Euryale Small Auxin Up RNA62 promotes cell elongation and seed size by altering the distribution of indole-3-acetic acid under the light

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Euryale (Euryale ferox Salisb.) is an aquatic crop used as both food and drug in Asia, but its utilization is seriously limited due to low yield. Previously, we hypothesized that Euryale small auxin up RNAs (EuSAURs) regulate seed size, but the underlying biological functions and molecular mechanisms remain unclear. Here, we observed that the hybrid Euryale lines (HL) generate larger seeds with higher indole-3-acetic acid (IAA) concentrations than those in the North Gordon Euryale (WT). Histological analysis suggested that a larger ovary in HL is attributed to longer cells around. Overexpression of EuSAUR62 in rice (Oryza sativa L.) resulted in larger glumes and grains and increased the length of glume cells. Immunofluorescence and protein interaction assays revealed that EuSAUR62 modulates IAA accumulation around the rice ovary by interacting with the rice PIN-FORMED 9, an auxin efflux carrier protein. Euryale basic region/leucine zipper 55 (EubZIP55), which was highly expressed in HL, directly binds to the EuSAUR62 promoter and activated the expression of EuSAUR62. Constant light increased the expression of both EubZIP55 and EuSAUR62 with auxin-mediated hook curvature in HL seedlings. Overall, we proposed that EuSAUR62 is a molecular bridge between light and IAA and plays a crucial role in regulating the size of the Euryale seed.

KEYWORDS
Euryale ferox Salisb., aquatic crop, seed size, EuSAUR62, IAA distribution, light

Introduction

Euryale ferox Salisb. is the only species in the genus Euryale in the botanical family Nymphaeaceae. It is widely distributed in the southern region of China and North Bihar, India, and is considered a staple food because of its high starch content (Jha et al., 2018; Biswas et al., 2020). Biological analysis has revealed that it has antidepression,
anti-oxidant, and anti-diabetic pharmacological properties; thus, it is used in traditional Chinese medicines as well (Song et al., 2011; Wu et al., 2017; Huang et al., 2018). However, the low yield of North Gordon Euryale (WT), the most widely distributed type, seriously inhibits the utilization and development of Euryale (Puste et al., 2005). Although all tissues have been developed as medicinal resources, livestock feeds, or non-staple foods, only the seed of Euryale is used as a staple food and as a major source of drugs (Wu et al., 2014). Among the many traits of seed, the size and weight of seed have been proposed as crucial contributors to yield in crop plants (Kesavan et al., 2013; Li and Li, 2015). Recently, we generated a new hybrid Euryale line (HL) with large and heavy seeds, providing a great opportunity to investigate the genetic factors and underlying molecular mechanisms in regulating the seed size of Euryale (Huang et al., 2020).

In China, Euryale is mainly divided into two ecological types: North Gordon Euryale (also known as ‘Ciqian’) and South Gordon Euryale (also known as ‘Suqian’) (Huang et al., 2020). Each Euryale develops ~18 gigantic floating leaves and ~20 round fruits at the mature stage. The first emerged reproductive tissue occurs 8 or 9 weeks after sowing, until then each plant generates about two reproductive tissues every week up to maturing in the 19th week. Before flowering above water, young reproductive tissue has an underwater developmental period. During this process, the energy and nutrition required for the growth of underwater reproductive tissues should be transported from leaves or stem tubers (Liu et al., 2018; Wu et al., 2022). In this case, both environment and metabolites play crucial roles in controlling Euryale fruit development.

Previous studies have shown that seed development is controlled by the integration of molecular regulatory networks coupled with the spatial and temporal distribution of multiple types of phytohormones, among which auxin is considered a dominant regulator in this process (Li et al., 2019; Cao et al., 2020). The spatial and temporal distribution of active auxin is dynamically mediated by polar auxin transport, biosynthesis, and signaling (Harrison, 2017; Teale and Palme, 2018). In our previous study, we proposed that small auxin up RNA (SAURs) may act as mediators of the auxin signaling pathway and affect the distribution of indole-3-acetic acid (IAA), whereby contributing to the larger seeds of HL (Huang et al., 2020). To date, many genes involved in IAA signal transduction have been documented, such as the SAUR, glycoside hydrolyase 3 (GH3), and auxin/indole-3-acetic acid (AUX/IAA) genes, but functional studies on SAURs have lagged behind those of the two primary auxin response genes (Hagen et al., 1984; Abel et al., 1994; Ren and Gray, 2015; Stortenbeker and Bemer, 2019). It has been reported that AtSAUR62 and AtSAUR75 play critical roles in pollen tube elongation and subsequent fertilization in Arabidopsis (He et al., 2018). AtSAUR63 promotes hypocotyl and stamen filament elongation in Arabidopsis (Chae et al., 2012). Auxin is enriched in immature seeds at several stages, especially at the ends of hypophysis and cotyledon primordia during somatic embryo development because of its transportability (Ni et al., 2001). In rice, endogenous IAA concentration also increased in the spikelets after pollination, and this process was positively correlated with IAA synthesis and transport in the ovary (Uchiumi and Okamoto, 2010; Nagasawa et al., 2013). Auxin transport is controlled by several types of influx and efflux carriers, including AUX1/LAX (AUXIN-RESISTANT 1/LIKE-AUX1), NITRATE TRANSPORTER 1.1 (NRT1.1), PIN-FORMED (PIN), PILS (PIN-LIKE), WALLS ARE THIN 1 (WAT1), and ABC transporters (Swarup et al., 2008; Peret et al., 2012; Barbosa et al., 2018). Among them, PIN proteins direct auxin transport by their coordinated polarized localizations to promote auxin accumulation locally and define sites of organogenesis (Zhou and Luo, 2018). In Arabidopsis thaliana, eight PIN proteins share two conserved, amino (N)-terminal and carboxyl (C)-terminal domains joined by a less conserved hydrophilic loop (Nodzynski et al., 2016). In maize, ZmPIN1a has been reported to regulate auxin spatiotemporal asymmetric distribution in multiple plant developmental processes (Li et al., 2018). Suppressing apple auxin efflux carrier MdPIN1 has an impact on leaf shape (Hu et al., 2020). A recent study in Nymphaea colorata showed that PIN1, PIN3, and PIN7 are involved in the regulatory network of flower development (Zhang et al., 2020). In Oryza sativa, OsPIN9 is monocot-specific PIN protein, and the rice genome contains one further group of two PINs (OsPIN10a and OsPIN10b) (Wang et al., 2009). Monocot-specific and organ-specific proteins exist and they have a distinct role in regulating auxin-induced organ development, such as PIN9 (Balzan et al., 2014). In sorghum, SbPIN3 and SbPIN9 are more highly expressed in flowers than in other organs, indicating their roles in regulating the development of reproductive tissues (Shen et al., 2010). Thus, we speculated that both SAURs and PINs may cooperatively regulate IAA-mediated seed size and yield in Euryale.

Normally, in the dark, dicotyledonous plants such as Arabidopsis, rapidly elongate the hypocotyl upward with tightly closed cotyledons, thereby forming an apical hook at the seedling stage, while apical hooks and cotyledons gradually open in response to light (Fankhauser and Chory, 1997; Zadnikova et al., 2016). However, contrary to this established notion, light exaggerates the apical hook curvature in the seedlings of Euryale. Similarly, it has been reported that light causes photoinhibition in Vallisneria natans and rice coleoptile, leading to a decrease in plant height and inhibition of coleoptile growth (Riemann et al., 2003; Li H. M. et al., 2020). This response is based on inhibition of cell elongation concomitant with a block of auxin transport (Riemann et al., 2003). We speculated that phototropism stimulates apical curvature formation in Euryale under light conditions according to the theory of polar location of IAA. The inner cells of the hook
region become enriched with higher auxin concentration and thereby inhibiting the growth of the inner side during hook formation, while seedlings showed a hookless phenotype when the asymmetrical accumulation of auxin was abolished (Lehman et al., 1996). Thus, the degree of apical curvature in Euryale seedlings under light conditions represents the levels of auxin transport and accumulation. Previous studies provided evidence that light turns off SAUR17 and SAUR50 on the inner side of the hook, leading to cell expansion and the opening of the hook, further indicating the role of SAURs in regulating auxin distribution (Lehman et al., 1996; Wang et al., 2020). Some basic leucine zipper (bZIP) proteins that may be able to specifically bind to G-boxes are considered G-box binding factors (GBFs), such as AtbZIP54 and AtbZIP55 (belonging to GBF2 and GBF3, respectively) (Jakoby et al., 2002). GFB genes from Arabidopsis are involved in ultraviolet and blue light signal transduction and the regulation of light-responsive promoters (Weisshaar et al., 1991). One cassava CPRF-2-Like bZIP gene is up-regulated during white light exposure, indicating its potential function in light response (Pontes et al., 2020). It has been reported that GBF2 and GBF3 are translocated into the nucleus upon light treatment, suggesting that they may act as photoreceptors for phototropism in Euryale seedlings and transactivate SAURs (Schindler et al., 1992; Terzaghi et al., 1997).

