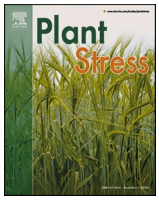
Plant Stress 16 (2025) 100824

Contents lists available at ScienceDirect

Plant Stress

journal homepage: www.sciencedirect.com/journal/plant-stress



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

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ARTICLE INFO

Keywords:
Crop nutrition
Root system architecture
Phosphate metabolism
Rice transcription factor
Gene expression

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-Martinez et al. 2019), which is then followed by a highly organized sequence of cell

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https://doi.org/10.1016/j.stress.2025.100824
Received 30 December 2024; Received in revised form 3 March 2025; Accepted 25 March 2025
Available online 26 March 2025

divisions and increasing expression of cell wall-related genes, such as those coding wall-associated kinases (WAK) and glucan endo-1, 3-beta-glucosidase (Xie et al. 2018; Chen et al. 2021).

Although many studies are being carried out to better understand the process of phosphate uptake in plants, there are important gaps. It was demonstrated that the *Arabidopsis thaliana sensitive to proton rhizotoxicity 1* (*AtSTOP1)* gene is the transcription factor responsible for starting the local phosphate starvation responses in Arabidopsis roots (Ham et al. 2018). On the other hand, in crop plants such as rice, soybeans, and maize, the transcription factor responsible for initiating this regulation is still unknown. In rice plants, the *AtSTOP1* orthologue is known as *Aluminum Resistance Transcription Factor 1* (*OsART1*) (Fan et al. 2016).

The OsART1 role in phosphate starvation responses has not yet been demonstrated. On the other hand, Arenhart et al. (2013, 2014) conducted extensive research on the role of *Abscisic acid, Stress, Ripening 5* (*OsASR5*) gene in rice, focusing on its involvement in aluminum (Al) tolerance mechanisms. ASR genes are unique to plants and function prominently in regulating responses to abiotic stresses, such as drought, salt, and high temperature, and are involved in developmental processes like fruit ripening and sugar metabolism. These genes have been identified in many plant families, including crops such as rice, tomato, and peanut, but they are absent in *Arabidopsis thaliana* and other members of the Brassicaceae (Cruciferae) family (Carrari et al. 2004). Both *ART1* and *ASR5* are involved in the genetic regulation of plant responses to aluminum toxicity. Together, *ART1* and *ASR5* may represent a coordinated response in rice and other plants to mitigate Al stress. In plants, aluminum (Al) and Pi interact in complex ways, often leading to challenges in nutrient absorption and plant growth. In acidic soils, Al ions (Al^{3+}) react readily with phosphate ions, forming aluminum-phosphate complexes. This reaction renders phosphate largely insoluble and unavailable to plants (Chen et al. 2022).

Since ASR5 acts in response to Al toxicity in an action complementary to ART1, our hypothesis is that ASR5 could also be regulating responses to phosphate deficiency. Therefore, this research aims to study the role of *ASR* genes, emphasizing the *OsASR5* gene, in responses to phosphate deficiency in rice plants. Our results suggest that ASR proteins are crucial for the complexity and robustness of the Pi depletion response pathway, and that *cis*-elements such as *P1BS* and *PHO* in its promoter region may be involved in the modification of the *OsASR5* expression pattern in response to low phosphate levels.

Material and methods

Plant material and treatments

Seeds from *O. sativa* cv. Nipponbare silenced for the *OsASR* genes (ASR-RNAi; Arenhart et al. 2013) and wild-type (WT) were surface sterilized by using ethanol 70 % and sodium hypochlorite 4 %, germinated on filter paper, soaked, and kept in water for 10 days at 20 ºC. Then, 20 seedlings of each genotype and with similar sizes were placed in a nutrient solution according to Yoshida et al. (1976), which was prepared by adding 91.4 g/L NH_4NO_3; 71.4 g/L K_2SO_4; 88.6 g/L $CaCl_2$; 324 g/L $MgSO_4 \cdot 7H_2O$; 1.5 g/L $MnCl_2 \cdot 4H_2O$; 0.074 g/L $(NH_4)_6{\cdot}Mo_7O_{24}{\cdot}4H_2O$; 0.93 g/L H_3BO_3; 0.035 g/L $ZnSO_4{\cdot}7H_2O$; 0.031 g/L $CuSO_4 \cdot 5H_2O$; 7.7 g/L $FeCl_3 \cdot 6H_2O$; 11.9 g/L $C_6H_8O_7 \cdot H_2O$, with different concentrations of phosphate (NaH_2PO_4), being 10 ASR-RNAi seedlings and 10 WT seedlings in 0.016 mM for low phosphate condition and 10 ASR-RNAi seedlings and 10 WT seedlings in 0.323 mM for high phosphate conditions phosphate. In both culture medium conditions, the pH was adjusted to 5.0 and the nutrient solution was changed every 3 days. The plants were kept in a culture room for 20 days at 28 °C with 12 h of light (200 Micromol/m^2/s) and 70 % humidity. Each experiment was repeated three times.

Plant roots from different treatments were photographed and measured using ImageJ software (Version 1.42q, NIH, http://rsb.info.nih.gov/ij/index.html). In the line-measured algorithm, the root length is calculated by counting the number of orthogonally and diagonally connected pairs of pixels.

Besides, another set of wild-type (WT) and ASR-RNAi seeds were surface sterilized using the same protocol and germinated in Petri dishes with filter paper. Later, seedlings were transferred to a hydroponic solution with (C, control) and without (-Pi) NaH_2PO_4 (Yoshida et al. 1976). After five days of treatment, where the plants were kept under the same conditions already described, the total RNA of the roots was extracted with the Direct-zol RNA MiniPrep Plus kit (Zymo Research, CA, US), according to the manufacturer's instructions. RNA samples with RNA Integrity Number (RIN) above 8 were sequenced on the Illumina® NovaSeq6000 system to generate 100 bp paired-end reads.

Analysis of GUS gene expression under the control of the OsASR5 promoter

GUS histochemical analysis was performed on the root system of transgenic rice plants according to Jefferson et al. (1987). Plants used in this work were previously obtained by our group (Arenhart et al. 2014). Briefly, a region of 2060 base pairs upstream of the start codon of the ASR5 gene was amplified using specific primers (Forward 5′-CACCGGACATACTTGCAATATCCTTCTT-3′ and Reverse 5′-AGCTAGAAGCTAGTGATGACAATTAGG-3′). The amplified product was cloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific) and recombined via an LR reaction into the pHGWFS7 vector (Karimi et al. 2002). The resulting plasmid was used to transform rice calli (Upadhyaya et al. 2000). Rice plants were cultivated in a hydroponic system (Yoshida et al. 1976), under the same conditions as described in the "Plant Material and Treatments" section. Rice roots were collected after 5 and 15 days of treatment and incubated in 1 mM X-Gluc, 100 mM phosphate buffer (pH 7.0), 2 mM KH2Fe and 0.5 % Triton X-100. Samples were incubated for 24 h at 37 ºC. After the reaction, the tissues were stored in 70 % ethanol and analyzed using a stereoscopic microscope with a Leica DFC500 camera.

Pre-Processing of RNA-seq data and differential expression analysis

The pre-processing and analysis of RNA-seq datasets were carried out following the methodology outlined by Vitoriano and Calixto (2021). Briefly, Trimmomatic version 0.39 (Bolger et al. 2014) was employed to trim residual adapter sequences from raw paired-end reads. For the production of transcript-specific expression data, the trimmed reads were pseudo-aligned to the rice reference transcriptome (comprising all cDNAs) from the MSU Rice Genome Annotation Project Database, Osa1 Release 7 (Kawahara et al. 2013), using Salmon version 1.4.0 (Patro et al. 2017).

Comprehensive analyses of differential gene expression (DE) were conducted using the 3D RNA-seq app (Guo et al. 2021). We initially produced read counts and Transcript per Million reads (TPM) information from each Salmon output file using the tximport R package version 1.10.0 and the lengthScaledTPM method. Subsequently, genes and transcripts exhibiting fewer than one count per million (CPM) in at least 11 of 12 samples were excluded. Later, the normalization of read counts was conducted across the samples utilizing the weighted Trimmed Mean of M-values (TMM) method. Finally, the voomWeights pipeline from the limma R package was used for DE analyses.

The specific thresholds for the DE analysis were: an absolute log2-fold change (L2FC) ≥ 1, an adjusted p-value (FDR) < 0.01, and an absolute change in percentage spliced (ΔPS) ≥ 0.1 (determined through F-test). In these analyses, the contrast groups were set as WT C vs. ASR-RNAi C, WT –Pi vs. ASR-RNAi –Pi, WT C vs. WT –Pi, and ASR-RNAi C vs. ASR-RNAi –Pi. The raw RNA-seq data and metadata are publicly available at BioProject accession PRJNA1210584.

Statistical analyses

The statistical analysis performed was either a Welch's *t*-test or Analysis of Variance (ANOVA), followed by Dunnett post hoc test, using the GraphPad Prism 9.0.0 (GraphPad Software Inc.) for Windows.

Results

Silencing of ASR genes delayed the early growth and development of rice seedling roots by compromising phosphate homeostasis

Plants silenced for ASR genes (hereafter ASR-RNAi), were previously obtained and studied in order to understand aluminum tolerance in rice (Arenhart et al. 2013; Arenhart et al. 2014). In control conditions, which corresponded to nutrient rich hydroponic solution, WT and ASR-RNAi plants showed no significant difference in root growth and development (Arenhart et al. 2014). However, when germinated on filter paper soaked only with water, phenotypic differences related to root architecture were observed between the two genotypes after seven days (Fig. 1A). The main root of WT plants was approximately twice as long compared to ASR-RNAi. Regarding adventitious roots, WT plants had an average of 2.5 roots per seedling, with each adventitious root being around 2.0 cm. In ASR-RNAi plants, a maximum of one adventitious root was observed per seedling with a length of <0.5 cm (Fig. 1B).

