

Correlation of expressional pattern of Ubiquitin activating gene with grain Fe content in rice

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ABSTRACT

Ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligases (E3) and 26S proteasome are the significant components of the ubiquitination process. The expression level of ubiquitin activating (UBA) gene was evaluated in 18 genotypes with differential grain iron (Fe) and zinc (Zn) (including landraces, high yielding and released biofortified varieties) at panicle initiation stage. The relative expression of the UBA gene was analysed with three yield checks- BPT 5204, MTU 1010 and Swarna and the highest gene expression level were found in land race Kalanamak followed by Jalpriya and Taroari Basmati. Significant positive correlation of expression of the UBA gene with grain Fe content suggests the possible role of ubiquitin activating enzymes in addition to the reported ubiquitin-conjugating and ligases in Fe homeostasis. The understanding of ubiquitination regulated nutrient transport mechanism is yet to be explored hence, more in-depth studies in future may provide better insight to understand the role of ubiquitination in nutrient homeostasis and develop better strategies for biofortification.

Key words: Ubiquitin activating (UBA) gene, rice, grain iron, expression, correlation

Ubiquitination, the post-translational modifier plays key role in regulatory mechanisms of plant growth, development and stress signalling (Sadanandom et al., 2012). Ubiquitin is a small globular protein of about 76 amino acids, highly conserved in all eukaryotic cells with a wide range of functions and it conjugates to a large range of proteins for degrading them through ubiquitin-proteasome pathway or ubiquitination process (Belknap & Garbarino, 1996). The three main enzymes involved in ubiquitination cascade are categorized as ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligases E3. The first step is the ubiquitin activation where E1 binds to MgATP and ubiquitin, and catalyzes ubiquitin C-terminal acyl-adenylation. In the next step, the catalytic cysteine (Cys) in the E1 attacks the ubiquitin~adenylate and forms the activated ubiquitin~E1 complex and passed it to Cys residue of E2 conjugating enzyme. In the last step, ubiquitin is passed to lysine (Lys) residue of the target protein through coordinated function of E3 ubiquitin

ligase (Zhiguo et al., 2015) either directly through Homology to the E6-associated protein C-terminus (HECT)-type E3s or indirectly through Really interesting new gene (RING), U-box and Cullin-based types E3s (Mandal et al., 2018). The polyubiquitinated-target protein is then degraded by the 26S proteasome. Ubiquitin mechanism plays vital role during several plant life processes from embryogenesis, hormone regulation, immune responses, DNA repair, chromatic remodeling to senescence (Yee & Goring, 2009). Ubiquitin-proteasome pathway is suggested to be one of the adaptation and survival strategies to different environmental stresses (Yang et al., 2010; Zhou et al., 2017). In rice, ubiquitination pathway was proposed as a probable target for improvement of abiotic stress tolerance (Dametto et al., 2015). Apart from its stress responsive mechanisms, ubiquitination has also been reported to have function in nutrient homeostasis in plants through control of plasma membrane proteins (Yates & Sadanandom, 2013). The proteasome-

