Spatial distribution of the lectin protein *Os*r40g3 determines its dual regulatory function in imparting salinity tolerance while impeding seed development in rice

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Abstract

While the role of *Os*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 presumably repressing it which led 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 persistently face an array of environmental challenges including various biotic and abiotic stress factors. Fascinatingly, they can counteract most these threats with their explicit defense signaling system and continue their growth (Muthamilasaram and Prasad 2013). Among the diverse protective machinery, plants have evolved an array of signaling proteins that recognize the environmental changes and subsequently regulate multiple 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 diverse stress responses in plants (Van Damme et al., 2008). The role of lectin proteins in modulating biotic stress responses in a variety of plant species has widely been reported (Ciopraga et al., 1999; Freire et al., 2002; Damico et al., 2003; Singh and Zimmerli 2013; Miyakawa et al., 2014). Involvement of lectin protein has been reported in regulating heat stress in *Dolichos biflorus* (Spadoro-Tank and Etzler, 1988). Recently, emerging evidences have highlighted their regulatory role in abiotic stress responses including drought and salinity tolerance (Hirano et al., 2000; Jiang et al., 2012a; He et al., 2016). 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 family members 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).

In modern agriculture, plant growth and development is one of the major concerns for the crop plants along with their stress-responsive potential. Crop productivity is greatly hampered when the plants are challenged with different environmental threats (Jiao et al., 2016). Several signaling proteins are reported to regulate the different phases of plant development. Among them, some proteins like Leafy Cotyledon (LFY), FUSCA3 (FUS3), and Abscisic Acid Insensitive 3 (ABI3) can critically modulate flowering as well as seed development in plants (Santos-Mendoza et al., 2008). In cereals, grain filling is directly associated with the productivity and yield which is considered as an important parameter of crop cultivation. Intriguingly, no lectin family members have identified so far that can regulate seed development in plants. In most of the studies, lectin proteins gain importance due to their stress-responsive role.

The *Os*R40 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). 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 widespread lectin protein member 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 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 increases 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). Similarly, several lines of evidences also 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 (data unpublished). However, the functional role of most other *Os*R40 proteins still remains to be investigated in detail. 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 rice and this function is precisely modulated by its tissue-specific expression pattern.

RESULTS

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

To investigate the salinity tolerance potential of selected 8 *indica* rice cultivars, the seedlings were exposed to salinity stress for 5 days (Fig 1A). Following stress treatment, several biochemical parameters such as chlorophyll content, osmolyte accumulation, and H_2O_2 content were analyzed. 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 Jaldi13 and MTU1010 cultivars (Fig. S1). Nonabokra and Vandana, on the other hand, displayed the least alterations in their morphology and chlorophyll content as compared to the other cultivars indicating their tolerant phenotype. Accumulation of the two well-known osmolytes, proline and glycine betaine, was also considered to evaluate their tolerance potential. The highest accumulation of these osmolytes in response to salinity stress was found in the Nonabokra and Vandana cultivars while the least accumulation was found in Jaldi13 and MTU1010 (Fig. S1). The rest of the four cultivars showed moderate levels of accumulation. Based on all these observations, the cultivars were arranged according to their salinity tolerance potential with the Nonabokhra and Vandana being the most tolerant while the Jaldi13 and MTU1010 were the most susceptible cultivars.

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 maximum under salinity stress (Fig S2). This prompted us to explore if the expression of *Osr40g3* gene can be correlated with the salinity tolerance potential in rice. As expected, the expression of this gene under salinity stress was indeed positively correlated with the salinity tolerance potential in the 8 rice cultivars with a 4.29-fold and 4-fold induction in the tolerant Nonabokhra and Vandana cultivars against a 2.429-fold and 2-fold induction in the susceptible Jaldi13 and MTU1010 cultivars respectively (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-specific marker. 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 WT and VC (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 to the WT and VC plants. Although the number of panicles per plant did not significantly differ from the WT and VC plants, the transgenic plants failed to produce seeds. Only 3 transgenic lines with comparatively lower *Osr40g3* expression levels could produce a few seeds.

However, when the plants were exposed to salinity stress, the transgenic plants displayed significantly improved tolerance as compared to the WT and the VC plants (Fig 3A). In response to salinity stress, the transgenic plants accumulated significantly higher levels of osmolytes as well as displayed lower chlorophyll degradation as compared to the WT and VC line (Fig S4). As expected, it was observed that the expression of the *Osr40g3* gene was significantly higher in the transgenic lines under control as well as salinity stressed conditions which could account for its tolerant phenotype (Fig 3B). The expression of the marker gene, *OsRAB16* was also strongly induced in the transgenic lines under salinity stress. The amount of sodium, potassium, and chloride ions in the soil before and after salt treatment was also measured (Table S2). 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). All the positive lines displayed no phenotypic abnormalities except a shorter plant height as compared to the WT and VC plants (Fig.4). Fascinatingly, unlike in rice, the transgenic *Arabidopsis* plants displayed no abnormality in seed development. In addition, no alteration in the 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 an increase in the *Osr40g3* transcript abundance under salinity stress condition (Fig. 5). These observations confirmed the

role of *Osr40g3* in imparting salinity tolerance but indicated that its interference with the seed development is rice-specific.

Osr40g3 overexpression induced severe 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. 6). 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 staining method. The pollen viability in all transgenic flowers was found to be drastically reduced as compared to the WT and VC flowers (Fig S6A). This observation directly supports the failure of the transgenic plants to set seeds. On contrary, transgenic *Arabidopsis* plants showed no alteration in floral morphology supporting their perfect seed development (Fig.7).

Wild-type 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 breeding 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 *Osr40g3* overexpressing 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 *OsR40g*3 overexpressing plants to set seed is 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 and 15, 60 and 120 days 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 for *Osr40g3* gene was observed in the leaves (or shoots) 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.

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.9B). Remarkably, the expression of *OsAGL*65, an important candidate for pollen sterility regulation, was not detected in the flowers and stamens of the transgenic plants while it is expressed specifically in the flowers including stamens in WT plants (Fig 9C). This signified the deleterious effect of *Osr40g3* expression in the stamens.

Overexpression of *Osr40g3* under control of its own promoter rescued the pollen sterility phenotype in rice

Since the *Osr40g3* gene follows a strict tissue-specific expression pattern, transgenic rice plants (*ox* lines) overexpressing *Osr40g3* gene under the control of its own promoter was generated. The positive transformed plants, harboring the recombinant *Osr40g3pro::Osr40g3* construct, were screened through genomic DNA PCR using *bar* gene-specific primers (Fig S7). Similar to the previous observation, these transgenic plants also exhibited a slightly dwarf phenotype compared to WT and VC line but displayed no abnormalities in seed development (Fig.10). Supporting this observation, the transgenic pollen grains also exhibited a similar viability percentage like that of the WT and VC plants (Fig 10C, Fig S6B). 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.

***Os*r40g3 interacts with *Os*GF14e to regulate pollen sterility while interacts 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 analysis against specific cDNA libraries. The *Os*r40g3 protein was found to interact with three non-redundant protein partners when expressed in the flower, namely *Os*GF14e (LOC4329775), *Os*AGL20 (LOCOs03g03070), and *Os*AP2 like proteins (LOCOs0107120) (Fig 11A). Next, the interaction of protein partners was confirmed by BiFC assay (Fig 11B). The strong yellow signal of Venus protein was observed in the nucleus

and cytoplasm in case of *Os*GF14e-*Os*r40g3 and *Os*AGL20-*Os*r40g3 interactions. However, no signal was obtained for *Os*AP2-*Os*r40g3 interaction. Among these partners, the GF14e protein has been reported to positively regulate seed development. These observations indicate 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 *Os*AGL20 can reveal interesting information as well which needs to be explored in future.

To identify the interacting protein partners of *Os*r40g3 in imparting salinity tolerance, a cDNA library from salinity stressed rice shoot was used for yeast two-hybrid assay. In this case the *Os*EG45-like (LOC4347346), a peroxisomal membrane protein, *Os*PMP22 (LOC4346360) and 60S ribosomal protein L35 (LOC4329423) were identified as interacting protein partners of *Os*r40g3 (Fig S8). Out of these partners, the *Os*EG45 is an expansin-like protein and was found to be regulated by salinity stress in rice (data not shown).

