Gnp4/LAX2, a RAWUL protein, interferes with the OsIAA3–OsARF25 interaction to regulate grain length via the auxin signaling pathway in rice

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Abstract

Grain length is one of the determinants of yield in rice and auxin plays an important role in regulating it by mediating cell growth. Although several genes in the auxin pathway are involved in regulating grain length, the underlying molecular mechanisms remain unclear. In this study we identify a RING-finger and wd40-associated ubiquitin-like (RAWUL) domain-containing protein, Gnp4/LAX2, with a hitherto unknown role in regulation of grain length by its influence on cell expansion. Gnp4/LAX2 is broadly expressed in the plant and subcellular localization analysis shows that it encodes a nuclear protein. Overexpression of Gnp4/LAX2 can significantly increase grain length and thousand-kernel weight. Moreover, Gnp4/LAX2 physically interacts with OsIAA3 and consequently interferes with the OsIAA3–OsARF25 interaction in vitro and in vivo. OsIAA3 RNAi plants consistently exhibit longer grains, while the mutant osarf25 has small grains. In addition, OsARF25 binds to the promoter of OsERF142/SMOS1, a regulator of organ size, and positively regulates its expression. Taken together, the results reveal that Gnp4/LAX2 functions as a regulator of grain length through participation in the OsIAA3–OsARF25–OsERF142 pathway and that it has potential value for molecular breeding in rice.

Keywords: Auxin, Gnp4/LAX2, grain length, rice, transcription regulation.

Introduction

Grain weight, grain number per panicle, and panicle number are major factors influencing yield in rice (Oryza sativa). Among them, grain weight is least affected by environmental factors (Sakamoto and Matsuoka, 2008). Nevertheless, grain weight remains a complex quantitative trait that is affected by multiple genes. Grain length, grain width, grain thickness, and
grain filling rate are contributory factors that determine grain weight (Xing and Zhang, 2010). Molecular studies of each of these characters are essential for a complete understanding of their potential roles in yield improvement.

To date, many genes contributing to grain weight in rice have been isolated, such as GS3, GW5, GS5, GL3.1, GW7/GL7, GW8, GLW7, OsGRF4, Big Grain1, XAO, SLG, OsLG3, and OsLG3b (Weng et al., 2008; Mao et al., 2010; Li et al., 2011b; Jiang et al., 2012; Wang et al., 2012, 2015; Zhang et al., 2012; Duan et al., 2015; Liu et al., 2015; Feng et al., 2016; Si et al., 2016; Yu et al., 2017, 2018). While they all ultimately affect cell expansion or proliferation, they can be classified into several groups according to the pathways involved, including the G-protein signaling pathway, the proteasomal degradation pathway, the transcriptional regulation-related pathway, and the plant hormone biosynthesis or signaling transduction pathways (Zuo and Li, 2014; Li and Li, 2016). Although these genes have been cloned and functionally characterized, knowledge of the underlying molecular mechanisms and genetic interaction networks remain elusive and fragmentary. Consequently, it is important to isolate novel grain size-associated regulators in order to understand the molecular mechanism behind grain weight in rice.

The RING-finger and ud40-associated ubiquitin-like (RAWUL) domain is a new member of the ubiquitin superfamily and has been found in the same polypeptide chain as the RING finger domain in the polycomb repressive complex 1 (PRC1) RING family, and in the same polypeptide domain as the WDR48-p80 protein family (Sanchez-Pulido et al., 2008). In Arabidopsis, two families of RING-finger proteins have been characterized as RAWUL domain-containing proteins, namely AtRING1A/B and AtBM1A/B/C (Xiao and Wagner, 2015), and play roles in developmental phase transitions, cell proliferation during organ growth, and water-stress responses. However, little is known about RAWUL domain-containing proteins in rice and other crop plants.

Indole-3-acetic acid (auxin) plays an important role in growth and development of plants by regulating many biological processes (Gallavotti, 2013; Ljung, 2013). The molecular mechanisms of auxin perception are relatively well understood in different plant species (Salehin et al., 2015). The auxin signaling transduction pathway consists of four components, namely the auxin receptors, the AUX/IAA repressors, the auxin response factors (ARFs), and the downstream target genes (Salehin et al., 2015). Signaling transduction is initiated by the perception of auxin by the TIR1/AFB receptors that encode F-box proteins and are components of an E3 SCF ubiquitin ligase complex (Dharmsirirat et al., 2005). AUX/IAA co-receptors function as repressors of the pathway by directly binding to the ARF transcription factors, and together with the co-repressor protein TOPESS they repress their activities (Szemenyei et al., 2008). Auxin triggers the formation of the AUX/IAA-SCF TIR1/AFB co-receptor complex and the degradation of the AUX/IAA protein in a 26S proteasome-dependent manner, which in turns results in the de-repression of the ARFs and thus the transcriptional activation of their target genes (Salehin et al., 2015; Dezfultan et al., 2016).

There are 31 AUX/IAAs and 25 ARF protein genes in the rice genome (Jain et al., 2006; Wang et al., 2007). To date, several rice AUX/IAAs have been associated with specific phenotypic effects, including root development, plant architecture, and biotic and abiotic stress responses. OsIAA3 (referred to as OsIAA31 by Jain et al., 2006) was the first functionally characterized AUX/IAA protein in rice and its gain-of-function causes growth defects in leaf blades and crown roots (Nakamura et al., 2006). Functional analysis of ARFs have been mainly based on studies of loss-of-function mutants. For example, loss of function of OsARF16 and OsARF12 lead to iron-deficiency responses (Qi et al., 2012; Shen et al., 2013), while OsARF23–OsARF24 has been shown to promote cell growth and morphogenesis by regulating RICE MORPHOLOGY DETERMINANT (RMD) expression (Li et al., 2014). These studies highlight many aspects of plant growth that are controlled by auxin-related pathways, but the underlying regulatory processes have only been identified in a few cases. Thus, it is still not clear how these AUX/IAA and ARF networks achieve target-specificity, and whether other factors or signaling proteins participate in this regulatory process.

