Role of *qGZn9a* in controlling grain zinc concentration in rice, *Oryza sativa* L.

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Abstract

Key message A candidate gene responsible for higher grain zinc accumulation in rice was identified, which was probably associated with a partial defect in anther dehiscence.

Abstract Zinc (Zn) is an essential mineral element in many organisms. Zn deficiency in humans causes various health problems; therefore, an adequate dietary Zn intake is required daily. Rice, *Oryza sativa*, is one of the main crops cultivated in Asian countries, and one of the breeding scopes of rice is to increase the grain Zn levels. Previously, we found that an Australian wild rice strain, *O. meridionalis* W1627, exhibits higher grain Zn levels than cultivated rice, *O. sativa* Nipponbare, and identified responsible genomic loci. An increase in grain Zn levels caused by one of the loci, *qGZn9a*, is associated with fertility reduction, but how this negative effect on grain productivity is regulated remains unknown. In this study, we artificially trimmed spikelets on the flowering day and found that a reduction in number of seeds was associated with an increase in the grain Zn levels. We also found that a partial defect in anther dehiscence correlated with the increase in grain Zn levels in plants carrying the W1627 chromosomal segment at *qGZn9a* in a Nipponbare genetic background. Among eight candidate genes in the *qGZn9a* region, three were absent from the corresponding region of W1627; one of these, *Os09g0384900*, encoding a DUF295 protein with an unknown function, was found to be specifically expressed in the developing anther, thereby suggesting that the gene may be involved in the regulation of anther dehiscence. As fertility and grain Zn levels are essential agronomic traits in rice, our results highlight the importance of balancing these two traits.

Keywords *Oryza sativa* · Grain zinc concentration · Fertility · Anther dehiscence

Introduction

Zinc (Zn) is one of the most essential nutrients in all organisms, including humans. Zn is involved in cell cycle progression, DNA replication, DNA damage repair, and apoptosis. Zn is essential for the activities of more than 300 enzymes, such as hydrolases, oxidoreductases, lyases, transferases, ligases, and isomerases. In humans, 2800 proteins, almost

10% of the human proteasome, have Zn-binding properties (McCall et al. 2000; Andreini et al. 2006; Yan et al. 2008). Owing to the multiple functions of Zn in the human body, Zn deficiency can cause serious health problems. Major damages caused by Zn deficiency are growth failure, hair loss, dermatitis, diarrhoea, appetite loss, and neurosensory, skeletal, reproductive, and immune system disorders (Roohani et al. 2013). In a recent report, Zn was found to be important for balancing immune responses against diseases such as COVID-19, as Zn deficiency is often observed in elderly individuals and individuals with chronic diseases (Vogel-González et al. 2021). Hence, the consistent intake of Zn through daily cereal consumption may be one of the most efficient ways to mitigate Zn deficiency.

Rice is a staple food for more than one-third of the global population (Khush 1997). Recently, how Zn is transported to different organs and tissues of rice has been intensively studied. The preferential root-to-grain distribution of Zn is

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mediated by a relay of various transporters localised in specific rice tissues (Huang et al. 2020). The primary Zn uptake from the soil is mediated by OsZIP9, which is localised at the exodermis and endodermis of mature roots (Huang et al. 2020). OsHMA3 (heavy metal P-type ATPase3) has been demonstrated to play a role in the sequestration of Zn to root vacuoles (Cai et al. 2019), and OsHMA2 was revealed to be responsible for root-to-shoot Zn translocation (Takahashi et al. 2012; Yamaji et al. 2013). Two transporters, OsZIP3 and OsHMA2 in nodes, are reported to play roles in distributing Zn to grains. Zn unloading from the xylem of enlarged vascular bundles is controlled by OsZIP3 (Sasaki et al. 2015), whereas Zn loading to the phloem region of diffuse vascular bundles and enlarged vascular bundles is controlled by OsHMA2 (Yamaji et al. 2013). OsZIP7 expressed in parenchyma cells of vascular bundles in roots and nodes was also implicated in the inter-vascular transfer of Zn to grains (Tan et al. 2019). In a recent study, OsZIP4 was found to transport Zn to the phloem of diffuse vascular bundles in the node, leading to the efficient distribution of Zn to tiller buds and other developing tissues (Mu et al. 2020). Additionally, two metallothionein genes, *OsMT2b* and *OsMT2c*, which are highly expressed in node I in rice, were demonstrated to distribute Zn to grains through chelation (Lei et al. 2021). These molecular studies uncovered the mechanism of Zn distribution in rice and will enable the fine tuning of the efficient distribution of Zn in the edible parts of many crops.