Given the limited development of root architecture, we hypothesized that ASR-RNAi plants were not responding to nutrient deficiency, specifically phosphate, as their root structure resembled that of Pi-insensitive plants (Péret et al. 2014). To assess whether ASR-RNAi plants could respond to Pi starvation, we cultivated them in a hydroponic solution with or without phosphate (Fig. 1C). While WT plants exhibited increased lateral root length under Pi starvation conditions, ASR-RNAi plants showed no response, maintaining their lateral roots significantly shorter (Fig. 1C and D), suggesting that lower levels of ASR could influence phosphate perception.

The OsASR promoter activity is responsive to phosphate starvation in rice seedling roots

Previous analyses using rice plants transformed for OsASR5 promoter-derived GUS expression (OsASR5pro:GUS) showed that the expression of this protein occurs mainly in the root apex (Arenhart et al. 2014). To obtain further evidence on the relationship between OsASR proteins and phosphate, we tested OsASR5pro:GUS roots to characterize the tissue-specific expression of this ASR homolog in the roots of rice plants under phosphate starvation. Our results show that the expression pattern was responsive to progressive exposure to stress (Fig. 2). Fig. 2A shows that, after 15 days of phosphate starvation, OsASR5 is highly expressed in lateral roots. The panels in the lower part of the figure display the time frame of the expression pattern in response to the treatment. At the beginning, as expected, the GUS reporter was expressed at the root apex (Fig. 2B). After five days of treatment with low phosphate concentration, GUS expression was noticed in the primordia of the lateral roots (Fig. 2C and D), and at the base of the emerging lateral roots (Fig. 2E). After 15 days of exposure to stress, the reporter gene expression showed a pattern throughout the root system, emphasizing the superficial lateral roots close to the branching region and the surface of the nutrient medium (Figs. 2F-H).

To further explore the relationship of ASR and phosphate, we performed *in silico* analyses of the promoter region of OsASR5. We identified conserved *cis*-regulatory binding elements for Phosphate Starvation Response 1 (OsPHR1), such as the P1BS region (Rubio et al. 2001). Besides, other frequent regulatory region elements of phosphate starvation-responsive genes were found, such as PHO, P responsive, TATA box-like, TC element, NIT 2-like, and helix-loop-helix (Table 1).

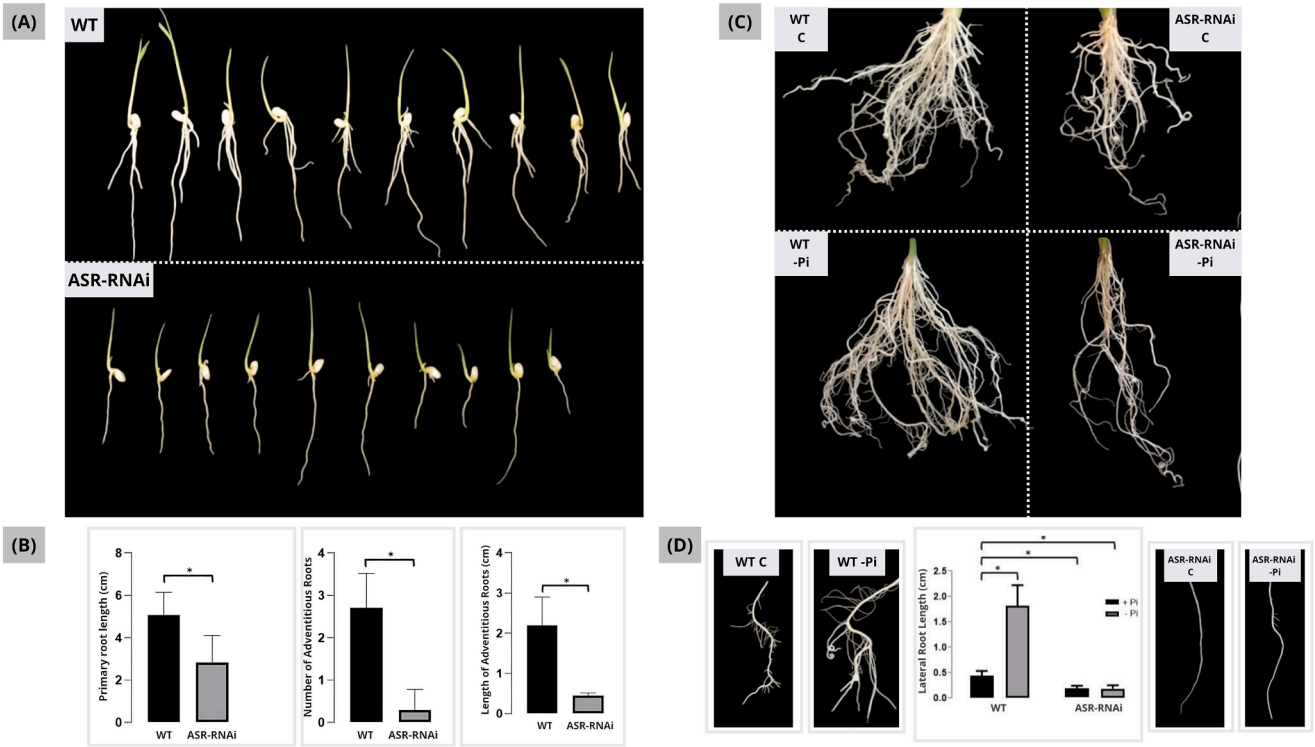

Fig. 1. Development of rice seedling roots under different conditions. (a) Visual comparison between the growth and development of primary and adventitious roots of WT and ASR-RNAi rice seedlings after seven days of growth on filter paper and water. (b) Plots displaying the length of primary roots, number of adventitious roots, and size of adventitious roots, respectively, of WT and ASR-RNAi rice seedlings under the already mentioned conditions. (c) Visual comparison between the growth and development of WT and ASR-RNAi rice roots after 15 days of Pi-sufficiency (C) or Pi-starvation (-Pi), (d) emphasizing the lateral root development in response to the stress.

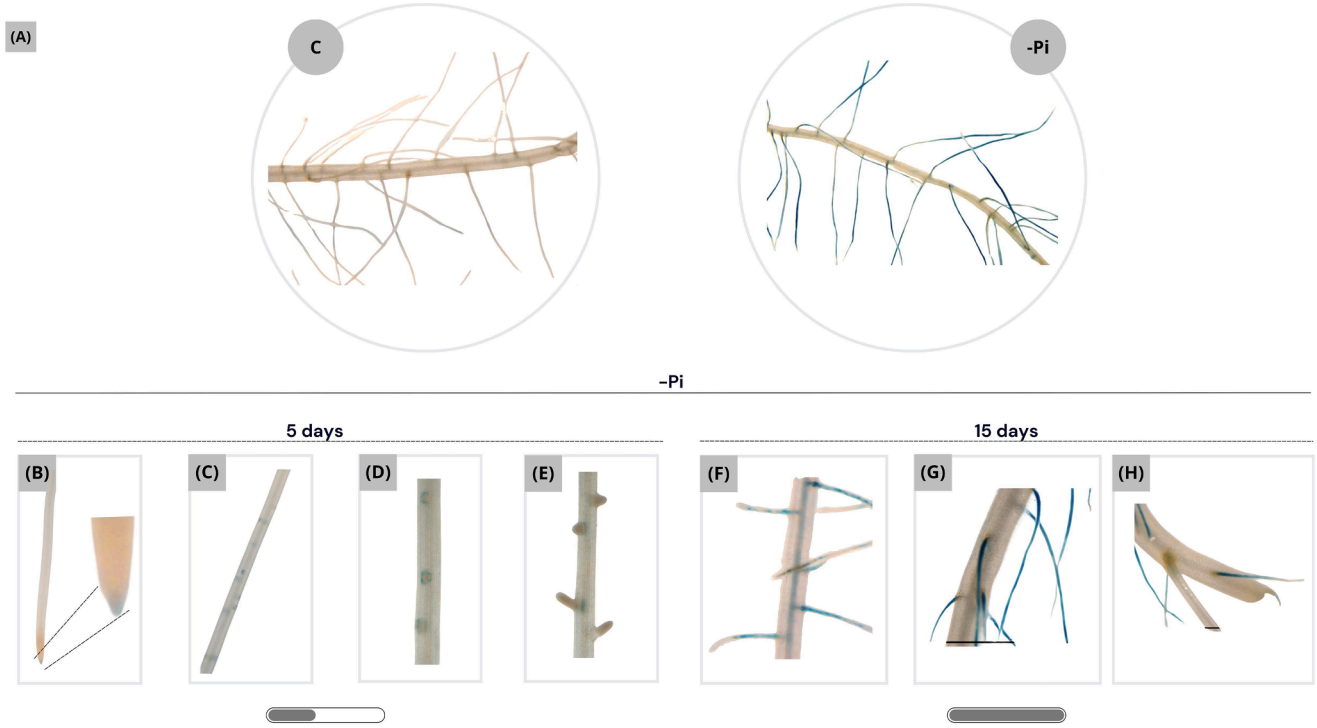

Fig. 2. Histochemical analysis of the roots of OsASR5pro:GUS rice plants under phosphate sufficiency (C) and starvation (-Pi) in a hydroponic system. (a) Under phosphate availability (C), absence of GUS expression in lateral roots, while the opposite occurred in response to nutritional stress (-Pi). After exposure to phosphate starvation for five days, the GUS expression was noticed in the (b) root tip, (c, d) lateral root primordia, and during the (e) lateral root emergence. After 15 days of -Pi treatment, the expression pattern of OsASR5pro:GUS changes, with emphasis on the (f, g, h) branching region.

