Spatial distribution of rice lectin protein r40g3 determines its dual regulatory role in imparting salinity tolerance while impeding seed development

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Abstract

While the role of R40 family of lectin proteins in osmotic stress has long been known, their mechanism of stress tolerance has not yet been explored. Among them, the expression of *Osr40g3* was most strongly induced in response to salt stress. Here, we report that *Osr40g3* plays a dual role in regulating salinity tolerance as well as seed development in rice. The overexpression of *Osr40g3* under control of a constitutive promoter significantly improved salinity tolerance but impaired seed development in transgenic rice. Remarkably, transgenic *Arabidopsis* ectopically expressing *Os*r40g3 exhibited enhanced salinity tolerance but produced seeds normally. It was further confirmed that this *Os*r40g3-mediated impairment in seed development was due to pollen sterility in the transgenic rice while the pistils remained unaffected. The gene further displayed a precise tissue-specific expression pattern which was essential for proper seed development in rice. The constitutive overexpression led to the interaction of *Os*r40g3 with *Os*GF14e in the stamens affecting its protein stability. This interaction further interfered with the gibberellic acid metabolism thus leading to pollen sterility. This interaction was, however, highly specific due to the structural distinctiveness of *Os*GF14e making the phenomenon rice-specific. Nevertheless, in salinity stressed leaves, *Os*r40g3 interacted with *Os*EG45 or its ortholog thus leading to salinity tolerance in both rice and *Arabidopsis*.

Keywords

Osr40g3, salinity tolerance, OsGF14e, seed development, pollen sterility, rice, Arabidopsis

INTRODUCTION

Plants continuously face a wide range of environmental challenges including various biotic and abiotic stress factors. Fascinatingly, they can counteract most of these threats with their intricate defense signaling network and maintain growth (Muthamilasaram and Prasad 2013). Among the diverse protective machineries, plants have evolved an array of signaling proteins that recognize the environmental changes and subsequently regulate downstream stress-responsive genes. Lectins are carbohydrate-binding proteins that serve as excellent candidates in the plant signaling network. It can bind to the exogenous as well as endogenous glycan molecules to regulate various stress responses in plants (Van Damme et al., 2008). The role of lectin proteins in modulating biotic stress responses has widely been reported in a variety of plant species (Ciopraga et al., 1999; Freire et al., 2002; Damico et al., 2003; Singh and Zimmerli 2013; Miyakawa et al., 2014). Emerging evidences have also highlighted their role in abiotic stress responses including heat, drought and salinity tolerance (Spadoro-Tank and Etzler, 1988, Hirano et al., 2000; Jiang et al., 2012a; He et al., 2016, Sahid et al., 2020).

In plants, 12 lectin protein families have been reported, out of which most of the members are expressed constitutively and show a positive regulatory role against pathogen attack (Jiang et al., 2010). Intriguingly, some lectin proteins which are localized in the nucleus and cytoplasm of the cell are highly accumulated in response to abiotic and biotic stress factors (Song et al., 2014). The R40 family is an interesting group of lectin proteins that are known to play important roles in regulating drought and salinity stress (Moons et al., 1995, 1997; Paul et al., 2015; Sahid et al., 2020). The group consists of five different members namely, *Os*r40c1, *Os*r40c2, *Os*r40g2, *Os*r40g3 and putative *Os*r40c1, in rice (Carpentier et al., 2007; Jiang et al., 2012b). The members of this R40 family possess a ricin-B like domain that specifically recognizes galactose or N-acetyl galactosamine residues. Ricin, a widely distributed lectin protein in plants, was first identified in castor bean seeds (*Ricinus communis*) and consists of two chains. Chain-A exhibits enzymatic activity whereas chain-B possesses duplicated ricin-B domain which determines the sugar-binding nature. A number of ricin-B domain containing proteins have been identified in different plant species (Van Damme et al., 2001; Cummings and Etzler 2009). It has been suggested that the ricin-B containing lectins mostly play major roles in biotic stress tolerance in plants. For example, the overexpression of *Solanum nigrum* SNA-I or SNA-IV in tobacco

increased the resistance against tobacco mosaic virus as well as certain pests like aphids (Chen et al., 2002; Vandenbusshe et al., 2004a, b; Sahidi-Noghabi et al., 2009). Again, several lines of evidences suggest that the members of *Os*R40 protein family, *Os*r40c1, *Os*r40g3 and *Os*r40c2 are accumulated at a high level in response to salinity and drought stress in plants (Moons et al., 1995; Jiang et al., 2012b; Paul et al., 2015). In addition, it has been demonstrated that the overexpression of *Os*r40c1 imparts drought tolerance in rice and tobacco suggesting its drought-tolerant activity in plants (Sahid et al., 2020). However, no members of R40 family have been identified so far that can regulate seed development in plants.

Seed development is a complex molecular phenomenon and is associated with intricate phytohormone signaling cross-talks. Among them, gibberellic acid (GA) critically regulates reproductive development, seed formation as well as germination of seeds (Lo et al., 2008). For example, exogenous GA application can induce development of male and female reproductive organs in different plant species. On contrary, GA deficiency can lead to pollen sterility thus hampering seed development (Goto and Pharis, 1998). Pollen germination is also hindered due to absence of GA (Chuun et al., 2007). In addition, several proteins interact with GA signaling proteins and modulate GA responses to alter pollen fertility and seed development (Lo et al., 2008 plant cell). GA when perceived by gibberellin insensitive dwarf 1 (GID1), forms a GA/GID1 complex and facilitates the degradation of DELLA repressor (Ueguchi-Tanaka et al., 2007). On the other hand, GAMYB acts as a positive regulator downstream of the GA signaling cascade. Earlier evidences suggest that GAMYB critically regulates the programmed cell death of tapetal cells, formation of pollen exine and Ubisch bodies in anthers (Aya et al., 2009).

In this study, we aimed to decipher the mechanism of how *Os*r40g3 protein regulates salinity tolerance in rice. Surprisingly, it was observed that *Os*r40g3 exhibits dual function in positively regulating salinity tolerance while negatively regulating pollen viability in a GA-dependent pathway. This function is precisely modulated by its tissue-specific expression pattern in rice.

RESULTS

Expression of *Osr40g3* positively correlated with the salinity tolerance potential in rice

To investigate the salinity tolerance potential of selected eight *indica* rice cultivars, the seedlings were exposed to salinity stress for 5 d (Fig 1A). Following stress treatment, the cultivars were

assessed according to standard evaluation system proposed by IRRI (2013) and several morphological parameters were recorded (Fig. S1A-D). In addition, different biochemical parameters such as chlorophyll content, osmolyte accumulation, and hydrogen peroxide (H_2O_2) content were analyzed (Fig. S1E-H). Chlorosis of leaves is one of the most common symptoms observed when plants are exposed to salinity stress which is a consequence of ion toxicity (Munns, 2002). Here, we observed the highest level of chlorosis in MTU1010 cultivar. Nonabokra, on the other hand, displayed the least alterations in the morphology and chlorophyll content compared with the other cultivars indicating its tolerant phenotype. The rest of the six cultivars showed moderate levels of sensitivity to salinity. Based on all these observations, the cultivars were arranged according to their salinity tolerance potential with the Nonabokhra being the most tolerant while the MTU1010 was the most sensitive cultivar.

Next, to investigate the stress-responsive roles of the *OsR40* genes, their expression patterns were analyzed in response to salinity stress. Interestingly, it was observed that among all the five *OsR40* genes, the expression of *Osr40g3* was the highest under salinity stress (Fig S2). This prompted us to explore if the expression of the *Osr40g3* gene can be correlated with the salinity tolerance potential in rice. The expression of this gene under salinity stress was indeed positively correlated with the salinity tolerance potential in the eight rice cultivars with a 4.29-fold induction in the tolerant Nonabokhra cultivar against a 2-fold induction in the sensitive MTU1010 cultivar (Fig 1B). The rest of the cultivars displayed a moderate level of *Osr40g3* induction in response to salinity stress. The *OsRAB16* gene was used as a salinity stress-specific marker (Ganguly et al., 2012). This observation strongly indicated that *Osr40g3* plays a crucial role in modulating salinity tolerance in rice.

Overexpression of *Osr40g3* improved salinity tolerance in rice but impaired seed development

To validate the function of *Osr40g3* in salinity tolerance, *Osr40g3* overexpressing transgenic rice plants (*ox* lines) harboring the recombinant *35S::Osr40g3* construct were generated. The positive transgenic plants were screened by genomic DNA PCR with *bar* gene-specific primers (Fig S3). The transgenic plants were analyzed to estimate the expression of *Osr40g3* gene and were grown under greenhouse conditions. The different morphological parameters like plant height, tiller numbers, shoot and root dry weight, number of panicles per plant, panicle length, number of

flowers per panicle, and number of grains per panicle were analyzed from the transgenic plants and compared with the wild-type (WT) and vector control (VC) plants (Fig 2). In the vegetative stage, the plants displayed no phenotypic abnormalities except a reduced plant height in most of the lines. Surprisingly, at the reproductive stage, all the transgenic lines developed a shorter panicle length in comparison with the WT and VC plants. Although the number of panicles per plant did not significantly differ, most of the transgenic lines failed to produce seeds. The plant transformation was carried out in three independent batches and only two transgenic plants from the first batch and three plants from each of the second and third batches produced 30-35 seeds per plant.

However, when the plants were exposed to salinity stress, the transgenic plants displayed significantly improved tolerance compared with the WT and VC plants (Fig 3A). The transgenic plants maintained lower percentage of rolled leaves and higher relative water content (RWC) over the WT and VC plants under salinity stress (Fig. S4). The transgenic plants also accumulated significantly elevated levels of osmolytes as well as displayed lower chlorophyll degradation. It was observed that the expression of the *Osr40g3* gene was significantly higher in the transgenic lines under control as well as salinity stress conditions which could account for their tolerant phenotype (Fig 3B). The amount of sodium (Na^+), potassium (K^+), and chloride (Cl^-) ions in the soil before and after salt treatment was also measured (Table S2). The lower accumulation of Na^+ in the transgenic plants compared with the WT and VC plants during salinity stress also indicated the tolerant phenotype of the transgenic plants (Fig. S4). Together, these observations suggested that *Osr40g3* overexpression could improve salinity tolerance but impaired seed development in the transgenic lines thus indicating its dual function.

Ectopic expression of *Osr40g3* improved salinity tolerance in *Arabidopsis* with no abnormality in seed development

To further confirm its dual function in a heterologous system, transgenic *Arabidopsis* plants (*ec* lines) harboring the recombinant *35S::Osr40g3* construct and ectopically expressing the *Osr40g3* gene were generated. The transgenic lines were confirmed by genomic DNA PCR using *bar* gene-specific primers (Fig S5). Surprisingly, unlike in rice, the transgenic *Arabidopsis* plants displayed no abnormality in seed development (Fig.4). In addition, no alteration in the rosette

diameter, plant height, silique number and length, as well as seed yield per plant, was observed in the transgenic plants. Yet, similar to rice, the transgenic *Arabidopsis* plants displayed significantly improved salinity tolerance which was accompanied by a higher RWC and chlorophyll content, lower Na^+ accumulation as well as an increased *Osr40g3* transcript abundance under salinity stress (Fig. 5, Fig. S6). These observations confirmed the role of *Osr40g3* in imparting salinity tolerance in plants but indicated that its interference with the seed development is rice-specific.

Osr40g3 overexpression resulted in pollen sterility in rice but not in _Arabidopsis_

To understand the differential interference of *Osr40g3* in seed development, we analyzed the floral morphology of both the rice and *Arabidopsis* transgenic plants. In rice, flowers of transgenic plants were found to be shorter than the WT and VC flowers (Fig. 6A-C). However, no other significant difference in the floral morphology was observed. Since impairment in seed development was observed, the pollen viability was tested using I_2-KI and fluorescein diacetate (FDA) staining methods. The pollen viability in all rice transgenic flowers was found to be drastically reduced compared with the WT and VC flowers (Fig. 6D-E, S7A). The *in vitro* pollen germination assay displayed that the most of the pollen grains from the rice transgenic plants failed to produce pollen tube after 24 h of pollen germination (Fig.6F). This observation directly supported the failure of the rice transgenic plants to set seeds. On contrary, the transgenic *Arabidopsis* plants showed no alteration in floral morphology, pollen viability and pollen germination supporting their perfect seed development (Fig.7).

