

**DIFFERENTIAL EXPRESSION OF
MICRONUTRIENT TRANSPORTER GENES IN
BIO-FORTIFIED RICE (*Oryza sativa* L.) GRAINS
AMONG SUPER-ELITE RICE ACCESSIONS
UNDER AEROBIC CONDITION**

MOUNASHREE, D. C.

PALB 5273

**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU – 560 065**

2017

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Thesis submitted to the
UNIVERSITY OF AGRICULTURAL SCIENCES, BENGALURU

*In partial fulfillment of the requirements
for the award of the degree of*

**MASTER OF SCIENCES (Agriculture)
in
PLANT BIOTECHNOLOGY**

BENGALURU

JULY, 2017



♥ Affectionately ♥ Dedicated to
♥ My Beloved ♥ Grand Father
♥ Sri Chikkae ♥ Gowda
♥ My Parents ♥ & Brother

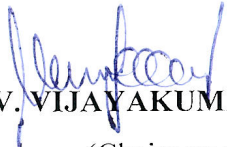
**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
GKVK, BENGALURU – 560 065**

CERTIFICATE

This is to certify that the thesis entitled, “**DIFFERENTIAL EXPRESSION OF MICRONUTRIENT TRANSPORTER GENES IN BIO-FORTIFIED RICE (*Oryza sativa* L.) GRAINS AMONG SUPER-ELITE RICE ACCESSIONS UNDER AEROBIC CONDITION** ” submitted in partial fulfilment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** to the University of Agricultural Sciences, GKVK, Bengaluru-560 065, is a bonafide record of research work done by **Ms. MOUNASHREE, D. C., ID. No. PALB 5273** during the period of her study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

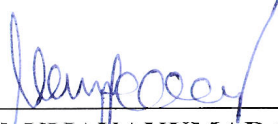
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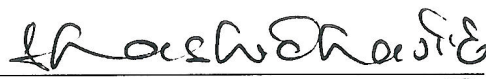
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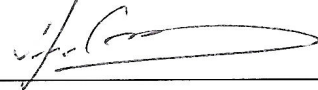

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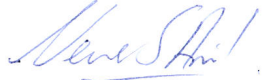
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Bengaluru,
July, 2017

(Mounashree, D. C.)

Differential Expression of Micronutrient Transporter Genes in Bio-fortified Rice (*Oryza sativa* L.) Grains among Super-elite Rice Accessions under Aerobic Condition

MOUNASHREE, D. C.

ABSTRACT

Ten super-elite genotypes were selected to discern the relative expression of micronutrient transporter gene in different parts of rice grain. The genotypes were grown under aerobic condition adopting RCBD design during *Kharif* -2016. High PCV, GCV coupled with high heritability and GAM was observed for plant height and white rice iron content. Significant positive correlation was observed for total number of tillers with iron and zinc productivity per day. Productivity per day, iron and zinc productivity per day revealed significant positive correlation with biomass and harvest index. Molecular analysis of relative expression of three seed specific transporters was done. AM-72 endosperm had relatively higher expression of OsZIP4a and OsYSL2b than in any other genotypes. Black rice suffered highest percent loss of zinc and iron due to polishing and the genotype had relatively higher expression of OsZIP4b in its embryo and endosperm when compared to other genotypes. ARB-6 manifested less percent loss of zinc due to polishing, had relatively higher expression of OsYSL2b and OsYSL15b in its endosperm and relatively lower or negligible expression of OsYSL15b in its embryo.

July 2017

Department of Plant Biotechnology
UAS, GKVK, Bengaluru

(H.V. Vijayakumar Swamy)
Major Advisor

ಏರೋಬಿಕ್ ಪರಿಸ್ಥಿತಿ ಅಡಿಯಲ್ಲಿ ಸೂಪರ್-ಗಣ್ಯ ಭತ್ತ ಪ್ರವೇಶಗಳ ನಡುವೆ ಜೈವಿಕ-
ಬಲವರ್ಧಿತ ಭತ್ತ (ಓರ್ಬಿಸಾ ಸಟೈವಾ ಎಲ್.) ಧಾನ್ಯಗಳಲ್ಲಿ ಸೂಕ್ಷ್ಮಪೋಷಕಾಂಶಗಳ
ವರ್ಗಾವಣೆ ವಂಶವಾಹಿನಿಗಳಲ್ಲಿ ವಿಭಿನ್ನ ಅಭಿವ್ಯಕ್ತಿತೆ

ಮೌನಶ್ರೀ ಡಿ ಸಿ

ಪ್ರಬಂಧ ಅಮೂರ್ತ

ಭತ್ತ ಧಾನ್ಯದ ವಿವಿಧ ಭಾಗಗಳಲ್ಲಿ ಸೂಕ್ಷ್ಮಪೋಷಕಾಂಶಗಳ ವರ್ಗಾವಣೆ ವಂಶವಾಹಿನಿ ಸಾಪೇಕ್ಷ ಅಭಿವ್ಯಕ್ತಿಗಳನ್ನು ಗ್ರಹಿಸಲು ಹತ್ತು ಸೂಪರ್-ಗಣ್ಯ ವಂಶವಾಹಿಗಳನ್ನು ಆಯ್ಕೆಮಾಡಲಾಯಿತು. ಮುಂಗಾರು-೨೦೧೬ ಅವಧಿಯಲ್ಲಿ ಆರ್ ಸಿ ಬಿ ಡಿ ವಿನ್ಯಾಸವನ್ನು ಅಳವಡಿಸಿ, ಏರೋಬಿಕ್ ಸ್ಥಿತಿಯಲ್ಲಿ ವಂಶವಾಹಿಗಳನ್ನು ಬೆಳೆಸಲಾಯಿತು. ಉನ್ನತ ಪಿಸಿವಿ, ಜಿಸಿವಿ ಹೆಚ್ಚಿನ ಅನುವಂಶಿಕತೆಯೊಂದಿಗೆ ಸೇರಿಕೊಂಡಿತ್ತು ಮತ್ತು ಸಸ್ಯ ಎತ್ತರ ಮತ್ತು ಬಿಳಿ ಅಕ್ಕಿ ಕಬ್ಬಿಣದ ಅಂಶಗಳಿಗೆ ಜಿ.ಎ.ಎಮ್ ಅನ್ನು ಗಮನಿಸಲಾಯಿತು. ದಿನಕ್ಕೆ ಕಬ್ಬಿಣದ ಮತ್ತು ಸತು ಉತ್ಪಾದಕತೆಯೊಂದಿಗೆ ಉಳುಮೆಗಳ ಒಟ್ಟು ಸಂಖ್ಯೆಗಳಿಗೆ ಗಮನಾರ್ಹವಾದ ಧನಾತ್ಮಕ ಪರಸ್ಪರ ಸಂಬಂಧವನ್ನು ಗಮನಿಸಲಾಯಿತು. ದಿನಕ್ಕೆ ಉತ್ಪಾದಕತೆ, ದಿನಕ್ಕೆ ಕಬ್ಬಿಣ ಮತ್ತು ಸತು ಉತ್ಪಾದಕತೆಯು ಜೀವರಾಶಿ ಮತ್ತು ಸುಗ್ಗಿಯ ಸೂಚ್ಯಂಕದೊಂದಿಗೆ ಗಮನಾರ್ಹ ಸಕಾರಾತ್ಮಕ ಸಂಬಂಧವನ್ನು ಬಹಿರಂಗಪಡಿಸಿತು. ಮೂರು ಬೀಜ ನಿರ್ದಿಷ್ಟ ಸಾಗಣೆದಾರರ ಸಾಪೇಕ್ಷ ಅಭಿವ್ಯಕ್ತಿಯ ಆಣ್ವಿಕ ವಿಶ್ಲೇಷಣೆ ಮಾಡಲಾಯಿತು. ಎಎಮ್-೭೨ ಎಂಡೋಸಲ್ಫೋಮ್ ಯಾವುದೇ ಇತರ ವಂಶವಾಹಿಗಳಿಗಿಂತ ಹೆಚ್ಚಾಗಿ ಓನ್ ಜೆಡ್‌ಐಪಿ೪೬ ಮತ್ತು ಓನ್ ವೈಎಸ್‌ಎಲ್‌೨ಬಿ ಯು ತುಲನಾತ್ಮಕವಾಗಿ ಹೆಚ್ಚಿನ ಅಭಿವ್ಯಕ್ತಿ ಹೊಂದಿತ್ತು. ಪಾಲಿಷ್ ಜಿನೋಟೈಪ್ ಮತ್ತು ಇತರ ಜೀನೋಟೈಪ್‌ಗಳಿಗೆ ಹೋಲಿಸಿದಾಗ ಅದರ ಭ್ರೂಣ ಮತ್ತು ಎಂಡೋಸಲ್ಫೋಮ್ ಓನ್ ಜೆಡ್‌ಐಪಿ೪ಬಿ ನಲ್ಲಿ ಹೆಚ್ಚಿನ ಅಭಿವ್ಯಕ್ತಿ ಹೊಂದಿದ್ದರಿಂದ ಕಂದು ಅಕ್ಕಿ, ಸತು ಮತ್ತು ಕಬ್ಬಿಣದ ಹೆಚ್ಚಿನ ಶೇಕಡ ನಷ್ಟವನ್ನು ಅನುಭವಿಸಿತು. ಹೊಳಪು ಕೊಡುವ ಕಾರಣದಿಂದ ಎ ಆರ್ ಬಿ-೬ ಸತುವು ಕಡಿಮೆ ಪ್ರಮಾಣದಲ್ಲಿ ನಷ್ಟವನ್ನು ತೋರಿಸಿದೆ, ಅದರ ಅಂತಃಸ್ರಾವಕದಲ್ಲಿ ಓನ್ ವೈಎಸ್‌ಎಲ್‌೨ಬಿ ಮತ್ತು ಓನ್ ವೈಎಸ್‌ಎಲ್‌೧೫ಬಿ ಯು ತುಲನಾತ್ಮಕವಾಗಿ ಹೆಚ್ಚಿನ ಅಭಿವ್ಯಕ್ತಿ ಮತ್ತು ಅದರ ಭ್ರೂಣದಲ್ಲಿ ಕಡಿಮೆ ಪ್ರಮಾಣದ ಅಥವಾ ಕಡಿಮೆಯಾದ ಓನ್ ವೈಎಸ್‌ಎಲ್‌೧೫ಬಿ ವ್ಯಕ್ತಪಡಿಸಿದೆ.

ಜುಲೈ-೨೦೧೭

ಸಸ್ಯ ಜೈವಿಕ ತಂತ್ರಜ್ಞಾನ ವಿಭಾಗ

ಗಾ, ಕೃ, ವಿ, ಕೆ, ಜಿಕೆವಿಕೆ, ಬೆಂಗಳೂರು

(ಎಚ್.ವಿ. ವಿಜಯಕುಮಾರ್ ಸ್ವಾಮಿ)

ಪ್ರಮುಖ ಸಲಹೆಗಾರರು



Differential Expression of Micronutrient Transporter Genes in Bio-fortified Rice (*Oryza Sativa* L.) Grains

Mounashree, D. C, Vijayakumar Swamy, H. V. and Shashidhar, H. E.

Department of Plant Biotechnology, UAS, GKVK, Bangalore-560 065



Introduction

- Rice is the staple food of India.
- It is the most important food crop with regards to human nutrition and calorie intake.
- India is the second largest producer and consumer of rice in the World. In India, the area under rice cultivation is around 44 M ha with production of 105 M T during 2015-16.
- Iron (Fe) and zinc (Zn) play major role in numerous physiological process in plants.
- Deficiency of iron and zinc causes decreased growth, chlorosis, yellowing etc., in plants.
- Majority of iron and zinc present in rice grain is reported to be lost during milling and polishing. Hence this study is being conducted to find a rice genotype, which contains higher iron & zinc and loses less during polishing and to find the expression of micronutrient transporter genes which makes endosperm nutritious.

Objectives

- Discerning iron and zinc distribution in different parts of the rice grain.

Material and Methods

- Plant material and area:** Ten super-elite rice genotypes differing in their iron and zinc content were selected and grown on main field under aerobic condition using RCBD design at Department of Plant Biotechnology, UAS, GKVK, Bangalore-65

1- ARB-6	3-Chittimuthayalu	5-Azucena
2- Black Rice	4-AM-65	6- AM-1
7- AM-27	8- AM-143	9- AM-94B
10- AM-72		

- Observations taken:** Plant height, number of tillers, number of productive tillers, panicle length, grain yield, brown and white rice iron and zinc content were taken after harvesting.

- Study the distribution of iron and zinc in different parts of the rice grain:** Grains were dehulled by using dehusker and brown rice were polished (120 sec) by using polisher (Mini lab rice polisher. Model – 710 - Non Ferrous Series) and iron and zinc content was estimated in clean brown and white rice by using XRF machine, at MSSRF, Chennai.

- Estimation of iron and zinc content:** Bran, brown and white rice was subjected to AAS analysis for assessing iron and zinc content, at Fisheries Research and Information Centre (FR&IC), Hebbal, Bangalore.

- Statistical analyses:** the readings were subjected to ANOVA. Correlation studies were done to establish relationship among various yield parameters.

Results

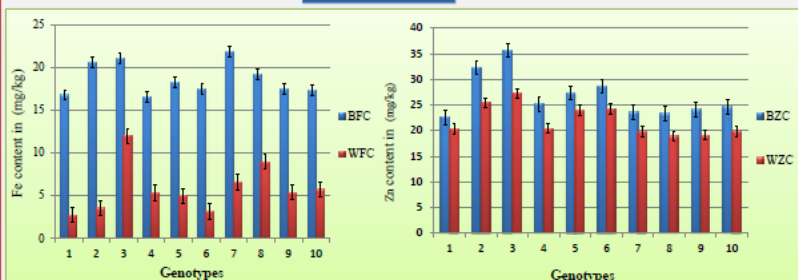


Fig1: Iron content in brown and white rice of ten genotypes (XRF method)

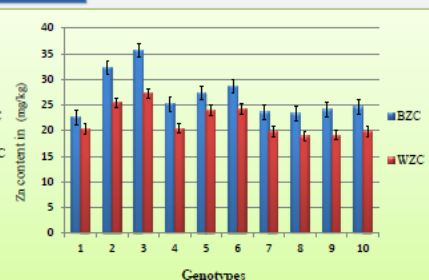


Fig2: Zinc content in brown and white rice of ten genotypes (XRF method)

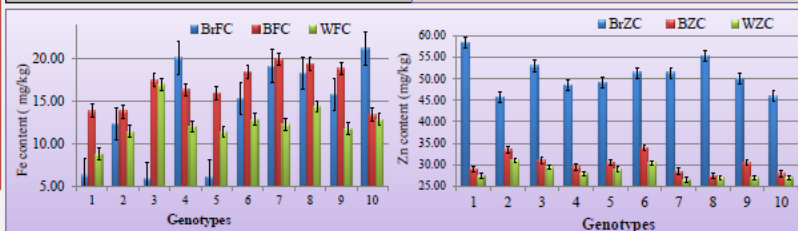


Fig 3: Iron content in bran, brown and white rice (AAS method)

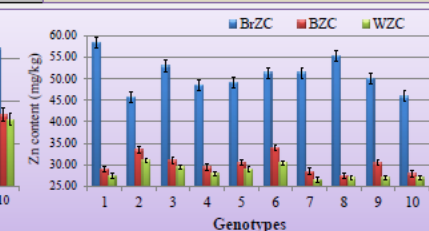


Fig 4: Zinc content in bran, brown and white rice (AAS method)

	PH	TNT	NPT	PL	DTM	DTF	DFF	HI	BFC	WFC	BZC	WZC
TNT	0.10	1.00										
NPT	0.19	0.86	1.00									
PL	0.87***	0.45*	0.42*	1.00								
DTM	0.32	-0.91	-0.10	0.33	1.00							
DTF	0.32	-0.21	-0.22	0.26	0.89***	1.00						
DFF	0.41	-0.35	-0.26	0.28	0.68***	0.75***	1.00					
HI	0.21	-0.20	-0.25	0.11	0.21	0.40*	0.34	1.00				
BFC	-0.08	0.36	0.32	0.01	-0.18	-0.39*	-0.18	-0.31	1.00			
WFC	0.13	-0.09	-0.17	0.04	-0.08	-0.08	0.16	0.10	0.51**	1.00		
BZC	0.25	0.12	0.21	0.23	0.00	-0.25	-0.01	-0.48**	0.45*	0.29	1.00	
WZC	0.30	0.09	0.27	0.25	0.01	-0.22	0.05	-0.37*	0.41*	0.16	0.84***	1.00

Table 1: Correlation analysis of different traits considered in the study with their correlation co-efficients

PH- Plant height, TNT- Total number of tillers, NPT- Number of productive tillers, PL- Panicle length, DTM- Days to maturity, DTF- Days to flowering, DFF- Days to fifty percent flowering, HI- Harvest index, BFC- Brown rice iron content, WFC- White rice iron content, BZC- Brown rice zinc content, WZC- White rice zinc content, BrFC- Bran iron content, BrZC- Bran zinc content.

Discussion

- Milling and polishing of rice grains is an inevitable process that has to be done to preserve the grains over long term.
- Some genotypes show high micronutrient content in grains even after polishing due to remobilization of seed micronutrient into endosperm.
- Highest iron and zinc content was found in Chittimuthayalu both in brown and white rice (35.6 & 27.26 mg/kg) respectively where as highest iron content in brown and white rice was observed in AM-27 and Chittimuthayalu (21.8 & 11.96 mg/kg) respectively.
- Lowest iron and zinc content was found in AM-143 in brown and white rice (23.3 & 18.9 mg/kg) and lowest iron content was found in AM-72 & ARB-6 in brown and white rice respectively.

