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SHORT COMMUNICATION

Functions of *OsWRKY24*, *OsWRKY70* and *OsWRKY53* in regulating grain size in rice

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Abstract

Main conclusion ***OsWRKY24 functions redundantly with OsWRKY53, while OsWRKY70 functions differently from OsWRKY53 in regulating grain size*.**

Abstract Grain size is a key agronomic trait that affects grain yield and quality in rice (*Oryza sativa* L.). The transcription factor *OsWRKY53* positively regulates grain size through brassinosteroid (BR) signaling and Mitogen-Activated Protein Kinase (MAPK) cascades. However, whether the *OsWRKY53* homologs *OsWRKY24* and *OsWRKY70* also contribute to grain size which remains unknown. Here, we report that grain size in *OsWRKY24* overexpression lines and *oswrky24* mutants is similar to that of the wild type. However, the *oswrky24 oswrky53* double mutant produced smaller grains than the *oswrky53* single mutant, indicating functional redundancy between *OsWRKY24* and *OsWRKY53*. In addition, *OsWRKY70* overexpression lines displayed an enlarged leaf angle, reduced plant height, longer grains, and higher BR sensitivity, phenotypes similar to those of *OsWRKY53* overexpression lines. Importantly, a systematic characterization of seed length in the *oswrky70* single, the *oswrky53 oswrky70* double and the *oswrky24 oswrky53 oswrky70* triple mutant indicated that loss of *OsWRKY70* also leads to increased seed length, suggesting that *OsWRKY70* might play a role distinct from that of *OsWRKY53* in regulating grain size. Taken together, these findings suggest that *OsWRKY24* and *OsWRKY70* regulate rice grain size redundantly and independently from *OsWRKY53*.

Keywords *OsWRKY70* · *OsWRKY24* · *OsWRKY53* · Rice · Grain size

Introduction

Rice (*Oryza sativa* L.) is an important food crop and the staple food for more than half of the world population (Takeda et al. 2008). Grain size is a key determinant of grain yield, was a key target trait of domestication, and is the subject of ongoing improvement in rice breeding programs (Xing et al. 2010; Zuo et al. 2014; Xia et al. 2018). Many quantitative trait loci (QTLs) have been identified for grain size, such as *GS2*, *GS3*, *GL3.1/qGL3*, *GS5*, *GW6a*, *GL7/GW7* and *GW8*. These genes play important roles in determining final rice grain size, and their favorable alleles have greatly contributed to improving rice yield (Che et al. 2016; Fan et al. 2006; Qi et al. 2012; Li et al. 2011; Song et al. 2015; Wang et al. 2015; Wang et al. 2012). However, there is still much room for improving rice yield, which necessitates the identification of more genes that influence grain size and the dissection of their underlying mechanisms.

Forward and reverse genetic approaches, including cloning the causal genes for grain size QTLs, have identified many genes that regulate grain size (length, width and thickness) through diverse pathways, including phytohormone signaling, Mitogen-Activated Protein Kinase (MAPK) signal transduction, regulation of transcription factors, G protein signaling and the ubiquitin proteasome pathway (Li et al. 2015; Li and Li 2016). For example, rice BIN2-like kinase *OsGSK2* is a core component of

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BR signaling, both gain and loss of function of *OsGSK2* cause dramatic phenotypic changes in plant height, leaf angle and seed size (Tong et al. 2012). MAPK kinase kinase 70 (*OsMKKK70*) and its homologs affect grain size and leaf angle by activating the MAPK kinase 4 (*OsMKK4*)-*OsMAPK6*-*OsWRKY53* signaling cascade (Liu et al. 2021). Likewise, the E3 ubiquitin-protein ligase Grain Width 2 (GW2) negatively regulates grain size by promoting cell division (Song et al. 2007).

WRKY transcription factors are one of the largest families of transcriptional regulators in plants and are involved in diverse growth and developmental processes and stress responses. WRKY members are classified into three groups based on the number of WRKY domains and the type of zinc finger-like motif they harbor. WRKY transcription factors regulate the expression of their target genes by directly binding to W-box (C/T)TGAC(T/C) elements in their target promoters (Yamasaki et al. 2005). The rice genome encodes at least 102 members of the WRKY family, of which only a few members have been functionally characterized. Overexpression of *OsWRKY45* resulted in enhanced salt and drought tolerance and increased disease resistance (Qiu et al. 2009). *OsWRKY51* and *OsWRKY71* were shown to regulate seed germination, and overexpression of *OsWRKY23* accelerates leaf senescence (Zhang et al. 2004; Xie et al. 2006; Jing et al. 2009). Lower transcript levels of *OsWRKY78* lead to smaller grains as a consequence of reduced cell length (Zhang et al. 2011). Conversely, *OsWRKY36* overexpression lines produce smaller grains, while *oswrky36* knockout plants have larger grains (Lan et al. 2020). Notably, whether other WRKY family members are involved in grain size control still needs to be explored.