In this study, we investigated the function of EuSAUR62 by constructing a gain-of-function strain of rice (Oryza sativa L.). The mechanism of EuSAUR62-mediated IAA distribution in rice was investigated by detecting the interaction between the EuSAUR62 and OsPIN9 proteins. In addition, differential expression levels of EuSAUR62 in HL and WT were studied based on the transcriptional regulation of the EubZIP55 on EuSAUR62. We also observed light-induced apical curvature to understand the regulation of IAA distribution by EuBZIP55 and EuSAUR62. Our findings highlighted that EuSAUR62 plays a crucial role in light-mediated regulation of seed size, which may be a crucial target for improving Euryale yields.

Materials and methods

Plant materials

The HL and WT, two varieties of Euryale used in this study, have been described previously (Huang et al., 2020). The plant materials were formally identified by Professor Qinan Wu based on morphology, and a voucher specimen of this material was been deposited at the Nanjing University of Chinese Medicine. Both HL and WT were sown in a completely randomized block design with three replications in the experimental field of Jiangsu Seed and Seed Breeding Base under natural conditions in April 2018. The transplanting period was carried out in early May. Each plot included 160 individual lines, each separated by 2 m from its neighboring lines. During the whole culture process, the water temperature ranges from 21 to 28°C. Before anthesis, young fruits at designed stages (developed from week 9 to week 12) were collected from a single plant and stored at –80°C for histological analysis with three replicates. After fruits ripened in September (the 20th or 21st week), seeds were collected for yield traits, including seed size, seed weight, and 100-seed weight.

A japonica rice variety (Oryza sativa L. ssp. Japonica cv. Nipponbare) was used for the transgenic experiments. The rice seeds used in this study were sourced from the WT and EuSAUR62 overexpressed (OE-EuSAUR62) plants. Plants were cultured in a glasshouse. A nursery bed was prepared to raise rice seedlings, and irrigation water was applied by sprinkling. Healthy seedlings were selected and transplanted to pots (one seedling in each pot) with 2 cm of standing water in April. During culture, the plants were supplied with compound fertilizer when needed. The image data of plants were recorded every 2 days after transplanting, and rice leaves were collected and stored at –80°C for histological analysis on days 10th and 60th after transplanting. Grains from each plant were harvested and kept separate for phenotypic analysis at maturity.

Metabolite analysis

Metabolites of mature Euryale seeds were detected by LC-MS as previously described (Wu et al., 2013; Wang et al., 2015). Briefly, 4 g powder of the sample was extracted in 40 ml extraction buffer (methanol: double distilled water = 4:1, v/v) by ultrasonication. Then, the samples were centrifuged at 10,000 rpm for 5 min; the upper phase was dried under a stream of nitrogen gas and redissolved with 1 ml chromatographic methanol. Each sample solution was injected into the reverse-phase C18 column (250 mm × 4.6 mm, 5 µm, Agilent, United States) for LC-MS analysis (Ultra-Fast Liquid Chromatography, Shimadzu, Japan; Triple TOFTM 5600, AB Sciex, United States). The qualitative analysis and the resolution of the chromatogram of the target components were carried out using the MS-DIAL software 3.98. The differences in metabolites were calculated using one-way ANOVA and post hoc statistical analysis.

Histological analysis

For section observation, fruit and leaf samples were cut into 30 µm sections with a microtome RM-2235 (Leica Microsystems, Germany) following a series of dehydration and infiltration steps. Sections were then stained with 1%
safranin and 1% Fast Green and imaged under a Carl Zeiss Axio Scope fluorescence microscope (ZEISS Göttingen, Germany). The cell lengths and widths were measured using the ImageJ 1.51 software.

For scanning electron microscopy (SEM) observations, glumes of rice were separately collected from the OE-EuSAUR62 and WT lines at the heading stage. After imaging the whole glume using a stereomicroscope (V20, ZEISS), samples were cut into small pieces and were gold plated followed by observation using a scanning electron microscope (LEO1530VP, ZEISS). The lengths and widths of the outer glume cells were measured using the ImageJ 1.51 software.

**Phylogenetic analysis**

Arabidopsis and rice SAUR protein sequences were downloaded from the Arabidopsis Information Resource and the China Rice Data Center websites, respectively. The phylogenetic tree was constructed using the MEGA 5.0 software via the neighbor-joining method.

**RNA extraction and qRT-PCR**

Total RNA was extracted from various tissues using TRIzol reagent (T9424, Sigma, United States) and 1 µg was used for cDNA synthesis with the PrimeScript RT Master Mix Kit (RR036A, TaKaRa, China). qRT-PCR was performed using a QuanStudio 3 instrument (Applied Biosystems, United States), and the β-actin was used as an internal control for normalization as described previously (Huang et al., 2020; Jing et al., 2021). At least three biological and three technical replicates were employed for all samples, and the expression data were generated using the comparative Ct (ΔΔCt) method after confirming a single product by disassociation curves.

**Subcellular localization assay**

The pCambia1300 vector was used for subcellular localization assays. The p35S:EuSAUR62-GFP, p35S:OsPIN9-GFP, p35S:EubZIP54-GFP, and p35S:EubZIP55-GFP vectors consisted of the full-length CDS of relative genes fused with the green fluorescent protein (GFP) reporter gene and was driven by the 35S promoter. The vectors were transformed into tobacco (Nicotiana tabacum L.) leaves, followed by overnight incubation staying in the dark, as previously described (Li et al., 2021). GFP signals were visualized using a laser confocal microscope (C3-ER, Nikon, Japan) after culturing under normal conditions for 3 days.

**Generation of transgenic plants**

A 429 bp cDNA without the stop codon was amplified from Euryale using the primers OE-EuSAUR62-F and OE-EuSAUR62-R (Supplementary Table 2) and inserted into the binary vector pCambia1300 via KpnI and XbaI sites. The p35S:EuSAUR62-GFP and p35S:empty-GFP plasmids were introduced into the Agrobacterium tumefaciens strain EHA105. The constructs were transferred into the wild rice by infecting calli, as previously reported (Dang et al., 2020).

For promoter activity analysis, a 1616 bp genomic fragment was amplified using EuSAUR62-p-F and EuSAUR62-p-R (Supplementary Table 2) and replaced the original 35S promoter of pCambia1301 via PstI and Ncol sites. The constructed plasmid was cloned into GV3101, and then the positive clones were injected into the leaves to construct transient expression tobacco.

**Western blot**

Rice leaves were lysed in protein extraction buffer containing 10 mM Tris–HCl (pH = 8), 1 mM Phenylmethylsulfonyl fluoride, 60 mM β-glycerophosphate, and 2% SDS. Samples were then rapidly homogenized and western blots were performed. Anti-GFP (66002-1-lg, Proteintech Group, China) and anti-β-actin (6008-1-lg, Proteintech Group, China) were used as primary antibodies. Horseradish peroxidase (HRP)-conjugated goat IgG (H + L) (SA00001-1, SA00001-2, Proteintech Group, China) was used as the secondary antibody. Blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). 1 WT was used as a negative control. All experiments were performed in triplicate.

