Ipa1 Improves Rice Drought Tolerance at Seedling Stage Mainly Through Activating Abscisic Acid Pathway

Menghao Zhu
Wuhan University
Yonggang He
Wuhan University
Mingqiang Zhu
Wuhan University
Ayaz Ahmad
Wuhan University
Shuang Xu
Wuhan University
Zijun He
Wuhan University
Shan Jiang
Wuhan University
Jinqiu Huang
Wuhan University
Zhihui Li
Wuhan University
Shaojia Liu
Wuhan University
Xin Hou
Wuhan University
Zhihong Zhang (zzh@whu.edu.cn)
Wuhan University https://orcid.org/0000-0002-1063-1186

Research Article

Keywords: Rice (Oryza sativa L.), Drought Tolerance, ipa1, Carbon-Nitrogen Metabolism, Abscisic Acid (ABA)

Posted Date: July 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-733123/v1

Version of Record: A version of this preprint was published at Plant Cell Reports on October 25th, 2021.
See the published version at https://doi.org/10.1007/s00299-021-02804-3.

Abstract

Drought is a major abiotic stress to crop production. *IPA1* (*IDEAL PLANT ARCHITECTURE 1*)/*OsSPL14* encodes a transcription factor and has been reported to function in both rice ideal plant architecture and biotic resistance. Here, with a pair of *IPA1*/*ipa1*-NILs (Near Iso-genic Lines), we found that *ipa1* could significantly improve rice drought tolerance at seedling stage. The *ipa1* plants had a better-developed root system and smaller leaf stomatal aperture. Analysis of carbon-nitrogen metabolism-associated enzyme activity, gene expression, and metabolic profile indicated that *ipa1* could tip the carbon-nitrogen metabolism balance towards an increased carbon metabolism pattern. In both the control and PEG-treated conditions, ABA content in the *ipa1* seedlings was significantly higher than that in the *IPA1* seedlings. Expression of the ABA biosynthesis genes was detected to be up-regulated, whereas the expression of ABA catabolism genes was down-regulated in the *ipa1* seedlings. In addition, based on yeast one-hybrid assay and dual-luciferase assay, IPA1 was found to directly activate the promoter activity of *OsHOX12*, a transcription factor promoting ABA biosynthesis, and *OsNAC52*, a positive regulator of the ABA pathway. The expression of *OsHOX12* and *OsNAC52* was significantly up-regulated in the *ipa1* plants. Combined with the previous studies, our results suggested that *ipa1* could improve rice seedling drought tolerance mainly through activating the ABA pathway and that regulation of the *ipa1*-mediated ABA pawthway will be an important strategy for improving drought resistance of rice.

Key Messages

ipa1 enhances rice drought tolerance mainly through activating the ABA pathway. It endows rice seedlings with a more developed root system, smaller leaf stomata aperture, and enhanced carbon metabolism.

Introduction

Plants live in fixed locations and face diverse abiotic stresses (such as drought, salinity, and cold) negatively affecting plant growth and seed production. To survive, plants have evolved high plasticity and complex mechanisms to respond to these stimuli over a long period of time (Hu and Xiong 2014). The understanding of plant responses to stresses in physiology, genetics, and molecular biology will be greatly helpful in improving the tolerance of plants to abiotic stresses through genetic engineering (Huang, et al. 2009).

Metabolic adaption to abiotic stress is important to plant surviving under unfavorable conditions(Barnaby, et al. 2019; Ma, et al. 2016). Generally, nitrogen promotes plant shoot growth rather than root, while carbon does oppositely, and high carbon/nitrogen (C/N) ratio enhances plant root development with a high root/shoot ratio (Osuna, et al. 2015), which allows plant root access to water profoundly and decreases shoot water losses. Accumulation of carbohydrates resulting from enhanced photosynthesis protects plants from membrane damage and accounts, in part, for the more vigorous growth during stress (Garg, et al. 2002). On the contrary, increasing nitrogen levels increased the degree

of water stress, resulting in decreased leaf water potential, especially when the total water applied was minimal (Aragon and De Datta 1982). Moreover, high C/N status may act as a stress condition, which induces a series of stress-related genes, including transcription factors such as *OsMYB4*, *CHS* (key enzyme in flavonoid biosynthesis) and genes involved in the jasmonate signaling pathway (Huang, et al. 2016).

Abscisic acid (ABA) is a multifunctional plant hormone that regulates many physiological processes, including seed dormancy and germination, stomatal movement, and plant responses to abiotic stress. NCED (9-*cis*-epoxycarotenoid dioxygenase) is the key rate-limiting enzyme in ABA biosynthetic pathway. Overexpression of *OsNCED3* in *Arabidopsis* results in increased accumulation of ABA, reduced relative water loss, delayed seed germination, and greater drought tolerance relative to that of wild-type (Hwang, et al. 2010). Rice *nced3* mutants had increased sensitivity to water and H_2O_2 stress, increased stomata aperture, delayed leaf senescence, and decreased ABA content, while overexpression of *OsNCED3* could enhance rice water stress tolerance, promote leaf senescence and increase ABA content (Huang, et al. 2018). ABA 8'-hydroxylase is considered as the main ABA catabolic enzyme. *OsABA8ox3* RNAi lines showed significant improvement in drought stress tolerance with increased ABA content. In contrast, overexpression seedlings were hypersensitive to drought stress with decreased ABA content, indicating *OsABA8ox3* gene plays an important role in controlling ABA level and drought stress resistance in rice (Cai, et al. 2015).

Stress response at the molecular level involves induction of stress-responsive and stress-tolerant genes. Many transcription factors have been identified to be involved in plant adaptation to abiotic stresses (Baillo, et al. 2019). SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) family transcription factors sharing a highly conserved SBP domain are plant-specific, and their functions are surprisingly diverse, covering virtually every aspect of plant growth and development and response to stresses (Wang and Wang 2015). In rice genome, about 19 *SPL* genes have been identified. Among these, as a multifunctional gene in regulating plant development, *IPA1*/ *OsSPL14* has attracted extensive attention. It was reported firstly to function in rice "Ideal Plant Architecture (IPA)" characterized by fewer unproductive tillers, larger panicles and stronger culms (Jiao, et al. 2010; Miura, et al. 2010). Since then, *IPA1* was also identified to play a vital role in rice biotic resistance (Liu, et al. 2019; Wang, et al. 2018). Overexpressing of *IPA1* could enhance rice resistance to *Xanthomonas oryzae pv. oryzae*, partially through gibberellin signaling, including interacting with SLR1 and enhancing GA metabolism by activating *EUI1* expression (Liu, et al. 2019).

Although great progress has been made in understanding the roles of *IPA1* in rice plant development and biotic resistance, its function in rice abiotic stresse tolerance is still unknown. Here, by using a pair of the *IPA1*/ *ipa1*-NILs (Near Iso-genic Lines), we found that *ipa1* could significantly improve rice drought tolerance at the seedling stage mainly through activating ABA pathway.

Materials And Methods

Plant materials

A pair of the *ipa1/IPA1*-NILs was developed from a cross of an *indica* cv. Yuetai B (*IPA1*/*IPA1*) and a *japonica* cv. Shaoniejing (SNJ) (*ipa1/ipa1*). Yuetai B was crossed with SNJ to develop F_1 plants, then the F_1 plants were backcrossed to Yuetai B to develop BC_1F_1 plants. The BC_1F_1 plants were self-crossed for six generations to develop F_7 plants, from which a plant with a genotype *IPA1*/*ipa1* was identified. Let this plant self-cross and, from its offspring, the plants with genotype *IPA1*/*IPA1* were identified as a *IPA1*-NIL, while the plants with genotype *ipa1*/*ipa1* as a *ipa1*-NIL. The degree of genomic similarity for the NILs is 95.8%. The *ipa1* plants showed fewer tillers and leaves, but more panicle branches (Supplementary table 1), as reported previously (Jiao, et al. 2010).

Hydroponic culture conditions

Seeds were disinfected in 20% sodium hypochlorite solution for 30 min, thoroughly washed with deionized water. Sterilized seeds were germinated in distilled water for 48–72 h at 30°C in darkness and then transferred to hydroponic culture solution as described previously (Wang, et al. 2018). Fresh solution was changed every 3 days, and pH was adjusted to 5.5 every day.

Drought stress and osmotic stress experiments

In soil drought experiments, two-leaf stage seedlings cultured in sandy soil were treated with dehydration by removing water with PVC pipes for 7 days and then re-watered for 5 days. For PEG treatment, two-leaf stage seedlings were transferred to culture solution containing 25% (w/v) PEG4000 for 5 days, and then recovered for 4 days.

Imaging of rice leaf stomata

Imaging of rice leaf stomata was conducted as described previously (Wang, et al. 2016) with some modifications. Leaves of 15 days seedlings under control and 6 h 25% PEG treatment conditions were immediately fixed by 2.5% glutaraldehyde, and stomatal pictures were obtained by scanning electron microscopy (JSM-6390LV, JEOL, Tokyo, Japan).