There have been several approaches to increase the grain Zn concentration in rice (Mahender et al. 2016; Swamy et al. 2016). Using the transgenic approach, the overexpression of the rice nicotianamine synthase (*OsNAS*) genes has been utilised to produce rice grains biofortified with Fe and Zn in rice (Johnson et al. 2011; Moreno-Moyano et al. 2016). The functional analysis of plant cadmium resistance 1 (PCR1) revealed that knockout and knockdown lines produce grains with higher Zn concentrations, although the grain size is smaller than that of the wild type (Song et al. 2015). Natural variations in wild relatives of cultivated rice are suggested to have great potential to confer better agricultural traits, including increased grain Zn concentrations, because of their large genetic variations and growing environments (Atwell et al. 2014; Ricachenevsky and Sperotto 2016). Several quantitative trait loci (QTL) related to grain Zn levels have been identified using various rice resources (Swamy et al. 2016). However, the introduction of wild genetic resources into cultivated rice can negatively affect yield. For example, fertility defects, as a reproductive barrier, are often observed in hybrids of cultivated and wild rice species (Doi et al. 2008; Koide 2020). These negative effects need to be controlled to improve the agricultural performances of rice breeding. Our previous study showed that wild rice, *Oryza meridionalis*, exhibited the highest grain Zn concentration among wild and cultivated rice species tested (Ishikawa et al.

2017). QTL analysis using segregating populations between *O. meridionalis* W1627 and *O. sativa* Nipponbare detected four loci involved in the differences in grain Zn concentrations. One of these loci, *qGZn9*, was found to contribute the most (Ishikawa et al. 2017). We found that the *O. meridionalis* chromosomal segment covering the *qGZn9* region increased the grain Zn concentration, and the region was further divided into two distinct loci, *qGZn9a* and *qGZn9b*. We also found that an increase in the grain Zn concentrations caused by *qGZn9a* is associated with a reduction in fertility. In this study, we focused on the relationship between grain Zn concentration and fertility to understand the effects of *qGZn9a* on rice breeding. We compared the grain Zn concentrations of plants with and without artificially trimmed fertilised seeds. Additionally, we investigated a possible step for partial sterility associated with *qGZn9a*. We also identified a gene responsible for *qGZn9a* that may be involved in fertility control.

Materials and methods

Plant materials and growth conditions

In this study, we used a *japonica* rice cultivar, *O. sativa* cv. Nipponbare. A near-isogenic line (NIL) with a chromosomal segment of Australian wild rice accession, *O. meridionalis* W1627, in the *qGZn9a* region was generated using one of the backcross recombinant lines with further crossing and genotyping (Ishikawa et al. 2017; Fig. 1a). Nipponbare and NIL(*qGZn9a*) were grown in a greenhouse or paddy field at Kobe University, Japan, during the summer. In the crossing experiment, an *indica* rice cultivar, *O. sativa* cv. IR36, was used as the pollen parent. Two *Tos17*-induced mutants of the candidate genes at *qGZn9a* (NC0405 and ND4542, Nipponbare) were obtained from the National Institute of Agrobiological Sciences of Japan (*Tos17* mutant panel project). The *Tos17* insertion into the genes was confirmed by PCR using genomic DNA with the primers presented in Table S1 online.