**Immunofluorescence staining**

Immunofluorescence staining for free-IAA was carried out on sections of the rice ovary as described previously (Nishimura and Koshiba, 2019; Huang et al., 2020), with some modifications: 80 µm sections were dehydrated for 5 min in ascending and descending 25, 50, 75, and 100% methanol solutions, incubated in 0.1% Sudan black b (dissolved in 70% methanol) for 10 min to remove the autofluorescence, washed three times for 5 min in 50% methanol. Sections were incubated in detergent solution (DMSO: Triton X-100: PBS = 10: 3: 87, v: v: v) for 30 min followed by washing three times. Then, sections were blocked with 5% (w/v) bovine serum albumin.
in PBS for 1 h, incubated with anti-IAA antibody (1:200, AS09421, Agrisera, Sweden) overnight. After washing three times with 0.1% (v/v) Tween 20 in PBS buffer for 5 min, sections were incubated with Alexa Fluor 488 [1:200, conjugated Goat Anti-Rabbit IgG (H + L), SA00006-2, Proteintech Group, United States] for 1 h in dark. Fluorescence signals (green) were imaged on a Carl Zeiss Axio Scope fluorescence microscope. PBS was added instead of anti-IAA antibodies as a negative control. All experiments were performed in triplicate.

Bimolecular fluorescence complementation assay

For the BiFC assay, the OsPIN9 CDS was inserted into the pFGC-YC155 vector to generate the OsPIN9-cYFP. The EuSAUR62 CDSs were inserted into the pFGC-YN173 vector to generate the EuSAUR62-nYFP. After transformation into Agrobacterium strain GV3101, all fusion proteins were co-expressed in tobacco leaves. After 3 days, the yellow fluorescence protein (YFP) signal was detected at a range of 530-630 nm using a laser scanning confocal microscope (SP8; Leica, Wetzlar, Germany).

Genome walking

The promoter of EuSAUR62 was obtained using a genome walking kit according to the manufacturer’s instructions (TaKaRa, Dalian, China). Random primers (AP1–AP4) were provided by the genome walking kit, and the specific primers were designed based on the known sequences (first round EuSAUR-r1; second round EuSAUR-r2; third round EuSAUR-r3, Supplementary Table 2). Specific PCR products were purified using the DNA Gel Extraction Kit (Axygen, United States), cloned into the pMD-18 vector system, and then sequenced.

β-glucuronidase staining

After growth under normal conditions for 3 days, the transgenic tobacco leaves were overnight stained with β-glucuronidase (GUS) staining buffer [50 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 1 mM K3[Fe(CN)6], 0.5 mM K4[Fe(CN)6], 3H2O; 0.1% (v/v) Triton X-100, 1 mg/ml X-Gluc] at 37°C. The samples were then washed with 70% ethanol to remove the chlorophyll. Images were captured using a stereomicroscope (V20, ZEISS).

DNA-pulldown assay

A ~1600 bp EuSAUR62 promoter (EuSAUR62pro) region was amplified using the biotinylated primers. This probe was then added to the fruit lysates of the HL and WT, respectively, and incubated at room temperature. Probe-protein complexes were precipitated with DynaBeads (Bever Biosciences Inc., United States), cloned into the pMD-18 vector system, and then sequenced. Random primers (AP1–AP4) were provided by the genome walking kit according to the manufacturer’s instructions (TaKaRa, Dalian, China). Random primers (AP1–AP4) were provided by the genome walking kit, and the specific primers were designed based on the known sequences (first round EuSAUR-r1; second round EuSAUR-r2; third round EuSAUR-r3, Supplementary Table 2). Specific PCR products were purified using the DNA Gel Extraction Kit (Axygen, United States), cloned into the pMD-18 vector system, and then sequenced.

Membrane yeast two-hybrid assay

The DUAL membrane system was used to assay the interactions between the EuSAUR62 and OsPIN9. Full-length EuSAUR62 and OsPIN9 were cloned into pBT3–SUC and pPR3–N, respectively. Transformation of NMY51 yeast strain was performed using standard procedures as described previously (Chen et al., 2021). The positive strains were transferred onto minimal SD agar base plates supplemented with DO Supplement (Chen et al., 2021). The interactions between the EuSAUR62 and OsPIN9. Full-length EuSAUR62pro was cloned into the pGADT7 vector at the same sites, whereas the EuSAUR62pro was inserted into the pHIS2.1 vector at the MluI and SacII sites. The resultant vectors

Yeast one-hybrid assay

The yeast one-hybrid (Y1H) was used to test direct interactions between EubZIPS and EuSAUR62pro as previously described (Xi et al., 2019). The CDS of EuSAUR62pro was inserted into the pGADT7 vector at the BanHI and Xhol sites, and EuSAUR62pro was inserted into the pGADT7 vector at the same sites, whereas the EuSAUR62pro was inserted into the pHIS2.1 vector at the MluI and SacII sites. The resultant vectors
were inserted into yeast strain Y187 cells, according to the manufacturers instructions (Clontech). Transformed yeast cells were grown on SD-T-L medium at 30°C for 3–5 days. The positive clones were transferred to SD-T-L-H but supplemented with increasing 3-AT concentration at 30°C for 3–5 days.

**Dual luciferase reporter assay**

To study the transcriptional activity of EubZIP54/55 on EuSAUR62, we performed a Dual luciferase reporter assay (LUC). The p35S:EubZIP54-GFP and p35S:EubZIP55-GFP were used as effector vectors. The p35S:empty-GFP was an empty effector vector. The EuSAUR62pro was inserted into the reporter vector pGreen II 0800-LUC. The effector vectors and reporter vectors were co-transfected into tobacco leaves. The reaction was performed using the Dual Luciferase Reporter Assay Kit (DL101-01, Vazyme, China) and luciferase activities were measured by the microplate reader (INFINITE2000, TECAN, Switzerland). All experiments were performed in triplicate.

**Seedlings treatment and hook curvature measurement**

Euryale seeds were cultured in water for sprouting in a plant growth chamber at a constant temperature (∼20°C) and a cycle of 14-h light/10-h dark. Two-week-old seedlings of HL and WT were randomly divided into four groups: (1) HL seedlings with constant light treatment for 7 days; (2)
the WT seedlings with constant light treatment for 7 days; (3) HL seedlings cultured in the dark for 7 days; (4) WT seedlings cultured in the dark for 7 days. During treatment, images of each seedling were captured using a digital single lens reflex camera (Canon, Tokyo) every day. Hook angles of individual seedlings were measured digitally. All experiments were performed in triplicate.

**Data analysis**

All data were expressed as the mean ± SD. Differences between two groups were analyzed using the t-test, while differences among groups were analyzed using one-way ANOVA, and \( p < 0.05 \) was represented as a statistically significant difference. Statistical analysis and frequency distribution analysis was carried out by GraphPad Prism 6.0 software (GraphPad Software, Inc.).

**Results**

**The cell elongation and ovary enlargement in hybrid Euryale lines were promoted by a high content of indole-3-acetic acid**

In September, fully mature seeds were collected for yield trait analysis. Compared to the WT, HL elevated yield traits of seeds in both size and weight (Figures 1A–E). The yield advantage of HL was also revealed by hundred-seed weight (Figure 1F), and the average yield (WT = 3500 kg/ha and HL = 4,875 kg/ha) in experimental plots. The growth of fruits in the two types showed that, compared to the difference in fruit numbers of each plant between these two varieties, HL obviously produces larger fruits since the 10th week after sowing (Supplementary Figure 1). The seed of HL is obviously rounder than that of WT (Figure 1D), leading us to speculate that there may be differences in fruit histology or seed metabolites between HL and WT.

To investigate the difference in metabolites in seeds of WT and HL, we performed untargeted metabolomics of both varieties using liquid chromatography-mass spectrometry (LC-MS) detection with negative ion mode. We detected 94 metabolites in total in mature seeds harvested at the 20th week from the two types (Table 1), and 50 different components between HL and WT, including phenolic acids, amino acids, flavonoids, organic acids, and their derivatives, are shown in Figure 2B. KEGG analysis revealed that these different metabolites are enriched in various pathways, such as metabolic pathways in the biosynthesis of alanine, aspartate, glutamate, phenylalanine, tyrosine, and tryptophan biosynthesis, and riboflavin metabolism (Figure 2A). A clear separation between HL and WT was observed using principal component analysis (PCA) and orthogonal projections to latent structures analysis (OPLS-DA), suggesting remarkably different metabolites in the seeds of two lines (Supplementary Figure 2). Among these metabolites, the indole-3-acetic acid (Figure 2B) and tryptophan metabolism pathway (Figure 2A) attracted our attention. IAA was significantly enriched in the seed of HL, indicating that IAA management between HL and WT is different during fruit growth. According to our previous study, there is no difference in IAA concentration between HL and WT fruits at the same stage, but the IAA distribution is different (Huang et al., 2020). Specifically, the WT allocates free-IAA to its pricky epicarp more frequently, whereas the fluorescence signal in fruits of HL is enriched in the ovary and stamen, indicating a potential role for IAA hemostasis in regulating fruit development.