Carbon-nitrogen metabolism-associated indexes measurement

After cultured for 20, 28, 36, 44 and 52 days in nutrient solution under natural conditions, the leaves of *IPA1/ipa1* seedlings were collected for carbon-nitrogen metabolism-associated indexes measurement. Soluble sugar and sucrose contents were determined using the anthrone method (Shields and Burnett 1960) and the resorcinol method (Han, et al. 2015), respectively. FBP (Fructose 1,6-bisphosphatase) activity was determined according to kit instructions (Solarbio, Beijing, China). PEPC (Phosphopyruvate carboxylase) activity was measured by the previously method (BLANKE and EBERT 1992). SPS (Sucrose Phosphate Synthase) and SS (Sucrose Synthase) activities were measured as described previously (Shi, et al. 2016).

Soluble protein and free amino acid contents were determined using the Bradford assay (Bradford 1976) and the ninhydrin method (Sun, et al. 2006), respectively. Nitrate content was measured as described previously (Doane and Horwáth 2003). NR (Nitrate reductase), GS (Glutamine synthase), GOGAT (Glutamate synthase), and GDH (Glutamate dehydrogenase) activities were estimated based on the previously methods (Li, et al. 2016).

Metabolomics analysis

The shoot bases of *IPA1/ipa1* seedlings grown for 21 days in nutrient solution under natural conditions were collected for metabolomics analysis. The sample preparation, extract analysis, metabolite identification and quantification were performed as described previously (Chen, et al. 2014) at Wuhan Metware Biotechnology Co., Ltd., Wuhan, China.

Phytohormone measurement

Leaves of 15 days seedlings under control and 6 h 25% PEG treatment conditions were collected for phytohormone measurement. Plant materials were ground into powder in liquid nitrogen, and extracted with 80% methanol at 4°C. The extract was centrifuged at 12,000 *g* under 4°C for 15 min. The supernatant was collected and evaporated to dryness under nitrogen gas stream, and then reconstituted in 30% methanol. The solution was centrifuged, and the supernatant was collected for LC-MS analysis. The LC-MS analysis was conducted with the API6500 QTRAP LC/MS/MS system, equipped with an ESI Turbo Ion-Spray interface, operating in a positive ion mode and controlled by Analyst 1.6 software (AB Sciex).

RNA extraction and real-time PCR

After treated with 25% PEG for 0, 3, 6 and 9 h, leaves of 15 days seedlings were collected for RNA extraction. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) reagent. According to the manufacturer, RNA sample (~ 2μg) was treated with DNaseI and then used for cDNA synthesis with the Super-Script III first-strand cDNA synthesis system (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using 2×SYBR Green PCR Master Mix (Takara, Dalian, China) in a CFX96TM Real-Time System (BIO-RAD, Hercules, CA, USA). Each experiment included three technical replicates and three biological replicates. *OsActin* was used as an internal control for normalization. Primers used are listed in Supplementary Table 2.

Dual-luciferase assays

The promoters of *OsHOX12* and *OsNAC52* were amplified by PCR from genomic DNA and cloned into pGreenII 0800-LUC reporter vectors in front of luciferase (LUC) gene. Besides, the Renilla luciferase (REN) reporter gene was driven by the CaMV 35S promoter as a control in each transformation. The coding region of *IPA1* was cloned behind the Ubi promoter into the effector vector pRGV (He, et al. 2018). The reporter and effector were transformed into in rice protoplasts. The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Beijing, China) and compared with empty vector-transformed plants.

Yeast one-hybrid assay

The coding region of $IPA1_{SBP}$ was amplified and cloned into the pGADT7 prey vector. The promoters of *OsHOX12* and *OsNAC52* were amplified and cloned into pAbAi bait vectors. The prey vectors, respectively, co-transformed with bait vector into Y1HGOLD strain. These transformed cells were grown on SD/-Leu/-Ura plates and then grown on SD/-Leu/-Ura/ AbAi plates at 28°C for 3–5 days.

Results

ipa1 enhances rice drought tolerance at seedling stage

To investigate the effect of *ipa1* on rice drought tolerance, a soil drought experiment was performed with a pair of the *IPA1*/*ipa1*-NILs. The *ipa1*-NIL obtained a survival rate of 83.5%, while it was only 20.8% for the *IPA1*-NIL (Fig. 1a, c). As treated with 25% PEG4000 (osmotic stress simulating drought stress), the *ipa1* seedlings exhibited a survival rate of 62.1%, while the *IPA1*-NIL showed a significantly lower survival rate of 43.5% (Fig. 1b, d). These results indicated that *ipa1* could significantly improve rice drought tolerance at seedling stage.

The characteristics of root and leaf stomata for the IPA1/ipa1 seedlings

As compared to that of the *IPA1* seedlings, the *ipa1* seedlings had significantly increased root length, root and shoot dry weight as well as the dry weight ratio of root to shoot (Fig. 2a-e).

As for leaf stomata, the *ipa1* seedlings showed a decreased stoma size as compared with that of the *IPA1* seedlings (Fig. 2f, g), despite no significant difference in stoma density between the NILs (Supplementary Fig. 1). Moreover, the *ipa1* plants displayed more stomata completely close (29.3%) and less stomata completely open (18.5%) than that of the *IPA1*-NIL (14.0% and 34.7%, respectively). When treated with PEG, more leaf stomata tended to close for both the *IPA1* and *ipa1* seedlings. Even so, there were still more stomata completely close (56.6%) and less stomata completely open (nearly 0.0%) for the *ipa1* seedlings than that for the *IPA1* seedlings (45.9% and 8.3%, respectively) (Fig. 2h). Consequently, the *ipa1* seedlings were found to lose less water (Fig. 2i).

The *ipa1* plants had a better developed root system conducive to enhancing their ability to absorb water from the soil and their leaves with smaller stomatal aperture were beneficial to enhance their moisturizing function, which could play a vital physiological role in improving their drought tolerance.

The ipa1 seedling could adjust its carbon-nitrogen metabolism balance to a metabolic pattern with a relatively strong carbon metabolism

It was found that the *ipa1* plants had a higher content for soluble sugar and sucrose than the *IPA1* plants in both leaves (Fig. 3a, b) and sheaths (Supplementary Fig. 2). Then, we measured the activity of several

carbon metabolism-related enzymes. Except for FBP, three carbon metabolism-related enzymes (PEPC, SPS, and SS) showed a activity level higher in the *ipa1* plants than in the *IPA1* plants (Fig. 3c-f).

As for the nitrogen metabolism, soluble protein and free amino acid contents for the *ipa1* seedlings were significantly lower than that for the *IPA1* plants (Fig. 4a, b). Meanwhile, the *ipa1* plants were found to be significantly higher in inorganic nitrate-nitrogen content than the *IPA1* plants (Fig. 4c). Four key nitrogen assimilation enzymes (NR, GS, GOGAT and GDH) were investigated and each of them showed a significantly lower activity level in the *ipa1* plants than in the *IPA1* plants (Fig. 4d-g). Accordingly, the expression of genes involved in nitrogen absorption, transport and assimilation was detected to be down-regulated, especially in the roots of the *ipa1* plants (Supplementary Fig. 3).

Combined with the above findings, it seems that the *ipa1* plant could change its carbon-nitrogen metabolism balance to a metabolic pattern with a relatively stronger carbon metabolism by enhancing its carbon metabolic activity and down-regulating its nitrogen metabolism, thus benefiting the accumulation of carbohydrates in the plant, which could provide a stronger material and energy basis for the plants to tolerate external abiotic stress.

Metabolic profile analysis of the IPA1/ipa1 plants

To further explore the effect of *ipa1* on plant metabolism, we analyzed the metabolic profiles with the *IPA1*/*ipa1*-NILs by using a liquid chromatography-electrospray ionization-tandem mass spectrometry. There were 357 compounds to be identified. These compounds covered the key components involved in metabolic pathways of sugars, amino acids, nucleotide, organic acids, fatty acids and others (Supplementary Table 3).

The majority of carbohydrates such as sucrose, trehalose 6-phosphate (T6P), glucosamine and glucarate o-phosphoric acid were detected to accumulate more in the *ipa1* plants than in the *IPA1* plants, except glucose (Fig. 5a). For the key metabolites involved in nitrogen assimilation, the *ipa1* plants showed a significantly decrease in the contents of amino acids Tyr and Trp (Fig. 5b) while their precursor shikimic acid mainly accumulated in the *ipa1* plants (Fig. 5c). Similarly, the levels of organic acids (2-OG, succinic acid, and malic acid) and amino acids (Gln, Glu, Asn and Asp, as the major forms of nitrogen in xylem sap of rice plant) were significantly decreased in the *ipa1* plants (Fig. 5b, c) while their precursor aconitic acid (a major element in TCA cycle) also showed to accumulate significantly in the *ipa1* plants (Fig. 5c). These results indicated that the carbon flux to nitrogenous compounds was depressed in the *ipa1* plants as compared to the *IPA1* plants. Besides that, most of the other identified amino acids, amino acid derivates and nucleotides were decreased to different degrees in the *ipa1* plants (Supplementary Fig. 4a, b). Therefore, all these results suggested that the gene *ipa1* could significantly influence the balance of carbon-nitrogen metabolism, tipping the carbon/nitrogen metabolism balance towards increased carbon metabolism.