Table 1
Phosphate starvation-responsive *cis*-elements in the OsASR5 promoter region according to positions and strands.

Sequence ID	Position	Strand	Sequence	-Pi-responsive binding sites	Description	Reference
	1792	+	AAATATCT	NIT 2-like	P responsive	
OsASR5	234	+	CACGTG	PHO	PHO element	Zeng et al. (2018)
	234	-	CACGTG	PHO	PHO element	
	233	–	CACGTGG	PHO-like	PHO element-like	
	1373	–	CAGATG	Helix-loop-helix	P responsive	
	1580	+	CAGATG	Helix-loop-helix	P responsive	
	268	–	CAGATG	Helix-loop-helix	P responsive	
	108	+	CATATG	Helix-loop-helix	P responsive	
	108	–	CATATG	Helix-loop-helix	P responsive	
	1373	+	CATCTG	Helix-loop-helix	P responsive	
	1580	–	CATCTG	Helix-loop-helix	P responsive	
	268	+	CATCTG	Helix-loop-helix	P responsive	
	1400	+	CATGTGG	PHO-like	PHO element-like	
	1030	+	GAATATAC	P1BS	PHR1 binding site	
	1030	–	GTATATTC	P1BS	PHR1 binding site	
	1541	+	TCTCTCT	TC element	P responsive	
	1543	+	TCTCTCT	TC element	P responsive	
	1545	+	TCTCTCT	TC element	P responsive	

These data, together with the promoter activity data, suggest that OsASR5 may play a role in rice root architecture during phosphate starvation.

Transcriptomic analysis reveals that some genes that respond exclusively to OsASR silencing are associated with root growth and development

The silencing of OsASR genes impaired changes in the root system architecture, such as the formation of lateral and adventitious roots, which are crucial for rice and other plants to cope with phosphate starvation. From this perspective, we performed a transcriptomic analysis aiming to investigate the effect of ASR gene silencing on the rice molecular network, under phosphate-sufficiency and -deficiency conditions. Thus, four experimental conditions were compared: WT control (C), WT –Pi, ASR-RNAi C, and ASR-RNAi –Pi. Three biological replicates were used for each sample type, generating a total of 12 RNA-seq libraries. Each sequenced library produced 24.2 million pairs of reads, on average, which were used to obtain transcript-specific expression data using Salmon (Patro et al. 2017). Approximately 89 % of the 200-bp read pairs were uniquely mapped to the rice reference transcriptome Osa1 Release 7 (Kawahara et al. 2013), as shown in Supplementary Table S1.

Before carrying out the differential gene expression analysis with the 3D RNA-seq app (Guo et al. 2021), read counts of 22,543 genes (29,586 transcripts) identified as expressed in our experiment were normalized across samples. Next, contrast groups were set to enable inter- and

intra-genotype comparisons, considering optimal condition and Pi starvation, namely WT C vs. ASR-RNAi C, WT –Pi vs. ASR-RNAi –Pi, WT C vs. WT –Pi, and ASR-RNAi C vs. ASR-RNAi –Pi.

Among the >800 differentially expressed genes in the four contrasts (Fig. 3) with log2FC ranging from −14 to 6.83, 114 genes (Table 2, Supplementary Table S2, Fig. 3A, Fig. 5B) were differentially expressed exclusively in response to *ASR* gene silencing (WT C vs. ASR-RNAi C), despite the nutritional stress, such as those encoding wall-associated kinase (OsWAK60, LOC_Os04g30240, log2FC 1.6), adenine nucleotide alpha hydrolase-like protein (LOC_Os06g18820, log2FC 1.13), OsPht1;9 transporter (LOC_Os06g21920, log2FC 2.2), flavonoid 3′-mono-oxygenase (LOC_Os08g01450, log2FC 1.05), 2′-deoxymugineic acid synthase (DMAS, LOC_Os10g02380, log2FC 1.17), protease inhibitor/seed storage/LTP family protein (LOC_Os10g40460, log2FC 2.02), and S-Domain Receptor Kinase 1–8 (SD1–8, LOC_Os11g17380, log2FC 2.1). These results are in agreement with the previous knowledge that ASR proteins are associated with vegetative and reproductive processes (Wang et al. 1998; Chen et al. 2011; Dominguez et al. 2013) since the knock-down of *ASR* modified the expression pattern of genes regulating root growth and plant development.

Under phosphate starvation, OsASR silencing changes the expression of stress-responsive genes in the roots of rice plants

Based on gene expression upregulation, transcriptome analysis suggests that *OsASR5* and *OsASR6* are the main players during phosphate starvation response among members of the rice ASR family (Fig. 4). Additionally, OsASR5 expression increases significantly in the absence of phosphorus. Hence, this transcription factor is possibly responsible for the transcriptional reprogramming noticed in ASR-RNAi roots in both conditions tested here.

The heat map (Fig. 5A) shows the transcriptional reprogramming taking place in DE genes upon phosphate starvation in rice. Considering this, classic phosphate starvation response genes (Fig. 5C) were identified, such as Pi transporters OsPHT1–4 (LOC_Os04g10750, log2FC −1.33), OsPHT1–9 (LOC_Os06g21920, log2FC −4.00), OsPHT1–10 (LOC_Os06g21950, log2FC −2.32), OsPHT1–6 (LOC_Os08g45000, log2FC −3.21) and OsPHT1–8 (LOC_Os10g30790, log2FC −1.56). Other canonical genes include purple acid phosphatases or PAP (LOC_Os01g56880, LOC_Os03g13540, LOC_Os08g17784, LOC_Os09g32840, LOC_Os11g05400, LOC_Os12g38750, LOC_Os12g44020, log2FC −2.60, −1.30, −2.96, −3.26, −1.45, −3.86, and −1.72, respectively) and soluble inorganic pyrophosphatase PPase 2 (LOC_Os02g47600, log2FC −1.66) and PPase 4 (LOC_Os05g02310, log2FC −1.48). Finally, transcriptional regulators such as OsIRO2 (LOC_Os01g72370, log2FC 1.36) and OsNIGT1 from the MYB family (LOC_Os02g22020, log2FC −1.62) and post-transcriptional regulators such as OsSPX family members (LOC_Os02g10780, LOC_Os03g29250, LOC_Os06g40120, LOC_Os10g25310, log2FC −2.30, −3.92, −2.28, and −4.61) were also regulated in response to Pi starvation. It is noticeable that the genes above mentioned were more up-regulated by the treatment in WT roots, while in ASR-RNAi the expression of classical response genes was two times lower (Fig. 5C). Besides, taking into account the knock-down of *ASR* genes and nutritional stress (WT –Pi vs. ASR-RNAi –Pi), simultaneously, 19 rice genes were differentially and exclusively regulated under exposure of the roots to this abiotic stress (Table 3, Supplementary Table S3, Fig. 3A).

OsASR silencing shrinks the molecular phosphate starvation response network by affecting cellular signaling

Fig. 3 suggests that the silencing limited the molecular response to phosphate starvation since, for example, the intra-genotype comparison WT C vs. WT -Pi resulted in approximately 26 times more DE genes than those grouped in WT -Pi vs. ASR-RNAi -Pi (Fig. 3) and about 257 more DE genes than ASR-RNAi C vs. ASR-RNAi -Pi (Fig. 3B). This hypothesis is supported by the distribution of GO categories since the common ones between WT C vs. WT -Pi and ASR-RNAi C vs. ASR-RNAi -Pi are the

