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SUPER STARCHY1/ONAC025 participates in rice grain filling

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Abstract

NAC transcription factors (TFs) are known for their role in development and stress. This article attempts to functionally validate the role of rice *SS1/ ONAC025* (LOC_Os11g31330) during seed development. The gene is seed-specific and its promoter directs reporter expression in the developing endosperm and embryo in rice transgenic plants. Furthermore, rice transgenic plants ectopically expressing *SS1/ ONAC025* have a plantlet lethal phenotype with hampered vegetative growth, but increased tillers and an altered shoot apical meristem structure. The vegetative cells of these plantlets are filled with distinct starch granules. RNAseq analysis of two independent plantlets reveals the differential expression of reproductive and photosynthetic genes. A comparison with seed development transcriptome indicates differential regulation of many seed-related genes by *SS1/ ONAC025*. Genes involved in starch biosynthesis, especially amylopectin and those encoding seed storage proteins, and regulating seed size are also differentially expressed. In conjunction, *SS1/ ONAC025* shows highest expression in *japonica* rice. As a TF, *SS1/ ONAC025* is a transcriptional repressor localized to endoplasmic reticulum and nucleus. The article shows that *SS1/ ONAC025* is a seed-specific gene promoting grain filling in rice, and negatively affecting vegetative growth.

KEYWORDS

amylopectin, grain, NAC, rice, seed development, transcription factor

1 | INTRODUCTION

NACs (NAM, ATAF and CUC, the name having been derived from the first three reported members of the family, NO APICAL MERISTEM/ NAM, *Arabidopsis thaliana* Activation Factor1 and 2/ ATAF1/2 and CUP-SHAPED COTYLEDON 2/ CUC2) are plant-specific TFs. These mutants show defective apical meristems (Souer et al., 1996; Aida et al., 1997). Since then, NACs have been characterized for their roles in both, stress responses and development, and are known to have evolved along with land plants (Mathew

and Agarwal, 2018). NAC TFs such as VNDs (VASCULAR-RELATED NAC-DOMAINS) and SNDs (SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN) are integral for secondary wall biosynthesis process (Yamaguchi et al., 2010; Agarwal et al., 2011; Li et al., 2012). NACs play key roles during embryogenesis, anther and seed dehiscence, nutrient remobilization (Mitsuda et al., 2005; Kunieda et al., 2008; Waters et al., 2009; Agarwal et al., 2011; Shih et al., 2014; Dalman et al., 2017; Mathew and Agarwal, 2018), and senescence programming (Kim et al., 2014; Ma et al., 2014). Wheat NAM1 positively regulates the nutrient

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efflux from vegetative tissues to the grains (Waters et al., 2009). TaNAC69 promotes increase of grain yield under dehydration stress (Xue et al., 2011). In maize, ZmNAC128 and ZmNAC130 regulate accumulation of starch and protein in seeds during endosperm development contributing to grain filling (Zhang et al., 2019). APN-1 is a NAC TF which shows high expression during maize endosperm development. It inhibits precocious germination by repressing the expression of genes encoding α -amylases, proteinases, and glucanases (Verza et al., 2011). Rice (*Oryza sativa*) represents the principal food for majority of the world's populations, especially in Asia and Africa. Coupled with an ever growing population, rice has always taken a key position in the research programs associated with crop improvement. There are 151 NAC encoding genes in rice (Nuruzzaman et al., 2010). We have previously shown that three rice NACs, ONAC020, 026, and 023 are associated with the grain size/weight trait (Mathew et al., 2016). Overexpression of OsNAP, delays senescence and results in increase in grain yield in rice (Liang, Wang et al., 2014). OsNAC10 accelerates leaf senescence and increases nutrient mobilization from leaves to developing seeds, thus, regulating grain filling process in rice (Sharma et al., 2019). Few NAC TFs are known which have negative effect on vegetative and reproductive development in rice. Overexpression of OsNAC2 reduces plant and spikelet height and is a negative regulator of flowering time (Chen et al., 2015). However, functional analysis of a NAC TF controlling rice grain development has been lacking till now.

Improving the nutritional quality of grains is important for attaining global food security. Nutritional quality of rice grains depends on the composition of oil, proteins, carbohydrates, and minerals (Zhu et al., 2003). The major bulk of the storage reserve in rice grains is constituted by carbohydrates, accumulated in the form of starch (90%) and proteins (10%). They serve as a source of energy and nitrogen to the population (Jiang et al., 2016). Accumulation of these storage components also has a great impact on the grain plumpness and grain weight properties (Zhou, Wang et al., 2017). Alteration in the level of storage starch and protein has been shown to affect the grain size and weight trait in rice. They also affect the nutritional aspects of grains (Peng et al., 2014; Zhou, Wang et al., 2017; Wang, Chao et al., 2018).

In our previous reports, rice seed development has been divided into five stages depending on the morphological and physiological changes. Grain filling occurring during the maturation phase of seed development determines the eventual grain composition. The regulatory proteins specifically expressed during these stages are essential for controlling the process (Agarwal et al., 2011; Sharma et al., 2012). *SS1/ ONAC025* shows high expression in ovary at 5–7 DAP and in endosperm at 7–42 DAP (Sato et al., 2011; Sharma et al., 2012). The present study aims to elucidate the role of seed-specific *SS1/ ONAC025* during rice grain development. The seed-specific expression was validated by promoter::*GUS* rice transgenic plants. Transcriptional properties of activation/repression and sub-cellular localization were analyzed. Rice transgenic plants ectopically expressing *SS1/ ONAC025* were raised. Since

the gene imparted a plantlet lethal phenotype, the changes were accounted for by histological experiments and RNAseq analysis. Furthermore, the transcriptome of these transgenic plants was compared with that of seed development transcriptome to elucidate the position of *SS1/ ONAC025* during seed development process. The expression of *SS1/ ONAC025* was also quantified in *japonica* and *indica* rice genotypes. Our study is the first report on the role of a NAC TF, *SS1/ ONAC025*, in the seed development process of rice.

2 | MATERIALS AND METHODS

2.1 | Generation of promoter::*GUS* rice transgenic plants and *in silico* promoter analysis

2.1.1 | Gene cloning

To check for the promoter guided expression of *SS1/ ONAC025*, 1997 bp upstream of *SS1/ ONAC025* CDS was amplified from *indica* rice IR64 genomic DNA and cloned in pMDC164 vector using Gateway® cloning technology. For this, the fragment was amplified with specific primers (Table S1) which fulfilled the minimum requirement for a CACC sequence at the 5' end of the forward primer. The resulting amplification products were gel purified and cloned into the Gateway® entry vector pENTR™ D-TOPO® (Invitrogen), with kanamycin selection, by mixing fresh PCR product with 20 ng of the TOPO® vector in a molar ratio ranging from 0.5:1 to 2:1. Ligated products were transformed into chemically competent *E.coli* DH5 α cells. A positive plasmid confirmed by restriction digestion and sequencing was then transferred into destination vector pMDC164 (Curtis and Grossniklaus, 2003), with kanamycin and hygromycin selection using Gateway® LR Clonase™ II enzyme mix (Invitrogen). It was then transformed into chemically competent *E.coli* DH5 α cells. Cloning in the Gateway® destination vector was confirmed by restriction digestion using *Ascl* and *PacI*, flanking the region of insert. Thus, a binary construct *pSS1/ ONAC025::GUS* expressing the *GUS* reporter gene under the control of the native promoter of *SS1/ ONAC025* was generated. These were transferred to chemically competent cells of *Agrobacterium* strain EHA105 by the freeze-thaw procedure (Xu and Qingshun, 2008). For this, *Agrobacterium* competent cells were thawed on ice and 1–2 μ g of the plasmid was added. The micro centrifuge tube containing the mixture was frozen in liquid nitrogen for 2 min, followed by a heat shock at 37°C for 5 min and immediate chilling on ice for 37°C for 10 min. The cells were then revived by the addition of 1 ml of LB broth with incubation at 200 rpm for overnight at 28°C. Next day, the cells were pelleted at 1,534 g for 5 min at 4°C and the pellet was resuspended in 100 μ l of LB. It was then plated on to the LB agar plate with the suitable media containing antibiotic for selecting the plasmid and 50 μ g/ml of rifampicin for selecting the *Agrobacterium*. The plates were incubated at 28°C for 2 days in dark conditions.

2.1.2 | Rice transgenic plants' development

The *pSS1/ ONAC025::GUS* construct was transferred into *Agrobacterium* strain EHA105 and was used for transforming rice (*indica* cv. PB1) using a scutellum culture methodology (Toki et al., 2006), with minor modifications. For this, rice seed sterilization was done using 0.1% HgCl_2 and 3–4 drops of Teepol detergent for 10 min, with continuous shaking, followed by washing with RO water, and soaking overnight in dark. Next day, after drying seeds on autoclaved filter paper, they were inoculated on NB media [N6B5 media contains N6 major (Stock I), B5 minor (Stock II), Fe-EDTA (Stock III), and organic source (Stock IV). Stock I (20X) was prepared by adding KNO_3 (56.6 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3.7 g/L), KH_2PO_4 (8 g/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (3.32 g/L), $(\text{NH}_4)_2\text{SO}_4$ (9.2 g/L). Stock II (1000X) was prepared by adding H_3BO_3 (3 g), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (10 g/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2 g/L), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.250 g/L), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.025 g/L), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.0250 g/L), KI (0.750 g/L). Stock III (100X) was prepared by adding $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.78 g/L) and Na₂EDTA (3.73 g/L). Stock IV (1000X) was prepared by adding glycine (2 g/L), myoinositol (100 g/L), nicotinic acid (0.5 g/L), pyridoxine HCl (0.5 g/L), thiamine HCl (0.1 g/L). For 1 L of N6B5 media; 50 ml stock I, 1 ml stock II, 10 ml stock III, 1 ml stock IV, 2.878 g L-proline, 500 mg L-glutamine, 300 mg CEH (casein enzyme hydrolysate), 30 g of sucrose, and 4 g phytigel per liter of solution with a pH-5.8, supplemented with 2 mg/ml of 2,4-D (2,4-dichlorophenoxyacetic acid) were added]. The plates were incubated for callus induction at 32°C in continuous light for 5 days. Primary culture of *Agrobacterium* harboring the construct was grown to obtain secondary culture with O.D₆₀₀ of 0.3. The resulting cells were pelleted at 599 g for 20 min at 25°C. The pellet was then resuspended in 11 ml of N6B5 liquid [at pH-5.2, without phytigel], supplemented with 100 μM acetosyringone. Rice embryogenic calli were then co-cultivated with N6B5 liquid containing *Agrobacterium* cells for 2 min. The co-cultivated calli after drying were transferred onto 90 mM Whatmann paper, pre-soaked in N6B5, placed on N6B5-AS medium [with 4 g phytigel per liter of solution, pH-5.2], supplemented with 2 mg/ml of 2, 4-D and 100 μM acetosyringone. The plates with the calli were incubated for two days at 28°C in dark conditions. After 48 hr of incubation, the co-cultivated calli were washed thoroughly in sterile RO water supplemented with 300 mg/ml of augmentin for 3–4 hr. The calli were then dried on sterile blotting paper and later transferred onto the selection media [50 ml stock I, 1 ml stock II, 10 ml stock III, 1 ml stock IV, 2.878 g L-proline, 500 mg L-glutamine, 300 mg CEH, 30 g of sucrose, and 4 g phytigel per liter of solution with a pH-5.8], supplemented with 2 mg/ml of 2,4-D, 300 mg/ml of augmentin and 50 μg /ml of hygromycin. These were further incubated at 32°C for 14 days in continuous light. Proliferating calli formed during selection were then transferred onto regeneration medium [50 ml stock I, 1 ml stock II, 10 ml stock III, 1 ml stock IV, 300 mg CEH, 30 g of sucrose, and 4 g phytigel per liter of solution with a pH-5.8], supplemented with 300 mg/ml of augmentin, 1 mg/l of BAP and 50 mg/l of hygromycin and were incubated at

28°C with 12:12 light and dark photoperiod conditions till regeneration. Once the shoots attained a length of 2–3 cm, they were transferred to the rooting media [25 ml stock I, 500 μl stock II, 5 ml stock III, 500 μl stock IV, 20 g of sucrose, and 2 g phytigel per liter of solution with a pH-5.8], supplemented with 300 mg/ml of augmentin and 50 mg/l of hygromycin. The tubes were incubated at 28°C with 12:12 light and dark photoperiod conditions for rooting. The transformed plantlets were hardened in hydroponics with Yoshida medium (Yoshida et al., 1971) and were transferred to pots in plant growth chamber (Conviron, Canada)/green house with relative humidity, 70%; light intensity, 220–350 $\mu\text{M}/\text{M}^2/\text{sec}$; 12:12 light and dark photoperiod; and temperature of 28°C during day and 23°C during night (Das et al., 2019).

2.1.3 | GUS staining of grains

Six transgenic lines were produced for promoter::GUS experiment. These transgenic lines expressing the *GUS* reporter gene driven by the native promoter of *SS1/ ONAC025* were screened for the integration of the transgene by performing *HYGROMYCIN PHOSPHOTRANSFERASEII/HPTII* amplification (primers in Table S1) with the genomic DNA of transgenic plants. Amplicon of 850 bp corresponding to *HPTII*, confirmed the integration of the transgene. Two independent transgenic lines, whose seeds showed 3:1 segregation in the T_1 generation were chosen and promoter activity of *SS1/ ONAC025* was analyzed by GUS staining (Chaudhury et al., 1995). Longitudinal sections of seeds (at least two seeds per seed development stage, i.e., S1-S5) and a small portion of leaf were immersed in GUS histochemical buffer (50 mM sodium phosphate, 50 mM EDTA pH 8.0, 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 0.1% Triton X-100, 1 mM X-Gluc) and incubated at 37°C. The incubation was carried out for 1–2 hr till the color developed, for seed sections, and overnight for leaves. Chlorophyll from the leaves was removed by repeated washing with decoloring solution (70% methanol and 30% acetone). The leaves were photographed using Nikon-5200 DSLR camera, and the seed sections were observed under Nikon AZ100 microscope.