Determination of mineral concentrations

Rice grains were de-hulled and dried in an oven at 70 °C. Then, they were digested with concentrated nitric acid (60% [w/v]) at up to 140 °C in a heating block as described previously (Sasaki et al. 2012). After dilution, the mineral concentrations (Zn, Cd, Cu, Fe, Mn, Ca, K, P, and Mg) were determined using inductively coupled plasma-mass spectrometry (ICP-MS, 7700X; Agilent Technologies, USA).

Fig. 1 Comparison of grain zinc (Zn) concentration and fertility of *Oryza sativa* Nipponbare and NIL(*qGZn9a*). **a** Graphical genotype of the NIL(*qGZn9a*). The positions of the markers flanking the W1627 chromosomal segments are displayed. **b** Grain Zn concentration of *O. sativa* Nipponbare and NIL(*qGZn9a*). Data are mean $\pm$ SD of five plants. **significance at the 1% level by unpaired Student's *t* test. **c** Panicle photographs of Nipponbare and NIL(*qGZn9a*). The white triangle indicates a sterile spikelet. Scale bar: 5 cm. **d** Seed fertility of *O. sativa* Nipponbare and NIL(*qGZn9a*). Data are mean $\pm$ SD of five plants. **significance at the 1% level by unpaired Student's *t* test

Evaluation of seed fertility, pollen fertility, and anther dehiscence

Grain fertility was determined based on the average number of filled and unfilled spikelets of five panicles and the average of the seed-setting ratios of five plants was used as a representative ratio in each line. Nipponbare and NIL(*qGZn9a*) panicles just before the flowering stage were fixed in 70% (wt/wt) ethanol and stored in a fridge to be used for pollen fertility analysis. We picked out six anthers from one spikelet, squashed their pollen from the anther on a glass slide, and stained it in 1% (wt/vol) iodine-potassium iodide (I_2 -KI) solution. The stained pollen grains were observed using an Olympus BH2-RFCA (Olympus, Tokyo, Japan). We took three photographs of each spikelet, and calculated the average quality of stained pollen. To determine anther dehiscence, we marked and collected 10 spikelets still opening at 13:00, when most *O. sativa* Nipponbare anthers were dehiscent. Then, we examined the dehiscence status of all six anthers in each spikelet, using a

stereoscopic microscope, and classified anthers in one of the three following categories; complete dehiscence: the anther slits were perfectly widened from apical to basal parts; partial dehiscence: the anther slits were widened only at apical and basal parts; closed: the anther slits were not observed at any part. The anther dehiscence ratio was calculated as the number of anthers classified in each of the aforementioned categories to the total number of anthers. In total, 50 spikelets (five panicles in each line) were evaluated.

Histological analysis of anther structure

Anthers for morphological observation were collected from spikelets of the main panicles just before flowering. They were fixed in an FAA solution (formaldehyde: acetic acid: 70% ethanol = 1:1:18 (volume ratio)) with vacuum infiltration and were preserved at 4 °C. An ethanol series (70%, 80% and 90% ethanol) was used to dehydrate the samples for 2 days at each stage. Then, the samples were embedded in Technovit 7100 resin (Heraeus Kulzer, Germany), according to manufacturer's instructions, and were cut into 3- μ m sections with a rotary microtome, RM1215RT (Leica Biosystems, Germany). The sections were then stained with toluidine blue O solution, observed under a microscope, and photographed with a digital camera using the imaging software.

RNA extraction and qRT-PCR analysis

For tissue and developmental stage-specific expression analysis of candidate genes at *qGZn9a*, *O. sativa* Nipponbare and NIL(*qGZn9a*) were grown in a greenhouse at Kobe University in the summer season. Five parts (flag leaf, lemma, palea, stamen, and pistil) were sampled at three developmental stages defined by the length of the anther (< 1.1 mm, 1.2–1.5 mm, and 1.7–2.0 mm; Fig. S1). Total RNA was extracted using the Plant Total RNA Mini Kit (VIOGENE, New Taipei City, Taiwan) and cDNA was prepared after a DNase I treatment using oligo dT primer and ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan). Quantitative real-time PCR analysis (qPCR) was performed using THUNDERBIRD[®] SYBR qPCR Mix (TOYOBO) with a Light Cycler 96 (Roche, Tokyo, Japan) and the gene-specific primer sets (Table S1). Actin and Ubiquitin were used as internal controls.