Summary

- Approximately 32 % of Zinc and 87 % of iron was lost due to polishing.
- RT-PCR for iron and zinc transporter genes will be done to establish relationship between micronutrient content and candidate gene marker at RNA level.

Advisory Committee

Chairperson : Dr. H. V. Vijayakumar Swamy
Members : Dr. H. E. Shashidhar
Dr. K. M. Harinikumar
Dr. M. K. Shivaprakash
Dr. Veena. S. Anil

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I INTRODUCTION

Rice is the staple food for more than 50 per cent of the world population. India is the second largest producer and consumer of rice globally. Area under rice in India is around 44 M ha with a production of about 105MT during 2015-16. At least 49 nutrients are required by humans for their normal growth and development (Welch & Graham, 2004), and the demand for most nutrients is generally supplied by cereals, particularly rice in the developing countries (Huang *et al.*, 2015).

Rice provides food and livelihood security to over half (~3.5 billion) of the global population. Approximately 150 million tons of rice is needed by 2030 to feed 1.378 billion Indian people (Goyal and Singh 2002) and ~850 million tons to feed 5 billion rice consumers in the world at least 1.1 % yield increase is required every year (Subudhi *et al.*, 2006).

Aerobic rice is a new concept of rice cultivation adopted in non-puddle and non-saturated soils with a water content of 70 per cent to 100 per cent of water holding capacity throughout a growing season. Aerobic rice varieties have the ability to maintain rapid growth in soils with moisture content at or below field capacity, and can produce yields of 4-6 t/ha with a moderate application of fertilizers under such soil water conditions and it saves around 50 per cent of irrigation water in comparison with lowland rice and also it saves 73 per cent of irrigation water for land preparation and 56 per cent during the crop growth period (Govindarasu *et al.*, 2015).

Rice is being staple food, provides most energy form, important component is, carbohydrate. The inner part of the grain, endosperm contains mostly starch and around 6 to 10 per cent protein. The bran is more diverse in its composition and contains protein, lipids, fibers, vitamins and minerals. Iron and zinc deficiencies have been reported to be a food-related primary health problem affecting nearly two billion people worldwide leads to micronutrient deficiency. The brown and red rice genotypes have high grain iron and zinc content.

The consumer's preference for milled rice therefore substantially reduces the availability of zinc. Conversely, brown rice can contribute appreciable amounts of zinc to the diet. The variability in zinc content among 3 different rice varieties is quite pronounced ranging from 14 to 59 mg/kg (Frei and Becker, 2005).

Iron (Fe), an essential nutrient for plants, plays a crucial role in a variety of cellular functions. The most widespread dietary problem in the world is Fe deficiency (World Health Organization, 2003). Such a deficiency also causes a metabolic imbalance deleterious to plant growth (e.g. impairing chlorophyll biosynthesis, chloroplast development, and photosynthesis). Therefore, Fe availability is directly correlated with plant productivity (Lee *et al.*, 2009).

Iron deficiency is considered to be one of the most wide-spread micronutrient disorders in the world. Rice can only contribute significantly to the iron supply if it is eaten as brown rice. Iron deficiency anemia occurs in a quarter of the world's population (An *et al.*, 2012).

There are two strategies for iron (Fe) acquisition from soil depending on the plant species. Dicots and non-gramineous monocots use Strategy I, which is characterized by the reduction of ferric to ferrous by ferric chelate reductase on the plasma membrane and subsequent uptake by the IRT1 transporter (Eide *et al.*, 1996; Robinson *et al.*, 1999). By contrast, graminaceous plants use Strategy II, which is characterized by the secretion of mugineic acid (MA) and subsequent uptake of the mugineic acid–ferric complex (Ma and Nomoto, 1996; Murata *et al.*, 2006).

Zinc (Zn) is essential for all organisms, and human. Zn deficiency ranks third in importance after iron and vitamin A deficiency (Hambidge, 2000). The expression of genes encoding the plant ZIP transporters appears to be induced by zinc or iron deprivation (Eide *et al.*, 1996; Grotz *et al.*, 1998). Some of the ZIP family members, such as IRT genes, are constitutively expressed, but expression levels increase with iron deprivation (Eckhardt *et al.*, 2001; Vert *et al.*, 2001).

Zn and Fe deficiency severely impaired physiological and biochemical reactions of plants. Therefore, controlled zinc and iron uptake and homeostasis network has been

evolved in plants. The Zinc-regulated transporters, Iron-regulated transporter-like Proteins (ZIP) are capable of uptaking and transporting divalent metal ion and are suggested to play critical roles in balancing metal uptake and homeostasis.

ZIP family takes its name from the first members to be identified. Around 25 ZIP family members have been identified which roughly fall into two subfamilies based on amino acid similarities. Most ZIP proteins are predicted to have eight potential trans membrane domains and a similar membrane topology in which the amino- and carboxyl-terminal ends of the protein are located on the outside surface of the plasma membrane. A member of yellow-stripe like (YSL) family of metal transporters play important roles in plant Fe homeostasis, and also plays an important role in long distant transport of metals complexed with nicotianamine (NA).

There are several seed specific transporters reported in rice and study of these transporters at molecular level would help to explain the differential distribution of micronutrients within rice grain. The temporal and spatial expression of micronutrient transporters imparts importance to the study of such transporters at the RNA level to explain the unequal distribution of micronutrients in different tissues of the same plant.

Hence, studying the relative expression of micronutrient transporters in different grain parts (embryo and endosperm) would help in revealing the specific transporters that play an important role in accumulation of Zn and Fe in the respective grain part.

Keeping in view the above points, an experiment was designed and conducted in the Aerobic rice laboratory of Department of Plant Biotechnology, to study the distribution of Zn and Fe among brown and white rice and relative expression of seed specific transporters in embryo and endosperm among the selected genotypes.

The present study was undertaken with following objectives;

1. Discerning zinc and iron distribution in different parts of the rice grain
2. Expression profiling of transporters across bio-fortified elite lines.

II REVIEW OF LITERATURE

In this chapter available literature pertaining to the present investigation has been reviewed and presented under the following headings.

- 2.1 Importance of zinc and iron in plants and humans
- 2.2 Aerobic rice
- 2.3 Bio-fortification of rice
- 2.4 Zinc and iron content in rice
- 2.5 Transporters linked with zinc and iron distribution in rice grain
- 2.6 Gene expression studies in rice grain

2.1 Importance of micronutrients in plants and humans

Zinc is the 23rd most abundant element with an atomic number 30 and atomic weight of 65.4. It is bluish-white metallic element which attributes 0.02 per cent of the earth's crust (Brown *et al.*, 2001).

Zinc plays multiple roles in plant biochemical and physiological processes, even slight deficiencies will cause a decrease in growth, yield, and zinc content of edible parts (Ramesh *et al.*, 2003)

A large proportion of the world's population is affected by deficiencies of bioavailable iron, zinc, and other essential minerals (Ghandilyan *et al.*, 2006).

Zn is one of the most important micronutrient required for almost all living organisms. It is one of the 49 nutrients required for human beings, which plays very important role in structure and function of large number of enzymes. Most critical Zn binding sites includes cysteine, histidine and glutamate or asparate residues (Welch, 2001, Welch and Graham, 2004).

More than two billion people are micronutrient deficient. Polished grains of popular rice varieties have concentration of approximately $2 \mu\text{g g}^{-1}$ iron (Fe) and $16 \mu\text{g g}^{-1}$ zinc (Zn) (Trijatmiko *et al.*, 2016).

Zinc deficiencies can affect plant by stunting its growth, decreasing number of tillers, chlorosis and smaller leaves, increasing crop maturity period, spikelet sterility and inferior quality of harvested products (Hafez *et al.*, 2013).

Sellappan *et al.*, (2009) reported that the iron and zinc content varies between polished and unpolished grain of rice. About 70% of micronutrients were lost during polishing, as most of the aleurone layer and embryo gets removed during polishing process.

Zinc is the only metal to be involved in all six classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Barak and Helmke, 1993). Zinc ions exist primarily as complexes with proteins and nucleic acids and participate in all aspects of intermediary metabolism (Tapeiro and Tew, 2003). Zinc ions are also neurotransmitters, and cells in the salivary glands, prostate, immune system and intestine use Zn signalling (Herschfinkel *et al.*, 2007). Zinc plays a key role in physical growth and development, the functioning of the immune system, reproductive health, sensory function and neurobehavioural development (Hotz and Brown, 2004).

Alloway (2009) listed eight major factors affecting Zn availability (and hence likely to be related to deficiency) which include low soil Zn content, soil with restricted root zones, calcareous soils, low soil organic matter, microbially inactivated Zn, cool soil temperatures, plant species and varieties, high levels of available phosphorus, and effect of nitrogen.

In order to boost Zn in edible parts of crops, scientists should exclude cadmium (Cd), which is a toxic Zn analogue. It causes cellular damage by binding readily to free sulfhydryl residues, e.g., cysteine and its derivatives such as glutathione, which leads to protein inactivation or denaturation (Martelli *et al.*, 2006; Bertin and Auerbeck, 2006). It has been also reported that Zn deficiency is aggravated by excess Ca, Mg, and Mn (Gupta

et al., 1993; Neue *et al.*, 1998). Sulfur crucially interacts with Zn for enzymatic catalysis. Zn–S interaction is also crucial for Zn-sensing transcription factors (Maret, 2004).

Symptoms found in crop plants suffering Zn deficiency occur at varying degrees of severity and in various combinations in different plant species. Severe Zn deficiency symptoms include impaired stem elongation leading to stunting of plants, root apex necrosis, heterogeneous or inter-veinal chlorosis, necrotic spots on leaves, bronzing of leaves, rosetting of leaves, leaf dwarfing, malformed leaves, development of reddish-brown or bronze tints, and inward curling of leaf lamina (Broadley *et al.*, 2007).

In humans, iron is needed to support the daily synthesis of the 200 billion red blood cells derived from reticulocytes, which synthesize massive quantities of haemoglobin. Maintenance of iron metabolism requires the uptake of only about 1–2 mg of iron from the diet each day; the remaining 25 mg of iron needed daily is captured and reused following the destruction of senescent red blood cells by macrophages. The intricate iron sensing system regulating iron uptake is beyond the scope of this short entry (Gkouvatos *et al.*, 2012).

The harvested paddy rice or rough rice is inedible, and undergoes de-husking and polishing before human consumption, leaving nearly only the endosperm. Thus, Fe biofortification efforts should focus primarily on increasing endosperm Fe bioavailability, and the major barriers for Fe entrance into the endosperm must be identified (Raul *et al.*, 2012).

Transporters contribute uptake from the soil *via* roots, control of transport from roots to above-ground parts of the plant, unloading of Fe from the xylem in above-ground parts, loading of Fe into mitochondria and plastids, transport of Fe to reproductive parts of the plant, and Fe mobilization during seed germination (Conte and Walker, 2011).

Iron is the most abundant element on Earth with a mass percentage of 32%, similar to oxygen at 30%, with carbon some 45-fold less abundant. However, in the ocean, iron is barely a trace element while oxygen is at 85%. Even without a consideration of biology, the reason for this discrepancy is obvious. Specifically, oxygen

favours the highly insoluble ferric (Fe^{3+}) over the reduced and reactive ferrous (Fe^{2+}) form. Hence, the human body which is largely comprised of water and high concentrations of oxygen is faced with the dilemma of how to utilize iron (Bashir *et al.*, 2013)

Zn deficiency limits the supply of substrate for carboxylation (Salama *et al.*, 2006). In the early stages of Zn deficiency, the younger leaves become yellow or even necrotic. Severely Zn-deficient plants are stunted, with reduced elongation and root tip growth. Zn can be acquired from the soil solution primarily as Zn^{2+} but also complexes with organic ligands (Kramer and Clemens, 2005).

2.2 Aerobic rice

Rice (*Oryza sativa* L.) is the staple food for more than 85 per cent of Asian population and it also the single biggest user of freshwater. It is mostly grown under submerged soil conditions and requires more water compared with other crops and it has been cultivated majorly in irrigated ecosystem as 57 per cent gives 75 per cent of total rice production (Bouman, 2001).

Aerobic rice is a new method of cultivating rice in less water than traditional flooded condition. Aerobic rice varieties are developed by crossing low land varieties with upland varieties and cultivated in irrigated but non flooded and non puddle soils (Bouman *et al.*, 2002; Bouman *et al.*, 2005).

Aerobic rice has efficient water use efficiency which saves about 45% of water utilization, decrease methane production; reduce production cost and eco-friendly compared to conventional irrigated rice (Shashidhar, 2007). Aerobic rice is an emerging agronomical production system that uses less water than conventional flooded rice (Tuong *et al.*, 2005).

Kadiyala *et al.*, (2012) has conducted field experiment at ANGR University Agricultural Research Station, Andhra Pradesh, India during 2009-2010 and 2010-2011 to compare crop growth, yield, and water savings under aerobic rice-maize (R-M) and

flooded R-M rotation systems. The effect of aerobic rice on the succeeding maize crop was also studied and reported higher water productivity and water savings up to 37 to 45% over flooded method of rice cultivation. Significant increase in yields in both systems with increased N rates up to 120 kg ha⁻¹. Significantly higher yields in no till maize grown subsequent to the aerobic rice than flooded rice was obtained possibly due to residual soil N and improved soil physical conditions.

Aerobic rice production systems eliminates continuous seepage and percolation losses, greatly reduces evaporation as no standing water is present at any time during the cropping season, and effectively uses the rainfall and thus helps in enhancing water productivity, concomitant loss of soil sediments, silt and fertility from the soil. Water requirement of low land rice varies from 1,650 to 3000 mm, aerobic rice system can save about 45 per cent of water, and minimizes methane production (Shashidhar, 2007).

Govindarasu *et al.*, (2015) report shows aerobic rice varieties have the ability to maintain rapid growth in soils with moisture content at or below field capacity, and can produce yields of 4-6 t/ha with a moderate application of fertilizers under such soil water conditions and it saves around 50 per cent of irrigation water in comparison with lowland rice and also it saves 73 per cent of irrigation water for land preparation and 56 per cent during the crop growth period.

The water use efficiencies of the aerobic varieties under aerobic conditions were higher than that of the lowland variety under lowland conditions. Aerobic rice maximizes water use in terms of yield and is a suitable crop for water limiting conditions. Aerobic rice cultivation will curb methane production and saves water without affecting the productivity. It is the time to save water from the irrigated system of rice cultivation by adopting the aerobic rice cultivation (Lal *et al.*, 2013).

Average yield under aerobic conditions was similar to or even higher than that achieved with flooded conditions (7.9 t ha⁻¹ in 2007 and 9.4 t ha⁻¹ in 2008 for aerobic versus 8.2 t ha⁻¹ for flooded). The average water productivity under aerobic conditions was 0.8– 1.0 kg grain m⁻³ water. The super-high-yielding cultivar Takanari yields greater

than 10 t ha⁻¹ with no yield penalty under aerobic conditions in high-productivity rice cultivation in aerobic condition (Kato *et al.*, 2009).

ARB-6 has manifested high degree of drought resistance and gives high yield, comparable to improved varieties when sufficient moisture is available. It has deep roots which are capable of extracting moisture from deeper layers of soil column. It grows fast and has high biomass (Gowda, 2010).

2.3 Bio-fortification of rice

Micronutrient deficiency (hidden hunger) is a major health problem which affects more than half of the world's population and contributing to high rate of children and women's mortality.

Rice contains less Fe levels, most of which is lost during processing. Populations with monotonous diets consisting mainly of cereals are especially prone to Fe deficiency. Fe bio-fortification in rice is done by generating cultivars that efficiently mobilize, uptake and translocation of Fe to the edible parts through conventional and transformation processes (Raul *et al.*, 2012).

Breeding of nutrient-rich staple food crops is indeed the main goal of different international consortia, exemplified by Harvest Plus (<http://www.harvestplus.org/>). The goal is to reduce micronutrient malnutrition (Provitamin A, zinc and iron) through different bio-fortification programs (Hotz *et al.*, 2007) and target different crops as a means to deliver the nutrients. In an effort coordinated by the Harvest Plus project, CGIAR (The Consultative Group on International Agricultural Research) Centers are taking a leading role in breeding for increasing concentration and bioavailable levels of Zn and Fe in seeds of major staple food crops (Pfeiffer and McClafferty, 2007).

Increasing body iron availability can treat iron deficiency anemia, but there is some evidence that it can also promote pathogen growth. An optimum level of iron status is largely achieved safely in developed countries. However, in developing countries, the widespread occurrence of infectious diseases and the consequent host-pathogen

competition for iron, makes the definition of guidelines for daily iron requirement more complex (Prentice *et al.*, 2008).

2.4 Zn and iron content in rice

Yang *et al.*, (1998) studied genotypic differences in concentration of Fe and Zn in polished rice grains. Totally 285 rice genotypes were considered. For all they estimated Fe and Zn concentrations along with concentrations of protein and lysine in polished rice grains. It was concluded that Zn concentrations in polished indica rice were about 2-fold higher than that in the polished japonica rice. But Fe concentrations of japonica rice were slightly higher than that in the indica rice. It was found that there was no close relationship between Zn and Fe concentration, protein and lysine concentration among the genotypes.