mediated degradation through ubiquitination and its role in iron (Fe) homeostasis has been widely studied in Arabidopsis (Eroglu & Aksoy, 2017). Post transcriptional ubiquitination found to help in the processing of IRON-REGULATED TRANSPORTER 1 (IRT1) (Kerkeb et al., 2008). Kobayashi et al., 2013 identified new Fe-binding regulator zinc (Zn)-finger protein 1 viz., *OsHRZ1* and *OsHRZ2* those also binds to Zn and have protein ubiquitination activity mediated by RING Zn-finger domains in rice. Differential expression of *OsHRZ1* and *OsHRZ2* under Fe deficiency and sufficient conditions was also reported (Kobayashi et al., 2014). Comparative transcriptome analysis of rice and wheat genotypes with differential grain Zn and Fe revealed enhanced ubiquitin related genes (Mishra et al., 2019; Neeraja et al., 2018). Globally, malnutrition is estimated to have affected 2 billion human population (FAO, 2018) and out of which about 1 billion is in India itself (Ritchie et al., 2018). Fe, Iodine, Zn and Vitamin A are among the most prevalent and widespread form of nutrient deficiencies affecting a larger proportion of the global population. Staple crops loses most of its Fe/Zn content during post-harvest milling and processing, that failing to provide an sufficient supply of Fe and Zn, leading to malnutrition in human population (Hefferon, 2018). Hence, biofortification is considered to be the most effective and sustainable strategy to enhance the micronutrient levels of Fe and Zn and its bioavailability in staple crops (Cheema et al., 2018; Wakeel et al., 2018). Rice biofortification is the desirable target among cereals, since it is one of the most consumed staple crops especially in Asia where it the central dietary source of carbohydrate (Kok et al., 2018). In popular rice varieties, the polished grains contain approximately 2 ppm of Fe and 16 ppm of Zn. The targeted concentration of Fe and Zn in polished rice grains is 13 ppm and 28 ppm respectively, in order to reach 30% estimated average requirement (EAR) (Trijatmiko et al., 2016). Although, majority of previous studies have identified several metal chelate-transporter and phytosiderophore biosynthesis pathway related enzymes coding genes which are responsible for improving Fe/Zn content but still some gaps remains to fully understand the mechanism of Fe/Zn uptake from roots (source), transport, translocation and loading onto the developing grains (sink) (Banerjee et al., 2010). Several novel mechanisms were proposed to play vital role in plant

mineral dynamics in addition to the known mechanisms. Ubiquitination was proposed to be one of the mechanisms associated with protein turnover and nutrient homeostasis by regulating the movement of membrane-bound transporters (Sperotto et al., 2014). The extent of ubiquitination in nutrient transport and compartmentalization is yet to be elucidated in cereal crops for their deployment in biofortification studies. Considering all these points, the current study, we have evaluated the expression level of ubiquitin-activating enzyme gene (*UBA*) in 18 genotypes (including high yielding and released biofortified varieties) at panicle initiation stage and correlated the expression level with the grain Fe and Zn content. Most of the studies have reported differential tissue specific-expression of ubiquitin conjugating and ubiquitin ligase genes under abiotic stresses such as drought, cold, salt stresses and hormone treatments such as IAA, 6-BA, GA and ABA in rice (Lourenço et al., 2013; Zhiguo et al., 2015), while a few groups studied on ubiquitin activating gene (Agrawal et al., 2016; Hatfield et al., 1997). In nutrient homeostasis of nitrogen, phosphorous and other minerals, the role of ubiquitin conjugating and ligases have been extensively reported (Kobayashi et al., 2014; Liu et al., 2017; Pan et al., 2019; Rodríguez-Celma et al., 2019; Yates & Sadanandom, 2013). Rice has six ubiquitin-activating, 39 ubiquitin-conjugating and over 1300 ubiquitin-ligase genes which is justified by their role in maintaining substrate specificity (Du et al., 2009; Zhiguo et al., 2015; Mandal et al., 2018). Ubiquitin-activating enzyme is 110-125 kD size having four conserved domain, plays the crucial initial step in the pathways regulating the rate of ubiquitination. *The UBA* genes have been studied in Arabidopsis, tobacco, wheat and soybean (Hatfield et al., 1997; Mandal et al., 2018; Takizawa et al., 2005). In rice roots, PEG induced drought stress was reported in 2-fold increase of ubiquitin activating enzyme (*OsC-4815*). Nutrient uptake and homeostasis studies have largely focussed on the genes encoding ubiquitin-conjugating and ubiquitin ligases, hence, we selected to study the expression of the *UBA* gene and to determine whether its relation to micronutrient homeostasis.

