The Plasmodesmal Protein Osger4 is Involved in Auxin Mediated Crown Root Development in Rice

Thi Trang Nguyen
University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology
The Dan Pham
University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology
Phat Tien Do
Institut of Biotechnology, Vietnam Academy of Science and Technology
Kieu Thi Xuan Vo
Graduate School of Green-Bio Science, Kyung Hee University
Anh Thi Van Le
University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology
Tuan Anh Tran
University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology
Hoang Ha Chu
Institut of Biotechnology, Vietnam Academy of Science and Technology
Jong-Seong Jeon
Graduate School of Green-Bio Science, Kyung Hee University
Thi Mai Huong To (to-thi-mai.huong@usth.edu.vn)
University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology
https://orcid.org/0000-0002-9435-2984

Research Article

Keywords: Auxin, crown root, Germin-like protein, plasmodesmata, rice

Posted Date: June 27th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3108300/v1

Abstract

In rice (*Oryza sativa* L.), the root system plays different essential roles, from water and nutrient uptake to responding to environmental signals. The mechanisms underlying root development are complex and involve many phytohormones, of which auxin is the most important. This study investigates the involvement of OsGER4, a putative Germin-like protein, in auxin-mediated crown root development in rice. The expression study of *OsGER4* in the *crl1* mutant confirms that OsGER4 is connected to the CRL1 signaling pathway- a master regulator for crown root development. Transgenic rice carrying the ProGER4::GUS reporter gene revealed that *OsGER4* is mainly expressed in the initiation and emergence zone of the crown and lateral root, such as epidermal cell, vasculature, and primordial under auxin treatment condition. Moreover, fewer crown roots of *osger4* knockout mutant lines than the wild-type under auxin treatment suggests that OsGER4 might function as a regulator limiting auxin flux to root growth regions under stress conditions. Besides, protein localization experiments confirmed that OsGER4 localizes to plasmodesmata, which are intercellular channels that could facilitate auxin transport. Our findings suggest that OsGER4 might play a substantial role in regulating plasmodesmata conformation to regulate auxin flow resulting in crown root developmental in rice under stress conditions.

Introduction

Rice (*Oryza sativa* L.) is an essential crop consumed by nearly half of the global population. However, its cultivation is heavily affected by adverse environmental conditions, such as drought, salt, and temperature stress, necessitating research into developing novel rice varieties better adapted to these conditions. The rice root system architecture (RSA) consists of a seminal embryonic root and various crown roots (CR) radiating from the rice stem base, with lateral roots (LR) found on both of them (Smith and de Smet 2012). Crown roots constitute the basic framework of the root system for most of the crop's life cycle (Meng et al. 2019) and are also inductively produced to help rice adapt to different stress conditions (Lakehal et al. 2019). Lateral roots develop from primordia initiated at pericycle cells of the seminal, crown, or lateral roots. They account for the extensive increase in the surface area of the root system, allowing for efficient water and nutrient uptake (Dubrovsky and Laskowski 2017). Due to the significance of crown root development in rice growth and stress response, research on the pathways that control this process is essential to developing new stress-resistant rice varieties.

Throughout the stages of crown root development, auxin serves as a significant regulator (Overvoorde et al. 2010). CR primordia initiation occurs at the ground meristem of the rice stem base (Itoh et al. 2005). Every process starts with auxin transport by a group of active efflux carriers named the PIN-FORMED proteins (OsPINs) (Krecek et al. 2009). They are asymmetrically distributed on cells, and the resulting polarity determines auxin flow, such as acropetal transport in the stele through the coordinated action of PIN 1,3,7 (Grunewald et al. 2007). In rice, several PINs were found to be closely involved in CR emergence, such as OsPIN1b and c in CR primordia and emerged CRs, OsPIN2 in epidermal cells, and OsPIN10a in CR tips during elongation (Lin and Sauter 2019). All mutants with disruptions in *OsPIN* expression displayed CR defects, such as the positive regulator knockout *oscrl4* (no CRs) (Kitomi et al. 2008) or the

overexpressed negative regulator OsRPK1 (underdeveloped CRs lacking LRs) (Zou et al. 2014). Consequent disruptions in auxin levels are perceived by a complex called TIR1-SCF. The TIR1 subunit senses the signal and binds with auxin, greatly enhancing the complex's affinity for Aux/IAAs (transcriptional repressors). Upon binding, Aux/IAAs are ubiquitinated and destroyed by 26S proteasome, which releases the transcriptional activity of different Auxin Response Factors (ARFs) that regulate auxin-responsive genes (da Costa et al. 2013), including those involved in CR development. For example, OsCRL1, which is regulated by Auxin Response Factor ARF1, is strictly required for CR initiation (Inukai et al. 2005; Liu et al. 2005). Another target of OsARF is OsCRL5, which indirectly promotes CR initiation by repressing cytokinin signaling through OsRR1 (Kitomi et al. 2011). OsRR1 itself can inhibit OsCKX4, a node that integrates auxin and cytokinin signaling in CR formation, with binding sites for response regulators of both hormones (ARF25, OsRR2, OsRR3) (Gao et al. 2014). *OsCKX4* expression is also indirectly activated by OsWOX11 (induced by auxin synthesis) through the latter's inhibition of OsRR2 (Zhao et al. 2009) and promoted during CR elongation by the OsWOX11-OsCRL1 complex (Zhang et al. 2018).

Despite our extensive knowledge of auxin's role in rice root development, there is a considerable gap in our understanding of CR primordia initiation at the stem base and subsequent maturation into emerged CRs, aside from a few studies focused on histological analyses of the stem base, a limited amount of research focused on the pathways that control this process. As CRs play a critical role in rice development and stress response, it is necessary to investigate and fill out the missing gaps in the early phases of their development. In a previous study, a hypothetical Germin-like protein named OsGER4 had been discovered to be linked to the induction of CR development in rice under exogenous Jasmonic acid treatment. OsGER4 was strongly induced in the stem base, and its expression pattern throughout the rice root system is highly similar to that of the auxin distribution pattern, suggesting a connection with auxin signaling. This hypothesis was supported by significant changes in the OsGER4 expression pattern when polar auxin transport was inhibited by NPA treatment. Furthermore, *in-silico* protein structure analysis showed that OsGER4 could be localized to plasmodesmata, which are intercellular channels that could accommodate a novel route of auxin transport (To et al., 2022). In this study, OsGER4's role in auxin-mediated rice root development was definitively confirmed. In addition, its localization at the plasmodesmata suggests the role in regulating the auxin flow as a stress response resulting in root developmental changes.

Materials and methods

Plant materials and growth conditions

The *osger4* mutant lines and the transgenic promoter lines (promGER4::GUS) were developed in the Kitaake background according to the process described in a previous study (To et al. 2022). For phenotyping, mature seeds were dried for two days at 42°C, hydrated to induce germination, then transferred to glass tubes (22x250mm) containing MS1/2 medium. The tubes were placed in a growth chamber for seven days at the 16-h (light, 28°C)/8-h (dark, 25°C) light cycle.

RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis

Seeds of *crl1* knockout mutant rice based on Taichung-65 (TC65) genetic background were gifted by Prof. Yoshiaki Inukai, Nagoya University (Inukai et al. 2005). Rice seedlings were grown in test tubes under mock (0 µM JA) or treated conditions (5 µM JA). After seven days, tissue from the stem base was collected for total mRNA extraction using TRIzol® Reagent (Thermo Fisher Scientific, US). The cDNA library was synthesized using the Maxima® First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). qPCR experiments were performed with a qTOWER³ (Jena Analytik, Germany) using GoTaq® qPCR master mix (Promega, US). Gene expression levels were normalized to the reference gene (Actin). The relative gene expression was calculated using the $2 - \Delta\Delta Ct$ method (Livak and Schmittgen 2001).

Phytohormone treatment and phenotyping of plant materials

In this study, three types of auxins (Indole-3-acetic acid or IAA, Indole-3-butyric acid or IBA, 1-Naphthaleneacetic acid or NAA) (Sigma Aldrich) were exogenously supplemented in MS/2 medium at a concentration of 5 µM. Changes in rice root system architecture were assessed by a plasticity index (I) was calculated as follows: *I= (NCR treatment-NCR control)/NCR control.*

Histological and anatomical analysis of rice crown root

ProOsGER4::GUS rice seedlings were grown for seven days under control or exogenous auxin treatment (IAA/IBA/NAA, 5 µM). Samples were incubated overnight with GUS staining solution at 37°C and processed according to a protocol described by Gallagher (Gallagher 1992). Stem base and root tissues were fixed in paraffin following a previously described protocol (Patel et al. 2016). The stained tissues' sections (6–10 µm) were performed using a microtome (HM 340E, Thermo Scientific).

In-silico prediction of NCR10 structure and function

The complete amino acid sequence of NCR10 was obtained from the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore/). Protein structure and function were predicted using the I-TASSER webserver (https://zhanglab.dcmb.med.umich.edu/I-TASSER/), UniProt (https://www.uniprot.org/uniprot/), and AlphaFold (https://alphafold.ebi.ac.uk/). Protein-substrate binding interactions were simulated with PyRx AutoDock Vina (v.1.2.0, Scripps Research Institute, United States) and visualized with PyMOL2 (v 2.0, Schrödinger Inc, United States).

Construction of OsGER4::RFP and AtFH11::GFP

The coding region of *OsGER4* was amplified using specific primers (*OsNCR-attB1-F*: 3'-AAAAAGCAGGCTTCATGGCGAGGGTACAGCTCTG-5', *OsNCR-attB1-R*: 3' AGAAAGCTGGGTGCTTGAACTTGGCCTTGATGGC-5'), then cloned into the pDONOR201 vector (Addgene, UK). After confirming the sequence, *OsGER4* was transferred to pH7RWG2 Karimi for C-terminal RFP (Red

Fluorescent Protein) fusion using the Gateway LR clonase (Invitrogen, USA). Similarly, the coding region of 282 amino acid N-terminus carrying transmembrane domain (TM) of *AtFH2*, which was previously reported to be a plasmodesmata-localized protein (Diao et al., 2018), was cloned into pH7FWG2 (Karimi et al. 2002) using specific primers (*AtFH2-attB1-F*: 3'- AAAAAGCAGGCTTCATGACTACAATACCCTTCTG –5', *AtFH2-attB1-R*: 3'- AGAAAGCTGGGTGAGACGACGAAGCTGGAGGAG-5') for C-terminal GFP (Green Fluorescent Protein).

Localization analysis

The constructs OsGER4::RFP and AtFH11::GFP were introduced to *Agrobacterium tumefaciens* GV3101 and co-infiltrated into 4-week-old *Nicotiana benthamiana* plants using 1-mL needleless syringes (Ma et al. 2012). The agro-infiltrated plants were kept at 25°C for four days. The localization of OsGER4-RFP was monitored using a confocal microscope (LSM510 META, Carl Zeiss, Germany).

Statistical Analysis

All presented data are the mean and standard deviation obtained from three replicates. Statistical differences between groups were checked by Student's t-test, Wilcoxon signed-rank test, and one-way analysis of variance with Tukey's test. All statistical analyses were performed in GraphPad Prism 7.

Results

The OsGER4 promoter contains binding motifs related to auxin and root development.

In this study, three relevant *cis*-acting regulatory elements (CAREs) were identified in the *OsGER4* promoter region: CRL1-box (CACAAC), LBD-box (GCGGCG), and small Auxin box (CATATG) (Fig. 1a). The first one is the binding target of CRL1- a master transcription factor essential for crown root initiation in rice which was discovered by Gonin et colleagues (Gonin et al. 2022). The second one, or LOB box (GCGGCG), is the binding target of the LBD transcription factor associated with root development in Arabidopsis (Husbands et al. 2007). The third one is GMSAUR-box or CATATG, the DNA binding site of genes traditionally induced by exogenous auxin (Stortenbeker and Bemer 2019). In addition, some transcriptional factor binding sites, such as the TGTCNN motif, can also be associated with auxin response were found (Zemlyanskaya et al. 2016). This DNA binding site occurs nine times on the *OsGER4* promoter region (Supplementary Fig. 1).

To investigate the possible involvement of OsGER4's in the CRL1 signaling pathway under abiotic stress, *OsGER4* expression levels were quantified in the stem base of both *crl1* knockout rice and the wild-type TC65 rice under mock and five µM JA treated conditions. As seen in Fig. 1c, in TC65 rice where CRL1 is intact and functional, the fold change was 2.57 after exogenous JA treatment, consistent that of with previous findings (To et al. 2022), while in the absence of CRL1 acting as a regulator, *OsGER4* was not induced by JA treatment at all. This finding indicates that OsGER4 is connected to the CRL1 signaling pathway and reinforces this gene's involvement in auxin signaling. Moreover, the *crl1* knockout mutant

line exhibited only one seminar root with many lateral roots from this embryonic root in both conditions implying that JA cannot recover the phenotype (Fig. 1b).

OsGER4 expression pattern in root tissue is significantly altered by exogenous auxin treatment.

Figure 2a shows the root system architecture and *OsGER4* expression patterns in rice seedlings, as reported by GUS, after treatment with three different types of auxin at five uM (IAA, IBA, and NAA). All treatments led to a significantly stronger *OsGER4* expression, and crown roots formed in shorter, denser clusters than control group seedlings. Throughout the stem base, *OsGER4* expression was almost undetectable (Fig. 2b, left). In crown roots, *OsGER4* expression was detected in every cell layer, concentrated strongly at the root tip (columella and lateral caps) and the two dermis layers (epi- and exo-) (Fig. 2b, middle). Cross sections of lateral roots emerging from crown roots showed a similar trend of prominent *OsGER4* expression at the root tips and in the central vascular tissues, where auxin transport may occur (Fig. 2b, right). Overall, enhanced *OsGER4* expression in response to auxin treatment and its unique expression patterns in the crown root and lateral roots at initiation and emergence zones reinforced OsGER4's involvement with auxin homeostasis, which is essential to rice root development.

OsGER4 knockout significantly affects rice root system architecture under stress conditions.