*Os*GF14e displayed structural distinctiveness from its *Arabidopsis* orthologs indicating its specificity towards *Os*r40g3

Although *Arabidopsis* has several GF14 orthologs, *Osr40g3* ectopic expression did not interfere with its seed development. Therefore, all the 14 GF14 family members in *Arabidopsis* were analyzed for their homology with *Os*GF14e protein. The multiple sequence alignment analysis revealed a highly conserved nature of the GF14 members except at their C-terminal domain (Fig S9). Consistent with this observation, it has been reported earlier that the diverse C-terminal domain determines the binding specificity of the GF14 family members which supports their diverse functions (Sehnke et al., 2006). Further, a conserved glycine or asparagine residue in the 8^{th} loop of the *Arabidopsis* GF14 proteins is replaced with a serine residue in *Os*r40g3 (Fig S10). This structural distinctiveness of *Os*GF14e presumably accounts for its binding specificity with *Os*r40g3 while its *Arabidopsis* orthologs fail to do so.

DISCUSSION

The *Os*R40 family of lectin proteins has long been known to play a vital role in regulating drought and salinity stress in plants (Moons et al., 1995; Jiang et al., 2012b). Among these proteins, the putative *Os*r40c1 has been found to play a fundamental role in imparting drought tolerance in rice and tobacco (data unpublished). However, the functional aspects of the other

*Os*R40 proteins have not been explored in detail. 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. This made us curious to explore how *Osr40g3* imparts salinity tolerance in plants. To achieve this, 8 *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 and Vandana cultivars and lowest expression in the susceptible Jaldi13 and MTU1010 cultivars. This strongly indicated a salinity-responsive role of *Osr40g3* in rice.

With this optimistic clue, *Osr40g3*-overexpressing transgenic rice lines were generated harboring the recombinant *35S::Osr40g3* construct. As expected, the transgenic lines exhibited significantly improved salinity tolerance in comparison to the WT and the VC plants. Surprisingly, however, all the transgenic lines revealed very poor seed setting suggesting a negative role of *Osr40g3* in seed development. In consistence with this observation, the transgenic rice flowers appeared to be shorter with mostly sterile pollen grains. On contrary, when the same construct was introduced into *Arabidopsis*, the transgenic lines ectopically expressing *Osr40g3* displayed improved salinity stress tolerance but no defect in seed development. Unlike rice, the floral morphology of the transgenic *Arabidopsis* lines displayed no phenotypic alteration supporting this unusual observation.

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 the 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. This observation strongly suggested that *Osr40g3* specifically acts as a negative regulator of pollen viability while the pistil remains unaffected.

Since *Osr40g3* gene was overexpressed under control of a constitutive promoter, the next obvious 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. Furthermore, the constitutive overexpression of *Osr40g3* in

flowers and stamens diminished the expression of one of the important regulators of pollen viability, *OsAGL65*. In line with this observation, the *Osr40g3* gene was next cloned under control of its own promoter (*Osr40g3pro::Osr40g3*) and transgenic rice lines were generated harboring this recombinant construct. Interestingly, in addition to salinity tolerance, all the transgenic lines showed proper seed development thus rescuing the pollen sterile phenotype. These observations indicated that *Osr40g3* follows 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 mechanism of the protein in rice. Intriguingly, it was identified that *Os*r40g3 interacts with separate sets of protein partners to execute its role in seed development and salinity stress. The *Os*GF14e protein was found to interact with *Os*r40g3 in the rice flowers. Fascinatingly, 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, two GF14 family members, *Os*GF14b and *Os*GF14e were demonstrated to positively regulate leaf blast resistance in plants (Manosalva et al., 2011). Also, the suppression of *Os*GF14e and *Os*GF14b could produce susceptibility towards panicle blast (Manosalva et al., 2011; Liu et al., 2015, 2016). In addition, *Os*GF14e can interact with DOF ZFP transcription factors that were activated by developmental signals in plants (Cooper et al., 2003). Interestingly, the silencing of *OsGF14e* displayed impaired seed development in rice which resembles our current observation (Manosalva et al., 2011; Liu et al., 2016). Moreover, earlier evidences suggested that the higher accumulation of GF14e protein was associated with fertile pollen grains of maize while lesser abundance of the protein was found in sterile pollens. It has also been depicted that maize GF14e can regulate the starch accumulation in pollen grains thus determining pollen viability (Datta et al., 2002). This certainly indicated a positive regulatory role of GF14e in pollen fertility. Accumulating evidences also suggested that rice GF14 family members also 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. Interestingly, it has also been demonstrated that out of eight GF14 family

members, *Os*GF14e and *Os*GF14f showed higher accumulation in flowers or panicles (Chen et al., 2006). With these in the background, it can be assumed that the *Os*r40g3 interacts with and represses *Os*GF14e in the stamens which ultimately lead to pollen sterility and impaired seed development in rice.

Although *Os*GF14e has several orthologs in *Arabidopsis*, the transgenic plants ectopically expressing *Osr40g3* displayed normal seed development. To understand this unusual observation, the sequences *Arabidopsis* GF14 proteins were analyzed. It has been reported that the *Arabidopsis* GF14 family can be broadly classified into two groups, the epsilon (ε) group and the non-epsilon group (Daugherty et al., 1996; Sehnke et al., 2006). The non-epsilon group members like omega (ω), psi (ψ), mu (μ), nu, chi (χ) showed close homology with the *Os*GF14e protein. Among them, the chi (χ) protein is known to be highly expressed in the *Arabidopsis* flowers including stamens (Daugherty et al., 1996). Intriguingly, the varied C-terminal domain of the GF14 family members has been reported to determine their functional variation and specificity in plants (Sehnke et al., 2006). Furthermore, the presence of a serine residue in the 8th loop of *Os*GF14e also differs from the conserved glycine or asparagine residue present in the *Arabidopsis* GF14 family members. This residue has also been considered to be crucial for target protein specificity (Sehnke et al., 2006). This structural distinctiveness of the *Os*GF14e from its *Arabidopsis* orthologs provided a possible answer why *Os*r40g3 failed to interact with the *Arabidopsis* GF14 proteins to impede seed development in *Arabidopsis.*

On the other hand, *Os*r40g3 was identified to interact with *Os*EG45 protein in the salinity stressed rice leaves. Interestingly, EG45 is a salinity stress-responsive expansin-like glycoprotein that shows endoglucanase-like function and helps in cell wall modification (Ludidi et al., 2002). Cell wall modification is a common phenomenon in the osmotic stressed cells (Gall et al., 2015). In addition, *Os*r40g3 also interacted with a peroxisomal membrane protein PMP22 and a 60S ribosomal protein L35. Since ectopic expression of *Os*r40g3 could impart salinity stress tolerance in *Arabidopsis* as well, it can be assumed that the *Os*r40g3 protein can interact with the orthologs of the partners in *Arabidopsis* to execute its salinity stress-responsive role.

In summary, the present investigation unraveled a dual regulatory function of *Os*r40g3 in imparting salinity tolerance as well as impeding the pollen fertility in rice (Fig 12). It was observed that the expression of *Osr40g3* positively correlated with the salinity tolerance potential

in rice. It was further observed that *Osr40g3* gene displayed a precise tissue-specific expression pattern which is essential for proper seed development. The *Os*r40g3 protein was found to specifically interact with the *Os*GF14e and presumably repress it. This led to pollen sterility in the constitutively *Osr40g3* overexpressing transgenic rice. Since, interaction with *Os*GF14e is determined by its structural distinctiveness from its *Arabidopsis* orthologs, the *Os*r40g3-mediated pollen sterility was observed in rice only. On the other hand, the protein was identified to interact with *Os*EG45 or its ortholog to impart salinity tolerance in both rice and *Arabidopsis*. Together, the current study highlighted an exciting dual role of *Os*r40g3 protein in positively regulating salinity tolerance while negatively modulating seed development 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. The plants were grown and maintained in the greenhouse under optimum conditions. For salinity stress, seeds were germinated and grown on on filter papers wetted with water for 15 days under optimum photoperiod at 30 ºC. The seedlings were treated with 200 mM NaCl solution for 5 days (Kurniasih et al., 2013). For salt treatment of mature soil-grown plants, the pots were watered with 200 ml of 200 mM NaCl solution per day and data was recorded after 5 days of salt treatment (Amirjani, 2012).

Arabidopsis seeds were procured from Nottingham Arabidopsis Stock Center (NASC), UK. *Arabidopsis* plants of Columbia ecotype (Col-0) were grown and maintained in growth chamber as standardized before (Datta et al., 2015). Plants were exposed to salinity stress using 200 mM NaCl solution as standardized before (Datta et al., 2015).