Then, we performed microscopic analysis on the 12-week-old fruits to investigate the difference in fruit histology between HL and WT. The longitudinal sections of both types of fruits are shown in Figure 3A, compared to the WT, the larger ovaries were observed in HL. We then measured the length of cells around the ovary by observing their autofluorescence (red, Figure 3B). As shown in Figure 3C, the cell length in HL and WT continuously varied from 18 to 24 µm and 16 to 20 µm, respectively. Therefore, we concluded that HL produces larger ovaries by distributing its auxin around the ovaries, thereby promoting the elongation of cells around them, which contributes to larger and rounder seeds.

**EuSAUR62 improved yield traits by increasing cell length in rice**

In a previous study, we found that some SAURs act as positive mediators of the auxin transduction pathway which might mediate the location of IAA (Huang et al., 2020). In this study, EuSAUR62, the most differently expressed in HL and WT, was selected to analyze its potential role in auxin regulation. Phylogenetic analysis revealed that EuSAUR62 was highly homologous to AtSAUR62 and AtSAUR75 (Figure 4A), which have been reported to promote cell elongation in Arabidopsis (He et al., 2018). Subcellular location assay showed that EuSAUR62 is located at the plasma membrane and nucleus (Figure 4B and Supplementary Figure 3A). As shown in Figure 4C, the expression level of EuSAUR62 in HL was increased about 2.5-fold compared to the WT. We speculated that it is the localization of auxin that causes the up-regulation of EuSAUR62 expression in HL, as its homologous gene belongs to an auxin-response SAUR-clade (van Mourik et al., 2017; Kathare et al., 2018). To verify the response manner of the EuSAUR62 gene, we analyzed the expression of EuSAUR62 in seedlings under exogenous IAA or NPA treatment as reported previously (Kant et al., 2009). It was found that EuSAUR62 is up-regulated from 0 to 60 min after 10 µm IAA treatment.
### TABLE 1 Metabolites of both WT and HL seeds.

| No. | Metabolite name                                      | Formula            | RT (min)       | Adduct type       |
|-----|------------------------------------------------------|--------------------|----------------|-------------------|
| 1   | Ala-Ala                                              | C₈H₁₄N₂O₃          | 1.995          | [M-H]             |
| 2   | Cytidine                                             | C₆H₁₂N₆O₩          | 2.017          | [M-H]             |
| 3   | Adrmine                                              | C₆H₁₂N₆             | 2.134          | [M-H]             |
| 4   | Pyridoxine                                           | C₆H₁₂N₆O₩          | 2.159          | [M-H]             |
| 5   | Serine                                               | C₆H₁₂N₆O₩          | 2.177          | [M-H]             |
| 6   | 1-Asparagine                                         | C₆H₁₄N₂O₩          | 2.186          | [M-H]             |
| 7   | Aspartic acid                                        | C₆H₁₂N₆O₩          | 2.305          | [M-H]             |
| 8   | Persitol                                             | C₃H₁₆O₄             | 2.368          | [M-H]             |
| 9   | 9-Fluorenone                                         | C₉H₈O               | 2.439          | [M-H]             |
| 10  | Sorbitol                                             | C₆H₁₄O₆             | 2.439          | [M-H]             |
| 11  | Arabitol(D)                                          | C₈H₁₂O₃             | 2.468          | [M-H]             |
| 12  | Sucrose                                              | C₆H₁₂O₁₁            | 2.485          | [M-H]             |
| 13  | Glucosamine                                          | C₆H₁₂O₆             | 2.566          | [M-H]             |
| 14  | Threonine                                            | C₆H₁₂O₅             | 2.673          | [M-H]             |
| 15  | 2-Ketogluconic acid                                  | C₂H₂₀O₃             | 2.676          | [M-H]             |
| 16  | Uridine                                              | C₆H₁₂N₆O₃          | 2.905          | [M-H]             |
| 17  | Guanosine                                            | C₈H₁₄N₆O₄          | 3.093          | [M-H]             |
| 18  | Ortophsphate                                         | H₂O₆P              | 3.539          | [M-H]             |
| 19  | Hyoxanthine                                          | C₆H₁₂N₄O₄          | 3.676          | [M-H]             |
| 20  | Tyrosine                                             | C₆H₁₂N₆O₃          | 3.734          | [M-H]             |
| 21  | Glucose-1-phosphate                                  | C₆H₁₄O₅P           | 4.023          | [M-H]             |
| 22  | Citrate                                              | C₆H₄O₇             | 4.275          | [M-H]             |
| 23  | Inosine                                              | C₆H₁₂N₄O₄          | 4.625          | [M-H]             |
| 24  | D-Pantothenic acid                                   | C₆H₁₂N₆O₄          | 6.105          | [M-H]             |
| 25  | Gallic acid                                           | C₆H₄O₅             | 6.556          | [M-H]             |
| 26  | Methylisunic acid                                    | C₂H₄O₄             | 6.711          | [M-H]             |
| 27  | Tryptophan                                           | C₃H₁₄N₄O₄          | 6.716          | [M-H]             |
| 28  | Homogenetic acid                                     | C₂H₄O₄             | 7.358          | [M-H]             |
| 29  | (2S,3S)-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol | C₁₇H₁₉O₇ | 8.533          | [M-H]             |
| 30  | N-Acetyl-l-D-methionine                              | C₆H₁₂N₆O₅S         | 8.973          | [M-H]             |
| 31  | 2,3-Dihydroxybenzoic acid                            | C₆H₄O₄             | 9.332          | [M-H]             |
| 32  | Epigallocatechin                                     | C₁₂H₂₀O₇            | 9.708          | [M-H]             |
| 33  | 2-Isopropylmalic acid                                | C₁₂H₁₄O₅            | 9.733          | [M-H]             |
| 34  | N-Isovalerylglycine                                  | C₁₂H₂₀N₂O₃          | 9.897          | [M-H]             |
| 35  | (-)-Riboflavin                                       | C₁₂H₁₄N₄O₃          | 10.018         | [M-H]             |
| 36  | Protocatechuic aldehyde                              | C₂H₄O₅             | 10.728         | [M-H]             |
| 37  | Methylgallate                                        | C₂H₄O₅             | 10.811         | [M-H]             |
| 38  | NGCG00385057-01!                                     | C₂H₂₀O₁₈            | 11.086         | [M-H]             |
| 39  | Catechol                                             | C₂H₂₀O₁₂            | 11.164         | [M-H]             |
| 40  | N-Acetylserine                                       | C₂H₂₀N₂O₃          | 11.571         | [M-H]             |
| 41  | Catechin                                             | C₂H₂₀O₆             | 11.763         | [M-H]             |
| 42  | 6,7-Dihydroxycoumarin                                | C₂H₄O₄             | 11.942         | [M-H]             |
| 43  | NGCG00347753-02_C19H18N4O7_9H- Purin-6-ol, 9-[1-(3-carboxyphenyl)-2,3-di] | C₁₈H₁₄N₂O₃         | 11.942         | [M-H]             |
| 44  | NGCG00384822-01([2R,3S;4,5R,6S]-3,4-dihydroxy-5,6-bis(3,4,5-trihydroxybenzoyloxy)oxan-2-yl)methyl 3,4,3-trihydroxybenzoate | C₂₇H₃₄O₄₈          | 12.057         | [M-H]             |
| 45  | 2-Hydroxy-4-methylpentanoic acid                     | C₂H₂₁O₁₃            | 12.126         | [M-H]             |
| 46  | Epigallocatechin-9-gallate                           | C₂H₂₀O₁₃            | 13.222         | [M-H]             |
| 47  | Myricetin-3-Galactoside                              | C₂H₂₀O₁₃            | 13.576         | [M-H]             |
| 48  | N-acetylphenylalanine                                | C₂H₂₁N₂O₃          | 13.981         | [M-H]             |
| 49  | 3-Phenylactic acid                                   | C₂H₂₀O₃             | 14.086         | [M-H]             |
| 50  | Indole-3-acetyl-l-glutamic acid                      | C₁₉H₂₃N₂O₃          | 14.443         | [M-H]             |
| 51  | Luteolin-7-O-glucoside                               | C₂₃H₂₀O₁₁           | 14.604         | [M-H]             |
| 52  | Hyperoside                                           | C₂₃H₂₀O₁₂           | 15.089         | [M-H]             |
| 53  | Epicatechin gallocate                                | C₂₂H₂₃O₁₀           | 15.336         | [M-H]             |

