biology

The impact of OsASR5 on rice microbiome: Implications for nutrient cycling and stress response

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Complete List of Authors:	Cadavid, Isabel; Universidade Federal do Rio Grande do Sul, Departmento de Biofísica e Centro de Biotecnologia Margis-Pinheiro, Marcia; Universidade Federal de Rio Grande do Sul, Genetics Dias-Oliveira, Mateus; Universidade Federal do Rio Grande do Sul, Departmento de Biofísica e Centro de Biotecnologia Ponte, Lucas; UFRGS, Botânica Ricachenevsky, Felipe; Universidade Federal do Rio Grande do Sul Instituto de Biociencias, Departamento de Botanica Margis, Rogerio; Universidade Federal do Rio Grande do Sul, Departmento de Biofísica e Centro de Biotecnologia
Keyword:	microbiome, phyllosphere, rhyzosphere, OsASR5, Bacterium

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CURRICULUM VITAE resumido

PONTE, L. R.

1. DADOS PESSOAIS

Nome: Lucas Roani Ponte

Local e data de nascimento: Santa Maria, Rio Grande do Sul, 15 de junho de 1996

Endereço profissional: Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Campus do Vale / Bloco 3 / Prédio 43423 / Sala 230

Telefone: (55) 981428262

E-mail: lucp109@gmail.com

2. FORMAÇÃO

Doutorado em Biologia Celular e Molecular
Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil – 2025
Período-sanduíche no Leibniz Institute of Plant Genetics and Crop Plant Research, Alemanha (Out 24 – Jul 25).
Tese:
Foco: Nutrição mineral de plantas; Homeostase de ferro; Arroz

Mestrado em Ciências Agrárias (Fisiologia Vegetal)
Universidade Federal de Viçosa, Viçosa, MG, Brasil – 2021
Dissertação: The antagonistic Effect of AtCCR4a Upon Drought or Heat Stress Conditions.
Foco: Estresses abióticos; Alta temperatura; Seca; Arabidopsis

Bacharelado em Agronomia
Universidade Federal de Santa Maria, Santa Maria, RS, Brasil – 2019
Foco: Nutrição mineral de plantas; Homeostase de ferro; Arroz

3. ESTÁGIOS

2018: Estágio curricular obrigatório realizado no Laboratório de Fisiologia Vegetal, pertencente ao Departamento de Botânica, do Centro de Biotecnologia e Instituto de Biociências da UFGRS, de março a junho de 2018 sob orientação do prof. Dr. Felipe Klein Ricachenevsky e supervisão da profa. Dra. Janette Palma Fett (carga horária total: 480 h). Acompanhei os experimentos e técnicas realizados pela doutoranda Andriele Wairich para elaboração da tese: Respostas à deficiência e excesso de ferro em espécies do gênero Oryza.

Tive a oportunidade de executar técnicas de biologia molecular (extração de RNA, síntese de cDNA e PCR quantitativo em tempo real), bioquímica vegetal (extração e quantificação de clorofilas) e medição de parâmetros fotossintéticos (uso do SPAD-502 e Analisador de Fotossíntese IRGA).

2017: Bolsista (FIPE Enxoval - PRPGP/UFSM) vinculado ao projeto Caracterização Ionômica de Plantas de Arroz (Oryza sativa) Mutantes para Genes Relacionados à Homeostase de Metais sob orientação do prof. Dr. Felipe Klein Ricachenevsky (carga horária total: 700 h). Realizei experimentos, em sala de crescimento, para avaliar o papel dos genes OsYSL13 e OsYSL15 na homeostase de Fe em plantas de arroz por meio da análise de parâmetros morfofisiológicos.

2015 – 2017: Estagiário voluntário vinculado ao projeto Caracterização Ionômica de Plantas de Arroz (Oryza sativa) Mutantes para Genes Relacionados à Homeostase de Metais sob orientação do prof. Dr. Felipe Klein Ricachenevsky (carga horária total: 344 h). Realizei experimentos, em sala de crescimento, para avaliar o papel dos genes OsYSL13 e OsYSL15 na homeostase de Fe em plantas de arroz por meio da análise de parâmetros morfofisiológicos.

2014 – 2015: Bolsista (PROBIC/FAPERGS/UFSM) vinculado ao projeto Otimização de Técnicas Experimentais em Cultivos Agrícolas sob orientação do prof. Dr. Sidinei José Lopes (carga horária total: 420 h). Auxiliei o mestrando Pedro Hernandez Padilha na realização de experimentos a campo para a elaboração da dissertação: Crescimento, desenvolvimento, dimensionamento amostral e atividade antiproliferativa de calêndula.

4. ARTIGOS COMPLETOS PUBLICADOS

Ponte, L. R., Alves, J. da S., Lima-Melo, Y., Menguer, P. K., Boulanger, H. G., Giehl, R. F. H., Calixto, C. P. G., Margis-Pinheiro, M., & Ricachenevsky, F. K. (2025). Abscisic acid, stress and ripening (ASR) proteins play a role in iron homeostasis in rice (Oryza sativa L.). Plant Physiology and Biochemistry, 223, 109882. https://doi.org/10.1016/j.plaphy.2025.109882

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5. RESUMOS E TRABALHOS APRESENTADOS EM EVENTOS CIENTÍFICOS

Ponte, L. R. Morad, M. M.; Eifler, J.; Sahr, M.; von Wiren, N.; Giehl, R. F. H. (2025). Root physiological and architectural characteristics of semi-dwarf rye (Secale cereale). Pôster apresentado no 20º Plant Science Student Conference, Gatersleben, Alemanha.

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