2.1.4 | Comparative promoter analysis

For promoter sequences of five genotypes, namely, SN, PB1, IR64, LGR, and NB, genomic DNA was isolated from four *indica* genotypes (Dellaporta et al., 1983; Mathew et al., 2016). Primers specific to the obtained promoter sequence (Table S1) were used to amplify *SS1/ ONAC025* promoter from all four genotypes. The amplicon obtained was cloned in pJET1.2 vector (Thermo scientific) and sequenced. The sequences from four *indica* genotypes and one *japonica* genotype NB were scanned through PLACE database (Higo et al., 1999). The output was compared to determine differences in *cis* elements. The sequences were also compared by ClustalX (Thompson et al., 1994; Thompson et al., 1997) to identify sequence differences.

2.2 | Transactivation and transrepression assays

For transactivation and transrepression assays, coding sequence of *SS1/ ONAC025* (NCBI BankIt accession ID AK107369.1) was cloned (primer sequences in Table S1) between *EcoRI* and *Sall* sites, in the pGBKT7 vector (TAKARA Bio USA, Inc. formerly Clontech) in-frame with the GAL4 DNA binding domain; and in the rGAL4 vector (Singh et al., 2019) in-frame with both activation and DNA binding domains of GAL4, respectively. These constructs were transformed into yeast strain AH109 using EZ-YeastTM Transformation Kit (MP). Overnight culture of AH109 in YPDA (1% yeast extract, 2% peptone, 2% glucose, and 0.2% adenine hemisulphate) with an O.D.₆₀₀ > 1 was pelleted for 1 min at 599 g. After removing the supernatant, the pellet was resuspended in 125 µl of EZ-transformation buffer (MP), 2.5 µl of carrier DNA and 2 µg of plasmid DNA by vortexing and incubated at 42°C for 30 min. The transformed yeast cells were selected by plating onto synthetic drop-out (SD) medium [0.667% yeast nitrogen base, 2% glucose, 2% agar with histidine, adenine, and leucine auxotrophic amino acid supplements], lacking tryptophan. The transactivation and transrepression properties of the constructs were determined by the differential growth of the yeast colonies on SD/-Trp/-His/-Ade media supplemented with 10 mM 3-AT (3-Amino-1,2,4-triazole). pGBKT7 vector was a negative control for activation assay while rGAL4 vector, a strong activator, was a positive control for repression assay (Mathew et al., 2016; Das et al., 2019; Singh et al., 2019).

The activation and repression properties were quantitated by β-galactosidase enzyme activity using ortho-nitrophenyl-β-galactoside/ONPG assay as described in Clontech[®] Yeast protocol hand book (TAKARA Bio USA, Inc. formerly Clontech; USA). β-galactosidase enzyme units were calculated for three colonies for each construct along with positive (rGAL4 vector) and negative (pGBKT7 vector) controls. Individual colonies of AH109 transformed with the construct were grown in liquid SD selection media at 30°C 12–16 hr. This was sub-cultured in YPD broth for secondary culture and was incubated for 3–5 hr until the cells reached mid-log phase as indicated by an absorbance O.D.₆₀₀ of 0.5 to 1. The culture was pelleted and then it was dissolved in Z-buffer [100 mM Na₂HPO₄·7H₂O, 25 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄·7H₂O with pH-7.0]. Suspension culture (100 µl) was transferred in three technical replicates in fresh tubes and freeze/thawed in liquid nitrogen/ 37°C water bath three times to break open the cells. Cell lysate was then mixed with Z-buffer along with β-mercaptoethanol. To this, the substrate 160 µl ONPG (ortho-Nitrophenyl-β-galactoside) was added. The reaction mixture was incubated at 30°C degrees till yellow color developed and the reaction was stopped by adding 1 M Na₂CO₃ (stop buffer) and total reaction time was noted. All the tubes were centrifuged for 10 minutes to remove any cell debris and supernatant was carefully transferred to fresh tubes and absorbance was measured at 420 nm. The β-galactosidase units were calculated for a minimum of three independent colonies for each construct, according to the formula $2000/t \times (OD_{420}/OD_{600})$, where t = time elapsed for incubation in minutes (Mathew et al., 2016; Singh et al., 2019).

2.3 | Sub-cellular localization by transient assay in onion peel cells and *Nicotiana benthamiana* leaves and Western blotting

To determine the sub-cellular localization of *SS1/ ONAC025*, CDS was amplified using gene-specific primers (Table S1) and cloned into the Gateway[®] entry vector, pENTRTM/D-TOPO[®] (InvitrogenTM). The resulting construct was transferred by an LR reaction into the destination vector pSITE-3CA (Chakrabarty et al., 2007) so as to produce *SS1/ ONAC025-YFP* fusion construct, under the control of a duplicated cauliflower mosaic virus (CaMV) 35S promoter. For co-localization studies, together with *SS1/ ONAC025-YFP* fusion construct AtWAK2-pBIN2 endoplasmic reticulum (ER)-mCherry marker, *GmMan1*-pBIN2 Golgi mCherry marker, *ScCOX4*-pBIN2 mitochondria mCherry marker and *prSSU*-pBIN2 plastid mCherry markers were used (Köhler et al., 1997; Dabney-Smith et al., 1999; He et al., 1999; Saint-Jore-Dupas et al., 2006). For particle bombardment, gold particles, as microcarriers, were sterilized with 70% ethanol and washed thrice with 100 µl of sterile MQ and 50% ethanol added to the pellet. The sterile gold particles were then coated with 5 µg of DNA in presence of 1 M CaCl₂ and 0.016 M spermidine with vortexing followed by brief centrifugation. The microcarriers were then washed once each with 70% ethanol and 100% ethanol. The pellet was resuspended in 48 µl of 100% ethanol and was proceeded for bombardment. Small square pieces of epidermal layers from bulbs of fresh spring onions were kept on MS media (Duchefa biochemie, Netherlands) containing plates. These were then co-bombarded with the fusion construct using Biolistic[®]-PDS-1000/He particle delivery system (Bio-Rad, CA) according to the manufacturer's protocol. Microcarriers were loaded at the center of the sterilized macrocarrier fitted inside the disc and placed in the launch assembly. The bombardment was performed at 27 mm Hg vacuum and 1100 psi pressure. Following bombardment, the plates were incubated at 28°C. After overnight incubation, the onion peels were observed in Leica TCS-SP8 confocal laser scanning microscope for YFP and mCherry signals at 514 nm and 594 nm, respectively. The nuclear-specific fluorophore, DAPI (4',6-diamidino-2-phenylindole), was observed at 351/364 nm wavelength.

For transient assay in *Nicotiana benthamiana* L., leaves of 2–4 weeks old wild type plants were used. *SS1/ ONAC025-YFP* fusion construct was transformed in *Agrobacterium tumefaciens* strain EHA105 (Elizabeth et al., 1993). The primary *Agrobacterium* culture was inoculated in LB with rifampicin (50 mg/L) and kanamycin (50 mg/L) and agroinfiltration buffer (0.5 M MES with pH: 6.0, 1M MgCl₂, 100 mM acetosyringone, 0.005% silvet, and 1% sucrose) and grown for 4–6 hr at 28°C. The cells were pelleted and then resuspended in agroinfiltration buffer so as to make the final O.D.₆₀₀ adjusted to 0.6. For agroinfiltration, this culture was gently injected by a 5 ml syringe on the abaxial side of the leaf. Following overnight incubation for 2–3 days at 28°C, the leaves were observed in Leica TCS-SP8 confocal laser scanning microscope for YFP at 514 nm. *Nicotiana benthamiana* leaves expressing *SS1/ ONAC025-YFP* were harvested without the main vein

at 36–48 hr after agroinfiltration infection and 1 g tissue was ground into a fine powder with liquid nitrogen. Extraction buffer (10% glycerol, 25 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 2% polyvinylpyrrolidone, 10 mM DTT, 1X protease inhibitor, and 1 mM PMSF) was added and the mixture pelleted. Total protein was collected in the supernatant and electrophoresed on a 10% acrylamide gel (ddH₂O, 30% Acrylamide: bis-acrylamide, 1.5 M Tris (pH: 8.8), 10% SDS, 10% ammonium persulfate and tetramethylethylenediamine) and stained with Coomassie brilliant blue R-250. The presence of a functional protein was confirmed through Western blot (Xu et al., 2015) using customized SS1/ ONAC025 rabbit IgG primary antibody (Imgenex, India) and mouse anti-rabbit IgG-HRP conjugate secondary antibody (Santa Cruz Biotechnology, CA).

2.4 | Tissue collection and QPCR

Rice plants (four *indica* genotypes, namely, Sonasal/ SN, Pusa Basmati1/ PB1, *indica* rice64/ IR64 and Long Grain Rice/ LGR; and a *japonica* genotype, Nipponbare/ NB) were cultivated in NIPGR fields during the rice growing season and the panicles were tagged on the day of pollination for collection of each DAP (days after pollination) seeds as specified earlier (Agarwal et al., 2007; Sharma et al., 2012; Mathew et al., 2016; Das et al., 2019; Malik et al., 2020). Seed samples, categorized into five groups, depending on the morphological changes (Agarwal et al., 2011), named, S1 (0–2 DAP), S2 (3–4 DAP), S3 (5–10 DAP), S4 (11–20 DAP), and S5 (21–29 DAP) and flag leaf tissues from all five genotypes were harvested. Total RNA was isolated from S1–S5 stages of seeds from five genotypes (SN, PB1, IR64, NB, and LGR) and from the leaf tissues, in biological triplicates. For RNA isolation from seed tissue, a modified protocol for starch rich seeds was used (Singh et al., 2003). Leaf RNA was isolated using TRIzol[®] reagent (Invitrogen Life Technologies). Subsequently, RNA was treated with RNase-free DNase treatment kit (Qiagen, Germany) and purified with RNeasy[®] MinElute Cleanup kit (Qiagen, Germany), according to the manufacturer's protocols. Transcript abundance of SS1/ ONAC025 in SN, PB1, IR64, NB, and LGR seeds was quantified by reverse transcription quantitative PCR (RT-qPCR) or quantitative real time PCR (QPCR) as mentioned earlier (Agarwal et al., 2007; Mathew et al., 2016). For each genotype, the fold changes of SS1/ ONAC025 in the five seed development stages was calculated with respect to flag leaf from that genotype. For this, SS1/ ONAC025-specific primers were designed (Table S1), using Primer Express version 3.0 (Applied Biosystems), with default parameters. Full length cDNA was prepared by High capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's protocol. For quantification by QPCR, Fast SYBR[®]Green master mix was used and the reaction was performed with default settings on 7500 Fast Real-Time PCR system (Applied Biosystems; USA). The expression data from the biological replicates was normalized with the rice actin gene, ACT1 and the fold change was calculated by 2^{−ΔΔCt} method (Livak and Schmittgen, 2001).

2.5 | Generation and analyses of rice transgenic plants ectopically expressing SS1/ ONAC025

For functional analysis of SS1/ ONAC025 in the native plant system, full length cDNA of SS1/ ONAC025 was over expressed (primers in Table S1) under the control of maize *UBIQUITIN* promoter in the binary vector, pB4NU by cloning in the sites *KpnI* and *BamHI*. This construct was used to generate rice transgenic plants (*indica* cv. PB1), OE_025, as mentioned above. The plants were photographed under Nikon-5200 DSLR camera and stereozoom microscope (AZ100, Nikon; Japan). The overexpression levels of SS1/ ONAC025 transcript was studied by QPCR in eight OE_025 transgenic plantlets (L_1 to L_8). All protocols from RNA isolation to data analysis were the same as mentioned above for QPCR in the five genotypes. Leaf tissue from 2.5-month-old plantlets was used. The fold change was calculated with respect to a wild type seedling of the same age grown alongside transgenic plants. Since each overexpression plant was just a few mm in length, and did not propagate ahead, biological replicates could not be used for the same and three technical replicates were used for calculation of standard error. For histological analysis, tissue samples fixed in FAE solution [10% formaldehyde, 5% acetic acid, and 50% absolute ethanol] were dehydrated in series of ethanol, which was gradually replaced by xylene and paraffin wax. The wax-infiltrated tissues were then paraplast embedded into molds (Yorko[®]; India) and incubated at 4°C overnight for solidification. Later, 8 μm thin sections were prepared using rotary microtome (Leica Biosystems, Germany). After removing the paraplast in the ribbon by two rounds of xylene treatment, the slides were then stained separately with Coomassie brilliant blue/CBB [0.25% CBB in 50% methanol and 10% acetic acid] or 0.25% Toluidine blue-O/TBO to observe shoot apical meristem (SAM) structure. Slides were stained with 2% KI/I₂ (Lugol's solution; Takeshita et al., 2015) to observe starch. Excessive staining was removed by dipping the slides in 50% ethanol. The tissue was mounted onto D.P.X. mountant (Himedia[®]) and sections were analyzed and captured under stereozoom microscope (AZ100, Nikon; Japan) and light microscope (Eclipse 80i, Nikon; Japan). Surface details of transgenic and wild type leaves were studied by scanning electron microscope (Evo LS10, Zeiss, Germany). Additionally, to compare the effect of the vector on rice transgenic plants, those harboring the empty vector (EV) pB4NU construct were raised using *Agrobacterium* mediated transformation as above and phenotyped.