We previously demonstrated that OsWRKY53 positively regulates leaf angle and seed size by integrating BR signaling and the MAPKKK10-MAPKK4-MAPK6 cascade (Tian et al. 2017, 2021). In addition, OsWRKY53 regulates plant-pathogen responses (Chujo et al. 2007; Xie et al. 2021; Gao et al. 2021) and herbivore resistance (Hu et al. 2015). *OsWRKY24*, *OsWRKY70* and *OsWRKY53* are close homologs and belong to the same subgroup Ia of WRKY family members (Xie et al. 2005; Zhang et al. 2015), raising the possibility that *OsWRKY24* and *OsWRKY70* might also regulate grain size. Here, we explored the role of *OsWRKY24* and *OsWRKY70* in grain size control. We discovered that loss of *OsWRKY24* is not sufficient to affect grain size on its own, but showed additional phenotypes when combined with the *oswrky53* mutant, while *OsWRKY70* played a complex role independently from *OsWRKY53* in regulating grain size.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* L.) cultivar Longjing 11 (LJ11) (*Oryza sativa ssp. japonica*) was used as the WT control and for generating overexpression transgenic plants and mutants and *oswrky53 and OsWRKY53-OE* were reported previously (Tian et al. 2017). The plants were cultivated in an experimental field (Heilongjiang Province, China) under natural long-day conditions.

Generation of transgenic plants, double mutants and triple mutant

To generate overexpression plants, the full-length coding region of *OsWRKY24* and *OsWRKY70* were amplified from LJ11 and cloned into *pC1390U*, in which *OsWRKY24* and *OsWRKY70* are directed by *Ubiquitin* promoter. To generate the *oswrky24*, *oswrky70*, *oswrky24 oswrky53*, *oswrky53 oswrky70 and oswrky24 oswrky53 oswrky70* mutants, the target sequence (Table S1) was synthesized, ligated with the respective sgRNA cassettes, and sequentially ligated into CRISPR/Cas9 binary vector *pYLCRISPR/Cas9Pubi-H* as described (Ma et al. 2015). All constructs were confirmed by sequencing. Rice cultivar LJ11 was used as the recipient for *Agrobacterium*-mediated transformation performed as described previously (Hiei et al. 1994).

Total RNA isolation and RT-qPCR analysis

Total RNA was extracted using TRIzol (Invitrogen) and treated with DNase I. cDNA was synthesized from 2 μg of total RNA using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed with Bio-Rad chromo 4 real-time PCR detector and SYBR Green PCR master mix (Takara). All expressions were calculated by normalizing against *ACTIN1* (*Os03g50885*). Three biological repeats were conducted for each analysis. Values are means ± SE of three biological repeats.

Brassinosteroid treatment assay

BR sensitivity assay of lamina joint was performed as described previously (Tong et al. 2009). Briefly, rice seeds were sterilized and germinated in 1/2 Murashige and Skoog (MS) medium (M519, Phytotech, China) and grown in the dark for 8 day at 30 °C. The uniform seedlings were selected and the entire segments including 1 cm of the leaf sheath, 1 cm of the second leaf blade and the lamina joint were cut and incubated in different concentrations of 24-epiBL (Sigma, E1641) for 72 h in dark. The angles of lamina joint bending were photographed and measured with ImageJ software (http://rsbweb.nih.gov/ij/).

Statistical analysis

For grain size and BR sensitivity statistics, 10 seeds or seedling were used. For plant height statistics, and leaf angle statistics, 25 and 30 plants were used to score and analyze.

The data were analyzed statistically by unpaired *t* test using GraphPad Prism ver. 8 (GraphPad Software, San Diego, CA, USA) and one-way ANOVA using Statistical Product and Service Solutions program (SPSS, version 19). The significance levels for *t* test are represented as *for $P \leq 0.05$, **for $P \leq 0.01$ and ns for no significance. The means and SE are based on independent biological samples. And $P < 0.05$, one-way ANOVA with Tukey's significant difference test.