Estimation of different biochemical compounds

Proline and glycine betaine contents were measured by spectrophotometric method from tissue samples collected from control and treated plants (Grieve and Grattan 1983; Woodrow *et al*., 2016). The chlorophyll content was measured according to Lichtenthaler (1987). The hydrogen peroxide (H_2O_2) content was measured following Yin et al. (2010). Briefly, the leaves were

homogenized with 0.1% (w/v) TCA solution. After centrifugation, the supernatant was collected the optical density has been measured through spectrophotometer (Hitachi) at 390 nm.

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 (BioRad) following the manufacture's protocol. The qRT PCR analysis was performed in CFX96 Real-time PCR (BioRad) using iTaq Universal SYBR Green Supermix (Biorad) and gene-specific primers (Table S1). The gene expression was normalized based on the relative quantification method referring three biological replicates. The *Actin* gene of rice and *Arabidopsis* was used as a reference gene.

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 pGEMT-Easy vector (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). 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 putative transformants were screened for herbicide resistance, positive lines were confirmed by genomic DNA PCR using *bar* gene-specific primers and were grown upto T_2 generation.

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. The amplified *OsR40g3* promoter was inserted between the *AgeI* and *PacI* RE sites of the recombinant vector *CaMV35S*::Os*r40g3* to generate the construct, *OsR40g3pro*::Os*r40g3*. The construct was used for *Agrobacterium*-mediated genetic transformation of rice as described above. The independent transgenic lines generated were maintained and grown for further generations under greenhouse conditions.

Morphological Analysis

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).

Pollen viability assay

For pollen viability evaluation, I_2-KI staining method was followed according to Chuun et al. (2007). Briefly, the anthers were collected before anthesis from spikelet, crushed into powder and were stained with 1% I_2 (v/v) and 3% KI 2% (w/v) solution. The samples were observed under bright field microscope (Leica DM IL LED S-80) to detect fertile and sterile pollen grains. The pollen grains which appeared black in color were considered as living or viable pollen grains. At least 500 pollen grains were considered for this study.

Hybridization of rice plants

The flowers of WT plants were emasculated and considered as female parent whereas the transgenic rice plants harboring the *35S::Osr40g3* construct were considered as male parent. In another set, the transgenic rice plants harboring the *35S::Osr40g3* construct were emasculated and considered as female parent and hybridized with WT as male parent. In both cases the number of hybrid seeds was measured.

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed to identify the different protein partners of *OsR40g3* protein. The *OsR40g3* gene was inserted into the *EcoRI* and *BamHI* RE sites of the bait vector, *pGBKT7* (Clontech). The recombinant *BD-Osr40g3* bait construct was transformed into yeast following manufacturer's protocol (Clontech). Rice cDNA library was prepared from flowers and ligated to *pGADT7-Rec* vector using Make Your Own "Mate and Plate" Library Manual (Clontech). To identify the interacting protein partners responsive for salinity stress tolerance

regulation, a second cDNA library was prepared from rice leaves under salinity stress condition. In yeast Y2H Gold strain, the recombinant plasmids were co-transformed according to the manufacturer's instructions (Clontech). 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 (Clontech) to identify the interacting protein partners. The *BD-p53* and *AD-T-antigen* interaction was used as a positive 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 genes of the identified partners like *OsGF14e*, *OsAGL20*, and *OsAP2* were cloned into *SpeI* and *BamHI* RE sites of *pVYNE* to generate *35S::OsGF14e-nVenus*, *35S::OsAGL20-nVenus*, *35S::OsAP2-nVenus* constructs respectively (Waadt et al. 2008). The recombinant constructs were transformed into *Agrobacterium tumifaciences* 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 laser scanning confocal microscope (Olympus FV1000-IX81).

Multiple sequence alignment and phylogenetic 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).

Statistical analysis

Three independent biological replicates were used for all experiments as applicable and data were represented as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism version 8.0.0 software (GraphPad Software, San Diego, California USA). The variation of morphological, and biochemical parameters as well as the relative transcript accumulation among genotypes and treatments were analyzed following two-way ANOVA followed by Sidak's multiple comparison tests. To identify the difference between two sets of data, the statistical significance at $p \leq 0.05$ was considered.

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: Biochemical estimation 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 under salinity stress condition.

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

Fig S4: Biochemical estimation 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 (*35S::Osr40g3*) using *bar* gene-specific primers.

Fig S6: Pollen viability percentage of WT, VC and transgenic rice lines harboring (A) *35S::Osr40g3* and (B) *Osr40g3pro::Osr40g3* constructs.

Fig S7: Genomic DNA PCR of *Osr40g3* overexpressing transgenic rice plants (*Osr40g3pro::Osr40g3*) using *bar* gene-specific primers.

Fig S8: (A) Yeast two-hybrid assay for *Os*r40g3 interaction against cDNA library from rice leaves under salinity stress. (B) BiFC analysis for interaction of *Os*r40g3 with *Os*EG45 like proteins, *Os*PMP22 and *Os*60SRPL5.

Fig S9: Multiple sequence alignment of *Os*GF14e protein with *Arabidopsis* GF14 family members showing structural variability at the C-terminal end.

Fig S10: Multiple sequence alignment of *Os*GF14e protein with *Arabidopsis* GF14 family member proteins showing substitution of a crucial serine residue in the *Os*GF14e by a glycine/asparagine residue in the 8th loop of *Arabidopsis* GF14 orthologs.

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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Figure Legends

Fig 1. Salinity stress assay of 8 *indica* rice cultivars. Fifteen days old seedlings were treated with 200 mM NaCl solution for 5 days 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. Results were represented as mean ± SEM of 3 biological samples. 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 independent transgenic lines harboring the *35S::Osr40g3* construct (*ox1, ox2* and *ox3*) were recorded and compared 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. 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 days 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. Results were represented as mean ± SEM of 3 biological samples. 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 shorter plant height with normal seed development. (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. 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 days 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. Results were represented as mean ± SEM of 3 biological samples. 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 independent transgenic lines harboring the *35S::Osr40g3* construct (*ox1*, *ox2* and *ox3*) were recorded and compared with WT and VC (*35S::GFP*) plants. The transgenic flowers appeared to be shorter than the WT and VC flowers with markedly reduced pollen viability. (A) Entire flower, (B) dissected flower, (C) stamens with pistil, and (D) pollen viability by I_2-KI staining method.

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) single stamen, and (D) pistil.

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.

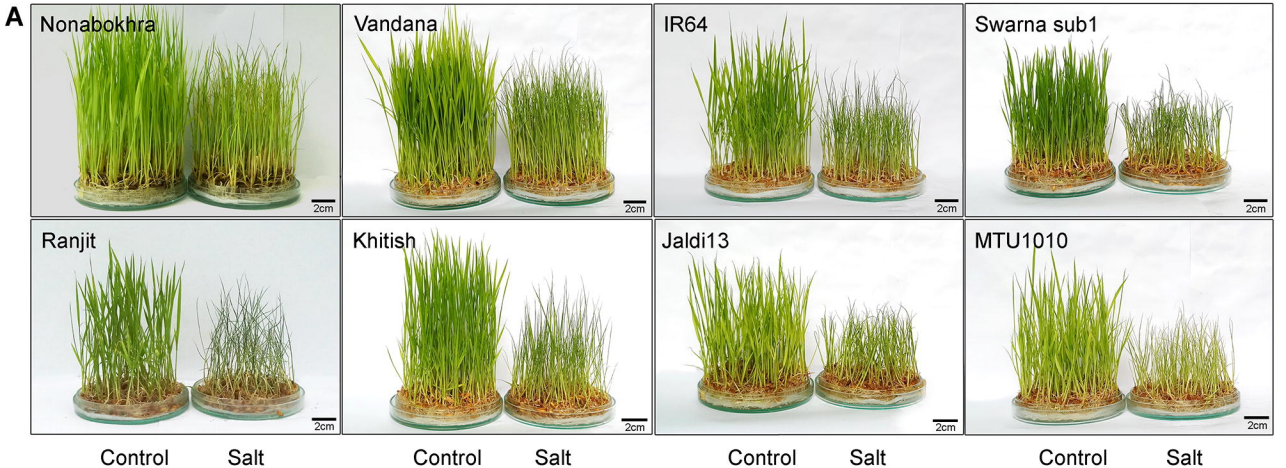
Fig 9. **Tissue-specific expression pattern of *Osr40g3* gene in rice.** (A) Relative transcript abundance of *Osr40g3* gene in different tissues of rice plants. Highest transcript abundance was observed in the leaves while no expression was detected in the stamen. Relative transcript abundance of (B) *Osr40g3* and (C) *OsAGL65* genes in the leaves, inflorescence and stamens of 3 independent lines of transgenic rice (*ox1*, *ox2*, and *ox3*) were compared with that in WT plant. The transcript abundance of *Osr40g3* was significantly increased in stamens while the accumulation of *OsAGL65* transcript was found to be absent in stamens of the transgenic lines. Results were represented as mean ± SEM of 3 biological samples. Statistical difference between the WT and transgenic lines was denoted by different letters at $p<0.05$ (a), $p<0.01$ (b), $p<0.001$ (c) and $p<0.0001$ (d).