Eighteen rice genotypes having different grain Zn and Fe content viz., Swarna, BPT 5204, Jaya, MTU 1010, Savitri, PR 116, Ranbir Basmati, Jalpriya, AkutPhou, ARB-45, High Iron Rice, Kadamkudy

Pokkali, Kalanamak, Kasturi, Mima Local, Seetasail, Taroari Basmati and Tilakkachari were selected and sown in pots arranged in a complete randomized block design with three biological replicates and maintained at nethouse of ICAR-Indian Institute of Rice Research (IIRR), Hyderabad. The grains were harvested from the *kharif* season of consecutive 3 years 2015-2017 for grain Zn/Fe estimation. The grains were dehusked using JLGJ4.5 testing rice husker (Jingjian Huayuan International Trade Co. Ltd) and polished using polisher (Krishi International India Ltd.) having non-ferrous and non-zinc components and was analysed by energy dispersive X-ray fluorescentspectrophotometer (ED-XRF) (OXFORD Instruments X-Supreme 8000) according to Rao et al., 2014. Total RNA was isolated using NucleoSpin RNA Plant kit (Macherey-Nagel, Germany) from the booting stage panicle tissues of the 18 genotypes and the RNA quality was assessed using Nanodrop® ND1000 spectrophotometer (Thermo Scientific, USA). cDNA synthesis was performed using transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) and diluted to 1:10 to use as template for qPCR. Each qPCR reaction was performed in three technical replicates using FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany) with the following program 95°C (2 min) followed by 40 cycles of 95°C (5 s), 58°C (30 s) with fluorescent signal recording and 72°C for 30 min LightCycler 96 system (Roche Diagnostics, Mannheim, Germany). The relative expression of the *UBA* gene was analysed using the $2^{-\Delta\Delta C_t}$ method according to (Schmittgen & Livak, 2008) with yield checks- BPT 5204, MTU 1010 and Swarna as control (Neeraja et al., 2018) and normalized using internal control gene *Memp* (Membrane protein) (Phule et al., 2018) (Table 1).

The mean values of grain Zn and Fe content in both brown and polished rice of 18 genotypes across three consecutive years (*Kharif* 2015-2017) were considered for the analyses (Table 2). Lack of consistent

Table 2. Fe and Zn content in brown and polished grains of all genotypes.

Sl no.	Genotype	Brown (ppm)		Polished (ppm)	
		Fe	Zn	Fe	Zn
1	AkutPhou (VR)	9.30	30.17	4.23	25.97
2	ARB-45 (BR)	9.93	28.87	3.67	23.73
3	BPT5204 (VR)	8.13	15.08	1.76	12.7
4	High Iron Rice (LR)	9.43	29.33	3.43	24.33
5	Jalpriya (VR)	12.3	26.4	3.3	13.2
6	Jaya (VR)	7.14	18.73	1.64	15.14
7	KadamkudyPokkali (LR)	9.17	29.23	2.47	23.17
8	Kalanamak (LR)	12.73	22.83	4.87	19.47
9	Kasturi (VR)	10.90	26.77	4.77	24.63
10	Mima (LR)	13.13	29.10	2.09	23.26
11	MTU 1010 (VR)	8.23	14.87	1.67	12.93
12	PR 116 (VR)	7.83	19.77	1.50	11.93
13	Ranbir Basmati (VR)	9.17	22.07	1.70	16.80
14	Savitri (VR)	8.53	21.60	2.80	16.70
15	Seetasail (LR)	10.80	28.83	4.10	22.80
16	Swarna (VR)	7.17	17.73	1.80	12.80
17	Taroari Basmati (PL)	9.83	31.30	4.60	25.70
18	Tilakkachari (LR)	8.00	26.93	4.03	21.20

VR: released varieties; BR: breeding lines; LR: landraces; PL: pure line

values in Fe/Zn content for particular genotypes have been reported in rice with variations being caused by several factors including environment and genotype interactions (Nachimuthu et al., 2014). The Fe content ranged from 7.14 ppm to 13.13 ppm in brown and 1.50 ppm to 4.87 ppm in polished grains, whereas Zn content ranged from 14.87 ppm to 31.30 ppm in brown and 11.93 ppm to 25.97 ppm in polished rice. Among all the genotypes, AkutPhou, Taroari Basmati and High Iron Rice were found to have higher Fe/Zn content in both dehusked and polished grains. The polishing of rice grains removes the bran which contains the aleurone layer containing more than 85% of the Fe content, the remaining starchy endosperm contains very low amount of Fe and Zn (Ishimaru et al., 2010; Sperotto et al., 2012). A similar trend was observed in genotypes Mima