Accession numbers

Gene sequence used in this study can be download in Rice Genome Annotation Project website via searching the following accession numbers: *OsWRKY53* (LOC_Os05g27730); *OsWRKY70* (LOC_Os05g39720); *OsWRKY24* (LOC_Os01g61080); *ACTIN1* (LOC_Os03g50885); *GA20ox1* (LOC_Os03g63970); *GA20ox2* (LOC_Os01g66100); *GA20ox3* (LOC_Os07g07420); *GA20ox4* (LOC_Os05g34854); *GA3ox1* (LOC_Os05g08540); *GA3ox2* (LOC_Os01g08220); *GA2ox1* (LOC_Os05g06670); *GA2ox5* (LOC_Os07g01340); *GA2ox6* (LOC_Os04g44150); *GA2ox7* (LOC_Os01g11150); *KO2* (LOC_Os06g37364); *SLR1* (LOC_Os03g49990).

Results and discussion

Overexpression or mutation of *OsWRKY24* does not affect grain size

We previous showed that plants overexpressing *OsWRKY53* form larger leaf angles, have larger grains, and are

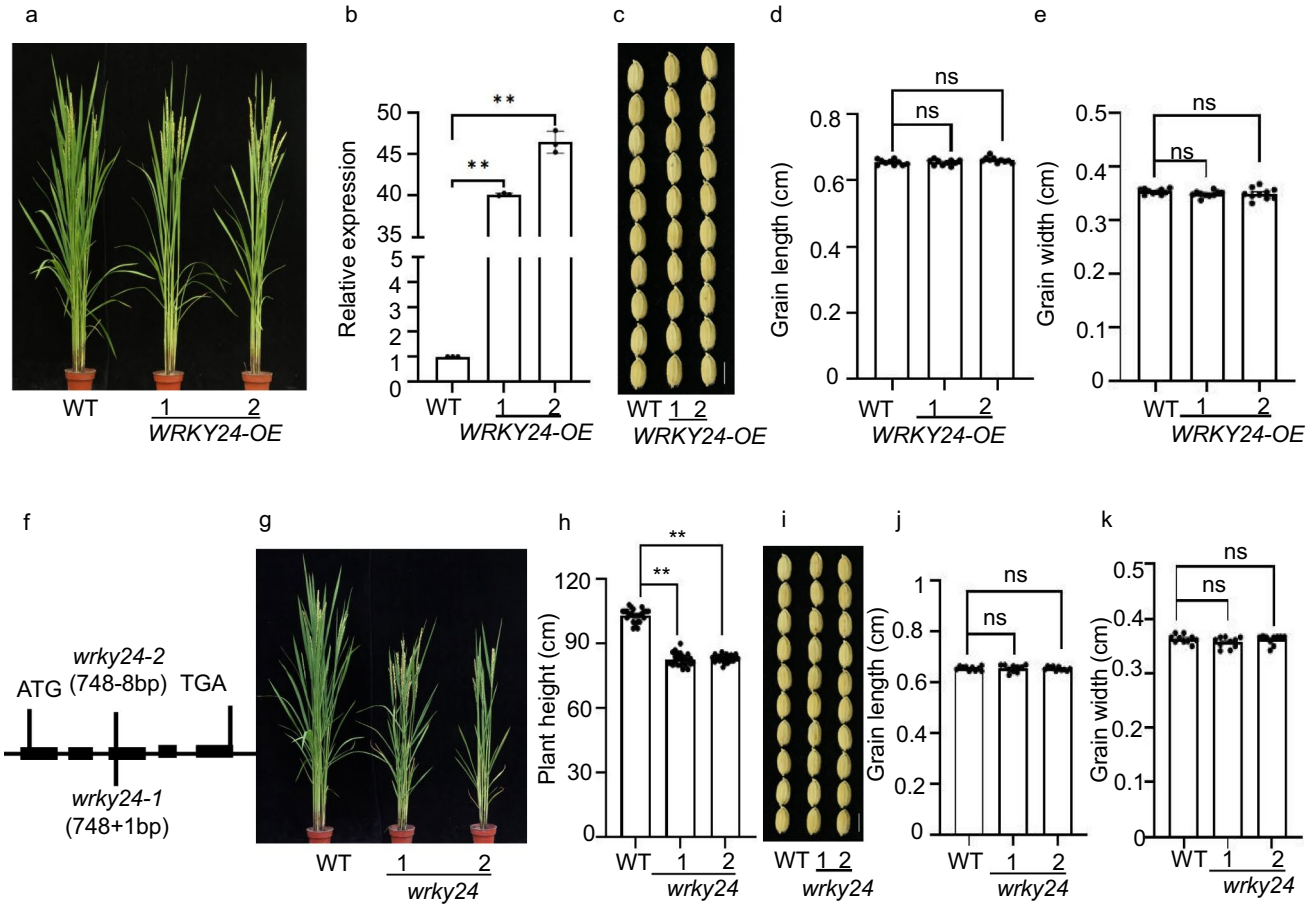
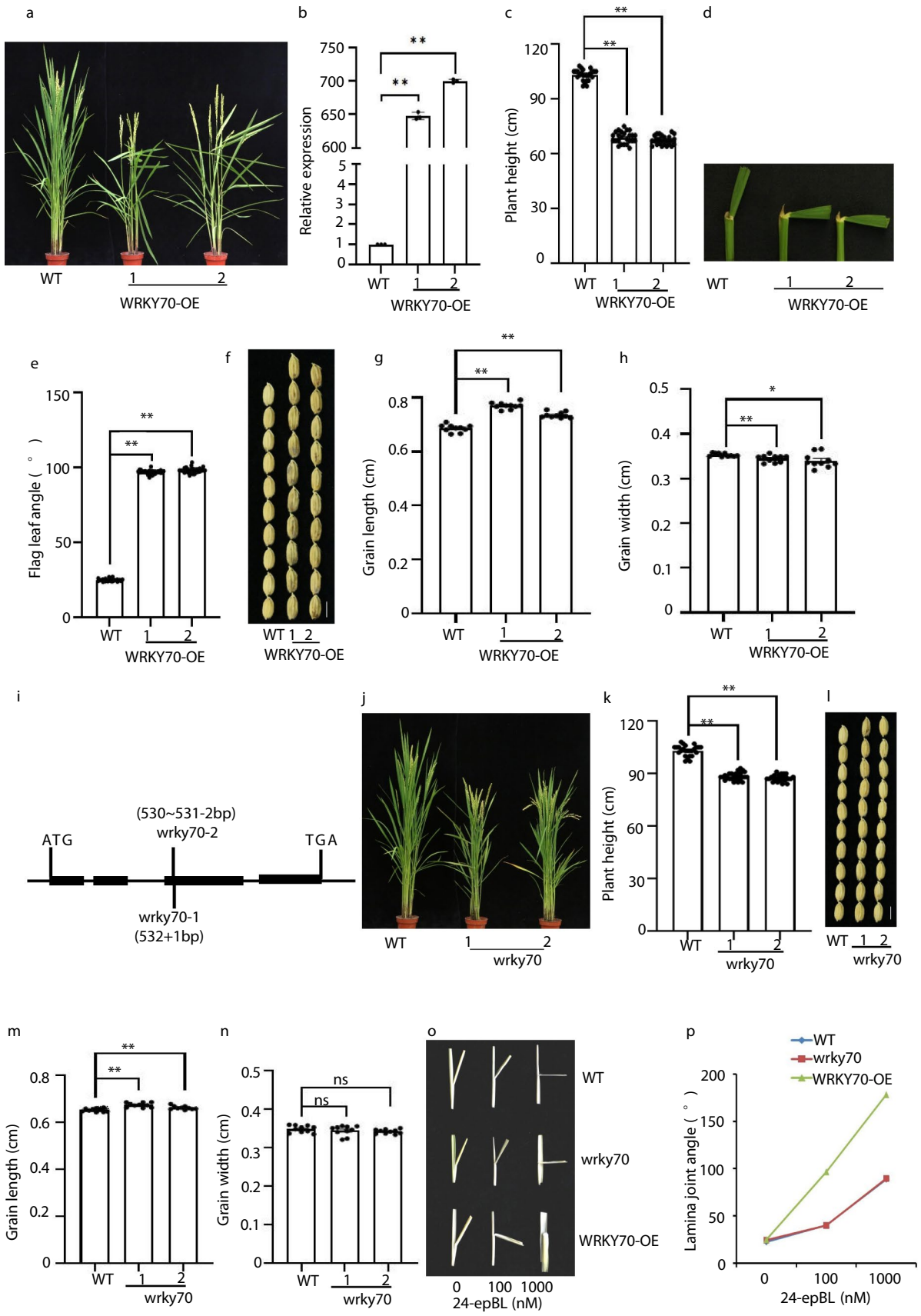