2.6 | Transcriptome analysis

To determine the genes affected by overexpression of SS1/ ONAC025, RNAseq analysis of *indica* PB1 plantlets ectopically expressing SS1/ ONAC025 was performed. Purified RNA isolated from 2.5-month-old two positive T₀ OE_025 plantlets (L_5 and L_6) and corresponding wild type (W) plantlet was used. This tissue was used since the plants could not be propagated further, and no seeds/next generation plants could be obtained. The integrity of RNA samples was checked



using Agilent 2100 Bioanalyzer. As per the protocol mentioned in Illumina® trueSeq® RNA sample preparation kit v.2 (Illumina; USA), poly-A mRNA was purified using oligo(dT)-attached magnetic beads. To this, 19.5 µl of Elute, Prime and Fragment Mix were added and kept at 94°C for 8 min for fragmentation and priming of the poly-A mRNA. cDNA was synthesized by SuperScript II Reverse transcriptase (Invitrogen™; USA), Second Strand Master Mix, End Repair Mix and Resuspension Buffer followed by adenylation by A-tailing Mix. Adapters were ligated to the double-stranded cDNA fragments followed by DNA fragment enrichment. The cDNA library produced was quantified according to Illumina sequencing library Q-PCR quantification. Size and purity of the library samples were checked by Agilent Technologies 2100 Bioanalyzer. Paired-end sequencing was performed with Illumina HiSeq™ 2000. The sequence generated was submitted in NCBI-SRA (sequence read archive) database (<https://www.ncbi.nlm.nih.gov/sra/PRJNA540785>) with SRA accession number PRJNA540785. The sequencing data was subjected to different tools for performing data pre-processing, alignment with reference, expression estimation and comparison analysis. The quality check on the fastq files was done by Burrows-Wheeler Alignment tool (Martin, 2013) and accuracy of the sequencing platform was assayed by Phred quality score/Q score with a cut off of Q30. Trimming and removal of low quality and other contaminating sequence reads were performed by, Cutadapt (v1.8.1; Martin, 2013), Bowtie2 version 2.1.0 (Langmead, 2010), in-house Perl scripts and Picard tools (version 1.100, <http://broadinstitute.github.io/picard>). High quality sequence reads, thus obtained were then aligned to the rice gene and genome model downloaded from MSU-RGAP 7 (Ouyang et al., 2007) using TopHat program version 2.1.1 (Trapnell et al., 2009). Estimation of the expression of genes and transcripts were derived in terms of FPKM (fragment per kilobase million), using Cufflinks program version 2.0.2 (Trapnell et al., 2012). Differential gene expression analysis was performed using Cuffdiff program with default settings (Trapnell et al., 2012). Genes showing a change in expression in two independent transgenic plantlets (L_5 and L_6) with $\geq 1.5 \log_2$ fold change, and $p \leq 0.05$, in comparison with the wild type were considered to be differentially expressed.

Furthermore, due to absence of biological replicates from the same plantlet, owing to lethality, differentially expressed genes (DEGs) amongst L_5 and L_6 were delineated by two analyses using CuffDiff (<http://cufflinks.cbc.umd.edu/>) and DeSeq2 (<https://bioconductor.org/packages/release/bioc/html/DESeq2.html>). In DeSeq2, the alignments in mapped.bam files were sorted using samtools (<http://samtools.sourceforge.net/>) and number of aligned reads mapped to particular gene, i.e., overlaps on its exon were calculated, for all samples using HTseq software (<https://pypi.org/project/HTSeq/>). These calculated reads across the samples were normalized and variance was calculated for each gene. Calling of differential expression was by performing negative binomial test in DeSeq package (Anders and Huber, 2010). Genes that were significant at 10% FDR correction and with $\geq 1.5 \log_2$ fold change, in comparison with the wild type were considered to be differentially expressed. To make the analysis more stringent, genes which were differentially expressed both by CuffDiff and

DeSeq2, in both transgenic plantlets, L_5 and L_6, were considered for analysis. Genes involved in various pathways/processes were functionally annotated using different online databases including MSU-RGAP 7 (Ouyang et al., 2007) and RicENCODE (<https://funricegenes.github.io/>). Differentially expressed TFs were analyzed using PlantTFDB 4.0 (Jin et al., 2017), rice TF database (<http://ricephylogenomics.ucdavis.edu/tf/>) and data available from previous study in the lab (Sharma et al., 2012). GO enrichment analysis of the DEGs was performed by The Biological Network Gene Ontology Tool (BiNGO) 3.0.3 (Maere et al., 2005) using MSU-RGAP 7 as the reference annotation set and AgriGO software v1.2 (Du et al., 2010). To map the genes involved in various pathways, MapMan 3.6.0RC1 (Thimm et al., 2004; Usadel et al., 2009), with mapping files from MSU-RGAP 7 was used. Heat maps were constructed using MeV Version 4.9.0 (Saeed et al., 2003).

2.7 | Statistical analysis

All the experiments were repeated at least twice and the biological mean was shown. The statistical significance of the results was checked by conducting student's t-test in Microsoft Excel®. The test was performed by assuming equal variances for the two samples and the number of distribution of tails was selected depending upon the experiments.

3 | RESULTS

3.1 | The promoter of SS1/ ONAC025 directs specific and high expression in five rice seed developmental stages

Seed development process in rice has been categorized previously into S1-S5 stages by us, depending upon the distinct morphological features during seed morphogenesis (Agarwal et al., 2007; Agarwal et al., 2011). Additionally, we have performed microarray analysis on *indica* rice IR64, from both reproductive (panicle and seed) and vegetative stages of rice plant, making a total of 19 stages. In this data, SS1/ ONAC025 was found to be a seed-specific gene. This meant it is expressed only during seed development stages of rice, without any expression in other 14 tissues comprising vegetative and panicle stages (Sharma et al., 2012). The expression values were also very high (Figure S1). In short, this was a seed-specific NAC gene like ONAC020, ONAC026, and ONAC023 (Mathew et al., 2016) and could be involved in an important aspect of development, like many other genes (Mathew and Agarwal, 2018). Since the promoter of a gene has a direct bearing on its expression, in light of the microarray data, the promoter (1997 bp) of SS1/ ONAC025 from IR64 was amplified and used to validate its seed-specific expression via generation of rice transgenic plants (Figure 1). Since PB1 is an easily and commonly transformable *indica* rice genotype, and is the most amenable to transformation, pSS1/ ONAC025::GUS plants were generated in this background. Due to its easy transformation and

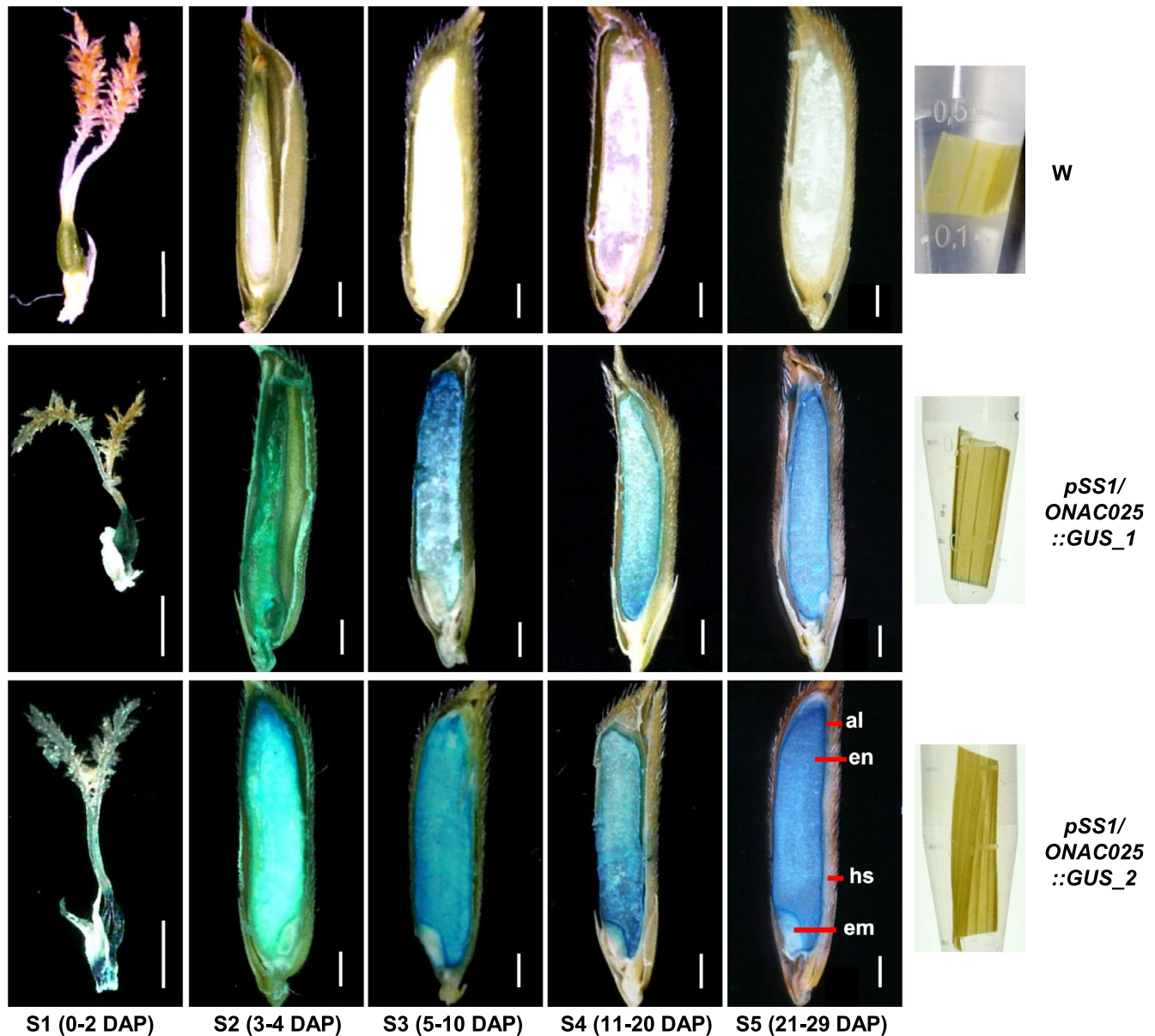


FIGURE 1 Promoter analysis of *SS1/ONAC025* in transgenic plants. Histological analysis of *GUS* expression in leaf and developing seed stages (S1-S5) in two lines of rice transgenic plants (*pSS1/ONAC025::GUS_1* and *pSS1/ONAC025::GUS_2*), and the wild type (W), as marked. Scale represents 1 mm. al, aleurone layer; en, endosperm; em, embryo; Hs, husk

high regeneration efficiency (Zhang, 1997), previously also, on the basis of IR64 microarray data, IR64 genomic DNA has been used as a template for the generation of tissue-specific promoter::reporter rice transgenic plants in PB1, and successfully used show the tissue specificity/functionality of the promoter (Sharma et al., 2012; Khurana et al., 2013; Nayar et al., 2013; Ranjan et al., 2017; Borah and Khurana, 2018; Burman et al., 2018; Das et al., 2019; Malik et al., 2020). In *pSS1/ONAC025::GUS* plants, seeds from all five developmental stages (S1-S5) from two independent transgenic lines (*pSS1/ONAC025::GUS_1* and *pSS1/ONAC025::GUS_2*) and leaf were checked for *GUS* expression. S1-S5 seeds exhibited intense *GUS* staining in the hand-cut sections from S1-S5 stages unlike the wild type (W) seeds. Both embryo and endosperm exhibited expression of *GUS* reporter. However, it was stronger and more uniform in the

endosperm. The gene did not express in the leaf (Figure 1). This validated the microarray data (Figure S1), indicating the specificity and high levels of expression of *SS1/ONAC025* in rice seed/grain. In short, the expression pattern of the reporter gene, driven by the promoter of *SS1/ONAC025*, indicated an important role of this gene in seed development, which has been explored in detail in this paper.