WT pollens could improve seed setting in _Osr40g3_ overexpressing rice plants

To check if the failure to produce seeds in the transgenic rice lines was due to pollen sterility, the *Osr40g3* overexpressing plants were crossed with the WT plants in two separate hybridization programs. When the WT plants were emasculated and the *Osr40g3* overexpressing plants were used as a male parent no seeds were produced which confirmed the pollen sterility in the transgenic plants. Interestingly, when the *Osr40g3* overexpressing plants were used as a female parent and crossed with the WT plant as a male parent, hybrid seeds were developed (Fig. 8). This finding confirmed that the failure of the transgenic plants to set seed was due to its pollen sterility with no defect in its female reproductive part.

Osr40g3 displayed a strict tissue-specific expression pattern in rice

To understand if the *Osr40g3* gene has any specific expression pattern in rice, its expression in the WT plant was studied from different tissues at different developmental stages. For this purpose, germinating seedlings, 60 d and 120 d old plants were used and samples were collected from the root, leaf, flag leaf, flower, stamen, and seeds under milk stage, dough stage as well as mature stage as applicable. The highest expression level of the *Osr40g3* gene was observed in the leaves under all developmental stages except for germinating seedlings where a low expression level was observed in both root and shoot (Fig 9A). Intriguingly, no expression of *Osr40g3* was detected in the stamen indicating its tissue-specific expression pattern. To further validate this observation, the promoter activity of the *Osr40g3* gene was analyzed by histochemical GUS assay. The transgenic plants harboring the recombinant *Osr40g3pro::gus* construct showed GUS expression in roots and leaves collected from different stages of development. However, in case of flower, GUS expression was observed in the pistil but not in the stamens further supporting the tissue specific expression of *Osr40g3* (Fig. 9B-C).

On the other hand, since a constitutive promoter was used to overexpress the *Osr40g3* gene, the stamens of the transgenic plants showed accumulation of *Osr40g3* transcript as expected (Fig.S8A). Remarkably, the expression of *OsAGL*65, an important regulator of pollen sterility, was not detected in the flowers and stamens of the transgenic plants while it was expressed specifically in the flowers including stamens of the WT plants (Fig S8B). This signified that the constitutive expression of the *Osr40g3* in stamens of transgenic rice induced pollen sterility.

Overexpression of *Osr40g3* under control of the native promoter imparted salinity tolerance and rescued pollen sterility in rice

Since the *Osr40g3* gene followed a strict tissue-specific expression pattern, transgenic rice plants (*ox* lines) overexpressing *Osr40g3* gene under the control of the native promoter were generated. The positive transformed plants, harboring the recombinant *Osr40g3pro::Osr40g3* construct, were screened through genomic DNA PCR using *bar* gene-specific primers (Fig S9). Similar to the previous observation, these transgenic plants also exhibited a slightly dwarf phenotype compared with the WT and VC plants but displayed no abnormalities in seed development (Fig.10). Supporting this observation, the transgenic pollen grains also exhibited a similar

viability and pollen germination efficiency like that of the WT and VC plants (Fig 10, S7B). These observations strongly suggested that the tissue-specific expression pattern of the *Osr40g3* gene is an important determinant of pollen viability and seed development in rice. Furthermore, no expression of *Osr40g3* was detected in stamens of these transgenic plants due to its tissue specific expression pattern. Concomitantly, the expression of *OsAGL65* was detected in the flowers including stamens similar to the WT plants (Fig. S10). On the other hand, these overexpression lines harboring the *Osr40g3pro::Osr40g3* construct also displayed a significant improvement in salinity tolerance and higher *Osr40g3* transcript abundance over the WT and VC plants (Fig. 11, Fig. S11).

_Os_r40g3 interacted with _Os_GF14e to regulate pollen sterility and with _Os_EG45-like protein to modulate salinity stress tolerance in rice

To gain some mechanistic insight into the function of *Osr40g3*, the interacting protein partners were identified by yeast two-hybrid (Y2H) analysis against specific cDNA libraries. The *Os*r40g3 protein was found to interact with three non-redundant protein partners, namely *Os*GF14e (LOC4329775), *Os*AGL20 (LOCOs03g03070), and *Os*AP2-like proteins (LOCOs0107120) when a cDNA library prepared from rice flowers was used (Fig 12A). Among these partners, the *Os*GF14e protein was reported to be associated with the regulation of seed development (Manosalva et al., 2011; Liu et al., 2016). We, therefore, confirmed the interaction of this protein partner by BiFC assay (Fig 12B). These observations indicated that *Os*R40g3 presumably regulate pollen sterility and seed development by modulating the activity of *Os*GF14e in rice. On the other hand, interaction of *Os*r40g3 with the other partners can reveal interesting information as well which needs to be explored in future.

To identify the protein partners of *Os*r40g3 involved in salinity tolerance, a cDNA library from salinity stressed rice shoot was used for Y2H assay. In this case, the *Os*EG45-like domain containing protein (LOC4347346), a peroxisomal membrane protein, *Os*PMP22 (LOC4346360) and 60S ribosomal protein L35 (LOC4329423) were identified as interacting partners of *Os*r40g3 (Fig S12). Out of these partners, the *Os*EG45, an expansin-like protein, was found to be up-regulated in response to salinity stress in rice (Fig S12).

The interactions of the *Os*r40g3-HA with *Os*GF14e-cMyc and *Os*EG45-cMyc proteins were further confirmed by co-immunoprecipitation assay (Co-IP). For this purpose, rice calli transiently expressing *Os*r40g3-HA and *Os*GF14e-cMyc/*Os*EG45-cMyc proteins singly or in combinations were used. The protein complexes were co-immunoprecipitated using anti-HA agarose beads followed by western blot analysis using anti-HA and anti-cMyc antibodies. Western blot from total protein was used as the input. Intriguingly, when western blot analysis was performed from total protein against anti-cMyc antibody, significantly lower band intensity was observed in case of calli co-expressing both *Os*r40g3-HA and *Os*GF14e-cMyc proteins (Fig. 12C). On the other hand, no such differential band intensity was observed for the *Os*r40g3-HA and *Os*EG45-cMyc combination (Fig S12C). This prompted us to explore if the binding of *Os*r40g3 triggers *Os*GF14e protein degradation. To investigate this possibility, we treated the transfected rice calli with a proteasomal degradation inhibitor, MG132. When western blot analysis was performed using anti-cMyc antibody, lower band intensity was observed in case of calli co-transfected with *Os*r40g3 and OsGF14e in absence of MG132 treatment. On the other hand, no reduction in band intensity was observed when the co-transfected calli were treated with MG132 thus demonstrating proteosomal degradation of *Os*GF14e protein in presence of *Os*r40g3 (Fig 12D).

Further, it was observed that *OsGF14e* was strongly expressed in flowers including stamens of the WT plants with comparatively lower transcript abundance in leaves and roots (Fig 12E). *Osr40g3*, on contrary, followed an opposite expression pattern. This tissue-specific expression of *Osr40g3* was perturbed when the gene was constitutively overexpressed under control of the *CaMV35S* promoter. This in turn, might have interfered with the protein stability of *Os*GF14e in flowers thus leading to pollen sterility in the transgenic lines.

_Os_r40g3 could not interact with _Os_GF14e orthologs in _Arabidopsis_ indicating its specificity towards _Os_GF14e

Although *Arabidopsis* has several GF14 orthologs, *Osr40g3* ectopic expression did not interfere with its seed development. Therefore, all the GF14 family members in *Arabidopsis* were analyzed for their interaction with *Os*r40g3 protein through Y2H analysis. Since we could not detect expression of *AtGF14-Pi* in flower, it was not included in this study. When Y2H analysis was performed using the *Os*r40g3 protein as bait and each of the *At*GF14 proteins as prey, no

interaction was evident (Fig.S13). On the other hand, the expansin-like B2 precursor protein (*At*EXLB2), an ortholog of *Os*EG45, was found to interact with the *Os*r40g3 protein. The multiple sequence alignment analysis revealed a highly conserved nature of the GF14 members except at their C-terminal domain (Fig S14). Consistent with this observation, it was reported earlier that the diverse C-terminal domain determines the binding specificity of the GF14 family members which supported their diverse functions (Sehnke et al., 2006).

In addition, the other members of *Os*r40 family lectin proteins like *Os*r40c1, *Os*r40g2, *Os*r40c2 and putative *Os*r40c1 were also considered to identify any interaction with OsGF14e protein. Interestingly, none of these members except *Os*r40g3 showed interaction with *Os*GF14e (Fig. S15). This structural distinctiveness of *Os*GF14e presumably accounted for its binding specificity with *Os*r40g3 while its *Arabidopsis* orthologs failed to do so.

Constitutive overexpression of *Os*r40g3 impaired GA metabolism in rice

We studied the germination efficiency of the few seeds that could be collected from the transgenic rice lines constitutively overexpressing the *Osr40g3* gene (*35S::Osr40g3*) and compared with the WT, VC and the transgenic lines overexpressing *Osr40g3* under control of the native promoter (*Osr40g3pro::Osr40g3*). It was observed that none of the seeds of the transgenic lines constitutively overexpressing the *Osr40g3* gene (*35S::Osr40g3*) germinated while the germination percentage for the other lines was more than 90 % (Fig.13A-B). Since GA had long been known to regulate dwarfism, pollen sterility and seed germination, we were curious whether the abnormalities in the transgenic lines (*35S::Osr40g3*) were mediated by GA pathway. Indeed, exogenous GA_3 treatment resulted in 90-95 % seed germination in the otherwise dormant transgenic (*35S::Osr40g3*) seeds. In addition, the α-amylase activity was found to be significantly low in the transgenic lines constitutively overexpressing *Osr40g3* which was improved in response to exogenous GA_3 treatment (Fig. 13C). It was further observed that the expression of two key enzymes of bioactive GA biosynthetic pathway, *GA3 oxidase 1* (*OsGA3ox1*) and *GA20 oxidase 1* (*OsGA20ox1*), was significantly down-regulated in the transgenic stamens (*35S::Osr40g3*) while the GA catabolism-related gene, *GA2 oxidase 1* (*OsGA2ox1*) was up-regulated (Fig. 13D). This observation strongly suggested that the *Os*r40g3-mediated degradation of *Os*GF14e in the transgenic lines (*35S::Osr40g3*) presumably hindered GA metabolism leading to pollen sterility and seed dormancy.

DISCUSSION

The R40 family of lectin proteins was known to play a vital role in regulating drought and salinity stress tolerance in plants (Moons et al., 1995; Jiang et al., 2012b). Among these proteins, the putative *Os*r40c1 was found to play a fundamental role in imparting drought tolerance in rice and tobacco (Sahid et al., 2020). However, the functional aspects of the other *Os*R40 proteins remain unexplored till date. In the present investigation, it was observed that among all the *Os*R40 family members, *Osr40g3* displayed the highest expression in response to salinity stress (Fig. S2). This prompted us to explore how *Osr40g3* imparts salinity tolerance in plants. To investigate this, eight *indica* rice cultivars were selected and analyzed for their salinity stress response. Excitingly, it was observed that the expression of *Osr40g3* positively correlated with the salinity tolerance potential with its highest expression in the tolerant Nonabokhra cultivar and lowest expression in the sensitive MTU1010 cultivar (Fig. 1, S1). This strongly indicated a salinity-responsive role of *Osr40g3* in rice.

To further validate this clue, *Osr40g3*-overexpressing transgenic rice lines were generated harboring the recombinant *35S::Osr40g3* construct. The resulting transgenic lines exhibited significantly improved salinity tolerance in comparison with the WT and VC plants (Fig. 3, S4). Surprisingly, however, most of the transgenic lines failed to set seeds suggesting a negative role of *Osr40g3* in seed development (Fig. 2). In consistence with this observation, the transgenic rice flowers appeared to be shorter with mostly sterile pollen grains (Fig. 6). On contrary, when the same construct was introduced into *Arabidopsis*, the transgenic lines ectopically expressing *Osr40g3* displayed improved salinity tolerance but no defect in seed development (Fig. 4-5, S6). Unlike rice, the floral morphology of the transgenic *Arabidopsis* lines also exhibited no phenotypic alteration (Fig. 7).

A failure to set seeds can result from a defect in the male part, female part or both in the flower. To check these possibilities, the WT rice plants were crossed with the *Osr40g3* overexpressing transgenic lines. Crossing the emasculated WT plants with the transgenic plants as a male parent failed to produce any seed thus confirming the pollen sterility in the transgenic lines. On contrary, when the emasculated transgenic plants were pollinated with the WT pollen grains, hybrid seeds were obtained indicating no defect in the pistil of the transgenic flowers (Fig. 8).