Fig. 1 Phenotypic of *OsWRKY24-OE*, *oswrky24* and WT plants. **a** Gross morphology of WT and *OsWRKY24-OE-1*, *OsWRKY24-OE-2* plants at the heading stage. **b** Relative expressions of *OsWRKY24* by RT-qPCR analysis in *OsWRKY24* of WT and *OsWRKY24-OE* two lines ($n=3$). **c** Grain phenotypes of WT and *OsWRKY24-OE-1, OsWRKY24-OE-2* plants (bar = 0.5 cm). **d–e** Quantification of grain length (**d**) and grain width (**e**) in **c** ($n=10$). Seeds from two overexpression lines showed similar results. **f** Identification of *oswrky24-1* and *oswrky24-2* mutants. ATG and TGA are initiation codon and stop codon, respectively. **g** Gross morphology of *oswrky24-1*, *oswrky24-2* and WT plants at the heading stage. **h** Quantification of plant height of *oswrky24* mutants and WT ($n=25$). **i** Grain phenotypes of *oswrky24* mutants and WT (bar = 0.5 cm). **j–k** Quantification of grain length (**j**) and grain width (**k**) in **i** ($n=10$). Seeds from two transgenic lines showed similar results. Each dot represents the result from one biological replicate; error bars indicate means ± SE. *P* values were determined by Student's *t* test, ** is $P<0.01$. *ns* means no significant difference


a
WT
1
2
WRKY70-OE
b
Relative expression
750
700
650
600
5
4
3
2
1
0
**
**
WT
1
2
WRKY70-OE
c
Plant height (cm)
120
90
60
30
0
**
**
WT
1
2
WRKY70-OE
d
WT
1
2
WRKY70-OE
e
Flag leaf angle (°)
150
100
50
0
**
**
WT
1
2
WRKY70-OE
f
WT 1 2
WRKY70-OE
g
Grain length (cm)
0.8
0.6
0.4
0.2
0
**
**
WT
1
2
WRKY70-OE
h
Grain width (cm)
0.5
0.4
0.3
0.2
0.1
0
*
**
WT
1
2
WRKY70-OE
i
ATG
(530~531-2bp)
wrky70-2
TGA
wrky70-1
(532+1bp)
j
WT
1
2
wrky70
k
Plant height (cm)
120
90
60
30
0
**
**
WT
1
2
wrky70
l
WT 1 2
wrky70
m
Grain length (cm)
0.8
0.6
0.4
0.2
0
**
**
WT
1
2
wrky70
n
Grain width (cm)
0.5
0.4
0.3
0.2
0.1
0
ns
ns
WT
1
2
wrky70
o
WT
wrky70
WRKY70-OE
0
100
1000
24-epBL (nM)
p
WT
wrky70
WRKY70-OE
Lamina joint angle (°)
200
150
100
50
0
0
100
1000
24-epBL (nM)

◂**Fig. 2** Phenotypic and BR sensitivity test of *OsWRKY70-OE*, *oswrky70* and WT plants. **a** Gross morphology of WT and two lines *OsWRKY70-OE-1*, *OsWRKY70-OE-2* plants at the heading stage. **b** Relative expressions of *OsWRKY70* by RT-qPCR analysis in WT and *OsWRKY70-OE-1*, *OsWRKY70-OE-2* plants ($n=3$). **c** Quantification of plant height in *OsWRKY70-OE* lines and WT ($n=25$). **d** Representative image of Lamina joints with flag leaves in WT and *OsWRKY70-OE* two lines. **e** Quantification of flag leaf angle in *OsWRKY70-OE* lines and WT ($n=30$). **f** Grain phenotypes of WT and *OsWRKY70-OE-1*, *OsWRKY70-OE-2* plants (bar = 0.5 cm). **g–h** Quantification of grain length (**g**) and grain width (**h**) in **f** ($n=10$). Seeds from two overexpression lines showed similar results. **i** Identification of *oswrky70-1* and *oswrky70-2* mutants. ATG and TGA are initiation codon and stop codon, respectively. **j** Gross morphology of *oswrky70-1*, *oswrky70-2* and WT plants at the heading stage. **k** Quantification of plant height in *oswrky70* mutants and WT ($n=25$). **l** Grain phenotypes of *oswrky70* mutants and WT (bar = 0.5 cm). **m–n** Quantification of grain length (**e**) and grain width (**f**) in ***i*** ($n=10$). Seeds from two transgenic lines showed similar results. **o** The leaf inclination of *OsWRKY70-OE, oswrky70* and WT in the presence of indicated concentration of 24-epiBL. **p** Statistical analysis of leaf inclination in **o** ($n=10$). Each dot represents the result from one biological replicate; error bars indicate means ± SE. *P* values were determined by Student's *t* test, ** is $P<0.01$. * is $P<0.05$. *ns* means no significant difference

hypersensitive to BR, while *oswrky53* mutants developed as erect plants with smaller grains and are insensitive to BR (Tian et al. 2017). We wished to determine whether *OsWRKY53* homologs might be involved in the same developmental processes. OsWRKY53 is highly similar to the group I WRKY members OsWRKY24 and OsWRKY70 (Zhang et al. 2015).