3.2 | *SS1/ONAC025* is a transcriptional repressor and localizes to endoplasmic reticulum and nucleus

SS1/ONAC025 is a typical NAC protein, which is 300 amino acids long, with an N-terminal NAC domain and a TRR at the C-terminal

end. N-terminal region has five sub domains, A-E, with NAC repression domain (NARD) present within the D sub-domain (Figure 2a). NARD has a hydrophobic LVFY motif which represses target genes (Hao et al., 2010). This has been shown for NAC50 from *Arabidopsis*

(Ning et al., 2015), OsNAP (Liang, Hirose et al., 2014) and many other NAC TFs, including those involved in development (Kim et al., 2007; Yang et al., 2011; Chen et al., 2016; Bhattacharjee et al., 2017; Mathew and Agarwal, 2018). Transactivation property of SS1/

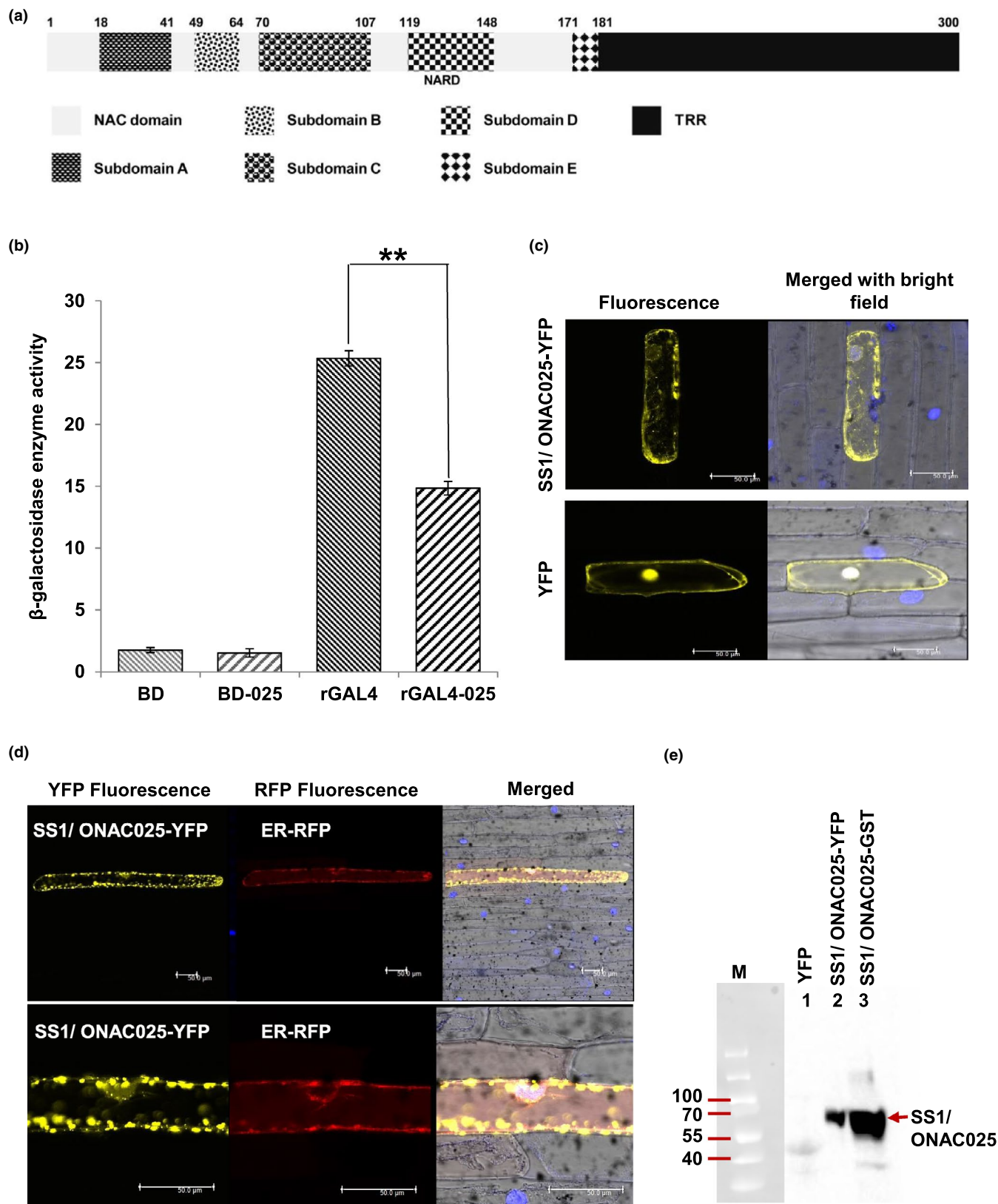


FIGURE 2 Transcriptional properties of SS1/ ONAC025. (a) Diagrammatic representation of SS1/ONAC025 having an N-terminal NAC domain with five sub-domains (a–e) and transcriptional regulatory region (TRR) at the C-terminal end, as marked. NAC repression motif, NARD is within the D sub-domain (b) β -GALACTOSIDASE enzyme activity of SS1/ ONAC025 after fusing with the binding domain of pGBKT7 (BD) vector and the reconstituted GAL4TF (rGAL4) for transactivation and transrepression assays, respectively. The enzyme activity calculated by the ONPG assays is graphically represented. The empty vector pGBKT7 (BD) and the reconstituted GAL4TF (rGAL4) act as the negative and positive controls for activation and repression assays, respectively. The values are the average of two independent biological replicates with the standard error bars as shown. The values which are significant with a $p \leq 0.005$ are shown with two asterisks. (c) Sub-cellular localization of SS1/ ONAC025-YFP fusion protein in onion peel cells by transient assay. The YFP images of the transfected cells observed at 514 nm are shown in the left panel, and their merger with the bright field images of the DAPI stained nucleus are shown in the right panel. Signal from SS1/ ONAC025-YFP fusion protein is in the upper panel and empty vector control showing YFP signal in the complete cell are in lower panel (d) Colocalization of SS1/ ONAC025-YFP signal with the red fluorescence from the ER marker is shown in the upper panel. The lower panel has zoomed-in images of the upper panel. Scale bars represent 50 μm . (e) Western blot analysis of *Nicotiana benthamiana* leaves expressing SS1/ ONAC025-YFP fusion protein with SS1/ ONAC025-specific antibody. Lane 1 is the protein isolated from individual *Nicotiana benthamiana* leaves expressing empty vector; lane 2 is the protein isolated from individual *Nicotiana benthamiana* leaves expressing SS1/ ONAC025-YFP fusion protein and lane 3 is the purified SS1/ ONAC025 protein with GST tag, taken as the positive control. M is the PageRuler™ pre-stained protein ladder

ONAC025 was assayed by its ability to activate reporter genes in yeast strain AH109 when fused with the binding domain of pGBKT7 (Figure 2b). SS1/ ONAC025 did not show any activation ability above the level of the vector control in ONPG assay, indicating that it is not a transcriptional activator. Since the presence of NARD suggested a possible repression property, the CDS of SS1/ ONAC025 was cloned in rGAL4 vector. Here, a repressor cloned in-frame with the reconstituted GAL4 TF will lead to a decrease in reporter gene expression (Mathew et al., 2016; Das et al., 2019; Singh et al., 2019). SS1/ ONAC025 significantly caused reduction of the reporter gene expression by 41% (Figure 2b). A repression in the activation ability of rGAL4 on fusion with SS1/ ONAC025 suggests that SS1/ ONAC025 functions as a transcriptional repressor in yeast. The validity of rGAL4 vector has been tested previously on the known SRDX repressor (Singh et al., 2019).

In terms of sub-cellular localization, SS1/ ONAC025 was predicted to be a cytoplasmic or nuclear protein by various online tools (Table S2). To validate this, ORF of SS1/ ONAC025 fused with the YFP reporter was expressed under the control of two copies of CaMV 35S promoter in the pSITE-3CA vector. This construct was used for transformation of both onion peel cells (Figure 2c) and *Nicotiana benthamiana* leaf cells (Figure S2a). In both cases, SS1/ ONAC025-YFP fusion protein was expressed primarily in a punctuate fashion throughout the cell. Faint YFP signal was also observed in the nucleus in both onion peel and *Nicotiana benthamiana* leaf cells, as indicated by overlap of the signal with DAPI stained nucleus (Figure 2c). Further, co-localization of SS1/ ONAC025-YFP protein was performed with organellar markers for endoplasmic reticulum (ER), Golgi bodies, mitochondria and plastid in onion peel cells (Figure 2d, Figure S2c). Merged images showed that the signal from SS1/ ONAC025-YFP overlapped with the signal from ER. It did not overlap with the other three organelle markers. Further, to confirm the expression of SS1/ ONAC025-YFP fusion protein, the total protein from transformed *Nicotiana benthamiana* leaves was isolated and used for Western blotting analysis using antibody specific to SS1/ ONAC025 (Figure 2e). The presence of a distinct band of 59.11 kDa specific to SS1/ ONAC025 was seen in the *Nicotiana benthamiana* leaf protein and that of 58.11 kDa was seen in the positive control

(SS1/ ONAC025-GST) indicating a functional SS1/ ONAC025-YFP protein, in which the YFP and SS1/ ONAC025 proteins have been fused in-frame (Figure S2c). In the Western blot (Figure 2e), marker line and sample line intensities are different because the same blot has been photographed in two different modes. For the protein sample, the mode is 'Chemi Hi Sensitivity' (Image Lab software, ChemiDoc™ MP, BIORAD) and for marker, the mode is "Stain Free Gel". In Chemi Hi Sensitivity, only proteins bound to antibodies are visible. For the Western blot analysis, both the photographs were merged and then checked for the appropriate protein band corresponding to the marker to confirm band size. Hence, the sub-cellular localization of SS1/ ONAC025 indicates its presence faintly in the nucleus and strongly in the ER (Figure 2d), and therefore, SS1/ ONAC025 is compartmentalized.

3.3 | Overexpression of SS1/ ONAC025 in rice leads to plantlet lethality and starch accumulation

SS1/ONAC025 was ectopically expressed under maize *UBIQUITIN* promoter to generate rice transgenic plants over expressing the gene in the *indica* cultivar PB1. This is a commonly used cultivar for functional characterization of genes since it has relatively high transformation efficiency (Zhang, 1997). All positive transgenic plants (~40 generated by multiple transformation events; OE_025) showed severe developmental defects, eventually leading to a plantlet lethal phenotype (Figure 3 and Figure S3). Data from 12 T₀ plantlets are presented in this paper. Multiple experiments (RNA isolations, histological sections with different stainings and RNAseq analysis) were performed to show the effect of SS1/ ONAC025. Since the plantlets were so small, it was not possible to use the same plantlet for different experiments. Hence, data from 12 plantlets, on which at least one experiment could be successfully performed and reported, has been shown in the paper. Therefore, OE_025 plantlets L_1 to L_8 were used for QPCR analysis, L_5 and L_6 were used for transcriptome analysis by RNAseq, L_9 to L_11 were utilized for preparing shoot apical meristem (SAM) histological sections, L_8, L_9, and L_11 histological sections were used for I₂/KI staining, and L_6, L_9, and

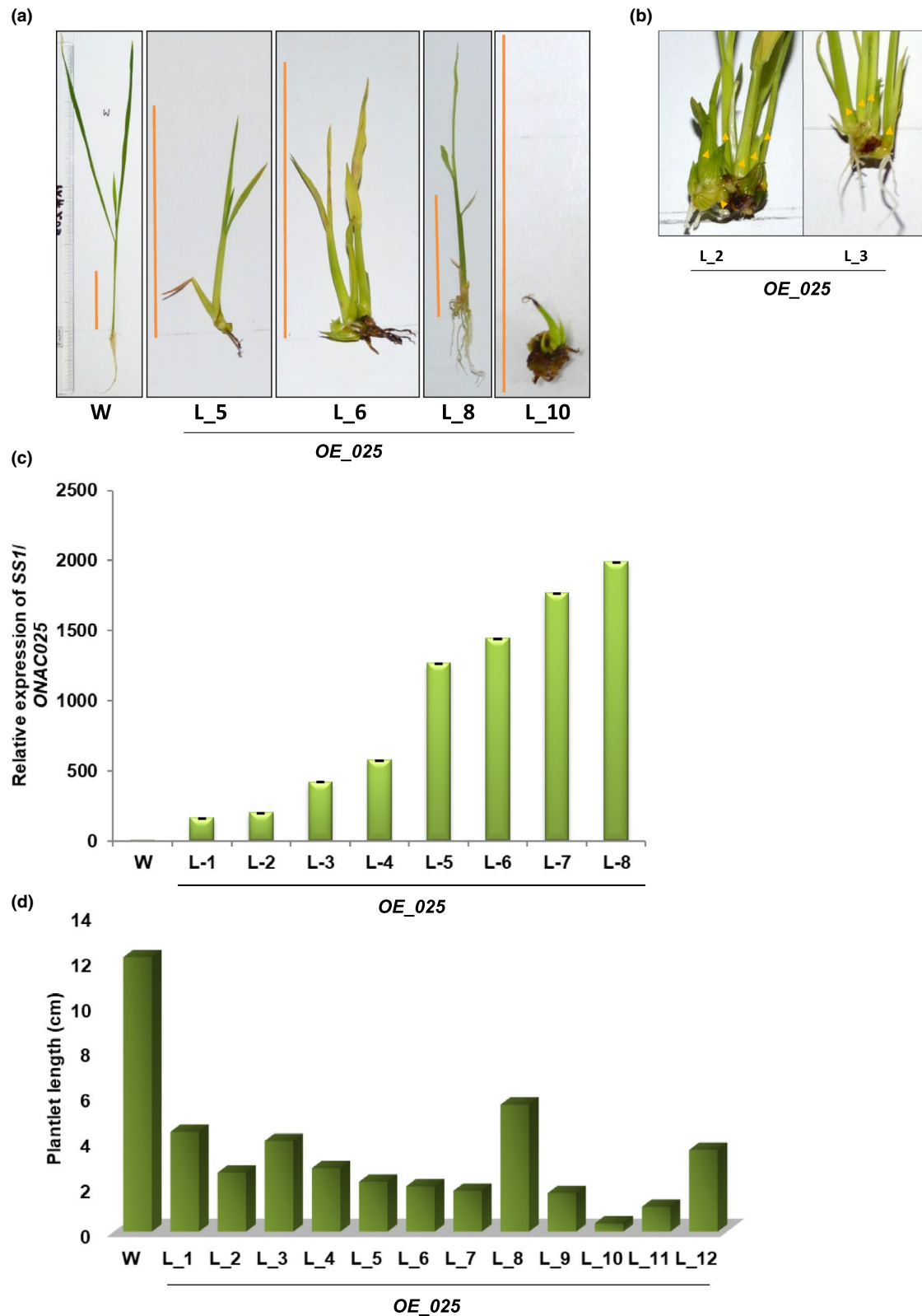


FIGURE 3 Phenotype of plantlets overexpressing *SS1/ ONAC025*. (a) Morphology of 2.5-month-old *OE_025* plantlets from L_5, L_6, L_8, and L_9, in comparison to a wild type plant (W). The orange vertical scale represents 2 cm. Plantlets L_5 and L_6 were used for transcriptome analysis. L_8 has the maximum length while L_10 has the minimum length. Note the difference in the length of the scale bar for W and transgenic plantlets. (b) Close-up photos of two plantlets, namely L_2 and L_3, showing multiple tillers originating in the same plant, as marked by yellow arrows. (c) Q-PCR analysis of eight plantlets (L_1 to L_8), showing increased expression of *SS1/ ONAC025* in transgenic plantlets compared to the wild type plant. Since each plantlet was very small, the standard error has been plotted on technical replicates. (d) Graphical representation of the plantlet length of 12 lines (L_1 to L_12), which have been used for multiple experiments in the paper. Note that Y-axis scale is in cm, and plantlets measuring a few mm are also recorded



L_12 were used for leaf surface scanning electron micrograph (SEM) photographs. Hence, the only plantlets used for more than one experiment were L_6 for RNAseq and leaf SEM; L_9 for SAM sections, KI/I₂ staining and leaf SEM. This was done to maintain an overlap of results between different plantlets used for various experiments, due to plantlet lethality phenotype.