Fig 10. **Morphological characterization of transgenic rice lines overexpressing *Osr40g3* under control of its own 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) grain morphology, (E) plant height, (F) number of tillers, (G) shoot dry weight, (H) root dry weight, (I) numbers of panicles/plant, (J) panicle length, (K) numbers of flowers/panicle, and (L) number of grains/panicle. Statistical difference between WT, VC and transgenic lines was denoted by 'c' at $p<0.001$.

Fig 11. 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. (B) BiFC analysis confirmed the interaction of *Os*r40g3 protein with *Os*GF14e and *Os*AGL20 in the nucleus and cytoplasm. Venus fluorescence, bright field, and merged images were represented for each set of constructs.

Fig 12. Model for dual function of *Os*r40g3 in regulating seed development and salinity stress tolerance. Salinity stress triggers the accumulation of *Os*r40g3 in cells. The expression of *OsEG45* like protein is also induced by salinity stress and it interacts with *Os*r40g3 to provide salinity tolerance in rice and *Arabidopsis*. On the other hand, in rice flowers, *Os*r40g3 can

interact with *Os*GF14e and negatively regulate pollen fertility and seed development presumably by suppressing the function of *Os*GF14e protein. This *Os*r40g3-*Os*GF14e interaction is specific to rice due to the structural distinctiveness of *Os*GF14e from its orthologs in *Arabidopsis*.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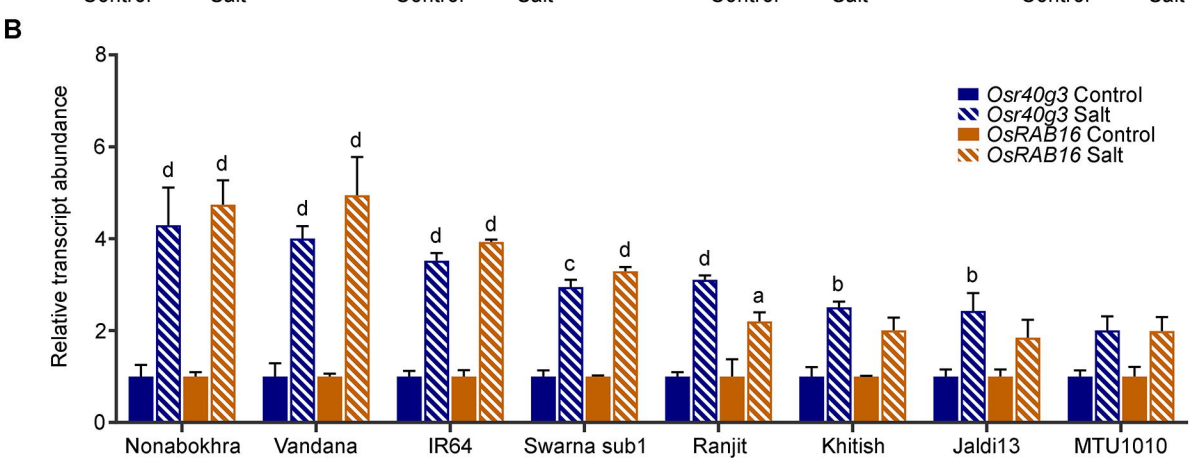
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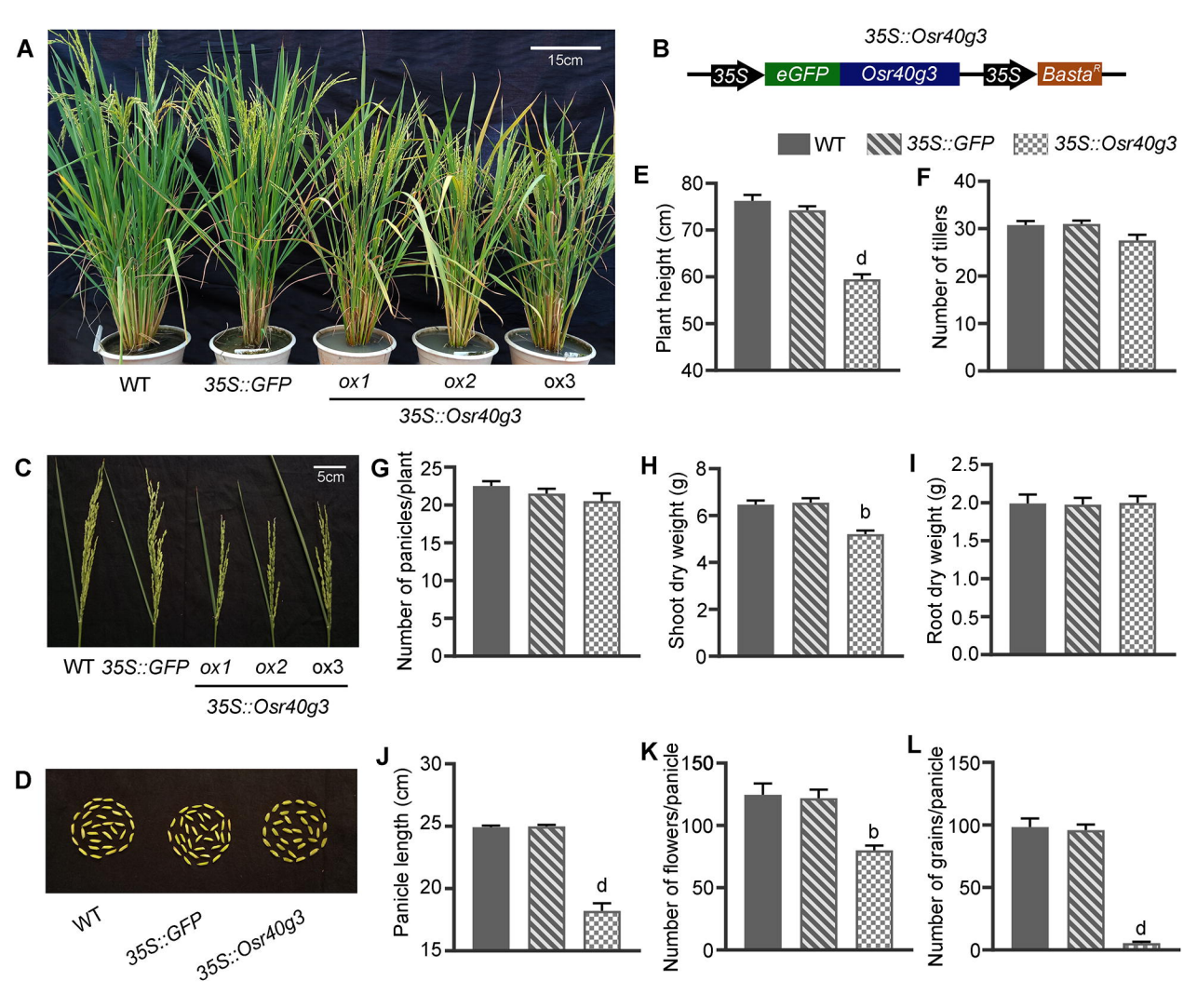
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Basta^R
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35S::GFP
35S::Osr40g3
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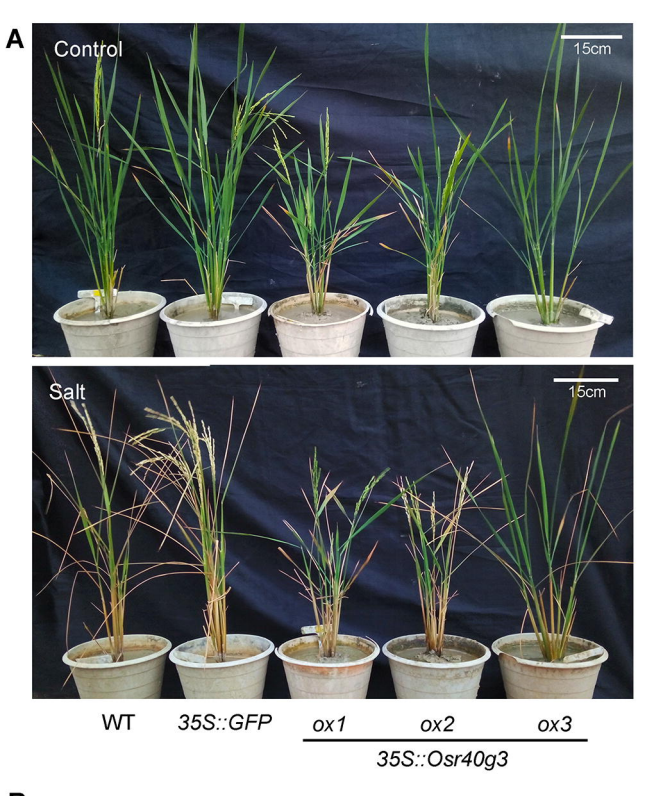
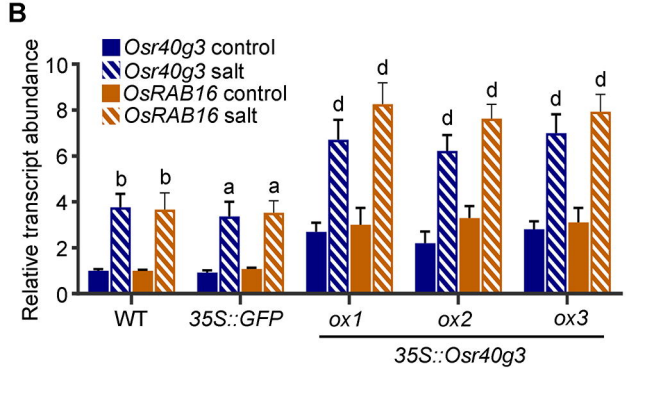
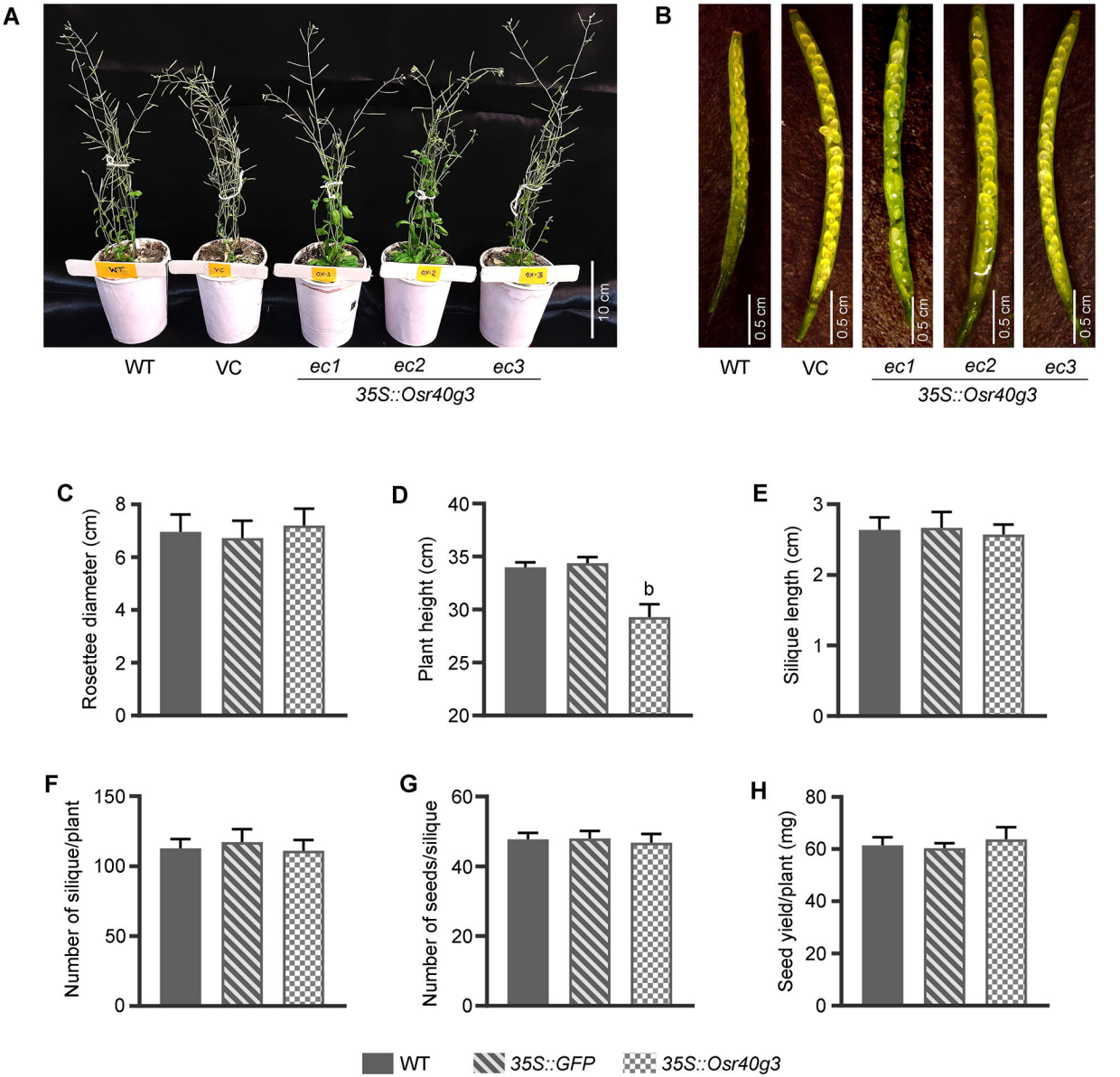
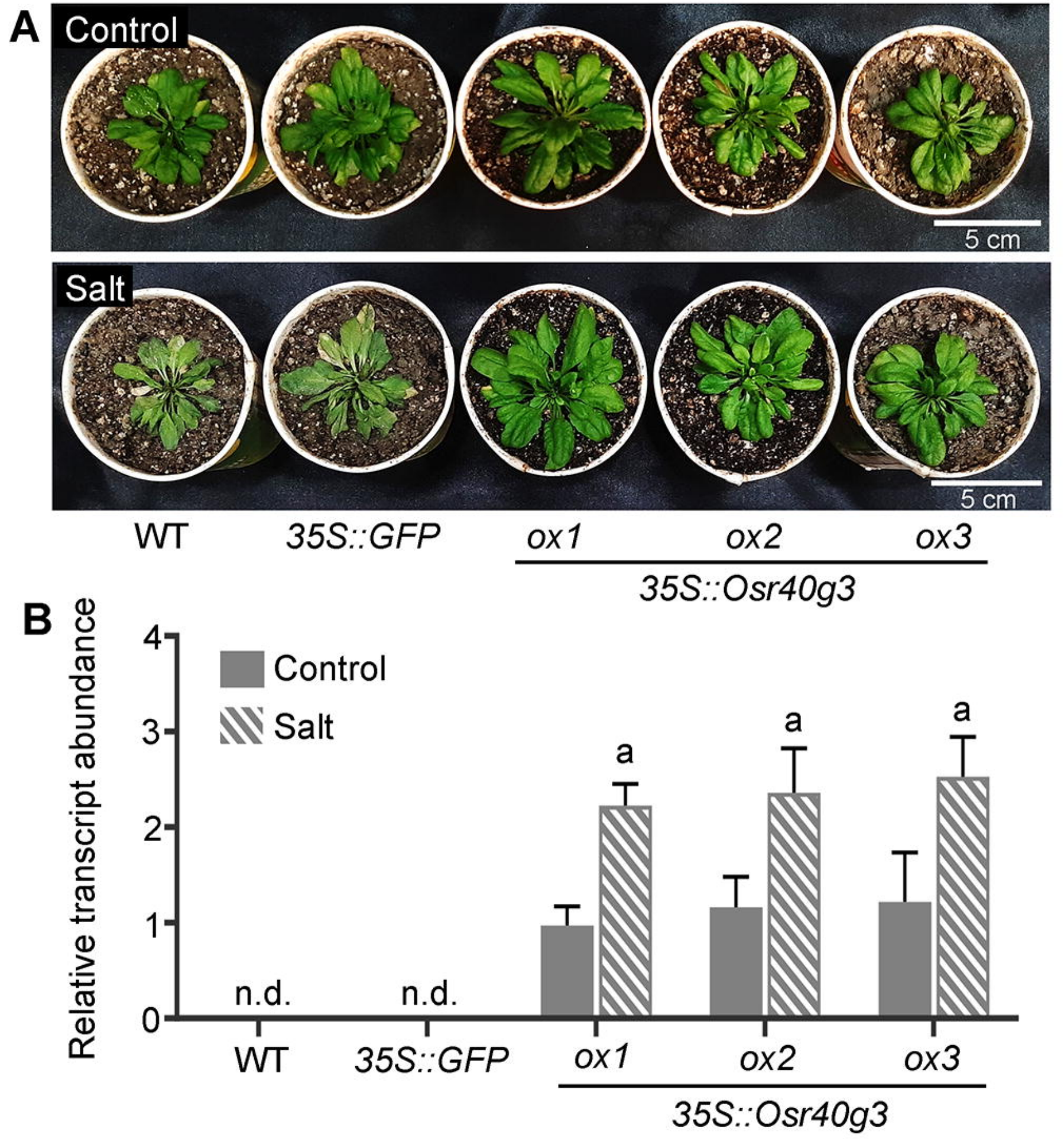
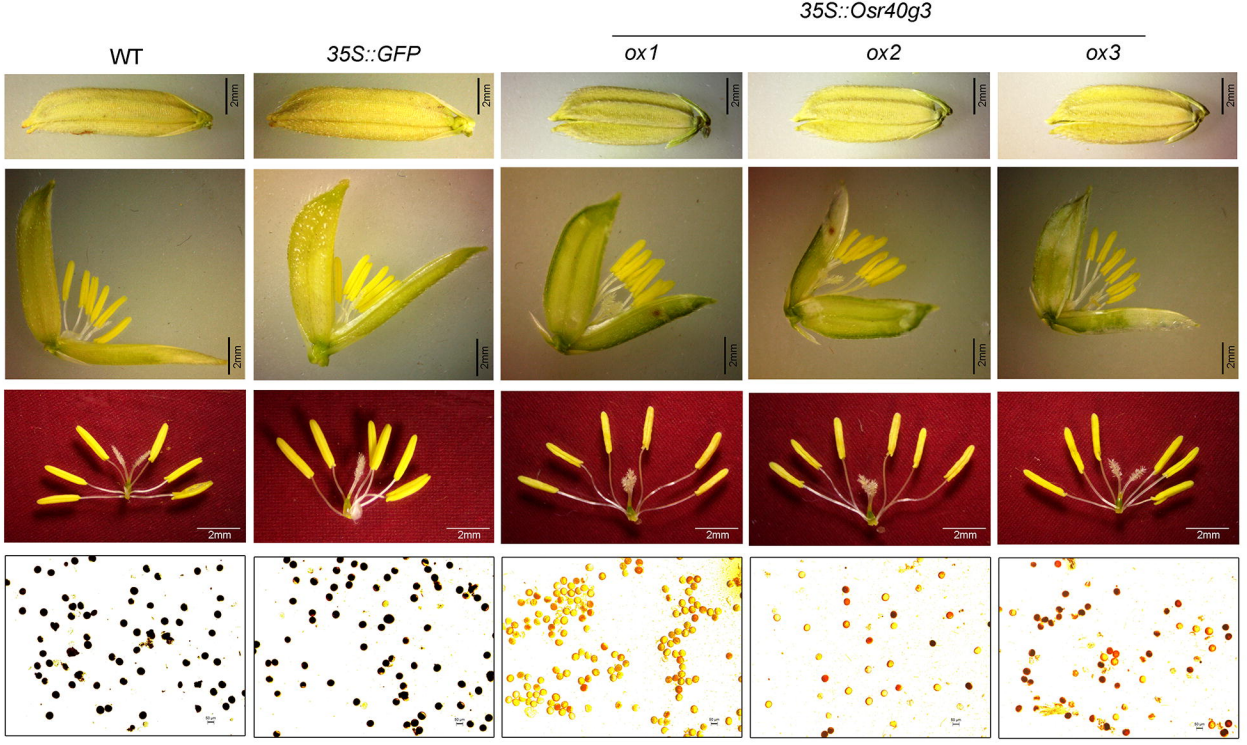
Number of grains/panicle
d