To investigate the biological function of OsWRKY24, we generated plants overexpressing *OsWRKY24* in the Longjing11 (LJ11) variety. We selected two independent lines (*OsWRKY24-OE1* and *OsWRKY24-OE2*) with a 40- and 46-fold increase in *OsWRKY24* transcript levels, respectively, relative to the wild type, as determined by reverse-transcription quantitative PCR (RT-qPCR) (Fig. 1a, b). Surprisingly, both lines showed similar morphological phenotypes to LJ11, including plant height, leaf angle and grain size (Fig. 1a–e). In a complementary approach, we obtained two independent *oswrky24* mutants, *oswrky24-1* and *oswrky24-2,* via CRISPR/Cas9-mediated genome editing in the LJ11 background. The *oswrky24-1* and *oswrky24-2* mutants harbored a 1-bp insertion and an 8-bp deletion, respectively, predicted to cause frameshifts (Fig. 1f; Fig. S1). Plants from both *oswrky24* mutants had a shorter stature relative to LJ11 (Fig. 1g, h). However, grain size did not appear to be affected in *oswrky24* mutants (Fig. 1i–k).

Overexpression and mutation of *OsWRKY70* increases grain length

To investigate the biological function of OsWRKY70, we generated *OsWRKY70* overexpression (*OsWRKY70-OE*) transgenic lines in LJ11. Relative *OsWRKY70* transcript levels rose by 650–700-fold in two independent *OsWRKY70-OE* lines, as shown by RT-qPCR (Fig. 2a, b). Plants from both *OsWRKY70-OE-1* and *OsWRKY70-OE-2* lines were shorter and formed a larger leaf angle than LJ11 (Fig. 2a–e). In addition, grains from *OsWRKY70-OE-1* and *OsWRKY70-OE-2* plants were longer but thinner compared to LJ11 grains (Fig. 2f–h). These results indicated that overexpression of *OsWRKY70* leads to reduced plant height, larger leaf angles and longer grains, all phenotypes that are exhibited by *OsWRKY53-OE* plants (Tian et al. 2017).

To further study the function of OsWRKY70 in regulating plant architecture and grain size, we generated *oswrky70* mutants in the LJ11 background via CRISPR/Cas9-mediated genome editing. We obtained two independent *oswrky70* mutant alleles, *oswrky70-1* and *oswrky70-2*, carrying a 1 bp insertion and a 2 bp deletion, respectively, in *OsWRKY70* (Fig. 2i; Fig. S2). The leaf angle of *oswrky70* mutants was similar to that of LJ11, while *oswrky70* plants were slightly shorter compared to LJ11 (Fig. 2j, k). Surprisingly, *oswrky70* grains were longer than those of LJ11, but grain width was not affected (Fig. 2l–n). These results suggested that loss of OsWRKY70 function also leads to longer grains.

The larger leaf angles and seeds seen in *OsWRKY70-OE* were reminiscent of mutants with enhanced BR signaling, such as *osbzr1-D* and *OsWRKY53-OE* (Qiao et al. 2017; Tian et al. 2017). We thus evaluated the sensitivity of *OsWRKY70-OE* and *oswrky70* mutant plants to BRs by performing a BR-induced lamina inclination assay. To this end, we treated plants with 100 nM or 1000 nM 24-epiBL for 3 day and measured the resulting leaf angles. We established that *OsWRKY70*-OE plants are much more sensitive to exogenous BR treatment than LJ11 (Fig. 2o, p). Indeed, leaf angles of *OsWRKY70-OE* plants were much larger than those in LJ11. By contrast, *oswrky70* mutants displayed a BR sensitivity comparable to that of LJ11 (Fig. 2o, p). Taken together, these results suggested that OsWRKY70 might positively regulate BR responses in rice.