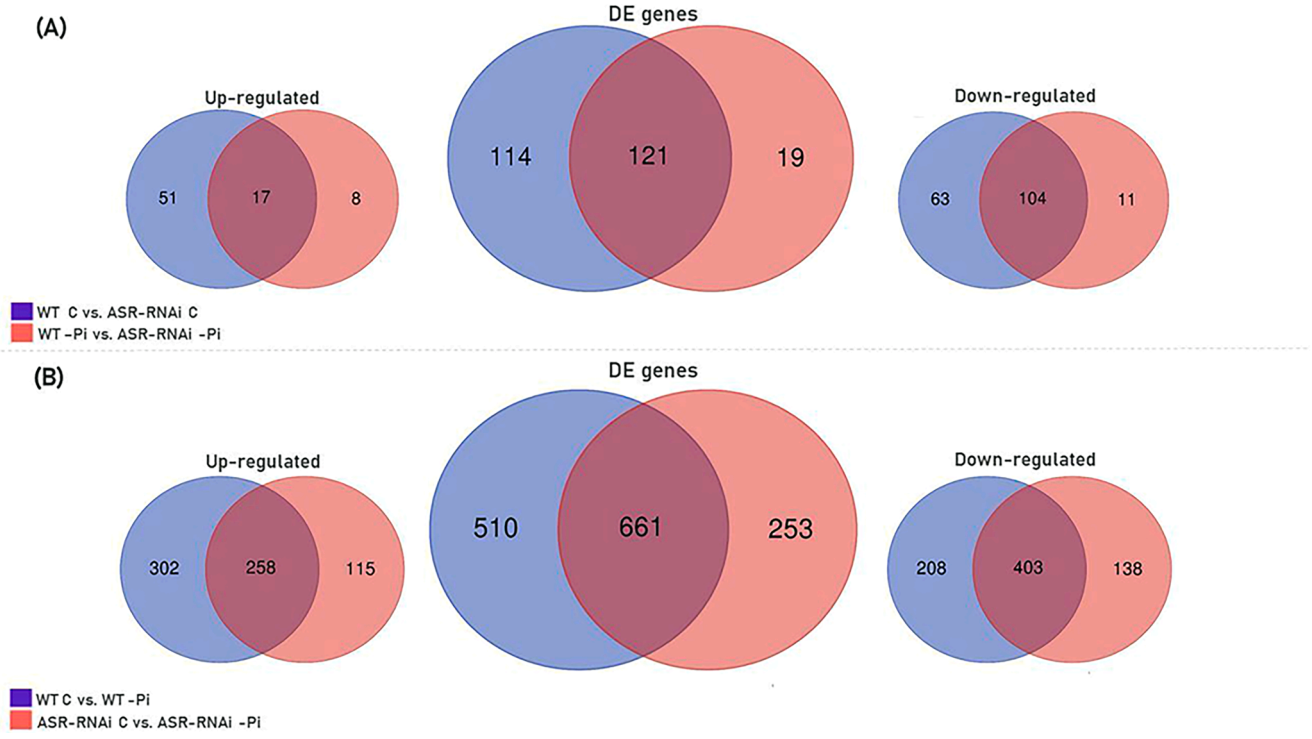

Fig. 3. Venn diagrams grouping the differentially expressed (DE) genes between the contrasts. (a) The total of genes differentially expressed in WT C vs. ASR-RNAi C (blue) compared to WT –Pi vs. ASR-RNAi –Pi (red), of which the number of those up- (left) or down-regulated (right) is shown in detail. (b) The total of genes differentially expressed in WT C vs. WT –Pi (blue) compared to ASR-RNAi C vs. ASR-RNAi –Pi (red) is illustrated, of which the number of those up- (left) or down-regulated (right) is highlighted.

Table 2
Genes up- or down-regulated exclusively in wild-type rice roots compared to ASR-RNAi ones under control conditions (WT C vs. ASR-RNAi C).

Gene ID	Adj.pval	log2FC	Expression	Description
LOC_Os01g01650	0.000726707	−1.09	Down-regulated	Isoflavone reductase homolog IRL, putative, expressed
LOC_Os01g08010	0.004768789	1.24	Up-regulated	Expressed protein
LOC_Os01g11830	0.000692424	1.12	Up-regulated	Oryzain alpha chain precursor, putative, expressed
LOC_Os01g22980	0.000330869	−1.28	Down-regulated	Osscp3 - Putative Serine Carboxypeptidase homologue, expressed
LOC_Os01g23370	0.004522604	−3.09	Down-regulated	Expressed protein
LOC_Os01g23440	9.53E-05	−2.44	Down-regulated	Expressed protein
LOC_Os01g27190	0.008777313	−1.71	Down-regulated	C2 domain containing protein, putative, expressed
LOC_Os01g29804	3.15E-06	−2.55	Down-regulated	Expressed protein
LOC_Os01g32439	0.000955919	−1.09	Down-regulated	Expressed protein
LOC_Os01g42350	0.001815662	1.85	Up-regulated	Pleiotropic drug resistance protein, putative, expressed
LOC_Os01g48620	0.001765192	−1.78	Down-regulated	Expressed protein
LOC_Os01g50680	0.001412855	1.64	Up-regulated	Ossub2 - Putative Subtilisin homologue, expressed
LOC_Os01g51140	0.006825651	1.1	Up-regulated	Helix-loop-helix DNA-binding domain containing protein, expressed
LOC_Os01g56450	0.007242506	1.27	Up-regulated	DUF567 domain containing protein, putative, expressed
LOC_Os01g68160	1.47E-05	1.09	Up-regulated	ZOS1–22 - C2H2 zinc finger protein, expressed
LOC_Os01g72910	0.004870476	1.2	Up-regulated	Abscisic stress-ripening, putative, expressed
LOC_Os01g74340	0.003543975	1.19	Up-regulated	RNA recognition motif containing protein, putative, expressed
LOC_Os02g09240	0.003389567	1.02	Up-regulated	Cytochrome P450 71D8, putative, expressed
LOC_Os02g09250	0.000695007	1	Up-regulated	Cytochrome P450 71D10, putative, expressed
LOC_Os02g11720	2.29E-05	−1.17	Down-regulated	Lipase, putative, expressed
LOC_Os02g11980	0.001200255	−1.86	Down-regulated	Receptor-like protein kinase precursor, putative, expressed
LOC_Os02g32950	0.002137318	−1.04	Down-regulated	RCN2 Centroradialis-like1 homologous to TFL1 gene; contains Pfam profile PF01161: Phosphatidylethanolamine-binding protein, expressed
LOC_Os02g40200	0.006356417	−2.12	Down-regulated	Receptor-like protein kinase precursor, putative, expressed
LOC_Os02g45930	6.58E-05	1.19	Up-regulated	Expressed protein
LOC_Os02g58360	0.000121243	−1.15	Down-regulated	Expressed protein
LOC_Os03g05334	0.000290785	1.08	Up-regulated	Expressed protein
LOC_Os03g39850	0.000527631	−1.19	Down-regulated	Glutathione S-transferase, putative, expressed
LOC_Os03g52180	0.000296745	−1.15	Down-regulated	4-hydroxy-3-methylbut-2-enyl diphosphate reductase, putative, expressed
LOC_Os03g52210	0.004039403	−1.2	Down-regulated	NA
LOC_Os04g05360	0.007545343	−3.11	Down-regulated	Expressed protein
LOC_Os04g13210	0.007410043	−1.01	Down-regulated	Multidrug resistance-associated protein, putative, expressed
LOC_Os04g17650	0.003055645	−2.38	Down-regulated	Sucrose synthase, putative, expressed
LOC_Os04g18770	0.000709008	−3.78	Down-regulated	NA
LOC_Os04g18780	0.001930911	−4.5	Down-regulated	NA
LOC_Os04g24319	0.002345226	1.14	Up-regulated	Jasmonate-induced protein, putative, expressed
LOC_Os04g24340	0.002137318	1.21	Up-regulated	Phytase, putative, expressed
LOC_Os04g30240	0.00050785	1.6	Up-regulated	OsWAK60 - oswak receptor-like protein kinase, expressed
LOC_Os04g34190	0.002159738	−3.79	Down-regulated	NA
LOC_Os04g34490	0.000373857	−2.54	Down-regulated	Nodulin, putative, expressed
LOC_Os04g49370	0.003233036	1.02	Up-regulated	Expressed protein
LOC_Os04g49500	0.008777313	1.16	Up-regulated	U-box domain-containing protein, putative, expressed
LOC_Os04g55740	0.0042538	−1.21	Down-regulated	Peroxidase precursor, putative, expressed
LOC_Os05g06920	0.003734073	2.37	Up-regulated	RelA-spot like protein RSH4, putative, expressed
LOC_Os05g08450	0.00019551	1.8	Up-regulated	Expressed protein
LOC_Os05g11580	0.000511517	1.25	Up-regulated	Expressed protein
LOC_Os05g12180	0.002231134	4.7	Up-regulated	Chalcone synthase, putative, expressed
LOC_Os05g23950	2.03E-06	−1.46	Down-regulated	TRAF-type zinc finger family protein, expressed
LOC_Os05g30310	0.000603545	−4.53	Down-regulated	NA

(*continued on next page*)

Table 2 (*continued*)