This observation strongly suggested that *Osr40g3* specifically affected the pollen viability while the pistil remained unaffected.

Since *Osr40g3* gene was overexpressed under control of a constitutive promoter, the next pertinent question was whether the gene possesses a tissue-specific expression pattern. Indeed, the gene displayed highest expression level in the leaves during most of the developmental phases with no expression in the stamen (Fig. 9). Furthermore, the constitutive overexpression of *Osr40g3* in flowers and stamens diminished the expression of one of the important regulators of pollen viability, *OsAGL65* (Benjamin et al. 2009). In line with this observation, the *Osr40g3* gene was next cloned under control of the native promoter (*Osr40g3pro::Osr40g3*) and transgenic rice lines were generated harboring this recombinant construct. Interestingly, in addition to salinity tolerance, all these transgenic lines showed proper seed development thus rescuing the pollen sterile phenotype (Fig. 10-11, S11). These observations indicated that *Osr40g3* followed a precise tissue-specific expression pattern which is crucial for proper pollen viability and seed development in rice.

Identifying the interacting protein partners of *Os*r40g3 was the next pertinent point in solving the dual regulatory role of the protein in rice. Intriguingly, it was identified that *Os*r40g3 interacted with separate sets of protein partners to execute its role in seed development and salinity stress. When a cDNA library from rice flowers was used, the *Os*r40g3 was found to interact with *Os*GF14e (Fig. 12). The *Os*GF14e is a member of the GF14 protein family (14-3-3 proteins) and modulates several stress responses as well as plant development (Chen et al., 2006). For example, the rice GF14 family proteins like *Os*GF14b, *Os*GF14c, *Os*GF14e and *Os*GF14f were found to interact with different target proteins to critically regulate the signaling responses during biotic stress (Chen et al., 2006). Furthermore, *Os*GF14e was demonstrated to negatively regulate sheath blight and bacterial blight resistance in rice (Manosalva et al., 2011). Also, the suppression of *Os*GF14e could produce susceptibility towards panicle blast (Manosalva et al., 2011; Liu et al., 2015, 2016). In addition, *Os*GF14e interacted with DOF ZFP transcription factors that were activated by developmental signals in plants (Cooper et al., 2003).

Interestingly, the silencing of *OsGF14e* gene displayed stunted growth and poor seed development in rice (Manosalva et al., 2011). A subsequent study by Liu et al. (2016) precisely demonstrated that all the *OsGF14e*-silenced lines failed to develop seeds except two lines with

less *OsGF14e* silencing. Similarly, in the present study, *Os*r40g3 was found to negatively regulate the stability of *Os*GF14e protein which presumably led to impaired seed development in the transgenic rice constitutively overexpressing *Osr40g3* (Fig. 12). Moreover, earlier evidences suggested that low accumulation of GF14e protein was associated with sterile pollen grains in maize (Datta et al., 2002). Accumulating evidences also suggested that rice GF14 family members interacted with site specific DNA binding proteins like *Em*BP1 and tissue specific transcription factor, VP1 to regulate signaling responses in a spatial manner (Schultz et al., 1998). This supported the highly precise and tissue-specific function of the GF14 proteins in plants. Consistent with these reports, out of the eight GF14 family members, the *Os*GF14e and *Os*GF14f showed higher accumulation in flowers and panicles which corroborated with our present observation (Chen et al., 2006).

Furthermore, the few seeds that developed in the transgenic rice lines constitutively overexpressing the *Osr40g3* gene showed complete inhibition of germination. Since, dwarfism, pollen sterility, and seed germination, are widely known to be regulated by GA, we assumed that these transgenic lines might have a defect in GA metabolism. Indeed, exogenous application of GA_3 could rescue the germination inhibition in the transgenic seeds supporting this hypothesis. In addition, the α-amylase activity was also found to be impaired in the transgenic seeds that were relieved by GA_3 treatment. The down-regulation of the *OsGA3ox1* and *OsGA20ox1* genes and up-regulation of the *OsGA2ox1* genes in the transgenic stamens further supported perturbation of the GA biosynthesis (Fig. 13). These observations corroborated with the previous report where the *OsGF14e*-silenced rice plants also exhibited a defect in GA metabolism (Liu et al., 2016). However, how *Os*GF14e integrates the GA pathway with pollen development is an exciting area that needs to be explored in the future.

Although *Os*GF14e has several orthologs in *Arabidopsis*, the transgenic plants ectopically expressing *Osr40g3* displayed normal seed development. To understand this unusual observation, we checked if these orthologs in *Arabidopsis* could interact with the *Os*r40g3 protein through Y2H analysis. However, none of them could interact with *Os*r40g3 suggesting a high specificity of the *Os*r40g3-*Os*GF14e interaction (Fig. S13). It was reported that the *Arabidopsis* GF14 family can be broadly classified into two groups, the epsilon group and the non-epsilon group (Daugherty et al., 1996; Sehnke et al., 2006). The non-epsilon group included

the omega, psi, mu, nu and chi proteins which showed close homology with *Os*GF14e. Among them, the *At*GF14-chi was reported to be highly expressed in the *Arabidopsis* flowers (Daugherty et al., 1996). Intriguingly, the varied C-terminal domain of the GF14 family members was reported to determine their functional variation and specificity in plants (Sehnke et al., 2006). This structural distinctiveness of *Os*GF14e from its *Arabidopsis* orthologs provided a possible answer why *Os*r40g3 ectopic expression failed to impede seed development in *Arabidopsis*. Interestingly, other members of *Os*r40 family also did not interact with *Os*GF14e suggesting the specific interaction of *Os*r40g3 with *Os*GF14e.

On the other hand, *Os*r40g3 was identified to interact with *Os*EG45-like domain containing protein in the salinity stressed rice leaves (Fig. S12). EG45 is a salinity stress-responsive expansin-like glycoprotein that showed endoglucanase-like function and helped in cell wall modification (Ludidi et al., 2002). Cell wall modification is a common phenomenon observed in the osmotically stressed cells (Gall et al., 2015). Several expansin genes were reported to be up-regulated under osmotic stress. For example, the expression of the rose expansin gene, *RhEXPA4*, was found to be induced in response to salinity stress and its ectopic expression in *Arabidopsis* led to salt-tolerant phenotype (Lü et al., 2013). Similarly, transgenic tobacco expressing the wheat expansin gene *TaEXPB23* displayed improved salinity tolerance (Han et al., 2012). In addition, up-regulation of expansin gene expression was also reported under water deficit conditions in many plants (Wu et al., 2001; Guo et al., 2011; Li et al., 2013). In the present investigation, overexpression of *Osr40g3* gene was found to significantly improve the salinity stress tolerance in rice by regulating the *Os*EG45-like domain containing protein. Since the *Os*r40g3 protein could also interact with *At*EXLB2, the *Arabidopsis* ortholog of *Os*EG45-like domain containing protein, these transgenic *Arabidopsis* lines displayed salinity stress tolerance.

The present investigation unraveled a dual regulatory function of *Os*r40g3 in imparting salinity tolerance while impeding the pollen viability in rice (Fig 14). This perturbation in pollen viability and improper seed development was mediated by its interaction with *Os*GF14e which led to a diminished *Os*GF14e protein stability and a subsequent defect in the GA metabolism. On the other hand, *Os*r40g3 imparted salinity tolerance via its interaction with the expansin protein, *Os*EG45. This dual role was largely mediated by a precise tissue-specific expression pattern of the *Os*r40g3 protein in rice.

MATERIALS AND METHODS

Plant material and stress treatment

The seeds of different *indica* rice cultivars like Nonabokra, Ranjit, Khitish, IR64, Swarna sub1, Jaldi13, MTU1010, and Vandana were procured from the Chinsurah Rice Research Station, West Bengal, India. Plants were grown in a greenhouse under the optimum condition at day/night temperature regime of 30°-35°C/22°-29°C and 60-65% relative humidity with 12 h photoperiod. For salinity stress, seeds were surface sterilized using 4 % sodium hypochlorite followed by 0.2 % bavistin treatment, germinated and grown on filter papers wetted with water for 15 d. Hundred seedlings of each cultivar were treated with 200 mM NaCl solution for 5 d as standardized before (Kurniasih et al., 2013; Sahid et al., 2020). For salt stress treatment of soil-grown plants, the pots were irrigated with 200 mL of 200 mM NaCl solution per day, and data was recorded after 5 d of salt treatment (Amirjani, 2012). For the control set, the same volume of distilled water was used.

A. thaliana seeds of Columbia ecotype (Col-0) were procured from Nottingham Arabidopsis Stock Center (NASC), UK and were grown and maintained in a growth chamber at 22°C under 16 h light/8 h dark cycle as standardized before (Datta et al., 2015). 30 d old plants were exposed to salinity stress by irrigating with 20 mL of 200 mM NaCl solution every alternate day as standardized before (Datta et al., 2015). For the control set, the same volume of distilled water was used.

Morphological analyses

Different morphological parameters like shoot and root length, seedling dry weight, and percentage of rolled leaves were analyzed under control and stressed conditions. Tissue was dried at 50°C for 3 d to measure the dry weight. The relative water content (RWC) was estimated after salinity stress exposure following Ma et al. (2018).

Estimation of different biochemical compounds

Proline and glycine betaine contents were measured by spectrophotometric method from tissue samples collected from control and treated plants according to Grieve and Grattan (1983) and

Woodrow *et al*. (2016) respectively. The chlorophyll content was measured according to Lichtenthaler (1987). The H_2O_2 content was measured following Yin et al. (2010).

RNA Extraction and qRT PCR Analysis

Total RNA was extracted using the Trizol method from tissues of rice and *Arabidopsis* plants. Complementary DNA (cDNA) was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacture's protocol. The qRT PCR analysis was performed in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-rad) and gene-specific primers (Table S1). The gene expression was normalized based on the relative quantification method. The *Actin* gene of rice and *Arabidopsis* was used as a reference gene. Three technical replicates and three biological replicates were considered for each analysis.

Vector Construction and Plant Transformation

For development of overexpression lines, the full-length gene of *Osr40g3* (LOC112936024) was amplified by PCR using gene specific primers (Table S1). The amplified product was inserted into pGEM®-T Easy Vector Systems (Promega) and subsequently sub-cloned between the *EcoRI* and *BamHI* restriction enzyme (RE) sites of *pEGAD* vector under the control of a constitutive *CaMV35S* promoter. The recombinant construct (*35S::Osr40g3*) was transformed into the background of *indica* rice cultivar khitish (IET4095) following *Agrobacterium*-mediated genetic transformation method (Datta et al., 2000; Sahid et al., 2020). The putative transformants were screened for herbicide resistance, and positive lines were confirmed by genomic DNA PCR using *bar* gene-specific primers. Positive lines, along with WT and VC plants, were grown in a greenhouse with optimum condition as described before. The same construct was used to transform *Arabidopsis* plants of Col-0 ecotype via *Agrobacterium*-mediated floral-dip method as standardized before (Clough and Bent, 1998; Datta et al., 2015). The independent transgenic lines generated were maintained and grown up to T_2 generations in a growth chamber as described above.

To clone the promoter region of *Osr40g3* gene, the 1,315 bp upstream sequences from +1 site of *OsR40g3* gene was selected. The fragment was amplified from rice genomic DNA by PCR using sequence specific primers. The amplified native promoter was inserted between the *AgeI* and

PacI RE sites of the recombinant vector *35S*::Os*r40g3* to generate the construct, *OsR40g3pro*::Os*r40g3*. The promoter fragment was also sub-cloned between the *BamHI* and *BglII* RE sites of *pCAMBIA1304* vector to generate the *OsR40g3pro*::*GUS* construct. The constructs were used for *Agrobacterium*-mediated genetic transformation of rice as described above. The independent transgenic lines generated were maintained and grown up to T_2 generations under greenhouse conditions as described above.

Morphological Analysis of Transgenic Plants

Several morphological parameters like plant height, number of tillers, shoot and root dry weights, panicle length, number of panicles/plant, number of flowers/panicle, number of grains/panicle, and floral morphology were analyzed from the WT, VC, and transgenic (*ox*) rice plants. In case of *Arabidopsis*, plant height, rossette diameter, floral morphology, silique length, number of silique/plant, number seeds/silique, and seed yield/plant were measured from WT, VC and transgenic (*ec*) lines. Floral morphology of both rice and *Arabidopsis* plants were studied and documented under zoom stereo trinocular microscope (Magnus MSZ-RR LED). For salt stress assay, different morphological parameters were analyzed under control and stressed conditions as described above.