Cysteine is the first carbon/nitrogen-reduced sulfur product resulting from the sulfate assimilation pathway. As a sulfur donor, it plays a major role in the growth and development of plant. Glutathione

derived from cysteine protects plants from reactive oxygen species (ROS) damage caused by abiotic stress (Droux 2004). In this study, a significantly increased cysteine content was found in the *ipa1* plants, coupled with an increase of reduced glutathione content and a decrease of oxidized glutathione content (Fig. 5d). Moreover, contents of the other antioxidants such as coumarin and curcumin raised dramatically in the *ipa1* plants (Supplementary Fig. 4c). The same situation also happened to glycerophospholipids (Supplementary Fig. 4d), the cell membrane major components. Therefore, *ipa1* may activate sulfate assimilation and the related defense mechanism, which plays an essential role in protecting the *ipa1* plants from ROS damage under abiotic stresses. In addition, ferulic acid is reported to be a marker metabolite for plant drought resistance and high photosynthesis (Ma, et al. 2016). The contents of ferulic acid-related metabolites were significantly upregulated in the *ipa1* seedlings (Supplementary Fig. 4e).

The enhanced drought tolerance in the ipa1 plants could be mediated by ABA accumulation

ABA and GAs are known to be primary phytohormones that antagonistically regulate plant abiotic stress resistance (Vishal and Kumar 2018). In this study, exogenous ABA application led to an obvious inhibition on plant height, whereas GA_3 treatment significantly promoted the trait for both the NILs under non-drought stress conditions (Supplementary Fig. 5). When PEG was applied to simulate drought conditions (osmotic stress), the ABA application significantly improved survival rates of seedlings, whereas GA_3 decreased survival rates for both the NILs (Fig. 6a, b). Although so, the two NILs showed significant differences in degree of response to ABA and GA_3 treatments. Comparatively, the *ipa1* seedlings were less sensitive to ABA or GA_3 treatment. Exogenous ABA treatment improved PEG resistance of the *IPA1* plants to a greater extent, resulting in a fact that survival rate of the *IPA1* plants was no longer different from that for the *ipa1* plants (Fig. 6a, b).

Then, we measured ABA and GAs contents of the NILs. In both control and PEG-treated conditions, ABA content in the *ipa1* seedlings was significantly higher than that in the *IPA1* seedlings (Fig. 6c). Meanwhile, the ABA biosynthesis genes such as *OsNCED1*, *OsNCED3*, and *OsNCED4* were detected to be up-regulated, whereas ABA catabolism genes were down-regulated in *ipa1*-NIL (Fig. 6e). *OsABI5*, *OsLEA3*, *OsLIP9*, and *OsRAB16A* are marker genes of the ABA pathway involved in abiotic stress response (Zhang, et al. 2015). In our study, all these marker genes were up-regulated significantly in the *ipa1* plants under the control and PEG-treatment conditions (Fig. 6f).

As for GAs, with an exception of a remarkable increase of the GA_4 content in *ipa1*-NIL under the control condition, no significant difference has been observed in the contents of GAs investigated between the two NILs under the control or PEG-treated conditions (Fig. 6d), although several genes for GA biosynthesis and catabolism showed some differences in expression levels between the two NILs (Supplementary Fig. 6).

The above results suggested that the enhanced drought tolerance of the *ipa1*-NIL could mainly result from a high level of ABA accumulation in the *ipa1* seedlings.

IPA1 directly activated the expression of OsHOX12 **and** OsNAC52

OsHOX12 is a transcription factor homologous with AtHB21, 40 and 53. It was reported to activate expression of *OsNCED1*, and promote ABA biosynthesis in rice (Liu, et al. 2020). OsNAC52, a transcription factor belonging to NAC family, potentially responds to ABA and confers drought tolerance in transgenic plants (Gao, et al. 2010). In addition, as a transcription activator, IPA1 can regulate its target gene by directly binding to the core motif GTAC or indirectly to the core motif TGGGCC/T of the target gene promoter (Lu, et al. 2013). Bioinformatics analysis identified twelve and three GTAC motifs in the promoters of *OsHOX12* and *OsNAC52*, respectively (Fig. 7a, b). We searched previously published ChIP-seq data of IPA1 (Lu, et al. 2013), and found that *OsHOX12* and *OsNAC52* were potential targets of IPA1, suggesting that IPA1 may directly activate the expression of *OsHOX12* and *OsNAC52*.

To test the hypothesis, we conducted a yeast one-hybrid assay. Cells co-transformed with bait vectors and prey vectors grew well on SD/-Leu/-Ura/AbAi plates, indicating that IPA1 can directly bind to the promoters of *OsHOX12* and *OsNAC52* (Fig. 7c, d). Then, we carried out a dual-luciferase assay using the full length of the *OsHOX12* and *OsNAC52* promoters in rice protoplasts. Co-transformed reporter vectors and effector vectors activated the expression of *LUC* gene, suggesting that IPA1 can significantly enhance the activity of the *OsHOX12* and *OsNAC52* promoters (Fig. 7e, f). Accordingly, the expression of *OsHOX12* was increased in the *ipa1* seedlings under PEG-treated conditions (Fig. 7h). Moreover, the expression of *OsNAC52* was also significantly up-regulated in the *ipa1* plants under both the control and PEG-treated conditions (Fig. 7i).

We also tested expression of the other genes involved in abiotic stresses in the NIL plants. *OsNAC5*, *OsNAC6*, and *OsNAC19* are three other NAC family transcription factors, and overexpression of each of those was reported to enhance rice resistance to abiotic stresses (Hu, et al. 2006; Takasaki, et al. 2010). The results depicted that, these genes' expression showed a significantly higher level in the *ipa1* plants than in the *IPA1* plants under control and PEG-treated conditions (Fig. 7j-l).

Discussion

IPA1/*OsSPL14* is one of the most concerned genes in current studies of rice functional genomics due to its multifunctions in regulating plant development (Wang, et al. 2018). In this study, we found that *ipa1* could significantly improve rice drought tolerance at seedling stage. The *ipa1* seedlings demonstrated a better-developed root system in terms of phenotypes, which helped to enhance their ability to absorb water from the soil. Their leaves with smaller stomatal aperture improved their moisturizing ability, which could play a crucial physiological role in enhancing their resistance to drought tolerance.

ABA is induced in response to adverse environmental conditions, and it plays a critical role in regulating abiotic stress response in plants (Cutler, et al. 2010). Deficit of ABA in *nced3* mutants increased rice sensitivity to water stress, while accumulation of ABA in *OsNCED3*-overexpressing seedings enhanced rice water stress tolerance (Huang, et al. 2018). In this study, the *ipa1* seedlings had a significantly higher ABA content than the *IPA1* plants (Fig. 6c). The ABA pathway marker genes were up-regulated by *ipa1*

(Fig. 6e). Exogenous ABA treatment largely promoted PEG resistance for the *IPA1* plants (Fig. 6a, b). These results seem to suggest that the improved drough tolerance might result from activation of the ABA pathway in the *ipa1* seedlings.

OsHOX12 and OsNAC52 are two of the transcription factors involved in ABA pathway in rice (Gao, et al. 2010; Liu, et al. 2020). Our yeast one-hybrid assay and dual-luciferase test indicated that IPA1 can directly bind to the promoters of *OsHOX12* and *OsNAC52* (Fig. 7c, d) and significantly enhance the activity of the *OsHOX12* and *OsNAC52* promoters. Therefore, IPA1 may directly activate *OsHOX12*, thus promoting ABA biosynthesis. Meanwhile, our study indicated that *ipa1* could enhance the ABA pathway by directly regulating *OsNAC52*, which was reported to be a positive regulator of the ABA pathway (Gao, et al. 2010). Additionally, Gonzalez-Grandio et al. (2017) reported that the TCP (TEOSINTE BRANCHED1, CYCLOIDEA, PCF) transcription factor BRANCHED1 (BRC1) in Arabidopsis binds to and positively regulates the transcription of three related Homeodomain leucine zipper protein (HD-ZIP) encoding genes HOMEOBOX PROTEIN 21 (HB21), HOMEOBOX PROTEIN 40 (HB40), and HOMEOBOX PROTEIN 53 (HB53), which together with BRC1, enhances *9-CIS-EPOXICAROTENOID DIOXIGENASE 3* (*NCED3*) expression, leading to ABA accumulation and triggering hormone response. In rice, Lu et al. (2013) revealed that IPA1 directly targets *OsTB1* (an ortholog of *BRC1*). Here, we showed that *OsTB1* was up-regulated significantly in the *ipa1* seedlings (Supplementary Fig. 7). Therefore, we speculate that, as in Arabidopsis, IPA1 could also target *OsTB1* to enhance the expression of the ABA biosynthesis genes, thus leading to ABA accumulation in *ipa1* seedlings.