Gene ID	Adj.pval	log2FC	Expression	Description
LOC_Os05g31160	0.00029176	−1.74	Down-regulated	Peptide chain release factor 2, putative, expressed
LOC_Os05g31890	0.003734073	2.16	Up-regulated	Aspartyl protease family protein, putative, expressed
LOC_Os06g08640	0.003733476	1.04	Up-regulated	Transferase family protein, putative, expressed
LOC_Os06g15750	0.000337127	−1.52	Down-regulated	NB-ARC domain containing protein, expressed
LOC_Os06g18820	0.000182215	1.13	Up-regulated	Serine threonine kinase, putative, expressed
LOC_Os06g21920	9.52E-05	2.2	Up-regulated	Inorganic phosphate transporter 1–9, putative, expressed
LOC_Os06g31280	0.001306145	3.08	Up-regulated	THION1 - Plant thionin family protein precursor, putative, expressed
LOC_Os06g31890	0.001047247	2.18	Up-regulated	THION3 - Plant thionin family protein precursor, expressed
LOC_Os06g44034	3.53E-05	−1.02	Down-regulated	Expressed protein
LOC_Os07g03040	0.007139603	−1.65	Down-regulated	Expressed protein
LOC_Os07g03780	0.006198718	−1.36	Down-regulated	Lectin-like receptor kinase, putative, expressed
LOC_Os07g14470	0.006693581	−2.17	Down-regulated	OsWAK67 - oswak short gene, expressed
LOC_Os07g24830	0.000252826	1.23	Up-regulated	Thionin-like peptide, putative, expressed
LOC_Os07g25060	0.009744829	4.07	Up-regulated	Thionin-like peptide, putative, expressed
LOC_Os07g25800	0.002689795	−3.05	Down-regulated	Osfbduf37 - F-box and DUF domain containing protein, expressed
LOC_Os07g27350	6.56E-08	−1.13	Down-regulated	atuA, putative, expressed
LOC_Os07g35560	7.20E-06	−1.06	Down-regulated	Glucan endo-1,3-beta-glucosidase precursor, putative, expressed
LOC_Os07g44920	0.000630223	1.07	Up-regulated	Expressed protein
LOC_Os07g46870	0.007122114	−1.27	Down-regulated	Sex determination protein tasselseed-2, putative, expressed
LOC_Os08g01450	0.000121765	1.05	Up-regulated	Cytochrome P450, putative, expressed
LOC_Os08g10244	0.001385404	−1.44	Down-regulated	NA
LOC_Os08g14880	0.004924905	−1.61	Down-regulated	NA
LOC_Os08g14950	0.00139043	−4.15	Down-regulated	Receptor-like protein kinase 2 precursor, putative, expressed
LOC_Os08g20420	0.000441245	1.61	Up-regulated	MGD2, putative, expressed
LOC_Os08g26350	0.006492827	1.14	Up-regulated	Expressed protein
LOC_Os08g26360	0.000943882	2.42	Up-regulated	Expressed protein
LOC_Os08g26560	0.007895201	3.49	Up-regulated	Dirigent, putative
LOC_Os08g27720	0.006679864	−1.2	Down-regulated	Pirin, putative, expressed
LOC_Os08g29560	0.000330869	−2.21	Down-regulated	NA
LOC_Os08g29900	0.000921469	−3.67	Down-regulated	Expressed protein
LOC_Os08g31170	0.002740319	1.8	Up-regulated	DC1 domain-containing protein, putative, expressed
LOC_Os08g40520	0.000885103	−3.94	Down-regulated	Expressed protein
LOC_Os09g04550	0.006735908	1.45	Up-regulated	NA
LOC_Os09g07290	0.001562858	−4.21	Down-regulated	GDSL-like lipase/acylhydrolase, putative, expressed
LOC_Os09g16520	0.007156612	−1.09	Down-regulated	Cytochrome b5-like Heme/Steroid binding domain containing protein, expressed
LOC_Os09g28489	0.002876507	−2.29	Down-regulated	Expressed protein
LOC_Os10g02380	0.00017791	1.17	Up-regulated	Oxidoreductase, aldo/keto reductase family protein, putative, expressed
LOC_Os10g04520	0.005121574	−1.32	Down-regulated	Expressed protein
LOC_Os10g04540	0.007871479	−1.1	Down-regulated	Expressed protein
LOC_Os10g07616	0.006198718	2.99	Up-regulated	Chalcone synthase, putative, expressed
LOC_Os10g09000	0.001538696	−3.09	Down-regulated	Expressed protein
LOC_Os10g30150	0.004255803	3.43	Up-regulated	Universal stress protein domain containing protein, putative, expressed
LOC_Os10g31330	0.001346465	−1.41	Down-regulated	NA
LOC_Os10g32930	0.002159738	1.23	Up-regulated	Expressed protein
LOC_Os10g36310	0.008663302	1.83	Up-regulated	Osfbx390 - F-box domain containing protein, expressed
LOC_Os10g39390	0.000295835	1.05	Up-regulated	Eukaryotic aspartyl protease domain containing protein, expressed
LOC_Os10g40460	0.000338753	2.02	Up-regulated	LTPL141 - Protease inhibitor/seed storage/LTP family protein precursor, expressed
LOC_Os10g41550	0.009637711	1.06	Up-regulated	Beta-amylase, putative, expressed
LOC_Os11g02100	0.003837253	−1.45	Down-regulated	Peroxidase precursor, putative, expressed
LOC_Os11g02720	0.000274358	−1.6	Down-regulated	Expressed protein

(*continued on next page*)

Table 2 (*continued*)

Gene ID	Adj.pval	log2FC	Expression	Description
LOC_Os11g13750	0.006492827	1.14	Up-regulated	Expressed protein
LOC_Os11g15250	0.001117187	2.85	Up-regulated	THION31 - Plant thionin family protein precursor, expressed
LOC_Os11g17380	5.90E-05	2.1	Up-regulated	Protein kinase domain containing protein, expressed
LOC_Os11g29920	0.000798942	−1.23	Down-regulated	NB-ARC domain containing protein, expressed
LOC_Os11g34570	0.005387075	−2.32	Down-regulated	Lysm domain-containing GPI-anchored protein precursor, putative, expressed
LOC_Os11g37940	0.007253692	−1.22	Down-regulated	WIP2 - Wound-induced protein precursor, expressed
LOC_Os11g40970	0.004140303	−1.79	Down-regulated	Receptor-like protein kinase precursor, putative, expressed
LOC_Os11g44990	0.000502953	−1.07	Down-regulated	NB-ARC domain containing protein, expressed
LOC_Os12g12010	0.004107677	−1.36	Down-regulated	Verticillium wilt disease resistance protein precursor, putative, expressed
LOC_Os12g12470	9.08E-05	1.04	Up-regulated	NADP-dependent oxidoreductase, putative, expressed
LOC_Os12g12600	0.002693457	1.89	Up-regulated	Dirigent, putative, expressed
LOC_Os12g15780	0.00013752	−2.36	Down-regulated	NA
LOC_Os12g25350	0.001562858	−2.59	Down-regulated	Expressed protein
LOC_Os12g36490	0.000128812	−1.28	Down-regulated	Expressed protein
LOC_Os12g39710	0.001275601	−1.07	Down-regulated	Expressed protein
LOC_Os12g42810	0.004924905	−1.49	Down-regulated	Mov34/MPN/PAD-1 family protein, expressed

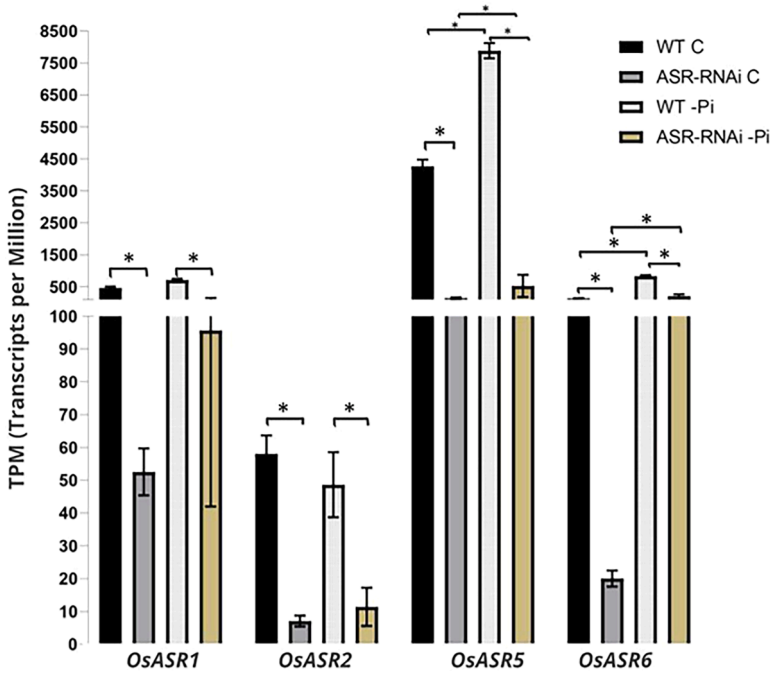

Fig. 4. Transcripts per Million (TPM) of reads corresponding to four of the six rice ASR family members in different libraries (WT C, ASR-RNAi C, WT -Pi, and ASR-RNAi -Pi). Asterisks represent statistical differences determined by 3D RNA-seq app (adjusted p-value < 0.01).

following: ATP-dependent activity (GO:0140,657), binding (GO:0005,488), catalytic activity (GO:0003,824), molecular function regulator (GO:0098,772), transcription regulator activity (GO:0140,110), and transporter activity (GO:0005,215). On the other hand, those exclusive to WT C vs. WT -Pi, such as molecular transducer activity (GO:0060,089) and structural molecule activity (GO:0005,198), suggest the effects of the ASR knock-down over the response to phosphate starvation.

OsASR potentially regulates phosphate responses through activation of OsDAHPS1

In order to better understand the potential direct regulation of OsASR5 over phosphate-related genes, we cross-referenced (Fig. 6) RNA-seq data with our previous OsASR5 ChIP-seq data produced under control conditions, when we found several OsASR5 targets and validated one of them by different approaches (Arenhart et al. 2014). The cross-referencing retrieved the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1 gene (*OsDAHPS1*, LOC_Os03g27230, log2FC −1.18) among the up-regulated ones (Table 4) in WT plants under