Figure 3a shows the root system architecture of Kitaake and one representative mutant line *osger4-3.1* under normal and auxin treatment conditions. Overall, auxin treatment negatively affects crown root development in the mutant line, with fewer crown roots produced, most prominently in the case of IBA treatment. The effect of different exogenous auxins (1 uM) on NCR was illustrated using an NCR plasticity index (I), calculated as the relative change determined in mutant lines compared to the Kitaake control (Fig. 3b). A plasticity index value of 1 would indicate that the NCR value had increased by 100%. As seen in Fig. 3b, the Kitaake control group produced more crown roots than all three *osger4* mutant lines (3.1, 5.2, and 6.1), most strongly under IBA treatment. While the effects of each auxin type on each mutant line varies, the overall trend is attenuated, but not utterly defunct auxin response in the case of *osger4* mutant line 6.1 under IBA, which suggests that *OsGER4* might function as a supporter downstream of the auxin regulator CRL1, especially under hormonal stress imbalance condition.

Auxin transport inhibition significantly affects CR development in osger4 mutants more than in wild-type rice.

Figure 4a shows the root system architecture of Kitaake compared to *osger4* mutant line 3.1 with and without polar auxin transport and signaling inhibitor NPA treatment. Under normal conditions, mutant seedlings produced more crown roots than wild-type ones. This observation was supported by NCR phenotyping results in the case of *osger4 3.1* (Fig. 4b). When auxin transport was inhibited through exogenous NPA treatment (Fig. 4a), the seminal root of Kitaake rice still produced very short, fine LRs, but overall NCR value significantly reduced. In comparison, treated *osger4* mutants were significantly more affected, producing fewer and shorter crown roots in all three *osger4* knockout mutant lines. Especially,

the mutant line *osger4 3.1* has 61% fewer CRs than Kitaake rice. Based on these findings, OsGER4 may reorganize auxin flux at the rice stem base under stress conditions.

OsGER4 is localized to plasmodesmata.

Results obtained from the protein structure prediction web server I-TASSER (Fig. 5a) predicted that OsGER4 consists of an α–helix at the N-terminal, followed by 12 β-strands of varying sizes, and three α-helices at the C-terminal. A disulfide bridge was present between residues CYS34 and CYS49. The first 24 residues comprised a signal peptide targeting the plasmodesmata while doubling as a transmembrane helix (4–22), and residues 25–215 included the protein's cytoplasmic section (Supplementary Table 1). The tertiary structure consisted of a central barrel-like cupin domain formed from 8 β-strands, with α-helices distributed near the two termini (Fig. 5a1). The surface representation is shown in Fig. 5a2. Regarding post-translation modifications, there were 2 N-glycosylated residues (ASN52 and 76), one of which (ASN76) lay on the cupin domain. *In-silico* predictions using the GPS-SUMO webserver suggested two sumoylation sites at LYS43 (central cupin domain) and 212 (α-helix), as well as a SUMO interaction motif (VAL-LEU-GLU-VAL) from residue 124 to 127 (Supplementary Table 1). The tertiary structure was predicted to dimerize, then trimerize to form a hexamer. Figure 5a3 and 5a4 represents a potential binding interaction between OsGER4 and an auxin via its central cavity, but the significance of this possibility is unknown. Consensus predictions with Gene Ontology (GO) terms by I-TASSER produced the following attributes: $Mn2+$ ion binding (molecular function); response to stress, cell wall organization (biological process); cell wall, apoplast (cellular component). These terms suggest that OsGER4's function could be related to cell wall modifications at the plasmodesmata in the rice root system as part of a stress response.

OsGER4 localization was assessed in *Nicotiana benthamiana* leaf cells expressing the *35S: OsGER4:RFP* construct (red signal). Fluorescence microscopy results (Fig. 5b, merge view) indicate that OsGER4 is exclusively localized to the cell wall in segments, which could be locations where plasmodesmata are located. As mentioned previously, a preliminary *in-silico* prediction that OsGER4 targets the plasmodesmata by a 24 amino acid signal peptide sequence/transmembrane helix at its N-terminal. Although the degree of overlapping localization is imperfect, several segments exhibiting comparatively strong fluorescence for OsGER4 and OsFH11 (yellow) were observed (Fig. 5b4).

Discussion

OsGER4 expression is dependent on crown root development regulator CRL1

It has been discovered that CRL1 can transactivate a set of at least five rice genes (*QHB, OsbHLH044, OsROP, ROC4, and OsHOX14*), of which *OsROP* and *OsBHLH044* are direct targets that are involved in promoting crown root formation (Gonin et al. 2022). In this study, the *OsGER4* promoter region also contains a CRL1 binding box, and this gene is also involved in crown root development under stress. Furthermore, the LBD box (GCGGCG) on the *OsGER4* promoter region suggests this gene could interact with LOB domain (LBD) proteins. Interestingly, the master regulator CRL1 is also an LBD protein (Liu et al.

2005), and it has been demonstrated *in planta* that CRL1 preferably binds to the LBD box (Gonin et al. 2022), thus reinforcing its potential involvement in *OsGER4* regulation during rice root development under stress. This gene probably interacts with LOB domain TFs that modulate CR formation by regulating cell division and cell wall modification (Li 2021). The possibility that *OsGER4* is a target of CRL1, a regulator through which auxin can promote rice crown root development in rice, is supported by previous results, in which OsGER4 was found to be expressed in root initiation or emergence zone regions. These regions also coincided with the auxin distribution pattern, and significant changes in *OsGER4* expression patterns following the disruption of polar auxin transport with naphthylphthalamic acid (NPA) treatment support this gene's involvement in auxin transport (To et al., 2022).

The OsGER4-CRL1-Auxin connection was finally demonstrated in this study by qPCR results, in which rice lacking *CRL1* was unable to induce *OsGER4* under JA treatment compared to TC65 control (Fig. 1c). The magnitude of this effect is highlighted in Supplementary Fig. 2, which shows the significantly diminished *OsGER4* expression level in *crl1* knockout mutants, at approximately 88.3% lower than the control group. In a previous study, it has been observed that under exogenous JA treatment, *OsGER4* is strongly induced, and an increase in NCR value occurred, though the placement of this phytohormone about auxin signaling and, by extension, CRL1 is unclear. The results above suggest that while JA signaling strongly influences OsGER4 expression and NCR, both still lie under the control of the crown root development regulator CRL1.

Overall, *OsGER4* promoter sequencing results revealed the presence of motifs closely associated with the transcription factor CRL1 (regulates various genes associated with crown root development) and response to auxin (integral to root development), which was confirmed by qPCR results showing a dependence of *OsGER4* expression on regulation by CRL1, thus supporting OsGER4's hypothesized role in rice crown root development under stress.

Close similarities between the OsGER4 expression pattern and auxin distribution pattern suggest its connection to auxin transport.