ABA is also a key regulator of plant stomatal aperture and root development. In response to drought stress, plants can synthesize ABA, which triggers closing of stomatal pores, thus reducing water loss (Schroeder, et al. 2001). The foliage-derived ABA promoted root growth relative to shoot growth but inhibited the development of lateral roots (McAdam, et al. 2016). Moreover, a very recent paper showed that moderate enhancement of ABA signaling helps maintain the RM (root meristem) size, sustaining root growth by antagonizing the GA-promoted degradation of OsSHR1 through the SnRK2-APC/CTE regulatory module, while mutants of *OsABA1* (a ABA biosynthesis gene) displayed a short root phenotype (Lin, et al. 2020). Therefore, the smaller stomatal and better-developed root system in *ipa1* seedlings could result from the accumulation of ABA.

The ABA pathway also plays a vital role in regulating plant metabolism. Treatment with low level ABA increases rice soluble sugar and decreases free amino acid contents (Zeng, et al. 2009). SnRK2s are key positive regulators of ABA signaling. Overexpression of *SnRK2.6* promotes plant carbon assimilation with drastically boosted sucrose and total soluble sugar levels in the leaves through increasing SPS activity (Zheng, et al. 2010). Moreover, *srk2d srk2e srk2i* (*snrk2.2/2.3/2.6*) showed enhanced TCA cycle (key source of C-skeletons for amino acid synthesis), leading to a higher carbon flux into amino acids and potentially proteins under non-stress conditions (Yoshida, et al. 2019). Our results indicated that *ipa1* plants could adjust the balance of carbon-nitrogen metabolism by enhancing their carbon metabolic activity, but relatively down-regulating their nitrogen metabolism (Fig. 3, 4). Metabolic profile analysis further supported such a change of carbon-nitrogen metabolism in *ipa1* plants (Fig. 5a, b). Thus, for the

ipa1 seedlings, tipping the carbon/nitrogen balance towards increased carbon metabolism might also result from the enhancement of ABA signaling, which contributes to their enhanced drought tolerance. Further detailed analyses are required to understand the interaction of ABA pathway and C/N metabolism balance in the *ipa1* plants.

It should be noted that, the results of the current study revealed the effect of the gene *ipa1* on drought tolerance of rice plants only at seedling stage. At this stage, *OsTB1* is the most important target of IPA1, and this target gene is mainly expressed at seedling stage (Lu, et al. 2013). As rice plants develop into panicle differentiation stage, the main targets of IPA1 turn into genes such as *OsDEP1* (Lu, et al. 2013). Accordingly, rice plants' hormone regulation and metabolic pattern could be changed remarkably, which is worthy of further study in the future. Additionally, the gene *ipa1* is one of the most essential yield-increasing genes reported in rice so far (Wang, et al. 2018; Yu, et al. 2020). It increases rice yield mainly by shaping ideal plant type with fewer tillers and larger panicles (Jiao, et al. 2010). In this study, the *ipa1* seedlings were observed to have a larger dry weight per plant (Fig. 2c, d), although their nitrogen metabolism was down-regulated relative to its WT seedlings. Obviously, the *ipa1* rice plants with larger biomass at the seedling stage would be more likely to develop larger panicles later, thus contributing to increased yields.

In conclusion, this study elucidated that *ipa1* could significantly improve rice drought tolerance at seedling stage. The *ipa1* plants had a better-developed root system and smaller leaf stomatal aperture. They could tip the carbon-nitrogen metabolism balance towards an increased carbon metabolism pattern. Meanwhile, the ABA biosynthesis genes were up-regulated, whereas the ABA catabolism genes were down-regulated in the *ipa1* seedlings, resulting in accumulation of endogenous ABA. Based on yeast one-hybrid assay and dual-luciferase assay, IPA1 was found to directly activate the expression of *OsHOX12* and *OsNAC52*, a transcription factor promoting ABA biosynthesis and a positive regulator of the ABA pathway, respectively. These results suggested that *ipa1* could improve rice seedling drought tolerance mainly through activating the ABA pathway, and that it may has potential applications in improving drought resistance of rice.

Declarations

Acknowledgements

The authors thank Dr. Q.Qian of the China National Rice Research Institute for providing the *ipa1* donor rice cv. Shaoniejing used in this particular research. This work was financed by the Special Transgenic Program of the Ministry of Agriculture in China (No. 2016ZX08001004-002), the National Natural Science Foundation of China (No.31901522), and the Collaborative Innovation Center of Hubei Province for Hybrid Rice.

Author contribution statement MZ and ZZ designed the experiments. MZ performed most experiments. MZ, YH and ZZ analyzed the data. MZ, AA, SX, ZH, SJ, JH, ZL and SL assisted in materials and data collection. MZ, XH and ZZ drafted the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no confict of interest.

References

1. Aragon EL, De Datta SK (1982) Drought response of rice at different nitrogen levels using line source sprinkler system. Irrig Sci 3:63–73
2. Baillo EH, Kimotho RN, Zhang Z, Xu P (2019) Transcription factors associated with abiotic and biotic stress tolerance and their potential for crops improvement. Genes (Basel) 10:771–793
3. Barnaby JY, Rohila JS, Henry CG, Sicher RC, Reddy VR, McClung AM (2019) Physiological and metabolic responses of rice to reduced soil moisture: relationship of water stress tolerance and grain production. Int J Mol Sci 20:1846
4. BLANKE MM, EBERT G (1992) Phosphoenolpyruvate carboxylase and carbon economy of apple seedlings. J Exp Bot 43:965–968
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
6. Cai S, Jiang G, Ye N, Chu Z, Xu X, Zhang J, Zhu G (2015) A key ABA catabolic gene, *OsABA8ox3*, is involved in drought stress resistance in rice. PLoS One 10:e0116646
7. Chen W, Gao Y, Xie W, Gong L, Lu K, Wang W, Li Y, Liu X, Zhang H, Dong H, Zhang W, Zhang L, Yu S, Wang G, Lian X, Luo J (2014) Genome-wide association analyses provide genetic and biochemical insights into natural variation in rice metabolism. Nat Genet 46:714–721
8. Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol 61:651–679
9. Doane TA, Horwáth WR (2003) Spectrophotometric determination of nitrate with a single reagent. Anal Lett 36:2713–2722
10. Droux M (2004) Sulfur assimilation and the role of sulfur in plant metabolism: a survey. Photosynth Res 79:331–348
11. Gao F, Xiong A, Peng R, Jin X, Xu J, Zhu B, Chen J, Yao Q (2010) *OsNAC52*, a rice NAC transcription factor, potentially responds to ABA and confers drought tolerance in transgenic plants. Plant Cell Tiss Organ Cult 100:255–262
12. Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 99:15898–15903
13. Gonzalez-Grandio E, Pajoro A, Franco-Zorrilla JM, Tarancon C, Immink RG, Cubas P (2017) Abscisic acid signaling is controlled by a *BRANCHED1/HD-ZIP I* cascade in Arabidopsis axillary buds. Proc Natl Acad Sci USA 114(2):E245–E254