OsWRKY24 is redundant with *OsWRKY53* for grain size

In light of the absence of seed size and leaf angle phenotypes in *OsWRKY24*-OE lines and *oswrky24* mutants (Fig. 1), we suspected that OsWRKY24 and OsWRKY53 might cooperate in the regulation of grain size. Accordingly, we generated *oswrky24 oswrky53* double mutant via CRISPR/Cas9-mediated genome editing (Fig. 3a; Fig. S3). While grain size in *oswrky24* and LJ11 was similar and *oswrky53* mutants had smaller grains than LJ11, the *oswrky24 oswrky53* double mutant produced significantly smaller seeds than the *oswrky53* single mutant, indicating that knocking out

a

	w24 53	w53 70	w24 70	w24 53 70
WRKY24	1-bp in (748)		1-bp in (748)	1-bp in (748)
WRKY53	1-bp in (349)	1-bp in (349)		1-bp in (349)
WRKY70		2-bp del (530-531)	1-bp in (532)	2-bp del (530-531)

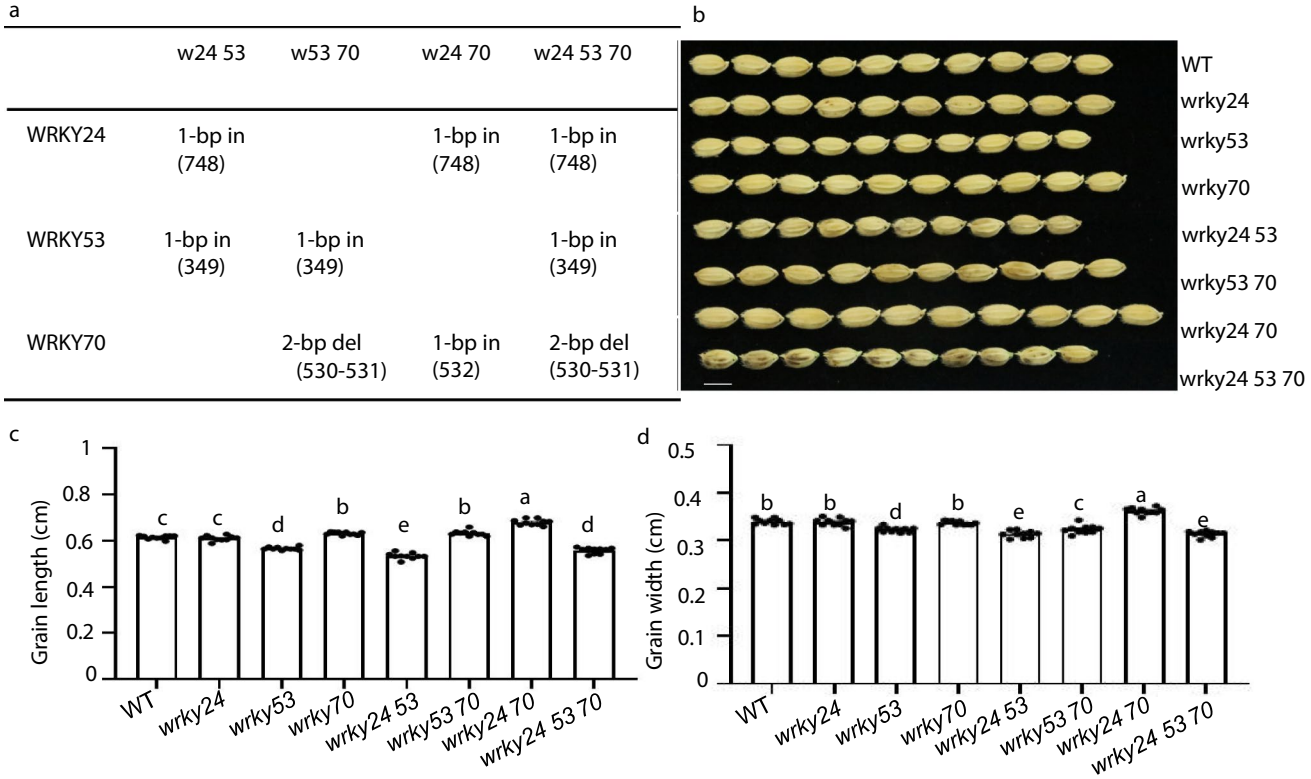


Fig. 3 Grain size analysis of double and triple mutants. **a** Identification of *oswrky24 oswrky53*, *oswrky53 oswrky70, oswrky24 oswrky70* and *oswrky24 oswrky53 oswrky70* mutants. The mutation of these three genes all leads to a frame shift. **b** Grain phenotypes of these relevant single, double, and triple mutants of *OsWRKY24*, *OsWRKY53*, *OsWRKY70* and WT. **c–d** Mean grain length (**c**) and grain width (**d**) with the relevant single, double and triple mutants of *OsWRKY24*, *OsWRKY53*, *OsWRKY70* and WT as shown in **b** ($n = 10$). Each dot represents the result from one biological replicate; error bars indicate means ± SE. Statistically significant differences are indicated by different lowercase letters ($P < 0.05$, one-way ANOVA with Tukey's significant difference test)

OsWRKY24 exacerbates the small grain phenotype of the *oswrky53* mutant (Fig. 3b–d). These results thus suggested that *OsWRKY24* might play a minor role in seed size control and function synergistically with *OsWRKY53*.

