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ARTICLE

The Endosperm-Specific Expression of *YUCCA* Genes Enhances Rice Grain Filling

Huijun Jiang[1,#], Kaien Zhai[3,#], Xiaofan Ye[3,#], Tianwei Hu[2], Jieming Jiang[4], Xiaoqiu Dong[1], Weihuai Pan[1], Jianwei Pan[2] and Jianxin Shou[1,*]

[1]College of Life Sciences, Shaoxing University, Shaoxing, 312000, China

[2]College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua, 321004, China

[3]School of Life Sciences, Lanzhou University, Lanzhou, 730000, China

[4]School of Life Science, South China Normal University, Guangzhou, 510631, China

[*]Corresponding Author: Jianxin Shou. Email: jxshou@126.com

[#]These authors contributed equally to this work

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ABSTRACT

Grain filling is a crucial process that affects yield in rice (*Oryza sativa* L.). Auxin biosynthesis and signaling are closely related to rice yield; therefore, it is important to understand the effects of auxin biosynthesis on rice grain filling to improve crop yield. In this study, we used physiological and molecular strategies to identify the roles of auxin in rice grain filling. Exogenous application of auxin (IAA) or auxin analogues (2, 4-D) to young spikelets and flag leaves improved the seed-setting rate and yield per spike. Furthermore, real-time quantitative PCR assays confirmed that nine members of the *OsYUCCA* family of auxin biosynthetic genes were upregulated during grain filling, implication that auxin biosynthesis plays a major role in grain development. The specific expression of either Arabidopsis *AtYUCCA1* or *OsYUCCA2* in the endosperm or leaves resulted in increased expression of *OsIAA* genes and auxin content of seeds, as well as increased grain filling and seed-setting rate. This result establishes that the auxin content in grains and leaves is important for grain development. Our findings further highlight the potential applications for improving rice yield by elevating targeted gene expression in specific tissues.

KEYWORDS

Auxin content; grain filling; IAA biosynthesis; rice; seed-setting rate; *YUCCA* genes

1 Introduction

Rice (*Oryza sativa*) is one of the most important food crops globally, and increasing its production has long been the primary goal of rice breeding. Grain filling and seed-setting rate are the most basic traits that determine rice yield [1–5]. Many factors affect these traits, including transcription factors, plant hormones, and signaling proteins [6–9]. Auxin was identified about 80 years ago to be an important regulator of seed development and seed weight [10,11]. Understanding the roles of auxin in determining rice yield is of great biological relevance.

Auxin is mainly biosynthesized by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA)/YUCCA flavin monooxygenase (YUC) pathway, which is highly conserved throughout the plant kingdom [12]. TAA1 catalyzes the conversion of tryptophan to indole-3-pyruvate (IPA), and then the flavin monooxygenase-like enzyme YUCCA converts IPA to indole-3-acetic acid (IAA) [13,14]. Auxin biosynthesized by the TAA/YUC pathway plays a critical role in determining rice yield, and auxin biosynthesis mediated by *OsYUCCA9* and *OsYUCCA11* regulates grain filling via the rice endosperm [6,15]. TILLERING AND SMALL GRAIN1 (*TSG1*) encodes a tryptophan aminotransferase in rice that promotes endogenous auxin levels, and loss of *TSG1* function decreases yield [16]. Furthermore, *OsYUCCA12* and *OsIAA29* are expressed transiently during early grain development, which suggests that *OsYUCCA12* may regulate grain filling in rice [15,17]. The fruit set of plants is highly dependent on auxin metabolism [18]. The gene TILLER ANGLE CONTROL4 (*TAC4*) regulates rice plant architecture and yield-related traits by affecting the endogenous auxin content and its asymmetrical distribution [19]. The AUXIN RESPONSE FACTOR (ARF)–mediated activation of NO_3-transporter and N-metabolism genes in response to auxin increases grain yield [20]. Moreover, *OsPIN5b* influences auxin levels, and its overexpression alters plant architecture and yield, leading to changes that include decreases in plant height, tiller number, leaf number, seed-setting rate, and the number of full grains per plant [8]. Collectively, these observations indicate that regulators of auxin signaling mediate rice yield via several different mechanisms.

Treatment with exogeneous auxin can improve the seed-setting rate in plants [21,22]. For example, treatment with the synthetic auxin 2, 4-dichlorophenoxyacetic acid (2, 4-D) or 1-naphthaleneacetic acid (NAA) or with IAA reverses male sterility and restores the seed-setting rate in barley [23]. Moreover, NAA significantly increases the yield of sweet potato under drought stress [24]. Application of exogenous IAA partially rescues the seed-setting rate defects in the Arabidopsis ADAPTOR PROTEIN COMPLEX 2 (AP2) mutant [25]. To date, more than 1,000 synthetic plant growth regulators have been used to improve food production. However, synthetic reagents may be harmful to the environment and human health; therefore, increasing the level of endogenous auxin is a more desirable option. Previous work suggested that transgenic expression of an auxin biosynthesis gene, *iaaM*, can increase the total IAA content in young flower buds of strawberry (*Fragaria* × *ananassa*) and raspberry (*Rubus idaeus*), as well as plant fecundity and fruit production [26,27]. Here, we explored the potential to increase rice yield via similar molecular approaches.

We determined that the application of exogenous auxin or elevation of the endogenous auxin level via overexpression of native or heterologous *YUCCA* genes in rice dramatically increased yield. We also established that treatment of spikelets and flag leaves of rice with IAA, 2, 4-D, or NAA increased the seed-setting rate and yield per spike, dependent on the concentration of auxin. Quantitative reverse transcription-PCR (qRT-PCR) assays revealed that nine *OsYUCCA* genes were upregulated and four *OsYUCCA* genes were downregulated, pointing to the importance of the spatiotemporal regulation of *YUCCA* expression during rice grain filling. Furthermore, expressing *AtYUCCA1* (as an alternative to *OsYUCCA1* because overexpression of the latter severely compromises viability) or *OsYUCCA2* under the control of endosperm-and leaf-specific promoters resulted in increased expression of *OsIAA* genes accompanied by an increased seed-setting rate. These results further support the notion that increasing in specific tissues of auxin concentrations is a promising method to increase rice yield.

2 Methods and Materials

2.1 Plant Materials and Growth Conditions

Grains of wild-type rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) and of transgenic plants carrying *pOsGt1::AtYUCCA1:GUS*, *pOsGluB::OsYUCCA2:GUS*, *pRubisco::AtYUCCA1:GUS*, and *pRubisco::OsYUCCA2:GUS* were surface sterilized with 70% ethanol for 10 min followed by 10% NaClO for

30 min and then rinsed eight times with water. Wild-type and transgenic plants were grown in the field under natural conditions in Jinhua and Sanya, China.