14. Han H, Tian Z, Fan Y, Cui Y, Cai J, Jiang D, Cao W, Dai T (2015) Water-deficit treatment followed by re-watering stimulates seminal root growth associated with hormone balance and photosynthesis in wheat (*Triticum aestivum L.*) seedlings. Plant Growth Regul 77:201–210
15. He F, Zhang F, Sun W, Ning Y, Wang GL (2018) A versatile vector toolkit for functional analysis of rice genes. Rice (N Y) 11:27
16. Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 103:12987–12992
17. Hu H, Xiong L (2014) Genetic engineering and breeding of drought-resistant crops. Annu Rev Plant Biol 65:715–741
18. Huang A, Sang Y, Sun W, Fu Y, Yang Z (2016) Transcriptomic analysis of responses to imbalanced carbon: nitrogen availabilities in rice seedlings. PLoS One 11:e0165732
19. Huang XY, Chao DY, Gao JP, Zhu MZ, Shi M, Lin HX (2009) A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. Genes Dev 23:1805–1817
20. Huang Y, Guo Y, Liu Y, Zhang F, Wang Z, Wang H, Wang F, Li D, Mao D, Luan S, Liang M, Chen L (2018) 9-*cis*-Epoxycarotenoid Dioxygenase 3 regulates plant growth and enhances multi-abiotic stress tolerance in rice. Front Plant Sci 9:162
21. Hwang SG, Chen HC, Huang WY, Chu YC, Shii CT, Cheng WH (2010) Ectopic expression of rice *OsNCED3* in Arabidopsis increases ABA level and alters leaf morphology. Plant Sci 178:12–22
22. Jiao Y, Wang Y, Xue D, Wang J, Yan M, Liu G, Dong G, Zeng D, Lu Z, Zhu X, Qian Q, Li J (2010) Regulation of *OsSPL14* by OsmiR156 defines ideal plant architecture in rice. Nat Genet 42:541–544
23. Li H, Liang Z, Ding G, Shi L, Xu F, Cai H (2016) A natural light/dark cycle regulation of carbon-nitrogen metabolism and gene expression in rice shoots. Front Plant Sci 7:1318
24. Lin Q, Zhang Z, Wu F, Feng M, Sun Y, Chen W, Cheng Z, Zhang X, Ren Y, Lei C, Zhu S, Wang J, Zhao Z, Guo X, Wang H, Wan J (2020) The APC/C(TE) E3 ubiquitin ligase complex mediates the antagonistic regulation of root growth and tillering by ABA and GA. Plant Cell 32:1973–1987
25. Liu M, Shi Z, Zhang X, Wang M, Zhang L, Zheng K, Liu J, Hu X, Di C, Qian Q, He Z, Yang DL (2019) Inducible overexpression of *Ideal Plant Architecture1* improves both yield and disease resistance in rice. Nat Plants 5:389–400
26. Liu X, Hu Q, Yan J, Sun K, Liang Y, Jia M, Meng X, Fang S, Wang Y, Jing Y, Liu G, Wu D, Chu C, Smith SM, Chu J, Wang Y, Li J, Wang B (2020) ζ-carotene isomerase suppresses tillering in rice through the coordinated biosynthesis of strigolactone and abscisic acid. Mol Plant 13:1784–1801
27. Lu Z, Yu H, Xiong G, Wang J, Jiao Y, Liu G, Jing Y, Meng X, Hu X, Qian Q, Fu X, Wang Y, Li J (2013) Genome-wide binding analysis of the transcription activator IDEAL PLANT ARCHITECTURE1 reveals a complex network regulating rice plant architecture. Plant Cell 25:3743–3759
28. Ma X, Xia H, Liu Y, Wei H, Zheng X, Song C, Chen L, Liu H, Luo L (2016) Transcriptomic and metabolomic studies disclose key metabolism pathways contributing to well-maintained

photosynthesis under the drought and the consequent drought-tolerance in rice. Front Plant Sci 7:1886

29. McAdam SA, Brodribb TJ, Ross JJ (2016) Shoot-derived abscisic acid promotes root growth. Plant Cell Environ 39:652–659
30. Miura K, Ikeda M, Matsubara A, Song XJ, Ito M, Asano K, Matsuoka M, Kitano H, Ashikari M (2010) *OsSPL14* promotes panicle branching and higher grain productivity in rice. Nat Genet 42:545–549
31. Osuna D, Prieto P, Aguilar M (2015) Control of seed germination and plant development by carbon and nitrogen availability. Front Plant Sci 6:1023
32. Schroeder JI, Kwak JM, Allen GJ (2001) Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature 410:327–330
33. Shi H, Wang B, Yang P, Li Y, Miao F (2016) Differences in sugar accumulation and mobilization between sequential and non-sequential senescence wheat cultivars under natural and drought conditions. PLoS One 11:e0166155
34. Shields R, Burnett W (1960) Determination of protein-bound carbohydrate in serum by modified anthrone method. Anal Chem 32:885–886
35. Sun S-W, Lin Y-C, Weng Y-M, Chen M-J (2006) Efficiency improvements on ninhydrin method for amino acid quantification. J Food Compos Anal 19:112–117
36. Takasaki H, Maruyama K, Kidokoro S, Ito Y, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K, Nakashima K (2010) The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. Mol Genet Genomics 284:173–183
37. Vishal B, Kumar PP (2018) Regulation of seed germination and abiotic stresses by gibberellins and abscisic acid. Front Plant Sci 9:838
38. Wang B, Smith SM, Li J (2018) Genetic regulation of shoot architecture. Annu Rev Plant Biol 69:437–468
39. Wang H, Wang H (2015) The miR156/SPL module, a regulatory hub and versatile toolbox, gears up crops for enhanced agronomic traits. Mol Plant 8:677–688
40. Wang J, Zhou L, Shi H, Chern M, Yu H, Yi H, He M, Yin J, Zhu X, Li Y, Li W, Liu J, Wang J, Chen X, Qing H, Wang Y, Liu G, Wang W, Li P, Wu X, Zhu L, Zhou J-M, Ronald PC, Li S, Li J, Chen X (2018) A single transcription factor promotes both yield and immunity in rice. science 361:1026–1028
41. Wang L, Yu C, Xu S, Zhu Y, Huang W (2016) OsDi19-4 acts downstream of OsCDPK14 to positively regulate ABA response in rice. Plant Cell Environ 39:2740–2753
42. Wang Q, Nian J, Xie X, Yu H, Zhang J, Bai J, Dong G, Hu J, Bai B, Chen L, Xie Q, Feng J, Yang X, Peng J, Chen F, Qian Q, Li J, Zuo J (2018) Genetic variations in *ARE1* mediate grain yield by modulating nitrogen utilization in rice. Nat Commun 9:735
43. Yoshida T, Obata T, Feil R, Lunn JE, Fujita Y, Yamaguchi-Shinozaki K, Fernie AR (2019) The role of abscisic acid signaling in maintaining the metabolic balance required for Arabidopsis growth under nonstress conditions. Plant Cell 31:84–105

44. Yu S, Ali J, Zhang C, Li Z, Zhang Q (2020) Genomic breeding of green super rice varieties and their deployment in Asia and Africa. Theor Appl Genet 133:1427–1442
45. Zeng Z, Yi Z, Wang G, Wang Y (2009) Influence of seed soaking with ABA on the physiological and yield-contributing traits of rice seedlings. J Southwest Univ (Nat Sci Ed) 31:52–56
46. Zhang DP, Zhou Y, Yin JF, Yan XJ, Lin S, Xu WF, Baluska F, Wang YP, Xia YJ, Liang GH, Liang JS (2015) Rice G-protein subunits *qPE9-1* and *RGB1* play distinct roles in abscisic acid responses and drought adaptation. J Exp Bot 66:6371–6384
47. Zheng Z, Xu X, Crosley RA, Greenwalt SA, Sun Y, Blakeslee B, Wang L, Ni W, Sopko MS, Yao C, Yau K, Burton S, Zhuang M, McCaskill DG, Gachotte D, Thompson M, Greene TW (2010) The protein kinase SnRK2.6 mediates the regulation of sucrose metabolism and plant growth in Arabidopsis. Plant physiol 153:99–113

Supplementry

Supplementary Table 1 is not available with this version.

Figures

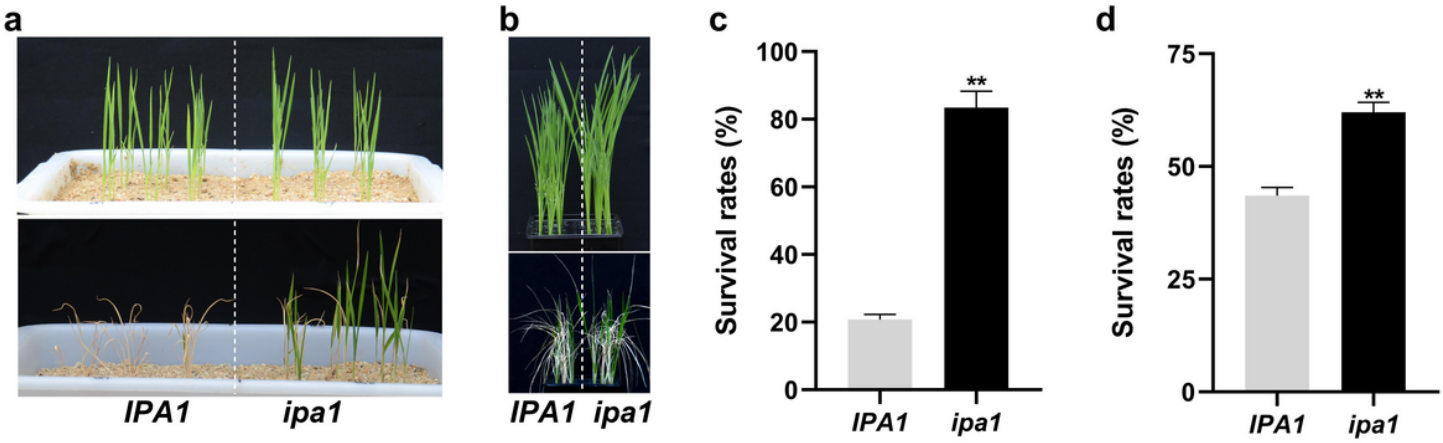

Figure 1

Drought tolerance of the IPA1/ipa1 seedlings. (a, b) Performance of the IPA1/ipa1 seedlings under soil drought and 25% PEG4000 treatments, respectively. (c, d) Survival rates of the IPA1/ipa1 seedlings treated with soil drought and 25% PEG4000, respectively. Each experiment has three replicates, and twenty-four (c) or thirty-six (d) seedlings were tested in each replicate. Data represent the means ± SE. *P<0.05, t-test. **P<0.01, t-test.