(Continued)
| Num. | Metabolite name                                      | Formula          | RT (min) | Adduct type         |
|------|-----------------------------------------------------|------------------|----------|---------------------|
| 54   | Indolelactic acid                                   | C_{11}H_{11}NO_3 | 15.43    | [M-H] −             |
| 55   | Ellagic Acid                                         | C_{14}H_{14}O_6  | 15.576   | [M-H] −             |
| 56   | Syringetin-3-O-glucoside                            | C_{22}H_{25}O_7  | 15.816   | [M-H] −             |
| 57   | Kaempferol-3-O-glucoside                            | C_{20}H_{21}O_7  | 16.238   | [M-H] −             |
| 58   | Taxifolin                                           | C_{18}H_{12}O_7  | 16.439   | [M-H] −             |
| 59   | 3-(2-Hydroxyphenyl)propanoic acid                   | C_{14}H_{14}O_3  | 16.448   | [M-H] −             |
| 60   | Seconolaricresinol                                  | C_{26}H_{27}O_8  | 16.682   | [M-H] −             |
| 61   | NCGC00169984-03!(3R,5R)-3,{[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4-dihydroxychlohexane-1-carboxylic acid | C_{25}H_{24}O_{12} | 16.983   | [M-H] −             |
| 62   | Indole-3-carboxaldehyde                              | C_{6}H_{7}NO     | 17.684   | [M-H] −             |
| 63   | Indole-3-acetic acid                                 | C_{10}H_{12}O_7  | 18.021   | [M-H] −             |
| 64   | Abscisic acid                                       | C_{12}H_{16}O_4  | 18.818   | [M-H] −             |
| 65   | Sebacic acid                                        | C_{16}H_{16}O_4  | 19.407   | [M-H] −             |
| 66   | Tricetin                                            | C_{13}H_{16}O_7  | 19.495   | [M-H] −             |
| 67   | Myricetin                                           | C_{13}H_{16}O_7  | 19.559   | [M-H] −             |
| 68   | Eriodictyol                                         | C_{13}H_{16}O_7  | 20.58    | [M-H] −             |
| 69   | 4-Nitrophenol                                       | C_{6}H_{5}NO_3   | 20.731   | [M-H] −             |
| 70   | Trans-Resveratrol                                   | C_{14}H_{13}O_2  | 20.737   | [M-H] −             |
| 71   | Luteolin                                            | C_{13}H_{15}O_6  | 21.967   | [M-H] −             |
| 72   | LPE 12.0                                            | C_{27}H_{28}O_7P | 22.044   | [M-H] −             |
| 73   | Quercetin                                           | C_{15}H_{16}O_7  | 22.306   | [M-H] −             |
| 74   | Phloretin                                           | C_{13}H_{15}O_7  | 22.484   | [M-H] −             |
| 75   | 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-2,3-dihydroxychro-4-one | C_{13}H_{15}O_6  | 22.542   | [M-H] −             |
| 76   | LPE 13.0                                            | C_{28}H_{31}O_7P | 23.221   | [M-H] −             |
| 77   | Aloe-emonin                                         | C_{13}H_{15}O_7  | 24.29    | [M-H] −             |
| 78   | LPE 15.1                                            | C_{28}H_{31}O_7P | 24.366   | [M-H] −             |
| 79   | Kaempferol                                          | C_{15}H_{16}O_7  | 24.882   | [M-H] −             |
| 80   | LPE 18.3                                            | C_{27}H_{28}O_7P | 24.969   | [M-H] −             |
| 81   | LPE 16.1                                            | C_{27}H_{28}O_7P | 25.132   | [M-H] −             |
| 82   | LPE 15.0                                            | C_{27}H_{28}O_7P | 25.328   | [M-H] −             |
| 83   | Sakuranetin                                         | C_{13}H_{15}O_7  | 25.49    | [M-H] −             |
| 84   | Pinocembrin                                         | C_{15}H_{15}O_7  | 26.071   | [M-H] −             |
| 85   | LPE 18.2                                            | C_{28}H_{31}O_7P | 26.184   | [M-H] −             |
| 86   | LPC 18.2                                            | C_{28}H_{31}O_7P | 26.282   | [M + FA-H] −        |
| 87   | Chrysian                                            | C_{15}H_{15}O_7  | 27.05    | [M-H] −             |
| 88   | LPE 16.0                                            | C_{28}H_{31}O_7P | 27.189   | [M-H] −             |
| 89   | LPC 16.0                                            | C_{28}H_{31}O_7P | 27.319   | [M + FA-H] −        |
| 90   | LPE 18.1                                            | C_{28}H_{31}O_7P | 27.786   | [M-H] −             |
| 91   | LPC 18.1                                            | C_{28}H_{31}O_7P | 27.912   | [M + FA-H] −        |
| 92   | LPE 17.0                                            | C_{28}H_{31}O_7P | 28.568   | [M-H] −             |
| 93   | Beta-Hydroxymyristic acid                           | C_{15}H_{15}O_7  | 30.224   | [M-H] −             |
| 94   | Octadecanedic acid                                  | C_{15}H_{15}O_7  | 32.584   | [M-H] −             |

(Figure 4E), while is down-regulated by application of 10 µm exogenous NPA (Figure 4G), an inhibitor of auxin transport, and the expression in both treatment groups gradually return to normal level after discontinuing treatment. As shown in Figures 4D,F, exogenous IAA and NPA dynamically increased and decreased the expression level of EuSAUR62, respectively.
and both showed dose-response relationships. In addition, an elevated expression of \textit{EuSAUR62} after treatment with 1-naphthaleneacetic acid (NAA) suggested that \textit{EuSAUR62} can positively respond to different types of auxins (Supplementary Figure 3B). Our results implied that \textit{EuSAUR62} responds to alteration of auxin concentration in a rapid manner.

Since it is difficult to perform the genetic transformation in Euryale, we used rice (\textit{Oryza sativa} L. ssp. \textit{japonica} cv. Nipponbare) as the recipient material for transformation to explore whether alterations in grain weight, grain size, and other phenotypes are associated with \textit{EuSAUR62}. The phenotypes of grains are shown in Figure 5A, grains from \textit{EuSAUR62} gain-of-function lines were more than 12% heavier and 10% longer ($p < 0.001$, Figures 5D,E), whereas there was no difference in grain width between the two lines ($p > 0.05$, Supplementary Figure 4B) compared to the WT, revealing that \textit{EuSAUR62} increases length and weight of grains. F2 populations of \textit{EuSAUR62} fit the expected 3:1 ratio ($\chi^2 = 0.0525$, df = 1, $p = 0.8187$) for a single segregating transgene. The \textit{EuSAUR62} protein was detected in larger grains by immunodetection using a GFP antibody, further supporting our results (Supplementary Figure 4A).

In addition to the increased grain size and weight observed in the \textit{OE-EuSAUR62}, the \textit{OE-EuSAUR62} also produced larger glumes (Figure 5B). Glumes in \textit{OE-EuSAUR62} lines had an average length of approximately 6.38 mm, which was significantly longer than that of the WT ($p < 0.01$, Figure 5F), whereas no difference was observed in the glume width between the two lines ($p > 0.05$, Supplementary Figure 4C).

Further histological analysis using SEM revealed an increase in the length of outer glume cells of \textit{OE-EuSAUR62} lines (Figures 5C,G), indicating that \textit{EuSAUR62} regulates grain size by modulating the longitudinal cell length in the lemma. Thus, our results provided evidence that \textit{EuSAUR62} positively regulates cell length and glume size.