In our previous studies, a natural mutant with defective development of lateral spikelets on the secondary panicle branches and increased grain length was characterized and the candidate gene was designated as Gap4 (Gain number per panicle 4), which shares the LAX2 (LAX PANICLE2) locus (Tabuchi et al., 2011; Zhang et al., 2011b). Here, we report that Gap4/LAX2 encodes a RAWUL domain-containing protein and has a hitherto unknown role in regulating grain length. Gnp4 functions as a regulator of grain length by participating in an OsAUX/IAA–OsARF25–OsERF142 pathway.

Materials and methods

Plant material

Seeds of Oryza sativa subsp. japonica cv. Nipponbare and transgenic lines used in this study were generated within our laboratory. All the transgenic plants used for phenotypic evaluation were more advanced than the T3 generation. Rice accessions used for haplotype analysis were selected from the rice mini core collection (Zhang et al., 2011a). The T-DNA insertion mutant Osarf25 and its wild-type (Hwayoung) were provided by Dr De’an Jiang (Zhejiang University) and the OsERF3-overexpression plants were provided by Dr Rongfeng Huang (Chinese Academy of Agricultural Sciences, Beijing).

Plasmid construction and rice transformation

To construct the overexpression plasmid Pmo35S::Gap4, the full coding sequence of Gap4 was amplified from the cDNA of Nipponbare, digested with AscI and SpeI, and cloned into the binary vector pMDC32 (Curts and Grossniklaus, 2003). For construction of the GUS (β-glucuronidase) plasmid, the 2-kb promoter region of Gap4 was amplified from the DNA of Nipponbare, digested with Pst and AscI, and cloned into the binary vector pMDC162 (Curts and Grossniklaus, 2003). For construction of GFP (green fluorescent protein) plasmids, the coding region of Gap4 was inserted into ProSuper1330::GFP vector, and OsIAA3 and OsIAA17 were amplified and digested with SpeI and AscI, and cloned into the binary vector pMDC83 (Curts and Grossniklaus, 2003). To construct an OsIAA3-RNAi vector, a 235-bp fragment containing part of the coding sequences and the 3′–UTR region was amplified from the cDNA of Nipponbare, digested with SalI and SpeI, and cloned into the pTCK303 vector (Wang et al., 2004) to generate the forward insertion. Next, a dsRNAi fragment obtained by digestion with BamHI and KpnI was cloned into the same vector to generate the reverse insertion.

To construct the overexpression plasmid Pmo35S::Gap4, the full coding sequence of Gap4 was amplified from the cDNA of Nipponbare, digested with AscI and SpeI, and cloned into the binary vector pMDC32 (Curts and Grossniklaus, 2003). For construction of the GUS (β-glucuronidase) plasmid, the 2-kb promoter region of Gap4 was amplified from the DNA of Nipponbare, digested with Pst and AscI, and cloned into the binary vector pMDC162 (Curts and Grossniklaus, 2003). For construction of GFP (green fluorescent protein) plasmids, the coding region of Gap4 was inserted into ProSuper1330::GFP vector, and OsIAA3 and OsIAA17 were amplified and digested with SpeI and AscI, and cloned into the binary vector pMDC83 (Curts and Grossniklaus, 2003). To construct an OsIAA3-RNAi vector, a 235-bp fragment containing part of the coding sequences and the 3′–UTR region was amplified from the cDNA of Nipponbare, digested with SalI and SpeI, and cloned into the pTCK303 vector (Wang et al., 2004) to generate the forward insertion. Next, a dsRNAi fragment obtained by digestion with BamHI and KpnI was cloned into the same vector to generate the reverse insertion.
All plasmids were introduced into *A. tumefaciens* EHA105. Rice transformation was conducted by the *Agrobacterium*-mediated method as previously described (Hiet *et al.*, 1994). A full list of primers used in this study can be found in Supplementary Dataset S5 at JXB online.

**Phylogenetic analysis**

The amino acid sequence of Gnp4 was used to BLAST search its closest homologous proteins from other plant species against databases in Uniprot (http://www.uniprot.org/). Multiple-sequence alignment was optimized with the Megalign program in the DNASTAR software package (http://dnastar.com). A neighbor-joining tree for homologous proteins was constructed using MEGA5.0 (Tamura *et al.*, 2011).

**GUS staining**

Tissues of transgenic plants containing the *ProGap4::GUS* vector sampled at different growth stages were fixed in GUS-staining solution (50 mM Na₂HPO₄, 10 mM Na₂EDTA, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.1% TritonX-100, 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid). After 12 h at 37 °C, the stained tissues were dehydrated in an ethanol series of (100%, 95%, 85%, 75%) to remove the chlorophyll, and photographed using a digital camera (Nikon D900).

**Total RNA extraction and qRT-PCR analysis**

Total RNA was extracted from different plant tissues using RNAiso Plus (Takara). First-strand cDNA was synthesized in 25 μl of reaction mixture containing 2 μg Dnase I-treated RNA, 200 U M-MLV reverse transcriptase (Takara), 40 U Recombinant RNase Inhibitor (Takara), and 0.1 μM oligo(dT). Quantitative RT-PCR was carried out in total volumes of 20 μl containing 10 μl SYBR EX Taq premix (Takara), 0.4 μl Rox Reference Dye II (Takara), 0.2 mM gene-specific primers, and 2 μl of first-strand cDNA on an ABI 7500 real time PCR system. *OsActin1* was used as a reference.