Table 1. Primer details of reference and target gene used in the qPCR

Sl no.	Gene name	Abbreviation	Primer sequence
1	Membrane Protein	<i>Memp</i>	GAGCGCAAAGTTCCAGAAGAA CGCCACTAGTTGCCGTCTGAT
2	Ubiquitin activating enzyme E1	<i>UBA</i>	CTGGTTTTGCTAACATGCGG ACCACCGAGTACCTTGACA

local, Jalpriya, Kalanamak and DRRDhan 45 where the reduction in grain Fe was found to be 84.08%, 73.17%, 61.74% and 71.53% respectively. For grain Zn content, the polishing was found to cause about ~15% reduction in most of the genotypes whereas in Jalpriya the reduction was observed more than 50%. Genotypic variation of grain Zn in polished rice has been reported to be differential translocation ability from aleurone to the endosperm (Bollinedi et al., 2020). Based on the Fe and Zn content, the 18 genotypes can be

divided into three groups as high, moderate and low. In Fe content, genotypes with lower than 2 ppm was considered as low (BPT 5204, Jaya, MTU 1010, PR 116, Ranbir Basmati and Swarna), with range 2 ppm - 3.9 ppm was categorized as moderate (ARB-45, High Iron rice, Jalpriya, KadamkudyPokkali, Mima Local and Savitri) and higher than 4 ppm was considered as high (Aktphou, Kalanamak, Kasturi, Seetasail, Taroari Basmati and Tilakkachari). In case of Zn content, within

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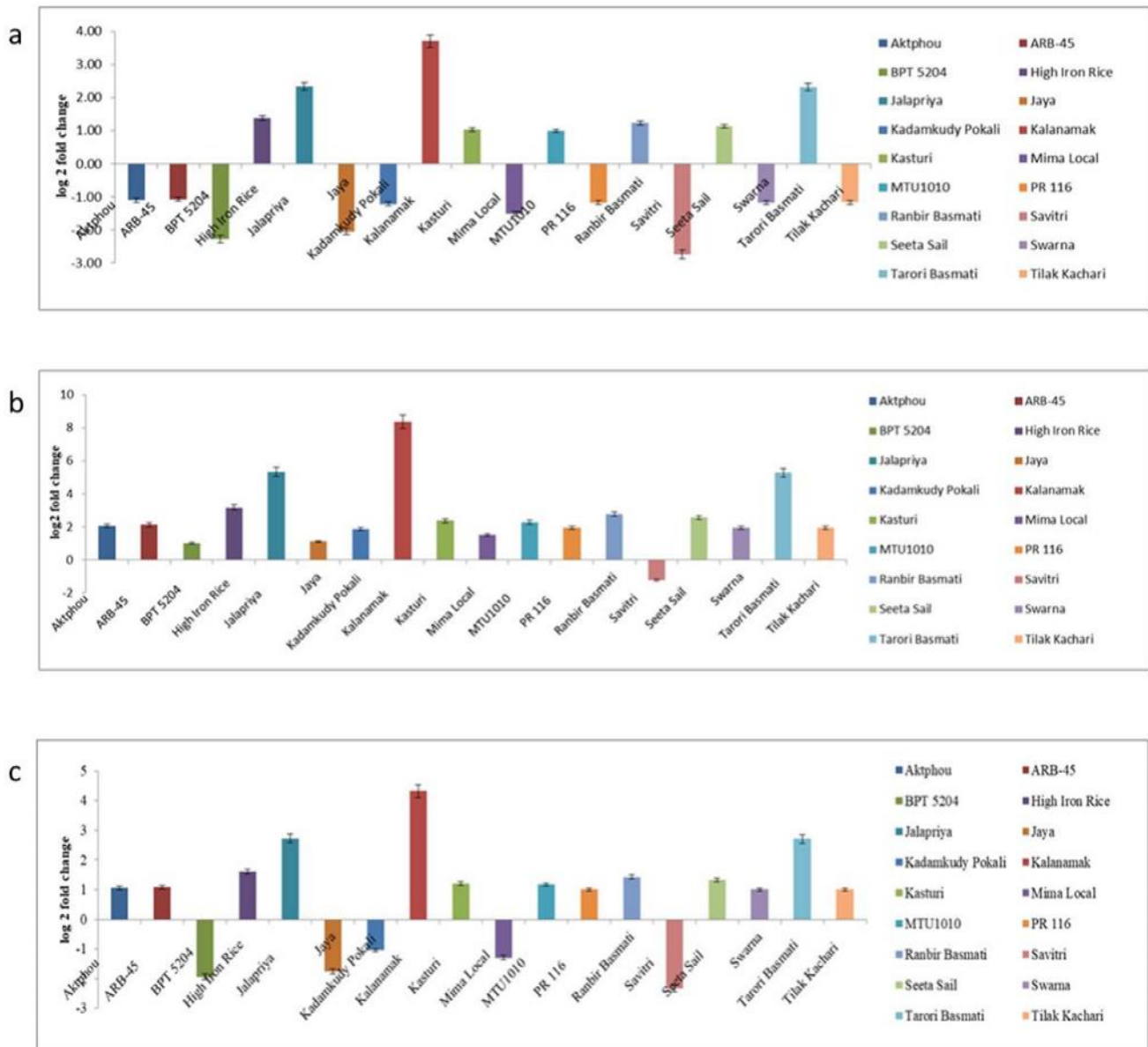