Oli *et al.*, (2016) studied on nutrient levels in parboiled rice grain. The rice kernels were analysed by Synchrotron X-Ray Fluorescence Microscopy (S-XFM) to determine the zinc and iron. The result showed that the bran and germ were rich in the minerals whereas endosperm had a lower concentration. The mineral diffusion to the endosperm from the bran layer and germ was increased by different hydrothermal treatment of kernels. The highest diffusion of zinc and iron was observed in the sample treated with high temperature hydration for a long time.

Huang *et al.*, (2015) studied 20 rice genotypes to investigate the effects of genotype, environment and their interaction on the micronutrient contents in brown rice. Analysis of variance showed that the zinc and iron were mainly affected by the environment variance. It showed that the interaction effect of genotype $\times$ environment for zinc was highly significant.

Bekele *et al.*, (2013) reported that in an experiment conducted with one hundred and seventy six Azucena $\times$ Moromutant, grain zinc concentration was negatively correlated with grain yield per plant.

It is noticeable that, AM 72 suffered highest percent loss of zinc and iron due to polishing and the genotype had relatively higher expression of OsYSL2b and OsYSL2c in its embryo and relatively lower expression of OsZIP4a in its endosperm when compared to other genotypes (Bhat, 2015).

It was reported that around 75 per cent of total grain Zn was present in the endosperm of brown rice (Jiang *et al.*, 2008). X-ray Fluorescence imaging revealed that Zn is abundant in embryo and in the aleurone layer (Takahashi *et al.*, 2009). Also, Fe has been localized by histochemical techniques in the aleurone layer and in the embryo, but not in the endosperm of rice seeds (Pro-u-thai *et al.*, 2003; Sivaprakash *et al.*, 2006; Sellappan *et al.*, 2009).

There is a significant positive correlation between grain Fe and Zn content. Fe content had significant positive correlation and Zn content had negative non-significant correlation with grain yield. Path coefficient analysis showed that there is no indirect and direct effect of grain Fe and Zn content on grain yield per plant (Patil *et al.*, 2015).

Gregorio *et al.*, (2000) reported iron content in brown and white rice. IR64, a popular commercial variety with the lowest iron in brown rice, the iron content dropped by more than one-third after 15 minutes of polishing. These observations suggest that much of the iron is in the outer layers of the grain.

Stangoulis *et al.*, (2007) noticed significant positive correlations between phytate and inorganic phosphorus (P), total P, Fe, Zn, Cu and Mn concentrations for both grain concentration and content. QTLs of Fe, Zn and Mn located on the same chromosomal regions, for phytate it was not found, suggesting that they were genetically different and thus using molecular markers in breeding and selection would modify the phytate level without affecting grain micronutrient density.

Total variability of a trait is divided into genotypic variability and phenotypic variability. The estimate of variability suggests the variation in heritable portion of a trait that could be transferred from parent to offspring in response to selection (Hallauer and Carena, 2009; Lakshmana *et al.*, 2009).

Bisne *et al.*, (2009) found high genotypic and phenotypic coefficient of variations for harvest index and hundred grain weight; high heritability coupled with high genetic advance for harvest index, grain yield per plant from a trial with four CMS lines, eight testers and thirty-two hybrids evaluated.

The range of iron and zinc concentration in brown rice is 6.3 to 24.4 µg/g and 13.5 to 28.4 µg/g respectively. There is approximately a fourfold difference in iron and two fold difference in zinc concentration, suggesting that some genetic potential to increase the concentration of these micronutrients in rice grains (Graham *et al.*, 1999)

Nine genotypes of rice were evaluated for iron and zinc content in rice grain at IRRI and found a range of 8.8 to 21.0 ppm, 14.0 to 40.0 ppm for iron, and zinc respectively (Martinez *et al.*, 2006). Zozali *et al.*, (2006) reported grain Zn concentration is substantially higher in certain landraces of Southeast Asia than in commonly grown high yielding rice varieties.

A breeding program to develop Zn rich genotypes can be initiated by screening of available germplasm accessions to identify the genetic variation for the trait that could serve as donors. Then high zinc containing genotypes can be crossed with high yield potential, tolerance to biotic and abiotic stresses, and good grain quality genotypes (Martinez *et al.*, 2010).

Swamy *et al.*, (2011) studied 126 accessions/varieties of rice samples including wild relatives *O. rufipogon* and *O. nivara*. The Fe concentration in brown rice ranged from 6 ppm (Athira) to 72 ppm (*O. nivara*). Zn concentration ranged from 27 ppm (Jyothi) to 67 ppm (*O. rufipogon*). Iron and zinc concentration varies between brown rice and polished rice. Also, when whole rice grains and broken rice grains were compared, there was variation in iron concentration but not in zinc concentration.

Anuradha *et al.*, (2012) analyzed 126 accessions of brown rice genotype including 7 accessions of two wild species for Fe and Zn concentration and reported that iron concentration ranged from 6.2 ppm to 71.6 ppm and zinc from 26.2 ppm to 67.3 ppm. Zn

concentration and grain elongation (-0.25) were significantly correlated. The wild accessions had the highest Fe and Zn.

Babu (2013) reported genotypic differences in grain quality including protein, micronutrient concentrations and few of the yield attributing parameters of twenty one popular rice hybrids. The data revealed huge variations in grain quality among the hybrids. Mean micronutrient iron concentration of hybrids (68.4 ppm) was found to be 1.83 times that of zinc (37.3 ppm). Two hybrids with both high iron and zinc concentration (DRRH-2 with 125.8 ppm and 43.8 ppm; and Sahyadri-4 with 104.8 and 43.0 ppm) were identified.

Susanto (2009) in IRRI reported that broad sense heritability of iron and zinc content in polished rice grains were 57.28 per cent and 76.36 per cent in population obtained from IR75862-206-2-8-3-B-B-B/IR64 parents.

Berhanu *et al.*, (2013) conducted a study to evaluate genetic variability parameters, correlations that exist for grain Zn concentration and yield related traits and identification of SSR markers linked to these traits in rice. Grain yield per plant and grain zinc concentration showed higher phenotypic and genotypic co-efficient of variation.

Analysis of variance revealed high significant variation for grain yield per plant and grain zinc content. High GCV, PCV (103.06, 109.16) was observed for grain yield per plant and moderate GCV, PCV (17.02, 18.39) was observed for grain zinc content (Naveen *et al.*, 2013).

All organisms possess a fine adjustment of metal homeostasis networks, which maintains concentrations of metal ions within physiological limits. Excess Zn can be toxic to cells, due to competition for binding sites with other biologically active ions.

2.5 Transporters linked with zinc and iron distribution in rice grain

Ishimaru *et al.*, (2011) reported that the transport of Zn in soil-root-shoot in the form of Zn^{2+} and ZnDMA at the root surface and Zn translocation to shoots.

Guerinot *et al.*, (1999) reported that how ZIP transporters functions and how each is controlled in response to nutrient availability may allow the manipulation of plant mineral status with an eye to creating food crops with enhanced mineral content, and developing crops that bioaccumulate or exclude toxic metals.

Forno *et al.*, (1975) reported that there is a 70% decrease in transport of Zn from the roots to shoots, by growing IR-8 genotype in the solution culture containing only 15 mM of bicarbonate. Under neutral to alkaline conditions, acetic acid at 10 me/1 produced only slight reductions in zinc uptake and transport to the shoots. At pH 4.5 the same concentrations of acetic acid reduced uptake by more than 90 per cent.

Single-Marker Analysis revealed that OsZIP6c was associated with culm zinc content and OsZIP5b is significantly associated with grain zinc content with twenty rice genotypes (Holla *et al.*, 2014).

The TF IDEF was focused to be necessary during the early onset of iron deficiency response and it was correlated with expression of OsIRO2, OsYSL15, OsIRT1, OsYSL2, OsNAS1, OsNAS2, OsNAS3, OsDMAS (Kobayashi *et al.*, 2009).

Alloway (2009) reported that soil factors like high pH, high calcite and organic matter contents and high concentrations of Na, Ca, Mg, bicarbonate and phosphate in the soil solution affecting the availability of Zn to plants.

Roy and Sharma (2014) conducted an experiment on 84 rice cultivars to assess the genetic diversity in rice germplasm based on agro morphological traits and iron-zinc content. Iron (Fe) and zinc (Zn) were estimated by using Atomic Absorption Spectrophotometric method. Iron concentration varies from 0.25 $\mu\text{g/g}$ to 34.8 $\mu\text{g/g}$ and zinc from 0.85 $\mu\text{g/g}$ to 195.3 $\mu\text{g/g}$ in the landraces.

Kobayashi *et al.*, (2014) experiment reported that the transcription factors IDEF1, IDEF2, and OsIRO2 are responsible for transcriptional induction, whereas the ubiquitin ligases OsHRZ1 and OsHRZ2, as well as the transcription factors OsIRO3 and OsbHLH133, are thought to mediate negative regulation of the genes involved in Fe uptake and translocation in response to Fe deficiency.

Inoue *et al.*, (2009) report suggest that *OsYSL15* is the dominant iron (III)-deoxymugineic acid transporter responsible for iron uptake from the rhizosphere and is also responsible for phloem transport of iron. They found that knockdown of *OsYSL15* in seedlings *showed* severe arrest in germination and early growth and were rescued by high iron supply.

Meng *et al.*, (2005) evaluated the iron content in different cereal foods (black rice, rice, red rice, sticky rice and millet) and different rice seeds as well as in the milling products. They found highest iron content in black rice than in the other rice types, and in rice chaff and husk the content is still fairly high. The iron content in rice and fine rice is also fairly low.

Lee *et al.*, (2009) experiment results demonstrate the roles of *OsYSL15* in Fe uptake and distribution in rice plants. Over expression of *OsYSL15* resulted in higher Fe concentrations in leaves and seeds, supporting that *OsYSL15* is a Fe transporter. Disruption or over expression of *OsYSL15* affected the concentration of Fe, but not Zn, Mn, or Cu, and also 20% reduction in Fe concentration under both sufficient and deficient conditions.

It was reported that constitutive over expression of *NAS* genes in rice was able to increase the Zn concentration in the rice grain by average two fold, also in the polished grain. The reason for this increase was also reported as enhanced level of nicotianamine increases the phloem mobility of Zn, thus enhancing the flow of Zn to the nucellar projection in the rice grain (Lee *et al.*, 2009; Masuda *et al.*, 2009; Johnson *et al.*, 2011; Lee *et al.*, 2011).

Bashir *et al.*, (2011) identified and characterize the rice mitochondrial Fe transporter (MIT). Homozygous knockout of *MIT* in this line resulted in a lethal

phenotype. Growth of *MIT*-knockdown (*mit-2*) plants shows significantly impaired despite abundant Fe accumulation. Further, the decrease in the activity of the mitochondrial and cytosolic Fe-S enzyme, aconitase, indicated that Fe-S (Iron-sulphur) cluster synthesis is affected in *mit-2* plants. Results indicate that MIT is a mitochondrial Fe transporter essential for rice growth and development.

Kavitha *et al.*, (2015) reported *OsZIP6* transporter was transcriptionally activated in shoot and root tissues in response to deficiency in Fe^{2+} , Zn^{2+} and Mn^{2+} . They noticed ion transport by *OsZIP6* was found to be p^{H} dependent and enhanced transport was observed at acidic p^{H} . Radioisotope uptake showed that, Co^{2+} competitively inhibits Fe^{2+} uptake by *OsZIP6*.

2.6 Gene expression studies in rice grain

Ishimaru *et al.*, (2005) conducted an experiment on Zn transporters in rice. They have reported that four genes *OsZIP4*, *OsZIP6*, *OsZIP5* and *OsZIP7* exhibit sequence similarity. Microarray analysis revealed that *OsZIP4* was expressed more in case of Zn deficiency in roots and shoots.

Twelve genotypes were evaluated for distribution of zinc and iron within different parts of rice grain and molecular characterization of seed specific micronutrient transporters was done for ten out of twelve genotypes. The report revealed that Moroberekan embryo and endosperm had relatively higher expression of *OsZIP4a*, *OsNAS2a*, and *OsYSL15b* than in any other genotypes. AM 72 embryo had relatively higher expression of *OsYSL2b* and *OsYSL2c* compared to other genotypes (Bhat, 2015).

Sunitha *et al.*, (2003) studied differential metal selectivity and gene expression of two zinc transporters (*OsZIP1* and *OsZIP3*) from rice. They found that, although both the transporters showed many similarities in function they differed in ionic selectivity and p^{H} optimum of activity. Expression patterns also differed between the two genes. Comparisons of the protein sequences, ionic selectivity, and gene expression patterns of the two transporters suggest that they may play different roles in the physiology of the whole plant.

Sasaki *et al.*, (2016) reported that OsZIP3 localized to the node is involved in the distribution of Zn to the developing tissues, rather than uptake. *OsZIP1* is expressed in the epidermis and vascular tissues of roots and leaves (Ramesh *et al.*, 2003; Bashir *et al.*, 2012).

OsIRT1 and OsIRT2 show a transport activity for ferrous Fe. *OsIRT1* is expressed in the root epidermis and stele and its expression is up-regulated by Fe deficiency (Ishimaru *et al.*, 2011) *OsYSL15* is expressed in the epidermis, endodermis, cortex, and vascular bundles of the roots as well as in the leaves under Fe deficiency. Knockout of *OsYSL15* decreased the Fe concentration in both the shoots and roots (Inoue *et al.*, 2009; Lee *et al.*, 2009).

Yokosho *et al.*, (2016) studied that *OsFRDL1* expressed in nodes is required for distribution of iron to grains in rice. The Knockout of *OsFRDL1* decreased pollen viability and grain fertility when grown in a paddy field. A stem-fed experiment with stable isotope Fe showed that the distribution of Fe in the anther and panicle decreased in the knockout line, but that in the flag leaf it increased compared with the wild-type rice. Overall report shows that *OsFRDL1* expressed in the upper nodes is required for the distribution of Fe in the panicles through solubilizing Fe deposited in the apoplastic part of nodes in rice.

Koike *et al.*, (2004) studied OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. They identified 18 putative yellow stripe 1 (YS1)-like genes (*OsYSLs*) in the rice genome. The phloem-specific expression of the OsYSL2 promoter suggests that OsYSL2 is involved in the phloem transport of iron. Electrophysiological measurements, showed that OsYSL2 transported iron(II)-nicotianamine (NA) and manganese(II)-NA, but did not transport iron(III)-phytosiderophore.

Li *et al.*, (2013) identified Nine ZIP-coding genes. They performed yeast complementation analysis to test the Zn or Fe transporter activity of ZmZIP proteins. Expression analysis showed that the ZmIRT1 transcripts were dramatically induced in

response to Zn- and Fe-deficiency. Expression patterns of ZmZIP genes were observed in different stages of embryo and endosperm development. Accumulations of ZmIRT1 and ZmZIP6 were increased in the late developmental stages of embryo, while ZmZIP4 was up-regulated during the early development of embryo.

Arabidopsis has 16 members in ZIP family (Maser *et al.*, 2001), including AtZIP1, AtZIP2, AtZIP3, AtZIP4, AtIRT1, AtIRT2 and AtIRT3 proteins. It is reported that in roots and/or shoots of Arabidopsis, under Zn limited levels, expression of several ZIP genes such as AtZIP1, AtZIP5, AtZIP9, AtZIP12 and AtIRT3 were increased (Kramer *et al.*, 2007).

Jean *et al.*, (2005) reported YSL1 as one of the better characterized members of Oligo-peptide (OTP) family of transporters, which localizes to the shoot vasculature as well as the siliques, pollen grains and the developing seeds. They also found that *ysl1* mutants accumulated reduced levels of Fe in the seeds, and these seeds showed germination defects on low-Fe soil, which suggest a role of YSL1 in metal loading of the seed.

As Zn is important for all living organisms, it needs to be present in cells in an adequate quantity but toxic levels of Zn are harmful. So organisms need to maintain the concentration of Zn. Thus a tightly regulated metal homeostasis network becomes necessary (Palmgren *et al.*, 2008).

Alexander *et al.*, (2011): Chun *et al.*, (2011) reported that the rice cultivars overexpressing single rice OsNAS genes showed higher levels of Fe and Zn in phosphorus-free regions of endosperm. They studied the effect of constitutive overexpression of OsNAS1, OsNAS2 and OsNAS3 genes on increasing Fe and Zn content in endosperm of three different populations of rice respectively. Four-fold increase in Fe content and two-fold increase in Zn content were observed in OsNAS2 over expressing genotype. In nutshell, this study revealed the enormous potential of OsNAS genes, OsNAS2 in particular, in increasing Fe and Zn content in polished rice.

III MATERIALS AND METHODS

The details of materials used, methods adopted, protocols followed and statistical tools employed for analysis of the experiment conducted in the aerobic rice laboratory of the Department of Plant Biotechnology, GKVK, Bangalore.

Experiment I

3.1 Discerning zinc and iron distribution in different parts of the rice grain

3.1.1 Experimental site

Ten genotypes were grown during *Kharif*-2016 at the fields of Aerobic Rice Research Laboratory at the Department of Plant Biotechnology, University of Agricultural Sciences, GKVK campus, Bangalore, India located at the latitude of 12°58' North; longitude 77°35' East and altitude of 930 meters above mean sea level (MSL).

3.1.2 Selection of genotypes

Ten super elite genotypes differing for grain Zn and Fe content were selected based on earlier studies of Narayanrao (2012) and Bekele (2012) using more than 1200 rice accessions. These studies were based on X-ray fluorescence (XRF) readings for Zn and Fe estimation. The list of the selected genotypes are given in Table 1.