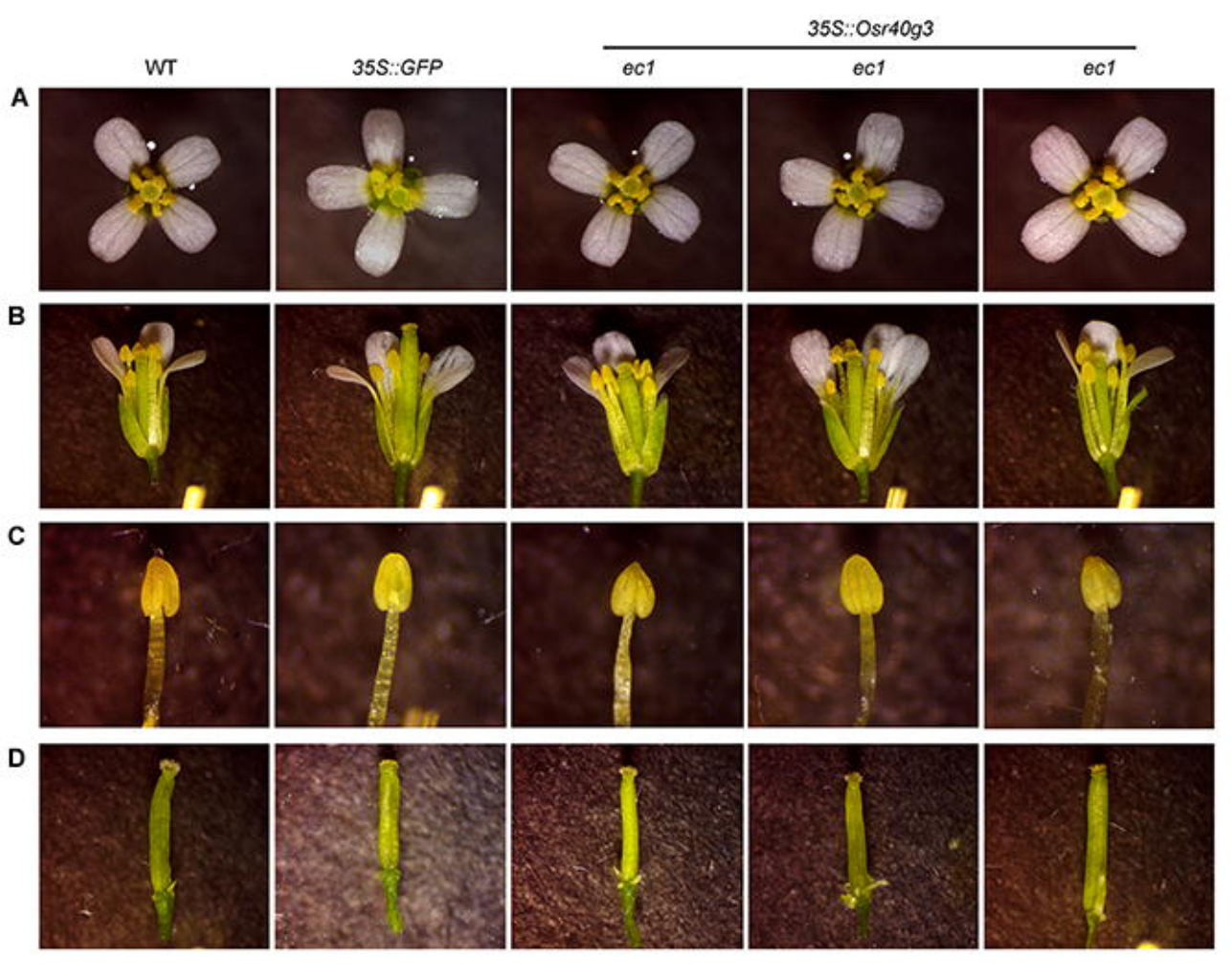

A
Control
15cm
Salt
15cm
WT
35S::GFP
ox1
ox2
ox3
35S::Osr40g3


B
Relative transcript abundance
Osr40g3 control
Osr40g3 salt
OsRAB16 control
OsRAB16 salt
0
2
4
6
8
10
b
b
a
a
d
d
d
d
d
d
WT
35S::GFP
ox1
ox2
ox3
35S::Osr40g3


A
WT
VC
ec1
ec2
ec3
35S::Osr40g3
10 cm
B
0.5 cm
C
Rosettee diameter (cm)
D
Plant height (cm)
b
E
Silique length (cm)
F
Number of silique/plant
G
Number of seeds/silique
H
Seed yield/plant (mg)
WT
35S::GFP
35S::Osr40g3