OsGER4 expression is significantly enhanced by exogenous auxin treatment (Fig. 2a). Histology results showed that the *OsGER4* expression pattern is characterized by strong localization in the root development zone, such as developing crown roots, lateral roots, and root tips, and in root vascular tissues (Fig. 2b). However, throughout the stem base (origin of crown roots), *OsGER4* is not expressed at significant levels, except in primordia that would eventually develop into crown roots. These observations support the theory that OsGER4 indirectly regulates auxin transport under stress. One point of note is that there are many similarities between the auxin distribution pattern and the *OsGER4* expression pattern in developing crown roots and lateral roots. For crown roots it was in root cap columella cells and the two dermis layers (Wang et al. 2018); lateral roots were in the basal cells, stele, and root tip cells (Kawai et al. 2022). Furthermore, the *OsGER4* expression pattern in crown roots coincides with that of the polar auxin transport route in rice roots, in which auxin synthesized by OsYUC8 travels down via stele tissues until it reaches the central columella cells of the root tip and gets cycled back up through cells of the exodermis,

facilitated by the transporter OsAUX1 (Huang et al. 2022), which also acts as a promoter of lateral root initiation (Zhao et al. 2015). This observation is reinforced by previous studies, in which many rice genes had been shown to display similar expression patterns and subcellular protein localization, such as the auxin influx transporters OsPIN1b (expressed in vascular tissues and crown root primordia in the stem base that regulates crown root emergence and development) (Xu et al. 2005), and OsPIN2 (expressed in lateral root tips and primordia, primary root tips, and the stem base) (Wang et al. 2018). This similarity in expression pattern also extends to transporter proteins in other families, such as OsABCB14 (in stele and root tips, the enhanced expression following treatment with IAA, Abscisic acid) (Xu et al. 2014), and OsBG1 (Liu et al. 2015). Overall, *OsGER4* showed a close association with auxin transport in rice roots. Its expression patterns in roots coincide with the auxin distribution pattern and several proteins localized to the plasma membrane that had been definitively proven to be tied to auxin transport.

Crown root defects in osger4 mutants under auxin treatment prove this gene's role in root development and support its involvement in auxin transport.

Compared to Kitaake rice under normal conditions, the *osger4* 3.1 mutant produced significantly longer crown roots that curl up at the tips from mechano-sensing (Fig. 4a). One case with a similar phenotype involves the acropetal auxin influx transporter OsABCB14, whose knockdown leads to mutants less sensitive to the auxins 2,4-D and IAA, with significantly longer roots under normal conditions (Xu et al. 2014). The increased CR length (Fig. 4a) and number (Fig. 4b) in *osger4* knockout mutants compared to Kitaake under normal conditions suggest that the priorities of auxin transport may have been affected, with more auxin accumulation at the root tips and stem base. Under exogenous treatment with auxin, Kitaake root development priorities were shifted, with more crown roots produced at the stem base that was much shorter and produced a higher percentage of very fine and short lateral roots. It can be inferred that the general effect of auxin treatment is a shift of auxin accumulation from the root tip to the stem base, thus prioritizing NCR over CR length. This was most clearly observed in the case of IBA-treated Kitaake, which features a markedly denser root mass compared to IAA treatment, as it has been demonstrated that IBA is more effective than IAA in root initiation (De Klerk et al. 1999), especially lateral root development (Chun et al. 2003). In *osger4* 3.1 mutants treated with different auxins, the number of CRs produced was significantly lower than Kitaake in all cases. This was most clearly observed under exogenous IBA treatment, in which CR length was also reduced (Fig. 3a). Figure 3b shows the plasticity index/relative NCR value of 3 different *osger4* knockout mutant lines compared to Kitaake under auxin treatment. It is clear from these results that the loss of *OsGER4* severely reduced the ability to increase crown root numbers in response to auxin treatment.

Exogenous NPA treatment was used to investigate the effects of auxin transport inhibition in rice lacking *OsGER4* and, by extension, the function of this gene. As described above, the loss of *OsGER4* exacerbated the effects of NPA on rice development, with all three knockout mutant lines producing significantly fewer CRs compared to Kitaake rice (Fig. 4b). This finding demonstrated that functionally, *OsGER4* plays a definite but unknown role in rice CR development driven by auxin signaling under stress. Several proteins that contribute to polar auxin transport in rice root growth also participate in stress responses, such as

OsAUX1 and 3 (LR initiation, phosphate and metal stress) (Feng et al. 2022) and OsPIN10a (CR development, drought stress) (Balzan et al. 2014). Thus, it is not unlikely that the OsGER4 signaling pathway could be connected to one or multiple proteins of the AUX and PIN families through a protein responsive to changes in auxin levels caused by polar transport.

Based on the substantial amount of evidence supporting the involvement of OsGER4 in regulating auxin distribution during root development (e.g., increased OsGER4 expression following auxin treatment, reduced sensitivity to auxin of *osger4* mutants, notably altered root system architecture compared to wild-type rice), and its similarities to several well-studied proteins, it is likely that OsGER4's function is connected to auxin transport, possibly associated with plasmodesmata, which are intercellular channels that connect adjacent plant cells.

Once localized at plasmodesmata, OsGER4 could regulate auxin passage through these channels.

How OsGER4 performs its function is unclear, but the encoded protein does possess an N-terminal signal peptide localized to plasmodesmata. These cytosolic bridges allow molecules to move between adjacent plant cells, ranging from small photosynthetic products to even mRNA and transcription factors. Protein localization analysis in *Nicotiana benthamiana* leaf cells revealed that OsGER4 was exclusively found in the plant cell wall, in distinct segments that resemble the distribution of plasmodesmata, similar to that of the Plasmodesmata protein OsPDLP1a, and confirmed by overlap (yellow) with the plasmodesmata marker OsFH11 (green). Many different phytohormones can diffuse through the Pd as part of their signaling pathway (Han and Kim 2016), such as salicylic acid (Shah and Zeier 2013), MeJA (Thorpe et al. 2007), and cytokinins (Bishopp et al. 2011). In a study on the *A. thaliana* mutant *gsl8*, it was found that auxin diffusion through the Pd could be enhanced by reducing callose (a cell wall polysaccharide) deposition at the narrow neck regions of Pd to increase permeability (Han et al. 2014). Another study in *A. thaliana* leaves concluded that Pd permeability could be regulated within a plant cell, and the resulting directionality of diffusion can affect tissue-specific auxin distribution patterns (Gao et al., 2020). Similarly, it was discovered in *A. thaliana* roots that auxin could travel through Pd to modify the auxin distribution pattern (created by efflux and influx carriers), thereby enabling its reflux and accumulation at the root tips (Mellor et al. 2020). Furthermore, regulating Pd permeability also shapes root architecture (Mellor et al. 2020). Aside from its plasmodesmata targeting signal peptide, OsGER4 has also been predicted to possess two sites for SUMOylation and 1 SUMO interaction motif, which could be related to its interaction with Pd, as a previous study in CMoV had shown that both of these attributes are required for efficient Pd targeting of the ORF4 movement protein (Jiang et al. 2021). Thus, according to our results and existing relevant bibliography, OsGER4 may be a supporter protein associated with Pd-mediated auxin transport as a stress response mechanism in rice roots. Regarding the processes are underlying.

Assuming that OsGER4 regulates transport through plasmodesmata, we hypothesized that it influences the auxin flow by blocking Pd at sites of auxin accumulation, thus indirectly promoting two processes dependent on high auxin concentrations in rice roots: initiation and elongation. With a fixed amount of available resources and auxin under stress, there is a trade-off in the rice root system between either

producing more roots (initiation) or increasing existing root length (elongation). In our experiments on rice seedlings, it is worth noting that CR initiation and development in the stem base always occur before CR elongation and LR initiation, shifting priorities towards generating CRs which could account for the higher number of CRs in *osger4* knockout mutants compared to Kitaake. Under an imbalance of auxin caused by exogenous treatment (stress), *osger4* mutants could not produce more crown roots, possibly due to their inability to adequately build up auxin at the stem base. While auxin treatment has been found to promote root initiation, we must also consider that without plasmodesmata-mediated transport and reflux, auxin cannot build up sufficiently at growth sites with just influx and reflux proteins (Mellor et al. 2020). Therefore, the need to efficiently retain auxin at the stem base, which OsGER4 is hypothesized to play a part in through plasmodesmata regulation, becomes critical when auxin imbalances occur due to stress. This is most clearly seen in the case of treatment with the auxin transport inhibitor NPA in *osger4* 3.1 (Fig. 4B).