The OE_025 rice plantlets were highly stunted and were a few mm in length even when they were 2.5 months old (see big scale bars for lines 1 to 12 in Figure 3a and Figure S3a). The tallest was L_8 at 5.6 cm, while the shortest was L_10 at 0.37 cm (Figure 3d). The mean length of 12 plantlets was 2.6 cm, with a median of 2.4 cm, and coefficient of variation as 0.5. The root growth was also hampered. Eventually, these plantlets senesced in the vegetative phase (a few cm in height) and did not produce any seeds. Additionally, these transgenic plantlets showed an increase in tiller number, all held together strongly at the base (Figure 3a,b, Figure S3a,b). The expression levels of *SS1/ONAC025* in the OE_025 transgenic plantlets (L_1 to L_8) were analyzed by QPCR and ranged from 100 to 2000 folds, approximately, amongst these eight independent transgenic plantlets (Figure 3c). This extreme phenotype was not the effect of the vector (Figure S4) as empty vector transgenic plants grew normally and produced seeds. All the transgenic plants ectopically expressing *SS1/ONAC025* showed severe developmental defects, irrespective of the huge variation in their expression levels. The expression level variation amongst the eight independent transgenic lines analyzed could be due to the number of copies inserted. Southern analysis, to check the stable integration of the transgene, was not performed due to limitation of tissue availability. Hence, a direct correlation between expression and phenotype intensity amongst these lines cannot be determined. The phenotype of lesser effect on height in L_8 may be due to a positional effect of the gene (Matzke and Matzke, 1998; Solís-Guzmán et al., 2017).

To get a better insight into the formation of multiple tillers, the longitudinal tissue sections of 2.5 months old three independent plantlets (L_9, L_10, and L_11, Figure 4) were analyzed. The sections revealed multiple alterations in comparison to a wild type SAM. The wild type SAM showed alternate phyllotaxy and independent leaves (Figure 4a,b; Giulini et al., 2004; Itoh et al., 2012). SAM sections from L_9, L_10, and L_11 plantlets of OE_025 showed increased vasculature (black triangle in Figure 4d,e,h,j,l). For L_9, a section of adjacent tiller buds (Figure 4d) was obtained, which showed vasculature connecting them. The structure appeared similar to a tiller bud meristem (Ohashi et al., 2018). At higher magnifications, the SAM appeared elongated (red line in Figure 4e,f,g,i,k,l,n,o), the first two leaves were not alternate (red and yellow stars in Figure 4e,f,g,i,k,l,n,o) and often appeared fused (red dot in Figure 4i,k,l,n,o). In two tiller buds from L_10 (Figure 4i,j), the leaves were rather tightly fused. Root vasculature initiation could also be observed (pink triangle in Figure 4g,l). Hence, the tillers in the transgenic plantlets appeared to emerge from a main basal region and were strongly connected by vasculature and the SAM was also elongated.

Additionally, histological sections showed that the cells of transgenic plantlets accumulated certain bodies (Figure 4p–r) which were absent in wild type cells (Figure 4c). Since *SS1/ONAC025* is a seed-specific gene, expressing at high levels during endosperm development

(Figure 1), in order to examine the nature of these bodies, histological sections of 2.5 month old transgenic plantlets from three lines (L_8, L_9, and L_11), were stained with KI/I₂ solution (Figure 5). The leaf cells of OE_025 plantlets showed deep brown stained bodies while negligible were present in the wild type plantlets grown under same conditions. This indicated the distinct presence of high numbers of starch granules. These granules were completely absent from the vasculature (Figure 5b–d,f) which has been mentioned above to be prominent in OE_025 plantlets (Figure 4). The deeply stained granules were prominent in leaves from transgenic plantlets (Figure 5f–h). At higher magnifications, the granules had the typical structure of an amyloplast rich in starch. Such a structure has been reported from developing rice seed (Toyosawa et al., 2016). The sections from OE_025 plantlets showed the presence of both compound type and spherical starch granules (Figure 5j–l). It is known that waxy or sticky or amylopectin-rich starch stains reddish brown while non-sticky or amylose rich starch stains blue (Bettge et al., 2000). In this case, the granules were reddish-brown. Since this was a very prominent overexpression phenotype, *SS1/ONAC025* was named as *SUPER STARCHY1/SS1*. Based on these observations, *SS1/ONAC025* can be hypothesized to be regulating starch biosynthesis, especially amylopectin. This has been given credence in experiments further ahead.

Since the plantlets overexpressing *SS1/ONAC025* were less soft to touch than wild type plants, abaxial leaf surfaces of three OE_025 plantlets were analyzed under SEM (Figure 6). L_6, L_9, and L_12 were used for the SEM analysis. L_6 was also used for RNAseq analysis. L_9 was also used for SAM sections and KI/I₂ staining. Both wild type and OE_025 leaf surfaces were typically covered with epicuticular wax. In wild type, both types of silica cells, i.e., protruding and circular were evenly spread over the leaf surface (Figure 6a,b). The silica cells on the transgenic OE_025 leaf surface (abaxial) appeared more cluttered due to an increase in their number. The size of some of the circular silica cells had also increased. This may be because of fusion of adjacent cells as reported earlier (Yang et al., 2015). This was observed in three independent plantlets tested (Figure 6c–h) and may have contributed to the harder plantlets. Silica provides structure to the plant (Sato et al., 2017) and resistance against biotic stress (Han et al., 2016).

3.4 | Ectopic expression of *SS1/ONAC025* disturbs the vegetative transcriptome and causes differential expression of seed transcriptome

In order to decipher genes whose expression had been altered in OE_025 plantlets, transcriptome analysis by RNAseq of two independent transgenic plantlets (L_5 and L_6) was performed. Their length is close to the median length and they have adjacent fold changes by QPCR (Figure 3c). Wild type plant (W) of the same age (2.5 months after regeneration from the callus tissue) was used as the control. For L_5, L_6 and control; 7.69, 11.06, and 10.94 Gb data, respectively, was generated. For validation, QPCR of *SS1/ONAC025* was performed (Figure 7a) in L_5 and L_6 plantlets and

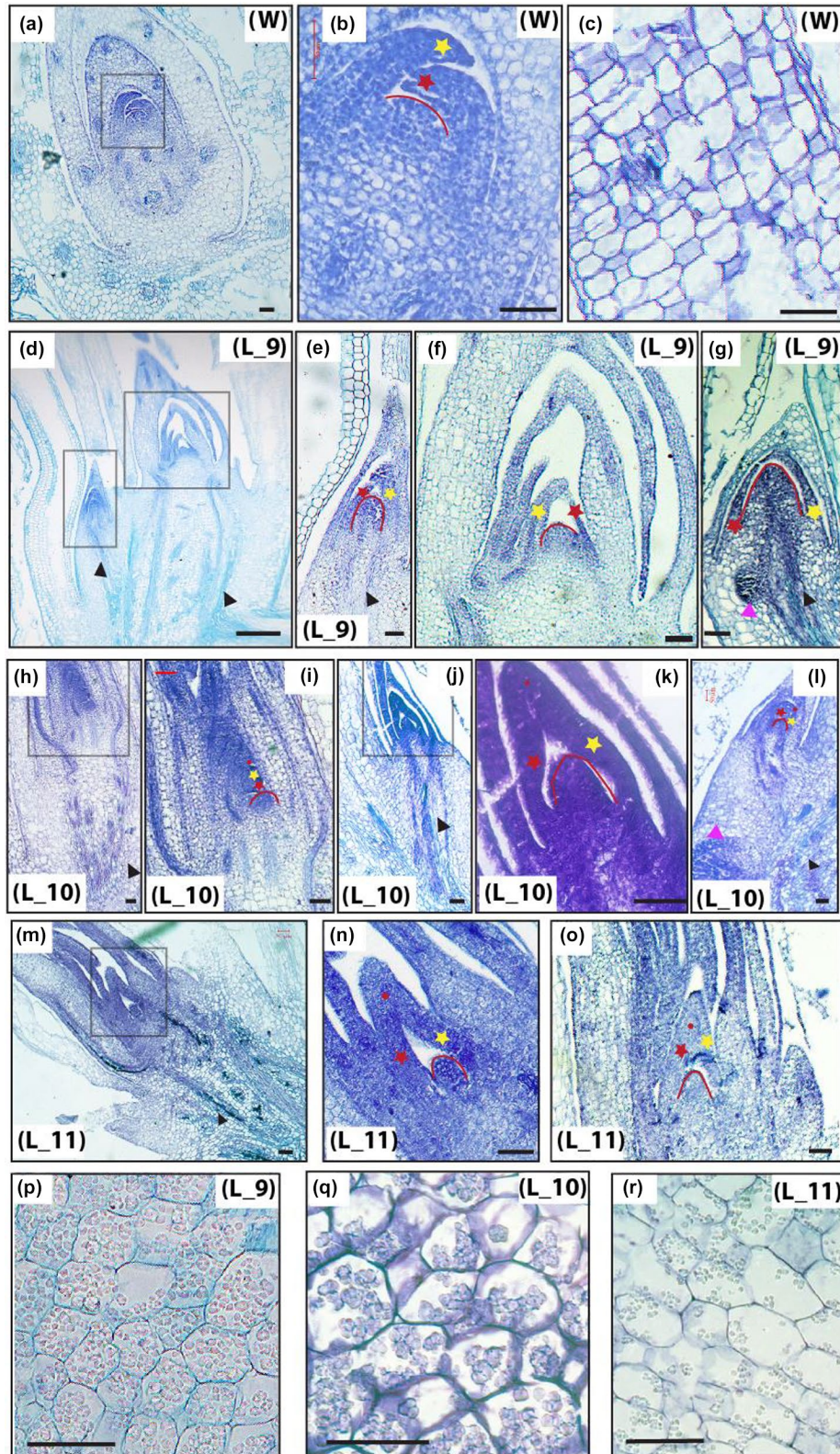


FIGURE 4 Histological sections showing modifications in vegetative SAM in 2.5-month-old OE_025 plantlets from L_9, L_10, and L_11. (a) Wild type (W) SAM and (b) magnified photo showing alternate first and second leaves. (c) Magnified photo from a W plant showing empty cells. Three plantlets, (d–g) L_9, (h–l) L_10, and (m–o) L_11 show the non-alternate arrangement of first two leaves (L1 marked with red star and L2 with yellow star) and their fusion (marked with red dot) as indicated. The SAM (marked with a red line) is elongated in transgenic plantlets. The vasculature of two tiller buds in (d) is connected and all tiller buds are supplied by vasculature as marked by black triangle (d, e, g, h, j, l, m). They also show root initiation as marked by pink triangle (g, i). (p–r) The cells in L_9, L_10, L_11, respectively, also show accumulation of certain bodies, which are absent in W (c). These sections were either stained with TBO or CBB for visualization. The empty black boxes marked in (a, d, h, j, and m) have been magnified in the subsequent adjacent figures. The scale bar in each figure represents 50 μm