**Subcellular localization**

The *ProSuper::Gnp4-GFP*, *Pro35S::OsIAA3-GFP*, or *Pro35S::OsIAA17-GFP* plasmids were transformed into *A. tumefaciens* EHA105 and co-infiltrated into tobacco epidermal leaf cells of *Nicotiana benthamiana*. After 3 d of incubation at 25 °C, the leaves were sampled for confocal microscopy (OlympusFV1000). The GFP and mCherry markers were excited with a 488-nm and 543-nm laser, respectively. Emission spectra were collected at 500–550 nm for GFP, and 565–615 nm for the mCherry marker.

**Yeast two-hybrid assays**

The full-length and truncated fragment series of *Gnp4* were amplified and recombined into a linearized pSPYCE(M) vector to construct *Gnp4*-YFP<sup>C</sup>. Similarly, the full-length coding sequences of *OsIAA3* and *OsIAA17* were cloned into pSPYNE173 to construct *OsIAA3*-YFP<sup>B</sup> and *OsIAA17*-YFP<sup>B</sup>, respectively. These plasmids were transformed into *A. tumefaciens* EHA105. For transient expression, the strains, together with the p19 strain and mCherry ER-rk CD5-959 (Nelson *et al.*, 2007), were co-infiltrated into 5–6-week-old *N. benthamiana* leaves. Tobacco epidermal leaf cells were observed with a confocal microscope (Olympus FV1000) 3 d after infiltration.

**Co-immunoprecipitation assays**

The full-length coding sequences of *OsIAA3* and *OsIAA17* were cloned into the *ProSuper::Myc* vector to construct *ProSuper::OsIAA3-Myc* and *ProSuper::OsIAA17-Myc*, respectively. The full-length coding sequence of *Gnp4* without the stop codon was amplified and recombined into a linearized *ProSuper::Myc* vector to construct *ProSuper::Gnp4-Myc*. Similarly, *Pro35S::HF-Gnp4* was constructed by recombining the full-length *Gnp4* with the *Pro35S::HF* vector. Co-immunoprecipitation was conducted as described previously (Zhang *et al.*, 2017).

**Yeast three-hybrid assays**

To construct the yeast three-hybrid plasmids, full-length *OsIAA3* and *OsIAA17* were amplified and recombined into the MCSI location of the pBridge vector in the EcoRI-BamHI site, resulting in Batl. For construction of BatII and BatIII, the truncated fragment and full-length *Gnp4* were amplified and recombined into the MCSII location of BatII in the Norl-BgII site, respectively. OsARF25-pGADT7 was used as prey. Bat and prey were co-introduced into yeast strain AH109 and incubated at 30 °C for 3–5 d, when equal optical-density yeast cells were plated out on selective medium. Qualitative evaluation was made of the interaction activity between bait and prey.

**Determination of β-galactosidase activity**

β-galactosidase activity assays with minor modification were conducted as previously described (Kippert, 1998). Yeast cells were collected and re-suspended in 800 μl Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol, pH 7.0), and placed on ice. β-galactosidase assays were conducted after equilibration at 30 °C for 15 min; 160 μl of 4 mg ml⁻¹ o-nitrophenyl-β-d-galactoside (ONPG) was added and the mixture was thoroughly vortexed before incubation at 30 °C. The reaction was stopped by addition of 400 μl 1M Na₂CO₃. The OD₄₅₀ and OD₄₂₀ values were determined. Three replicates were performed, each with five technical replicates.

**Scanning electron microscopy**

Samples were fixed in 2.5 % glutaraldehyde and vacuumized for 30 min, then stored overnight at 4 °C. The samples were subjected to dehydration in an ethanol gradient series: 50% ×2–3 times, 70%, 80%, 90%, and 95%, then stored overnight at 4 °C. The samples were subjected to dehydration in an ethanol gradient series: 50% ×2–3 times, 70%, 80%, 90%, and 95%, and then stored overnight at 4 °C. The samples were treated with a mixture of equal volumes of ethanol and isoamyl acetate for 30 min and with isoamyl acetate for 1–2 h. After critical-point drying they were coated with gold using ion-beam sputtering for deposition and observed using a S-3000N scanning electron microscope (Hitachi, Tokyo, Japan).

**Transient transcriptional activity assays**

The effector, reporter, and internal control plasmids were transformed into rice protoplasts using the PEG-mediated method. Total proteins were extracted using lysis buffer (Promega, E4550) after incubation at 25 °C for 12–16 h. GUS and firefly luciferase (LUC) activities were assessed as previously described (Zhang *et al.*, 2017).
Transcriptome analysis

High-quality total RNA was extracted from 10 young panicles (1 cm in length) of Gnp4-overexpression and wild-type plants. Illumina sequencing was performed using a HiSeq2000 system at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Gene Ontology (GO) analysis was conducted by searching the differentially expressed genes (DEGs) against the AgriGO database of Oryza sativa subsp. japonica (http://bioinfo.cau.edu.cn/agriGO/index.php). Analysis of the significantly enriched pathways was conducted using the KEGG database (http://genome.jp/kegg/). Protein domain analyses of DEGs were conducted at DAVID (https://david.ncifcrf.gov/tools.jsp). The file containing all the 2-kb promoter sequences of the rice genome was downloaded from RAP-DB (http://rapdb.dna.affrc.go.jp/) and filtered to obtain sub-files for AuxRE cis-element analysis using Perl script.