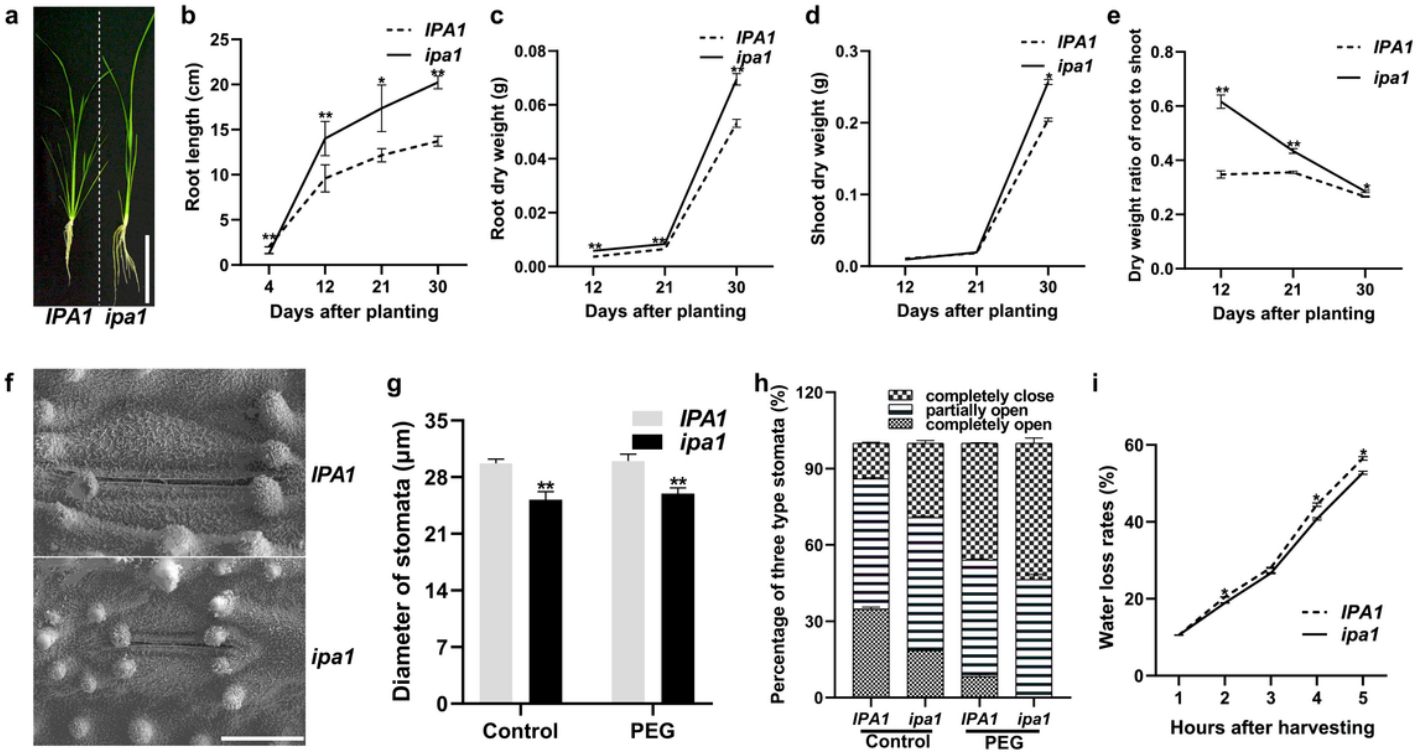

Figure 2

Characteristics of root and leaf stomata for the IPA1/ipa1 seedlings. (a) Plant architecture of the IPA1/ipa1 seedlings twenty-five days after planting. Bar, 10 cm. (b-e) Root length, Root dry weight, Shoot dry weight and Dry weight ratio of root to shoot, respectively. b-e, n=18. (f) Scanning electron microscopy (SEM) images of IPA1/ipa1 seedlings. Bar, 10 μm. (g) The stomatal diameter (n=12, derived from three seedlings). (h) Percentage of three types of stomata. Forty-seven to fifty-two stomata from three seedlings were calculated. (i) The water loss rates, n=15. PEG, 25% PEG4000 treatment condition. Data represent the means ± SE. *P<0.05, t-test. **P<0.01, t-test.

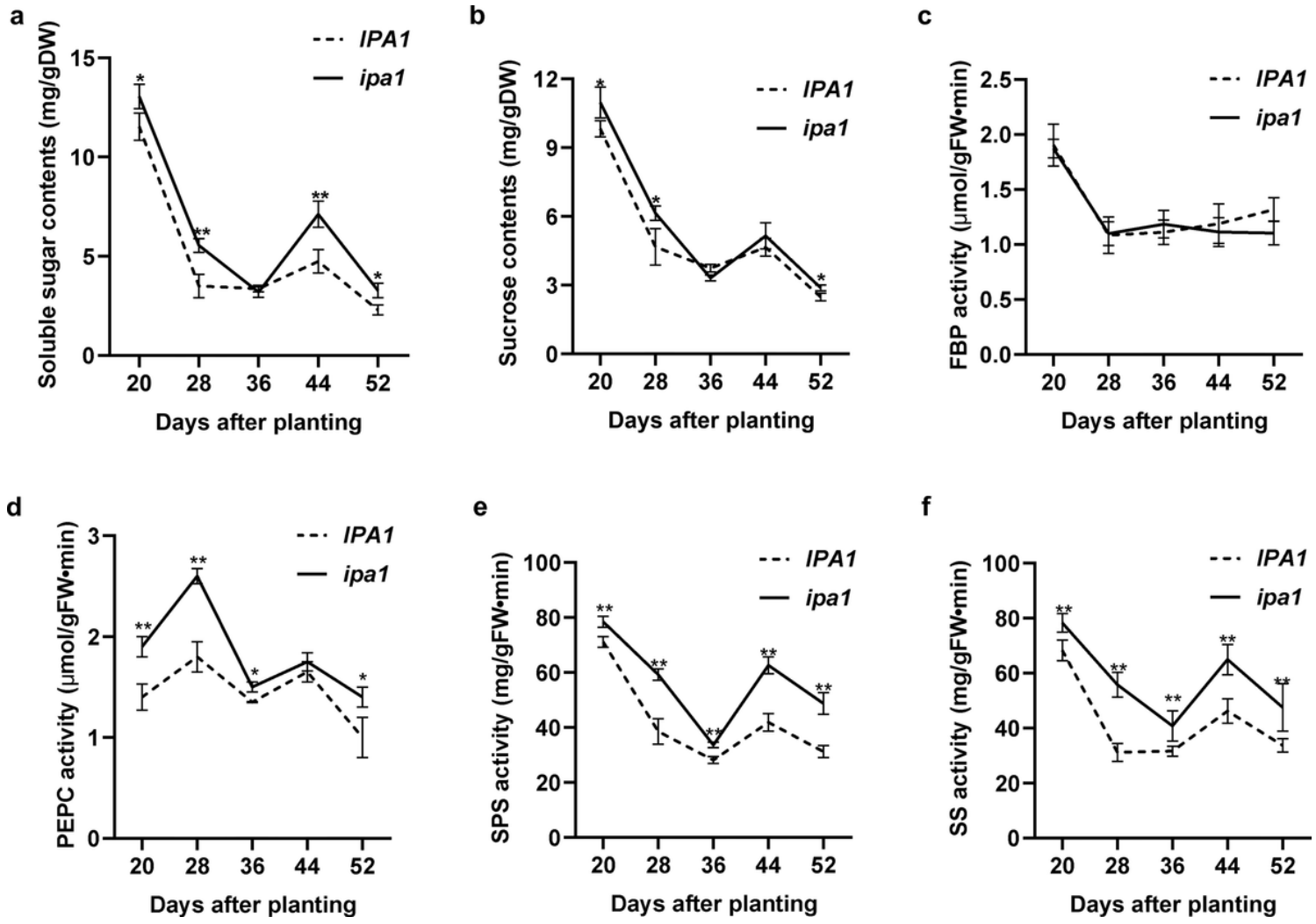

Figure 3

Carbon metabolism-associated indices of the IPA1/ipa1 seedlings. (a, b) Contents of soluble sugar and sucrose, respectively, in the IPA1/ipa1 seedling leaves. (c-f) Activity of FBP, PEPC, SPS and SS, respectively, in the IPA1/ipa1 seedlings. Data represent the means ± SE (n=3). *P<0.05, t-test. **P<0.01, t-test.