3.1.3 Layout of the experiment

The experiment was laid out in Randomized Complete Block Design (RCBD) with three replications. Each genotype was grown in three replications with 10 plants per row plant in field. Field view of the experimental plot is given in Plate 1. The plants were grown by sowing the seeds directly into the field, maintaining a spacing of 30 X 10 cm. To meet the nutrient demand of the plants, fertilizers were provided. Nitrogen was provided as urea at basal, 30 and 60 days after sowing. Phosphorus was provided as single super phosphate and potassium was provided as murate of potash. Irrigation was done in regular basis on alternate days. All necessary measures were taken to control pest and disease infestation. The soil at the experimental site had 1.62 ppm for Zn, 21.96 ppm for Fe, a p^H 6.1 and 7.4 g/kg of organic carbon.

Table 1: List of super elite genotypes selected for the study

Sl. No.	Variety name	Pedigree
1.	AM-1	Azucena × Moromutant
2.	AM-27	Azucena × Moromutant
3.	AM-65	Azucena × Moromutant
4.	AM-72	Azucena × Moromutant
5.	AM-94B	Azucena × Moromutant
6.	AM-143	Azucena × Moromutant
7.	ARB-6	Budha × IR-64
8.	Azucena	Exotic Local accession
9.	Black rice	Local accession
10.	Chittimuthyalu	Indigenous Local accession



Plate 1: A view of experimental plot

3.1.4 Recording the observations

Five plants from each replication were randomly selected for recording the observations. Later the mean of the observations recorded was calculated and considered for analysis. The details of phenotypic field traits considered for recording observations is as follows.

3.1.4.1 Days for fifty percent flowering

The number of days taken for each entry from sowing to opening of flower in fifty percent of the plants was recorded.

3.1.4.2 Days to maturity

The number of days taken for each entry from sowing to maturity was recorded.

3.1.4.3 Plant height at maturity

The height of the plant from base of the plant to tip of the longest plant part was measured in terms of cm and recorded as plant height.

3.1.4.4 Number of tillers per plant maturity

Total number of tillers (both productive and non-productive) per plant was counted at the time of harvest and recorded.

3.1.4.5 Number of productive tillers at maturity

Number of productive tillers was counted at the time of maturity.

3.1.4.6 Panicle length (cm)

Length of a randomly selected panicle from a plant was measured from base of the panicle to its tip in terms of cm and recorded.

3.1.4.7 Plant biomass (g)

The total dry weight of the plant parts above ground (only straw) was recorded.

3.1.4.8 Grain yield per plant (g)

Total weight of all the filled grains of a plant was measured in terms of grams and recorded.

3.1.4.9 Productivity per day per plant

The growth of the plant was measured on per day basis to evaluate the growth habits of the crop. The ratio of the grain yield per plant of the genotype to the maturity duration was considered as productivity.

3.1.4.10 Harvest index

The ratio of the grain yield to the biomass (shoot dry weight) of the plant was calculated and recorded as harvest index.

3.1.4.11 Brown rice Zn and Fe content

The harvested grain samples were hand threshed manually to avoid unnecessary contamination. The healthy grains were then preserved for Zn and Fe estimation.

Healthy grains were dehusked by dehusker. Such dehusked samples were cleaned for presence of whole paddy or deformed grains. Zn and Fe content of the brown rice samples was estimated using XRF (X-Ray Fluorescence) machine at MSSRF Chennai. The instrument was switched on 24 hours before hand. After calibration, Zn and Fe content of around five grams of brown rice samples were estimated (ppm). Three replications were averaged to get the final value. A picture of the XRF instrument used to estimate Zn and Fe in different parts of rice grain is given in Plate 2.

3.1.4.12 Polished rice Zn and Fe content

Brown rice samples subjected to XRF analysis were polished for 150 seconds in non-ferrous polisher at Aerobic Rice Research Laboratory at the Department of Plant Biotechnology, University of Agricultural Sciences, GKVK campus, Bangalore, (Mini Lab Rice Polisher. Model: K-710-Non Ferrous Series). Deformed and half cut grains were excluded.



Plate 2: X-Ray Fluorescence machine for estimation of zinc and iron in rice grain

About five grams of polished grain samples were then used to estimate the Zn and Fe content of the samples (mg/kg). Three replications were taken and averaged to get the final value.

3.1.4.13 Bran Zn and Fe content estimated by AAS (Atomic Absorption Spectrophotometer)

The bran samples were collected at the time of polishing. They were filtered thoroughly in water then were again subjected to washing with distilled water, after which they were dried and subjected to acid digestion. The final filtrate obtained was collected for individual samples and taken to AAS facility at FR&IC, Hebbal, Bengaluru. The readings were noted down and the total Fe and Zn content was calculated later on.

3.1.4.14 Zn and Fe content in grains (ppm) estimated by AAS

The brown and polished rice grains were washed and dried after which they were subjected to acid digestion. The final filtrate obtained for each of the samples was taken to FR&IC (Fisheries Research and Information Centre), Hebbal, Bengaluru, where the Fe and Zn content was estimated using the AAS facility. The readings were recorded and the total Fe and Zn was calculated.

3.1.4.15 Acid digestion and estimation of Fe & Zn using AAS

3.1.4.1 Materials required

- 0.1N HCl
- Nitric acid (HNO₃)
- Perchloric acid (HClO₄)
- Distilled water

3.1.4.2. Protocol

- ❖ 0.5g of the sample (grain, Bran, Brown and Polished rice) was washed in 0.1N HCl twice and once with distilled water.
- ❖ The samples were then left to dry for 1 day.

- ❖ The dried samples were transferred to mortar and were powdered using liquid nitrogen.
- ❖ The powdered samples were transferred to conical flask and 15 ml of concentrated HCl was added and left undisturbed overnight for cold digestion.
- ❖ The next day, 10:4 ratios of concentrated nitric acid and perchloric acid was added and placed over hot pan for hot digestion.
- ❖ The samples were removed from hot pan once they become colourless.
- ❖ The colourless digested samples were left to cool after which they were filtered using wattman filter papers.
- ❖ The volume of the filtrate was made up to 50 ml with distilled water and used for further estimation procedures in AAS.

3.1.4.3 Estimation of Fe & Zn in AAS

AAS was switched on and lamps were also turned on. The standards of Fe and Zn were made ready. Three standards were fed into AAS each for iron and zinc prior to the samples. The standard curve was obtained. The samples were fed one by one and the readings were noted down.

$$\text{Total concentration of Fe/Zn in sample (ppm)} = \frac{\text{Reading} \times \text{Dilution factor}}{0.5}$$

3.1.5 Statistical analyses

Mean values of five plants used for recording the observations were computed for different plant characters for each of the genotypes. Three replications were considered. The phenotypic data for all the genotypes for each character was subjected to statistical analysis.

3.1.6 Analysis of variance

The analysis of variance for different characters was used to partition the variance due to different sources following the method given by Panse and Sukhatme (1964).

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-ratio
Replication	(r-1)	rSS		
Genotype	(g-1)	gSS	$\frac{gSS}{g-1} = gMS$	$\frac{gMS}{EMS}$
Error	(r-1)(g-1)	ESS	$\frac{ESS}{(r-1)(g-1)} = EMS$	
Total	(rg-1)	TSS		

Where,

r = Number of Replications

g = Number of Genotypes

The significance was tested by comparing with the table values as given by Yates (1965). Standard error of means (SEM) and Critical difference (CD) were worked out using appropriate formula for comparing individual line means.

3.1.7 Phenotypic and Genotypic coefficient of variation (PCV and GCV)

The phenotypic and genotypic coefficient of variation was computed as per Burton and Dewane (1953) for low moisture stress.

$$PCV = \frac{P}{X} \times 100$$

$$GCV = \frac{G}{X} \times 100$$

Wherein,

P = Phenotypic standard deviation

G = Genotypic standard deviation

X = Grand mean of character

PCV = Phenotypic coefficient of variation

GCV = Genotypic coefficient of variation

PCV and GCV were classified according to Robinson *et al.*, (1949). 0-10 per cent was considered as low, 10-20 per cent as moderate and more than 20 per cent as high.

3.1.8 Heritability (h^2)

Broad Sense Heritability was calculated using the formula (Hanson *et al.*, 1956).

$$h^2 \% = \frac{V_g}{V_p} \times 100$$

Where,

h^2 % = Heritability percentage

V_g = Genotypic variance

V_p = Phenotypic variance

Heritability percentage was categorized as follows (Robinson *et al.*, 1949).

0-30 percent was considered as low,

30-60 percent as moderate and more than 60 per cent as high.

3.1.9 Genetic advance (GA)

Genetic advance was calculated by using formula given by Johnson *et al.*, (1955).

$$GA = h^2 \times \sigma_p \times K$$

Wherein,

h^2 = Heritability (Broad sense)

σ_p = Phenotypic standard deviation

K = Selection differential which is 2.06 at 5 per cent intensity of selection (Lush, 1949).

3.1.10 Genetic advance as percent mean

$$GAM = \frac{GA}{X} \times 100$$

Wherein,

GA = Genetic advance and

X = Treatment mean for the character.

The GA as per cent mean was classified (Johnson *et al.*, 1955) as given below.

0-10 per cent was considered as Low;

10-20 per cent as Moderate and more than 20 per cent as high.

3.1.11 Correlation analysis

To estimate the degree of association between the traits studied, phenotypic correlation was computed by using the formula given by Webber and Moorthy (1952).

$$r_p = \frac{\text{COV (X,Y)}}{[V(X).V(Y)]^{1/2}}$$

Wherein,

r_p = phenotypic correlation co-efficient.

COV (X, Y) = Phenotypic covariance.

V(X) and V(Y) = Phenotypic variances of the traits X and Y.

3.2 Study the distribution of Zn and Fe in different parts of rice grain

3.2.1 Selection of genotypes

Same as mentioned in 3.1.2.

3.2.2 Layout of the experiment

Same as mentioned in 3.1.3.

3.2.3 Zn and Fe content of brown rice (ppm)

Same as mentioned in 3.1.3.11.

3.2.4 Zn and Fe content of polished rice (ppm)

Same as mentioned in 3.1.3.12.

3.2.5 Distribution of Zn and Fe in different parts of rice grain

Brown rice is botanically nothing but aleurone layer, embryo and endosperm. Polished rice is typically a part of endosperm. By comparing the Zn and Fe content values of brown and polished rice samples for different genotypes, distribution of Zn and Fe in different parts of rice grain can be assessed.

Brown rice = Aleurone layer + Embryo + Endosperm.

Polished rice = Endosperm (Gunstone, 2011)

By deducing the Zn and Fe content values of polished rice from that of brown rice, distribution of micronutrients in aleurone layer, embryo and endosperm was assessed. Meanwhile, per cent loss in Zn and Fe content due to polishing was calculated using the following formula,

$$\% \text{ loss of Zn or Fe} = \frac{(BZ-WZ)}{BZ} \text{ or } \frac{(BF-WF)}{BF} \times 100$$

3.3 Molecular characterization of Zn and Fe transporters in rice grains

3.3.1 Selection of genotypes

As mentioned in 3.1.2.

3.3.2 Layout of the experiment

Same as mentioned in 3.1.3.

3.3.3 Selecting differentially expressed transporter genes in rice grain

3.3.4 Collecting grain samples for RNA isolation

Grain samples were collected from the genotypes under study at dough stage of grain development. Dough stage is a reproductive stage in rice plant where the milky portion of the grain first turns into soft dough and later into hard dough and the grains in the panicle begin to change from green to yellow. The grain samples were washed in running tap water and then in nuclease free water. Excess moisture was removed by

wiping with dry filter paper. Since RNA is highly sensitive to ubiquitously present RNase, RNA activity in the grain was preserved by storing the grain samples at -80 °C immediately after cleaning. This process of sampling, cleaning and storage was done quickly to avoid RNA degradation.

3.3.5 Total RNA isolation from different parts of rice grain

Individual rice grains were dehusked and separated into embryo and endosperm. Total RNA was isolated from both parts of rice grains using modified protocol. Total RNA isolated was re-suspended in nuclease free water (0.1 % DEPC treated water), extreme care was taken during RNA isolation and further steps to avoid RNase contamination.

3.3.5.1 Requirements for total RNA isolation

- Seed samples were collected from all the ten study genotypes at early dough stage. Grains were separated into embryo and endosperm under aseptic condition in Laminar Air Flow (LAF).
- Extraction buffer – I

Tris-HCl (pH 7.0)	100 mM
NaCl	500 mM
EDTA (pH 8)	10 Mm
SDS	2% (w/v)
β -mercaptoethanol	4% (v/v)(freshly added before use).
- Phenol (water saturated):chloroform:isoamyl alcohol (25:24:1, v/v/v).
- Chloroform:isoamyl alcohol (24:1, v/v).
- 6 M and 2 M LiCl.
- Ice cold 70% ethanol (v/v)
- RNase free sterile water (0.1% DEPC—treated autoclaved water).

RNase free water was prepared by adding 1 per cent DEPC in double distilled water, keeping it overnight for dissolving and then removing excess DEPC by autoclaving.

3.3.5.2 Protocol for RNA isolation.

- Grain samples were ground to powder form in pre-chilled mortar and homogenized in 1 ml extraction buffer.
- 1 ml of phenol (water saturated): chloroform: isoamyl alcohol mixture was added and mixed thoroughly. The suspension appeared milky polished in color.
- The mixture was transferred to a microfuge tube and centrifuge at 15,000 rpm for 15 min. The top colorless aqueous phase containing nucleic acids was transferred to a microfuge tube.
- Equal volume of chloroform: isoamyl alcohol mixture was added, and mixed thoroughly by inverting the tube for 15 to 20 times.
- The mixture was then centrifuged at 15,000 rpm for 10 min at 4°C, and the aqueous phase was transferred to a microfuge tube.
- 6 M LiCl was added to a final concentration of 3 M and incubated in –80°C for 5 h or –20°C for 10 h.
- The solution was then centrifuged at 15,000 rpm for 30 min at 4°C to collect RNA as precipitate.
- The precipitate was re-suspended in 400 to 600 µl of 2M LiCl and centrifuged at 15000 rpm for 20 min at 4°C.
- The supernatant was discarded and the RNA pellet was washed with 70% ice cold ethanol.
- The pellet was then air dried and alcohol free pellet was dissolved in 25 to 50 µl of DEPC treated sterile water and stored at -80 °C for using further downstream processes.

3.3.6 Reverse transcription

The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA). *M-MuLV* Reverse Transcriptase was used. *Moloney Murine Leukemia Virus* Reverse Transcriptase (*M-MuLV* RT) is an RNA-dependent DNA polymerase which uses single stranded RNA in the presence of a primer to synthesize a complementary DNA strand. The resulting cDNA is single-stranded and the process is called first strand cDNA synthesis (up to 7 kb) which can be used directly as a template. This enzyme is isolated from *E.coli* expressing a portion of the *pol* gene of *M-MuLV* on a plasmid.

The components and the protocol for reverse transcription (RT) and PCR is as follows,

Hi-cDNA synthesis kit components

1. Reverse transcription buffer.
2. 10X solution.
3. *M-MuLV* Reverse transcriptase.
4. RNA template.
5. Molecular biology grade water.

PCR amplification conditions for preparation of cDNA

I-Step	II-Step	III-Step
50°C	42°C	4°C
15 min	2 min 30 sec	Final hold

cDNA can be further used to perform PCR assay.

3.3.6.1 Protocol for PCR

Taq buffer	1.0 µl
DNTPs	0.5 µl
MgCl ₂	0.5 µl
Forward gene specific primer	0.5 µl
Reverse gene specific primer	1.0 µl
<i>Taq</i> DNA polymerase	0.5 µl
cDNA	3.0 µl
Water	3.0 µl
Total	10 µl

In this process, synthesized first strand cDNA is used as template for PCR amplification. Zn and Fe gene specific primers were used (Bhat, 2015). The PCR mix was made fresh in bulk depending on the number of samples each time. Each 10 µL mix contained.

PCR amplification condition

I-Step	II-Step			III-Step	IV-Step
95°C	94°C	T°C**	72°C	72°C	4°C
5 min	1 min	45 sec	45 sec	3 min	Final hold

N cycles**

** T °C and N Cycles vary from one primer to other

3.3.7 Gel electrophoresis

The PCR products were loaded on to Ethidium Bromide stained 1 to 2 per cent Agarose gels in TAE. A 1 kb or 100 bp DNA ladder molecular weight marker was run on every gel to confirm the expected molecular weight of the amplification product.

IV EXPERIMENTAL RESULTS

The genotypes selected for the study were evaluated during *Kharif*-2016 to know the distribution of micronutrients and relative expression levels of different micronutrient transporters in different grain parts.

The experimental results obtained from the present study are presented in this chapter with following sub-headings.

4.1 Discerning zinc and iron distribution in different parts of the rice grain

4.1.1 Performance of genotypes with respect to morphological traits

4.1.2 Zn and Fe content in different parts of rice grain

4.1.3 Statistical analysis of data

4.2 Expression profiling of transporter across bio-fortified elite lines

4.2.1 Zn and Fe transporters

4.2.2 Total RNA isolation from different parts of rice grain

4.2.3 Reverse transcription of RNA and PCR

4.2.4 Relative expression of Zn and Fe transporters in different parts of grain

4.1 Discerning zinc and iron distribution in different parts of the rice grain

4.1.1 Performance of genotypes with respect to morphological traits

Statistical parameters namely minimum, maximum, mean, genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), broad sense heritability (h^2) and genetic advance as percent mean for recorded for shoot morphological characters and grain Zn and Fe content in all genotypes grown in *Kharif*-2016 are presented in Table 2.