Yeast one-hybrid assays

To construct the ProOsERF142::LacZ and ProOsERF3::LacZ reporters, different promoter regions were recombined into the EcoRI and Xhol sites of the pLacZi2μ vector, respectively. For construction of pB42AD-OsARF25, full-length OsARF25 was recombined into the EcoRI and Xhol sites of the pB42AD (pG4-5) vector. The pB42AD-OsARF25 plasmid and the reporter constructs were co-transformed into the yeast EGY48 strain. Transformants were grown on SD/–Trp–Ura drop-out plates containing X-β-gal for blue colour development to detect the interaction. The ProFHY1::LacZ reporter and pB42AD-FHY3 were used as positive controls (Li et al., 2010).

ChIP-qPCR assays

The young panicles of Pro35S::OsARF25-FLAG plants were harvested and cross-linked, and then assays were conducted using a ChIP Assay Kit (P2078, Beyotime, China) according to the manufacturer’s instructions. The enriched DNA fragments were analysed by qRT-PCR using ABI7500 system and Software v2.0.5.

Accession numbers

Sequence data from this work can be found in the GenBank/EMBL databases under following accession numbers: Gnp4/LAX2 (Os04g0396500, KY673700), OsIAA3 (Os01g0231000), OsIAA17 (Os05g0230700), OsARF25 (Os12g0613700), OsERF142/SMOS1 (Os05g0389000), and OsERF3 (Os01g0797600).

Results

The gnp4 mutant shows longer grain length

In our previous work, Gnp4 was narrowed to a 10.7-kb region on chromosome 4, in which there was one predicted ORF (Os04g0396500). However, there was no DNA sequence difference between wild-type (WT) Nipponbare and the gnp4 mutant except for different DNA methylation levels of several nucleotides in the promoter region (Zhang et al., 2011b). We found that the increased grain length and reduced grain number were closely linked. (Fig. 1A, B). The F1 seeds from the cross between gnp4 and the WT or gnp4 and te, a rice tillering mutant (Lin et al., 2012), showed similar grain length to gnp4 (Fig. 1C, D). The expression level of Gnp4 was consistently higher in the gnp4 mutant than the WT and te, and in the

![Fig. 1](https://example.com/f1.png)

**Fig. 1.** High expression of Gnp4 correlates with longer grain length. (A) Grain morphologies of wild-type Nipponbare (Nip) and five recessive F2 segregates (R1-R5) of the cross between Nip and gnp4. (B) Grain length and grain number for Nip and the five recessive plants. Data are means (±s.e.m.) (n=50 grains from three panicles). (C, D) Grain morphology (C) and grain length (D) for Nip, gnp4, te, and F1 plants (n=50 grains). (E) The relative expression of Gnp4 in Nip, gnp4, and te (n=4 plants). (F) Genotype analysis of gnp4/gnp4, Gnp4/gnp4, and Gnp4/Gnp4 individuals using SDS-PAGE. (G) The relative expression of Gnp4 in gnp4/gnp4, Gnp4/gnp4, and Gnp4/Gnp4 individuals. Data are means (±s.e.m.) (n=5 plants). Different letters indicate significant differences between means according to LSD tests (P>0.05). (This figure is available in colour at JXB online.)
gnp4/gnp4 and Gnp4/gnp4 individuals compared with Gnp4/
Gnp4 individuals (Fig. 1E–G).

Next, we examined correlations between the expression lev-
elos of Gnp4 and grain length and grain number per panicle in
17 japonica accessions randomly selected from the rice mini
core collection (Zhang et al., 2011a). We found that grain length
but not grain number per panicle was correlated with Gnp4
expression levels over 3 years (Supplementary Fig. S1A, B).
These results indicated that the gnp4 mutation was an epigen-
etic change that might be correlated with longer grain length
with higher expression levels of Gnp4.

Overexpression of Gnp4/LAX2 increases grain length

To confirm whether the expression level of Gnp4 corre-
related with the grain length, an overexpression construct
(Pro35S::Gnp4) was introduced into the Nipponbare wild-
type (WT). Three overexpression lines (OE1, OE2, and OE3)
showed significantly increased grain length compared to WT
plants (Fig. 2A–C). The mean grain length in the lines OE1,
OE2, and OE3 were about 7.41 mm, 7.51 mm, and 7.66 mm,
respectively, compared to 7.09 mm in WT plants (Fig. 2D). In
addition, thousand-kernel weight was increased by 9.5%, 11%,
and 12.8% in the OE1, OE2, and OE3 lines, respectively, com-
pared to WT plants, but there was little change in grain width
(Fig. 2E, F). However, there was reduced yield per plant as seed
setting was reduced in the Gnp4 overexpression plants com-
pared with the WT and there were no significant differences in
grain number per panicle (except for OE1) and panicle num-
ber (Fig. 2G–J).

The spikelet glumes in rice set a limit to the final grain
size, and this is determined by co-ordinated cell expansion and
cell proliferation (Li and Li, 2016). We found that the mean
cell length in Gnp4 overexpression plants was significantly
increased compared to the WT, but the number of epidermal
cells in outer glume region decreased (Fig. 2K–O). Thus, the
longer grains in Gnp4 overexpression plants mainly resulted
from enhanced cell expansion in the spikelet hulls. In addi-
tion, the expression levels of several genes conferring larger
grain size by cell expansion were much higher in Gnp4 over-
expression plants than that the WT, including GL7, GLW7,
POSITIVE REGULATOR OF GRAIN LENGTH 1 (PGL1),
and POSITIVE REGULATOR OF GRAIN LENGTH 2
(PGL2) (Supplementary Fig. S2; Heang and Sassa, 2012; Wang
et al., 2015). Taken together, these results showed that overex-
pression of Gnp4 could increase rice grain length by promo-
tion of cell expansion.