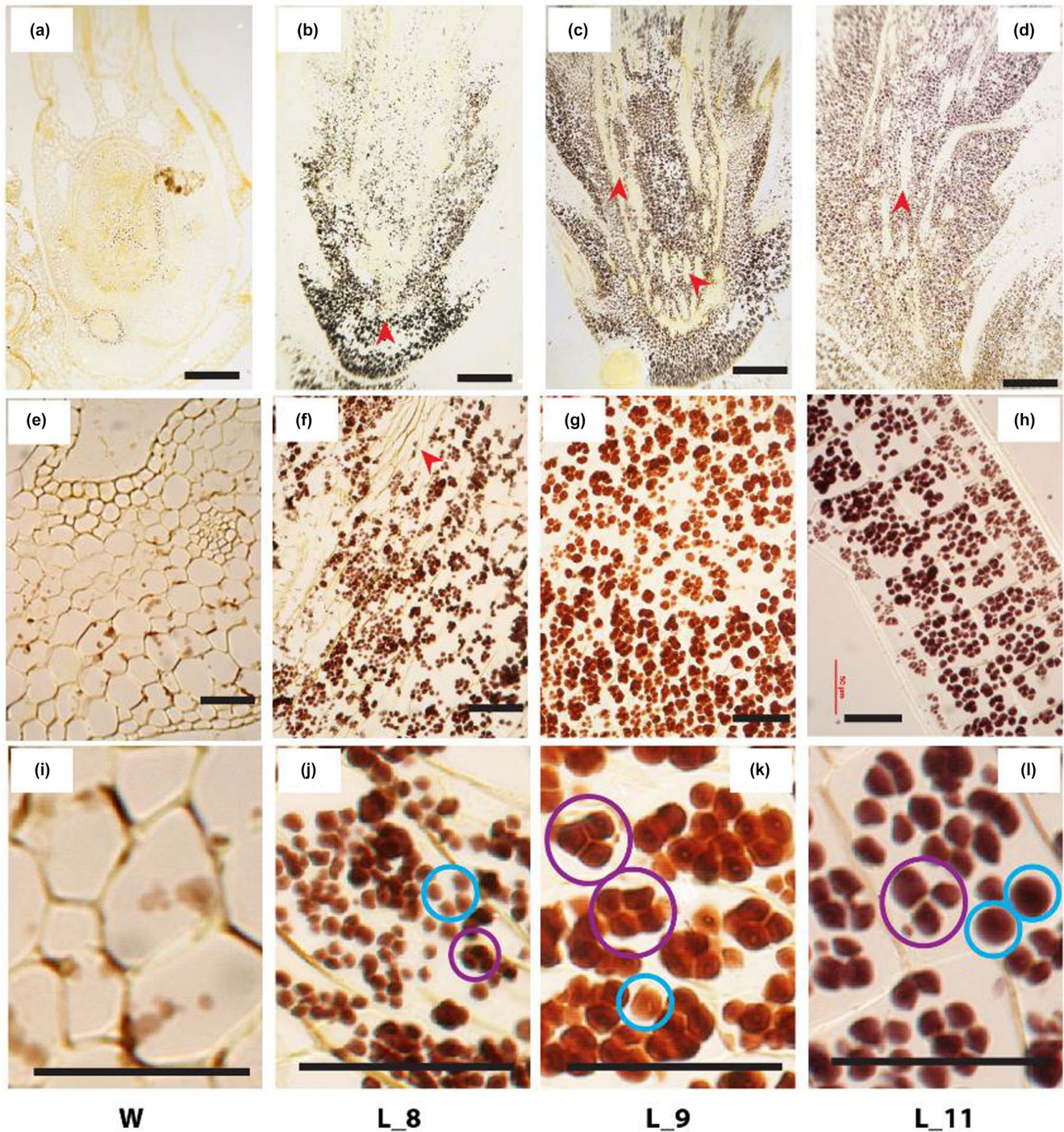


FIGURE 5 Starch deposition in 2.5-months-old OE_025 plantlets. Sections from wild type (W) plants (a, e, i), three OE_025 plantlets L_8 (b, f, j), L_9 (c, g, k), and L_11 (d, h, l), have been stained with KI/I₂ solution and examined at increasing magnifications from top to lower most panel. The blue circles represent spherical starch granules while purple circles indicate compound type starch granules. The red arrows indicate vasculature. Scale bar represents 50 μ m

they showed a fold change of 1269 and 1445, respectively. This pattern was comparable to the RNAseq values, where the gene was more upregulated in plantlet L_6 (Figure 7a). Furthermore, validation of RNAseq data was done for two downregulated genes (Figure 7b,c), LOC_Os11g42220 and LOC_Os12g43130. Both genes showed a trend similar to the RNAseq data, where they were more downregulated in plantlet L_6 (Figure 7b,c). It was delineated that

145 genes were upregulated while 992 genes were downregulated in both L_5 and L_6. For upregulated genes, significant enrichment was seen for GO terms and pathway categories including reproduction, post-embryonic development, and epigenetic regulation under biological processes (Figure 7d) and for term cell under cellular processes (Figures S5, and S6a). No enrichment was seen for metabolic processes probably due to lesser number of genes. Two

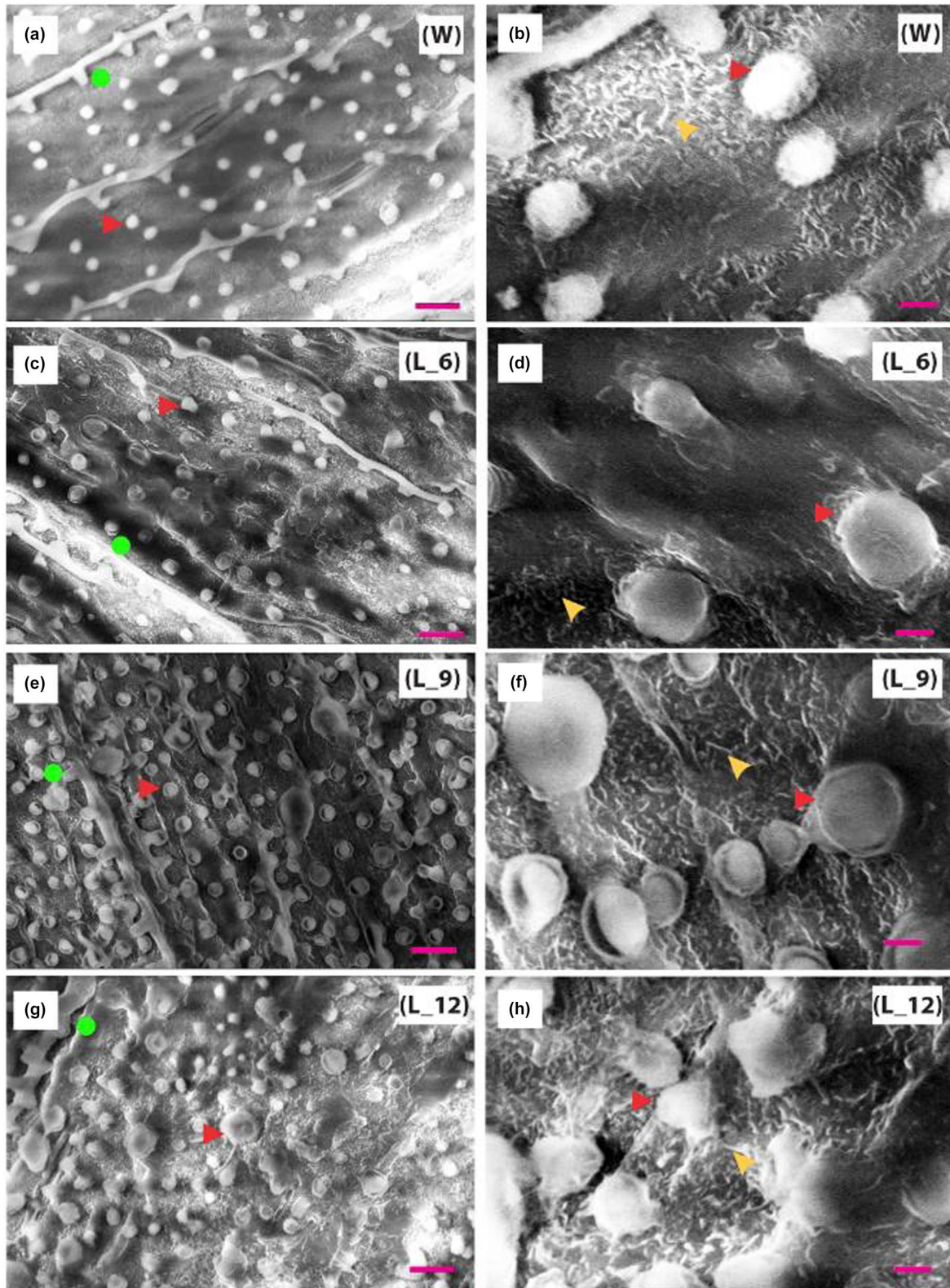


FIGURE 6 Scanning electron micrographs of leaf abaxial surface of 2.5-months-old OE_025 plantlets. The surface shows the presence of round (red arrow heads) and protruding silica bodies (green circles), and epicuticular wax (yellow arrow heads). The scale in the left panel represents 10 μ m and that on the right panel is 2 μ m, right panel images are magnifications of those on the left panel. The images have been taken from wild type plants, W (a, b), and transgenic OE_025 plantlets L_6 (c, d), L_9 (e, f), and L_12 (g, h)

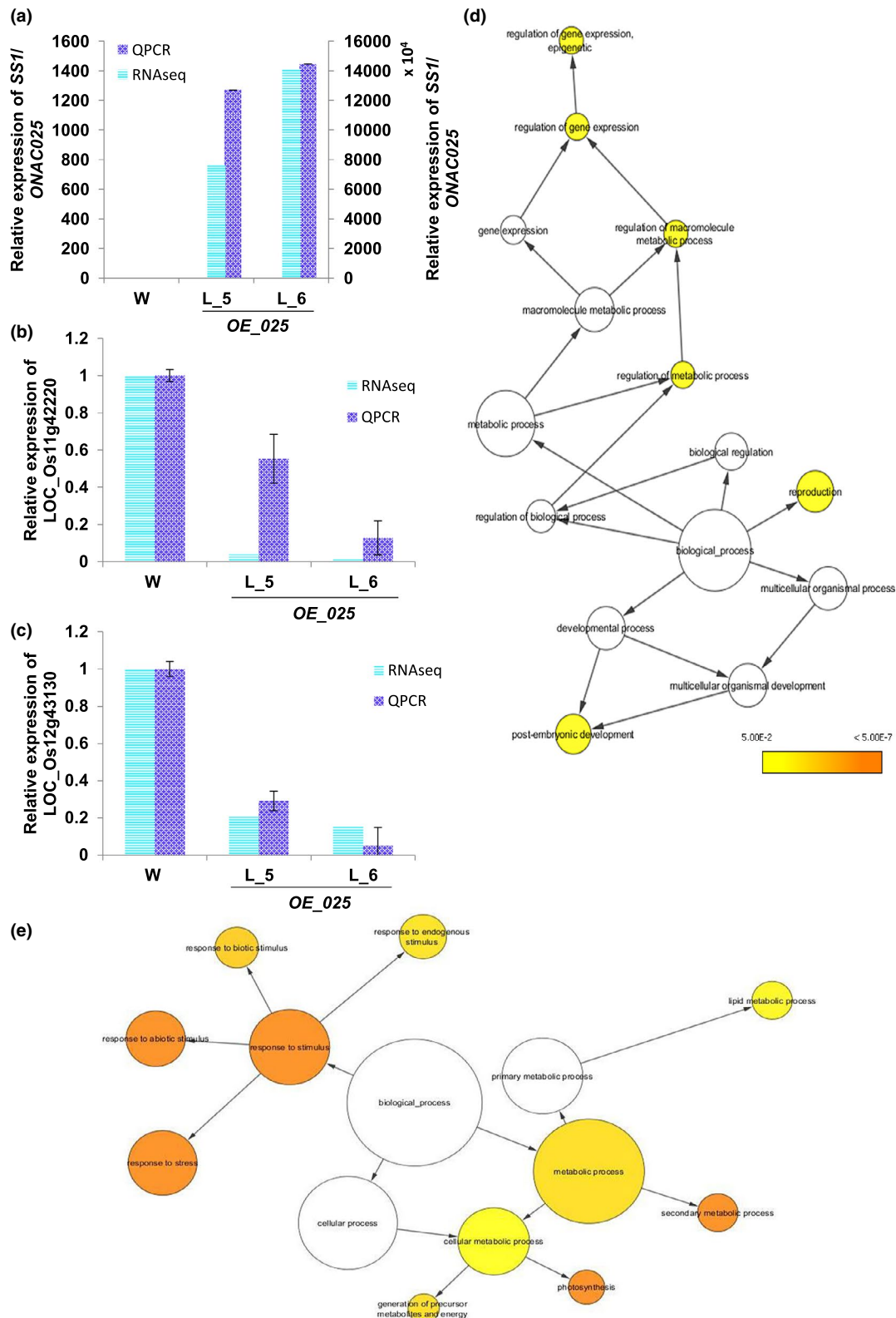


FIGURE 7 Transcriptome analysis of 2.5-months-old OE_025 (L_5 and L_6) plantlets. Validation of transcriptome analysis by QPCR of (a) *SS1/ONAC025* and two downregulated genes, (b) *LOC_Os11g42220*, and (c) *LOC_Os12g43130*. As shown in (b, c), the blue bars represent the RNAseq fold change values in L_5 and L_6 for (a–c), while purple bars represent fold changes by QPCR. The normalization genes used for QPCR was rice actin gene, *ACT1*. The RNAseq FPKM value for *SS1/ONAC025* in W in (a) is 1×10^{-5} , hence results in very high fold changes in L_5 and L_6, which has been plotted on secondary Y-axis. The QPCR error bars have been plotted on technical replicates. (d) GO enrichment analysis for the functional categorization of DEGs in total upregulated genes (e) and total downregulated genes using BiNGO 3.0.3 and visualized using Cytoscape version 3.4.0. Node size is equivalent to the total number of genes falling in that category and the color bar indicates the p-value significance as shown in (d). Yellow implies less enriched genes and orange implies more genes enriched in that node



genes each in post-embryonic development and secondary metabolism (Figure S5a,e), most in nucleic acid metabolism (Figure 5c) and all genes in epigenetic regulation and reproduction (Figure 5d,f) were highly upregulated. For the genes downregulated in L_5 and L_6 plantlets (Figure 7e), biological processes as response to stimulus, photosynthesis, secondary metabolic processes, generation of precursor metabolites and energy were enriched. Cellular components, such as, cell wall, thylakoid and plastids (Figure S6b), and molecular functions, namely, catalytic activity, oxygen binding and transcription factor activity (Figure S6c) were also enriched. There was downregulation of genes involved in post-embryonic development, cell wall, and secondary metabolism (Figure S5a–c,e). Pathway categorization by RicENCODE (Table S3) also had comparable results. In terms of TFs, seven TF encoding genes were upregulated while 92 were downregulated in OE_025. Most of these TFs belonged to AP2, bHLH, MADS, bZIP, Myb, Homebox, Co-like, WRKY, C₂H₂, Zim, and NAM families (Figure S7).