4.1.1.1 Plant height (PH)

Among the selected genotypes, minimum and maximum plant height was found to be 50.06 cm (AM-143) and 114.67 cm (Azucena) respectively with an average of

Table 2: Genetic parameters for yield and its associated traits among selected rice accessions

Traits	Mean $\pm$ SE	Min.	Max.	PCV (%)	GCV (%)	h^2 (%)	GAM (%)
PH (cm)	76.83 $\pm$ 2.04	53.03	114.66	30.2	29.85	97.6	60.77
TNT (no.)	17.6 $\pm$ 2.66	7.2	25.86	39.23	29.16	55.2	44.66
NPT (no.)	16.7 $\pm$ 3.02	9.53	25.2	38.96	23.2	35.6	28.62
PL (cm)	18.61 $\pm$ 1.29	14.26	22.11	18.35	13.83	56.8	21.48
DTM (days)	149.1 $\pm$ 2.33	143.3	171.6	6.23	5.61	81.04	10.41
DFPF (days)	112.3 $\pm$ 2.34	96	123.6	7.77	7.04	82.2	13.16
DFF (days)	100.4 $\pm$ 2.12	89	117.6	8.6	7.59	77.8	13.8
BM (gm)	22.89 $\pm$ 4.28	13.01	32.36	39.14	21.93	31.39	25.31
GYP (gm)	11.43 $\pm$ 3.10	3.86	22.37	58.16	34.24	34.6	41.54
HI	0.52 $\pm$ 0.11	0.18	0.77	44.93	25.75	32.86	30.41
PDP	3.81 $\pm$ 1.0	1.2	7.4	58.16	34.27	34.73	41.64
ZDP	0.65 $\pm$ 0.15	0.28	1.14	50.32	28.33	31.71	32.87
FDP	0.46 $\pm$ 0.11	0.18	0.83	51.03	24.35	22.7	23.94

DFF=Days for first flowering

DTM= Days to maturity

TNT= Total numbers of tillers

PL= Panicle length (cm)

HI= Harvest index

PDP= Productivity per day per plant

FPD= Iron productivity per day

GCV= Genotypic coefficient of variation

GAM= Genetic advance as per mean

DFPF= Days for fifty percent flowering

PH= Plant height (cm)

NPT= Number of productive tillers

GYP= Grain Yield per Plant (g)

BM= Biomass (g)

ZPD= Zinc productivity per day

PCV= Phenotypic coefficient of variation

h^2 = Heritability in broad sense

76.83 cm. The trait also possessed high PCV of 30.2, GCV of 29.85, heritability of 97.6 per cent Genetic advance as per mean (GAM) of 60.77 per cent.

4.1.1.2 Number of tillers (NT)

Minimum number of tillers was recorded in Azucena (7.20), whereas maximum number of tillers was observed in ARB-6 (25.87) with a mean value of 17.6. The trait had high PCV of 39.23, GCV of 29.16, moderate heritability (55.2) and moderate GAM (44.66 per cent).

4.1.1.4 Number of productive tillers (NPT)

Average value for number of productive tillers in these selected genotypes was 16.7 with a range of 9.53 (Azucena) to 25.60 (ARB-6). High PCV of 38.96, GCV of 23.2, moderate heritability of 35.6 and high GAM of 28.62 was recorded for this trait.

4.1.1.5 Days for first flowering (DFF)

Minimum of 89 days (Black rice), maximum of 117 days (Azucena) and average days for first flowering being 100.4 days among the genotypes. Indeed, a low PCV of 8.6, low GCV of 7.59, high heritability of 77.8 per cent and moderate GAM of 13.8 per cent was recorded for this trait among the genotypes under study.

4.1.1.6 Days for fifty percent flowering (DFPF)

Days for fifty percent flowering ranged from 95 days (Black rice) to 126 days (Azucena) with a mean of 112.3 days. The trait exhibited low PCV of 7.77, low GCV of 7.04, high heritability of 82.2 per cent, and moderate GAM of 13.16 per cent among the genotypes selected.

4.1.1.7 Days to maturity (DTM)

The trait ranged from 143.33 days (AM-1) to 171.67 days (Azucena) with an average of 149.1 days. A low PCV of 6.23, high GCV of 5.61, high heritability of 81.04 per cent and moderate GAM of 10.41 per cent was observed for the trait among the selected rice accessions under study.

4.1.1.8 Panicle length (PL)

Maximum panicle length of 22.11 cm (Azucena), minimum panicle length of 14.27 cm (AM-143) with average of 18.61 cm was recorded among the genotypes. The trait possessed a moderate PCV of 18.35, GCV of 13.83, and moderate heritability of 56.8 percent and high GAM of 21.48 per cent.

4.1.1.9 Biomass (g)

Minimum and maximum biomass was found to be 13.01 g (AM-143) and 32.36 g (ARB-6) respectively with an average of 22.89. The trait possessed high PCV of 39.14, high GCV of 21.93, moderate heritability of 31.39 per cent, and high GAM of 25.31 per cent.

4.1.1.10 Grain yield (g)

Average value for grain yield per plant recorded in these selected genotypes was 11.43 g/plant with a range of 3.86 g/plant (Black rice) to 16.17 g/plant (AM-65). High PCV of 58.16, moderate GCV of 34.24, moderate heritability of 34.6 percent and high GAM of 41.54 per cent.

4.1.1.11 Harvest index (HI)

An average of 0.52 was observed for this trait with a range of 0.18 (Black rice) to 0.77 (AM-143). High PCV of 44.93, GCV of 25.75 and moderate heritability of 32.86 percent and high GAM of 30.41 per cent.

4.1.1.12 Productivity per day per plant (PDP)

The productivity per day per plant of rice plants ranged from 1.29 g/day/plant (Black rice) to 7.46 g/day/plant (ARB-6) with an average of 3.81. High PCV of 58.16, GCV of 34.27, moderate heritability of 34.73 per cent, and high GAM of 41.64 per cent.

4.1.1.13 Zinc productivity per day (ZPD)

The trait ranged from 0.28 mg/kg/day (Black rice) to 1.14 mg/kg/day (ARB-6) with an average of 0.65. High PCV of 50.32, GCV of 28.33, and moderate heritability of 31.71 per cent, high GAM of 32.87 per cent.

4.1.1.14 Iron productivity per day (FPD)

Minimum Fe productivity was found in 0.18 mg/kg/day (Black rice) to 0.83mg/kg/day (ARB-6). The average Fe productivity among the genotypes calculated to be 0.45 mg/kg/day. The trait exhibited high PCV of 51.03, GCV of 24.35 and moderate heritability and high GAM of 22.7 per cent and 22.94 per cent respectively.

4.1.2 Zn and Fe content in different parts of rice grain

4.1.2.1 Zn and Fe content in different parts of rice grain

Average Zn and Fe content in different parts of rice grain among genotypes studied are presented in Table 3. Various genetic parameters were imposed on Zn and Fe content in different parts of rice grain among selected genotypes to know the variability of the trait. The results obtained are represented in Table 4.

4.1.2.2 Brown rice zinc content (BZC)

An average of 26.72 ppm was observed for this trait with a range of 22.56 ppm (ARB-6) to 35.6 ppm (Chittimuthyalu). Moderate PCV of 18.11, GCV of 14.99, high heritability of 68.5 per cent and high GAM of 25.56 percent was recorded for this trait in these genotypes (Fig. 1 and 3).

4.1.2.3 Polished rice zinc content (PZC)

The trait had a minimum value of 18.90 ppm (AM-143) and a maximum value of 27.27 ppm (Chittimuthyalu) with an average of 21.92 ppm. A moderate GCV, moderate PCV, moderate heritability and moderate GAM (16.39, 12.16, 55.1 % and 18.6 % respectively) was found for this trait (Fig. 1 and 4).

Table 3: Average Zn and Fe content in different parts of rice grain among selected genotypes

Genotypes	BFC	BZC	PFC	PZC
AM-1	17.46	28.63	3.13	24.2
AM-27	21.8	23.56	6.56	19.8
AM-65	16.53	25.2	5.33	20.43
AM-72	17.3	24.6	5.7	19.8
AM-94B	17.5	24.1	5.36	19.1
AM-143	19.16	23.3	8.93	18.9
ARB-6	16.73	22.56	2.73	20.3
Azucena	18.2	27.33	4.96	23.96
Black rice	20.53	32.3	3.53	25.46
Chittimuthyalu	21	35.6	11.96	27.26

Table 4: Genetic parameters for zinc and iron content in different parts of the rice grain traits among selected genotypes

Traits	Mean $\pm$ SE	Min.	Max.	PCV (%)	GCV (%)	h^2 (%)	GAM (%)
BFC	18.62 $\pm$ 0.88	16.53	21.8	12.17	8.96	54.24	13.6
PFC	5.82 $\pm$ 1.18	2.7	11.96	56.24	43.78	60.6	70.21
BZC	26.72 $\pm$ 1.56	22.56	35.6	18.11	14.99	68.5	25.56
PZC	21.92 $\pm$ 1.39	18.9	27.26	16.39	12.16	55.1	18.6

BRF=Brown rice iron content
PFC= Polished rice iron content

BRZ= Brown rice zinc content
PZC= Polished rice zinc content

4.1.2.4 Brown rice iron content (BFC)

Minimum Fe content in brown rice was 16.53 ppm (AM-65); maximum was 21.80 ppm (AM-72) with an average of 18.62 ppm. A moderate PCV, low GCV, moderate heritability, and moderate GAM (12.17, 8.96, 54.24 % and 13.6 % respectively) was observed for this trait (Fig. 2 and 5).

4.1.2.5 Polished rice iron content (PFC)

For this trait, minimum reading obtained was 2.7 ppm (ARB-6), and maximum readings recorded were 11.96 ppm (Chittimuthyalu) with a mean value of 5.82 ppm. A high PCV, high GCV, high heritability, and high GAM (56.24, 43.78, 60.6 % and 70.21 % respectively) were recorded for this trait when selected genotypes were compared (Fig. 2 and 6).

4.1.3 Statistical analysis of data

4.1.3.1 Analysis of variance

The mean sum of squares due to various sources of variation for yield and its associated traits, brown rice Zn and Fe content are estimated.

4.1.3.1.1 Analysis of variance for yield and its associated traits

Mean sum of squares value for the yield and its associated traits are represented in (Table 5 and 6). Most highly significant differences among the genotypes under study were found for four traits viz., plant height, days for first flowering, days for fifty percent flowering, days to maturity, at <0.01 per cent level of significance (LOS), total number of tillers, number of productive tillers, panicle length, grain yield, harvest index, showed highly significant difference among the genotypes at 1% and 5% level of significance (LOS) indicating a wide variance among the genotypes for these traits. There was no significant difference among the genotypes for three traits viz., biomass, zinc productivity per day and iron productivity per day.

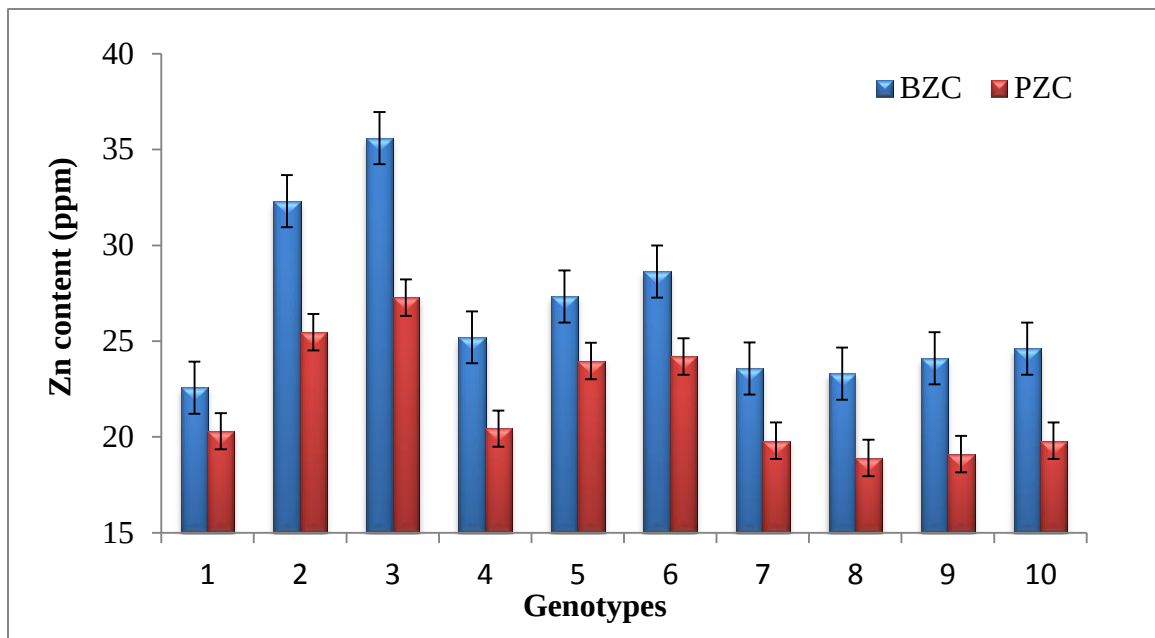


Fig. 1: Zinc content in brown and polished rice samples of selected genotypes

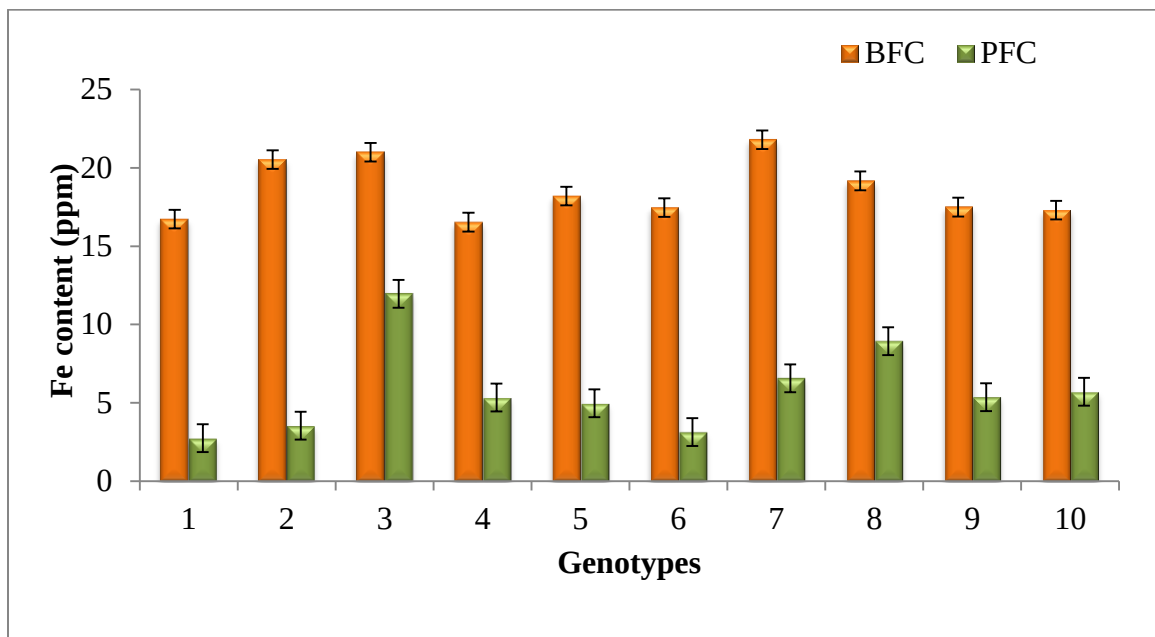


Fig. 2: Iron content in brown and polished rice samples of selected genotypes

Legends:

1- ARB-6,	2- Black rice,	3- Chittimuthyalu,	4- AM-65,	5- Azucena,
6- AM-1,	7- AM-27,	8- AM-143,	9- AM-94B,	10- AM-72
BFC- Brown rice iron content,		PFC- Polished rice iron content		
BZC- Brown rice zinc content		PZC- Polished rice zinc content		