Conclusion and perspectives

In conclusion, the results obtained from this study have confirmed definitively that *OsGER4* plays a role in the development of crown roots and auxin signaling in rice plants under stress. We've also confirmed that OsGER4 is localized to plasmodesmata through its N-terminal peptide sequence. Regarding the significance of this observation, we hypothesized that OsGER4 might function as part of an unknown auxiliary pathway that regulates the auxin transport through the blockade of plasmodesmata for auxin to sufficiently build-up at sites of root growth/development, such as the stem base. Further investigations on correlations between OsGER4 expression, plasmodesmata permeability, and auxin flow at root growth sites would help elucidate the exact function of this protein as part of the rice stress response.

Declarations

Competing Interests: The authors declare that they have no competing interests

Ethical Approval and Consent to Participate: Not applicable

Authors' contributions: TT Nguyen, TD Pham, TP Do, TVA Le, TA Tran, and HH Chu performed the phenotyping and qPCR experiments. JS Jeon and TXK Vo conducted the localization study. TD Pham and HTM To wrote the main manuscript. All authors reviewed the manuscript and approved the submission.

Funding: This research was supported by the Vietnam Academy of Science and Technology through grant number: VAST 02.05/22-23

Data availability statement: All relevant data can be found within the manuscript and its supporting materials.

Acknowledgment: We want to thank Ms. Nguyen Thi Hue, Ms. Dinh Thao Phuong, Ms. Hoang Thi Huyen Trang, and Ms. Nguyen Thi Linh, Nguyen Van Doai, Nguyen Hong Nhung for the technical support—a special thanks to Prof. Jea Yean Kim (Gyeongsang National University, KR) for suggesting the PD markers, to Dr. Antony Champion (IRD, FR) for CRL1 network suggestion.

We are very grateful to Prof. Yoshiaki Inukai at the International Center for Research and Education in Agriculture Graduate School of Bio agricultural Sciences, Nagoya University, for his generous gift of TC65 and *crl1* seeds, and the Intellectual Property Office, Academic Research & Industry-Academia-Government Collaboration, THERS National University Corporation, Tokai National Higher Education and Research System for the MTA administrative support.

References

1. Balzan S, Johal GS, Carraro N (2014) The role of auxin transporters in monocots development. Frontiers in Plant Science 5:
2. Bishopp A, Lehesranta S, Vatén A, et al. (2011) Phloem-transported cytokinin regulates polar auxin transport and maintains vascular pattern in the root meristem. Current biology : CB 21:927–932. https://doi.org/10.1016/J.CUB.2011.04.049
3. Chhun T, Taketa S, Tsurumi S, Ichii M (2003) The effects of auxin on lateral root initiation and root gravitropism in a lateral rootless mutant Lrt1 of rice (Oryza sativa L.). Plant Growth Regulation 2003 39:2 39:161–170. https://doi.org/10.1023/A:1022592511387
4. Da Costa CT, de Almeida MR, Ruedell CM, et al (2013) When stress and development go hand in hand: Main hormonal controls of adventitious rooting in cuttings. Frontiers in Plant Science 4:133. https://doi.org/10.3389/FPLS.2013.00133/BIBTEX
5. De Klerk GJ, Van Der Krieken W, De Jong JC (1999) Review the formation of adventitious roots: New concepts, new possibilities, i Vitro Cellular & Developmental Biology - Plant 1999 35:3 35:189–199. https://doi.org/10.1007/S11627-999-0076-Z
6. Dubrovsky JG, Laskowski M (2017) Lateral Root Initiation. Encyclopedia of Applied Plant Sciences 1:256–264. https://doi.org/10.1016/B978-0-12-394807-6.00126-X
7. Feng Y, Bayaer E, Qi Y (2022) Advances in the Biological Functions of Auxin Transporters in Rice. Agriculture 12:989. https://doi.org/10.3390/agriculture12070989
8. Gallagher SR (1992) GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression - Google Sách. Academic Press, San Diego
9. Gao C, Liu X, De Storme N, et al (2020) Directionality of Plasmodesmata-Mediated Transport in Arabidopsis Leaves Supports Auxin Channeling. Current biology : CB 30:1970-1977.e4. https://doi.org/10.1016/J.CUB.2020.03.014
10. Gao S, Fang J, Xu F, et al (2014) CYTOKININ OXIDASE/DEHYDROGENASE4 Integrates Cytokinin and Auxin Signaling to Control Rice Crown Root Formation. Plant Physiol 165:1035–1046. https://doi.org/10.1104/pp.114.238584

11. Gonin M, Jeong K, Coudert Y, et al (2022) CROWN ROOTLESS1 binds DNA with a relaxed specificity and activates OsROP and OsbHLH044 genes involved in crown root formation in rice. The Plant journal : for cell and molecular biology 111:546–566. https://doi.org/10.1111/TPJ.15838
12. Grunewald W, Parizot B, Inzé D, et al (2007) Developmental Biology of Roots: One Common Pathway for All Angiosperms?
13. Han X, Hyun TK, Zhang M, et al (2014) Auxin-callose-mediated plasmodesmal gating is essential for tropic auxin gradient formation and signaling. Developmental cell 28:132–146. https://doi.org/10.1016/J.DEVCEL.2013.12.008
14. Han X, Kim JY (2016) Integrating Hormone- and Micromolecule-Mediated Signaling with Plasmodesmal Communication. Molecular Plant 9:46–56. https://doi.org/10.1016/J.MOLP.2015.08.015
15. Hochholdinger F, Zimmermann R (2008) Conserved and diverse mechanisms in root development. Current opinion in plant biology 11:70–74. https://doi.org/10.1016/J.PBI.2007.10.002
16. Huang G, Kilic A, Karady M, et al (2022) Ethylene inhibits rice root elongation in compacted soil via ABA- and auxin-mediated mechanisms. Proceedings of the National Academy of Sciences of the United States of America 119:e2201072119. https://doi.org/10.1073/PNAS.2201072119/SUPPL_FILE/PNAS.2201072119.SAPP.PDF
17. Inukai Y, Sakamoto T, Ueguchi-Tanaka M, et al (2005) Crown rootless1, which is essential for crown root formation in rice, is a target of an AUXIN RESPONSE FACTOR in auxin signaling. The Plant cell 17:1387–1396. https://doi.org/10.1105/TPC.105.030981
18. Itoh J-I, Nonomura K-I, Ikeda K, et al (2005) Rice plant development: from zygote to spikelet. Plant Cell Physiol 46:23–47. https://doi.org/10.1093/pcp/pci501
19. Jiang J, Kuo Y-W, Salem N, et al (2021) Carrot mottle virus ORF4 movement protein targets plasmodesmata by interacting with the host cell SUMOylation system. New Phytologist 231:382–398. https://doi.org/10.1111/nph.17370
20. Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7:193–195. https://doi.org/10.1016/s1360-1385(02)02251-3
21. Kawai T, Akahoshi R, Shelley IJ, et al (2022) Auxin Distribution in Lateral Root Primordium Development Affects the Size and Lateral Root Diameter of Rice. Frontiers in Plant Science 13:832. https://doi.org/10.3389/FPLS.2022.834378/BIBTEX
22. Kitomi Y, Ito H, Hobo T, et al (2011) The auxin responsive AP2/ERF transcription factor CROWN ROOTLESS5 is involved in crown root initiation in rice through the induction of OsRR1, a type-A response regulator of cytokinin signaling. Plant J 67:472–484. https://doi.org/10.1111/j.1365-313X.2011.04610.x
23. Kitomi Y, Ogawa A, Kitano H, Inukai Y (2008) CRL4 regulates crown root formation through auxin transport in rice. Plant Root 2:. https://doi.org/10.3117/plantroot.2.19
24. Krecek P, Skůpa P, Libus J, et al (2009) The PIN-formed (PIN) protein family of auxin transporters. Genome biology 10:249. https://doi.org/10.1186/gb-2009-10-12-249