Since SS1/ ONAC025 is a seed-specific gene (Figure 1, Figure S1) and its ectopic expression led to a plantlet lethal phenotype (Figure 3, Figure S3), the differential transcriptome data generated from L_5 and L_6 in *indica* rice (mentioned above) was compared with seed development transcriptome data to have an idea about its putative role during the process. The seed development transcriptome data was previously generated by us (Sharma et al., 2012) on *indica* rice IR64, and included seed development stages, S1–S5, which are the same in which the promoter::GUS analysis was done (Figure 1). Since both PB1 and IR64 are *indica* rice, and have comparable patterns of SS1/ ONAC025 expression (Figure 9), the transcriptome data from the two were compared (Figure 8, Figure S8) to elucidate if any DEGs by SS1/ ONAC025 are also DEGs during seed development. The microarray data had been generated on Affymetrix GeneChip®. The genes there are represented by a probe set ID. Out of 145 genes upregulated in both L_5 and L_6, by both CuffDiff and DeSeq2, 121 had a corresponding probe set ID, out of which 35 genes were also upregulated during any one stage of seed development (Figure 8a, Table S4). Out of these, six genes were seed-specific (Figure 8c), which means they were expressed exclusively during rice seed development. These hinted at the regulation of seed development transcriptome by SS1/ ONAC025. Inversely, there were only two genes which were up in L_5 and L_6 but were downregulated during rice seed development (Figure 8a, Figure S8c, Table S4). Out of 992 genes downregulated in L_5 and L_6, 898 had corresponding probe set IDs. Out of these, 101 genes were commonly downregulated (Figure 8a, Figure S8a, Table S5). Inversely, there were 65 genes which were upregulated at least during any one stage of seed development (Figure 8a, Figure S8b, Table S5) but downregulated in L_5 and L_6.

Furthermore, DEGs generated by CuffDiff, which had a fold change cut-off of 1.5 and *p*-value cut-off at 0.05, in L_5 and L_6, could explain the phenotype, described above (Figures 3–6). Three genes regulating tillering (Mimura et al., 2012; Paul et al., 2012; Jung et al., 2015), two genes involved in root meristem development (Ge et al., 2004; Zhou, Jiang et al., 2017), seven genes controlling

meristem development and transition (Yang et al., 2006; Ishikawa et al., 2011; Tanaka et al., 2011; Khanday et al., 2013; Ren et al., 2013; Yoshida et al., 2013; Ma et al., 2017), were differentially expressed (roles detailed in Table 1) in L_5 and L_6, which may be responsible for the multiple tillering, elongated SAM and decreased root phenotypes described for Figures 3, 4 and Figure S3. Additionally, the *onion/oni* mutants also have fused first and second leaves as observed for OE_25 plantlets (Figure 4) and have a phenotype similar to SS1/ ONAC025. These genes code for 3-ketoacyl-CoA synthase (Ito et al., 2011; Tsuda et al., 2013; Akiba et al., 2014). Out of 28 genes delineated to be coding for 3-ketoacyl-CoA synthase at MSU (Ouyang et al., 2007), two showed significant downregulation in both the OE_025 plantlets (L_5 and L_6) used for RNAseq analysis (Table S6). To conclusively deduce the effect of SS1/ ONAC025 on the regulation of the above mentioned DEGs, this needs to be examined further.

To understand the accumulation of starch granules (Figure 4), genes involved in starch biosynthesis, specifically amylopectin (Figure 8b) were found to be upregulated in L_5 and L_6. These included *ADP-GLUCOSE PHOSPHORYLASES LIKE/OsAPL3* and *OsAPS1/OsAGP*, *DPE1/PLASTIDIAL DISPROPORTIONATING ENZYME1* and *OsBEI1b/Oryza sativa STARCH BRANCHING ENZYME11b* (Figure 8b). *OsAPL3* and *OsAPS1/OsAGP* constitute the large and small subunit of rice AGPase, respectively (Akihiro et al., 2005; Lee et al., 2007). In rice, six different AGPase encoding genes are expressed specifically in seeds. It is known that *OsAPS2*, *OsAPL2* and *OsAPL3* are extra-plastidial forms and *OsAPS1*, *OsAPL1*, and *OsAPL2* are plastidial forms (Akihiro et al., 2005). In cereal endosperm, major cytosolic form and minor plastidial form of AGPase are involved in synthesizing starch (Nagai et al., 2009). Our study reports the upregulation of *OsAPS1* (plastidial) and *OsAPL3* (extra-plastidial) in plantlets overexpressing SS1/ ONAC025. Additionally, the starch granules formed in OE_025 leaves (Figure 5) resemble amyloplasts (Toyosawa et al., 2016), supporting starch production by AGPase there. In leaves, AGPases are known to be plastidial (Pérez et al., 2018). Furthermore, it has been shown that the grain size is altered when the starch structure changes (Matsushima et al., 2016; Wang, Zhang et al., 2018; Jiang et al., 2018). Hence, the expression of genes known to regulate seed size was examined in L_5 and L_6. Two genes enhancing grain size were downregulated (Table 2). Alongside, upregulation of SSP (seed storage proteins) encoding genes was also seen both by QPCR (Figure 8d) and RNAseq (Figure 8e). Out of 65 SSPs in rice, nine were upregulated in the vegetative tissues ectopically expressing SS1/ ONAC025 including four globulin genes, three glutenin genes and two albumin genes (Figure 8d,e). SSPs are expressed during grain filling stages (Agarwal et al., 2011; Zhou, Wang et al., 2017). The differential expression of genes controlling specific aspects of grain filling in OE_025 plantlets indicates their regulation by SS1/ ONAC025, an aspect which should be examined in greater detail, perhaps in plants with altered expression of SS1/ ONAC025 which produce seeds, to confirm the same.

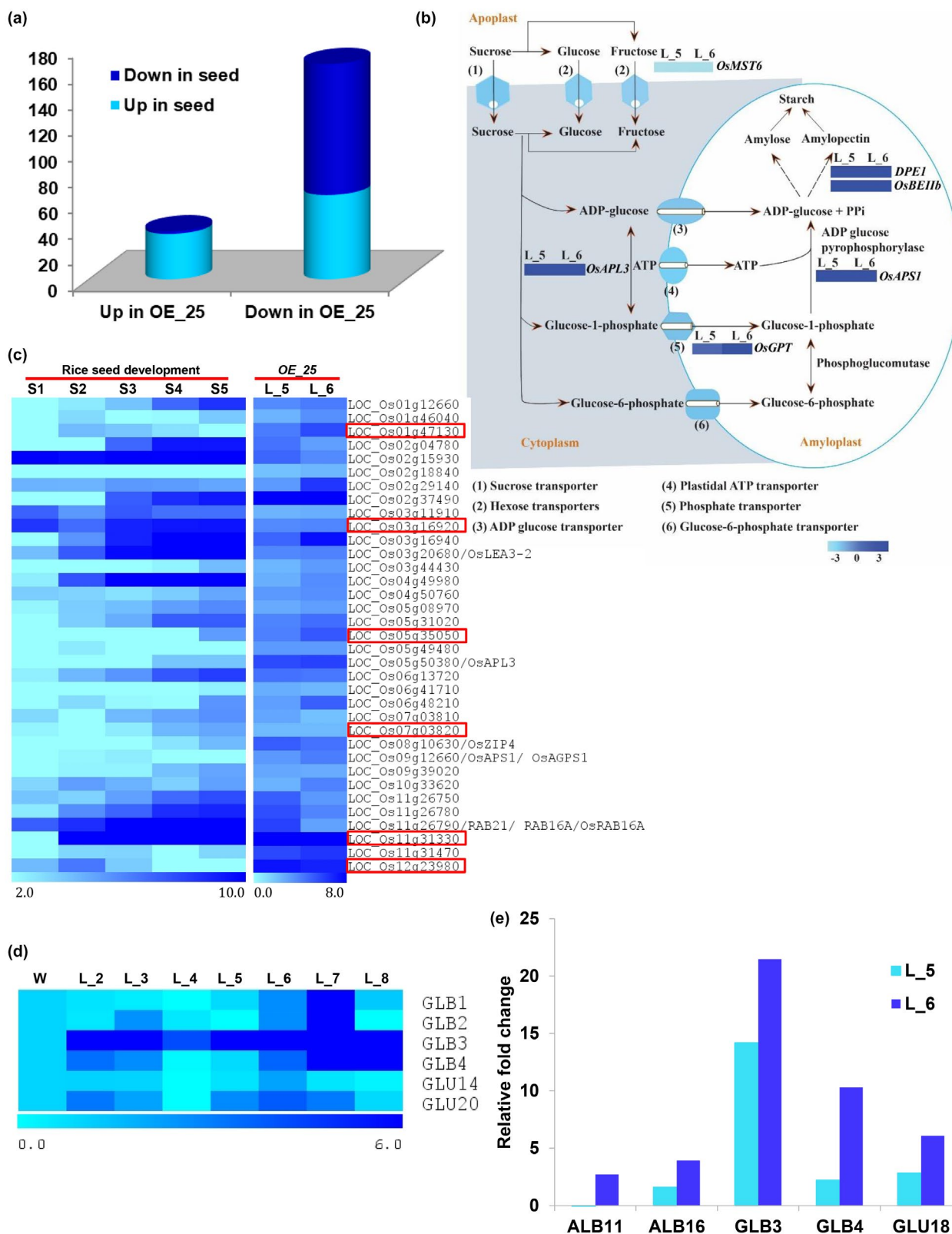


FIGURE 8 Comparison of IR64 seed development (S1-S5) transcriptome with OE_025 plantlet (L_5 and L_6) transcriptome. Genes showing differential expression in both OE_025 plantlets have been compared with the differentially expressed genes in the five development stages of rice seeds, which have been obtained from previous microarray analysis (Sharma et al., 2012). Genes showing up/downregulation in both OE_025 plantlets (compared to the corresponding wild type) and any seed stage (compared with four vegetative controls) have been considered for analysis. (a) Graph representing the number of genes differentially expressed in OE_025 plantlets, which are also DEGs during rice seed development. (b) Starch biosynthetic genes showing differential expression in both OE_025 plantlets. Various transporters involved in starch biosynthesis are numbered (1)-(6) and detailed in the legend. The diagram is not drawn to scale and the model of the starch biosynthetic pathway in cereal endosperm has been adapted (Akihiro et al., 2005; Li et al., 2014; Cakir et al., 2016). The color bars for heat maps have been shown. The expression values have been plotted as $\log_2$ fold change. (c) Heat map representing the expression levels of genes upregulated in both L_5 and L_6 plantlets ($\log_2$ FPKM values) and any seed development stage ($\log_2$ GCRMA values). The tissues analyzed have been mentioned on top of the heat map (S1-S5 stages of rice seed development; and L_5 and L_6). Seed-specific genes have been boxed in red. (d) Q-PCR analysis of six different SSPs in individual plantlets (L_2 to L_8), displayed as a heat map. Each column indicates the plantlets analyzed, as mentioned above the heat map. (e) Relative levels of five SSPs upregulated in L_5 and L_6 plantlets as quantified by RNAseq analysis

TABLE 1 Log₂ fold change values of genes known to be crucial for tillering and meristem development, in L_5 and L_6 plantlets of OE 025

Locus IDs/gene names	Regulatory role in tillering and phase transition	Log ₂ fold change in L_5	Log ₂ fold change in L_6	Reference
Genes involved in tillering				
LOC_Os01g68000/ <i>PLA2/LHD2</i>	Positively regulates tillering	12.84	13.25	Mimura et al. (2012)
LOC_Os02g04160/ <i>OsTEF1</i>	Positively regulates tillering	1.54	1.66	Paul et al. (2012)
LOC_Os01g53880/ <i>IAA6/OsIAA6</i>	Negatively regulates tillering	-1.52	-2.76	Jung et al. (2015)
Genes involved in root meristem development				
LOC_Os01g15340/ <i>OsRAA1</i>	Mediates auxin induced root responses	-1.84	-2.42	Ge et al. (2004)
LOC_Os07g48560/ <i>WOX11</i>	Essential for cell division and root growth	-3.86	-1.73	Zhou, Jiang et al. (2017)
Genes involved in meristem development and transition				
LOC_Os05g15630/ <i>OsBLE3</i>	Positive regulates plant growth	-1.58	-1.53	Yang et al. (2006)
LOC_Os12g01550/ <i>LBD12-1</i>	Positively regulates SAM development	-2.56	-2.88	Ma et al. (2017)
LOC_Os02g53140/ <i>COP1/PPS</i>	Involved in vegetative to reproductive phase transition	-1.54	-1.51	Tanaka et al. (2011)
LOC_Os05g41760/ <i>MFS1</i>	Positively regulates spikelet meristem transition	-3.99	-2.94	Ren et al. (2013)
LOC_Os06g16370/ <i>HD1</i>	Positively regulates floral transition	2.53	3.64	Ishikawa et al. (2011)
LOC_Os12g06340/ <i>OsBLH1</i>	Positively regulates floret specification and development	2.38	2.80	Khanday et al. (2013)
LOC_Os10g33780/ <i>TAW1</i>	Suppresses meristem phase transition	1.95	3.41	Yoshida et al. (2013)

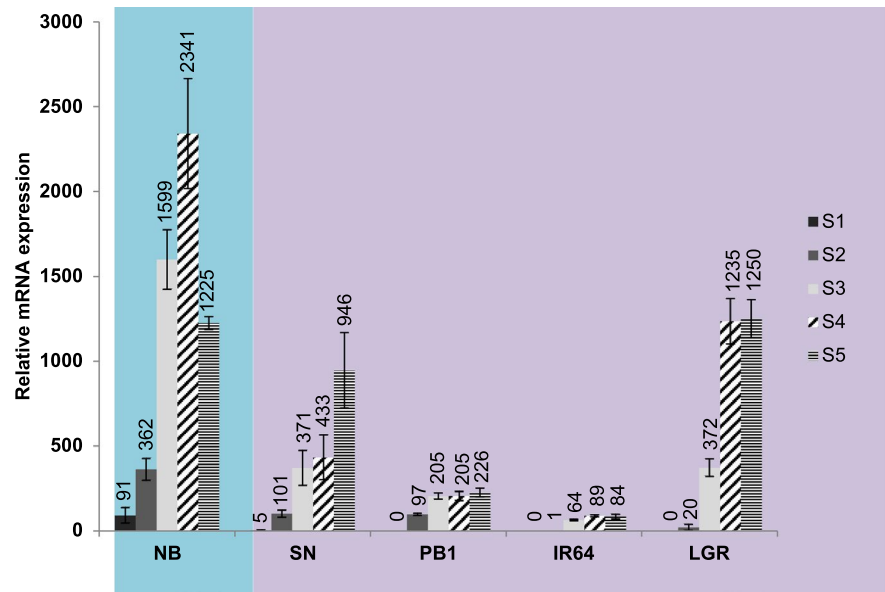
Abbreviations: COP1, CONSTITUTIVE PHOTOMORPHOGENIC1; HD1, HEADING DATE1; LBD12-1, LATERAL ORGAN BOUNDARIES DOMAIN; LHD2, LEAFY HEAD2; MFS1, MULTI-FLORET SPIKELET1; OsBLE3, *Oryza sativa* BRASSINOLIDE ENHANCED GENE3; OsBLH1, *Oryza sativa* BEL1-LIKE HOMEODOMAIN MEMBER; OsIAA6, *Oryza sativa* INDOLE-3-ACETIC ACID6; OsRAA1, *Oryza sativa* ROOT ARCHITECTURE ASSOCIATED1; OsTEF1, *Oryza sativa* TRANSCRIPTION ELONGATION FACTOR1; PLA2, PLASTOCHRON2; PPS, PETER PAN SYNDROME-1; TAW1, TAWAWA1.; WOX11, WUSCHEL-RELATED HOMEODOMAIN.

TABLE 2 Log₂ fold change values of genes related to seed size in L_5 and L_6 plantlets of OE_025 plants

Locus IDs/gene names	Role in seed development	Log ₂ fold change in L_5	Log ₂ fold change in L_6	References
LOC_Os06g12210/BU1	Enhances grain size	-2.97	-1.63	Tanaka et al. (2009)
LOC_Os07g41200/GW7/GL7/SLG7	Enhances grain length	-3.23	-2.88	Wang et al. (2015)
LOC_Os03g12660/OsDWARF4	Reduces grain yield	-3.24	-1.87	Sakamoto et al. (2006)

Abbreviations: BU1, BRASSINOSTEROID UPREGULATED1; GW7/GL7/SLG7, GRAIN LENGTH ON CHROMOSOME 7.

FIGURE 9 Relative expression of *SS1/ ONAC025* in five rice genotypes, namely, NB, SN, PB1, IR64, and LGR, arranged according to their increasing weights, in the five developing seed stages (S1-S5). The data is an average of three independent biological replicates and the standard error bar is shown. The *japonica* genotype has a blue background, while *indica* genotypes have a purple background



This was even higher than our earlier documented expression in IR64. Interestingly, the expression was maximum in *japonica* rice in comparison with four *indica* genotypes, and was highest in Nipponbare S4 stage. Even in S1 stage of Nipponbare, there was considerable expression, not seen in *indica* genotypes (Figure 9). As the expression of a gene is majorly controlled by the presence of specific *cis*-elements on the upstream promoter region (Porto et al., 2014), the 2 kb upstream promoter region of *SS1/ ONAC025* was amplified and sequenced from the five genotypes and compared (Table S7). The sequences had multiple SNPs at the rate of 49 SNP/1000 bp among the genotypes, with the region of variability increasing further away from the start codon. Also, sequence from LGR was more diversified. Element E2FCONSENSUS, MYBCOREATCYCB1 were absent in NB and present in *indica* genotypes while PREATPROD was present only in NB. The promoter sequences had 12 known seed-specific elements, which may be responsible for its seed-specific and high expression (Figure 1, Figure S1). Amongst seed-specific elements, for -300CORE, -300ELEMENT, AMYBOX1, CAATBOX1, EMHVCHORD, PROLAMINBOXOSGLUB1, PYRIMIDINEBOXOSRAMY1A, SEF1MOTIF, SEF4MOTIFGM7S, the numbers were same in NB and four *indica* genotypes, except LGR. For seed-specific elements EBOXBNNAPA and TATABOX4, numbers were same for NB and LGR. Thus, though the identification of elements on the promoter sequences of the five genotypes could justify for the high seed-specific expression, they could not fully explain the variation in expression levels amongst them (Figure 9), which may be more of a genotypic effect under the control of other upstream factors, also seen for other seed-specific NAC genes from rice (Mathew et al., 2016).

4 | DISCUSSION

NAC TFs have been rather well elucidated for their roles in stress responses in various plant species. However, there is limited knowledge

about their functions in developmental processes, including rice seed development (Mathew and Agarwal, 2018). Since NAC emerged as a family with members having high and seed-specific expression during the process of rice seed development by microarray analysis (Sharma et al., 2012), *SS1/ ONAC025* was selected from the data for determination of its role during rice seed development. The 2 kb upstream promoter of *SS1/ ONAC025* was abundant with seed-specific elements and directed reporter *GUS* expression specifically throughout the endosperm tissue and embryo, during all five stages of rice seed development (Figure 1). The promoter::reporter expression is often correlated with the tissue where the gene operates. For example, *GW2* regulates seed size in rice and its promoter expresses in the aleurone layer (Lee et al., 2018). Sucrose transporters *SWEET11* and *15* import nutrients during grain filling. The promoters of both of these genes express in the nucellus during early stages of seed development (Yang et al., 2018). Hence, the reporter expression in rice promoter::*GUS* plants points toward the role of *SS1/ ONAC025* in rice seed development, and also validates the microarray data.

Rice transgenic plants with ectopic expression of *SS1/ ONAC025* had a plantlet lethal phenotype (Figure 3). The plantlet transcriptome data from L_5 and L_6 showed enriched upregulation of genes involved in reproductive development and downregulation of those involved in photosynthesis and cell wall formation (Figure 7). Suppression of vegetative pathways can cause a lethal phenotype (Suzaki et al., 2008). OE_25 plantlets had stunted height but increased tiller number. Emergent tillers in transgenic plantlets shared vasculature showing their origin from the main plant. This was supported by upregulation of the positive regulators of tillering, and down regulation of a negative regulator (Table 1). Furthermore, tillering is a meristem activity (Fletcher, 2018) and a cytochrome P450 encoding gene, *GIANT EMBRYO*, promotes both tillering and SAM maintenance in the embryo (Yang et al., 2013). Different genes involved in meristematic transition of vegetative to reproductive and spikelet growth were also differentially expressed in OE_025 plants.



Thus, elongation of shoot meristem (Figure 4) would have resulted from the downregulation of various genes involved in SAM development and upregulation of those involved in floral transition. Hence, the phenotype and transcriptome analysis show that *SS1/ ONAC025* when overexpressed in vegetative tissues, causes the differential expression of genes involved in meristematic transitions/development, eventually leading to a plantlet lethal phenotype. Also, the production of starch and SSPs in the vegetative stages would have further contributed to the plantlet lethal phenotype since the presence of degradation resistant amylopectin hampers seedling growth (Pan et al., 2018). Basically, *SS1/ ONAC025* was suppressing vegetative growth and promoting a seed like environment in the transgenic plantlets.

The distinct accumulation of starch granules in vegetative tissues of *OE_25* plantlets (Figure 5) was explained by upregulation of genes involved in starch biosynthesis many of whose expression patterns overlapped with that of *SS1/ ONAC025*. An interesting phenotype of *OE_25* plantlets has been the distinct presence of starch granules (Figure 5), which appear similar to that reported from rice endosperm (Matsushima and Hisano, 2019). The observation is not due to starch produced as a result of photosynthesis because the granules in the wild type plant grown on the same medium and in same conditions are very less. This is because the starch produced in photosynthetic tissues is transitory. In both leaves and endosperm, starch is produced from sucrose, eventually in plastids, it being chloroplast in leaves and amyloplast in endosperm. ADP glucose pyrophosphorylase is the main enzyme in both cases (Baroja-Fernández et al., 2004), though the subunits localization differs. Since both its subunits were upregulated in *OE_25* plantlets (Figure 8), the observed starch accumulation can be said to be linked to the process of seed development. In a similar observation as ours, in cassava, where root is the place for starch storage, the knock down of an enzyme causing starch accumulation in root, resulted in its higher accumulation in leaves, leading to growth retardation (Zhou He, et al., 2017). This has also been reported in rice (Liang, Hirose et al., 2014). The production of transitory and storage starch has been aptly called as “two sides of the same coin” due to similarity in pathways (Lloyd and Kossmann, 2015). Also, the starch granules stained reddish-brown, indicating a bent towards amylopectin biosynthesis (Bettge et al., 2000) as also seen in the transcriptome data. Enhanced expression of *DPE1* and *OsBE1b* implies increased amylopectin biosynthesis. Overexpression of *DPE1* reduces amylose content which results in small and tightly packed starch granules and affects degree of polymerization (Dong et al., 2015; Hwang et al., 2016). *OsBE1b* negatively regulates the amylose content and positively affects amylopectin synthesis, by causing the formation of short chains of amylopectin clusters (Tanaka et al., 2004; Lu and Park, 2012; Nakata et al., 2018; Wang, Chao et al., 2018). The mutant of this gene is characterized by erect leaf phenotype with short plant stature and is associated with enhanced grain yields (Sakamoto et al., 2006). Amylopectin content is high in *japonica* rice which is often sticky to cook (Inukai, 2017) and is favored by people of Northeast Asia (Sun et al., 2011). Interestingly, the expression of *SS1/ ONAC025* was the highest in *japonica* rice NB

(Figure 9). This rice is often short and round (Nagata et al., 2015) and genes positively controlling seed length and width were downregulated in *OE_25* plants (Table 2). Of these, plants overexpressing *BU1* are characterized by the increased bending of lamina joint and enhanced grain size (Tanaka et al., 2009) and *GW7* enhances cell division in longitudinal direction resulting in more slender grains (Wang et al., 2015; Zhou et al., 2015). Since seed size and number are inversely correlated (Gupta et al., 2006), *OsDWARF4* which reduces yield was also downregulated (Table 2).

The transcript patterns vary with the tissue types and conditions. In this study, we had a seed-specific gene whose overexpression did not generate seeds to help elucidate its function. Hence, the challenge was to devise a way to understand its role in rice seed development. First, the transcriptome of *OE_25* plantlets was compared to a WT plantlet to elucidate DEGs due to overexpression of *SS1/ ONAC025*. These DEGs were then compared to the developing seed transcriptome of rice, to find out common DEGs. These genes would be the ones which are also downstream to *SS1/ ONAC025* in rice seed, by virtue of their differential expression in both kinds of tissues. This methodology is supported by the fact that seed-specific genes, were also upregulated in *OE_25* plantlets (Figure 8), indicating an activation of seed transcriptome by *SS1/ ONAC025*. The biggest number of genes in this comparison were the ones downregulated both during seed development and in *OE_25* plantlets. There were also genes which were upregulated during seed development but downregulated in *OE_25* plantlets. This downregulation of genes involved in seed development by *SS1/ ONAC025* could be an effect of its transrepression property (Figure 2). This differential expression of seed-related genes, though in plantlets, hints at a role of *SS1/ ONAC025* in rice seed development, which needs to be explored further in detail. The presence of starch granules is not enough proof for the same function in the grains, the plantlet being an entirely different developmental tissue. A major down side of such a plantlet lethal phenotype is that the transgenic plants did not mature to produce next generation seeds. So, the actual effect of increased expression of *SS1/ ONAC025* in seeds could not be observed. A more pragmatic approach in this situation is to use an endosperm/seed-specific promoter to overexpress the gene specifically in the seed (Liu et al., 2016). This will obliterate the hampering effect of a reproductive gene on the vegetative growth, and exhibit the effect of the gene in the requisite tissue only.

To the best of our knowledge, this is the first report of the functional characterization of a NAC TF from rice, which regulates aspects of seed/grain development. The promoter::reporter rice plants and high seed-specific expression along with presence of starch (amylopectin) granules, highest expression in *japonica* rice, activation of seed transcriptome and suppression of vegetative transcriptome indicate towards the probable roles of *SS1/ ONAC025* in rice seed development. However, the occurrence of these changes in overexpression plantlets and the absence of a reproductive phase in them strongly implies the need to examine the exact functional role of this gene in much greater details.



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CONFLICT OF INTEREST

The authors declare no conflict of interest with the work described in the manuscript.

AUTHOR CONTRIBUTION

IEM, RP, AM, and PJ performed various experiments. IEM, RP, and PA wrote the manuscript. PA formulated the study and experiments. SK gave regular scientific inputs. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

RNAseq was performed for one wild type and two independent transgenic plantlets (L₅ and L₆) using Illumina® trueSeq® RNA sample preparation kit v.2 (Illumina; USA). The sequences generated were submitted to NCBI SRA database (<https://www.ncbi.nlm.nih.gov/sra/PRJNA540785>) with SRA accession number PRJNA540785. Biosample accession numbers for individual samples are SRR9005258_WT, SRR9005257_L₅, and SRR9005256_L₆.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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