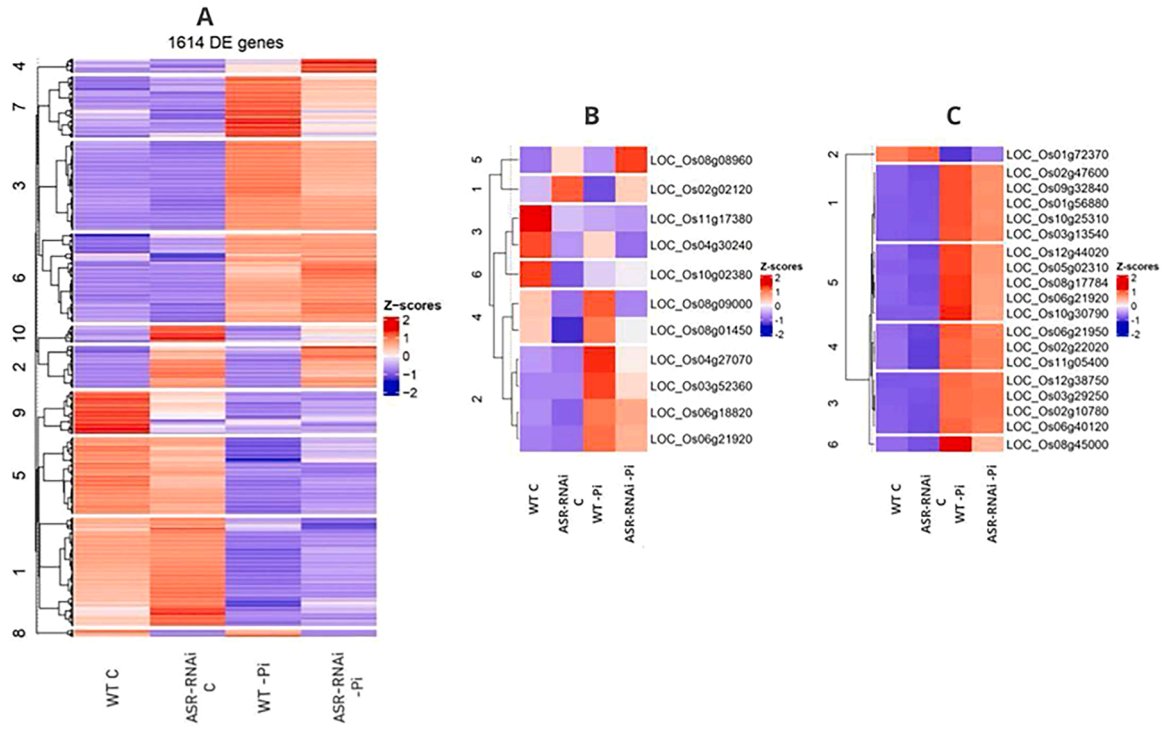

Fig. 5. Z-score clustering heat map of genes of interest across the different genotypes and conditions analyzed in this study. The heat map in (a) displays all DE genes identified in our study. Panel (b) includes only a subset of DE genes that were specifically associated with root growth and development, as well as non-canonical genes responsive to phosphate starvation. Panel (c) includes the classic phosphate-starvation responsive genes, which were not identified as DE in our study. The red and blue colors indicate an increase or decrease in the gene expression levels, respectively, based on the z-score scale. White color represents values equal to zero.

phosphate starvation conditions, but not in ASR-RNAi plants, suggesting that OsASR5 binds to and potentially upregulates *OsDAHPS1* expression upon Pi starvation. This gene is involved with the biosynthesis of aromatic amino acids followed by the release of phosphate.

Discussion

Despite the well-established involvement of ASR proteins from different crops with the responses to abiotic stresses such as drought and salinity (Yacoubi et al. 2022; Yang et al. 2024), our group was among the first to associate rice tolerance to Al toxicity with the OsASR5 transcription factor activity. Considering this, the silencing of *OsASR* genes modified the expression of genes and proteins, including direct targets via a proposed *cis*-element, resulting in an Al-sensitive phenotype (Arenhart et al. 2013, 2014). Building on this understanding, our current study shifts focus to another significant constraint on crop productivity: the nutritional limitations posed by the physicochemical properties of the macronutrient phosphate.

It is reasonable to propose that the noticed phenotypic differences observed between ASR-RNAi and WT plants germinated in water are associated with low levels of ASR transcripts, as members of this family are involved with plants' vegetative and reproductive growth (Wang et al. 1998; Chen et al. 2011; Dominguez et al. 2013). It has been demonstrated that, without exposure to stresses, expression of *OsASR6* promoted *Arabidopsis* root growth by modifying the lignification pattern to increase the longitudinal xylem cell growth and improve hydraulic conductance (Agarwal et al. 2019). Besides, *VvMSA* (a grape ASR ortholog) silencing changed the expression of several classes of proteins crucial for endogenous developmental programming, including those associated with mRNA processing and stability, cell division and differentiation, and epigenetic regulation (Atanassov et al. 2022).

Considering this, the decreased occurrence of histone marks that represent high transcriptional activity in *VvMSA*-RNAi cells (Atanassov et al. 2022) indicates that the knock-down of rice *ASR* transcripts may have hampered the progressive transition between developmental phases, resulting in a decreased length and number of primary and adventitious roots, respectively, compared to WT seedlings (Fig. 1A and B). Accordingly, our data suggest that ASR proteins regulate growth-promoting target genes in rice roots, whose transcripts were exclusively down-regulated in response to silencing of these transcription factors (Table 2).

Since the phenotypes described above were influenced by *OsASR* silencing, we hypothesized that these transcription factors might be linked to the nutritional status of rice seedlings. To explore this, we prioritized investigating phosphorus (P), given its critical role in metabolism. Phosphorus is essential for ATP production, signal transduction via phosphorylation and dephosphorylation, water conductance, and sugar metabolism (Blevins, 1999). Particularly, one of the most well-characterized mechanisms involving ASR proteins relates to their role in sugar metabolism (Parrilla et al. 2022).

When germinating plants on water-soaked paper, the limited availability of nutrients means that most nutrients used for sprouting come from the reserves stored within the seed. As the plants grow, their root systems develop in search of external nutrients, resembling the root architecture of wild-type (WT) plants (Fig. 1A). However, the root architecture observed in ASR-RNAi plants was similar to plants unable to respond to limited phosphate (Pi) supply or mutants deficient in Pi-starvation responses (Ruan et al. 2017). This similarity suggests that ASR-RNAi plants may exhibit an impaired phosphate starvation response.

During the controlled Pi-starvation experiment, ASR-RNAi plants displayed similar lateral root lengths in both control and stress conditions (Fig. 1D), suggesting that these plants indeed could have impaired Pi-starvation responses. This phenotype was similar to *osarf16, osacs1*, and *osmyb5p* plants, which have limited lateral root growth when grown in low phosphate conditions (Shen et al. 2013; Yang et al. 2018; Lee et al. 2019). Our GUS analyses, using OsASR5pro:GUS plants, showed that *OsASR5* expression is highly induced during Pi-starvation stress,

Table 3
Genes up- or down-regulated exclusively in wild-type rice roots compared to ASR-RNAi ones under phosphate starvation (WT -Pi vs. ASR-RNAi -Pi).

Gene ID	Adj.pval	log2FC	Expression	Description
LOC_Os01g08600	0.006839412	−1.21	Down-regulated	Expressed protein
LOC_Os01g51180	0.008294009	5.49	Up-regulated	NA
LOC_Os01g52514	0.002778929	−1.02	Down-regulated	B3 DNA binding domain containing protein, expressed
LOC_Os01g56620	2.79E-06	−1.11	Down-regulated	Pseudouridylate synthase, putative, expressed
LOC_Os01g73700	0.003967964	1.16	Up-regulated	RCLEA2 - Root cap and Late embryogenesis related family protein precursor, putative, expressed
LOC_Os02g02120	6.15E-05	−1.22	Down-regulated	OsWAK11 - OsWAK receptor-like protein kinase, expressed
LOC_Os02g33070	0.007458995	−1.05	Down-regulated	Expressed protein
LOC_Os02g37260	0.000357653	1.04	Up-regulated	Hypothetical protein
LOC_Os03g29150	0.002547351	−2.36	Down-regulated	NAD dependent epimerase/ dehydratase family protein, putative, expressed
LOC_Os03g52360	6.49E-05	1.03	Up-regulated	PIII3 - Proteinase inhibitor II family protein precursor, putative, expressed
LOC_Os04g27070	5.00E-05	1.07	Up-regulated	Terpene synthase, putative, expressed
LOC_Os05g40790	0.007952142	−1.60	Down-regulated	CCR4-NOT transcription factor, putative, expressed
LOC_Os06g06850	0.000474028	−1.02	Down-regulated	Resistance protein LR10, putative, expressed
LOC_Os07g25030	0.00051047	4.40	Up-regulated	NA
LOC_Os08g08960	0.000133388	−1.22	Down-regulated	Cupin domain containing protein, expressed
LOC_Os08g09000	1.29E-05	1.01	Up-regulated	Cupin domain containing protein, expressed
LOC_Os09g15830	0.004174775	−2.01	Down-regulated	Expressed protein
LOC_Os10g18870	5.52E-06	1.33	Up-regulated	Dirigent, putative, expressed
LOC_Os11g06730	0.000396378	−3.57	Down-regulated	ECAGL2 - ECA1 gametogenesis related family protein precursor, expressed

increasing over time (Fig. 2), suggesting that OsASR5 may regulate those responses.

Transcriptome analysis confirms that among members of the rice ASR family affected by silencing, *OsASR5,* and *OsASR6* are components of the stress response (Fig. 4). This study is the first to scrutinize the relationship between these proteins and phosphate starvation. RT-qPCR data previously indicated that *OsASR5* is the most highly expressed *ASR* gene in rice roots under control conditions (Arenhart et al. 2013). Besides, the results presented here suggest that OsASR5 is also the most induced in the absence of phosphorus in a hydroponic solution. These results indicate that OsASR5 may play a key role in the transcriptional

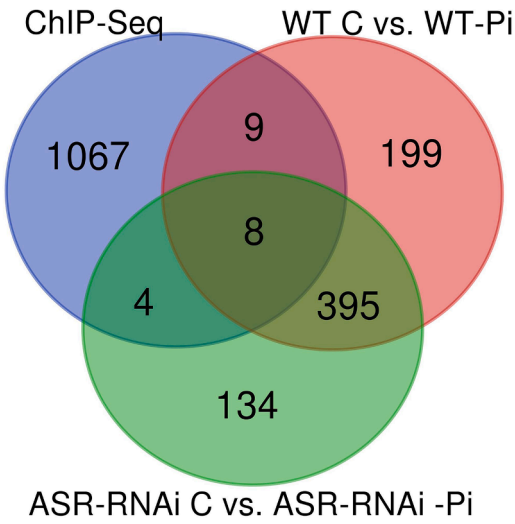

Fig. 6. Venn diagram grouping the up-regulated genes between the contrasts (control conditions vs. phosphate-starvation conditions), comparing the total of genes differentially expressed in WT C vs. WT -Pi experiment to ASR-RNAi C vs. ASR-RNAi -Pi, and ChIP-seq data.

reprogramming observed in ASR-RNAi roots under both tested conditions.