Subcellular localization and expression patterns of
Gnp4/LAX2

Gnp4 is predicted to encode a 394-amino acid protein. We
identified six Gnp4 paralogs in rice, and five orthologues in
Arabidopsis, one in maize, one in sorghum, and one in brachyl-
odium distachyon (Fig. 3A). In all cases, the proteins con-
tained a conserved RAWUL domain at the C-terminus,
and they could be classified into two groups, namely Gnp4-
LIKE1 (Gnp4-L1) and Gnp4-LIKE2 (Gnp4-L2) according
to the amino acid sequences at the N-termini (Fig. 3B, C).
A C3H4-type zinc ring finger was present in the Gnp4-L2
group, whereas the Gnp4-L1 group was characterized by
numerous stretches of the same amino acid residue, such as
Arg, His, and Ser. Gnp4 belonged to the Gnp4-L1 group
(Supplementary Dataset S1).

Nicotiana benthamiana leaves infiltrated with Agrobacterium
haboring the ProSuper::Gnp4-GFP construct showed clear
GFP signals in the nuclei, indicating that Gnp4 is a nuclear
protein (Fig. 4A), which was consistent with the subcellu-
lar location of LAX2 in rice root cells (Tabuchi et al., 2011).
Histochemical analysis of different tissues from ProGnp4::GUS
transgenic plants showed that Gnp4 was widely expressed in
both the vegetative and reproductive tissues, but was especially
higher in stems and young panicles (Fig. 4B), consistent with
previous results (Supplementary Fig. S3). RNA from roots,
leaves, stems, sheaths, and panicles of different lengths were
isolated and used for quantitative RT-PCR analysis of Gnp4
expression, and the results were in agreement with the histo-
chemical analysis (Fig. 4C). The expression pattern of Gnp4/
LAX2 was thus consistent with a role in regulating grain length.

Gnp4/LAX2 interacts with OsIAA3 and OsIAA17 in
yeast and plant cells

To elucidate the potential mechanism by which Gnp4 influ-
ences grain length, a yeast two-hybrid assay was performed
to identify interacting proteins. Auto-transcriptional activa-
tion activity was not detected with the full-length and trun-
cated fragment constructs of Gnp4 (Supplementary Fig. S4).
Consequently, the full-length Gnp4 was used as a bait to
screen two yeast prey cDNA libraries. A total of 23 candi-
date interacting proteins were isolated, among which OsIAA3
(Os01g0231000, referred to as OsIAA3 by Jain et al., 2006) and
OsIAA17 (Os05g0230700), the nearest homologous protein
of OsIAA3 in rice, were identified (Fig. 5A; Supplementary
Fig. S5; Supplementary Table S1). Moreover, subcellular local-
ization showed that OsIAA3 and OsIAA17 also localized in
the nucleus and showed similar expression patterns to Gnp4
(Supplementary Fig. S6).

Next, we found that constructs containing the RAWUL
domain of Gnp4 (amino acids 101 to 394, 205 to 394, 224 to
394, 285 to 394, and 224 to 390) interacted with OsIAA3 and
OsIAA17, but no interaction was observed with the constructs
lacking the region corresponding to amino acids 380 to 390
(Fig. 5A). These results suggested that these 11 amino acids at
the C-terminus were required for interaction between Gnp4 and
OsIAA3 and OsIAA17. Furthermore, Tabuchi et al. (2011)
identified the last 15 amino acids of LAX2 as being important
for its interaction with LAX1, indicating the potentially critical
function of the RAWUL domain for protein–protein inter-
action. However, LAX1 was not pulled down by Gnp4 in our
yeast two-hybrid assays.

To further characterize the interactions between Gnp4 and
OsIAA3 and OsIAA17 in plant cells, a BiFC assay was con-
ducted by transient expression in tobacco leaf cells. As antici-
pated, fluorescence signals were observed in the nuclei of leaf
cells where Gnp4-YFP was co-expressed with OsIAA3-YFP.
or OsIAA17-YFP, but not with YFP alone (Fig. 5B). We also found that HF-Gnp4 proteins interacted with OsIAA3-Myc and OsIAA17-Myc proteins in tobacco leaves, but not HF proteins alone (Fig. 5C). Collectively, these results indicated that Gnp4 interacted with OsIAA3 and OsIAA17 in plant cells as well as in yeast cells.
Gnp4/LAX2 forms a dimer and interferes with the interaction between OsAUX/IAA and OsARF