25. Lakehal A, Chaabouni S, Cavel E, et al (2019) A Molecular Framework for the Control of Adventitious Rooting by TIR1/AFB2-Aux/IAA-Dependent Auxin Signaling in Arabidopsis. Molecular Plant 12:1499–1514. https://doi.org/10.1016/J.MOLP.2019.09.001/ATTACHMENT/DDE9FEAA-AA8A-4EBB-A73E-28CECF9C6C9E/MMC1.PDF
26. Li S-W (2021) Molecular Bases for the Regulation of Adventitious Root Generation in Plants. Frontiers in Plant Science 12:
27. Lin C, Sauter M (2019) Polar Auxin Transport Determines Adventitious Root Emergence and Growth in Rice. Front Plant Sci 10:444. https://doi.org/10.3389/fpls.2019.00444
28. Liu H, Wang S, Yu X, et al (2005) ARL1, a LOB-domain protein required for adventitious root formation in rice. Plant Journal 43:47–56. https://doi.org/10.1111/J.1365-313X.2005.02434.X
29. Liu L, Tong H, Xiao Y, et al (2015) Activation of Big Grain1 significantly improves grain size by regulating auxin transport in rice. Proceedings of the National Academy of Sciences of the United States of America 112:11102–11107. https://doi.org/10.1073/PNAS.1512748112/SUPPL_FILE/PNAS.201512748SI.PDF
30. Liu W, Yu J, Ge Y, et al (2018) Pivotal role of LBD16 in root and root-like organ initiation. Cellular and molecular life sciences : CMLS 75:3329–3338. https://doi.org/10.1007/S00018-018-2861-5
31. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262
32. Ma L, Lukasik E, Gawehns F, Takken FLW (2012) The Use of Agroinfiltration for Transient Expression of Plant Resistance and Fungal Effector Proteins in Nicotiana benthamiana Leaves. In: Bolton MD, Thomma BPHJ (eds) Plant Fungal Pathogens: Methods and Protocols. Humana Press, Totowa, NJ, pp 61–74
33. Mellor NL, Voß U, Janes G, et al (2020) Auxin fluxes through plasmodesmata modify root-tip auxin distribution. Development 147:dev181669. https://doi.org/10.1242/dev.181669
34. Meng F, Xiang D, Zhu J, et al (2019) Molecular Mechanisms of Root Development in Rice. Rice 12:. https://doi.org/10.1186/S12284-018-0262-X
35. Overvoorde P, Fukaki H, Beeckman T (2010) Auxin control of root development. Cold Spring Harb Perspect Biol 2:a001537. https://doi.org/10.1101/cshperspect.a001537
36. Patel PG, Selvarajah S, Boursalie S, et al (2016) Preparation of Formalin-fixed Paraffin-embedded Tissue Cores for both RNA and DNA Extraction. JoVE (Journal of Visualized Experiments) 2016:e54299. https://doi.org/10.3791/54299
37. Shah J, Zeier J (2013) Long-distance communication and signal amplification in systemic acquired resistance. Frontiers in plant science 4:. https://doi.org/10.3389/FPLS.2013.00030
38. Smith S, de Smet I (2012) Root system architecture: insights from Arabidopsis and cereal crops. Philosophical Transactions of the Royal Society B: Biological Sciences 367:1441–1452. https://doi.org/10.1098/RSTB.2011.0234

39. Stortenbeker N, Bemer M (2019) The SAUR gene family: the plant's toolbox for adaptation of growth and development. Journal of Experimental Botany 70:17–27. https://doi.org/10.1093/JXB/ERY332
40. Thorpe MR, Ferrieri AP, Herth MM, Ferrieri RA (2007) 11C-imaging: methyl jasmonate moves in both phloem and xylem, promotes transport of jasmonate, and of photoassimilate even after proton transport is decoupled. Planta 226:541–551. https://doi.org/10.1007/S00425-007-0503-5
41. To HTM, Pham DT, Thi VAL, et al (2022) The Germin-like protein OsGER4 is involved in promoting crown root development under exogenous jasmonic acid treatment in rice. The Plant Journal 112:860–874. https://doi.org/10.1111/TPJ.15987
42. Wang L, Guo M, Li Y, et al (2018) LARGE ROOT ANGLE1, encoding OsPIN2, is involved in root system architecture in rice. Journal of Experimental Botany 69:385–397. https://doi.org/10.1093/JXB/ERX427
43. Xu M, Zhu L, Shou H, Wu P (2005) A PIN1 Family Gene, OsPIN1, involved in Auxin-dependent Adventitious Root Emergence and Tillering in Rice. Plant and Cell Physiology 46:1674–1681. https://doi.org/10.1093/PCP/PCI183
44. Xu Y, Zhang S, Guo H, et al (2014) OsABCB14 functions in auxin transport and iron homeostasis in rice (Oryza sativa L.). The Plant Journal 79:106–117. https://doi.org/10.1111/TPJ.12544
45. Zemlyanskaya EV, Wiebe DS, Omelyanchuk NA, et al (2016) Meta-analysis of transcriptome data identified TGTCNN motif variants associated with the response to plant hormone auxin in Arabidopsis thaliana L. Journal of Bioinformatics and Computational Biology 14:. https://doi.org/10.1142/S0219720016410092
46. Zhang T, Li R, Xing J, et al (2018) The YUCCA-Auxin-WOX11 Module Controls Crown Root Development in Rice. Front Plant Sci 9:523. https://doi.org/10.3389/fpls.2018.00523
47. Zhang Y, Li Z, Ma B, et al (2020) Phylogeny and Functions of LOB Domain Proteins in Plants. International Journal of Molecular Sciences 21:. https://doi.org/10.3390/IJMS21072278
48. Zhao H, Ma T, Wang X, et al (2015) OsAUX1 controls lateral root initiation in rice (Oryza sativaL.). Plant Cell and Environment 38:2208–2222. https://doi.org/10.1111/PCE.12467/SUPPINFO
49. Zhao Y, Hu Y, Dai M, et al (2009) The WUSCHEL-related homeobox gene WOX11 is required to activate shoot-borne crown root development in rice. Plant Cell 21:736–748. https://doi.org/10.1105/tpc.108.061655
50. Zou Y, Liu X, Wang Q, et al (2014) OsRPK1, a Novel Leucine-Rich Repeat Receptor-like Kinase, Negatively Regulates Polar Auxin Transport and Root Development in Rice. Biochimica et biophysica acta 1840:. https://doi.org/10.1016/j.bbagen.2014.01.003