2.2 Chemicals and Treatments

The chemicals 2, 4-D, IAA, 1-NAA, and Tween-20 were obtained from Sigma-Aldrich. Stock solutions were prepared as follows: 10 mM stock solutions of 2, 4-D, IAA, and 1-NAA were made by dissolving each reagent in a few drops of 1 M KOH and diluting with ddH_2O. The stock solutions were diluted to 50, 100, and 200 μM with ddH_2O and 0.5% (v/v) Tween-20. Distilled water with 0.5% (v/v) Tween-20 was used as a control.

Spikelets and flag leaves were treated at 15:00 every other day, and the treatment was performed three times in total. The seed-setting rate, yield per spike, and 1,000-grain weight were measured after harvest for three seasons, from 2012 to 2014, as follows: seed setting rate (%) = (total grain number – shell number)/total grain number; yield per spike = the weight of the full seed per spike; yield per plant = the weight of the full seed per plant; and 1,000-grain weight = 100-grain weight × 10. Statistical analyses were performed using two-tailed Student's *t*-tests for data from three independent experiments.

2.3 Quantitative Reverse Transcription-PCR (qRT-PCR)

To quantify the expression levels of endogenous *OsYUCCA* genes in wild-type grains and of *OsIAA* genes in the grains of transgenic plants, total RNA was isolated from grains using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized with a SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed with Thunderbird SYBR qPCR mix (Toyobo) and a StepOnePlus Real-Time PCR System (Applied Biosystems). The reactions were performed in a 20 μL volume containing 10 μL 2 × SYBR qPCR mix (Toyobo), 10 ng cDNA, and 1 μM of each gene-specific primer (Table S1). The PCR cycles were performed as follows: one cycle of 95°C for 3 min and 40 cycles of 95°C for 5 s and 60°C for 50 s. The resulting data were analyzed using StepOne Software v2.1. The transcript levels were normalized to that of the housekeeping gene *OsACTIN2* (Table S1). For statistical analysis, the transcript levels from three independent experiments were analyzed with two-tailed Student's *t*-tests.

2.4 Construct Generation, Transformation, and Molecular Identification in Rice

To specifically overexpress the *AtYUCCA1* and *OsYUCCA2* genes in rice, constructs were generated using PCR and restriction digestion and were ligated with the plant transformation vector *pCAMBIA2300S-GUS* containing the *OsGt1*, *OsGluB*, and *Rubisco* promoters. The resulting constructs were confirmed by sequencing. All primer sequences for the constructs are indicated in Table S2.

Rice transformation was conducted as described previously [28]. To determine whether the transgenic plants harboring the constructs expressed the constructs, RT-PCR and GUS staining were performed in T_1 plants. RNA isolation and cDNA synthesis were conducted as described above. RT-PCR primers were designed to amplify the coding sequences of *AtYUCCA1*and *OsYUCCA2*. Expression of rice *OsACTIN2*, a housekeeping gene, was used as an internal control and wild-type Nipponbare cDNA served as a negative control (NC). Twenty-seven cycles of PCR were used to amplify *AtYUCCA1*, *OsYUCCA2*, and *OsACTIN2* from the rice transgenic lines. Homozygous transgenic lines that overexpressed the *AtYUCCA1* or *OsYUCCA2* constructs were recovered in the T_3 generation via selection for G418 resistance. To stain for GUS, samples were immersed in GUS staining solution, placed under vacuum for 30 min, incubated at 37°C overnight, and destained with solution (3:1 ethyl alcohol:acetic acid [v/v]) for 16 h.

3 Results

3.1 Treating Rice with Exogenous Auxin Increases Yield

Auxin affects grain filling, photosynthesis, and photosynthate distribution in rice and consequently can enhance seed-setting rate and yield. To further demonstrate the function of auxin in rice, we treated young

spikelets and flag leaves with exogenous IAA or its analogs 2, 4-D and NAA and analyzed how auxin affected yield. Young spikelets (2 days after full heading) were treated with different concentrations of IAA, 2, 4-D, and NAA (0, 50, 100, or 200 μM). Analysis of the seed-setting rate, yield per spike, and 1,000-grain weight showed that the 50 and 100 μM IAA treatments caused a significant increase in yield per spike, and 50, 100, and 200 μM IAA treatments increased the seed-setting rate, compared with those of the mock-treated control (0 μM) (Fig. 1A). Similarly, treatment with 50, 100, or 200 μM 2, 4-D improved the seed-setting rate, yield per spike, and 1,000-grain weight relative to the control values (Fig. 1B). Treatment with NAA caused an increase in seed-setting rate, although it did not significantly change the yield per spike or the 1,000-grain weight (Fig. 1C). These results demonstrate that exogenous auxin effectively increases rice yield.

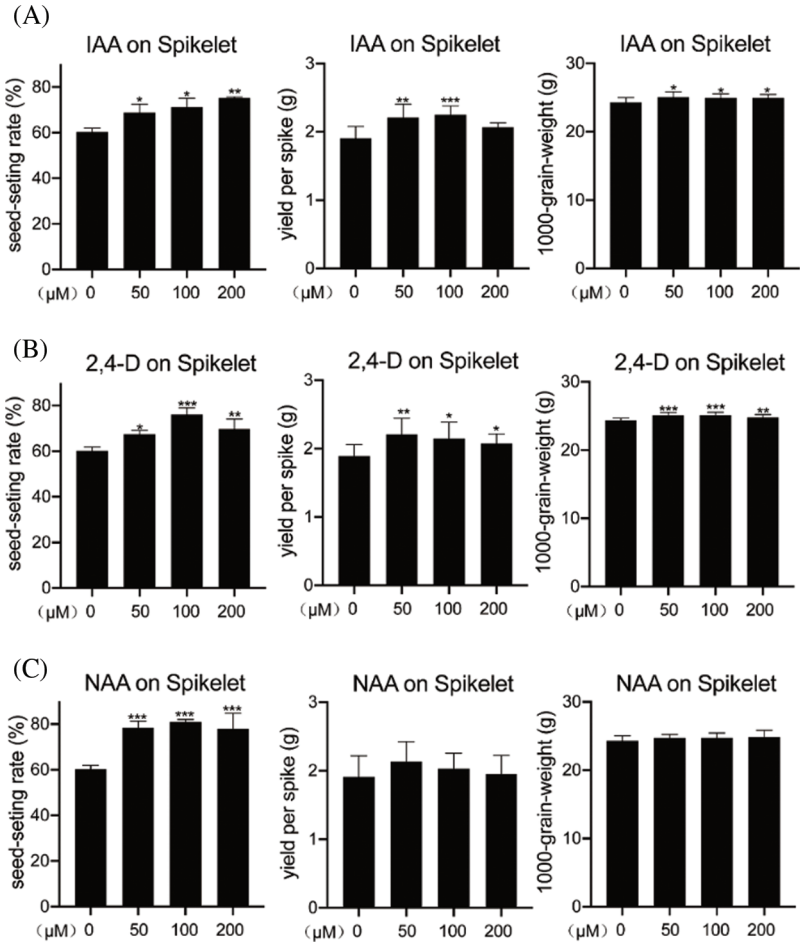

Figure 1: Effect of treating spikelets with exogenous auxin on rice yield traits. Effect of IAA (A), 2, 4-D (B), and NAA (C) treatment on seed-setting rate (%), yield per spike (gram), and 1,000-grain weight (gram) in rice spikelets. The concentrations of 2, 4-D, IAA, and NAA were 0 (mock), 50,100, and 200 μM. Values are means ± SD. Statistically significant differences at $P < 0.05$, 0.01, and 0.001 are indicated by *, **, and ***, respectively (Student's *t*-test; compared with the corresponding mock control)

Flag leaves provide the greatest nutrition for the spikelets during grain development; therefore, we also treated flag leaves with 50, 100, or 200 μM of 2, 4-D, IAA, and NAA. Similar to the results for spikelets, treatment of flag leaves with 50,100, or 200 μM auxin dramatically increased the seed-setting rate (by

12.4%, 11.3%, and 10.8% with IAA; 18.1%, 17.2%, and 17.4% with 2, 4-D; and 12.3%, 10.5%, and 9.6% with NAA) compared with the control values (Figs. S1A–S1C). Additionally, the yield per spike increased following treatment with 50 or 100 μM IAA as well as with 50, 100, or 200 μM 2, 4-D (Figs. S1A and S1B). However, 1,000-grain weight was not affected by auxin treatment (Figs. S1A–S1C). Collectively, these results confirm that treating different rice tissues with exogenous auxin effectively increases yield in a concentration-dependent manner.

3.2 OsYUCCA Genes are Differentially Expressed during Grain Filling

OsYUCCA genes are involved in IAA biosynthesis and are expressed in almost all organs, including roots and leaves, and in vascular tissues [29]. Rice contains 14 *OsYUCCA* homologs [22]. Our finding that auxin promotes rice grain filling prompted us to test whether *OsYUCCA* genes are also involved in rice grain filling. To this end, we quantified the expression levels of *OsYUCCA1–OsYUCCA14* in rice kernels during grain filling at 0, 5, 10, 15, and 20 days after flowering by qRT-PCR. The time-course analysis revealed that the expression of nine genes (*OsYUCCA1*, *OsYUCCA2*, *OsYUCCA3*, *OsYUCCA5*, *OsYUCCA7*, *OsYUCCA8*, *OsYUCCA9*, *OsYUCCA11*, and *OsYUCCA12*) increased, particularly starting from day 10, whereas the expression of three genes (*OsYUCCA4*, *OsYUCCA6*, and *OsYUCCA10*) decreased as grain filling progressed, and *OsYUCCA13* and *OsYUCCA14* were not detected (Fig. 2). Among the upregulated genes, *OsYUCCA5*, *OsYUCCA7*, *OsYUCCA8*, and *OsYUCCA12* were primarily expressed in the early stage of grain filling (by day 5), and the other five genes were upregulated in the middle or late stages. Similarly, *OsYUCCA9* and *OsYUCCA11* were previously reported to play important roles in rice grain filling [6]. These results suggest that YUCCA-dependent auxin biosynthesis is involved in rice grain filling, which prompted us to continue to analyze the potential of *YUCCA* genes to improve rice yield.

3.3 Confirmation of the Tissue-Specific Overexpression of YUCCA Genes in Transgenic Rice

The YUCCA enzymes represent the rate-limiting step auxin biosynthesis, and in Arabidopsis, their overexpression dramatically increases the concentration of IAA [30]. Plants expressing *35S:OsYUCCA1* accumulate high levels of auxin but are very difficult to regenerate due to abnormal organ development [29]. Therefore, we used the Arabidopsis homolog *AtYUCCA1* in place of *OsYUCCA1*. We selected the promoters of *Glutelin 1* (*OsGt1*) and *OsGluB* from rice and of the ribulose 1, 5-bisphosphate carboxylase/oxygenase small subunit promoter from Arabidopsis (*Rubisco*) to drive the tissue-specific expression of the *YUCCA* genes. *OsGt1* and *OsGluB* are specifically expressed in the endosperm [31,32], and *Rubisco* is specifically expressed in leaves [33]. To further investigate the role of auxin biosynthesis genes in rice grain development, we generated transgenic plants that expressed *AtYUCCA1* or *OsYUCCA2* fused to the β-glucuronidase (*GUS*) reporter gene and driven by the tissue-specific *OsGt1* and *OsGluB* promoters from rice and the *Rubisco* promoter from Arabidopsis (Fig. S2A).

We performed GUS staining and RT-PCR assays to examine the expression patterns and levels of the *AtYUCCA1* and *OsYUCCA2* transgenes in the endosperm and leaves of T_1 plants. GUS staining was observed in the endosperm of *pOsGt1::AtYUCCA1:GUS* plants (lines #1, #2, #3, and #4) and *pOsGluB::OsYUCCA2:GUS* plants (lines #1, #2, #3, and #4) (Fig. 3A), whereas *pRubisco::AtYUCCA1:GUS* #1 and *pRubisco::OsYUCCA2:GUS* lines #1/#2 showed GUS staining in the leaves (Fig. 3C). RT-PCR assays confirmed that these lines showed higher tissue-specific expression of *YUCCA1* and *YUCCA2* than the non-transgenic plants (Fig. 3B). In the T_3 generation, we selected homozygous transgenic lines according to their segregation for G418 resistance. The homozygous lines grew on agar supplemented with G418, whereas the root growth of wild-type plants was inhibited (Fig. S2B).

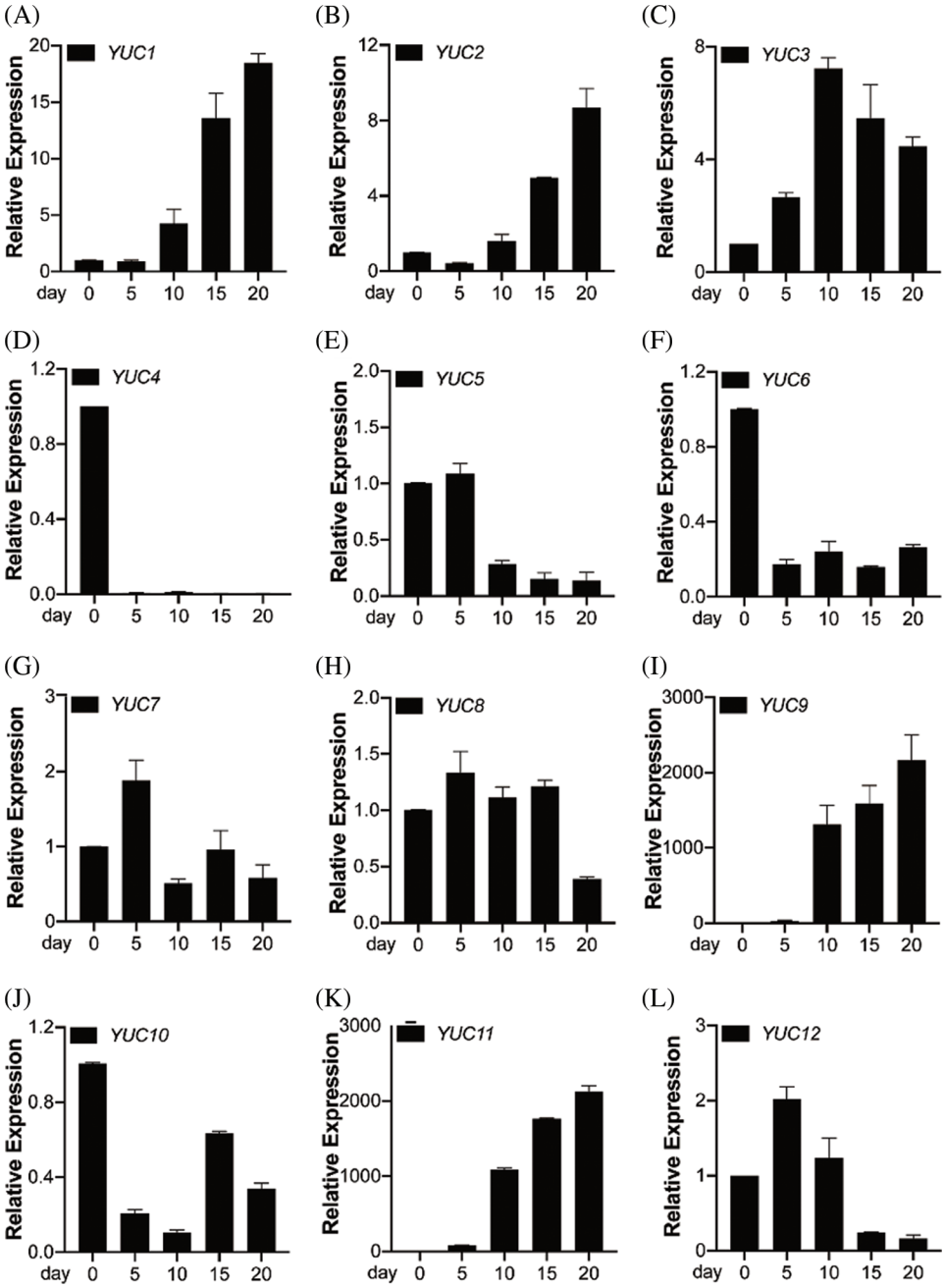

Figure 2: qRT-PCR analysis of transcript levels of *OsYUCCA* genes during grain filling. Expression of *OsYUCCA* genes in rice grains 0, 5, 10, 15, and 20 days after flowering. *OsACTIN* was used as an internal control. For each gene, the transcript level was normalized to the level at 0 day after flowering. Values shown are means ± SD

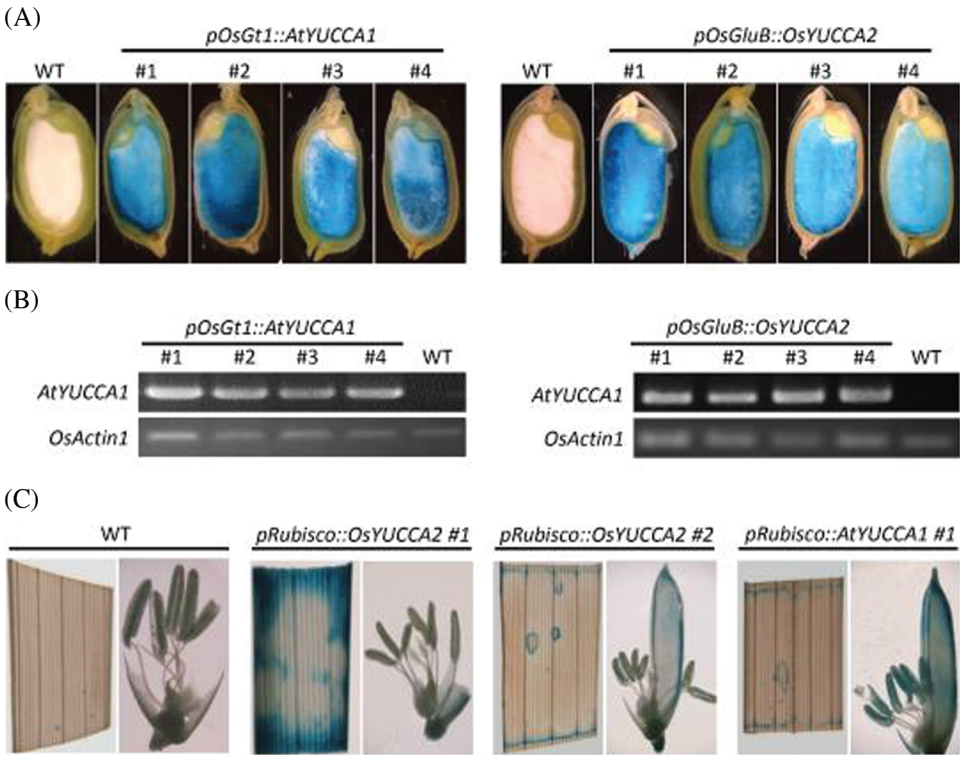

Figure 3: Tissue-specific expression of *AtYUCCA1* or *OsYUCCA2* in transgenic expression lines. (A) β-Glucuronidase (GUS) staining of transgenic lines expressing *AtYUCCA1* or *OsYUCCA2* driven by the endosperm-specific promoter *pOsGt1::AtYUCCA1:GUS* or *pOsGluB::OsYUCCA2:GUS*. Bar = 4 mm. (B) RT-PCR assays in individual T_1 G418-resistant transgenic lines expressing *AtYUCCA1* or *OsYUCCA2* under the control of endosperm-specific promoters. Numbers indicate different transgenic lines. *OsACTIN1* was used as an internal control. (C) GUS staining of transgenic lines expressing *OsYUCCA2* and *AtYUCCA1* driven by the leaf-specific promoter *pRubisco*

3.4 Tissue-Specific Expression of AtYUCCA1 and OsYUCCA2 Increases OsIAA Transcript Levels

Overexpression of *YUCCA* genes in Arabidopsis resulted in altered auxin phenotypes [13,30]. Rice contains 31 *OsIAA* homologs, which show different spatiotemporal expression patterns [34]. Treatment with exogenous IAA promotes the expression of *OsIAA9* and *OsIAA30* in young panicles and *OsIAA1*, *OsIAA6*, and *OsIAA10* in leaves [34]. To determine the effect of tissue-specific *AtYUCCA1* and *OsYUCCA2* expression on the expression levels of endogenous *OsIAA* genes, we analyzed *OsIAA* gene expression in the young panicles of transgenic plants using qRT-PCR. The expression of both *OsIAA9* and *OsIAA30* was higher in caryopses of *pOsGt1::AtYUCCA1:GUS* and *pOsGluB::OsYUCCA2:GUS* transgenic lines compared to the wild type (Fig. 4A). These results demonstrate that the endosperm-specific expression of either *AtYUCCA1* or *OsYUCCA2* increases the expression of *OsIAA9* and *OsIAA30*. We also analyzed the levels of *OsIAA1*, *OsIAA6*, and *OsIAA10* expression in transgenic rice plants that carried the leaf-specific *pRubisco::OsYUCCA2:GUS* construct and observed that all three *OsIAA* genes were dramatically upregulated in lines #1 and #2 (Fig. 4B). In addition, IAA content determination of seeds indicated that *pOsGt1::AtYUCCA1:GUS* and *pOsGluB::OsYUCCA2:GUS* material content was increased compared to the wild type (Fig. 4C). These results further confirmed that the transgenic expression of *AtYUCCA1* and *OsYUCCA2* increased the expression of *OsIAA* genes in rice.

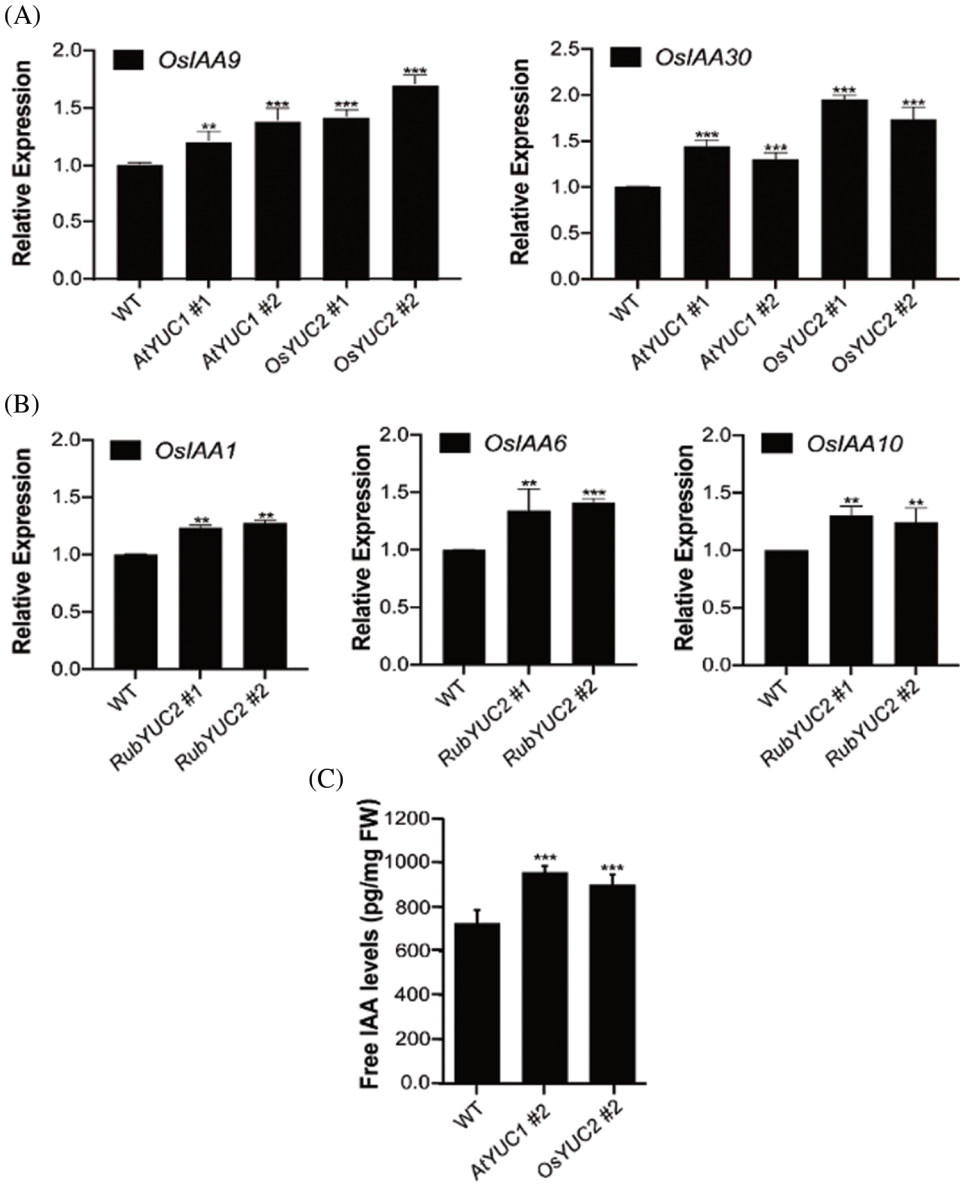

Figure 4: Tissue-specific overexpression of *YUCCA1* or *YUCCA2* increases *OsIAA* transcript levels. (A) Transcript levels of *OsIAA9* and *OsIAA30* determined by qRT-PCR in *pOsGt1::AtYUCCA1:GUS* (*AtYUC1*) and *pOsGluB::OsYUCCA2:GUS* (*OsYUC2*) transgenic lines. (B) qRT-PCR assays in transcript levels of *OsIAA1* and *OsIAA6* in *pRubisco::OsYUCCA2:GUS* (*RubYUC2*) transgenic lines. Wild-type Nipponbare was used as the negative control. Numbers indicate different transgenic lines. *OsACTIN* was used as an internal control. (C) Measurement of auxin transport in wild-type, *pOsGt1::AtYUCCA1:GUS* (*AtYUC1*) and *pOsGluB::OsYUCCA2:GUS* (*OsYUC2*) plants. Values are means ± SD. Statistically significant differences at $P < 0.01$ and 0.001 are indicated by ** and ***, respectively (Student's *t*-test; compared with the wild type)

3.5 Transgenic Expression of AtYUCCA1 or OsYUCCA2 Improves Grain Yield

Next, we quantified grain yield traits in the transgenic expression lines to confirm the effect of endogenous auxin on rice yield. In the endosperm-specific expression lines *pOsGt1::AtYUCCA1:GUS*

and *pOsGluB::OsYUCCA2:GUS*, the seed-setting rate was notably elevated (Figs. 5A, 5D), and the yield per spike was increased relative to the wild type (Figs. 5B, 5E). However, no significant differences in 1,000-grain weight were observed between the wild type and the endoderm-specific transgenic lines (Figs. 5C, 5F). Similarly, seed-setting rate and yield per plant, but not 1,000-grain weight, were increased in lines that expressed *pRubisco::OsYUCCA2:GUS* specifically in the leaves (Figs. 5G–5I). These results agree with previous findings that exogenous auxin promotes grain yield. Collectively, these results supported the notion that YUCCAs play a critical role in rice grain filling.

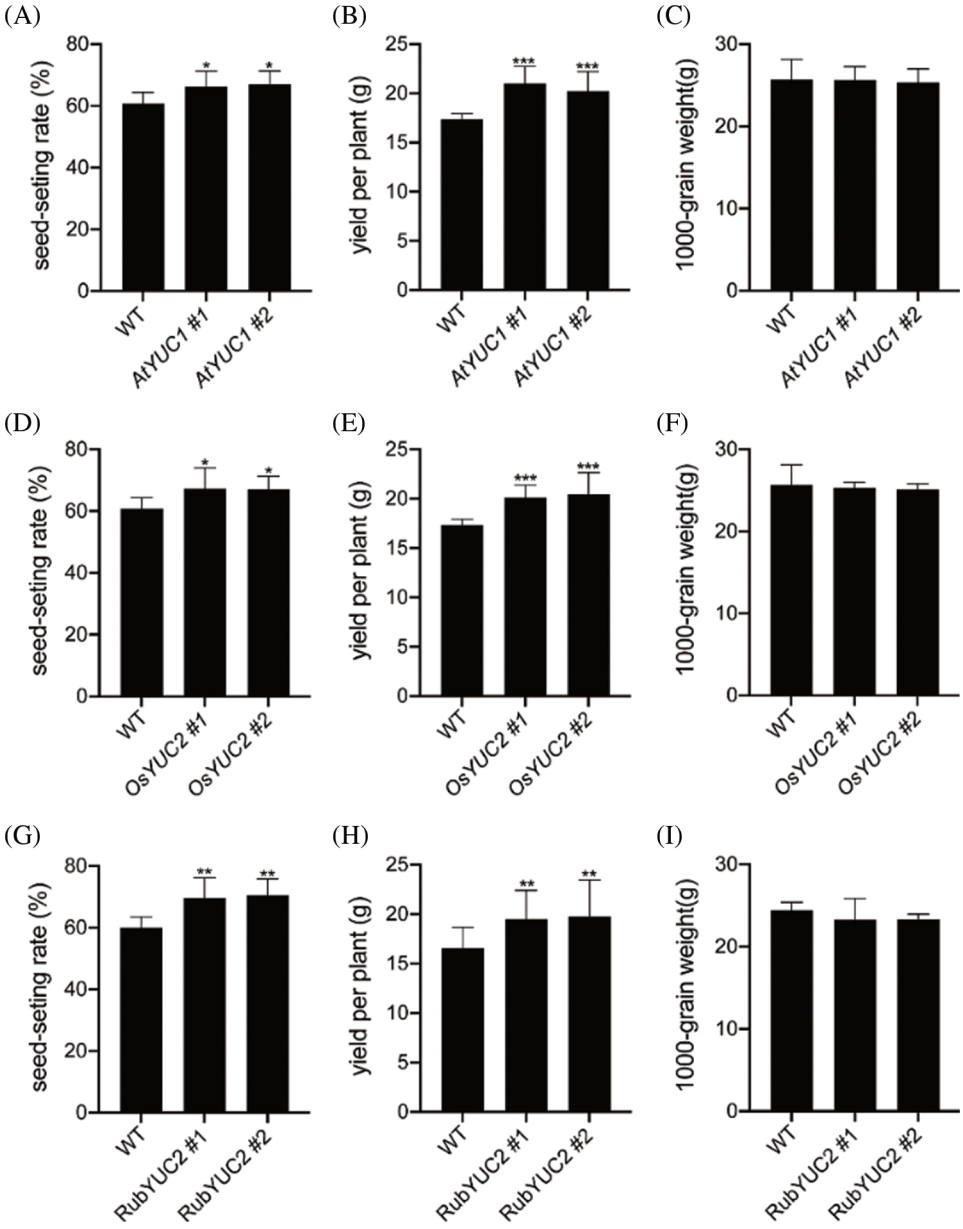

Figure 5: Analysis of yield traits in rice transgenic lines transgenically expressing *AtYUCCA1* or *OsYUCCA2*. Seed-setting rate (%), yield per plant, and 1,000-grain weight in *pOsGt1::AtYUCCA1:GUS* (*AtYUC1*) (A–C), *pOsGluB::OsYUCCA2:GUS* (*OsYUC2*) (D–F), and *pRubisco::OsYUCCA2:GUS* (*RubYUC2*) (G–I) transgenic lines. CK, non-transgenic lines. Values are means ± SD. Statistically significant differences at $P < 0.05$, 0.01, and 0.001 are indicated by *, **, and ***, respectively (Student's *t*-test; compared with the non-transgenic lines)

4 Discussion

Plant hormones are critical in the regulation of seed set, and studies suggest that auxin can substitute for pollination and fertilization signals to promote seed growth [35]. In rice, the TAA/YUCCA pathway is essential for IAA biosynthesis during grain filling, and the IAA concentration is strongly correlated with the expression of IAA biosynthesis genes, including *OsYUCCA9*, *OsYUCC11*, and *OsTAR1* [36]. Here, we confirmed that increasing the auxin level via both exogenous and endogenous methods improves grain yield in rice.

4.1 Exogenous Auxin Treatments Increase Rice Yield

Exogenous plant growth regulators are commonly applied to crops: For example, exogenous application of the gibberellin GA_3 affects the deposition of storage compounds in seeds during seed filling in oilseed rape [37]. Auxins such as IAA repress the germination of soybean seeds by mediating the synthesis of abscisic acid and GA [21]. Consistent with this, our results indicated that exogenous auxin treatment promoted the seed-setting rate and yield per spike in rice (Fig. 1). When we treated young spikelets and flag leaves with 2, 4-D, IAA, or NAA, treatment of the flag leaf was more effective in promoting yield (Fig. S1). In particular, the seed-setting rates were substantially higher when the flag leaf, compared with the spikelet, was treated with different concentrations of 2, 4-D, IAA, or NAA. Notably, treating flag leaves with exogenous auxin mainly promotes photosynthesis in those leaves (Watson, 1952); by contrast, treating the young spikelet promotes the transport of photosynthetic products to the grain. This is why improving the photosynthetic efficiency of rice leaves is the main factor involved in increasing rice yield under conditions that limit photosynthetic capacity. Moreover, the effect of different exogenous auxins on crop yield is variable and may depend on environmental conditions, genotype, or the properties of the auxins. Collectively, these results confirm the important roles of exogenous auxins in promoting seed yield in rice.