Our RNA-seq data confirmed that some genes that respond exclusively to *OsASR* silencing are associated with root growth and development. Particularly, genes encoding a protease inhibitor/seed storage/LTP family protein (LOC_Os10g40460, log2FC 2.02) and a OsPht1;9 transporter (LOC_Os06g21920, log2FC 2.20) were down-regulated in ASR-RNAi control roots compared to the WT roots. Accordingly, Ye et al. (2015) reported that, under Pi-sufficiency conditions, a Pi transporter from the Pht1 family is expressed both in the tips and in the maturation zone of rice roots, and its silencing resulted in a decline in the primary root number compared to WT plants. Similarly, in our study, the primary roots of ASR-RNAi seedlings differed from WT in both length and expression pattern of OsPht1;9. Moreover, Takehisa et al. (2012) demonstrated that a protease inhibitor/seed storage/LTP family was up-regulated in the elongation zone and associated with rice root development. However, our results point out that this gene was down-regulated in the roots of mutant seedlings (Table 2 and Fig. 5B). This downregulation, triggered by OsASR silencing, likely contributed to the observed reduction in root number and length in ASR-RNAi transgenic plants compared to WT.

Signaling regulated by Wall-Associated Kinase (WAK) proteins is also crucial for cell expansion. A significant reduction in the transcript levels of a WAK family member has been shown to dramatically alter the length, thickness, uniformity, and surface area of rice roots, resulting in smaller roots compared to wild-type (WT) plants and the absence of root hairs (Kanneganti and Gupta, 2011). Root growth is also modulated by cell wall loosening and lignification pattern, which was modified by the expression of an *OsASR6* in *A. thaliana*, favoring the root development (Agarwal et al. 2019). Based on these findings, it is plausible that the knock-down of *OsASR* genes may delay root growth and development via inhibition of genes such as OsWAK60 (LOC_Os04g30240, log2FC 1.60) (Table 2 and Fig. 5B).

Regarding post-translational modifications, as reported by Samuel et al. (2008), the SD1 promotes phosphorylation of an *A. thaliana* Ubiquitin Ligase and may contribute to a functional switch or migration of the target. Also, an Arabidopsis adenine nucleotide alpha hydrolase-like protein was phosphorylated in response to brassinolide (BL) treatment and thus included in a network related to phytohormone-regulated growth (Lin et al. 2015). Taking it into account, in ASR-RNAi rice seedling roots, DE genes such as SD1–8

Table 4
Up-regulated genes in WT C vs. WT - Pi in overlap with ChIP-seq data.

Gene ID	Adj.pval	log2FC	Expression	Description
LOC_Os07g22510	4.17281551014883e-07	1.43	up-regulated	uncharacterized protein
LOC_Os04g35270	3.45257669273454e-05	1.07	up-regulated	
LOC_Os02g36030	0.00309162666424859	1.95	up-regulated	putative, expressed Cytochrome P450 76C2
LOC_Os12g08850	0.00106385255622571	2.08	up-regulated	expressed protein
LOC_Os01g71830	3.57549857298648e-08	1.01	up-regulated	glycosyl hydrolases family 17, putative, expressed
LOC_Os09g22000	9.2177207127523e-08	1.34	up-regulated	
LOC_Os02g36020	0.00770177498345753	3.77	up-regulated	
LOC_Os03g27230	2.29912528249765e-10	1.18	up-regulated	3-deoxy-d-arabino-heptulosonate 7-phosphate synthase 1
LOC_Os01g11160	1.81783262750885e-05	1.11	up-regulated	cationic amino acid transporter

(LOC_Os11g17380, log2FC 2.10) and an ortholog of adenine nucleotide alpha hydrolase-like protein (LOC_Os06g18820, log2FC 1.13) were downregulated at the transcript level (Table 2 and Fig. 5B), suggesting that root growth may have been compromised by alteration of signal perception even under optimal conditions.

Other down-regulated genes include the flavonoid 3′-mono-oxygenase (LOC_Os08g01450, log2FC 1.05), which was up-regulated in sugarcane, and the flavonoid biosynthesis was associated with the adventitious root formation in response to auxin (Li et al. 2020). Along with previously discussed data, the down-regulation of these genes (Fig. 5B) provides molecular evidence that helps explain the observed phenotype, in which ASR-RNAi seedlings exhibited primary roots approximately two times shorter and adventitious roots four times shorter than those of the wild-type (Fig. 1).

The serine-type endopeptidase inhibitor (LOC_Os03g52360, log2FC 1.03) was up-regulated in WT –Pi plants compared to ASR-RNAi –Pi, suggesting that only WT may have properly readjusted growth under Pi starvation, as observed in maize plants (Sun et al. 2016). A gene encoding a cupin domain-containing protein (LOC_Os08g09000, log2FC 1.01) was also up-regulated in rice roots in response to nutrient deficiency and during Pi re-supply (Secco and Whelan, 2014). Also, the gene encoding a terpene synthase (LOC_Os04g27070, log2FC 1.07) was up-regulated only in WT –Pi plants after both a five-day root-treatment (as applied here) and after 21 days of stress involving both roots and shoots (Secco et al. 2013). The gene encoding a dirigent protein (LOC_Os10g18870, log2FC 1.33) was up-regulated in WT –Pi rice plants and also in Arabidopsis (Misson et al. 2005) under phosphate starvation, but this response is not present in ASR-RNAi. Finally, wall-associated kinase (LOC_Os02g02120, log2FC −1.22) up-regulation highlights that ASR transcription factors can directly or indirectly regulate this gene. This kinase participates in wall pectin-mediated responses to P starvation and barley root growth (Tripathi et al. 2021).

Notably, during exposure to stress, both WT and ASR-RNAi plants activate pathways that include kinases, transcription factors, genes involved with lipid and carbohydrate metabolism, those associated with phytohormone biosynthesis and signaling, flavonoid production, cytochromes P450, and phosphate starvation response genes (Supplementary Table S4 and S5).

However, there was a conspicuous up-regulation of kinases and transcription factors in WT rice roots compared to ASR-RNAi under stress (WT C vs. WT -Pi x ASR-RNAi C vs. ASR-RNAi -Pi). This was particularly evident in members of the MYB family (LOC_Os01g50720, LOC_Os04g45020, LOC_Os04g50770, and LOC_Os08g37970, log2FC 1.05, −1.25, −1.27, and −1.12, respectively). The GO terms support this observation, as the category *molecular transducer activity* (GO:0060,089) is identified only in the first contrast, alongside a greater abundance of the term *transcription regulator activity* (GO:0140,110).

Also, genes such as lactate/malate dehydrogenase (LOC_Os06g01590, log2FC −1.67) and one involved with lignin degradation (laccase precursor protein, LOC_Os01g61160, log2FC −1.12) were up-regulated only in WT -Pi roots. Regarding phytohormones, genes associated with strigolactone signaling (hydrolase, alpha/beta fold family domain-containing protein, LOC_Os01g62010, log2FC −1.03, and LOC_Os10g38860, log2FC −1.12) were specifically upregulated in WT roots in response to nutrient deficiency (WT C vs. WT -Pi). Similarly, a gene encoding a BAK1 kinase (LOC_Os11g31540, log2FC −2.45), a component upstream of the brassinosteroid signaling pathway, was also upregulated. Interestingly, in rice WT cv. Shiokari roots, the expression of a hydrolase (*DWARF14*) associated with strigolactone signaling was repressed after three and seven days of phosphate starvation (Haider et al. 2023), suggesting cultivar-specific responses. On the other hand, the up-regulation of *BAK1*, which inactivates a GSK component downstream to BR signaling, aligns with the recent model proposing that GSK impairs the phosphate starvation responses regulated by a major transcription factor PHR (Zhang et al. 2024). Otherwise, ASR-RNAi (ASR-RNAi C vs. ASR-RNAi -Pi) up-regulated two cytokinin-O-glucosyltransferase 2 (LOC_Os04g25370, log2FC −1.48, and LOC_Os04g25980, log2FC −1.23).

Regarding transport molecules, genes encoding proteins involved in iron and citrate transport, such as citrate transporter (LOC_Os10g31040, log2FC −1.39) (Panchal et al. 2023) and MATE efflux (LOC_Os12g03200, log2FC −1.16), were up-regulated only in ASR-RNAi –Pi (ASR-RNAi C vs. ASR-RNAi -Pi) roots. This expression pattern might indicate that *OsASR* knock-down plants are highly sensitive to the treatment since the exudation of organic acids tends to increase with the severity of the stress. Besides, it could point out a different strategy compared to rice WT plants regarding the timing and relocation of carbon resources (Tiziani et al. 2020) at the expense of root growth since smaller roots exude less citrate (Chen et al. 2013).