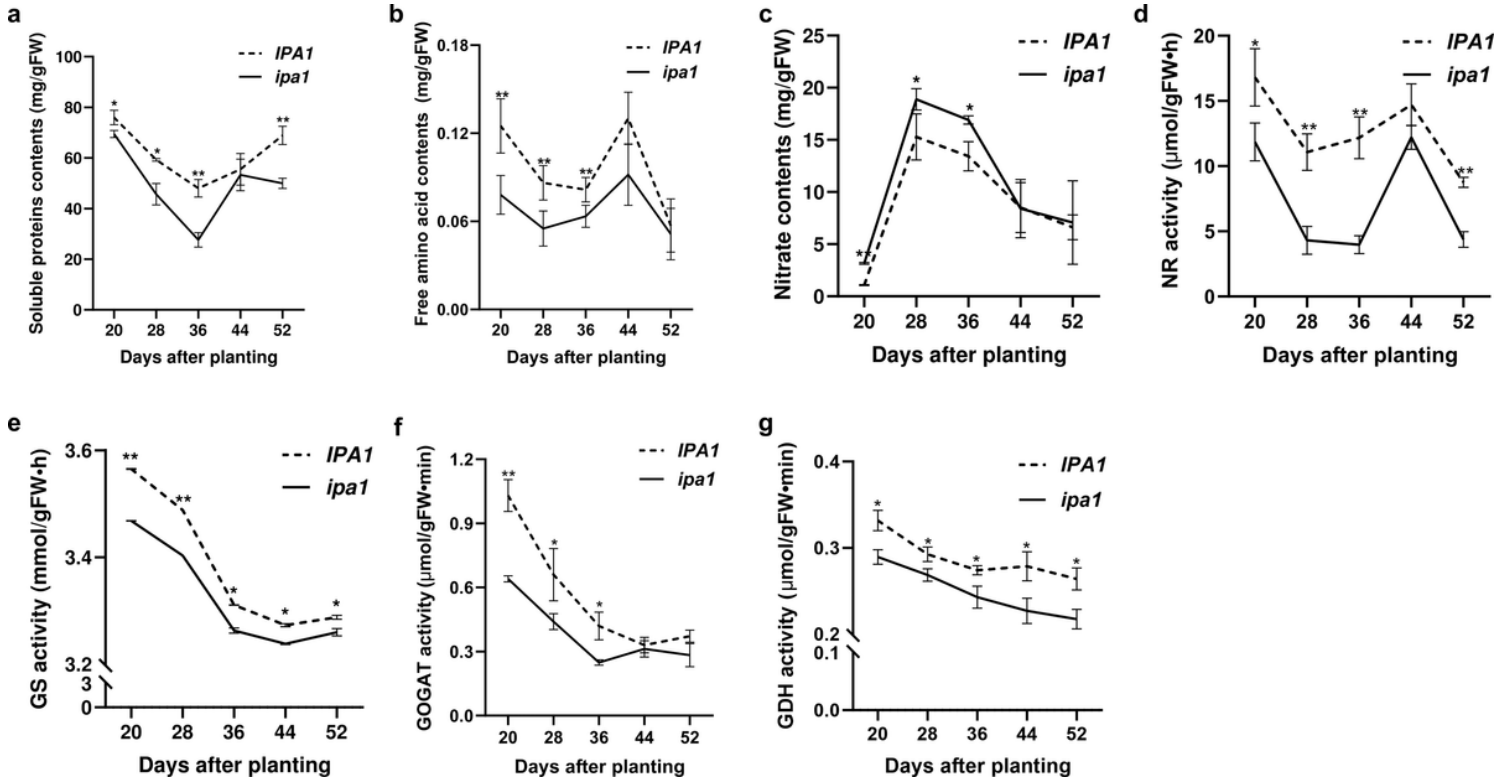

Figure 4

Nitrogen metabolism-associated indices of the IPA1/ipa1 seedlings. (a-c) Contents of soluble proteins, free amino acid and nitrate, respectively, in leaves of the IPA1/ipa1 seedlings. (d-g) Activity of NR, GS, GOGAT and GDH, respectively, in the IPA1/ipa1 seedlings. Data represent the means ± SE (n=3). *P<0.05, t-test. **P<0.01, t-test.

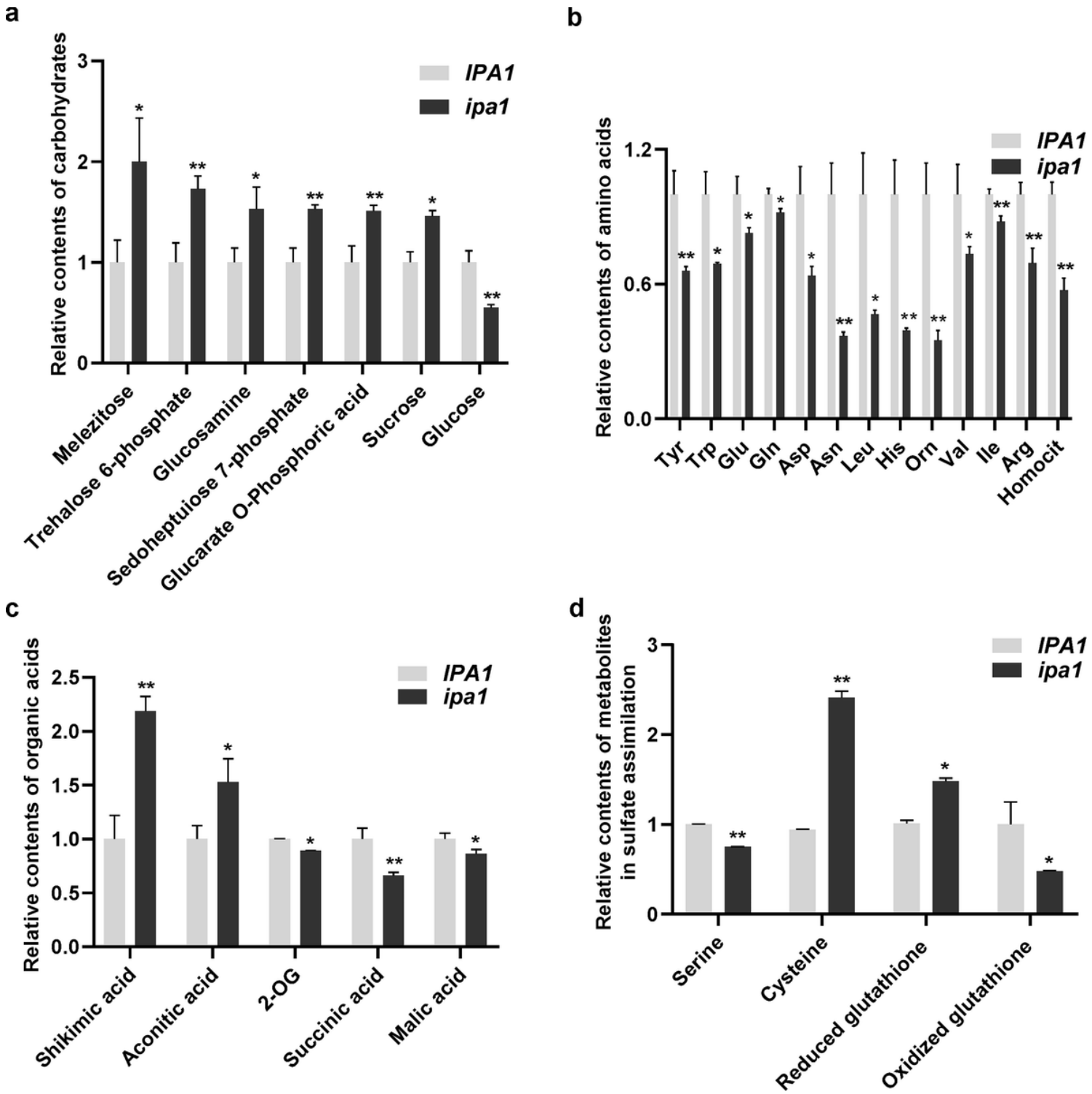

Figure 5

Contents of key metabolites in primary metabolism of the IPA1/ipa1 seedlings. (a-d) Contents of carbohydrates, amino acids, organic acids and metabolites in sulfate primary assimilation processes, respectively, in the IPA1/ipa1 seedlings. Data represent the means ± SE (n=3). * VIP≥0.5, P<0.05, t-test. ** VIP≥0.5, P<0.01, t-test.