Fig. 1. Relative expression of *UBA* gene among 18 genotypes compared to yield checks viz., (a) MTU 1010, (b) BPT 5204 and (c) Swarna was normalized using *Memp* as internal control.

range 24 ppm - 25 ppm was categorized as high (AkutPhou, High Iron rice, Kasturi and Taroari Basmati), within range 20 ppm - 24 ppm was considered as moderate (ARB-45, KadamkudyPokali, Mima Local, Seetasail and Tilakkachari) and below 20 ppm was considered as low (BPT 5204, Jalpriya, Jaya, Kalanamak, MTU 1010, PR116, Ranbir Basmati, Savitri and Swarna). The differential expression of the *UBA* gene was obtained by quantitative real time PCR and the relative fold change was calculated using the $\Delta\Delta C_t$ method by comparing the fold change relative to high yield checks- MTU 1010, BPT 5204 and Swarna (Fig. 1 a, b,c & 2 a, b). The comparative expression of *UBA* with MTU 1010 was found to be up regulated in genotypes Kalanamak (3.71), Jalpriya (2.34), Taroari Basmati (2.33), High Iron Rice (1.39), Seetasail (1.13), Ranbir Basmati (1.23) whereas with BPT 5204, the gene expression was found to be up-regulated in all genotypes except Savitri (-1.21) and highest in Kalanamak (8.37) (Supp Table 1). The expression relative to yield check Swarna a few genotypes BPT 5204 (-1.94), Jaya (-1.75), KadamkudyPokkali (-1.04), Mima Local (-1.29) and Savitri (-2.33) were found to have negative fold change of the *UBA* gene (Supplementary Table 1). The genotypes having higher expression of *UBA* compared with all the three yield checks were found to be highest in Kalanamak followed by Jalpriya and Taroari Basmati and moderate expression was found in High Iron Rice, Ranbir Basmati, Kasturi, and Seetasail (Fig. 1 a, b, c). The correlation between the fold change of *UBA* gene and grain Fe/Zn content was determined using R software version 3.2.4. The Pearson correlation between fold changes relative to BPT 5204 to grain Fe/Zn content found to be 0.62

and 0.57 with Fe content in brown and polished grains respectively (Fig. 3 a & d). The correlation of Fe content in brown and polished grains with fold change relative to yield check MTU 1010 was 0.59 and 0.51 whereas 0.45 and 0.59 with yield check Swarna respectively (Fig. 3 b, c, e & f). The correlation of fold change with grain Zn content was not found to be significant. The correlation study of the expression pattern of *UBA* gene with grain Fe content shows the probable role of ubiquitin activating enzyme E1 in Fe homeostasis.