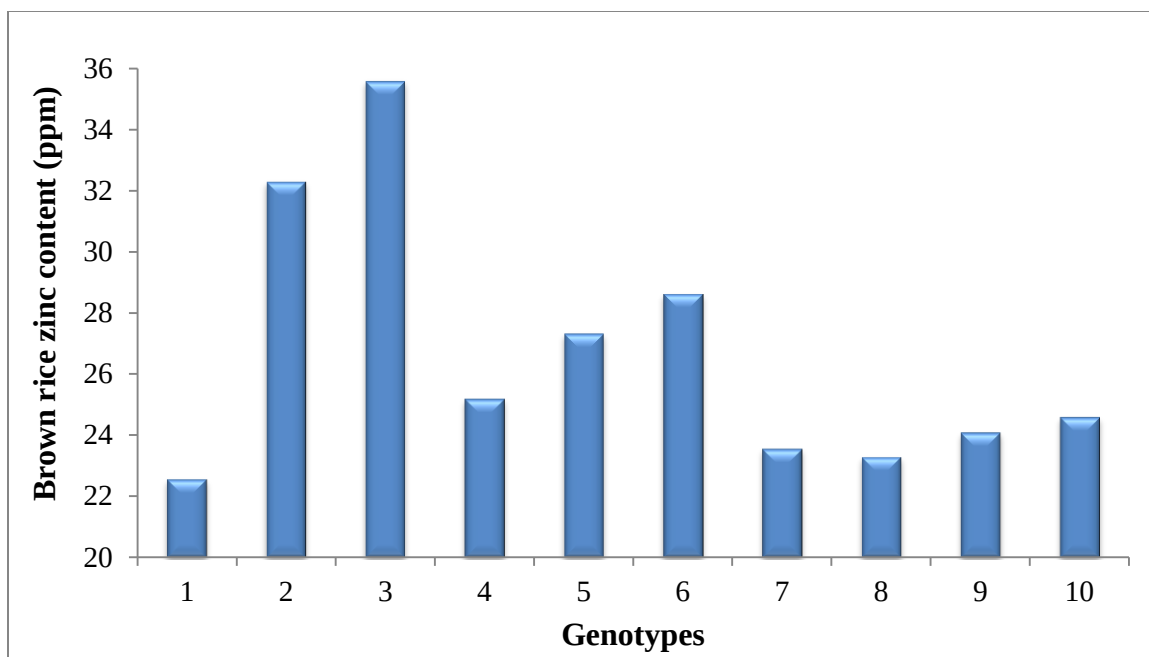


Fig. 3: Zinc content in brown rice samples of selected genotypes

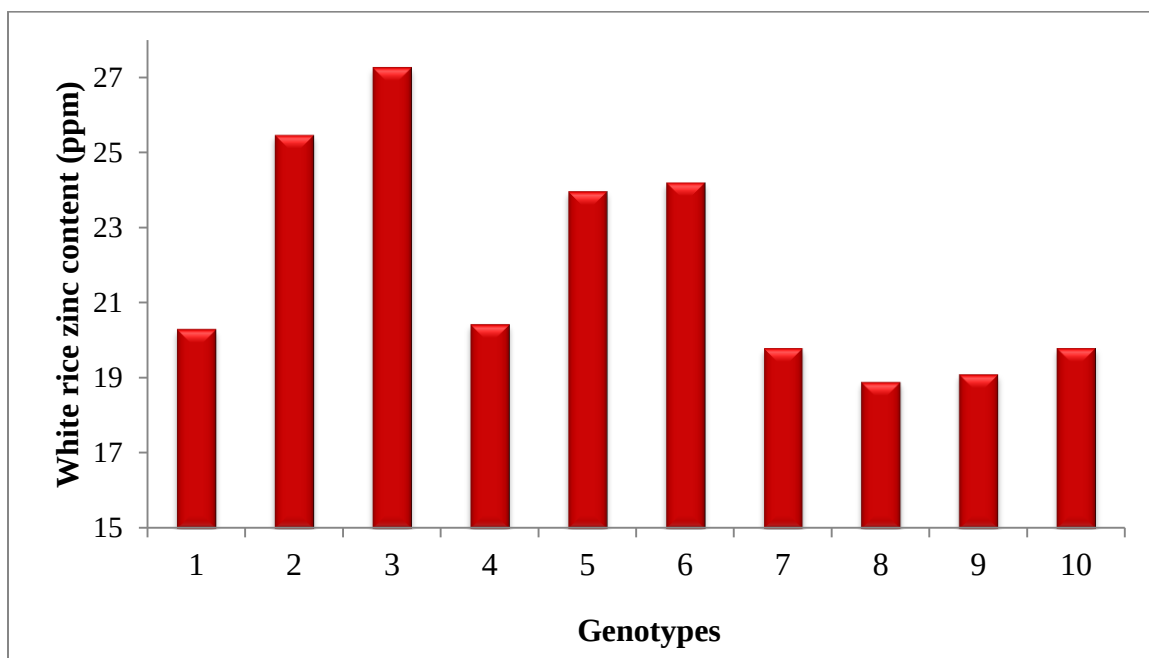


Fig. 4: Zinc content in polished rice samples of selected genotypes

Legends:

1- ARB-6,	2- Black rice,	3- Chittimuthyalu,	4- AM-65,	5- Azucena,
6- AM-	7- AM-27,	8- AM-143,	9- AM-94B,	10- AM-72

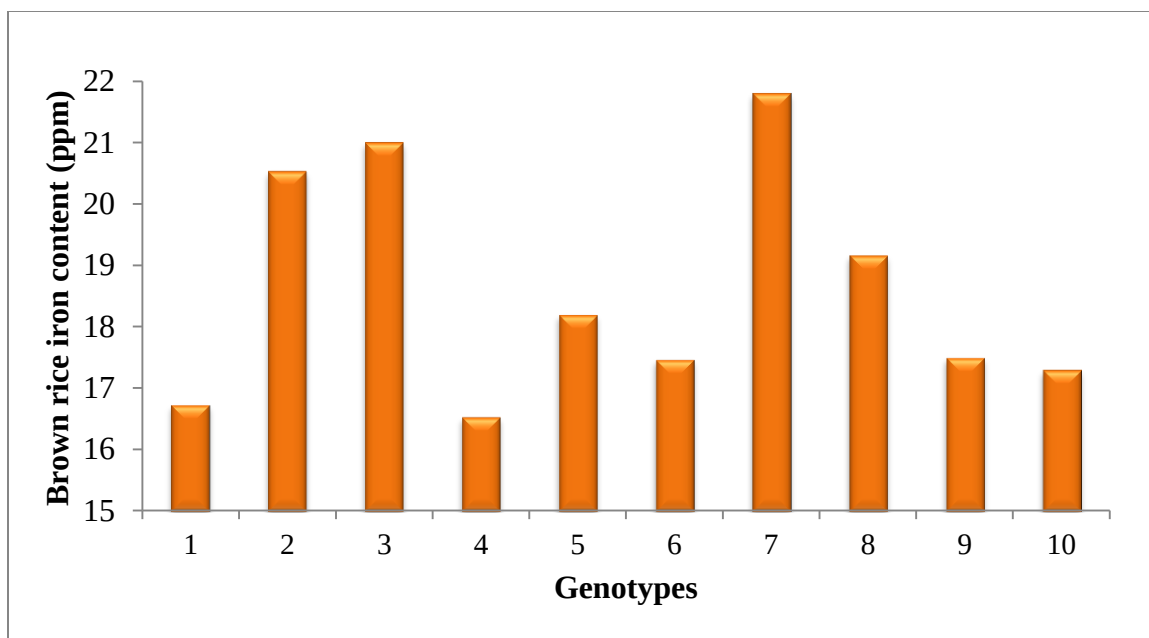


Fig. 5: Iron content in brown rice samples of selected genotypes

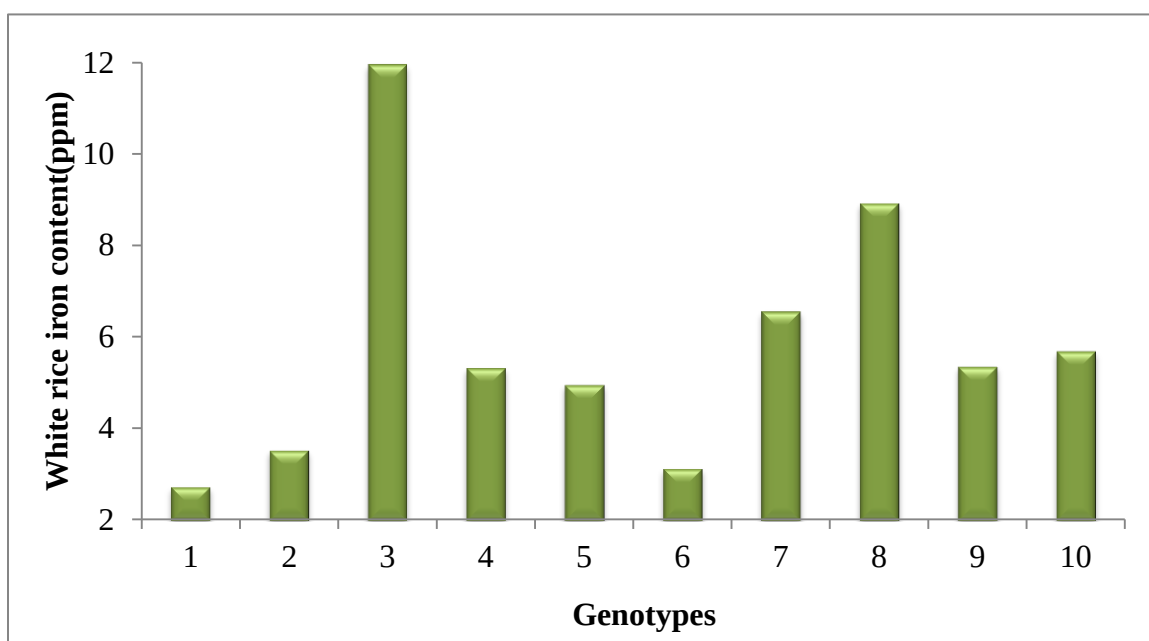


Fig 6: Iron content in polished rice samples of selected genotype

Legends:

1- ARB-6,	2- Black rice,	3- Chittimuthyalu,	4- AM-65,	5- Azucena,
6- AM-1,	7- AM-27,	8- AM-143,	9- AM-94B,	10- AM-72

Table 5: ANOVA for seven yield and its associated traits in selected rice accessions

Source of variation	df	Mean Sum of Squares						
		PH	TNT	NPT	GY	BM	ZPD	FPD
Genotypes	9	1591.0***	100.38**	73.30*	74.96*	130.71 ^{NS}	0.17 ^{NS}	0.08 ^{NS}
Error	18	12.52	21.33	27.54	28.92	55.09	0.07	0.04
CD @ 5%		6.07	7.92	9.00	9.22	12.7	0.46	0.35
CD @ 1%		8.31	10.85	12.33	12.63	17.4	0.64	0.48
CV		4.60	26.24	31.25	47.01	32.42	41.58	44.85

Table 6: ANOVA for six yield and its associated traits in selected rice accessions

Source of variation	df	Mean Sum of Squares					
		HI	PL	DFF	DFPF	DTM	PDP
Genotypes	9	0.09*	24.92**	191.11***	201.81***	226.90***	8.33*
Error	18	0.03	5.03	16.52	13.56	16.41	3.20
CD @ 5%		0.33	3.84	6.97	6.31	6.95	3.07
CD @ 1%		0.45	5.27	9.55	8.65	9.52	4.20
CV		36.81	12.05	4.04	3.27	2.71	46.98

* Significant at 5% ** Significant at 1% *** Significant at < 0.01%

NS=Non-significant

PH= Plant height, **TNT**= Total number of tillers, **NPT**= Number of productive tillers

GY= Grain yield, **ZPD**=Zinc productivity per day, **FPD**=Iron productivity per day

HI= Harvest index, **PL**= Plant height, **DFF**=Days for first flowering,

DFPF= Days for fifty percent flowering, **DTM**=Days to maturity, **PDP**= Productivity per day per plant, **BM**= Biomass

4.1.3.1.2 Analysis of variance for Zn and Fe content in different parts of rice grain among selected genotypes

Analysis of variance was performed for Zn and Fe content in different parts of rice grain and the results obtained are presented in Table 7.

There was very high significant difference among the study genotypes for all the traits for brown rice Zinc and polished rice iron content at 0.1 per cent LOS. For brown rice iron and polished rice zinc content, highly significant difference was found among the genotypes at 1 per cent LOS.

4.1.3.2 Correlation studies

The results of the correlation analysis for yield and its associated traits and grain Zn and Fe content are presented in the Table 8.

4.1.3.2.1 Correlation analysis for yield and its associated traits

Total number of tillers had a significant positive correlation with number of tillers (0.84), productivity per day (0.38), iron productivity per day (0.49), and zinc productivity per day (0.41) and in contrary, showed a significant negative correlation with days to maturity (-0.60), days for fifty percent flowering (-0.52), and days for first flowering (-0.39).

Panicle length (0.74), days to maturity (0.52), days for fifty percent flowering (0.41), and biomass (0.37) showed significant positive correlation with plant height, number of productive tillers (-0.57), and total number of tillers (-0.66) revealed a significant negative correlation with plant height.

Number of productive tillers (0.46), days for fifty percent flowering (0.92), and days for first flowering (0.68) tillers had a significant positive correlation with days to maturity (0.46) and significant negative correlation with panicle length (-0.46), and days for fifty percent flowering (-0.39).

Table 7: ANOVA for zinc and iron content in different parts of the rice grain among rice genotypes

Source of variation	df	Mean sum of squares			
		BFC	BZC	PFC	PZC
Genotype	9	10.71**	55.52***	23.73***	27.14**
Error	18	2.35	7.37	4.22	579
CD at 5%		2.63	4.65	3.52	4.13
CD at 1%		3.60	6.38	4.83	5.65
CV		8.23	10.16	35.30	10.98

BFC= Brown rice iron content

PFC= Polished rice iron content

*** Significant at <0.01 %

BZC= Brown rice zinc content

PZC= Polished rice iron content

** Significant at 1%

Table 8: Phenotypic correlation coefficients for yield and its associated traits among selected genotypes

	PH	TNT	NPT	PL	DTM	DFPF	DFF	BM	HI	PDP	FPD	ZPD
TNT	-0.66***	1.00										
NPT	-0.57***	0.84***	1.00									
PL	0.74***	-0.34	-0.46*	1.00								
DTM	0.52**	-0.60***	0.46**	0.23	1.00							
DFPF	0.41*	-0.52**	-0.39*	0.21	0.92***	1.00						
DFF	0.26	-0.39*	-0.30	0.21	0.68***	0.81***	1.00					
BM	0.37*	0.22	0.14	0.50**	-0.16	-0.10	-0.15	1.00				
HI	-0.225	0.16	0.09	-0.20	0.21	0.35	0.34	-0.30	1.00			
PDP	0.08	0.38*	0.25	0.21	0.13	0.30	0.21	0.57***	0.52**	1.00		
FPD	-0.03	0.49**	0.32	0.15	0.01	0.18	0.16	0.57***	0.49**	0.96***	1.00	
ZPD	0.07	0.41*	0.24	0.24	0.06	0.23	0.24	0.59***	0.43*	0.95***	0.95***	1.00
GY	0.08*	0.38	0.25	0.21	0.13	0.30	0.21	0.57***	0.52**	1.00	0.96***	0.95***

*** Significant at <0.01 %

** Significant at 1%

PH= Plant height **TNT**= Total number of tillers **NPT**= Number of productive tillers **GY**= Grain yield **ZPD**=Zinc productivity per day
FPD= Iron productivity per day **HI**= Harvest index **PL**= Plant height **DFF**=Days for first flowering
DFPF= Days for fifty percent flowering **DTM**=Days to maturity **PDP**= Productivity per day per plant **BM**=Biomass.

Panicle length revealed a significant positive correlation with biomass (0.50). Productivity per day per pant (0.52), iron productivity per day (0.49), and zinc productivity per day (0.43) disclosed a significant positive correlation with biomass and harvest index.

Iron productivity per day (0.96), and zinc productivity per day (0.95) revealed a significant positive correlation with productivity per day per plant. Productivity per day per plant (0.95), and iron productivity per day (0.95) had a significant positive correlation with zinc productivity per day.

4.1.3.2.2 Correlation analysis for grain Zn and Fe content

The results of correlation coefficients for Zn and Fe content in different parts of rice grain are presented in Table 9.

Brown rice zinc content had a significant positive correlation with brown rice iron content (0.45), and polished rice zinc content (0.84). Brown rice iron content showed a significant positive correlation with polished rice zinc content (0.41), and polished rice iron content (0.5) while correlation between other trait combinations were found to be statistically insignificant.

4.1.3.3 Loss of Zn and Fe due to the process of polishing

Loss of Zn and Fe in terms of percent is tabulated in Table 10. Average percent loss of Zn for all the genotypes studied was found to be 32 per cent, and it was 87 per cent for Fe. Highest loss of Zn after polishing was found for Chittimuthyalu (23.58%) and lowest loss was found to be for ARB-6 (10.08 %). In case of Fe, highest percent loss of Fe was found to be for ARB-6 (83.71 %) and lowest for Chittimuthyalu (43 %). Also, variance analysis (ANOVA) suggested significant difference for percent loss of Fe among the selected genotypes, however non significance for percent loss of Zn. Results obtained for ANOVA for per cent loss is presented in Table 11. Correlation coefficients for per cent loss of Zn and Fe are tabulated in Table 12.

Table 9: Correlation coefficients for distribution of zinc and iron among selected genotypes

	BZC	BFC	PZC
BFC	0.45*	1.00	
PZC	0.84***	0.41*	1.00
PFC	0.29	0.51**	0.16

*** Significant at <0.01 %

** Significant at 1%

*Significant at 5%

BFC= Brown rice iron content

BZC= Brown rice zinc content

PFC= Polished rice iron content

PZC= Polished rice iron content

Table 10: Per cent loss of zinc and iron from selected genotypes due to polishing

Genotypes	PLF	PLZ
AM-1	82.04	15.63
AM-27	69.92	15.97
AM-65	68.29	18.87
AM-72	66.62	18.06
AM-143	54.57	17.17
AM-94B	70.39	19.95
ARB-6	83.71	10.08
Azucena	72.45	12.54
Black rice	82.69	21.07
Chittimuthyalu	43.00	23.58

Table 11: ANOVA for loss of zinc and iron from selected genotypes due to polishing

Source of variation	df	Mean sum of squares	
		PLF	PLZ
Genotype	9	492.40**	74.92 ^{NS}
Error	18	93.19	47.68
CD at 5%		16.56	14.84
CD at 1%		22.68	20.34
CV		13.91	50.05

**= Significant at 1%

NS= Non-significant

PLF= Per cent loss of iron

PLZ= Per cent loss of zinc

Table 12: Correlation coefficients indicating correlation of per cent zinc and iron lost due to polishing with harvest index

	HI	PFC	PLF	PLZ
PFC	0.10	1.00		
PLF	-0.17	-0.97***	1.00	
PLZ	-0.30	0.20	-0.116	1.00

***=Significant at <0.01%

HI=Harvest index

PFC= Polished rice iron content

PLF= Per cent loss of iron content

PLZ= Per cent loss of zinc content

4.2 Expression profiling of transporter across bio-fortified elite lines

4.2.1 Zn and Fe transporters

A list of all the rice micronutrient transporters explored from several literatures is shown in Table 13.