Estimation of sodium (Na^+) content

The Na^+ content was measured by ICP-OES (iCAP 6300 Duo ICP-OES, Thermo Scientific) following Jiang et al (2007). Briefly, samples collected from control and stressed plants, weighed, and incubated in Hot Air Oven at 55°C for 3 d. Ash was prepared from the dried samples and digested with 0.5 M HNO_3. The solution was filtered, volume adjusted to 25 mL with 0.5 M HNO_3, and analyzed.

Pollen viability assay

For I_2-KI staining, mature pollen grains of rice were stained with 2% I_2-KI (w/v) solution and observed under bright field microscope (Leica DM IL LED S-80) according to Chuun et al. (2007). For FDA staining, a pollen viability solution (PVS) was prepared with 0.001 % (w/v) fluorescein diacetate, 290 mM sucrose, 1 mM KNO_3, 0.16 mM boric acid, and 1.27 mM $Ca(NO_3)_2$. The mature pollen grains of rice and *Arabidopsis* were incubated in PVS at dark for 15 min at 28°C and centrifuged. The pellet was resuspended in PVS without FDA and observed

under fluorescence microscope (Leica DM IL LED S-80) using 488 nm excitation and 510 nm emission wavelengths (Muhlemann et al. 2018). For *Arabidopsis* pollen grains, Alexander staining was performed according to Schofta et al. (2011) and examined under bright field microscope (Leica DM IL LED S-80). At least 500 pollen grains were considered for each sample.

In vitro pollen germination assay

The *in vitro* pollen germination assay was performed according to Li et al. (2018). Briefly, the mature pollen grains collected from rice flowers were spread onto pollen germination medium, pH 6.0, containing of 15 % (w/v) sucrose, 0.01 % (w/v) boric acid, 1 mM $Ca(NO_3)_2$, and 0.3 % (w/v) low melting agarose and were incubated at 25°C under humid condition. After 4 h of incubation, pollen germination was analyzed and documented under bright field microscope (Leica DM IL LED S-80).

For *in vitro* pollen germination of *Arabidopsis* a solid pollen germination medium was used. The mature pollen grains were collected and spread on the pollen germination medium containing 0.01 % H_3BO_4 (w/v), 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgSO_4$, 10 % sucrose (w/v), and 0.1 % agarose (Boavida and McCormick, 2007). The pollen germination was examined under bright field microscope (Leica DM IL LED S-80) after 6 h incubation.

Hybridization of rice plants

The flowers of WT plants were emasculated and considered as female parent whereas the transgenic rice plants (*ox*) harboring the *35S::Osr40g3* construct were considered as male parent. In another set, the transgenic rice plants (*ox*) harboring the *35S::Osr40g3* construct were emasculated and considered as female parent and hybridized with WT as male parent. Hybridization was carried out as standardized before (Paul et al. 2014). In both cases the number of hybrid seeds was measured.

Histochemical GUS assay

Samples from different tissues under various developmental stages were collected from the transgenic rice lines harboring the *Osr40g3pro::GUS* construct. Samples were stained with GUS staining solution (50mM sodium phosphate,pH7.0, 10mM EDTA, 0.5mM $K_3[Fe(CN)_6]$, 0.5mM

$K_4[Fe(CN)_6]$, 0.1% (v/v) Triton X-100, and 1mM X-glucuronide) following Jefferson et al.1987 The stained samples were visualized under zoom stereo trinocular microscope (Magnus MSZ-RR LED).

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed to identify the protein partners of *OsR40g3* protein using the Matchmaker® Gold Yeast Two-Hybrid System (Takara) following the manufacturer's protocol. The *OsR40g3* gene was inserted into the *EcoRI* and *BamHI* RE sites of the bait vector, *pGBKT7* (Takara). The recombinant *BD-Osr40g3* bait construct was transformed into yeast following manufacturer's protocol. The other members of *Os*r40 family like *Os*r40c1, *Os*r40g2, *Os*r40c2 and putative *Os*r40c1 were also cloned into the bait vector pGBKT7 and used as recombinant bait constructs. The cDNA library was prepared from rice flowers and ligated to *pGADT7-Rec* vector using Make Your Own "Mate and Plate" Library System (Takara). To identify the interacting protein partners responsive for salinity stress tolerance, a second cDNA library was prepared from rice leaves under salinity stress condition. The recombinant plasmids were co-transformed into yeast Y2H Gold strain (Takara). The transformed yeast strains were grown on DDO (SD/-Leu/-Trp) and QDO/X/A (SD/–Ade/–His/–Leu/–Trp/X-α-Gal/Aureobasidin-A) media. Positive clones were selected and analyzed by sequencing following the manufacturer's instructions to identify the interacting protein partners. To analyze the interaction of *Os*r40g3 with the orthologs of *Os*EG45 and *Os*GF14e in *Arabidopsis*, the *At*EXLB2 and 12 *At*GF14 proteins were cloned into the *pGADT7-Rec* vector and used as prey for the *BD-Osr40g3* bait. Y2H analysis was performed as describes above. The *BD-p53* and *AD-T-antigen* interaction was used as a positive control. The empty *BD* and *AD-T-antigen* interaction was used as a negative control.

Bimolecular Fluorescence Complementation (BiFC) assay

For BiFC assay, the *OsR40g3* gene without stop codon was inserted into the vector *pVYCE* between *ApaI* and *BamHI* RE sites to obtain *35S::Osr40g3-cVenus* construct. The *OsGF14e* gene was cloned into *SpeI* and *BamHI* RE sites of *pVYNE* to generate *35S::OsGF14e-nVenus* construct (Waadt et al. 2008). The recombinant constructs were transformed into *Agrobacterium tumifaciens* strain GV3101. Then, the *Agrobacterium* strain was used to infiltrate onion epidermal cells (Yang et al., 2014). The fluorescence signal for Venus protein was detected at the

excitation wavelength of 514 nm using a confocal laser scanning microscope (Olympus FluoView™ FV1000). The *35S::Osr40g3-cVenus* construct with empty *35S::nVenus* vector was considered as negative control.

Co-immuno precipitation (Co-IP) and immunoblot analysis

The rice embryo calli transformed with *35S::Osr40g3-HA* and/or *35S::OsGF14e-cMyc* were used for total protein isolation (Chen et al. 2019). The samples were extracted in lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 % Triton-X-100 (v/v) and protease inhibitor, Thermo Scientific) at 4°C and centrifuged at 13000 rpm for 30 min at 4°C. Supernatants were collected and mixed with 20 µL anti-HA-agarose beads and incubated overnight following the manufacturer's instructions (Pierce™ HA-Tag IP/Co-IP Kit, Thermo Fisher Scientific). The HA-tagged protein was eluted and quantified by Bradford method (Bradford, 1976). Equal amount of protein was used for immunoblot analysis using anti-HA antibody (1:1000 dilution, Sigma) and anti-cMYC antibody (1:1000 dilution, Agrisera). Immunoblot against anti-α-Tubulin antibody (1:1000 dilution, Agrisera) was used as loading control. For proteasomal degradation inhibitor assay, the transformed rice calli were incubated in dark for 10 d at 37°C. The calli were then incubated with 10 µM MG132. After 4 h of incubation, proteins were isolated from the calli and used for immune blot analysis as described above (Garcia-Cano et al. 2014).

Multiple sequence alignment analysis

The protein sequences of *Arabidopsis* GF14 family members and *Os*GF14e were retrieved from NCBI database. The sequences were subjected for multiple sequence alignment using PRALINE multiple sequence alignment tool (Simossis and Heringa, 2005).

Seed germination and α-amylase assay

The seeds collected from WT, VC, *ox* lines (*35s::Osr40g3* and *OsR40g3pro*::O*sr40g3*) were surface sterilized with 4 % sodium hypochlorite solution followed by 0.2 % bavistin treatment and placed on MS medium with and without GA_3 (5uM/ml) for germination under dark for 7 d. Estimation of α-amylase activity was carried out following Shen et al. (2019).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.3.0 software (GraphPad Software, San Diego, California USA). The variation of morphological, and biochemical parameters as well as the relative transcript abundance among genotypes and treatments were analyzed following two-way ANOVA followed by Sidak's multiple comparison tests. To compare the morphological parameters between WT, VC and *ox*/*ec* lines, one-way ANOVA followed by Dunnett's multiple comparison tests were performed. To identify the difference between two sets of data, the statistical significance at $P \leq 0.05$ was considered. Details of replicates, sample size, and the significance levels of P-values were indicated in respective figure legends. The data were represented as mean ± standard error of mean (SEM).

Supplementary data

Table S1: Primers used in this study

Table S2: Concentration of sodium, potassium and chloride ions in soil before and after salt treatment.

Fig S1: Morphological and biochemical analyses of the 8 *indica* rice cultivars in response to salinity stress.

Fig S2: Relative transcript abundance of 5 different *Os*R40 family genes from rice shoots in response to salinity stress condition.

Fig S3: Genomic DNA PCR of *Osr40g3* overexpressing transgenic rice plants (*35S::Osr40g3*) using *bar* gene-specific primers.

Fig S4: Morphological and biochemical analyses of the *Osr40g3* overexpressing transgenic rice lines, VC and WT in response to salinity stress.

Fig S5: Genomic DNA PCR of transgenic *Arabidopsis* ectopically expressing *Osr40g3* gene using *bar* gene-specific primers.

Fig S6: Morphological and biochemical analyses of the transgenic *Arabidopsis* ectopically expressing *Osr40g3* gene, VC and WT in response to salinity stress.

Fig S7: Pollen viability percentage of WT, VC and transgenic rice lines harboring (A) *35S::Osr40g3* and (B) *Osr40g3pro::Osr40g3* constructs.

Fig S8: Relative transcript abundance of *Osr40g3* and *OsAGL65* genes in the WT and transgenic rice lines harboring the *35S::Osr40g3* construct from leaf, flower and stamen tissues.

Fig S9: Genomic DNA PCR of *Osr40g3* overexpressing transgenic rice plants (*Osr40g3pro::Osr40g3*) using *bar* gene-specific primers.

Fig S10: Relative transcript abundance of *Osr40g3* and *OsAGL65* genes in transgenic rice line (*ox1*) harboring the *Osr40g3pro::Osr40g3*construct from leaf, flower and stamen tissues.

Fig S11: Morphological and biochemical analyses of the transgenic rice lines harboring the *Osr40g3pro::Osr40g3*construct, VC and WT in response to salinity stress.

Fig S12: (A) Yeast two-hybrid assay for *Os*r40g3 interaction against cDNA library from rice leaves under salinity stress. (B) Relative transcript abundance of the partners in response to salt stress. (C) Co-immunoprecipitation analysis for interaction of *Os*r40g3 with *Os*EG45 like protein.

Fig S13: Yeast two-hybrid assay for the interaction of *Os*r40g3 with the *Arabidopsis* orthologs of *Os*GF14e and *Os*EG45 proteins.

Fig S14: Multiple sequence alignment of *Os*GF14e protein with *Arabidopsis* GF14 family members showing structural variability at the C-terminal end.

Fig S15: Yeast two-hybrid assay for the interaction of *Os*GF14e with all the *Os*r40 family proteins.

AUTHOR CONTRIBUTIONS

RD and SP conceived and designed the original research plan; CR and SS performed the experiments; RD and SP analyzed the data; RD and SP wrote the manuscript with contributions from CR and SS.