OsWRKY53 and *OsWRKY70* have different functions in the regulation of grain size

Although *OsWRKY70* positively regulated BR responses, both the overexpression and mutation of *OsWRKY70* resulted in longer grains. To further explore the biological function of *OsWRKY70*, we generated the *oswrky53 oswrky70*, *oswrky24 oswrky70* double mutants and the *oswrky24 oswrky53 oswrky70* triple mutant via CRISPR/Cas9-mediated genome editing (Fig. 3a; Fig. S4). We determined that the *oswrky70* single mutant and the *oswrky53 oswrky70* double mutant have longer grains compared to the *oswrky53* single mutant (Fig. 3b–d). Notably, grain length in the *oswrky24 oswrky53 oswrky70* triple mutant was intermediate between the shorter grains of the *oswrky24 oswrky53* double mutant and the longer grains of the *oswrky53 oswrky70* double mutant (Fig. 3b–d). These results thus demonstrated that knocking out *OsWRKY70* leads to larger grains, which was distinct from the phenotypes seen in the *oswrky53* mutant, suggesting that OsWRKY70 might play a complex role in regulating grain size (Fig. 3b–d).

Overexpression of *OsWRKY53* increased grain size, while *oswrky53* mutants had smaller grains (Tian et al. 2017). However, both the gain and the loss of OsWRKY70 function resulted in longer grains, in contrast to the regulatory function of OsWRKY53 (Figs. 2, 3). Given that there might have regulatory relationship among these WRKYs, we examined the expression of *OsWRKY24* and *OsWRKY53* in *oswrky70* mutant, and found that *OsWRKY53* expression was increased and *OsWRKY24* expression has no change in *oswrky70* mutant relative to WT (Fig. S5a). In addition, *oswrky24* single mutant did not affect grain size while *oswrky24 oswrky53* developed smaller seeds than *oswrky53* single mutant. However, *oswrky24 oswrky70* showed bigger size than *oswrky70* single mutant (Fig. 3b–d). There might exist a complex regulatory relationship among three WRKYs, which resulted in that the function of WRKYs have slight divergence in different *wrky* mutant background.

Previous studies indicated that several members of the WRKY family of transcription factors regulate plant height and grain size in rice. Among them, OsWRKY36 and OsWRKY70 negatively regulate plant height by suppressing gibberellic acid (GA) signaling and biosynthesis, respectively (Li et al. 2015; Lan et al. 2020). In terms of grain size, overexpression of *OsWRKY36* results in smaller grains, while *oswrky36* mutants produce larger grains. OsWRKY36 was shown to modulate grain size by inhibiting GA signaling via an upregulation of *SLENDER RICE1* (*SLR1*) transcription and preventing GA-mediated SLR1 degradation (Lan et al. 2020). To test whether OsWRKY70 was involved in GA signaling, we examined the expression of GA signaling-related genes in *oswrky70* mutant. We found that expression of some GA biosynthesis genes such as *OsGA20ox3*, *OsGA3ox1* and *OsGA3ox2* were reduced in *oswrky70* mutant, whereas GA metabolized genes such as *OsGA2ox1* was upregulated in *oswrky70* mutant, but *OsGA2ox5* and *OsGA2ox6* were downregulated compared with WT (Fig. S5b). These results imply that OsWRKY70 might also regulating seed size via the GA signaling pathway. And we suspect that *OsWRKY24* and *OsWRKY70* might employ OsWRKY53-independent (e.g. *OsWRKY70* can regulate GA signaling) or OsWRKY53-dependent manner (e.g. *OsWRKY70* negatively regulating the expression of *OsWRKY53*) to regulate seed size (Fig. S5). It will be worth investigating the mechanism by which *OsWRKY70* regulates grain size independently of *OsWRKY53* in the future.

Author contribution statement XJT conceived the research plan; JQT, EYM and MLH performed the experiments; JQT, QYB, and XJT analyzed the data and wrote the article.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00425-022-03871-w.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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