The vital role of ubiquitination in Fe homeostasis through endocytosis processing of IRT1 which in turn plays vital role in Fe uptake at the transcriptional level and post-transcriptionally ubiquitination has already been demonstrated (Kerkeb et al., 2008). In the root epidermal cells of Arabidopsis, IRT1 was found localized to early endosomes/trans-Golgi network (EE/TGN) for taking up Fe from the soil and its rapid recycling to the plasma membrane. Mono-ubiquitination of Lys residues on IRT1 resulted in vacuole sorting while mutation in these Lys residues was found to stabilize the plasma membrane leading to extreme lethality (Barberon et al., 2011). Shin et al. (2013) identified a RING-type E3 ubiquitin ligase, designated as IRT1 DEGRADATION FACTOR 1 (IDF1), was found to be involved in IRT1 degradation process. A similar finding was reported in Arabidopsis BOR1 boron transporter, at high boron concentrations mono- or diubiquitination induced BOR1 was observed and mutation of single Lys residue affected the vacuole trafficking of BOR1 (Takano et al., 2005; Zelazny et al., 2011). Kobayashi et al., 2013 identified new Fe-binding regulators zinc (Zn)-finger

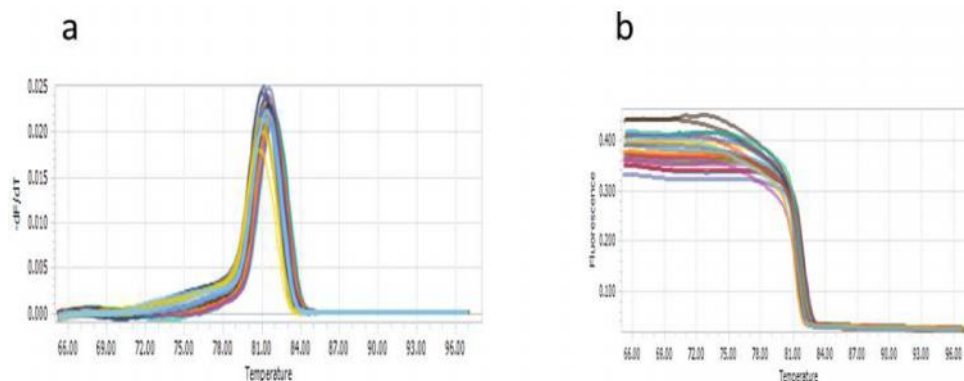


Fig. 2. (a) Melting peak and (b) melting curve of *UBA* gene showing T_m at 82°C.