Figures

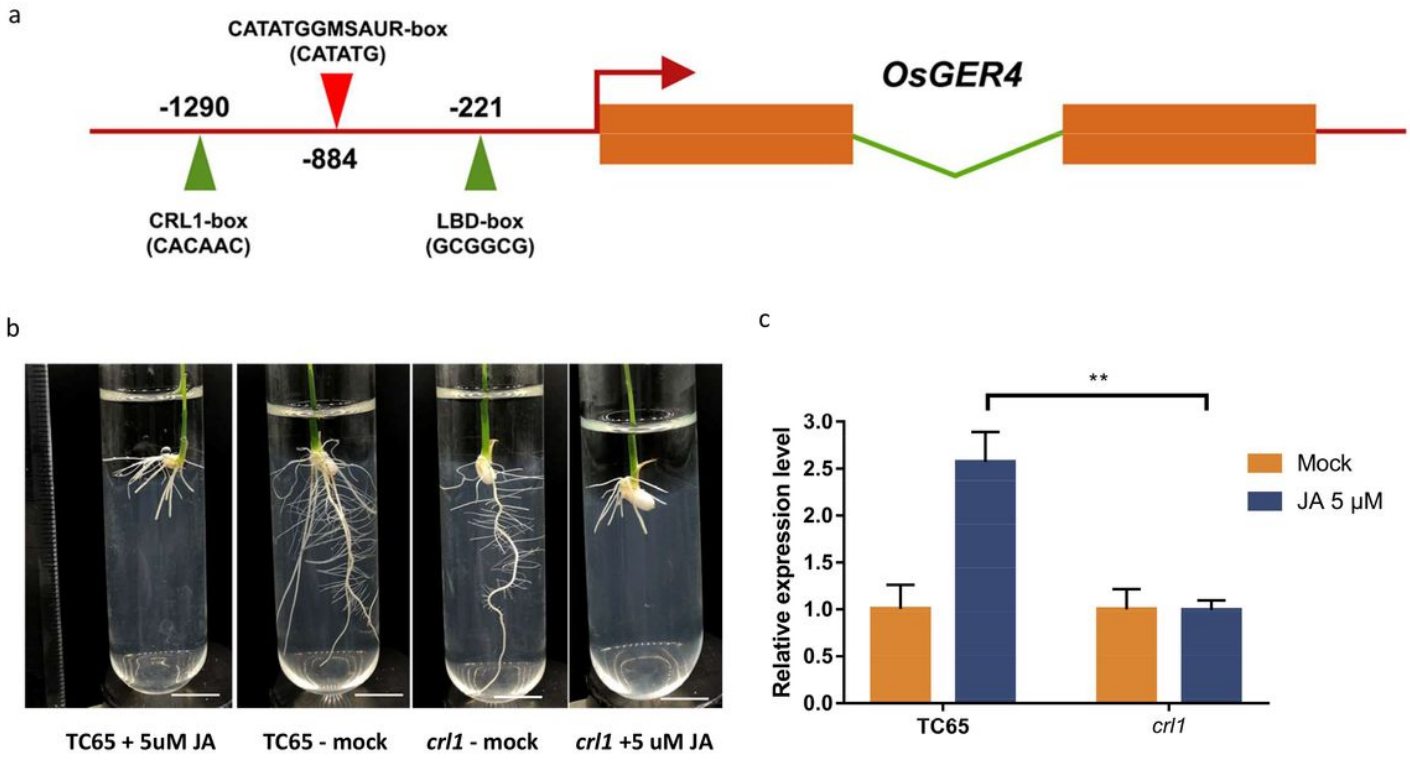

Figure 1

a Transcription factor binding motifs of interest on the *OsGER4* promoter region. **b** Rice root system architecture of *crl1* knockout mutants with and without JA treatment, compared to TC65 genetic background, scale bar = 1cm. **c** *OsGER4* expression levels in *crl1* knockout mutant under JA treatment compared to TC65 under similar conditions

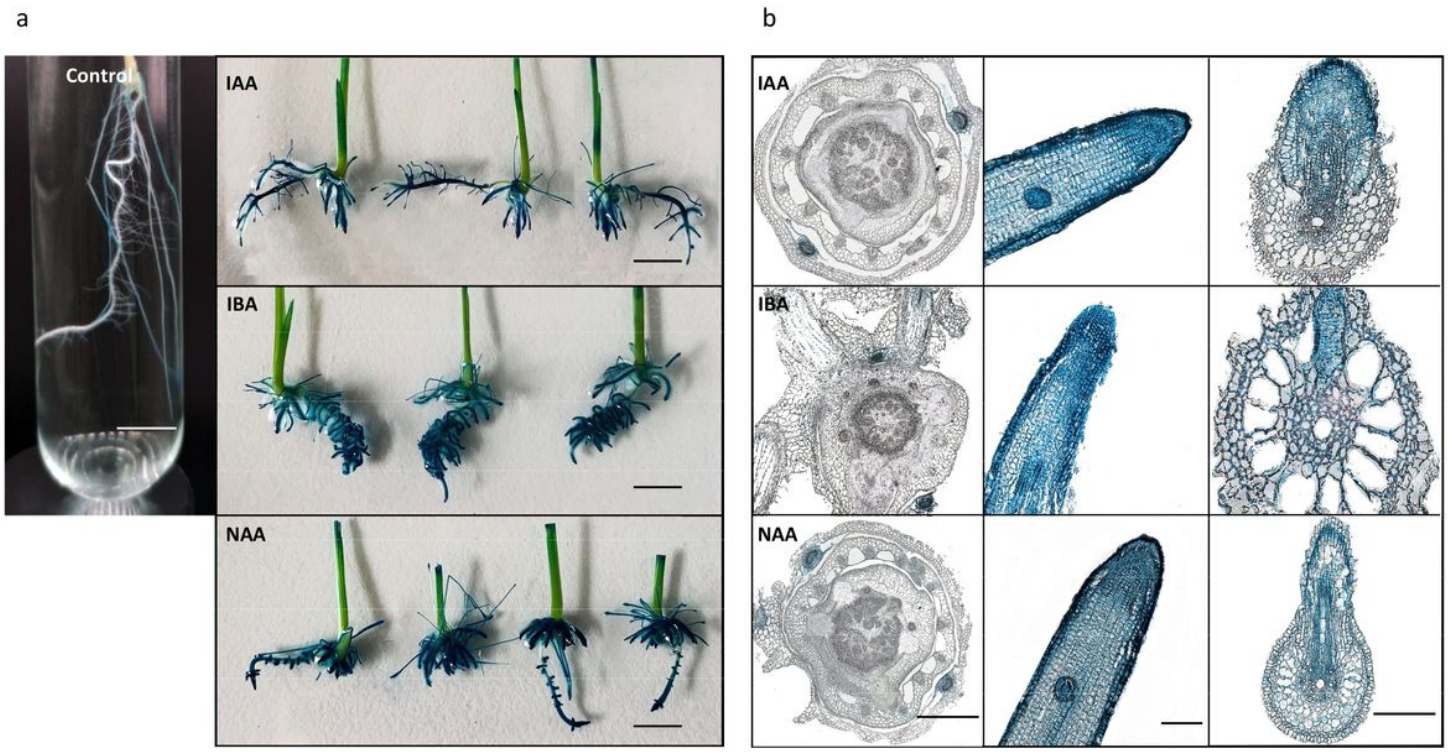

Figure 2

a Root system architecture of Kitaake seedlings on the 7th day after treatment with 3 different auxins at 5 µM for each type, scale bar = 1 cm**. b** Histology of GUS stained stem base and crown roots (longitudinal and cross sections) from Kitaake seedlings on the 7th day after treatment with 3 different auxins at 5 µM for each type, scale bar = 500 µm