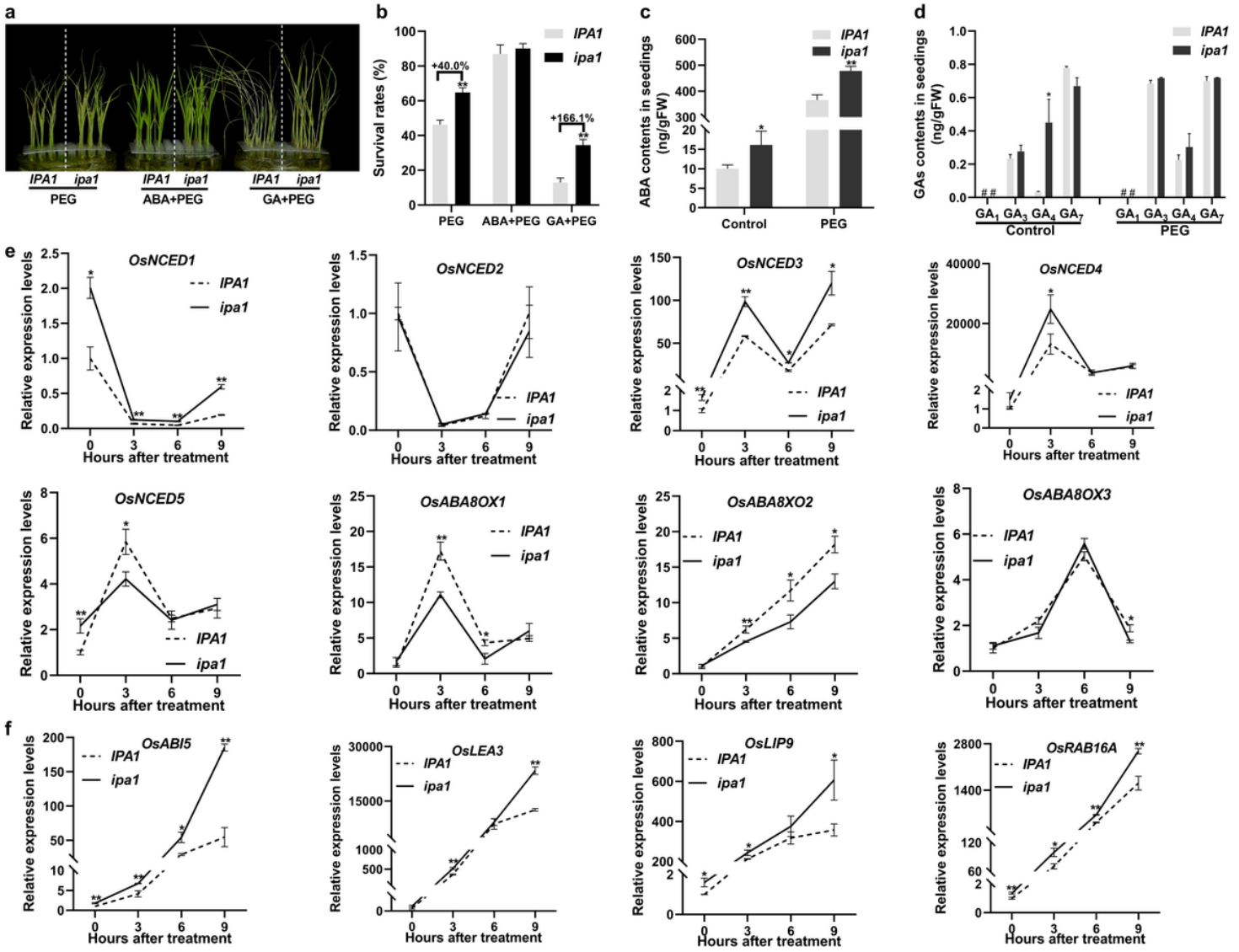

Figure 6

PEG tolerance response to exogenous ABA and GA3 treatments, endogenous ABA and GAs contents of the IPA1/ipa1 seedlings and the expression of ABA-associated genes in the IPA1/ipa1 seedlings. (a) Phenotypes of the IPA1/ipa1 seedlings treated by ABA and GA3 with PEG. The IPA1/ipa1 seedlings cultured in normal condition were treated with ABA (5 μmol) and GA3 (10 μmol), respectively, then transmitted to culture solution with 25% PEG. (b) Survival rates of the IPA1/ipa1 seedlings in corresponding conditions. Each experiment has three replicates, and eighteen seedlings were tested in each replicate. (c, d) ABA and GAs contents in the IPA1/ipa1 seedlings, respectively. (e) The expression levels of genes involved in ABA biosynthesis and catabolism in the IPA1/ipa1 seedlings. (f) The expression levels of marker genes of the ABA pathway. Data represent the means ± SE. *P<0.05, t-test. **P<0.01, t-test.

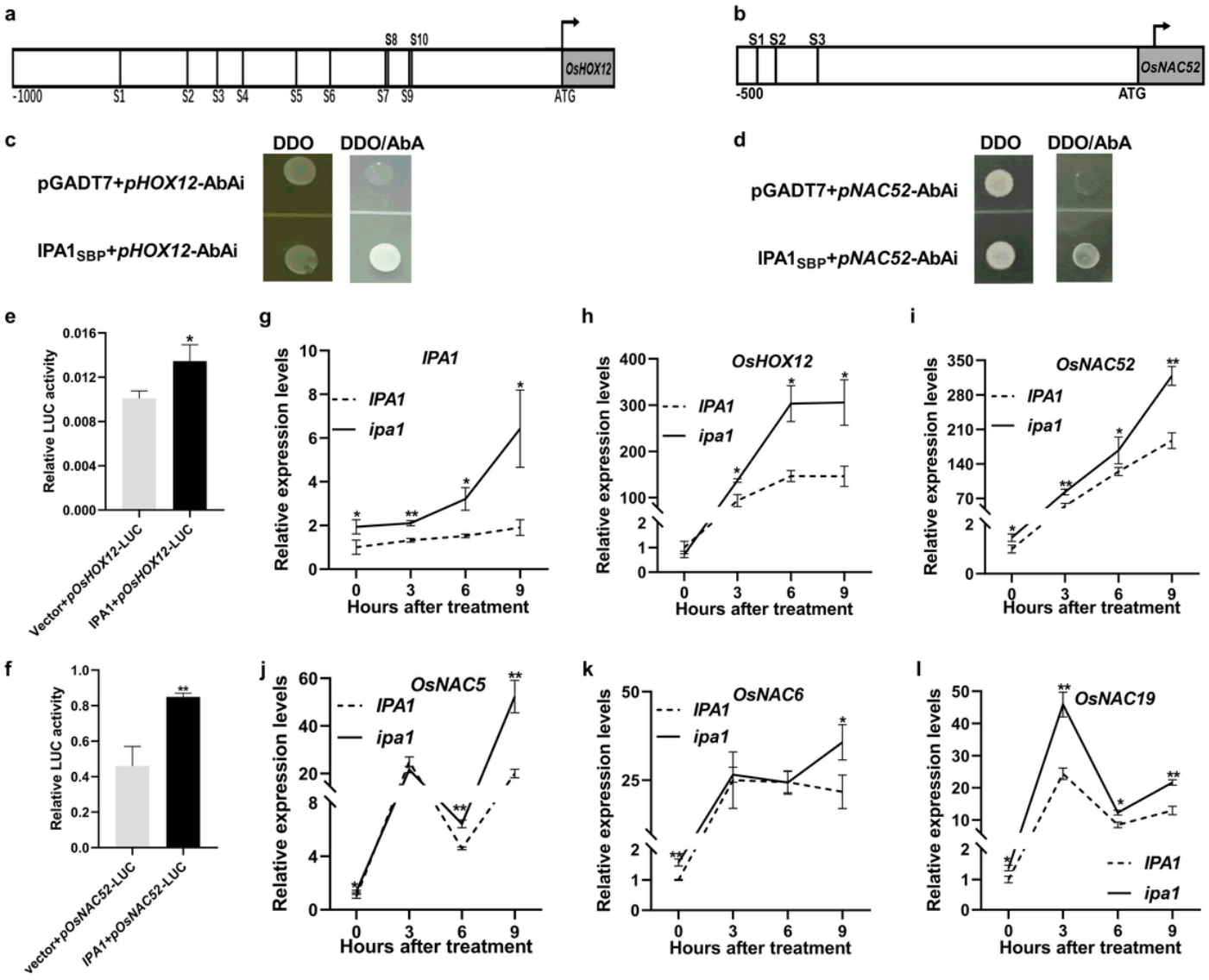

Figure 7

IPA1 directly activates the promoter activity of OsHOX12 and OsNAC52, and up-regulates the expression of the other NAC family genes. (a) Schematic of OsHOX12 promoter. S6 sequence is GTACGTACGTAC, and the other sites sequence is GTAC. (b) Yeast one-hybrid analysis with the fragment containing S1-S10 as bait. (c) Schematic of OsNAC52 promoter. The S1-S3 sequence is GTAC. (d) Yeast one-hybrid analysis with the fragment containing S1-S3 as bait. (e, f) Relative LUC activity in transient transactivation assays. (g-l) Relative expression levels of IPA1, OsHOX12, OsNAC52, OsNAC5, OsNAC6 and OsNAC19, respectively. Data represent the means ± SE (n=3). *P<0.05, t-test. **P<0.01, t-test.

Supplementary Files

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

- SupplementaryFigures.docx

- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx