



Pluronic F-68 Improves Root Growth of Recalcitrant Rice Cultivar Through Enhanced Auxin Biosynthesis

Authors:

Andrew De-Xian Kok, Janna Ong-Abdullah, Amanda Shen-Yee Kong, Rogayah Sekeli, Chien-Yeong Wee, Swee-Hua Erin Lim, Wan-Hee Cheng, Jiun-Yan Loh* and Kok-Song Lai*

***Correspondence:** james.loh@jcu.edu.au; lkoksong@hct.ac.ae

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Highlights:

- The 0.04% PF-68 significantly increased both the length and number of roots in the recalcitrant MR 219 rice.
- PF-68 treatment of MR 219 rice resulted in elevated indole-3-acetic acid concentrations, providing further evidence of its role in auxin biosynthesis.
- PF-68 has the potential to stimulate root growth, thereby enhancing rice production.

EARLY VIEW

Pluronic F-68 Improves Root Growth of Recalcitrant Rice Cultivar Through Enhanced Auxin Biosynthesis

¹Andrew De-Xian Kok, ¹Janna Ong-Abdullah, ²Amanda Shen-Yee Kong, ³Rogayah Sekeli, ³Chien-Yeong Wee, ⁴Swee-Hua Erin Lim, ⁵Wan-Hee Cheng, ⁶Jiun-Yan Loh* and ⁴Kok-Song Lai*

¹Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

² School of Pharmacy, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Subang Jaya 47500, Malaysia

³ Biotechnology and Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), Kuala Lumpur, Malaysia

⁴ Health Sciences Division, Abu Dhabi Women's College, Higher Colleges of Technology, Abu Dhabi, United Arab Emirates

⁵ Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Putra Nilai, Nilai 71800, Negeri Sembilan, Malaysia

⁶Tropical Futures Institute, James Cook University Singapore, 149 Sims Drive, 387380, Singapore

*Corresponding authors: james.loh@jcu.edu.au; lkoksong@hct.ac.ae

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Abstract. In plants, roots play a vital role in crop performance and yield that impact the agricultural productivity. Pluronic F-68 (PF-68) is a type of non-ionic surfactant that is typically utilized as a plant growth additive. There is a lack of studies on the impact of PF-68 on root growth. This work

aims to assess the impacts of PF-68 on recalcitrant MR 219 rice root growth. Supplementation of 0.04% PF-68 enhanced the length (18.50%) and number of roots (15.87%) of MR 219 rice. The PF-68-treated MR 219 rice also showed a significant increment in sugar accumulation (1.73 mg/mL) and glutamate synthase activity (0.88 $\mu\text{mol/g}$ protein). Consistent with the root growth enhancement, MR 219 rice supplemented with PF-68 recorded an increased in transcription levels of *Indole-3-Acetic Acid 23* (*OsIAA23*) (1.84-folds) and *WUSCHEL-Related Homeobox 11* (*OsWOX11*) (2.00-folds). Moreover, the PF-68-treated MR 219 rice also exhibited an enhancement of indole acetic acid (IAA) concentrations (27.33 ng/g FW), further suggesting its role in auxin biosynthesis. Taken together, our study revealed that the introduction of PF-68 enhanced the root growth of MR 219 rice through improved sugar accumulation, glutamate synthase activity, and auxin biosynthesis.

Keywords: Auxin Biosynthesis, Root Growth, Pluronic F-68, Recalcitrant indica cv. MR 219

Abstrak. Dalam tumbuhan, akar memainkan peranan penting dalam prestasi tanaman dan hasil yang memberi kesan kepada produktiviti pertanian. Pluronic F-68 (PF-68) adalah sejenis surfaktan bukan ionik yang biasanya digunakan sebagai bahan tambahan pertumbuhan pokok. Tidak banyak kajian dibuat berkenaan kesan PF-68 terhadap pertumbuhan akar. Kajian ini bertujuan untuk menilai kesan PF-68 terhadap pertumbuhan akar padi MR 219 yang rekalsitrant. Penambahan 0.04% PF-68 meningkatkan kepanjangan (18.50%) dan bilangan (15.87%) akar pokok padi MR 219. Pokok yang dirawat juga menunjukkan peningkatan yang ketara dalam pengumpulan gula (1.73 mg/mL) dan aktiviti sintase glutamat (0.88 $\mu\text{mol/g}$ protein). Selaras dengan peningkatan pertumbuhan akar, pokok-pokok tersebut juga merekodkan peningkatan tahap transkripsi Indole-3-Acetic Acid 23 (*OsIAA23*) (1.84 kali ganda) dan *WUSCHEL-Related Homeobox 11* (*OsWOX11*) (2.00 kali ganda). Tambahan pula, pokok MR 219 yang dirawat PF-68 juga menunjukkan peningkatan kepekatan asid indole asetik (IAA) (27.33 ng/g FW), seterusnya mencadangkan peranannya dalam biosintesis auksin. Secara keseluruhan, kajian kami mendedahkan bahawa pengenalan PF-68 meningkatkan pertumbuhan akar pokok MR 219 melalui peningkatan pengumpulan gula, aktiviti sintase glutamat, dan biosintesis auksin.

Kata Kunci: Biosintesis Auksin, Pertumbuhan Akar, Pluronic F-68, Indika Rekalsitrant cv. MR 219

INTRODUCTION

Rice stands as the paramount staple for over half the global population. Its cultivation spans approximately 154 million hectares annually, primarily in Asia. Predictions by the United Nations initially suggested an 8-billion-person world by 2025 (Khush 2005). To meet the escalating demand driven by population growth, rice production needs a 40% boost (Low *et al.* 2018). However, this improvement must occur amid constraints like limited land, reduced water, labour, and fertilizer usage (Coudert *et al.* 2010; Kok *et al.* 2018). Enhancing root growth becomes pivotal for amping up rice production. A larger root system facilitates greater soil access, crucial for water and nutrient absorption at varying depths (Meng *et al.* 2019).

As a non-ionic surfactant, Pluronic F-68 (PF-68) finds application in plant and animal cultures (Barbulescu *et al.* 2011; Meier *et al.* 1999). In animal cell cultures, PF-68 aids cell growth stimulation, protection, and the repair of damaged cells in suspension (Meier *et al.* 1999). In plant studies, PF-68 has shown efficacy in enhancing multiple shoot regeneration in various species like *Pyrus communis* (Dashti *et al.* 2012), *Ricinus communis* L. (Kulathuran & Narayanasamy 2015), as well as *Abelmoschus esculentus* L. (Irshad *et al.* 2018). Furthermore, it has been reported to bolster root growth in *Solanum dulcamara* (Kumar *et al.* 1990) and *Corchorus capsularis* L. (Khatun *et al.* 1993). Recent studies have highlighted PF-68's potential in promoting callus proliferation in recalcitrant indica rice (Kok *et al.* 2021; Kok *et al.* 2020). In addition, PF-68-treated callus also showed increased root formation, suggesting its potential role in stimulating rice root growth. (Kok *et al.* 2021; Kok *et al.* 2020).

Although PF-68's effects on plants have been extensively studied, its underlying mechanism and specific impact on root growth remain largely unexplored, especially concerning rice, a crucial global food crop. Moreover, a better understanding of the role of PF-68 in root growth will allow its use to enhance crop growth and improve food security. Therefore, this investigation aims to assess PF-68's influence on the root growth of a challenging rice variety.

MATERIALS AND METHODS

For this study, seeds from the Malaysian rice cultivar MR 219 were utilized. Analytical-grade PF-68 (10%) from Thermo Fisher Scientific, USA, was employed. Seed surface sterilization followed a previously outlined procedure with minor adaptations (Lim & Lai 2017). In brief, mature seeds underwent de-husking and were surface-sterilized using 70% ethanol for 1 min, and then 50% Clorox for 30 min. Post-sterilization, the seeds were rinsed with distilled water and air-dried. These

sterilized seeds were cultured on shoot induction medium consisting of Gamborg's B5 basal medium (Gamborg *et al.* 1968) supplemented with specific nutrients and hormones under controlled conditions (Low *et al.* 2019). After one week, approximately 1 cm of shoot apices were excised and cultured on a root growth medium containing Murashige and Skoog medium (Murashige & Skoog 1962) with varying PF-68 concentrations [0.02, 0.04, 0.06, 0.08, and 0.10% (v/v)]. The medium without PF-68 [0% (v/v)] served as a control. The rooted shoot apices were then incubated under specific light and temperature conditions for three weeks, following which root length and number were recorded. Each treatment was replicated thrice with ten samples per replicate (n=10).

Moreover, approximately 0.5 g of root samples underwent soluble sugar measurement using the phenol-sulphuric acid method (Terzi *et al.* 2014), measuring absorbance at 565 nm. Additionally, glutamate synthase (GOGAT) activity was assessed following Ertani *et al.*'s method (2011) (Ertani *et al.* 2011). Root samples (approximately 0.5 g) were ground into powder with liquid nitrogen for GOGAT activity analysis, measuring absorbance at 340 nm.

RNA isolation from plant sample powder subjected to different treatments (control and 0.04% PF-68) followed Lai and Masatsugu's protocol (2013) using the RNeasy Plant Mini Kit (Qiagen, Germany) (Lai & Masatsugu 2013). For first-strand cDNA synthesis, 1 µg of extracted total RNA was processed with the QuantiNova Reverse Transcription Kit (Qiagen, Germany). The primers were designed using the Primer-Blast from the National Center for Biotechnology Information (NCBI) (Supplementary Table 1) and synthesized by Integrated DNA Technologies (IDT, USA). Real-time PCR was executed on a Bio-Rad CFX96 system (Bio-Rad, US) with QuantiNova SYBR Green PCR (Qiagen, Germany), following the methodology outlined by Lai *et al.* (2011) (Lai *et al.* 2011). PCR conditions comprised an initial step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 5 s. Each sample underwent three technical replicates across three biological replicates. Data analysis was carried out using Bio-rad CFX Manager 3.1 software, and relative expression levels ($2^{-\Delta\Delta CT}$) were calculated using Livak's method (Livak & Schmittgen 2001). The *rice cyclophilin* (*OsCYC*) and *ubiquitin 5* (*OsUBQ5*) were employed as reference genes in this study.

To assess indole-3-acetic acid (IAA) levels in both control and 0.04% PF-68-treated roots, the method outlined by Pan *et al.* (2010) was followed. Analysis was conducted using Agilent 1100 HPLC (Agilent Technologies, United States), and IAA levels were quantified using an external standard method (ng/g FW) with three biological replicates (Pan *et al.* 2010). All data presented are the mean $\pm$ standard error of the mean (SEM) from three biological replicates, each with three technical replicates. Statistical analysis, conducted using one-way analysis of variance

($p < 0.05$) between treatments, was performed utilizing the Statistical Package for the Social Sciences version 20 (IBM 2016).

RESULT AND DISCUSSIONS

This research showcases the efficacy of PF-68 in augmenting root growth in MR 219 rice (Fig. 1a-c). Specifically, the addition of 0.04% and 0.06% PF-68 significantly bolstered ($p < 0.05$) root count by 42.85% and 38.89%, respectively. Conversely, 0.10% PF-68 exhibited the least stimulating effect on root production (15.87%) (Fig. 1a). Moreover, 0.04% PF-68 significantly enhanced ($p < 0.05$) root length by 18.50% (Fig. 1a), while 0.10% PF-68 displayed minimal impact on root length (0.55%) (Fig. 1a). Notably, the lower concentration (0.04%) of PF-68 demonstrated more favourable effects on root growth compared to the higher concentration (0.10%) (Fig. 1a).

(a)

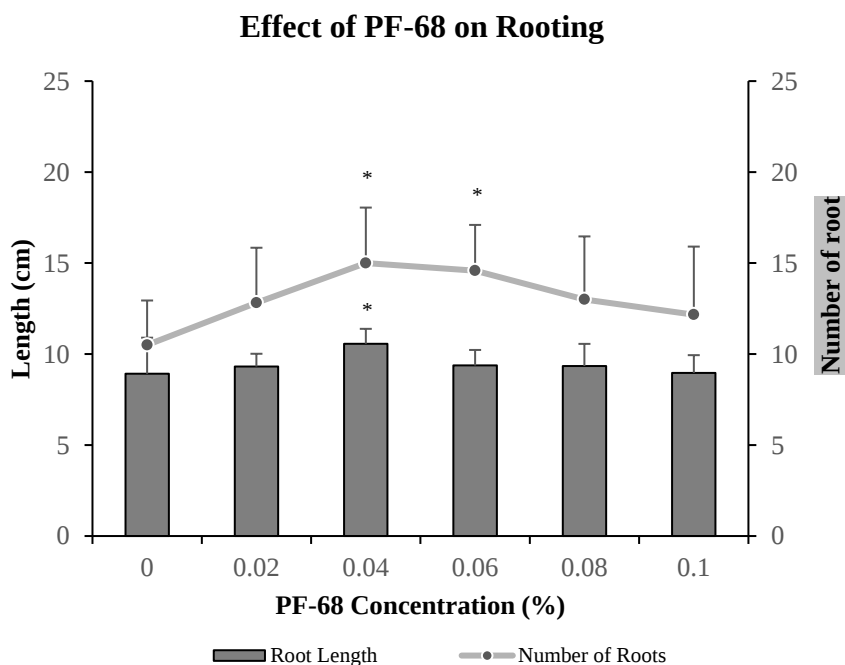




Figure 1. Effects of different concentrations of PF-68 on root growth. **(a)** Root length and number of roots recorded after four weeks of incubation. Morphology of MR 219 shoot apex grown on **(b)** control and **(c)** medium supplemented with 0.04 % PF-68. Data shows mean of three biological replicates ($n = 10$). Asterisks indicate statistical significance difference at $p < 0.05$ compared to control. Error bars represent standard error mean. Scale bar represents 0.5 cm.

Biochemical assessments on control and 0.04% PF-68-treated rice explants revealed a substantial increase in total sugar content (1.73 mg/mL) and GOGAT activity ($0.88 \mu\text{mol/g protein}$) in the treated MR 219 rice compared to the control (Fig. 2a-b). This suggests that the presence of PF-68 promotes root growth via heightened sugar accumulation and GOGAT activity. Further gene expression analysis via real-time PCR focused on three target genes involved in auxin biosynthesis (Fig. 2c). Notably, 0.04% PF-68 treatment resulted in significant increments of *WUSCHEL-Related Homeobox 11* (*OsWOX11*) (2.00-folds) and *indole-3-acetic acid 23* (*OsIAA23*) (1.84-folds) transcripts compared to the control. Additionally, IAA quantification revealed higher content in 0.04% PF-68-treated rice explants ($27.33 \pm 2.08 \text{ ng/g FW}$) versus the control ($22.67 \pm 1.53 \text{ ng/g FW}$) (Fig. 2d), indicating PF-68's potential role in auxin biosynthesis. While these results may not be statistically significant, they could be due to limitations in sample size and uncontrollable variability. However, it's important to note that statistical significance does

not necessarily imply a lack of effect. Hence, we could not completely rule out the positive effect that PF-68 has on the IAA.

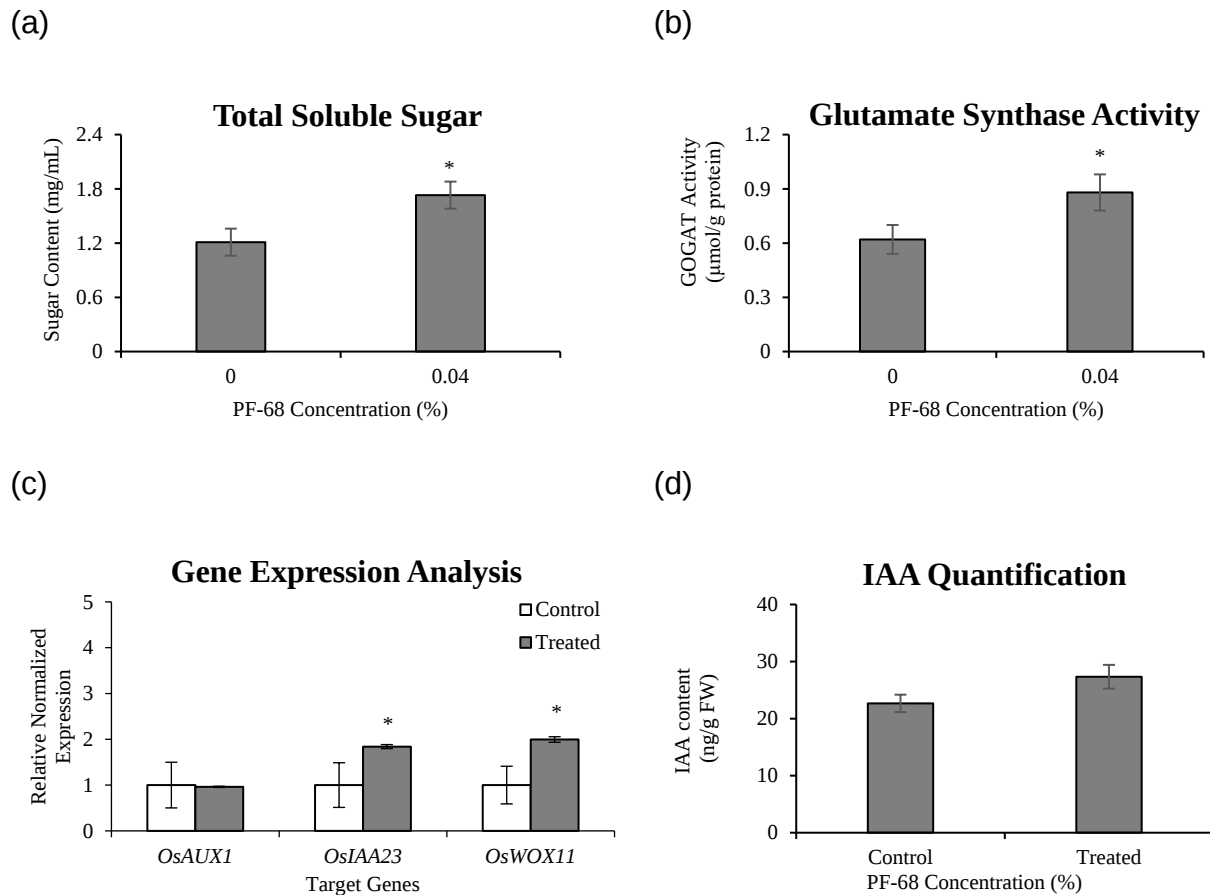


Figure 2. Biochemical assays, gene expression analysis and indole acetic acid (IAA) quantification performed on control and 0.04% PF-68 treated roots. (a) Total sugar content, (b) GOGAT activity, (c) Normalized relative gene expression of selected genes (*OsAUX1*, *OsIAA23*, and *OsWOX11*) and (d) IAA quantification. Data shows the mean of three biological replicates. Asterisk indicates statistically significant at $p < 0.05$ compared to control (0% PF-68). Error bars represent standard error mean.

Root development significantly impacts plant growth and nutrient absorption. This study demonstrated that 0.04% PF-68 supplementation effectively enhanced MR 219 rice root growth (Fig. 1). Considering the fixed external nutrient supply in each treatment, this suggests that PF-68 might facilitate root growth in MR 219 by improving nutrient acquisition. Earlier reports also

align with our findings, indicating PF-68's ability to enhance root growth at lower concentrations, with diminishing effects as the concentration increases (Kumar *et al.* 1990).

Soluble sugars play a pivotal role in plant functions, influencing metabolism, growth, and development. For instance, Eveland and Jackson (2011) highlighted how sugar accumulation fosters cell growth by fuelling carbon and energy production through carbohydrate metabolism (Eveland & Jackson 2011). Both carbon and nitrogen assimilation are vital for overall plant development and are intricately interconnected (Jiang *et al.* 2024). Studies have shown that a decrease in soluble sugar content significantly hampers protein synthesis and diminishes nitrogen utilization efficiency in plants (Hao *et al.* 2020). This reduction in carbon and nitrogen metabolism affects amino acid biosynthesis, crucial as nitrogen serves as a fundamental building block for amino acids (Lehmeier *et al.* 2013). GOGAT, a key player in nitrogen metabolism, exhibited heightened activity in 0.04% PF-68-treated explants, suggesting an enhancement in nitrogen metabolism. The observed increase in soluble sugar accumulation and GOGAT activity (Fig. 2) in these explants indicates PF-68's pivotal role in promoting root growth through carbohydrate and nitrogen metabolisms.