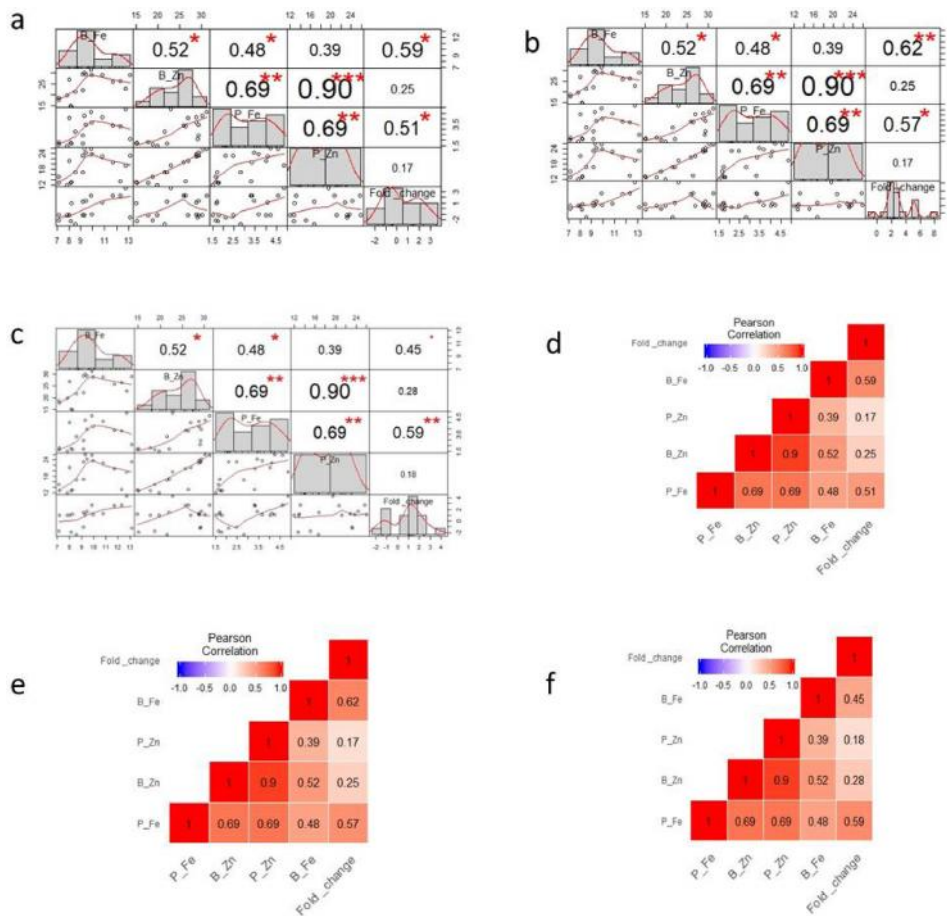


Fig. 3. Pearson correlation of fold change relative to (a) BPT 5204, (b) MTU 1010 and (c) Swarna with grain Fe/Zn content in all genotypes. The GGplot graph showing correlation of fold change with grain Fe/Zn content relative to (d) BPT 5204, (e) MTU 1010 and (f) Swarna.

protein 1 (*OsHRZ1*) and *OsHRZ2* binding to Zn and have protein ubiquitination activity mediated by RING Zn-finger domains. Under Fe deficiency conditions, the expression of *OsHRZ1* and *OsHRZ2* was found to be induced, while under Fe sufficient conditions, *OsHRZ1* and *OsHRZ2* acts as negative regulators preventing excess Fe uptake (Kobayashi et al., 2014). Interestingly, Yang et al., 2018 revealed differential expression of ubiquitin/proteasome 26S system by transcriptome comparison of two contrasting ecotypes of Zn/cadmium (Cd) hyper accumulator *Sedum alfredii* Hance species. Comparative transcriptome analysis of four high- and three low-grain Zn and Fe containing wheat genotypes showed two ubiquitin related genes (E3 ligase and ubiquitin dependent protein) specific to the genotypes with high nutrients (Mishra et al., 2019). Panicle transcriptome study on two landraces and a widely

grown popular variety BPT with differential Zn in the polished rice reported higher fold change of a gene encoding putative ubiquitin conjugating enzyme 7 interacting protein (Neeraja et al., 2018). In the present study, the role of ubiquitin activating enzyme has been shown to be associated with the nutrient metabolism in addition to the reported roles of ubiquitin conjugating and ligating enzymes. To enhance the grain Fe/Zn content and its bioavailability in staple crops using conventional or transgenic approach requires the understanding of the mechanisms of the nutrient uptake, translocation and loading of the minerals - Fe/Zn. Genetic variability having varying Fe/Zn content has been observed in diverse genotypes of rice which if exploited by genetic strategies can increase Fe/Zn concentrations in sink tissues (grains). Studies have established the role of ubiquitination and ubiquitin

enzymes in the uptake and trafficking of essential micronutrients in plants but mechanism needs to be deciphered in detail. In our study, we have observed positive correlation of grain Fe content with expression of ubiquitin activating gene *UBA* in rice landraces, suggesting the potential role of *UBA* in grain Fe homeostasis. However, the understanding of ubiquitination regulated nutrient transport and homeostasis is still in its infancy depth study in this field may provide better insight to understand and develop better strategies for biofortification.

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