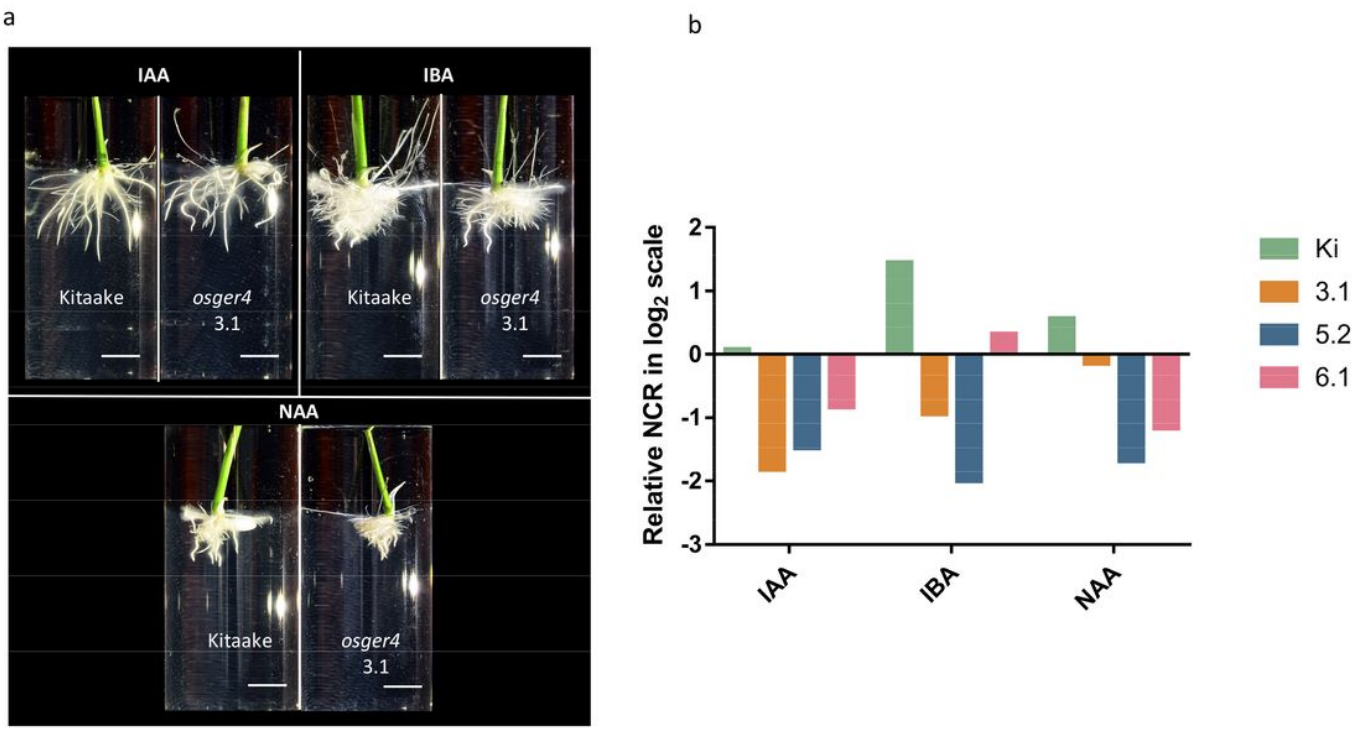

Figure 3

a Rice root system architecture of Kitaake and OsGER4 knockout mutant 3.1) under auxin-treated conditions (5 μM for each type). Scale bar = 1 cm. **b** NCR plasticity index of wild-type rice compared to three different osger4 knockout mutants (3.1, 5.2, 7.2) under auxin-treated conditions (1 μM for each type)

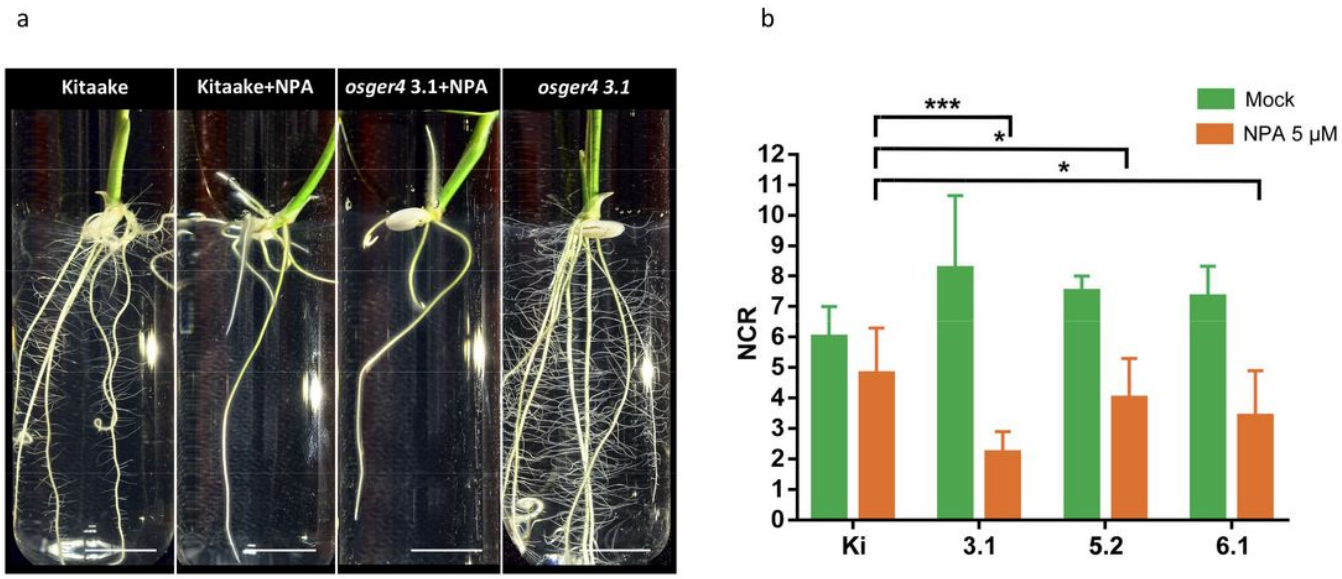

Figure 4

a Rice root system architecture of Kitaake and osger4 knockout mutant 3.1 under normal and NPA 5 µM treated conditions, scale bar = 1 cm. **b** NCR values of osger4 knockout mutants compared to Kitaake control under treatment with the auxin transport inhibitor NPA (5 µM). Asterisks represent different levels of significance as determined by one-way ANOVA and Tukey's test (**P < 0.01, ***P < 0.001)

Figure 5

a OsGER4 structure predicted by I-TASSER. (1) Overview (2) Surface view, (3) Binding mode between OsGER4 and NAA, with detected cavities, (4) Surface view of the binding interaction. **b**Co-localization of OsGER4:RFP with plasmodesmata (PD) marker AtFH2:GFP in N. benthamiana leaves, scale bars = 10 µm. Arrow indicates the signals that intersect

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfiguresandtables.pdf