4.2.2 Total RNA isolation from different parts of rice grain

Total RNA isolated from ten different genotypes is presented in Plate 3a and 3b. Total RNA from endosperm and embryo was isolated separately.

Rice seeds were collected during late dough stage of seed development and stored at -80°C. Rice seeds were separated into embryo and endosperm under aseptic condition and nuclease free condition as shown in Plate 4. Total RNA was isolated from embryo and endosperm using modified protocol (Sajeevan *et al.*, 2014).

Total RNA isolated were quantified using nanodrop (Eppendorf Biospectrometer). Nanodrop readings for embryo and endosperm were tabulated in Table 14.

4.2.3 Reverse transcription of RNA and PCR

Extracted RNA was converted to c-DNA by using *M-MuLV* Reverse transcription kit. Relative expression of three genes (*OsZIP* 4, *OsYSL* 2, and *OsYSL* 15) by using five primers (*OsZIP*4a, *OsZIP* 4b, *OsYSL* 2b, *OsYSL* 2c, and *OsYSL* 15b). Actin (Lee *et al.*, 2007) gene which act as an internal control for relative expressions of genes. Primers specific to the nucleotide sequence of cDNA of transporters are given in Table 15.

4.2.4 Relative expression of Zn and Fe transporters in different parts of grain

Totally five primers were studied to know the relative expression of three genes. Results obtained for five primers are presented below as subheadings. For each primer, the band intensity values were generated by using Gel Analyzer software (Version: 2010a freeware). These band intensity values for individual genotypes under study were compared to reveal the relative expression of individual genes in embryo and endosperm.

Table 13: List of micronutrient transporters explored for the present study from literature

Sl. No.	Transporter	Gene	Gene Id	Chrm.	NCBI ref seq.	Reference
1	OsZIP4	Os08g0207500	47169686	8	NC_008401.2	Chen <i>et al.</i> , 2008
2	OsYSL2	Os02g0649900	297599682	2	NM_001054121.2	Conte and Walker, 2011
3	OsYSL15	Os02g0650300	297721466	2	NM_001186167.1	Inoue <i>et al.</i> , 2009

Table 14: Nanodrop readings for total RNA isolated from embryo and endosperm of selected genotypes

Genotypes	Concentration (µg/mL)		A ₂₆₀		A ₂₆₀ /A ₂₈₀	
	Embryo	Endosperm	Embryo	Endosperm	Embryo	Endosperm
AM 1	55.2	15.3	2.35	0.59	2.00	1.89
AM 27	25.3	50.6	0.63	1.26	2.03	2.01
AM 65	25.4	38.2	0.63	0.95	2.07	2.01
AM 72	84.6	87.0	2.16	2.17	2.05	1.93
AM 94B	83.5	118.2	2.10	0.29	2.01	1.93
AM 143	56.9	31.7	1.19	1.26	1.97	1.89
ARB 6	61.2	74.9	1.53	1.87	1.90	2.00
Azucena	64.2	50.4	1.62	1.26	1.90	1.94
Black rice	30.6	65.5	0.76	1.63	2.07	2.04
Chittimuthyalu	85.3	36.5	2.75	1.28	2.02	1.96

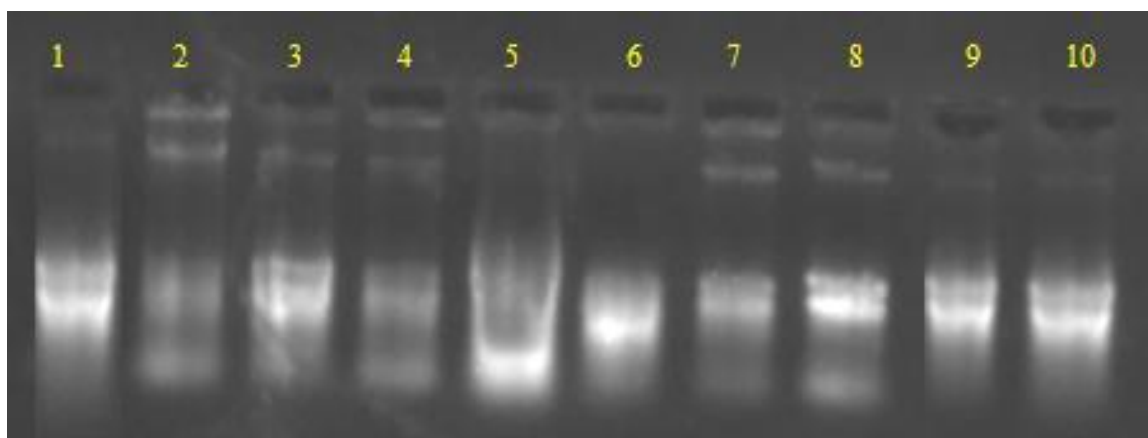


Plate 3a: Total RNA isolation from endosperm of selected rice genotypes

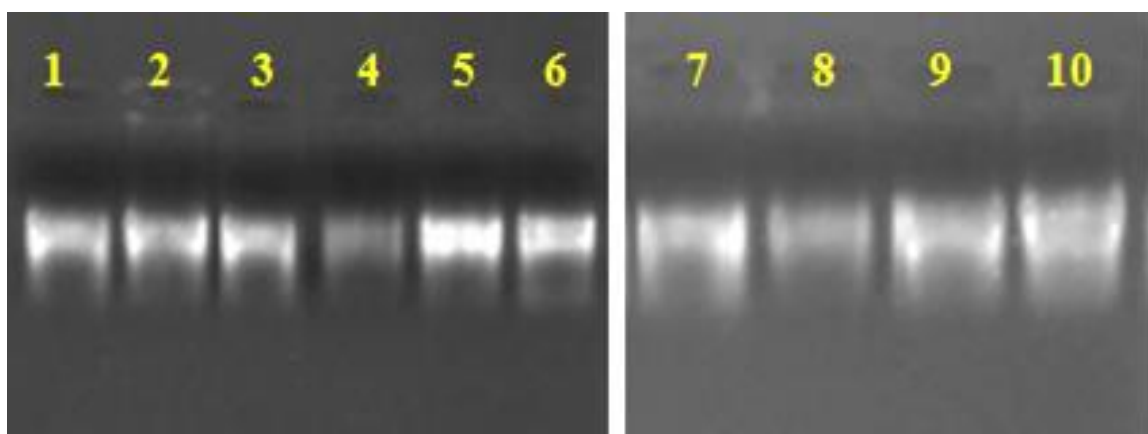


Plate 3b: Total RNA isolation from embryo of selected rice genotypes

Legends:

1- ARB-6,	2- Black rice,	3- Chittimuthyalu,	4- AM-65,	5- Azucena,
6- AM-1	7- AM-27,	8- AM-143,	9- AM-94B,	10- AM-72

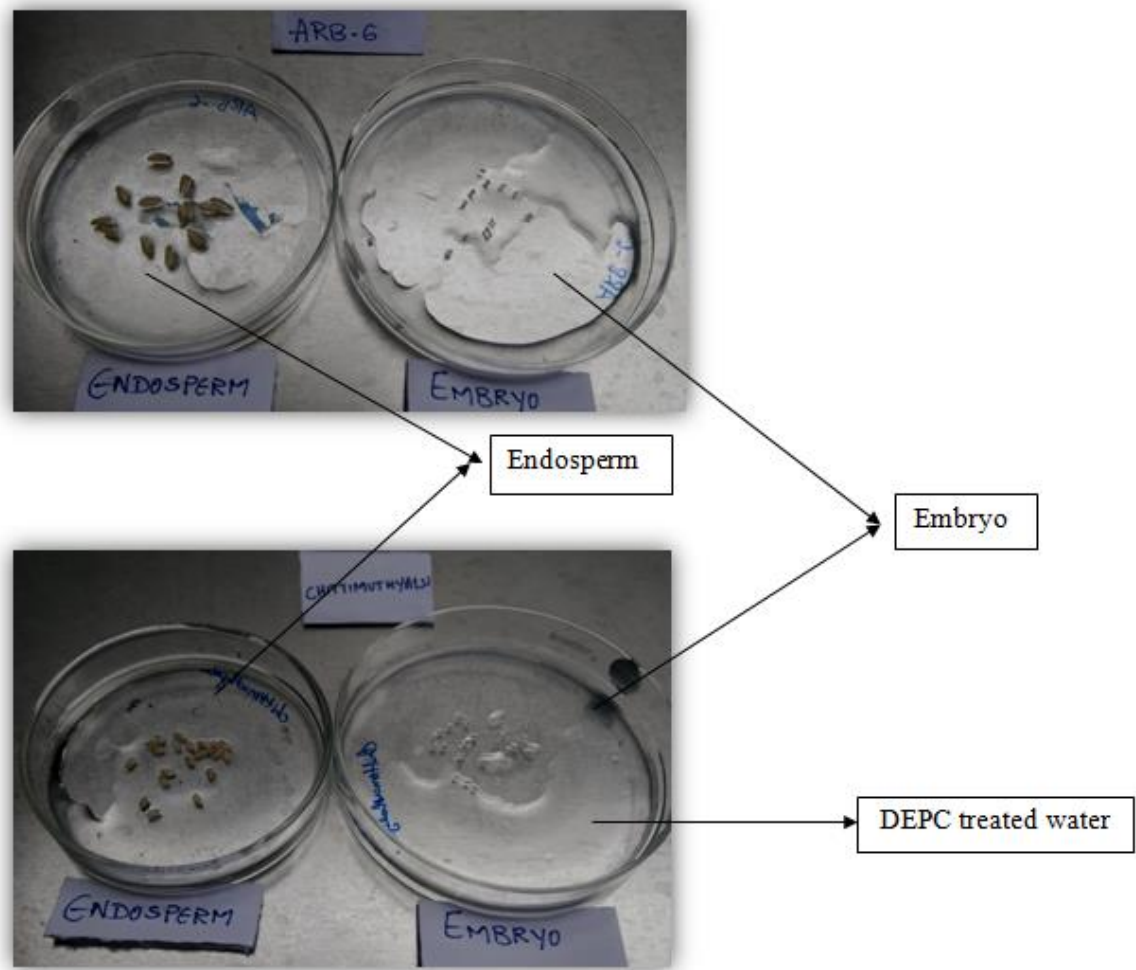


Plate 4: Separation of embryo and endosperm under aseptic and nuclease-free condition

Table 15: Details of the gene specific primers used

Sl. No.	Primer name	Forward primer sequence(5'-3')	bp	Reverse specific primer(5'-3')	bp	Product size	Annealing temperature
1.	OsZIP4a	CAGGCAAATTTTAAGGCGAAAGC	23	GACCAGGGACATGTAGATCAGTAT	24	186	54.6
2.	OsZIP4b	AATCTCATCCAGCTATAGCAAGCAC	25	GCTGAAGCTTTGTGTTTGTGTTGAAG	25	158	55.2
3.	OsYSL2b	GTAATTGCGATACCCATAATGTTCC	25	CTTGTACTAGCTGCTTCACTATTC	23	235	54.2
4.	OsYSL2c	TTCTTATAAACGGATTCCATACCCC	25	GAACATTTCCAGGATACCACTTAC	24	520	54.2
5.	OsYSL15b	GATGATTTGCTCACACCTTG	20	CGTAGGCTATGATCACGTAG	20	670	50.8
6.	OsAct1	GTATCCATGAGACTACATACAACT	24	TACTCAGCCTTGGCAATCCACA	22	348	53.5

4.2.4.1 OsZIP4a

The results obtained from RT-PCR analysis for expression of *OsZIP 4a* in endosperm of selected genotypes is displayed in Plate 5a and 5b respectively.

Expression of *OsZIP4a* was found only in endosperm. There is no or nil expression in embryo.

In endosperm relative expressions of *OsZIP4a* was found higher in AM-72 and relatively lower in ARB-6.

4.2.4.2 OsZIP4b

Relative expression of *OsZIP4a* obtained from RT-PCR analysis for endosperm and embryo are presented in Plate 6a and 6b respectively.

Expression of *OsZIP4a* was found to be relatively higher in embryo than in endosperm in all selected genotypes except in AM-65 where expression was higher in endosperm than in embryo

Both in embryo and endosperm, the band intensity values of genotypes under study for *OsZIP4b* generated using Gel Analyzer software revealed expression of *OsZIP4b* in both tissues was relatively higher in Black rice and lower expression in AM-72.

4.2.4.3 OsYSL2b

The results for relative expression study done for *OsYSL 2b* in endosperm and of selected genotypes is shown in Plate 7a and 7b respectively.

Expression of *OsYSL2b* was found only in endosperm. There is no or nil expression in embryo.

In endosperm relative expressions of *OsYSL2b* was found higher in Black rice and relatively lower in AM-72.

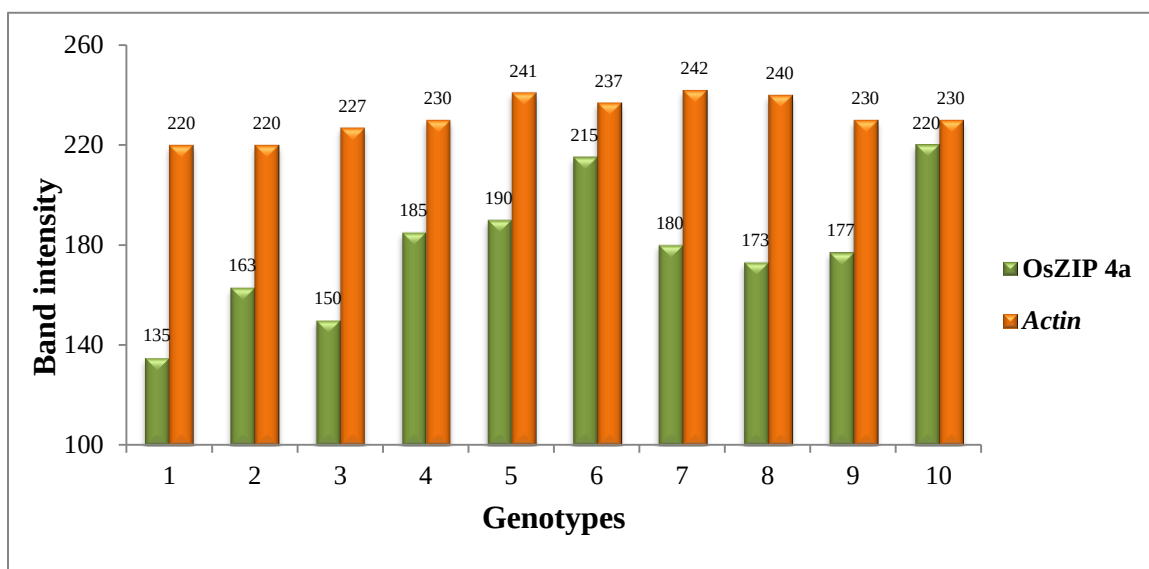
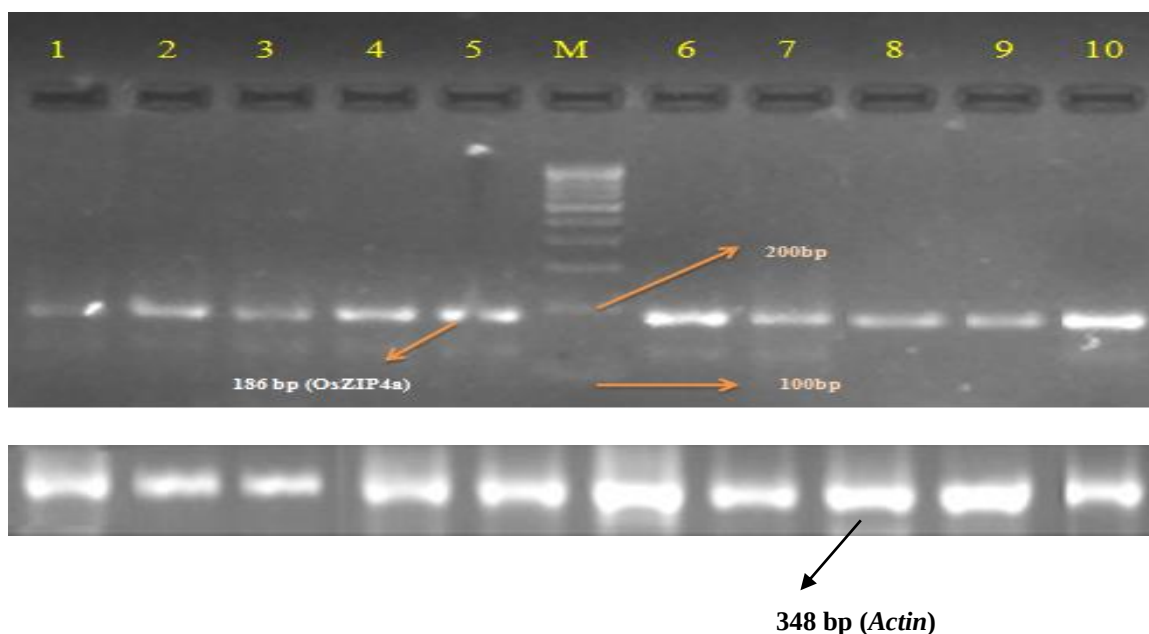