Results

Effect of *qGZn9a* on grain Zn concentration

We previously identified *qGZn9* as a QTL for grain Zn concentration detected between *O. sativa* Nipponbare and *O.*

meridionalis W1627 (Ishikawa et al. 2017). The genetic dissection at *qGZn9* resulted in estimating two linked loci, *qGZn9a* and *qGZn9b*, within the 190 kb and 950 kb regions, respectively. Furthermore, plants with a wild chromosomal segment covering *qGZn9a*, but not *qGZn9b*, were associated with fertility reduction. To further understand the relationship between fertility and grain Zn concentration, we first produced a NIL(*qGZn9a*) carrying a 190 kb-chromosomal segment of *O. meridionalis* W1627 covering *qGZn9a* in a Nipponbare genetic background (Fig. 1a). The grain Zn level of NIL(*qGZn9a*) was approximately 1.2 times higher than that of Nipponbare (Fig. 1b). Fertility reduction was observed for NIL(*qGZn9a*) compared with Nipponbare (Fig. 1c and d). These results confirm that the *qGZn9a* region harbours either a gene controlling both grain Zn levels and fertility, or two linked genes individually controlling the two traits.

Fertility reduction was associated with an increase in grain Zn concentration in rice

To investigate whether lower fertility caused an increase in grain Zn levels because of distribution balance, we compared the grain Zn levels of Nipponbare with or without their spikelets cut at the flowering stage. A couple of days after the first spikelet flowered, spikelets were cut using scissors on the flowering day, and we counted the number of seeds in panicles against the total spikelets (Fig. 2a). This cutting treatment artificially inhibited seed setting on the flowering day, leading to a reduction in fertilised seeds. Approximately 40–70% of spikelets were left in the treated panicle (Fig. 2a).

Next, we compared the grain Zn levels of Nipponbare plants based on whether they received the cutting treatment. The average fertility of plants that received the cutting treatment was 54.0%, ranging from 38.4 to 68.7%, which was significantly lower than that without the cutting treatment (83.3%; Fig. 2b). Conversely, the average grain Zn level of Nipponbare with spikelet cut was 46.0 mg kg⁻¹, which was significantly higher than that of plants that did not receive the treatment (38.6 mg kg⁻¹; Fig. 2c). This result implicated that the fertility reduction may lead to an increase in grain Zn levels in rice.

Fertility reduction in NIL(*qGZn9a*) was caused by paternal factor

We found that reduced fertility was associated with an increase in Zn levels (Fig. 2). Next, we analysed a possible cause of the fertility reduction in NIL(*qGZn9a*). We carried out a crossing experiment of Nipponbare and NIL(*qGZn9a*) pollinated with IR36, an *indica* rice cultivar. The average fertility of self-pollination in Nipponbare and NIL(*qGZn9a*) was 93.0% and 74.1%, respectively, with a significant difference (Table 1). However, we found that NIL(*qGZn9a*) pollinated with IR36 had similar fertility levels (90.8%) to Nipponbare (93.4%; Table 1), thereby indicating that the female gametes of NIL(*qGZn9a*) were normal. This crossing experiment indicated that *qGZn9a* did not affect female gamete fertility, and pistils could accept fertile pollen and produce mature seeds. Therefore, the fertility reduction observed for NIL(*qGZn9a*) was possibly male factor-induced.