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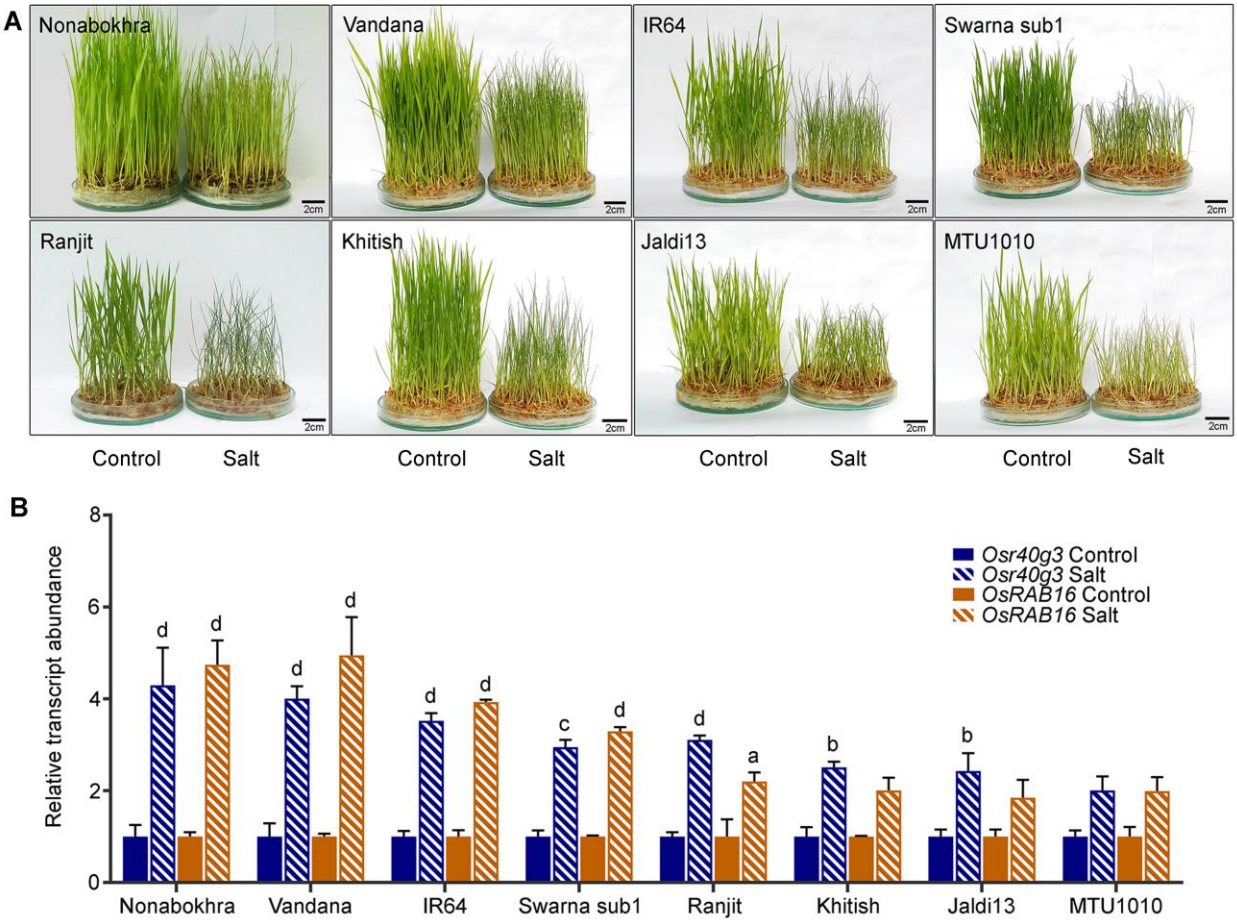

Fig 1. **Salinity stress assay of 8 *indica* rice cultivars**. 15 d old seedlings were treated with 200 mM NaCl solution for 5 d and morphological changes were analyzed. (A) Morphology of 8 *indica* rice cultivars in response to salinity stress. (B) After salt treatment, samples were used for qRT-PCR analysis to study the relative transcript abundance for *OsRAB16* and *Osr40g3* genes. The experiment was independently repeated thrice and results were represented as mean ± SEM. Statistical difference between samples under control and salinity stress was denoted by different letters at $p<0.05$ (a), $p<0.01$ (b), $p<0.001$ (c) and $p<0.0001$ (d).

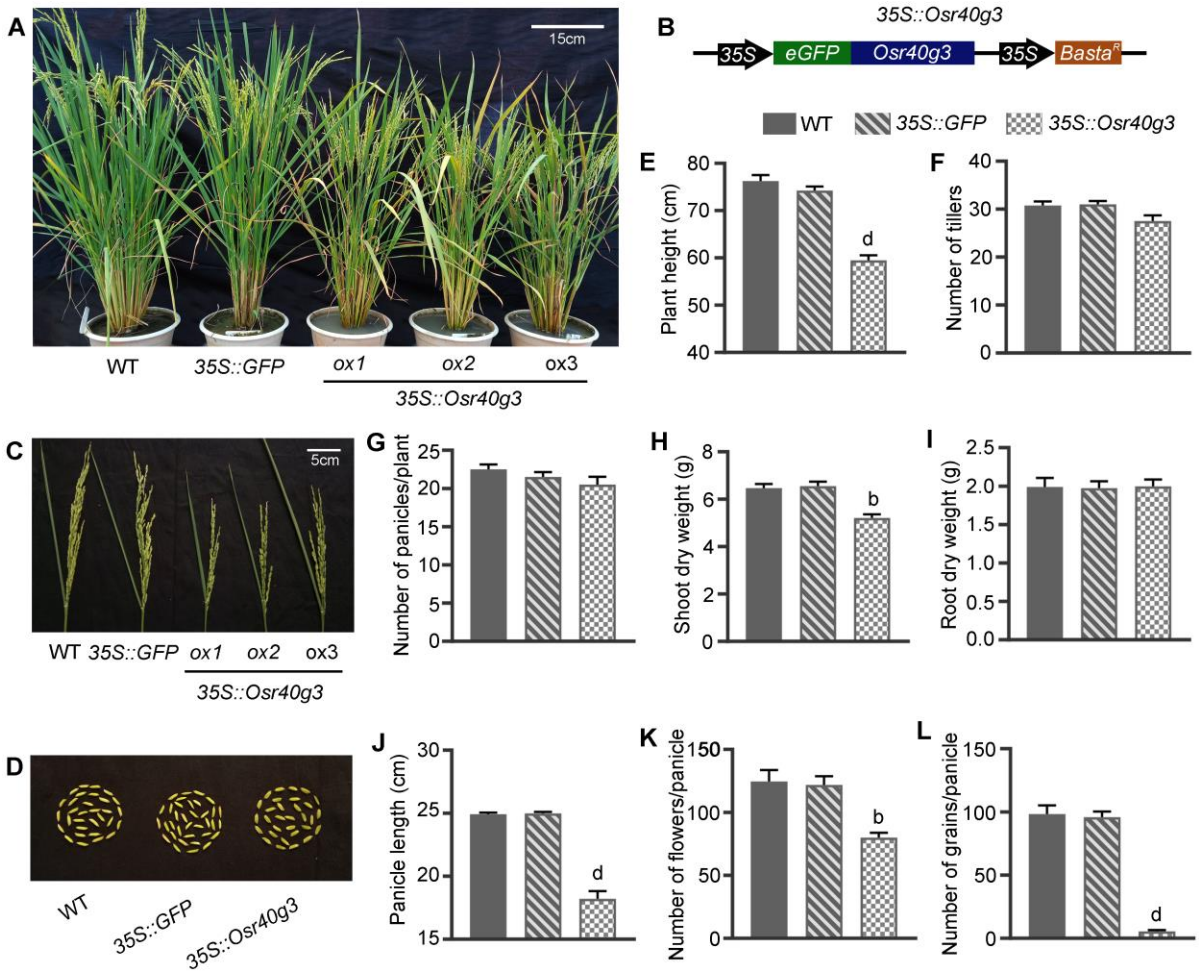

Fig 2. Morphological characterization of *Osr40g3* overexpressing transgenic rice lines. Different morphological parameters of 3 representative independent transgenic lines harboring the *35S::Osr40g3* construct (*ox1*, *ox2* and *ox3*) along with WT and VC (*35S::GFP*) plants. The transgenic lines exhibited shorter plant height with impaired seed development as compared to the WT and VC plants. (A) WT, VC and transgenic lines at reproductive stage, (B) overexpression construct, (C) panicles, (D) grain morphology (empty grains in transgenic lines), (E) plant height, (F) number of tillers, (G) numbers of panicles/plant, (H) shoot dry weight, (I) root dry weight, (J) panicle length, (K) numbers of flowers/panicle, and (L) number of grains/panicle. Ten independent transgenic lines were considered for morphological analysis. For panicle length, numbers of flowers/panicle, and number of grains/panicle, 3 panicles from each line were considered. Statistical difference between WT, VC and transgenic lines was denoted by different letters at $p<0.01$ (b) and $p<0.0001$ (d).

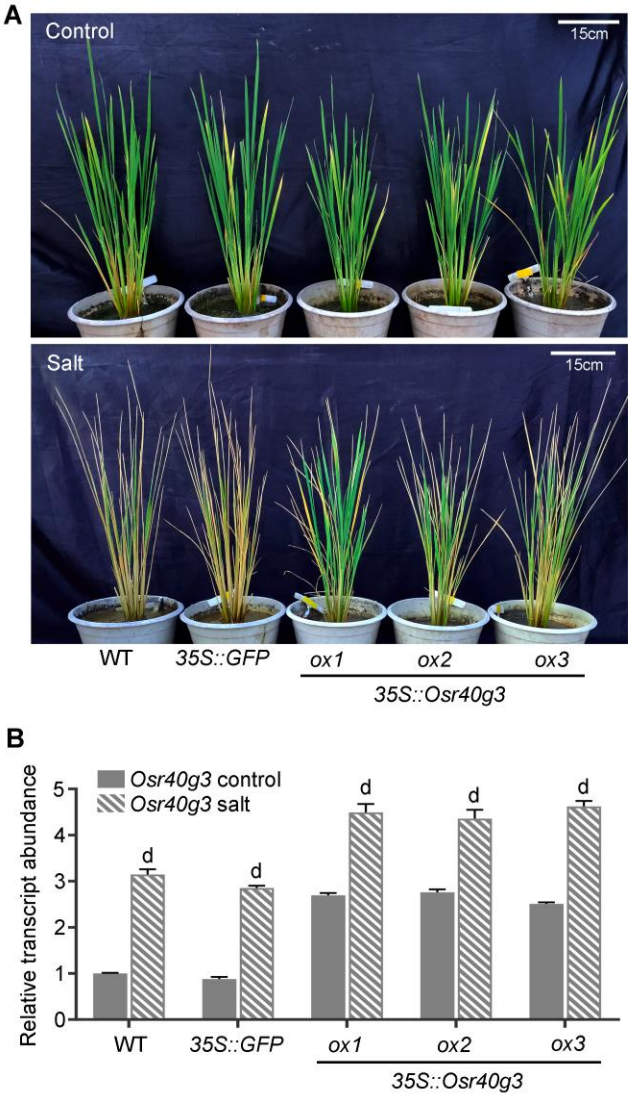

Fig 3. **Response of transgenic rice plants under salinity stress condition.** The WT, VC (*35S::GFP*) and 3 independent transgenic lines harboring the *35S::Osr40g3* construct (*ox1, ox2* and *ox3*) were exposed under salinity stress for 5 d and analyzed. The transgenic lines indicated improved salinity stress tolerance over WT and VC lines. (A) Morphological response under salinity stress, (B) relative transcript abundance of *Osr40g3* and *OsRAB16* genes in response to salinity. The experiment was independently repeated thrice and results were represented as mean ± SEM. Statistical difference of the samples under control and salinity stress was denoted by different letters at $p<0.05$ (a), $p<0.01$ (b) and $p<0.0001$ (d).