Moreover, we detected other altered phenotypes in \textit{OE-EuSAUR62} plants, including plant height and leaf angle. For the plant height, we found that, on day 10, after transplantation, young leaves of \textit{OE-EuSAUR62} lines grow much faster than WT (Supplementary Figures 5A,C). We also observed a significantly enlarged leaf angle in the bottom leaves of \textit{OE-EuSAUR62} plants on the 60th day (Supplementary Figures 5E,G). Further histological observation of leaf parenchyma cells and pulvinus cells by autofluorescence revealed that most parenchyma cells in \textit{OE-EuSAUR62} lines and WT are distributed at 44–74 and 34–64 µm, respectively (Supplementary Figures 5B,D), and there are more pulvinus cells above 64 µm in the \textit{OE-EuSAUR62} lines than in the WT (Supplementary Figures 5F,H). Collectively, these results suggested that \textit{EuSAUR62} promotes the elongation of both parenchyma and pulvinus cells, strongly confirming its role for \textit{EuSAUR62} in promoting cell length.

As previously reported, IAA distribution is significantly different in two Euryale (Huang et al., 2020). We speculated that \textit{EuSAUR62} mediates IAA distribution during fruit development. Thus, to determine whether \textit{EuSAUR62} regulates the distribution of IAA in rice, we performed an immunofluorescence assay with an anti-IAA antibody on
reproductive tissue sections at the flowering phase. After excluding the background fluorescence in the negative control, which was incubated with 1% BSA instead of the primary antibody (Supplementary Figure 4D), we found that OE-EuSAUR62 lines allocate free-IAA to the ovary more frequently compared to the WT (Figure 5H), indicating that EuSAUR62 regulates the location of IAA in reproductive tissues. The expressed EuSAUR62 protein was also detected in OE-EuSAUR62 rice by western blot assay (Figure 5I).

EuSAUR62 directly interacted with OsPIN9 protein

Indole-3-acetic acid homeostasis is controlled by both local IAA biosynthesis and polar IAA transport, but there is no significant change in IAA synthesis genes between the WT and HL at the same growth stage according to our transcriptome data, suggesting that the accumulation of IAA was induced not by local biosynthesis (Supplementary Figure 6). Based on network analysis in the STRING database (the OsSAUR39 was the most homologous to EuSAUR62 in the database), OsPIN9 was screened as a candidate that interacts with the OsSAUR39 protein (Supplementary Figure 7A), further indicating that the accumulation of IAA was mediated by polar transport. Polar IAA transport is controlled by several carrier proteins, such as AUX/LAX, PIN, and ABC transporters (Kamimoto et al., 2012; He et al., 2017). Transient expression of OsPIN9 in tobacco leaves revealed that it is expressed at the plasma membrane (Supplementary Figure 7B). As both EuSAUR62 and OsPIN9 can express at the plasma membrane (Supplementary Figure 3A), we speculated that
there could be an interaction between EuSAUR62 and OsPIN9 proteins. To verify our hypothesis, we completed bimolecular fluorescence complementation (BiFC) and membrane yeast two-hybrid assays (mY2H) assays. The physical interaction between EuSAUR62 and OsPIN9 was confirmed in the BiFC assay, as YFP signals could only be detected in samples expressing both EuSAUR62 and OsPIN9 (Figure 6A). We used the DUAL membrane system to analyze the interaction between EuSAUR62 and OsPIN9 on the cellular membrane as previously described (Chen et al., 2021). As shown in Figure 6B, the direct interaction was confirmed in this assay while expressing EuSAUR62 and OsPIN9 in pBT3-SUC and pPR3-N vectors, respectively. However, no interaction was observed when expressing EuSAUR62 and OsPIN9 in the pPR3-N vector and pBT3-SUC vector, respectively, suggesting EuSAUR62 positioned incorrectly by itself in yeast (pBT3-SUC vector expresses an extra signal peptide). Co-immunoprecipitation (Co-IP) assay also revealed the interaction between EuSAUR62 and OsPIN9 in vivo (Figure 6C). These results suggested that EuSAUR62 interacts with OsPIN9 and affects the auxin distribution.

EubZIP55 up-regulated the expression of EuSAUR62 and mediated indole-3-acetic acid accumulation under light conditions

After confirming the function of EuSAUR62 in seed size, the question remained as to why EuSAUR62 is differentially expressed in HL and WT. As the CDS sequences of EuSAUR62 in two types were proven to be the same, we used genome walking to sequence the promoter (≈1.6 kb) of EuSAUR62 in HL and
FIGURE 5
Grain size and histology comparison of the WT and OE-EuSAUR62 rice. After harvest, (A) morphologies of the mature grains produced by OE-EuSAUR62 and WT were observed. The grains generated by the OE-EuSAUR62 were significantly longer than that of WT. Scale bar = 1 cm. Histological analysis of the glumes (B) and outer glume cells (C) at heading stage. The glumes of WT and transformant were observed using a stereo microscope (Scale bar = 4 mm). The outer glume cells were observed under a scanning electron microscope (Scale bar = 100 µm). After harvest, 10 grains per line were collected randomly for size and weight measurement. OE-EuSAUR62 significantly improved the grains in weight (D) and length (E) compared with the WT. The glume length of OE-EuSAUR62 rice was longer than the WT (F). Length of the outer glume cells was longer in OE-EuSAUR62 rice compared to the WT (G). Statistical count is shown as the frequency distribution of the cell length. (H) Localization of IAA in the rice ovary (white arrow) of WT and OE-EuSAUR62 at the flowering stage (Scale bar = 500 µm). (I) The expression of EuSAUR62 was detected using western blot analysis with an anti-GFP antibody. The β-actin was used as an internal control. The negative controls are represented in the supplementary material. Data are mean ± SD. One-way ANOVA and post hoc statistical analysis was performed (**p < 0.01, ***p < 0.001). OE-EuSAUR62, EuSAUR62 overexpression lines; WT, wild type rice.
WT. However, there was no difference in promoter sequences between the two types (Supplementary Figure 8), suggesting that the different expression of EuSAUR62 between HL and WT is not attributable to mutations in the gene sequence. Therefore, we focused on transcription factors that regulate EuSAUR62 expression in Euryale. Then, we used the JASPAR database and DNA-pulldown to screen the transcription factors (TFs) that potentially bind to the promoter of EuSAUR62. By setting the relative profile score threshold to 100%, we found that its promoter contains specific motifs that can be bound by five types of TFs (Supplementary Table 1), indicating that different proteins bind to the promoter of EuSAUR62 (EuSAUR62pro) between HL and WT. Next, products of DNA pulldown were electrophoresed by SDS-PAGE and silver stained, obviously different protein bands were detected (Supplementary Figure 9A), indicating that different proteins bind to the promoter of EuSAUR62 (EuSAUR62pro) between HL and WT. Next, products of DNA pulldown were identif...
asymmetrical distribution of IAA, we treated HL and WT seedlings under constant light or dark conditions to observe the hook curvature phenotype. The degree of curvature was represented as a perpendicular line in the upper right of Figures 8C,D, during 3 days light treatment, apical hooks of HL were observed to be more curved than that of WT.