Fig. 2 Reduction in number of seeds correlated with an increase in grain Zn level. **a** Panicle photographs of *Oryza sativa* Nipponbare with or without their spikelets cut at flowering. The white triangle indicates a spikelet that received a cutting treatment. Scale bar: 5 cm. **b** Fertility of Nipponbare with and without spikelet cut. The seed set-

ting ratio (%) is the number of seeds to the total number of spikelets. Data are mean $\pm$ SD of five plants. **significance at the 1% level by unpaired Student's *t* test. **c** Grain Zn concentrations of treated and untreated Nipponbare. Data are mean $\pm$ SD of five plants. **significance at the 1% level by unpaired Student's *t* test

Table 1 Seed fertility of Nipponbare and NIL(*qGZn9a*) for self-pollination or cross-pollination with IR36

Cross-combination	Plant	Seed fertility (%) for each plant				Average seed fertility (%) ^a	<i>t</i> test ^b
		1	2	3	4		
Self (control)	Nipponbare	93.8	86.4	98.8	92.9	93.0 ± 5.1	**
	NIL(<i>qGZn9a</i>)	71.1	85.7	65.3	74.3	74.1 ± 8.6	
IR36 pollen	Nipponbare	88.2	95.5	95.8	94.1	93.4 ± 3.5	n.s.
	NIL(<i>qGZn9a</i>)	92.7	91.3	94.4	84.6	90.8 ± 4.3	

^aData are mean ± S.D. of four plants^b** and n.s. indicate significant at the 1% level and not significant by unpaired Student's *t* test, respectively

Partial defect in anther dehiscence observed for NIL(*qGZn9a*)

Fertility reduction is often caused by the imperfection of male organs, especially in pollen and anthers (Zhou et al. 2011). To further explore a possible step leading to reduced fertility in NIL(*qGZn9a*), we compared the morphologies of male gametes of Nipponbare and NIL(*qGZn9a*). First, pollen function was investigated by iodine-potassium iodide (I₂-KI) staining (Fig. 3a). We found that both Nipponbare and NIL(*qGZn9a*) had normal pollen contents of 92.0% and 91.3%, respectively (Fig. 3b), demonstrating that pollen function is not affected in NIL(*qGZn9a*). Anther function is also a key factor for proper pollination in many flowering plants (Wilson et al. 2011). The rice anther consists of two bending thecae bound to each other by connecting tissues. Each theca has short and long locules and is connected to the stomium and septum, which are small epidermal cells (Matsui et al. 1999). The dehiscence cavity surrounded by the stomium and septum is the starting point of anther dehiscence. We compared the anther dehiscence status of Nipponbare and NIL(*qGZn9a*) on a flowering day. The dehiscence status of spikelets collected on the day of flowering was determined under a microscope (Fig. 3c). More than 50% of anthers were classified as completely dehiscent in Nipponbare, whereas less than 30% of anthers were classified as completely dehiscent in NIL(*qGZn9a*). Conversely, 70.0% of anthers were classified as partially dehiscent in NIL(*qGZn9a*), which was higher than that in Nipponbare (45.7%; Fig. 3d). In both plant types, the percentages of anthers classified as closed were relatively low. These results indicated that Nipponbare and NIL(*qGZn9a*) exhibited differences in their anther dehiscence status. To further explore anther dehiscence differences, the thickness of the anther locule walls and the smallest number of cells separating the anther dehiscence cavity and the locule were measured based on transverse sections (Fig. 3e–g). No apparent differences in terms of the thickness of the anther locule walls and the number of cells were observed at any of the

three parts in anther (apical part, middle part, and basal part; Fig. 3e–g). Moreover, degradation of the tapetum layer, which is one of the necessary components of pollen development (Yu et al. 2017), was observed in both Nipponbare and NIL(*qGZn9a*) (Fig. 3e). These results demonstrated that the anthers of both Nipponbare and NIL(*qGZn9a*) developed normally and with no apparent phenotypic abnormalities, thereby suggesting that the development of anthers may not be the cause of differences in anther dehiscence rates and that the opening of the anther may have a defect.