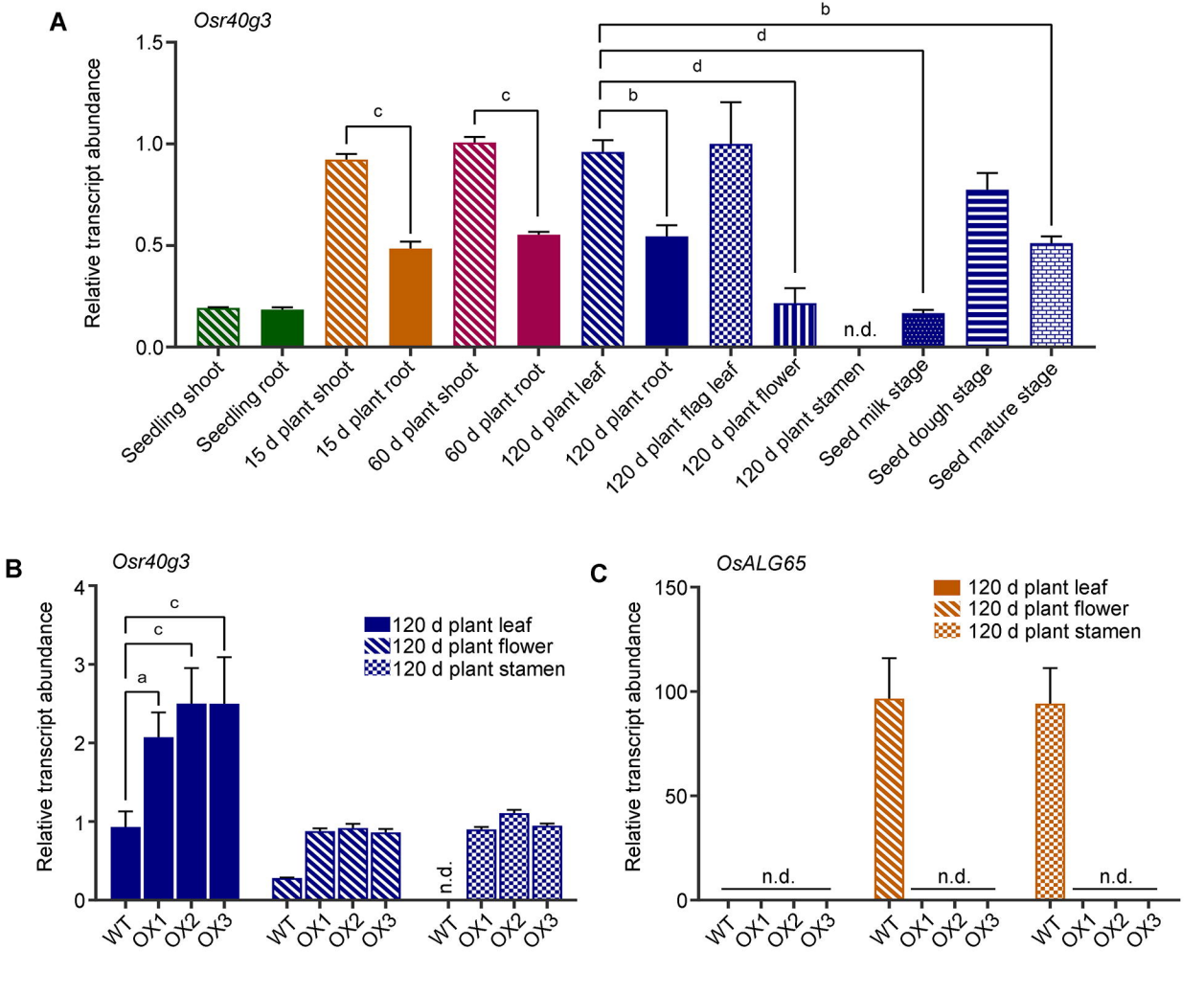

A
Control
5 cm
Salt
5 cm
WT
35S::GFP
ox1
ox2
ox3
35S::Osr40g3
B
Relative transcript abundance
4
3
2
1
0
Control
Salt
n.d.
n.d.
a
a
a
WT
35S::GFP
ox1
ox2
ox3
35S::Osr40g3