4.2 Endosperm-Specific Expression of AtYUCCA1 and OsYUCCA2 Increases Rice Yield

Grain filling is the critical period that determines rice yield and quality [38]. The main component of rice grains is starch, and starch content and composition directly influence rice yield and quality. IAA affects the activities of enzymes involved in the conversion of sugar into starch. The protein products of *TAA1* and the *YUCCA* genes are required to catalyze the biosynthesis of IAA from tryptophan. A recent study suggested that loss of *OsYUCCA11* function reduces the seed weight and decreases the size of starch granules compared with wild-type plants [6], indicating that auxin biosynthesis genes in rice critically affect yield. Therefore, we investigated the expression of 14 *OsYUCCA* genes in kernels during grain filling and observed differential expression patterns. The expression of nine *OsYUCCA* genes was upregulated during grain filling, with two showing expression peaks at day 5 and the others late in grain filling (Fig. 2). By contrast, three *OsYUCCA* genes showed decreasing expression throughout grain filling, and two showed no expression during this process, implying that different *OsYUCCA* genes have stage-or tissue-specific functional roles and may be functionally redundant. This is consistent with previous findings that *OsYUCCA7* is highly expressed early in grain development (2 days after pollination, DAP) and *OsYUCCA9* and *OsYUCCA11* are highly expressed at 7 DAP, whereas *OsYUCCA12* shows a transient peak in expression at 3–4 DAP [36]. Furthermore, recent studies have reported that *OsYUCCA1*, *OsYUCCA9*, and *OsYUCCA11* expression gradually increases during endosperm development, whereas *OsYUCCA12* is specifically expressed in the endosperm at 2 DAP, and its expression then decreases [39]. These results suggest that the YUCCA pathway is critical for auxin biosynthesis and, therefore, for rice grain development.

Exogenous plant growth regulators can be expensive or harmful to the environment, and genetic modification technologies have been widely used in agriculture to improve crop performance (Phillips, 2010). The epidermis-specific promoter of FLORAL BINDING PROTEIN 7 (FBP7) from petunia has been used to drive the tissue-specific expression of an IAA biosynthesis gene, *iaaM*, in ovule epidermal

cells, and this specifically increased IAA levels and substantially increased the number of lint fibers in cotton [40]. To further dissect the role of the auxin biosynthesis genes *AtYUCCA1* and *OsYUCCA2*, we expressed them from the promoters of endosperm-and leaf-specific genes. We detected GUS signals in the endosperm and leaves of the transgenic plants (Fig. 3) and observed increased levels of *OsIAA* transcripts compared with those in wild-type plants (Fig. 4). The seed-setting rate and yield per spike were notably greater in the transgenic plants, whereas 1,000-grain weight was not affected (Fig. 5). The absence of an effect on 1,000-grain weight might arise mainly because an increased concentration of IAA causes more photosynthates to be transported to the smaller grains and less to the larger grains. Grain quality was improved by the endosperm-specific overexpression of *iaaM* in rice [39]. We also observed that in some plants with a high seed-setting rate, the yield per spike did not significantly increase because the yield per plant was also affected by the panicle number and total grain number per plant. It will be important to determine which other factors regulate crop yield, as well as to determine the most appropriate IAA concentration to increase yield.

Overexpression of *AtYUCCA1* and *iaaM* leads to dramatic auxin overproduction phenotypes in Arabidopsis [41]; similarly, overexpression of *OsYUCCA1* causes abnormal leaf, root, and stem development in rice [29]. Furthermore, rice lines constitutively expressing *35S::OsYUCCA1* are extremely difficult to regenerate and grow to seed set because they have defects in organ development. Therefore, by contrast with constitutive expression, the elevation of phytohormone levels at specific developmental stages or in specific tissues from tissue-specific promoters represents a potential and feasible method to improve crop yield. Here, we used endosperm-and leaf-specific promoters to express *AtYUCCA1* and *OsYUCCA2* to increase the IAA level, and subsequently grain yield, without adversely affecting plant development.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

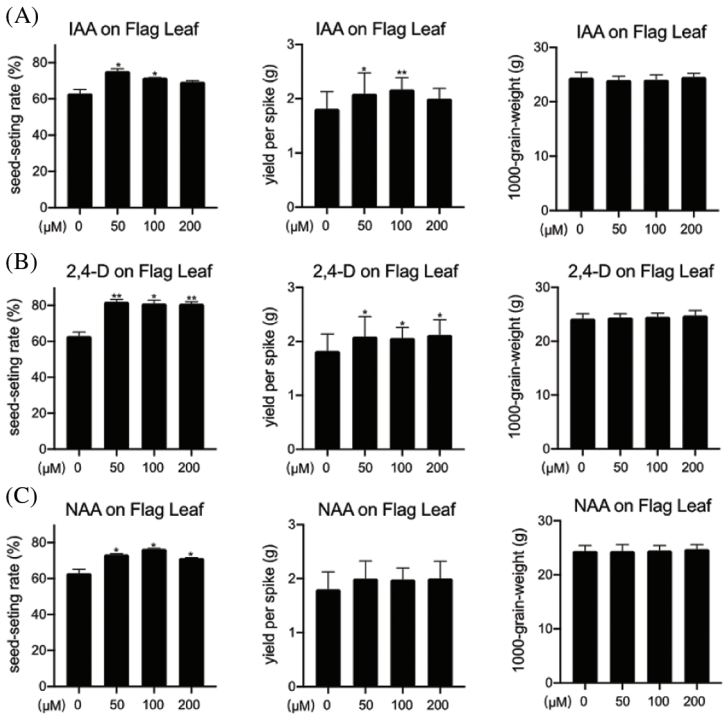

Figure S1: Effect of auxins on rice yield traits in the flag leaf. Effects of IAA (A), 2, 4-D (B), and NAA (C) treatments on seed-setting rate (%), yield per spike (gram), and 1,000-grain weight (gram) in the flag leaf. Concentrations of 2, 4-D, IAA, and NAA were 0 (mock), 50, 100, and 200 μM. Values shown are means ± SD. Statistically significant differences at $P < 0.05$ and 0.01 are indicated by * and **, respectively (Student's *t*-test; compared with the corresponding mock control)