Although Gnp4 is a nuclear protein, no transcriptional activity or recognizable DNA binding domain were found, indicating that it might not regulate transcription directly. We found that Gnp4 could form dimers in yeast and plant cells (Supplementary Fig. S7). Aux/IAA proteins are well established as transcriptional repressors of the ARFs that play a key regulatory role in plant growth and development (Salehin et al., 2015). Thus, we speculated that Gnp4 might function by modulating the interaction between OsAUX/IAA and OsARF. It is well documented that OsIAA3 and OsIAA17 interact with eight OsARF activators (Shen et al., 2010), among which OsARF25 showed a similar expression pattern to Gnp4 and high expression levels during inflorescence development (Supplementary Fig. S8). Next, a yeast three-hybrid assay was performed to explore the effect of Gnp4 on the OsARF25–OsIAA3 and OsARF25–OsIAA17 interactions. We constructed three kinds of bait (Bait I, II, and III) to test for interactions with OsARF25 as prey. Bait I contained only the full-length OsIAA3 or OsIAA17, Bait II contained the C terminus-truncated Gnp4 (Gnp4∆C), and Bait III contained an entire Gnp4 (Fig. 5D). Transcription of Gnp4∆C and Gnp4 can be conditionally regulated from the Met25 promoter (ProMet25), which is actively repressed in the presence of methionine but not in its absence. We found that yeast harboring Bait III grew much more slowly than that containing Baits I or II on selective medium lacking methionine (Fig. 5E). Moreover, β-galactosidase assays showed that the OsARF25–OsIAA3 and OsARF25–OsIAA17 interactions were suppressed when Gnp4 was expressed, but not when Gnp4∆C was expressed (Fig. 5E), suggesting that Gnp4 functioned as a ‘blocker’ of the interactions, at least in yeast cells.

A transient transcriptional activity assay was then conducted to confirm that Gnp4 suppressed the OsARF25–OsIAA3 and OsARF25–OsIAA17 interactions in planta. We found that the relative GUS activity increased significantly when the reporter was co-expressed with Pro35S::Gnp4-Myc (Fig. 5F, G).
Together, these results indicated that Gnp4 interfered with the OsIAA3–OsARF25 and OsIAA17–OsARF25 interactions, which may have de-repressed OsARF25 and enhanced the transcription of its downstream target genes in plant cells.

**Characterization of grain length in OsIAA3-RNAi and osarf25 plants**

We next reasoned that the reduced expression of the OsAUX/LA4 genes might mimic Gnp4/LAX2 overexpression. We constructed a RNAi vector of OsIAA3 and transformed it into the Nipponbare wild-type (WT). Two independent OsIAA3-RNAi plants, namely Ri31 and Ri33, were selected for detailed phenotypic analysis (Fig. 6A, B). We also checked the expression levels of several near-homologous genes of OsIAA3 and found no significant differences between the WT and Ri31 or Ri33, except that OsIAA19 was down-regulated in Ri31 (Supplementary Figs S5, S9). Compared with WT plants, grain length significantly increased in Ri31 and Ri33, and wider grains were observed in Ri33 (Fig. 6C–F). The thousand-kernel weights were increased by 4.7% and 8.0% in Ri31 and Ri33 plants, respectively, compared to the WT (Fig. 6G). Similar to Gnp4-overexpression plants, Ri31 and Ri33 showed significantly decreased seed setting, but similar grain numbers per panicle (except for Ri33), and panicle numbers (Fig. 6H–J). These data suggested that the knockdown of OsIAA3 expression had a positive effect on grain length.

To clarify the function of OsARF25, we obtained a T-DNA insertion mutant, osarf25, in the variety Hwayoung (HY) background. Homozygous mutant plants were identified by genomic DNA and mRNA levels (Supplementary Fig. S10). Phenotypic analysis showed that osarf25 had smaller grains and panicles. The thousand-kernel weight and grain numbers per panicle were significantly reduced in osarf25 relative to HY (Fig. 7). In addition, we found that the outer glume cell length, but not cell number, was significantly lower in osarf25 than in HY (Supplementary Fig. S11). Hence, we concluded that OsARF25 modulated grain length by cell expansion, as does Gnp4.