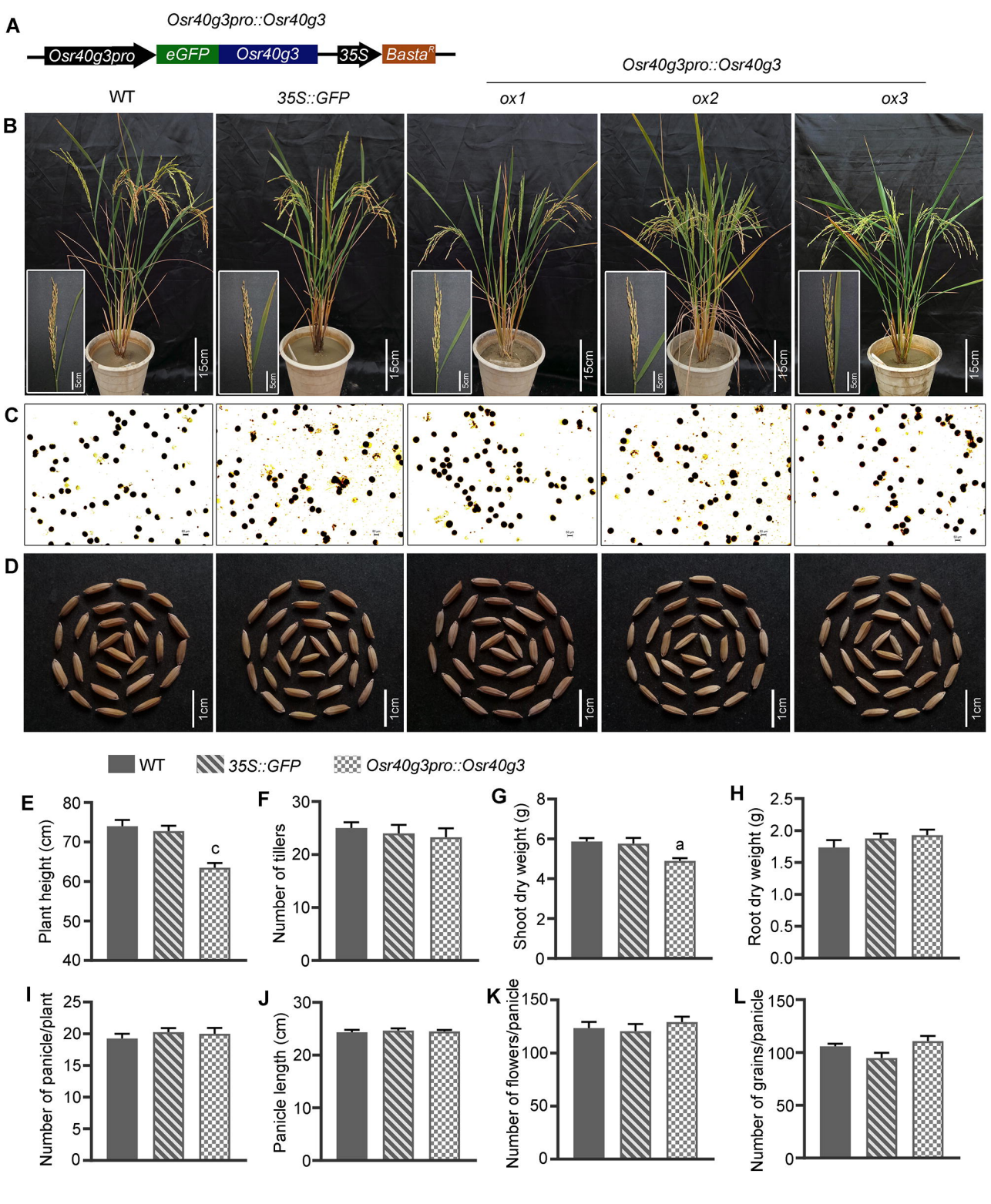

35S::Osr40g3
WT
35S::GFP
ox1
ox2
ox3
A
B
C
D
2mm

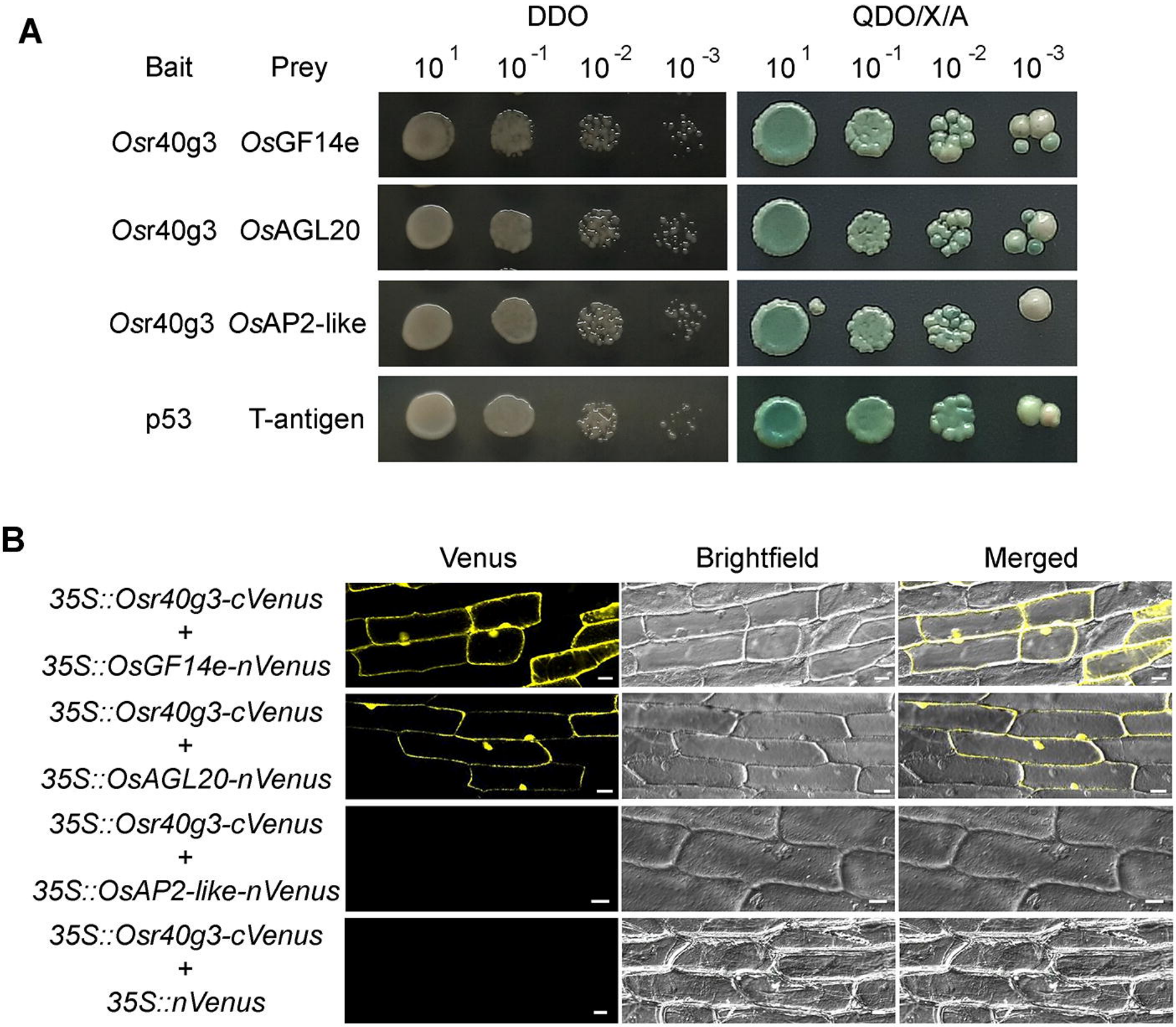

35S::Osr40g3
WT
35S::GFP
ec1
ec1
ec1
A
B
C
D

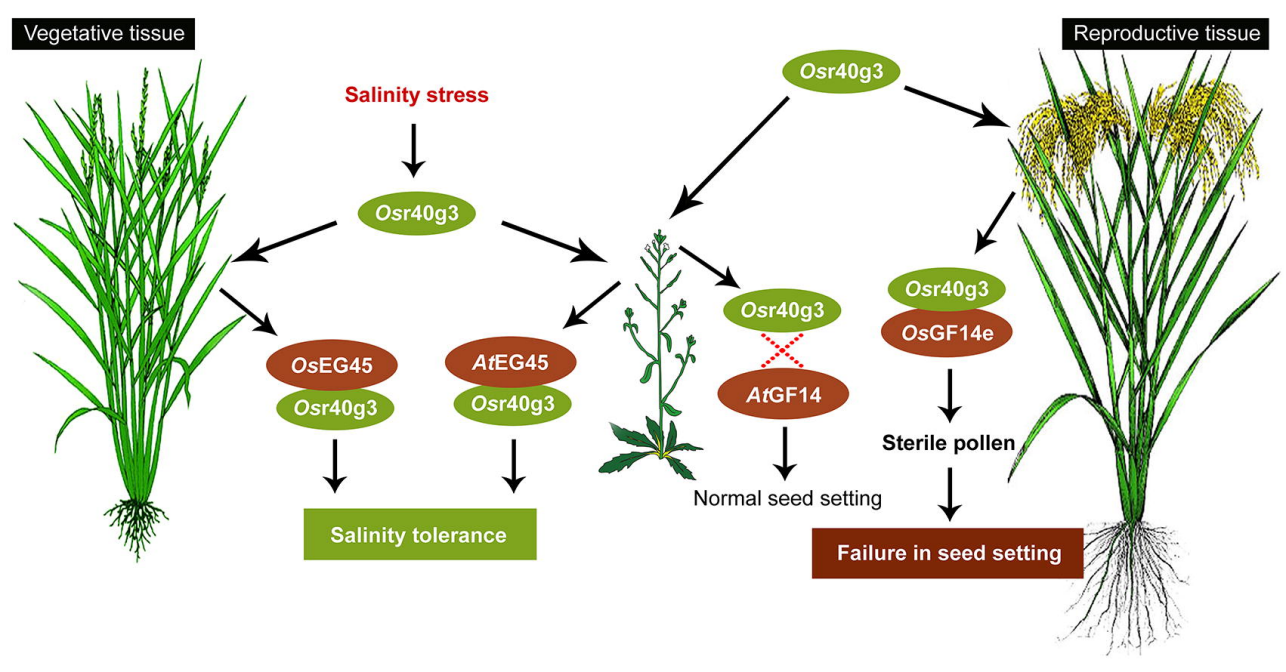
35S::Osr40g3 ♀
+
WT ♂

WT ♀
+
35S::Osr40g3 ♂


A
Osr40g3
Relative transcript abundance
1.5
1.0
0.5
0.0
b
d
d
b
c
c
n.d.
Seedling shoot
Seedling root
15 d plant shoot
15 d plant root
60 d plant shoot
60 d plant root
120 d plant leaf
120 d plant root
120 d plant flag leaf
120 d plant flower
120 d plant stamen
Seed milk stage
Seed dough stage
Seed mature stage
B
Osr40g3
Relative transcript abundance
4
3
2
1
0
c
c
a
120 d plant leaf
120 d plant flower
120 d plant stamen
n.d.
WT
OX1
OX2
OX3
C
OsALG65
Relative transcript abundance
150
100
50
0
120 d plant leaf
120 d plant flower
120 d plant stamen
n.d.
n.d.
n.d.
WT
OX1
OX2
OX3


A
Osr40g3pro::Osr40g3
Osr40g3pro
eGFP
Osr40g3
35S
Basta^R
Osr40g3pro::Osr40g3
WT
35S::GFP
ox1
ox2
ox3
B
15cm
5cm
C
D
1cm
WT
35S::GFP
Osr40g3pro::Osr40g3
E
Plant height (cm)
c
F
Number of tillers
G
Shoot dry weight (g)
a
H
Root dry weight (g)
I
Number of panicle/plant
J
Panicle length (cm)
K
Number of flowers/panicle
L
Number of grains/panicle


A
DDO
QDO/X/A
Bait
Prey
10 1
10 -1
10 -2
10 -3
Osr40g3
OsGF14e
Osr40g3
OsAGL20
Osr40g3
OsAP2-like
p53
T-antigen
B
Venus
Brightfield
Merged
35S::Osr40g3-cVenus
+
35S::OsGF14e-nVenus
35S::Osr40g3-cVenus
+
35S::OsAGL20-nVenus
35S::Osr40g3-cVenus
+
35S::OsAP2-like-nVenus
35S::Osr40g3-cVenus
+
35S::nVenus


Vegetative tissue
Salinity stress
Osr40g3
OsEG45
Osr40g3
AtEG45
Osr40g3
Salinity tolerance
Osr40g3
AtGF14
Normal seed setting
Osr40g3
Reproductive tissue
Osr40g3
OsGF14e
Sterile pollen
Failure in seed setting