### Table 2: Proteins from DNA-pulldown were identified by LC-MS/MS.

| Group                  | Names1                   | Coverage (%) | Unused2 | Reference organism | Unique PepSeq |
|------------------------|--------------------------|--------------|---------|--------------------|---------------|
| WT                     | Histone H2A              | 19.72        | 4.29    | Arabidopsis        | NKGIDGIGASQGE, HQQLAVR, AGLQFPVGR |
|                        | DNAse I-like superfamily protein | 4.71 | 2.26    | Arabidopsis        | VSTKWNLSLR, QHETYTRGLR |
|                        | Putative lar-red impaired response | 1.13 | 2       | Oryza sativa       | KAFNLSGNIKAK   |
|                        | Nucleosome assembly protein 1 | 3.18 | 2       | Oryza sativa       | AGTEHIVYPGK   |
|                        | Beta-glucosidase-like protein | 1.60 | 2       | Arabidopsis        | IGEATALEVR   |
|                        | Alpha/beta-Hydrolases superfamily protein | 2.65 | 2       | Arabidopsis        | AGFAGDDAPR   |
|                        | NAC-A/B domain-containing protein | 6.90 | 2       | Arabidopsis        | SPASDTYIFGEAK |
|                        | Histone H2A              | 6.51         | 4       | Oryza sativa       | INPVLLFK       |
|                        | Calcium-dependent lipid-binding (CalB domain) family protein | 1.52 | 2       | Arabidopsis        | GSSSVSVDK     |
|                        | 60S ribosomal protein L23A | 8.55 | 2       | Arabidopsis        | LTPDYDIALDVK   |
|                        | Phytoclome kinase substrate 1 | 5.14 | 2       | Arabidopsis        | LMEPNTLNSIPK   |
| HL                     | Histone H2B              | 24.19        | 6.35    | Oryza sativa       | LVLPGEAK, QVHPDGIESSK, YNKPITPITSR |
|                        | ATP synthase subunit alpha | 6.48 | 6.21    | Oryza sativa       | VYDALGVPIEGK, TGGVVDVPGK, AVDSUVPGK |
|                        | Histone H4               | 31.07        | 6       | Oryza sativa       | DNNQGRTKPAIR, DAVTTYTEHAR, DGLIYEER |
|                        | ATP synthase subunit beta | 5.88 | 5.22    | Oryza sativa       | IGLPFGGAGVYK, VLNTGSFFTYPVGR |
|                        | Eukaryotic translation initiation factor 4A-1 | 6.03 | 4.74    | Arabidopsis        | GLDVYQQAQSTGK, YLITTDLAK |
|                        | Elongation factor 1-alpha | 4.70 | 4.69    | Oryza sativa       | IKGIGTVYPGR, STTGHLYK |
|                        | HSP90.1                  | 3.40         | 4.56    | Arabidopsis        | GVDSDDLPLNSIR, AVENSFPLER |
|                        | HSP70.1                  | 4.30         | 4.44    | Arabidopsis        | NDTVYFPHNDSQR, TFPVYAFTDSER |
|                        | Fructose-bisphosphate aldolase | 7.26 | 4.44    | Oryza sativa       | VAPEVIAFYTIR, GIALAESTGIGK |
|                        | Actin-1                  | 7.96         | 4.26    | Oryza sativa       | VAPEFHPFILTEAPLPK, DAYVGEAQSKER |
| T-complex protein 1 subunit delta | 4.49 | 4       | Oryza sativa       | GINQLVIDEAEAR, LGGTVTDITELIR |
|                        | Histone H3               | 10.65        | 3.06    | Oryza sativa       | STELLIR, EIAQFDKTDLR |
|                        | NAC-A/B domain-containing protein | 13.30 | 2.98    | Arabidopsis        | DIEIUVMTQAVGSR, SPASDTYFIPGK |
|                        | Sucrose synthase         | 1.36         | 2.27    | Arabidopsis        | IKQQQNLNTPR |
|                        | Calnexin                 | 2.42         | 2.26    | Arabidopsis        | LONGLECGGAYKL |
|                        | Terminal flower 1        | 7.91         | 2.24    | Arabidopsis        | VVGYDLFDFFTPK |
|                        | 40S ribosomal protein     | 2.62         | 2.23    | Arabidopsis        | LLIDTDPR    |
|                        | Nucleic acid-binding     | 8.28         | 2.21    | Arabidopsis        | DYOQDKADVLK |
|                        | Biotin carbonyl carrier protein of acetyl-CoA carboxylase | 4.31 | 2.11    | Arabidopsis        | SPQGEPFFVKY |
|                        | Mov34/MPN/PAD-1 family protein | 3.02 | 2.04    | Arabidopsis        | AVAVYVDPFPQK |
|                        | Histone H2A              | 6.00         | 2.03    | Arabidopsis        | AGLQFPVGR   |
|                        | 265 proteasome regulatory particle triple-A ATPase subunit4 | 3.00 | 2.01    | Arabidopsis        | GYVLGYGPQK |
|                        | ATP synthase             | 1.93         | 2.01    | Oryza sativa       | LAADIPLTFLQQR |
|                        | Glycin-rich RNA-binding protein | 5.56 | 2       | Oryza sativa       | DAIEMGNIK |
|                        | Ras-related protein      | 5.31         | 2       | Oryza sativa       | LQIWWTAGQER |
|                        | L11 domain containing ribosomal protein | 5.42 | 2       | Oryza sativa       | IGPILGIPK |
|                        | Glycolyl hydrolase family 3 N terminal-domain containing protein | 1.60 | 2       | Oryza sativa       | IGATEALYR |
|                        | G-box binding factor     | 4.69         | 2       | Oryza sativa       | TYPDVEELYTEER |
|                        | Glycoconyltransferase    | 2.78         | 2       | Arabidopsis        | TVYDAMMTIPEK |
|                        | Phosphoserine aminotransferase | 2.35 | 2       | Oryza sativa       | FGLIYAIAGQK |
|                        | NF22                     | 3.31         | 2       | Arabidopsis        | ALGTEIEWYPGK |

1 "Names" were re-annotated in Uniprot database, and "uncharacterized protein" were deleted. 2 Only unused ≥ 2 were shown.
FIGURE 7
EubZIP55 activates expression of EuSAUR62. (A) The subcellular localization of EubZIP54/55 in tobacco leaves. EubZIP54/55 were fused to GFP and transfected into tobacco leaves. Merged images including bright field, H2B-mCherry, and GFP fluorescence were observed using a confocal laser scanning microscope. Relative expression of EubZIP54 (B) and EubZIP55 (C) in the fruits of WT and HL was detected using qRT-PCR. (D) Yeast one-hybrid assay for the DNA-protein interaction between the EuSAUR62pro and EubZIP54 or EubZIP55. The indicated constructs were transformed into yeast reporter strain Y187 and grown on SD–L-T-H media with different concentrations of 3-AT. pGADm53 vs. pHIS-p53 was used as a positive control, and pGADT7 vs. pHIS-EuSAUR62pro was used as a negative control. Schematic design is shown on the left (E). Transcription repression and activation activity of EubZIP54 (F) and EubZIP55 (G) on EuSAUR62, respectively, which is expressed by the ratio of firefly luciferase to Renilla luciferase (LUC/REN). (H) GUS activity of EuSAUR62pro and 35S promoter was compared in tobacco leaves (Scale bar = 2 mm). p35S:GUS was used as a positive control, and empty:GUS was used as a negative control. Two-tail Student’s t-test was used for statistical analysis; Data are mean ± SD. *p < 0.05, **p < 0.01. EuSAUR62pro, EuSAUR62 promoter; HL, hybrid Euryale lines; WT, wild type (North Gordon Euryale).

Although dark treatment inhibited the hook curvature in both types, HL still generated hooks with a slightly more pronounced curve than WT in the dark, indicating that the auxin location was remarkably changed in HL under light conditions. We speculated that the stronger hook curvature in HL was attributed to the high expression of EuSAUR62 and EubZIP55 under light conditions. Thus, the expression levels of EuSAUR62 and EubZIP55 in these seedlings were determined by qRT-PCR. As expected, it was found that their expression in HL is significantly elevated following light treatment for 3 days compared to that in the dark, and both EuSAUR62 and EubZIP55 are more highly expressed in HL than in WT under constant light or dark conditions (Figures 8A,B). Overall, our results suggested that EubZIP55 up-regulates the expression of EuSAUR62 and alters the distribution of IAA under light stimulation.

Discussion

Hybrid Euryale lines promote the enlargement of cells by distributing indole-3-acetic acid around the ovary, which contributes to larger seeds

Euryale is an economic crop, providing food and medicine resources for many people in China and India (Jha and Sharma, 2010; Jha et al., 2018; Zhang et al., 2019). Biomass
is a crucial agronomic trait in Euryale, but the molecular mechanisms that regulate it remain unclear. To improve the biomass and yield of Euryale, we generated HL after selecting dominant lines and crossbreeding for many years (Huang et al., 2020). Compared to the WT, HL produced larger and heavier seeds (Figure 1) with a 35% elevation in yield. Seeds of HL also retained the potential medical effects, as some metabolites related to the pharmacologic effects of *Euryale ferox* remained unchanged, such as catechin and hyperoside (Table 1; Wu et al., 2013). Comparing the metabolites between two lines, distinct in the tryptophan metabolism pathway and concentration of IAA forced us to focus on the IAA homeostasis in Euryale (Figure 2). Our previous study showed that the concentration of IAA in fruits is similar at the same growth stages in two lines, while the location of IAA is different. In particular, IAA is mainly distributed in the exocarp in WT and is enriched around the ovary in HL during reproductive growth (Huang et al., 2020). In this study, our histological analysis revealed that cells around the ovary are longer in HL, which supports our opinion that HL distributes its IAA around the ovary to promote cell elongation and ovary enlargement during reproductive growth, thereby resulting in larger seeds (Figure 3).