When analyzing the down-regulated differentially expressed (DE) genes resulting from stress exposure in the two genotypes, again, kinases and transcription factors were among the most affected by the starvation in WT plants (WT C vs. WT -Pi) and, comparatively, these groups of genes were less impacted in the ASR-RNAi response i (ASR-RNAi C vs. ASR-RNAi -Pi). Besides, genes related to the photosynthetic system and those encoding proteins with the zinc-finger domain, such as LOC_Os06g34430, LOC_Os09g32730, and LOC_Os03g28080 (log2FC 1.62, 1.24, and 1.24, respectively) were predominantly down-regulated in the Pi starvation response of WT plants.

Likewise, several genes encoding F-box domain-containing protein (LOC_Os02g33240, LOC_Os03g02550, and LOC_Os10g03850, log2FC 1.08, 1.15, and 1.00, respectively), which are involved in post-translational modifications, were repressed in these roots. In contrast, a kinase associated with brassinosteroid signaling (serine/threonine-protein kinase BRI1-like precursor 2, LOC_Os06g47700, log2FC 1.05) was exclusively down-regulated in the roots of ASR-RNAi plants. Additionally, sulfate (LOC_Os03g09940, log2FC 1.02), iron (metal-nicotianamine transporter YSL16-related, LOC_Os04g45900, log2FC 1.23) and citrate (MATE efflux family protein, LOC_Os03g64150, log2FC 1.02) transporters were also specifically down-regulated in ASR-RNAi roots.

Studies on *A. thaliana* orthologs demonstrated that the *DAHPS1* gene (At4g39980) is related to the biosynthesis of aromatic amino acids. The first step in this pathway involves the condensation of phosphoenol-pyruvate (PEP) and erythrose-4-P to 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) with the release of Pi. This reaction is catalysed by DAHP synthases, which are induced under P limitation and regulated by

PHR1 (Pant et al. 2015). In *A. thaliana* plants, it was shown that At4g39980 was induced under phosphate starvation conditions. The same study assessed the expression of a key regulator in the phosphate pathway known as Phosphate starvation response 1 (PHR1). In PHR mutant plants, the expression of *At4g39980* was repressed in both shoots and roots, confirming that the At4g39980 gene is a direct target of PHR1 (Pant et al. 2015). Corroborating this study, Hammond and White (2008) evaluated a cluster of nine genes, whose expression increased within 4 and 28 h of phosphate deficiency. Among these, the gene At4g39980 displayed a significantly increased expression.

Fig. 7 summarizes the proposed role of OsASR proteins in rice roots under phosphate-depleted conditions. The knock-down of *OsASR* transcripts disrupts the molecular network required for an effective response, impairing root development. As a result, unlike the WT, mutant plants exhibit a highly sensitive phenotype, unable to modify their root system architecture (RSA) to increase nutrient acquisition.

Conclusions

The silencing of *OsASR* disrupted phosphate homeostasis, compromising the early vegetative growth of rice roots. Future research should focus on identifying the developmental stages at which ASR proteins interact with phosphate, to determine whether they regulate phosphate uptake, remobilization to seeds, or efficient utilization from the endosperm. Besides, phosphate starvation treatments confirmed that the *OsASR5* promoter is stress-responsive, consistent with *in silico* analyses of this region. Moreover, the expression pattern of the reporter gene points out that one of the pathways regulated by OsASR5 for the response to phosphate starvation may involve auxin accumulation.

At the molecular level, transcriptomic analysis confirmed that genes associated with root development were down-regulated in ASR-RNAi plants under optimal conditions. On the other hand, exposure to stress triggered a transcriptional reprogramming of the metabolism, perception, and signal transduction pathways. Classical genes such as phosphate transporters and phosphatases were induced to only half the extent in ASR-RNAi roots compared to WT roots. These genes may be directly regulated by OsASR5 since, according to our results, this was the homolog most recruited by WT roots to cope with stress. Additionally, the promoter region of OsASR5 contains stress-responsive *cis*-elements, and key phosphate-starvation-responsive genes, such as *LPR* and *ALMT1*, contain binding sites for ASR5 protein in their regulatory regions (data not shown).

Our findings position ASR proteins at the core of regulatory pathways governing pleiotropic responses to aluminum and phosphorus. However, the potential influence of other nutrients cannot be ignored. Nitrogen, for instance, plays a critical role in root emergence (Murata and Matsushima, 1975), while phosphorus is essential for sustaining growth rates (Rees and Raven, 2021). Future research should investigate whether ASR proteins are also involved in responses to nitrogen imbalance and explore how ASR coordinates the regulation of these essential nutrients along with aluminum stress.

Funding statement

This work has been financially supported by grant provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Proc.409813/2021–4). NLFB is supported by CNPq Grant number 140,586/2021–0. TST is supported by FAPERGS (PDTI2 - Proc. 23/2551–0001,893–4). CPGC is supported by the São Paulo Research Foundation (FAPESP), grants number 2019/13158-8 and 2021/000394-5, and Institute Serrapilheira, grant number R-2011-37880. The funding bodies had no role in the design of the study, data analysis, interpretation, and writing the manuscript.

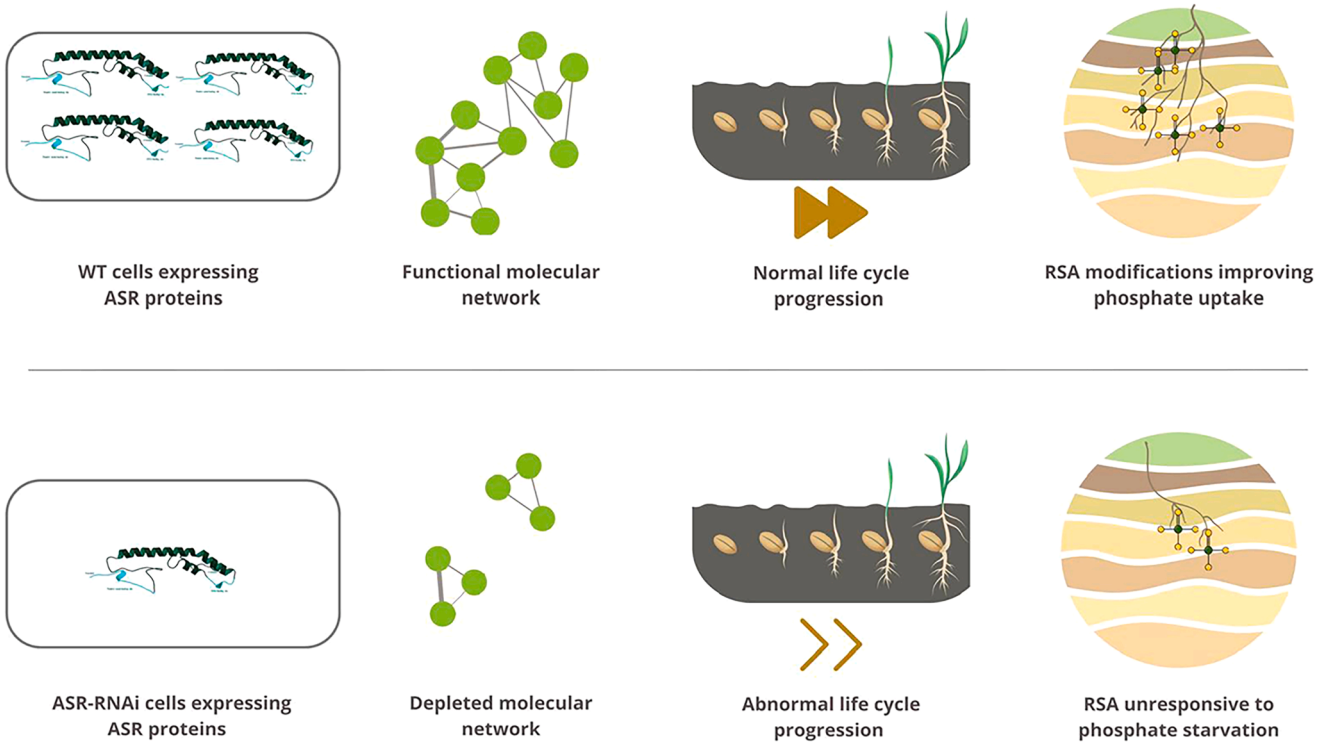

Fig. 7. Schematic illustration displaying the involvement of OsASR proteins with rice phosphate-starvation response. The knock-down of *OsASR* transcripts depleted the molecular network triggered by this condition, hampering root development and resulting in seedlings highly sensitive to this nutritional deficiency. In comparison to wild-type (WT) plants, the mutant ones are unable to modify the root system architecture (RSA) to increase nutrient acquisition.

CRediT authorship contribution statement

Nicolle Louise Ferreira Barros: Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization. **Breno Xavier Gonçalves:** Methodology, Formal analysis, Data curation, Conceptualization. **Thomaz Stumpf Trenz:** Writing – review & editing, Methodology. **Paloma Koprovski Menguer:** Supervision, Methodology, Conceptualization. **Lucas Roani Ponte:** Methodology. **Cristiane P.G. Calixto:** Formal analysis, Writing – review & editing. **Felipe Klein Ricachenevsky:** Writing – review & editing, Conceptualization. **Marcia Margis-Pinheiro:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Leticia Gal for technical assistance in the laboratory.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2025.100824.

Data availability

The raw RNA-seq data and metadata are publicly available at BioProject accession PRJNA1210584.

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