**The grain-size regulators OsERF142/SMOS1 and OsERF3 function downstream of Gnp4/LAX2 and OsARF25**

To further investigate the downstream targets regulated by Gnp4, transcriptome analysis was performed for Gnp4-overexpression and wild-type plants. A total 846 and 449 genes were up- and down-regulated, respectively, in Gnp4-overexpression plants compared to the WT (Supplementary Dataset S2). The expression levels of several genes were quantified by qRT-PCR to confirm the RNA-seq results (Supplementary Fig. S12). Gene ontology (GO) analysis showed that genes affected by the overexpression of Gnp4 were significantly enriched in 40 GO terms (Supplementary Table S2). Among these terms, several specific GO keywords were consistent with the molecular function of Gnp4, such as GO:0045449 (regulation of transcription), GO:0003700 (transcription factor activity), GO:0030528 (transcription regulator activity), and GO:0005634 (nucleus) (Supplementary Fig. S13). Two KEGG pathways were enriched, namely ko04075 (plant hormone signal transduction) and ko04626 (plant–pathogen interaction) (Supplementary Fig. S14). These results were consistent with our proposed role for Gnp4 in the regulation of grain size.
Fig. 5. Gnp4/LAX2 interferes with the OsIAA3–OsARF25 and OsIAA17–OsARF25 interactions. (A) Gnp4 interacts with OsIAA3 and OsIAA17 in yeast cells. The RAWUL domain of Gnp4 is indicated by the gray boxes. SD/–T–L, selective medium lacking Trp and Leu; SD/–T–L–H–A, selective medium lacking Trp, Leu, His, and Ade. (B) BiFC assays showing the interactions between Gnp4 and OsIAA3 and OsIAA17 in tobacco leaf epidermal cells. CD3-959 is a mCherry marker (ER-rk CD3-959). Scale bars are 25 μm. (C) Co-immunoprecipitation assays showing that HF-Gnp4 interacts with OsIAA3-Myc and OsIAA17-Myc in plant cells. (D) Schematics of baits and prey used for yeast three-hybrid assays. (E) Yeast three-hybrid assays showing that the OsIAA3–OsARF25 (top panel) and OsIAA17–OsARF25 (bottom panel) interactions were suppressed in the presence of full-length Gnp4. Relative interaction activities were evaluated using β-galactosidase assays. Data are means (±s.e.m.). Three replicates were performed, each with five technical replicates. Significant differences were determined using Student’s t-test: **P<0.01. (F, G) Transient assays showing the effect of Gnp4 on the OsIAA3–OsARF25 and OsIAA17–OsARF25 interactions. Relative GUS and LUC activities were measured and normalized to LUC activity. Data are means (±s.e.m.), four replicates were performed, each with five technical replicates. Different letters indicate significant differences between means according to LSD tests (P>0.05).
We also analysed the gene functional categories of the up- and down-regulated genes using the DAVID database. Five terms were significantly enriched for the up-regulated genes and one term was enriched for down-regulated genes (Supplementary Table S3). Among the up-regulated genes, the most significantly enriched was the AP2 (APETALA2) domain, which plays roles in various biological processes of plant development and abiotic and biotic stress responses (Licausi et al., 2013). These results provided hints on the downstream targets of Gnp4 that are potentially involved in regulating grain length. A total of 28 genes encoding AP2 domain-containing proteins were found to be up-regulated in Gnp4-overexpression plants and were selected for further investigation. Interestingly, the AuxRE (TGTCTC) cis-element was found in the 2-kb promoter region of 18 of these genes (Supplementary Table S4). Among them, OsERF142 (also known as SMOS1 and SHB) is well known to influence organ size through the auxin signaling pathway, and to modulate root meristem size by influencing GA biosynthesis (Aya et al., 2014; Li et al., 2015), and OsERF3 is known to be involved in root development, drought tolerance, and defense responses in rice (Lu et al., 2011; Wan et al., 2011; Zhao et al., 2015). A recently isolated novel AP2 domain-containing protein, OsLG3/OsERF62, has been shown to be a positive regulator of grain length (Yu et al., 2017). Next, we generated OsERF3-overexpression plants in the Nipponbare background and found that several independent transgenic lines showed significant increases in grain length and thousand-kernel weight compared to the WT plants, indicating that OsERF3 was a positive regulator of grain length (Supplementary Fig. S15). We thus speculated that OsERF142 and OsERF3 might be common targets of OsARF25 and act downstream of Gnp4.
To test the hypothesis, we first checked the expression levels of OsERF142 and OsERF3, and found that the mRNA abundance was much higher in Gnp4-overexpression plants than in the WT (Fig. 8A, B). In contrast, reduced expression of OsARF25 led to significantly decreased expression levels of OsERF142 and OsERF3 in the osarf25 mutant in the HY background (Fig. 8C, D). Next, yeast one-hybrid and CHIP-qPCR assays confirmed that OsARF25 binds to the P2 region of ProOsERF142. However, we could not detect any interactions between OsARF25-pB42AD and fragments of ProOsERF3 in yeast cells (Fig. 8H, I). In addition, we found elevated GUS activity driven by the OsERF142 promoter when Gnp4 was co-expressed compared with controls (Fig. 8J, K). These results collectively indicated that OsERF142 and OsERF3 function downstream of Gnp4 and OsARF25, and that OsARF25 binds to the promoter of OsERF142/SMOS1 and positively regulates its expression.

Nucleotide diversity and haplotype analysis of Gnp4/LAX2

In previous studies, several quantitative trait loci located near Gnp4 conferring grain length have been isolated in different rice cultivars, such as GWT4a, qLWR4, and qGL4b (Lin et al., 1996; Ying et al., 2012; Segami et al., 2016). To investigate the natural variation of Gnp4 in different germplasm types, we further analysed the sequences of Gnp4 in 259 cultivated and nine wild rice types and found 17 haplotypes (Hap) based on 46 SNPs. Hap1-Hap5 and Hap8 were mainly present in the indica subpopulation (sub-I), Hap6, Hap7, Hap9, and Hap10 were mainly present in the japonica subpopulation (sub-J), and Hap11-Hap17 were present in wild rice (Supplementary Fig. S16; Supplementary Dataset S3). This analysis showed an obvious diversification of the Gnp4 locus in the indica and japonica subspecies. Furthermore, we found that the nucleotide diversity of Gnp4 in japonica (π=0.00016) was much lower than that in indica (π=0.00093) and in wild rice (π=0.0021). Significant Tajima’s D and Fu Li’s D values were also observed in japonica (Supplementary Table S5). These results indicated that Gnp4 alleles in the japonica subpopulation might have been selected during domestication.