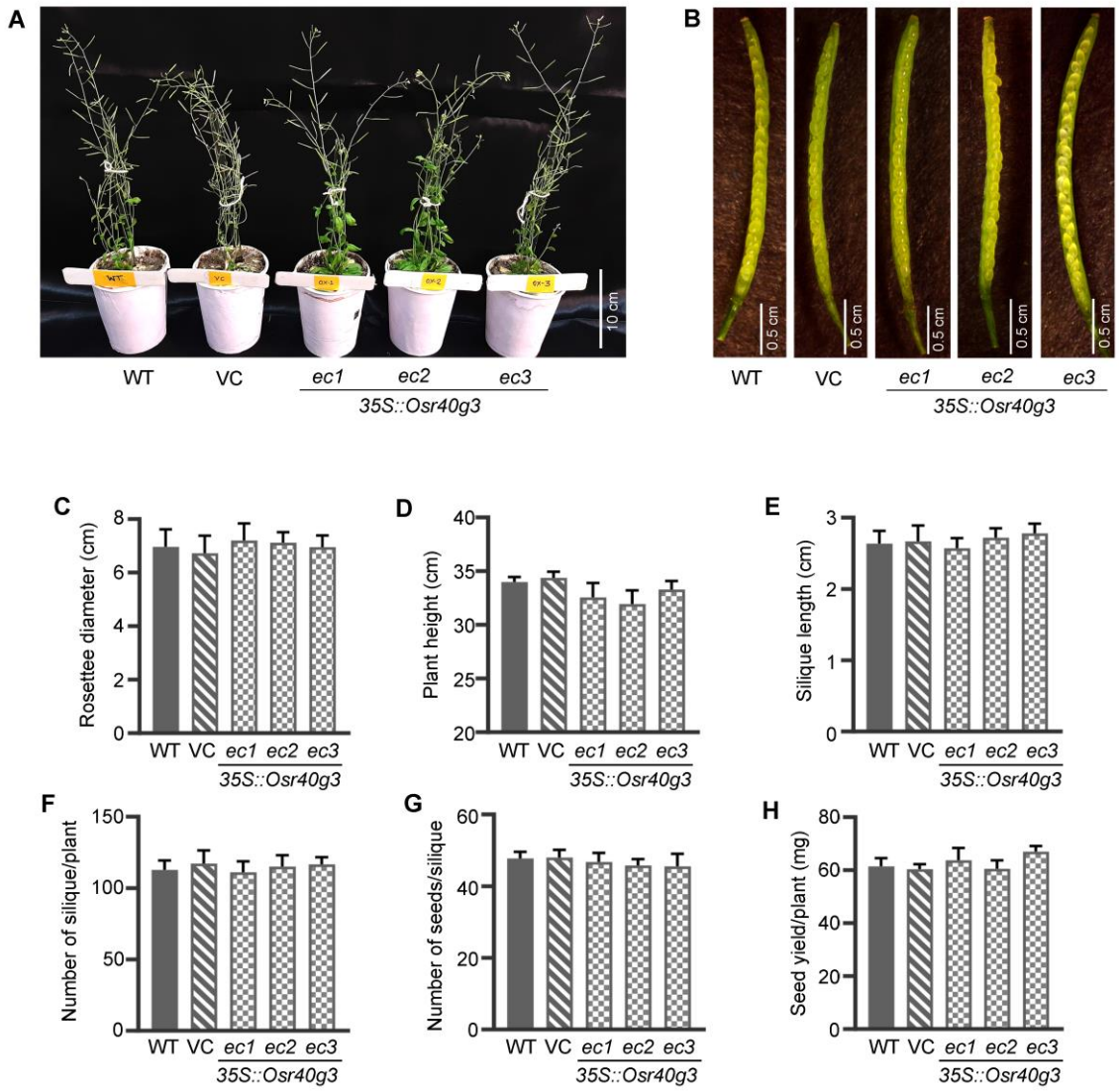

Fig 4. **Morphological characterization of transgenic *Arabidopsis* lines ectopically expressing *Osr40g3***. Different morphological parameters of 3 independent transgenic lines harboring the *35S::Osr40g3* construct (*ec1*, *ec2* and *ec3*) were recorded and compared with WT and VC (*35S::GFP*) plants. The transgenic lines exhibited no phenotypic abnormalities. (A) WT, VC and transgenic lines at reproductive stage, (B) siliques, (C) rosette diameter, (D) plant height, (E) silique length, (F) number of siliques/plant, (G) number of seeds/silique, and (H) seed yield. Five biological replicates were considered for each line. Statistical difference between WT, VC and transgenic lines was denoted by 'b' at $p<0.01$.

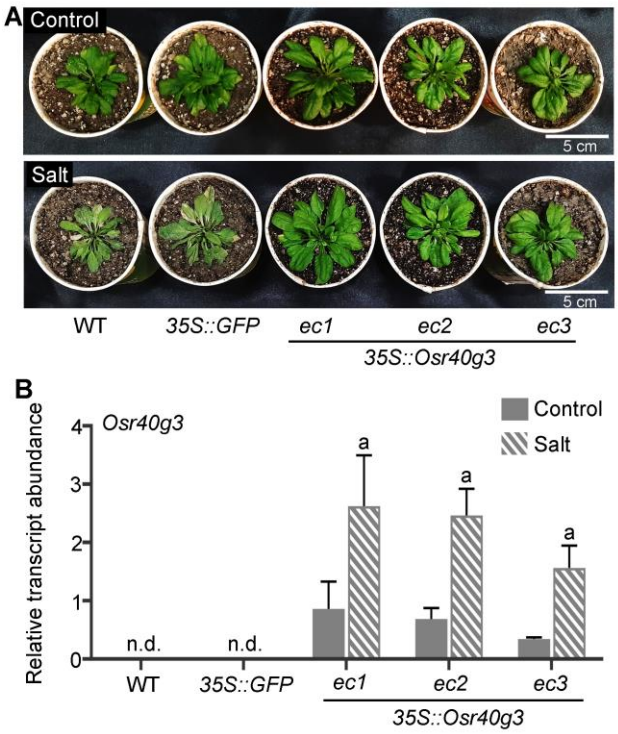

Fig 5. Response of transgenic *Arabidopsis* lines ectopically expressing *Osr40g3* gene under salinity stress condition. The WT, VC (*35S::GFP*) and 3 independent transgenic lines harboring the *35S::Osr40g3* construct (*ec1*, *ec2* and *ec3*) were exposed under salinity stress for 5 d and analyzed. The transgenic lines exhibited improved salinity stress tolerance over WT and VC lines. (A) Morphological response under salinity stress, (B) relative transcript abundance of *Osr40g3* gene in response to salinity. The experiment was independently repeated thrice and results were represented as mean ± SEM. Statistical difference of the samples under control and salinity stress was denoted by 'a' at $p<0.05$.

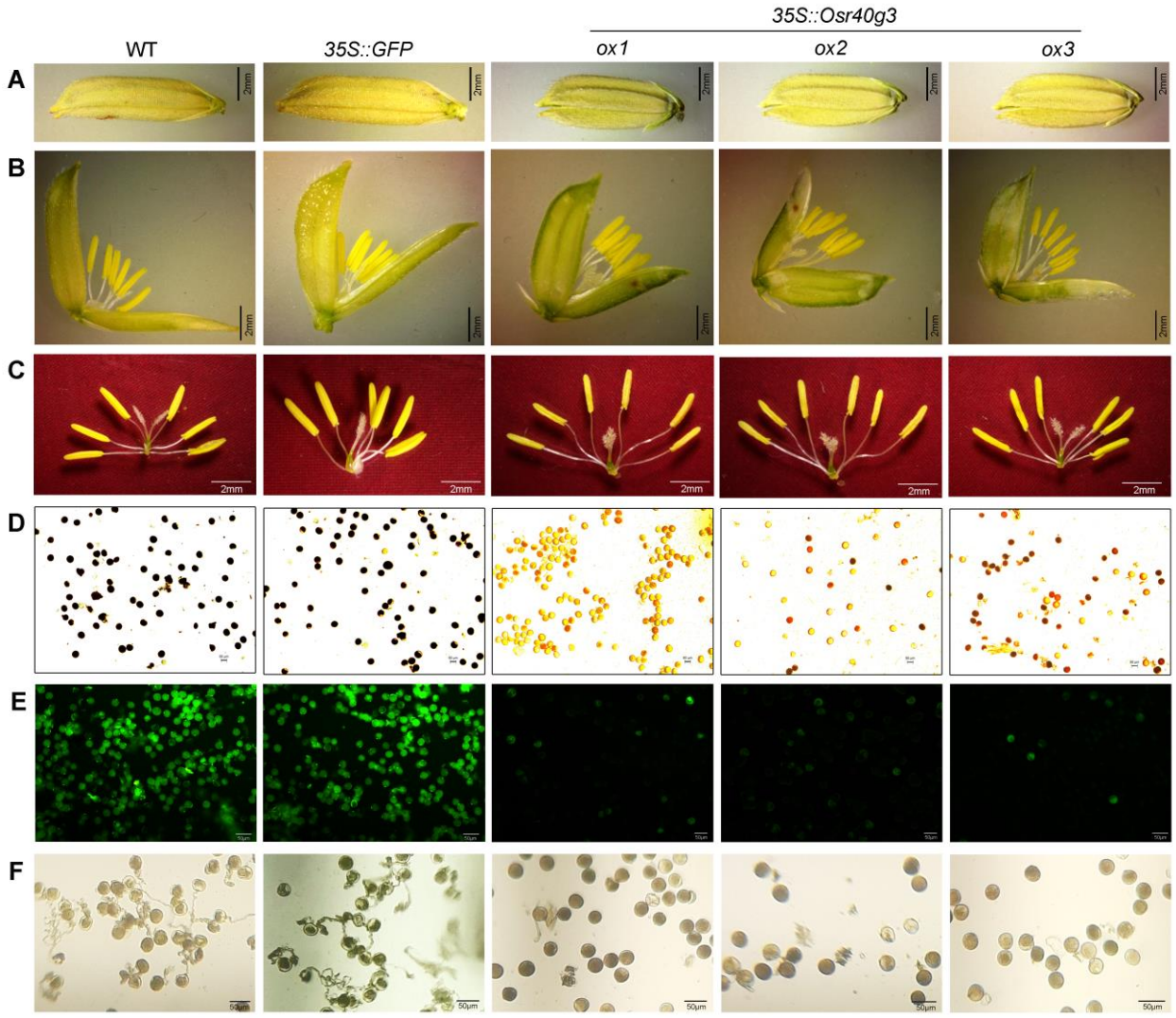

Fig 6. **Floral morphology of *Osr40g3* overexpressing transgenic rice lines.** The floral morphology and pollen viability of 3 representative independent transgenic lines harboring the *35S::Osr40g3* construct (*ox1*, *ox2* and *ox3*) along with the WT and VC (*35S::GFP*) plants. The transgenic flowers appeared to be shorter than the WT and VC flowers with markedly reduced pollen viability and germination. (A) Entire flower, (B) dissected flower, (C) stamens with pistil, (D) pollen viability by I_2-KI staining method, (E) pollen viability by FDA staining method, and (F) *in vitro* pollen germination assay. For floral morphology, 3 flowers from each of 10 independent transgenic lines were considered. For pollen viability and *in vitro* germination assay, at least 500 pollen grains from each line were studied.

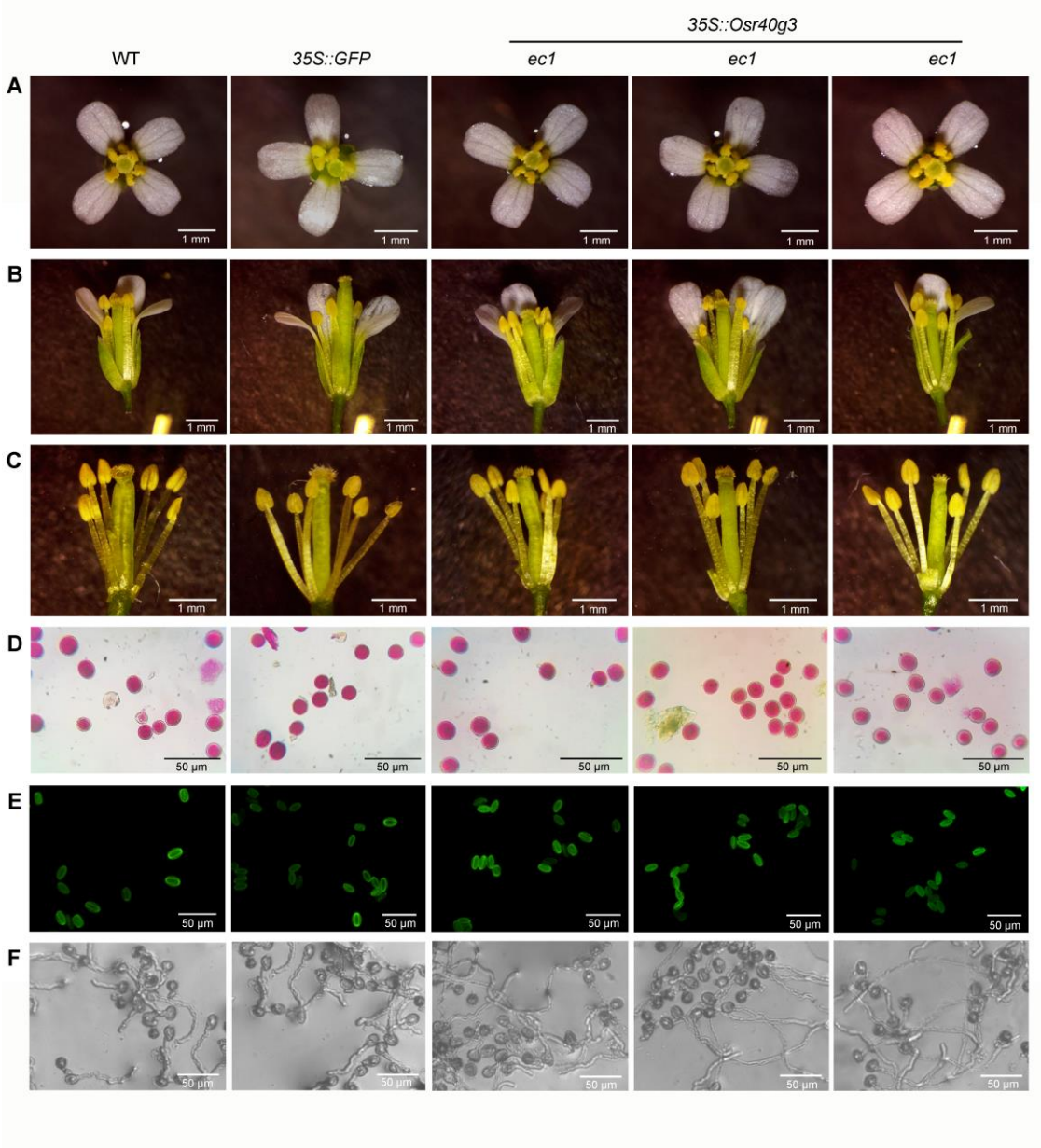

Fig 7. **Floral morphology of transgenic *Arabidopsis* lines ectopically expressing *Osr40g3*.** The floral morphology of 3 independent transgenic lines harboring the *35S::Osr40g3* construct (*ec1*, *ec2* and *ec3*) were recorded and compared with WT and VC (*35S::GFP*) plants. No morphological alteration was observed between the transgenic, VC and WT flowers. (A) Entire flower, (B) dissected flower, (C) stamens with pistil, (D) pollen viability assay by alexander staining method, (E) pollen viability assay by FDA staining method, and (F) *in vitro* pollen germination assay. Five biological replicates were considered for each line. For pollen viability and *in vitro* germination assay, at least 500 pollen grains from each line were studied.