Estimation of gene responsible for *qGZn9a*

To identify the gene responsible for *qGZn9a*, we further narrowed down the candidate region between RM24211 and RM24218 (Ishikawa et al. 2017). First, two additional DNA markers estimated that the possible candidate region for *qGZn9a* was between RM24211 and MG15, a 148.6 kb region (Fig. 4a). We also carried out further fine-mapping analysis using 3,354 plants; however, no plants with a recombination between the two markers, RM24211 and MG15, were identified, indicating that recombination may be suppressed in the region. Based on the Nipponbare reference sequence, eight genes were found in the region (Table 2; Fig. 4a). First, we performed a sequencing analysis of these genes and found three genes (*Os09g0383000*, *Os09g0384601*, and *Os09g0384900*) that were deleted in the *qGZn9a* candidate region in W1627 (Table 2; Fig. 4a). We investigated DNA polymorphisms between the five genes of Nipponbare and W1627. Polymorphisms were found to cause amino acid substitutions, but none were found that caused a premature stop codon. We also searched for *Tos17*-inserted mutants at these candidate genes (Miyao et al. 2003). Two genes (*Os09g0382300* and *Os09g0383300*) were found to carry *Tos17* insertion at the exon regions resulting in disruptions of gene function. Their grain Zn levels were similar to those of the Nipponbare controls, suggesting that the two genes are not likely to be involved in Zn regulation (Fig. 4b). We then analysed the expression levels of the eight genes by RT-PCR using Nipponbare and

Fig. 3 Comparison between male reproductive tissues of Nipponbare and NIL(*qGZn9a*). **a** I_2 -KI-stained pollen of Nipponbare and NIL(*qGZn9a*). The red triangle indicates a defective aborted pollen grain (Scale bar: 100 μm). **b** Pollen fertility of Nipponbare and NIL(*qGZn9a*). Values displayed are the mean $\pm$ SD ($n=5$). n.s. indicates no significance by unpaired Student's *t* test. **c** Anther of Nipponbare and NIL(*qGZn9a*) on the flowering day subjected to evaluate anther dehiscence ratio. **d** Anther dehiscence ratio (%) of Nipponbare and NIL(*qGZn9a*). After the spikelets opened, the anther dehiscence status was classified into three categories (complete dehiscence,

partial dehiscence, and closed). Data are mean $\pm$ SD of 5 plants. * and n.s. indicate significance at the 5% level and no significance by unpaired Student's *t* test, respectively. **e** Anther structure by transverse sections with three parts (apical part, middle part, and basal part) in Nipponbare and NIL(*qGZn9a*) (Scale bar: 50 μm). **f, g** The morphological characters of anther transverse sections in Nipponbare and NIL(*qGZn9a*). **f** The thickness of the locule wall in three parts. **g** The number of cell layers separating the cavity for dehiscence and the locule at the two parts. Data are mean $\pm$ SD of five plants. n.s. indicates no significance by unpaired Student's *t* test

NIL(*qGZn9a*) spikelet samples. Five genes (*Os09g0382300*, *Os09g0382400*, *Os09g0382500*, *Os09g0383300*, and *Os09g0383400*) were broadly expressed in all tissues tested, whereas the other two genes (*Os09g0383000* and *Os09g0384601*) were mostly silenced in all spikelet tissues of Nipponbare and NIL(*qGZn9a*) (Fig. 5a, Fig. S2). Interestingly, we found that *Os09g0384900* was specifically expressed in young stamens measuring less than 1.1 mm in Nipponbare (Fig. 5). This result agrees with the gene expression analysis of Nipponbare in the public database

(Rice XPro, Fig. S3). We further carried out qRT-PCR analysis of the eight candidate genes in stamen and confirmed that *Os09g0384900* demonstrated high expression levels specific to the stamen tissue less than 1.1 mm, but not in NIL(*qGZn9a*) (Fig. 5b), as the gene is completely deleted (Fig. 4a). Although other genes were still possible candidates, the specific expression of *Os09g0384900* in the anthers and the deletion in the region in W1627 suggested that *Os09g0384900* was likely to be responsible for low fertility in NIL(*qGZn9a*).