Discussion

Gnp4/LAX2 functions as a subset of the auxin response pathway

Although the biosynthesis, transportation, and signal transduction processes of auxin have been well studied, the regulators of components of the auxin pathway are largely unknown. In this
study, we found a component of auxin signaling involved in the regulation of grain length in rice. Gnp4/LAX2 physically interacted with OsIAA3 and OsIAA17, and affected the interaction between them with OsARF25 (Fig. 5). OsIAA3-RNAi plants displayed a phenotype with longer grain length similar to that of Gnp4-overexpression plants, while the loss-of-function osarf25 mutant had small grains (Fig. 7), leading us to propose that Gnp4 interfered with the OsIAA3–OsARF25 interaction and increased the expression of OsARF25 target genes, including OsERF142 and OsERF3. The rice smos1 (oserf142) mutant has small organ size due to decreased cell size (Aya et al., 2014). SMOS1 (OsERF142) interacts with SMOS2 (also known as Dwarf and Low Tillering, DLT) to form a complex that regulates the expression of its direct target, OsPHI-1, which is involved in cell expansion (Hirano et al., 2017). In addition, expression levels of OsERF142 and OsPHI-1 were decreased in plants overexpressing OsIAA3 (P58L, a constitutively active form of OsIAA31 named by Jain et al., 2006) (Hirano et al., 2017). We found that OsERF142 was up-regulated in Gnp4-overexpression plants compared to the Nipponbare wild-type (WT), but was down-regulated in osarf25 compared to the HY wild-type (Fig. 8A, C). In addition, up-regulation of OsPHI-1 and three OsPHI-1-like genes in Gnp4-overexpression plants were detected in our RNA-seq data (Supplementary Dataset S24). Moreover, microscopy showed that the cell length increased significantly in Gnp4-overexpression plants but decreased in osarf25, resulting from changes in cell expansion (Fig. 2K–O; Supplementary Fig. S11). Based on our results and previous reports, we propose that Gnp4 might function in an OsIAA3–OsARF25–OsERF142 pathway to regulate grain length as shown in Fig. 9.
core sequence ‘LGLE’ of the PDZ motif, including OsIAA3 (Supplementary Dataset S4). Together, our results showed that Gnp4 forms a dimer and functions as a regulator of protein–protein interactions (Fig. 5; Supplementary Fig. S7).

**Manipulation of the expression level of Gnp4/LAX2 has the potential to improve grain yield**

Our results demonstrated that Gnp4 functions as a regulator of grain length. Overexpression of Gnp4 significantly increased the grain length and thousand-kernel weight of rice, indicating its potential value for breeding. However, we found that seed setting was decreased in Gnp4-overexpression plants (Fig. 2). When we evaluated correlations of Gnp4 expression levels with seed setting and yield per plant in 17 japonica accessions, we found that accessions with 7–10-fold increases in Gnp4 expression levels relative to Nipponbare exhibited high yields, implying that there is an appropriate level of Gnp4 expression that might be required for yield improvement (Supplementary Fig. S1). Constitutive overexpression of CBP1 (CLUSTERED PRIMARY BRANCH 1) under the control of the maize ubiquitin promoter has been shown to increase grain length but not yield per plant, a result of other unfavorable agronomic traits; however, optimized expression of CBP1 using a panicle-specific promoter did result in improved yield in rice (Wu et al., 2016). In a similar way, it would be interesting to test whether controlled expression of Gnp4 in specific tissues could be used to improve yields in rice.

**Supplementary data**

Supplementary data are available at JXB online.

- Fig. S1. Analysis of the correlation between Gnp4/LAX2 mRNA levels and several agronomic traits in 17 japonica accessions.
- Fig. S2. Relative expression levels of several genes related to grain length in rice.
- Fig. S3. In silico expression analysis of Gnp4/LAX2.
- Fig. S4. Auto-transcriptional activation activity analysis of Gnp4/LAX2 in yeast cells.
- Fig. S5. Phylogenetic tree of Aux/IAA proteins in rice.
- Fig. S6. Subcellular localization and expression pattern of OsIAA3 and OsIAA17.
- Fig. S7. Gnp4 forms a dimer in yeast and plant cells.
- Fig. S8. Expression pattern analysis of OsARF25.
- Fig. S9. Relative expression levels of nearest homologous genes of OsIAA3 in wild-type Nipponbare and OsIAA3-RNAi plants.
- Fig. S10. Identification of osarf25.
- Fig. S11. Scanning electron microscopy of glumes of wild-type Hwayoung and osarf25.
- Fig. S12. Validation of transcriptome data by qRT-PCR.
- Fig. S13. Gene ontology analysis of DEGs.
- Fig. S14. KEGG pathway analysis of DEGs.
- Fig. S15. Phenotypic analysis of OsERF3-overexpression and wild-type plants.
- Fig. S16. Haplotype analysis of Gnp4/LAX2.
Table S1. Gnp4/LAX2-interacting proteins isolated by yeast two-hybrid assays.

Table S2. Enriched GO terms in significant DEGs.

Table S3. Significantly enriched protein domains of up- and down-regulated genes in Gnp4/LAX2-overexpression compared to wild-type plants.

Table S4. DEGs containing AP2 domains in Gnp4/LAX2-overexpression compared to wild-type plants.

Table S5. The nucleotide diversity of Gnp4/LAX2.

Dataset S1. Amino acid alignments of Gnp4/LAX2 and its homologous proteins.

Dataset S2. DEGs in Gnp4/LAX2-overexpression compared to wild-type plants.

Dataset S3. Details of *Oryza sativa* varieties and wild rice genotypes.

Dataset S4. Proteins in rice containing the core sequence ‘LGLE’ of the PDZ motif.

Dataset S5. Primers used in this study.

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Author contributions

ZZ designed and performed the research and wrote the article; Jinjie Li contributed to supervising the research and revising the manuscript; ZT contributed to the transcriptome experiment; XS contributed to helping with the transgenic experiment; HZ supervised the research; JY contributed to helping with the haplotype analysis; CY, GL, HG, JL, WH, HH, YX, and ZY contributed to the preparation of samples or reagents; GY, GL, HG, JL, WH, HH, YX, and ZY contributed to the preparation of samples or reagents; GY, GL, HG, JL, WH, HH, YX, and ZY contributed to the preparation of samples or reagents; YQ contributed to the identification of the osarf25 mutant; RH contributed to the transcription of the osarf25 mutant; RH contributed to the Pro35S::OsERF3 transgenic formation; WY contributed to transcriptome experiment; and ZL conceived the research and assisted in writing the manuscript.

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