Multiple genes associated with plant root growth, including *OsAUX1*, *OsIAA23*, and *OsWOX11*, underwent scrutiny via gene expression analysis. *OsAUX1* manages auxin transport, pivotal for root growth and lateral root development (Péret *et al.* 2012; Wiśniewska *et al.* 2024). It also facilitates the accumulation of IAA in the root apex, aiding lateral root development (Wiśniewska *et al.* 2024). Mutations in *aux1* have revealed developmental issues linked to auxin, like extended primary roots and shortened root hairs in rice (Yu *et al.* 2015). Agravitropic roots and diminished lateral root initiation have been observed in the mutant *Osaux1* (Swarup *et al.* 2005; Zhao *et al.* 2015). Nonetheless, scrutiny of *OsAUX1* transcript in 0.04% PF-68-treated samples (Fig. 2d) indicated no alterations, suggesting that PF-68 application does not influence *AUX1*, the auxin transporter's regulation.

In plant, the importance of auxin biosynthesis in root development has been extensively documented. IAA, the primary natural auxin, chiefly originates from tryptophan via Trp-dependent and Trp-independent pathways (Zhao *et al.*, 2015). Various studies have highlighted the impact of hindering Trp production on root development (Nishimura *et al.* 2014; Soeno *et al.* 2010). For instance, inhibiting Trp aminotransferase with L-amino-oxyphenylpropionic acid (AOPP) in *Arabidopsis* led to deficiencies in root growth and development (Soeno *et al.* 2010). Similarly, moderate aluminium supply to tea plant roots significantly boosted their IAA content, resulting in notable improvements in lateral root number and length (Gao *et al.* 2022). In this study, the

increase in IAA content (Fig. 2d) in 0.04% PF-68-treated samples aligned with the rise in both root number and length (Fig. 1).

Crown roots are pivotal components of the fibrous root system in rice (Zhao et al. 2015). Genes such as *IAA23* and *WOX11* contribute to crown root development in plants (Islam et al. 2021; Jun et al. 2011). *IAA23* expression is specific to quiescent center cells during various root developments (Meng et al. 2019). Loss of *Osiaa23* function in rice resulted in root cap disintegration and halted root growth (Jun et al. 2011). *WOX11* expression in early crown root primordia and the root meristem's cell division zone play crucial roles (Coudert et al. 2010). Zhao et al. (2009) demonstrated that auxin treatment failed to induce crown root production in rice *wox11* mutants, while overexpressing *WOX11* led to early crown root growth and increased root biomass (Zhao et al. 2009). Enhanced expression of *OsIAA23* and *OsWOX11* transcripts in 0.04% PF-68-treated samples (Fig. 2c) corresponded with increased root number (Fig. 1a). These results support PF-68's capacity to enhance root growth by regulating *OsIAA23* and *OsWOX11* transcripts.

In summary, PF-68 application significantly bolstered root growth in the MR 219 cultivar. Enhanced carbon and nitrogen metabolism in 0.04% PF-68-treated samples, indicated by carbohydrate accumulation and increased GOGAT activity, were evident. Furthermore, upregulated genes involved in crown root development and elevated IAA content suggest increased auxin biosynthesis in 0.04% PF-68-treated samples. Overall, PF-68's efficacy is concentration-dependent, making it a valuable plant supplement for stimulating root growth.

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AUTHORS' CONTRIBUTIONS

Andrew De-Xian Kok: Formal analysis and investigation, Writing – original draft preparation

Janna Ong-Abdullah: Writing – review and editing

Amanda Shen-Yee Kong: Writing – review and editing

Rogayah Sekeli: Writing – review and editing

Chien-Yeong Wee: Writing – review and editing

Swee-Hua Erin Lim: Writing – review and editing

Wan-Hee Cheng: Funding acquisition
Jiun-Yan Loh: Conceptualization
Kok-Song Lai: Conceptualization, Methodology

All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

Table S1: Primers used for respective genes.

Target Genes	Primer Sequence 5`–3`	
OsAUX1	Forward	GGTAGAAGAAGAAGAGGGC
	Reverse	CCAAACAAACACAAGGACA
OsIAA23	Forward	GATCCTACCACACGCAACGA
	Reverse	GCCGTCGTCCAAAAACCAA
OsWOX11	Forward	CCAGATGGGCGAGAGCTACT
	Reverse	CGTTGCCATCGATCAATCAA
Housekeeping genes	Primer Sequence 5`–3`	
OsCYC	Forward	GGTGTCATCATGACTTCTG
	Reverse	GCCCATCCGAAACGATAC
OsUBQ5	Forward	TAGGCGTAGGCTCCTGTTCT
	Reverse	ACAGAGGTGATGCTAAGGTGT