Fig. 4 Candidate genes at *qGZn9a*. **a** Candidate region of *qGZn9a* on chromosome 9. The positions of eight genes are displayed below the chromosome based on the Rice Annotation Project Database. Grey-coloured genes are missing in the corresponding region of *qGZn9a* in W1627. **b** Grain Zn concentration of two *Tos17*-inserted mutants,

NC0405, the insertion on *Os09g0382300*, and ND4542, the insertion on *Os09g0383300*, were obtained. Data are mean $\pm$ SD of 4 and 6 plants for NC0405 and ND4542 compared with Nipponbare wild-type control in 2014 and 2020, respectively. n.s. indicates no significance by unpaired Student's *t* test

Table 2 List of the candidate genes for *qGZn9a*

Gene locus ID ^a	Description ^a	Sequence (Nipponbare and W1627)
<i>Os09g0382300</i>	Cyclin-D2-1	+ 3 V, V22F, + 45G, + 46G, P72R, N99Y, -74S, -75A, A76S, R304S
<i>Os09g0382400</i>	Conserved hypothetical protein	V10A, + 39G
<i>Os09g0382500</i>	Conserved hypothetical protein	A9S, P19A, P29L, G74S
<i>Os09g0383000</i>	Plant disease resistance response protein domain containing protein	Deletion in W1627
<i>Os09g0383300</i>	Hypothetical conserved gene	N191H, P238S
<i>Os09g0383400</i>	DEAD-like helicase, N-terminal domain containing protein	R18H, + 25 T, + 31R, + 32R, R180C, V186I, Q300G, N415S
<i>Os09g0384601</i>	Hypothetical gene	Deletion in W1627
<i>Os09g0384900</i>	Protein of unknown function DUF295 family protein	Deletion in W1627

^aGene locus ID and description are based on the Rice Annotation Project Database (Sakai et al. 2013)

Discussion

Partial sterility of NIL(*qGZn9a*)

In this study, we found that the anther dehiscence ratio of NIL(*qGZn9a*) was lower than that of Nipponbare (Fig. 3d). As the pollen function of NIL(*qGZn9a*) was standard as compared with that of Nipponbare (Fig. 3a and b), the slight difference in anther dehiscence may cause partial sterility. The timing of anther dehiscence is critical for fertilisation. A delay in anther dehiscence may result in an increase in inactivated pollen before pollination owing to the shortness of longevity (Pacini and Dolferus 2019). Therefore, the limitation of functional pollen for fertilisation may be the cause of partial sterility. Several other traits related to anther dehiscence may also be involved. For example, pollen swelling is an essential factor in anther dehiscence. Matsui et al. (2000) revealed that the pollen grain diameter that increased

by swelling after flowering was affected by the degree of anther dehiscence in two-rowed barley. Rapid pollen swelling presses the anther locule walls, leading to the breaking of the stomium. Further morphological analyses of pollen and anthers are required to understand the possible step for partial sterility caused by *qGZn9a*.

Estimation of gene for *qGZn9a*

Among the eight genes located in the *qGZn9a* candidate region, *Os09g0383000*, *Os09g0384601*, and *Os09g0384900* were not present in the *qGZn9a* region of *O. meridionalis* W1627, suggesting that one of them is a gene responsible for grain Zn levels via fertility regulation (Table 2; Fig. 4a). We searched for *Tos17*-inserted mutants for the eight genes and found that *Os09g0382300* and *Os09g0383300* had a *Tos17* insertion at their exons. Comparing the grain Zn levels of *Tos17* inserted lines and Nipponbare revealed no apparent