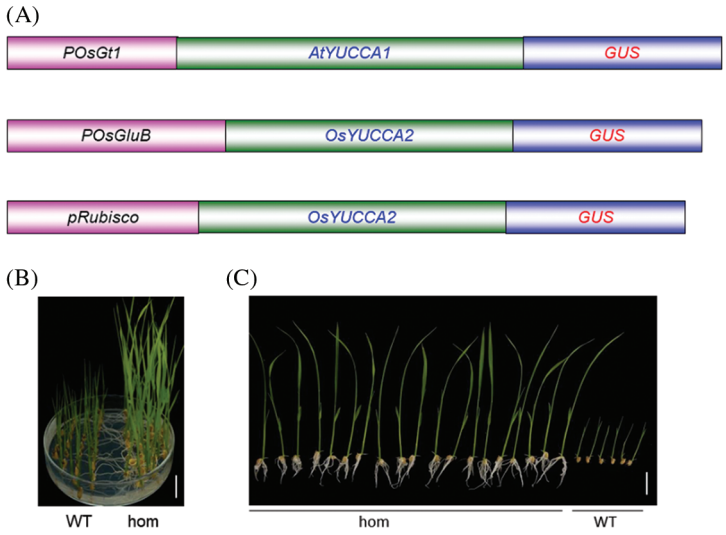

Figure S2: Molecular cloning and identification of homozygous transgenes. (A) Constructs used for the tissue-specific expression of *AtYUCCA1/OsYUCCA2*. The promoters *pOsGt1* and *pOsGluB* from rice were used to drive endosperm-specific expression, and Arabidopsis *pRubisco* was used for leaf-specific expression. (B) and (C) Identification of homozygous lines in the T_3 generation. B: Left, wild-type, and right, homozygous lines, Bar = 1 cm; C: Left, homozygous, and right, wild-type lines, bar = 2 cm

Table S1: Primer sequences for quantitative reverse transcription-PCR

Primer	Sequences information 5'-3'
OsYUCCA1-realF	TCATCGGACGCCCTCAACGTCGC
OsYUCCA1-realR	GGCAGAGCAAGATTATCAGTC
OsYUCCA2-realF	GTCCAAAGGGAGGAGTCGTCCAG
OsYUCCA2-realR	GCATGATGTTTACACCCGGCCTT
OsYUCCA3-realF	GTGAGAACGGGCTCTACTCGGTCG
OsYUCCA3-realR	GCTTATGCATGACCGATGAACACG
OsYUCCA4-realF	GCAGAATGGCCTGTACGCTGTTGG
OsYUCCA4-realR	CAGACCAGCACATGACGTGTCTAC
OsYUCCA5-realF	ACCTCCTACGACGCCGCCATGATC
OsYUCCA5-realR	CTCCCAACACAGCGACGACAGAAC
OsYUCCA6-realF	CCATTCCCAGATGGTTGGAAGG
OsYUCCA6-realR	CATGTTGCGCCTCAAGATATTTG
OsYUCCA7-realF	CACTGCTGTGTCCTACAATATCAC
OsYUCCA7-realR	GGAGGTGCATCTCCGTCATCTTC
OsYUCCA8-realF	CCGGGAGGTGGCAGGAGACGCAGCA
OsYUCCA8-realR	ATGTTGCCATGCATGCGAGCGAGAG
OsYUCCA9-realF	AGCAGCAGCAAGCCTACCCACAACA
OsYUCCA9-realR	AATCAAAGACCACCCAAGGGCAAGT
OsYUCCA10-realF	GGATGGTGAGGAGGGGAATCTACGG
OsYUCCA10-realR	TGGACGTCAAAGTGAACAGGGCCTA
OsYUCCA11-realF	GATTATCTGGTATTGCTCATGACGC
OsYUCCA11-realR	AGCTTGCTTCAAACATAATGTAACA
OsYUCCA12-realF	GAGATAAGGGAAAACCCAAAAGCAA
OsYUCCA12-realR	CCCAGATATATACATAGTGGCCAAA
OsYUCCA13-realF	AAGGAATGCCATTACCTCCATACAA
OsYUCCA13-realR	CCGCTCCTCTTCTCTCTTCTCATTT
OsYUCCA14-realF	GAAAAATATTGCAAATGACATCGTG
OsYUCCA14-realR	AAGTTGGATTTTACACGAGCTGAAG
OsIAA1-realF	ACCAAGAGCCGCTCAATGAG
OsIAA1-realR	ATCACACGTGGGCGAACATC
OsIAA6-realF	GGCTATCGTCAGCTGTCAAAC
OsIAA6-realR	GCAATTTGCGCATTAGTTTGG
OsIAA9-realF	CGAGAAGAAAATGGCCAATGA
OsIAA9-realR	ATCCCCATCACCATCCTCGTA
OsIAA10-realF	CACATCTGAAACCGACACCAA
OsIAA10-realR	CTTTTCGCCCTCCTCCTTGT

(Continued)

Table S1 (continued)

Primer	Sequences information 5'-3'
OsIAA30-realF	CAGCTCCTTCACCATTGGAAA
OsIAA30-realR	CAGAGCCGTTGAGCAGATCA
OsACTIN-realF	TGGCATCTCTCAGCACATTCC
OsACTIN-realR	TGCACAATGGATGGGTCAGA

Table S2: Primers used for construction of *YUCCA* expression vectors with different promoters

Primer	Sequences information 5'-3'
OsGt1-*Hind*III-F	GAAAGCTTAGGTCATAGGGAGAGGGAGCT
OsGt1-*Kpn*I-R	AGGGTACCGTTGTTGTAGGACTAATGAACTG
pOsGluB-1-157-*Hind*III-F	TGGAAGCTTGACCAAGGAAAAGCTCGTATTAGTGAGTAC
pOsGluB-1-1553-*Kpn*I-R	GAGGGTACCCTATTTGTACTTGCTTATGGAAACTTAAGCTAA
pRubisco-Pro-183-*Hind*III-F	TAGAAGCTTAAGACCAAATCCTCTGTTTTAGAT
pRubisco-Pro-1522-*Kpn*I-R	ATCGGTACCTACTTCTTCTTGTTGTTTCTCTTCTTCTTT
GUS-244-R	ATCGAAACGCAGCACGATACGCTG
OsYUC2-*Kpn*I-F	TGGGGTACCATGCTTGTTTGGGTTCAAGGGCCAATAGTT
OsYUC2-*BamH*I-R	CGAGGATCCGGAAAAGAAATACTGAAATTCTTCTACAGC
AtYUC1-32540-*Kpn*I-F2	CAGGGTACCATGGAGTCTCATCCTCACAACAAAACTGAC
AtYUC1-32540-*BamH*I-R2	CGAGGATCCGGATTTAGAGGTAAAGACAAAACGAGAACT