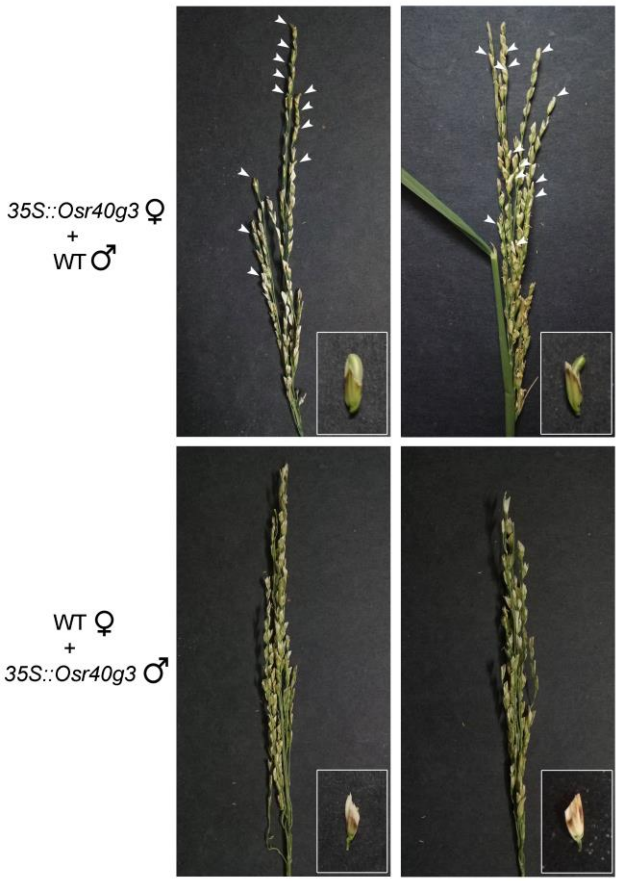

Fig 8. **Crossing of WT plants with *Osr40g3* overexpressing transgenic rice harboring *35S::Osr40g3* construct**. (A) Crossing of emasculated transgenic plants with WT pollen grains produced hybrid seeds while (B) crossing the emasculated WT plants with the pollens from transgenic plants failed to produce seeds. The experiment was independently repeated thrice.

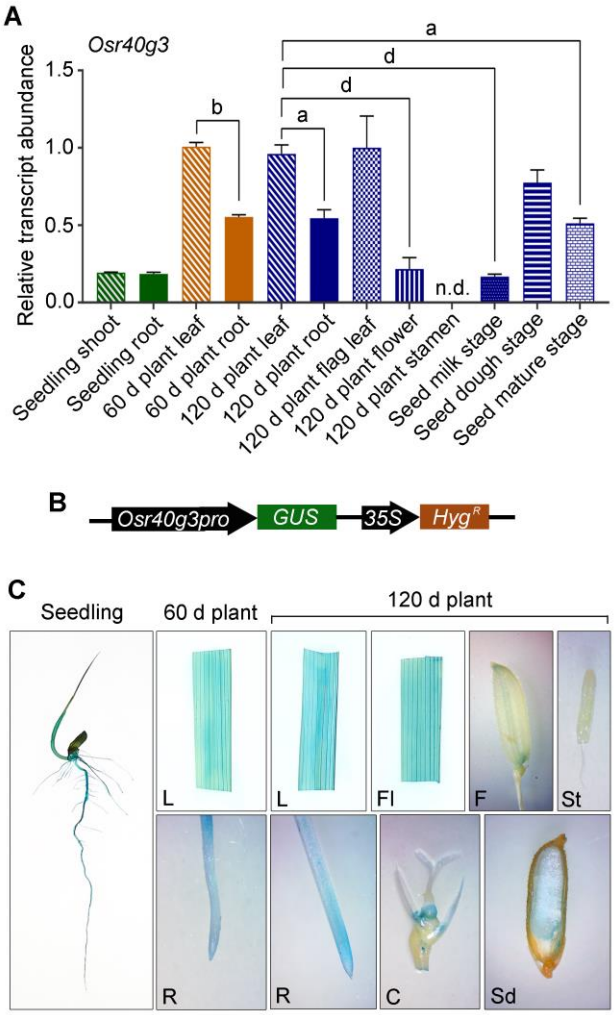

Fig 9. **Tissue-specific expression pattern of *Osr40g3* gene in rice.** (A) Relative transcript abundance of *Osr40g3* gene in different tissues of WT rice plants. Highest transcript abundance was observed in the leaves while no expression was detected in the stamen. The experiment was independently repeated thrice and results were represented as mean ± SEM. Statistical difference between different samples was denoted by different letters at $p<0.05$ (a), $p<0.01$ (b), $p<0.001$ (c) and $p<0.0001$ (d). (B) The *Osr40g3pro::GUS* construct. (C) Representative image of histochemical GUS staining showing Osr40g3 promoter activity in different tissues under various developmental stages. The experiment was independently repeated thrice.

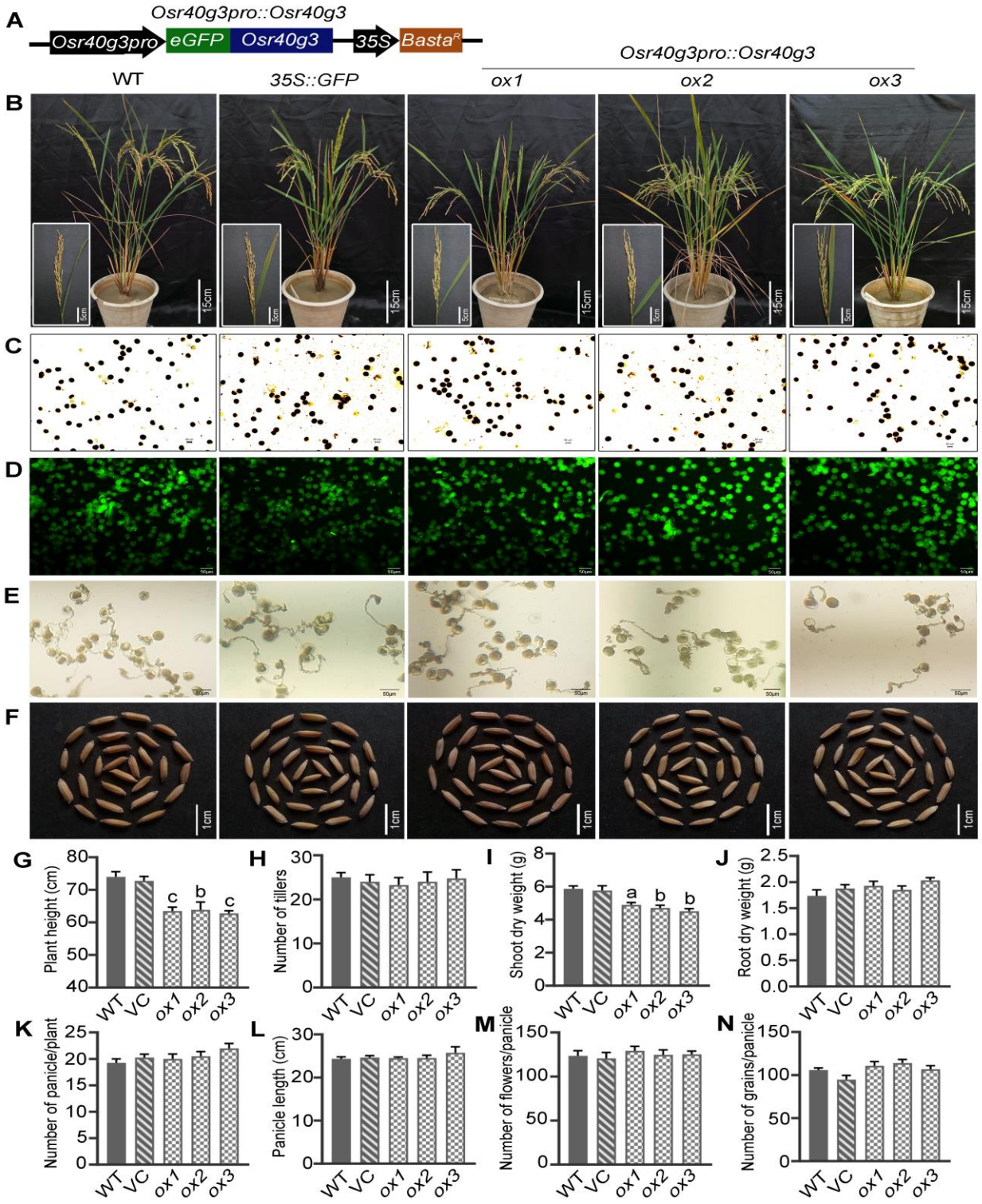

Fig 10. **Morphological characterization of transgenic rice lines overexpressing *Osr40g3* under control the native promoter**. Different morphological parameters of 3 independent transgenic lines harboring the *Osr40g3pro::Osr40g3* construct (*ox1*, *ox2* and *ox3*) were recorded

and compared with WT and VC (*35S::GFP*) plants. No abnormality in the floral morphology and pollen viability was observed. (A) Overexpression construct, (B) WT, VC and transgenic lines at reproductive stage with panicles in the inset, (C) pollen viability by I_2-KI staining method, (D) pollen viability by FDA staining method, (E) in vitro pollen germination assay, (F) grain morphology, (G) plant height, (H) number of tillers, (I) shoot dry weight, (J) root dry weight, (K) numbers of panicles/plant, (L) panicle length, (M) numbers of flowers/panicle, and (N) number of grains/panicle. Five biological replicates were considered for each line. For pollen viability and *in vitro* germination assay, at least 500 pollen grains from each line were studied. Statistical difference between WT, VC and transgenic lines was denoted by 'c' at $p<0.001$.