Fig. 5 Expression analysis of the candidate genes at *qGZn9a*. **a** RT-PCR analysis of eight candidate genes in spikelet tissues of Nipponbare based on three developmental stages of stamen. **b** Gene

expression levels of eight candidate genes at *qGZn9a* based on three developmental stages of stamen in Nipponbare and NIL(*qGZn9a*) analysed by qRT-PCR. Data are mean $\pm$ SD of four plants

differences, indicating that the two genes are unlikely to be the candidate gene at *qGZn9a* (Fig. 4b). We also compared sequences of the other three genes and found several polymorphisms between Nipponbare and W1627 (Table 2). Although the polymorphisms caused amino acid changes, they did not cause any premature stop codons. Among the three genes missing at the *qGZn9a* region in W1627, we noticed that *Os09g0384900* is specifically expressed in the developing anther in Nipponbare (Fig. 5a and b). Although the function of the gene remains to be elucidated, its specific expression in anthers and deletion in W1627 suggest that *Os09g0384900* is likely to be a candidate gene at *qGZn9a*. As W1627 demonstrates normal fertility, the gene may be translocated in other chromosomal regions apart from *qGZn9a* in W1627. According to the annotation of *Os09g0384900* in the Rice Annotation Project Database (Sakai et al. 2013), the gene encodes a protein of the domain of unknown function DUF295. A recent genome-wide study of the DUF295 domain in *Arabidopsis* revealed that the DUF295 domain likely originated in angiosperms (Lama et al. 2019). Furthermore, domain structure analysis of proteins carrying DUF295 demonstrated that the domain is in the C-terminal region and accompanied by the F-box domain in the N-terminal region, suggesting that the proteins are involved in the protein degradation pathway. The DUF295 gene family consists of 94 genes in *Arabidopsis*, especially expanded in Brassicaceae. Notably, most of the DUF295 genes in *Arabidopsis* were found to be expressed under specific tissues or conditions. Of these specifically expressed genes, approximately 20 genes were upregulated in the anther (Lama et al. 2019), as observed for *Os09g0384900*, a possible candidate gene for *qGZn9a*. In *Arabidopsis*, *KIB1*, which encodes a protein containing F-box and DUF295 domains, was recently identified through the functional analysis of a kink suppressed in a *bzr1-1D* mutant and found to be involved in the brassinosteroid-mediated signalling pathway (Zhu et al. 2017). *KIB1* was demonstrated to bind BIN2 and promote its degradation, which prevents BIN2-substrate interactions. These studies implied that the DUF295 proteins coupled with the F-box domain may degrade specific target proteins in specific tissues to control plant development and signalling pathways. Although the confirmation of the involvement of *Os09g0384900* in the *qGZn9a* effect by complementation (or producing a knockout line) is required, a functional analysis of *Os09g0384900* regarding rice fertility and grain Zn levels will be interesting and critical in future research.

Balancing grain Zn concentration in rice

In this study, we found that lower fertility correlated with the upregulation of grain Zn levels. In an additional experiment, we found that when Nipponbare spikelets

were artificially cut on the flowering day, seeds had a higher Zn concentration than the control (Fig. 2). The survey of additional mineral elements (Cd, Cu, Fe, Mn, Ca, K, P, and Mg) in the artificial cutting of Nipponbare spikelets found that only Zn concentration was significantly different from controls (Table S2). Currently, we do not know the reason why Zn is specifically affected by fertility levels, but this could be attributed to an attempt to balance the grain number and Zn distribution. One possible explanation for the relationship between fertility and grain Zn concentrations may be a trade-off between grain numbers and Zn distribution. Based on the hypothesis that a reduction in fertile spikelets causes an accumulation of Zn in each grain, a high grain number per panicle may cause the downregulation of grain Zn levels. Much effort has been put into increasing grain numbers to support the growing population during rice domestication (Zuo and Li 2014; Ishikawa et al. 2020), and this goal will be among the future breeding scope. It would be of interest to compare the grain Zn levels of high-yielding varieties. Although in this study we aimed to identify the causal gene at *qGZn9a* and one possible candidate was estimated, our results also highlighted the important regulatory relationship between the number of grains and Zn levels in rice. It would be desirable to have high-yielding rice cultivars without reduced Zn quantities in the future.

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Author contribution's statement RI conceived and designed the study. MO, NM, GM, KT, SL, MI and RI performed the experiments. MO, NM, GM, KT, MI, TI, JFM and RI analysed the data. MO and RI prepared the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interests: The authors declare no conflicts of interest.

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