### EuSAUR62 regulates indole-3-acetic acid distribution by interacting with PINs

As we concluded above, IAA homeostasis is regulated differently in WT and HL during fruit growth. The IAA homeostasis depends on a complex interplay between IAA metabolism and IAA transport (Rosquete et al., 2012). To understand the molecular mechanisms underlying this regulation, *SAUR* family genes were analyzed because some of them have been reported to mediate both auxin synthesis and its transport in rice (Xu et al., 2017). Among *SAURs* in Euryale, *EuSAUR62* was selected due to its differential expression in HL and WT. *SAURs* are the largest family of early auxin response genes, but there have been few functional analyses to date (Ren and Gray, 2015). Previous studies have implicated that AtSAUR62 promotes cell expansion and proliferation in Arabidopsis (He et al., 2018). Therefore, we speculated that *EuSAUR62* may promote cell elongation due to its high homology with AtSAUR62 (Figure 4A). To confirm whether *EuSAUR62* regulates seed size by mediating the distribution of IAA in Euryale, we generated *EuSAUR62* gain-of-function rice. *OE-EuSAUR62* lines produced longer and heavier grains than
WT, but had no effect on the grain width (Figures 5A,D,E and Supplementary Figure 4B), indicating a role for EuSAUR62 in promoting cell elongation. To further confirm its functions, glumes and outer glume cells were observed. Compared to the WT, glumes in HL were longer and their outer glume cells were significantly elongated (Figures 5B,C,F,G). In addition, EuSAUR62 also increased plant height and leaf angle by promoting the elongation of parenchyma and pulvinus cells (Supplementary Figure 5). Based on these results, we confirmed that EuSAUR62 promotes cell elongation during plant growth.

Among all plant hormones, auxin has a unique property, as it undergoes directional, cell-to-cell transport facilitated by auxin carrier proteins (Harrison, 2017). Among these proteins, the PIN family plays a prominent role in regulating auxin efflux due to the asymmetric subcellular localization of their proteins (Hernandez-Hernandez et al., 2018). As we concluded previously, HL distributes its IAA around the ovary to promote seed development, we subsequently asked if EuSAUR62 mediates localization of IAA. Then, the immunofluorescence assay confirmed that IAA was much more enriched in the ovary in OE-EuSAUR62 lines compared to the WT (Figure 5H). Further, prediction from the STRING database suggested that EuSAUR62 regulates IAA location in rice by interacting with OsPIN9 proteins, as it has not been reported that SAURs directly regulate IAA distribution. The BiFC, mY2H, and Co-IP assays confirmed their interaction (Figure 6). Although the function of OsPIN9 has not been reported yet, the expression pattern in tissues and phylogenetic analysis indicated that it plays a distinct role in regulating auxin-driven organ development (Balzan et al., 2014; Bennett et al., 2014). We speculated that EuSAUR62 might affect the phosphorylation level of OsPIN9, which affects the IAA distribution in rice. However, post-translational regulation between these two proteins needs further investigation. In this study, we did not apply NPA to verify our hypothesis, that is because, although NPA leads to inhibition of auxin export activity by interacting directly in a high-affinity manner with PINs, it also influences the expression level or stability of EuSAUR62 mRNA (Figures 4F,G; Abas et al., 2021). Genes involved in IAA biosynthesis are also important for its distribution, such as amidase 1 (AMI1), tryptophan aminotransferase of Arabidopsis 1 (TAA1), nitrilase (NIT), YUCCAs (YUC), but did not express differently between the WT and HL at the same growth stage (Supplementary Figure 6). The fact that OsPIN9 is not induced by IAA suggests that some signaling molecules may be involved in the IAA response and transport of OsPIN9 (Wang et al., 2009). Rapid response of EuSAUR62 in IAA concentration (Figures 4D,E), and interaction between EuSAUR62 and OsPIN9 proteins (Figure 6) make us conclude that EuSAUR62 functions as a signaling molecule linking IAA and OsPIN9 at the protein level. Specifically, a slight alteration in IAA level induces changes in expression of EuSAUR62, which affects IAA distribution by interacting with PIN-FORMED proteins. However, the function of OsPIN9 should be investigated further.

**EubZIPS regulate the expression levels of EuSAUR62 in light conditions**

To understand the underlying regulation in EuSAUR62 expression, we first confirmed that there is no mutant in EuSAUR62 sequences between HL and WT (Supplementary Figure 8). Then, we speculated that the different expression of EuSAUR62 was induced by up-stream TFs. Thus, we performed a DNA-pulldown assay to investigate the TFs that directly bind to EuSAUR62pro. A large number of histone modification-relative proteins were detected in LC-MS/MS assay (Table 2), indicating that epigenetic modification mediates the expression of EuSAUR62. Normally, histone modification is recruited by TFs to enhance or repress its function in regulating gene expression (Field and Adelman, 2020), further suggesting the existence of at least one TF in regulating the expression of EuSAUR62. Combined with JASPAR and transcriptome data, EubZIP55 and EubZIP54, detected in the DNA-pulldown assay, were chosen for further analysis. Expression of both EubZIP55 and EubZIP54 were much higher in HL compared to the WT (Figures 7B,C). The Y1H assay indicated a strong interaction between EubZIP55 and the EuSAUR62pro (Figure 7D). Interestingly, EubZIP55 activated the expression of EuSAUR62, but EubZIP54 repressed it (Figures 7E,G). In
Specifically, the light activates the expression of *EubZIP55* in the exocarp. The presumed model is shown in Figure 9, which potentially results in the development of pricks on seed size. However, this molecular mechanism is not activated by *EubZIP55* proteins, thereby promoting ovary enlargement and increasing a repressor to keep the expression of *EuSAUR62* at a normal level.

A previous study placed the AtbZIP proteins into ten groups: A to I, and S (Jakoby et al., 2002). Both AtbZIP55 and AtbZIP54 belong to the G group, denoting that light activation has been proven to stimulate their subcellular localization into the nucleus (Terzaghi et al., 1997). It has been reported that apical hook development is a suitable model for further promoting the mediation of light and the location of IAA on plant growth regulation, as auxin-response and auxin-transport proteins jointly define the spatial and temporal accumulation of auxin during apical hook development (Vandenbussche et al., 2010; Zadnikova et al., 2010, 2016; Beziat et al., 2017; Beziat and Kleine-Vehn, 2018). Euryale is an aquatic plant whose seedlings exhibit auxin-mediated apical curvature under light conditions. We speculated that the *EubZIP55-EuSAUR62* module might enhance apical curvature in HL under light treatment. Thus, we treated HL and WT seedlings under constant light or dark conditions to observe the degree of the hook curve. As expected, light significantly enhanced the degree of hook curve (Figures 8C,D) and up-regulated the expression of *EubZIP55* and *EuSAUR62* in HL, while this up-regulation in WT was much lower (Figures 8A,B). In addition, *EubZIP55* and *EuSAUR62* were more highly expressed in HL under light or dark conditions than in the WT (Figures 8A,B). Results revealed that the light-mediated apical hook of Euryale is regulated by the polar distribution of IAA, which is controlled by *EubZIP55* and *EuSAUR62*. Therefore, in this study, we found that it is *EubZIP55* that mediates light-induced expression of *EuSAUR62* in Euryale. We did not focus on the repressor factor *EubZIP54* because the underlying mechanism involved in the competition of *EubZIP55* and *EubZIP54* needs to be further clarified. Extensive studies have reported that the ELONGATED HYPOCOTYL5 (HY5), a bZIP-type transcription factor, tightly controls the light response and photomorphogenesis (Nawkar et al., 2017; Li H. M. et al., 2020; Ortigosa et al., 2020). GBF3 mRNA is accumulated by light exposure in a similar manner to HY5, suggesting that it also participates in the early light response (Kurihara et al., 2020). However, the underlying mechanism of light response of *EubZIP55* in Euryale needs further investigation.

Overall, we highlighted a molecular bridge role for *EuSAUR62* between light and IAA distribution in Euryale. Specifically, the light activates the expression of *EubZIP55* and subsequently up-regulates the expression of *EuSAUR62*, which affects the distribution of IAA by interacting with PIN family proteins, thereby promoting ovary enlargement and increasing seed size. However, this molecular mechanism is not activated in WT, which potentially results in the development of pricks on the exocarp. The presumed model is shown in Figure 9.
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