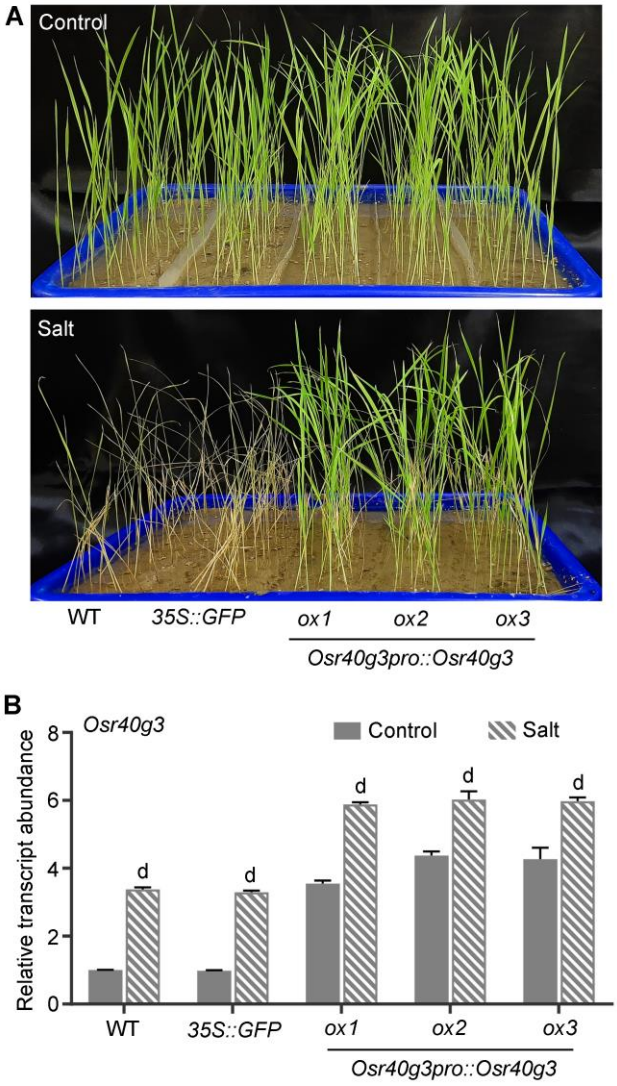

Fig 11. Response of transgenic rice plants overexpressing *Osr40g3* under control of the native promoter in response to salinity stress. 20 plants of each of the WT, VC (*35S::GFP*) and 3 independent transgenic lines harboring the *Osr40g3pro::Osr40g3* construct (*ox1*, *ox2* and *ox3*) were exposed under salinity stress for 5 d and analyzed. The transgenic lines indicated improved salinity stress tolerance over WT and VC lines. (A) Morphological response under salinity stress, (B) relative transcript abundance of *Osr40g3* gene in response to salinity. The experiment was independently repeated thrice and results were represented as mean ± SEM. Statistical difference of the samples under control and salinity stress was denoted by different letters at $p<0.05$ (a), $p<0.01$ (b) and $p<0.0001$ (d).

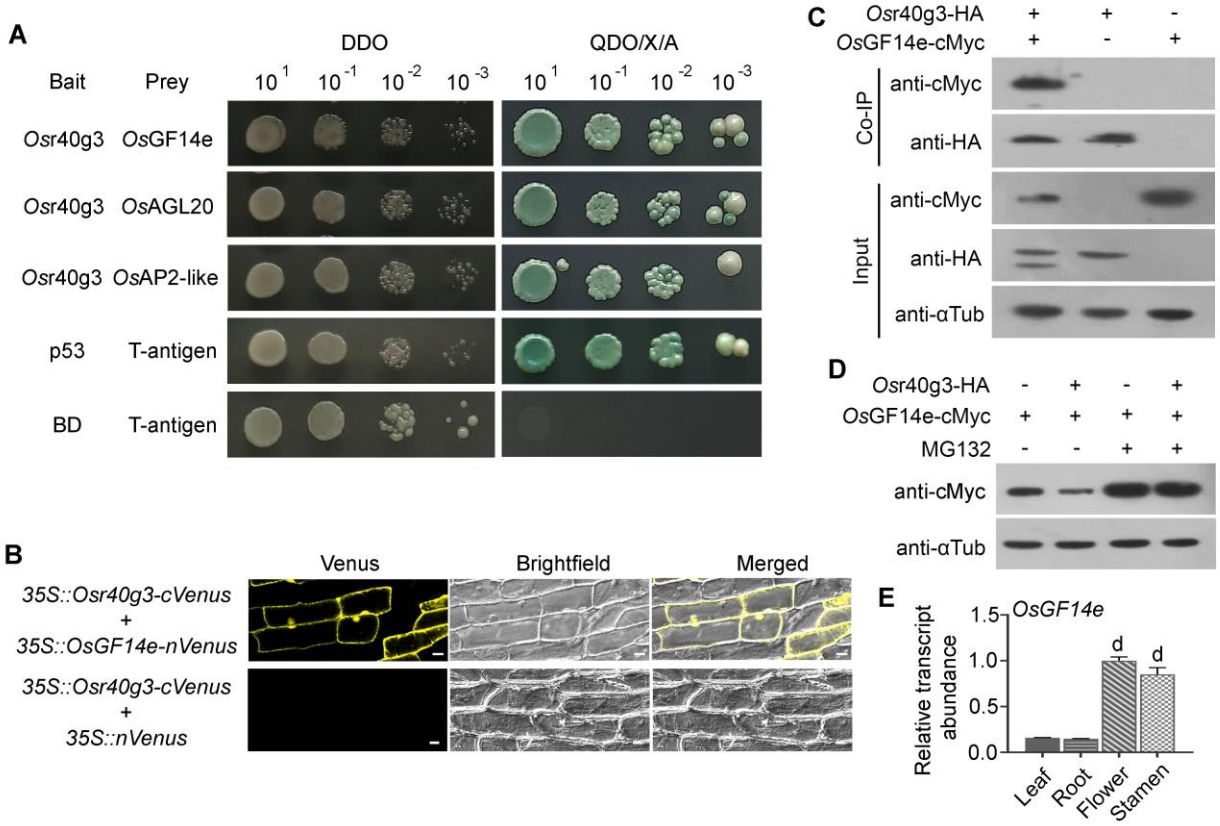

Fig 12. Identification of interacting protein partners of *Os*r40g3 in flowers. (A) Yeast two-hybrid analysis identified the interaction of *Os*r40g3 with *Os*GF14e, *Os*AGL20 and *Os*AP2-like proteins. The interaction of p53 protein with T-antigen was used as a positive control. The interaction of the empty BD with T-antigen was used as a negative control. (B) BiFC analysis confirmed the interaction of *Os*r40g3 protein with *Os*GF14e. Venus fluorescence, bright field, and merged images were represented for each set of constructs. (C) Interaction of *Os*GF14e-cMyc with *Os*r40g3-HA was confirmed through co-immunoiprecipitation assay followed by immunoblot analysis using anti-HA and anti-cMyc antibody. The experiment was independently repeated thrice. (D) Proteasomal degradation assay of *Os*GF14e-cMyc was performed using a proteasomal inhibitor, MG132 followed by immunoblot analysis using anti-cMyc and anti-HA antibody. The amount of *Os*GF14e-cMyc was reduced to a significant level when coexpressed with *Os*r40g3-HA. The experiment was independently repeated thrice. (E) Relative transcript abundance of *OsGF14e* in leaf, root, flower and stamen of WT plant. The experiment was independently repeated thrice and results were represented as mean ± SEM. Statistical difference of the samples was denoted by letter at $p<0.0001$ (d).

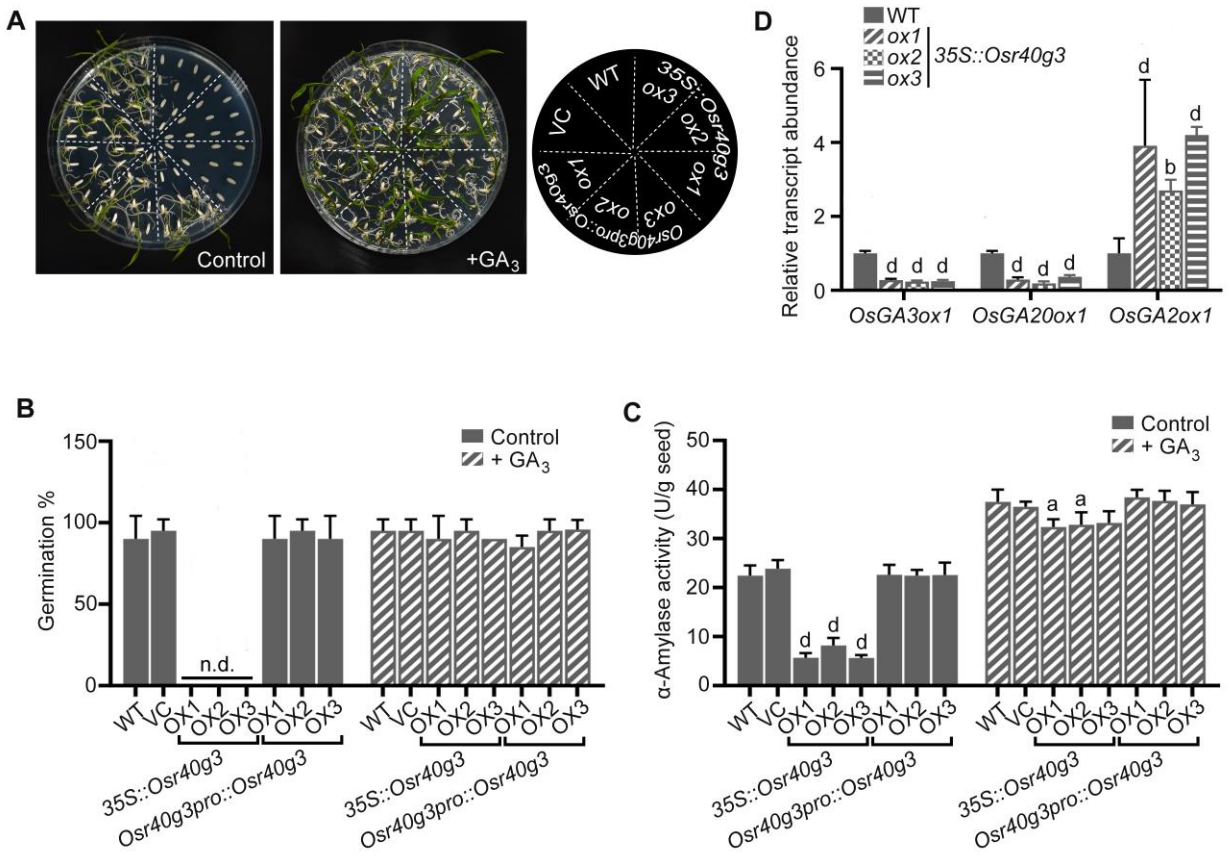

Fig 13. Constitutive overexpression of *Osr40g3* interfered with GA metabolism in rice (A) Seed germination assay for WT, VC and transgenic rice lines harboring *35S::Osr40g3* and *Osr40g3pro::Osr40g3* constructs under control and GA_3 treated conditions. (B) Seed germination percentage, and (C) α-amylase assay for the same. Exogenous GA_3 treatment significantly improved seed germination percentage as well as α-amylase activity in the otherwise dormant transgenic lines harboring the *35S::Osr40g3* construct. The experiment was independently repeated twice with 10 seeds of each line for each set. (D) Relative transcript abundance of *OsGA3ox1*, *OsGA20ox1* and *OsGA2ox1* genes in the stamen of WT and transgenic rice lines harboring the *35S::Osr40g3* construct. The expression of *OsGA3ox1* and *OsGA20ox1* genes were significantly down-regulated while *OsGA2ox1* was up-regulated in the transgenic stamens as compared with the WT stamen. The experiment was independently repeated thrice and results were represented as mean ± SEM. Statistical difference of the samples was denoted by letter at $p<0.05$ (a), $p<0.01$ (b), and $p<0.0001$ (d).

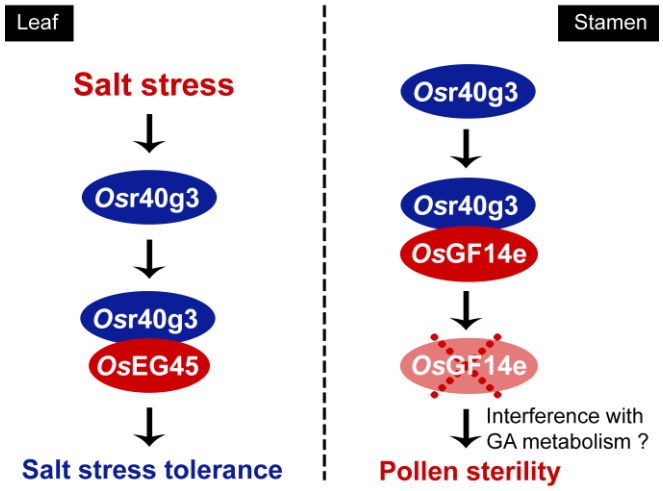

Fig 14. Dual function of *Os*r40g3 in regulating seed development and salinity stress tolerance in rice. Salinity stress triggers the accumulation of *Os*r40g3 in cells. It then interacts with *Os*EG45-like protein to provide salinity tolerance. On the other hand, in rice stamen, *Os*r40g3 can interact with *Os*GF14e and negatively regulate pollen fertility and seed development by affecting the stability of *Os*GF14e protein. This *Os*r40g3-*Os*GF14e interaction interferes with GA metabolism leading to pollen sterility.