Plate 5: Relative expression of OsZIP4a in endosperm of selected genotypes

Legends:

1- ARB-6, 2- Black rice, 3- Chittimuthyalu, 4- AM-65, 5- Azucena,
6- AM-1 7- AM-27, 8- AM-143, 9- AM-94B, 10- AM-72

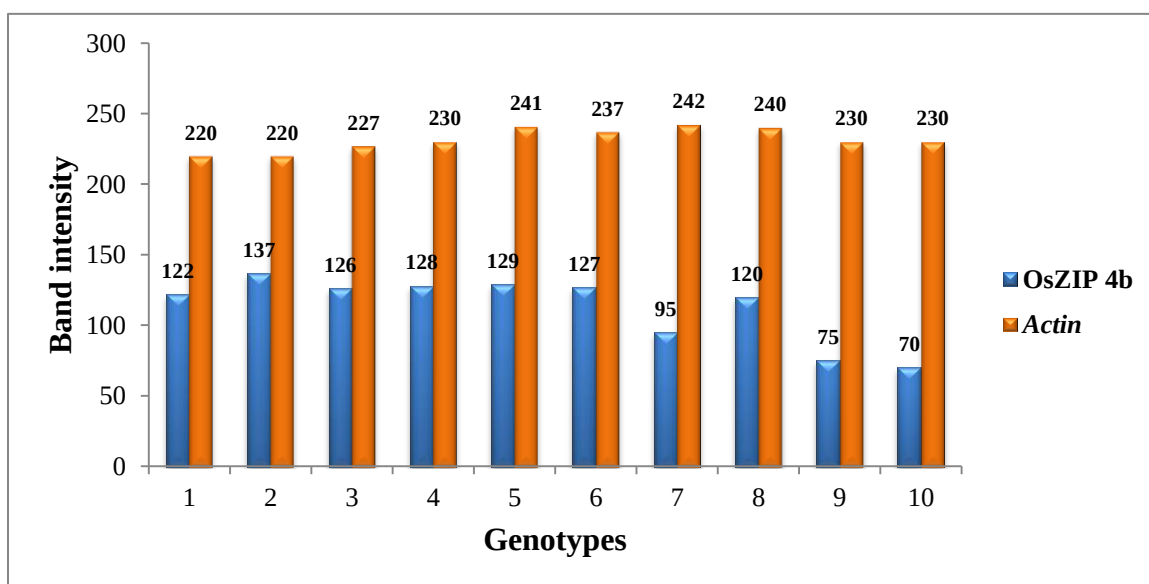
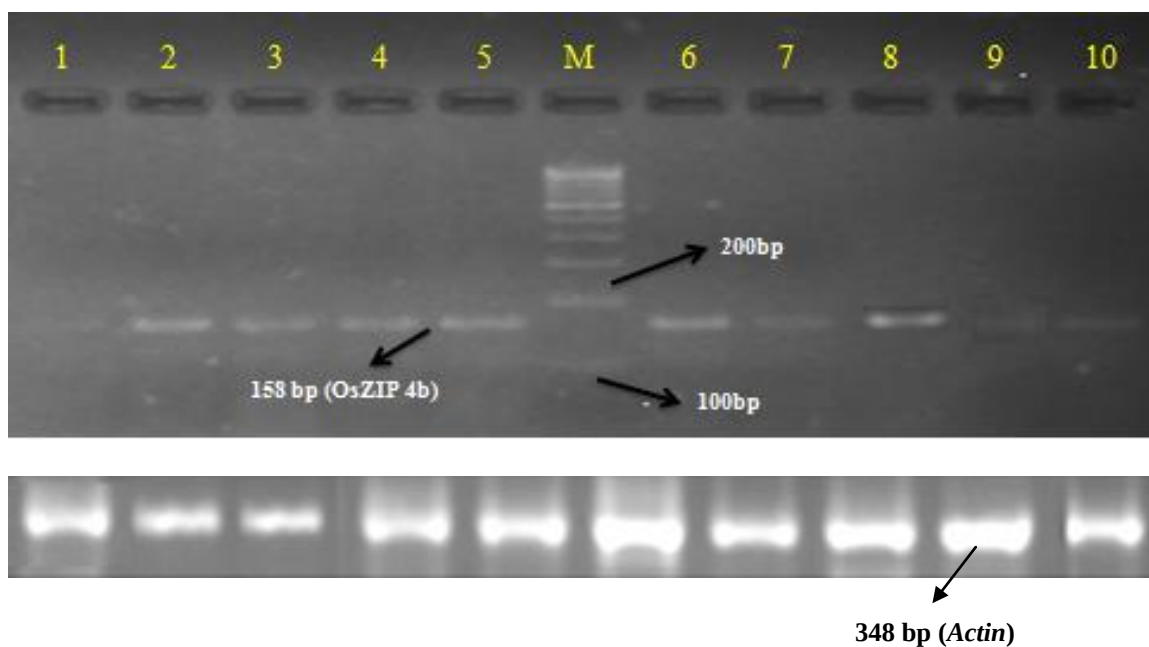


Plate 6a: Relative expression of OsZIP4b in endosperm of selected genotypes

Legends:

1- ARB-6, 2- Black rice, 3- Chittimuthyalu, 4- AM-65, 5- Azucena,
6- AM-1 7- AM-27, 8- AM-143, 9- AM-94B, 10- AM-72

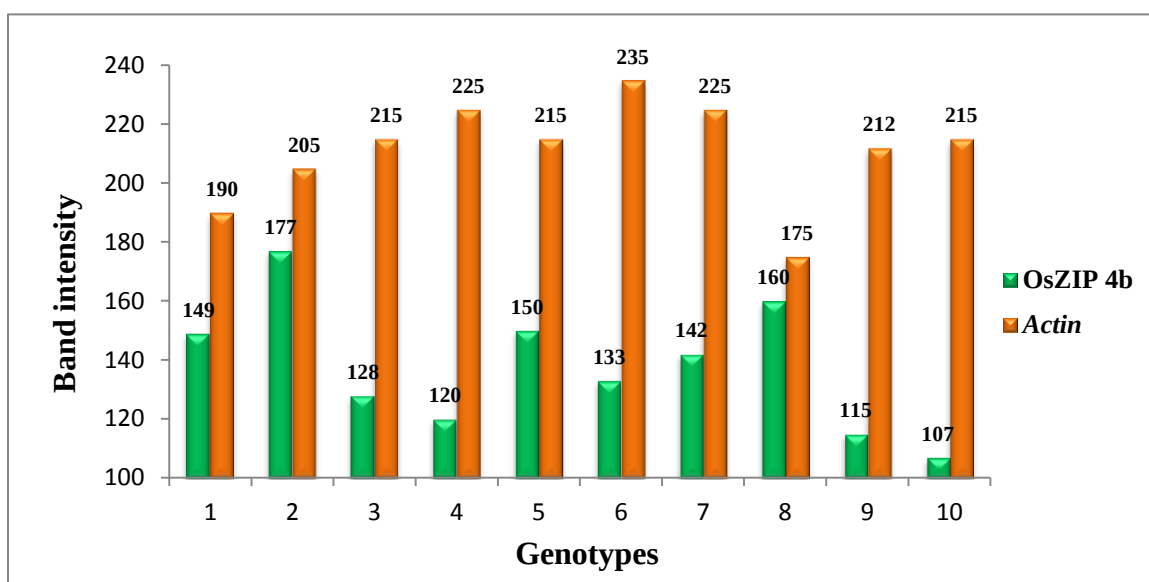
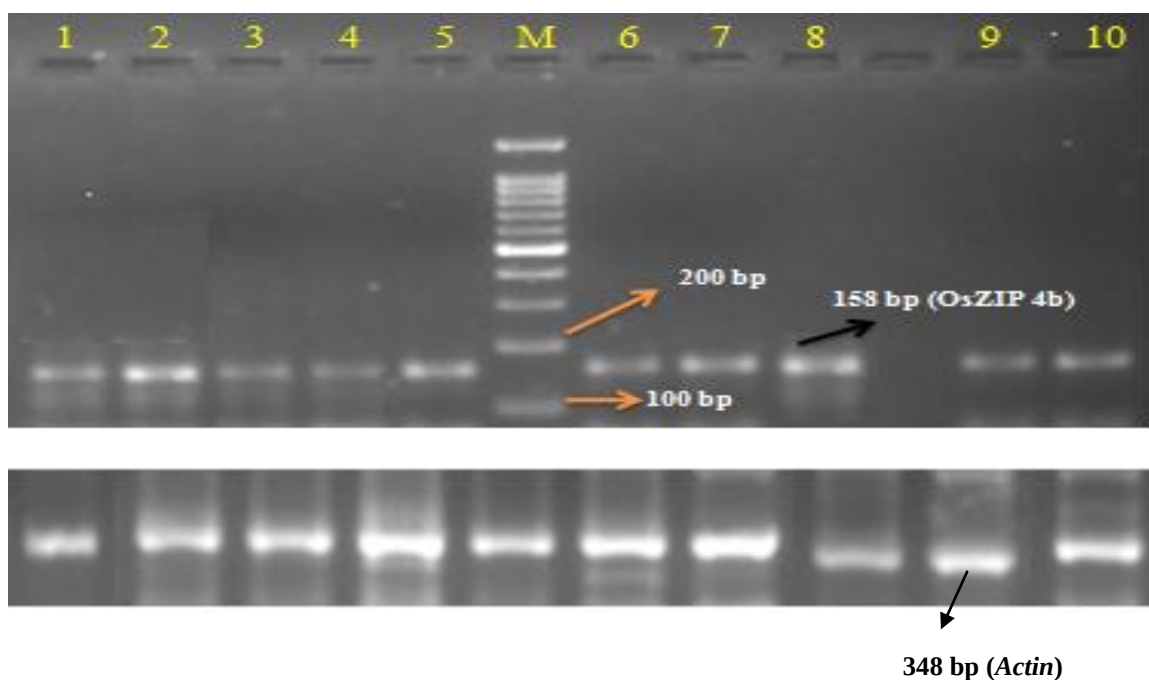


Plate 6b: Relative expression of OsZIP4b in embryo of selected genotypes

Legends:

1- ARB-6, 2- Black rice, 3- Chittimuthyalu, 4- AM-65, 5- Azucena,
6- AM-1 7- AM-27, 8- AM-143, 9- AM-94B, 10- AM-72

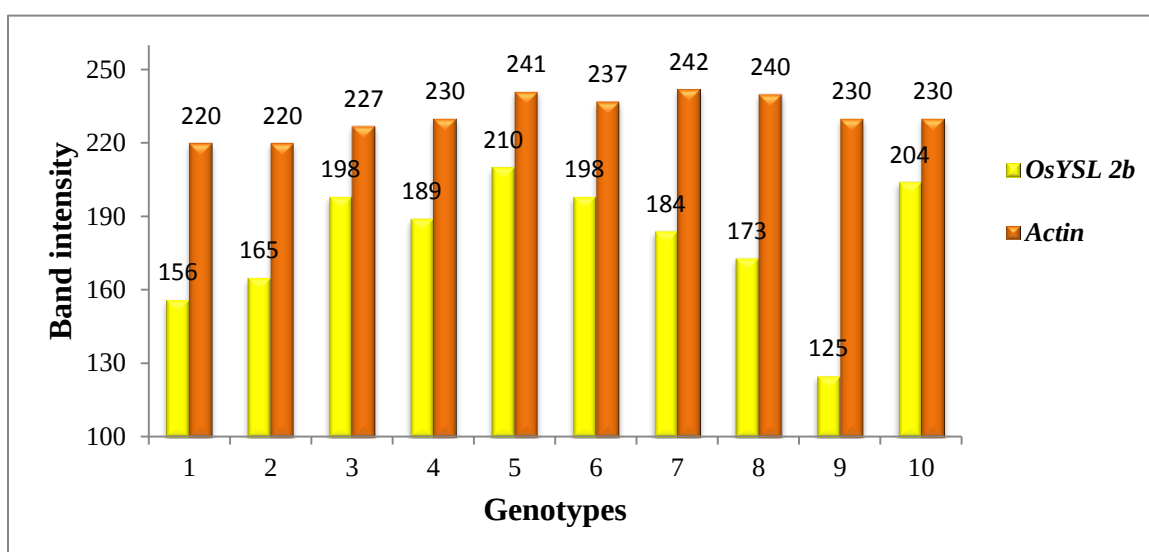
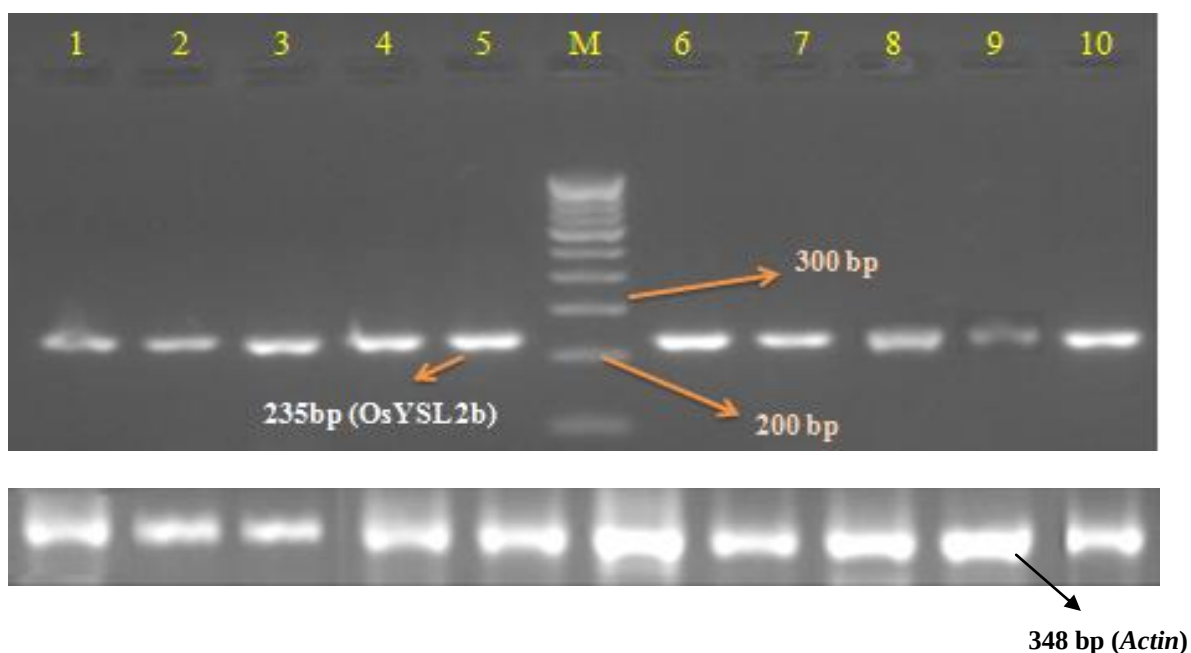


Plate 7: Relative expression of OsYSL2b in endosperm of selected genotypes

Legends:

1- ARB-6, 2- Black rice, 3- Chittimuthyalu, 4- AM-65, 5- Azucena,
6- AM-1 7- AM-27, 8- AM-143, 9- AM-94B, 10- AM-72

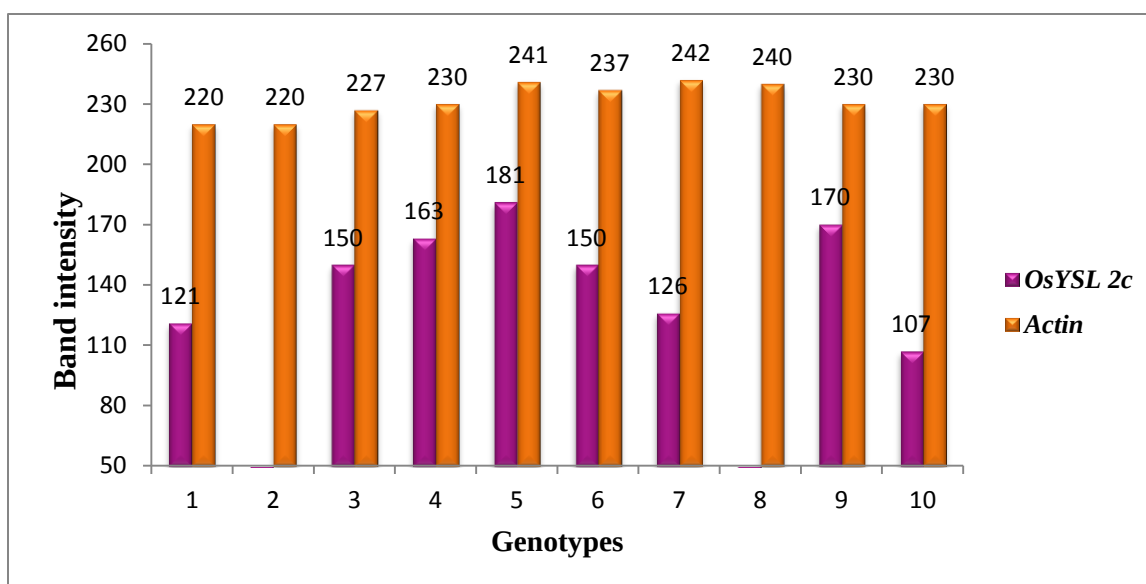
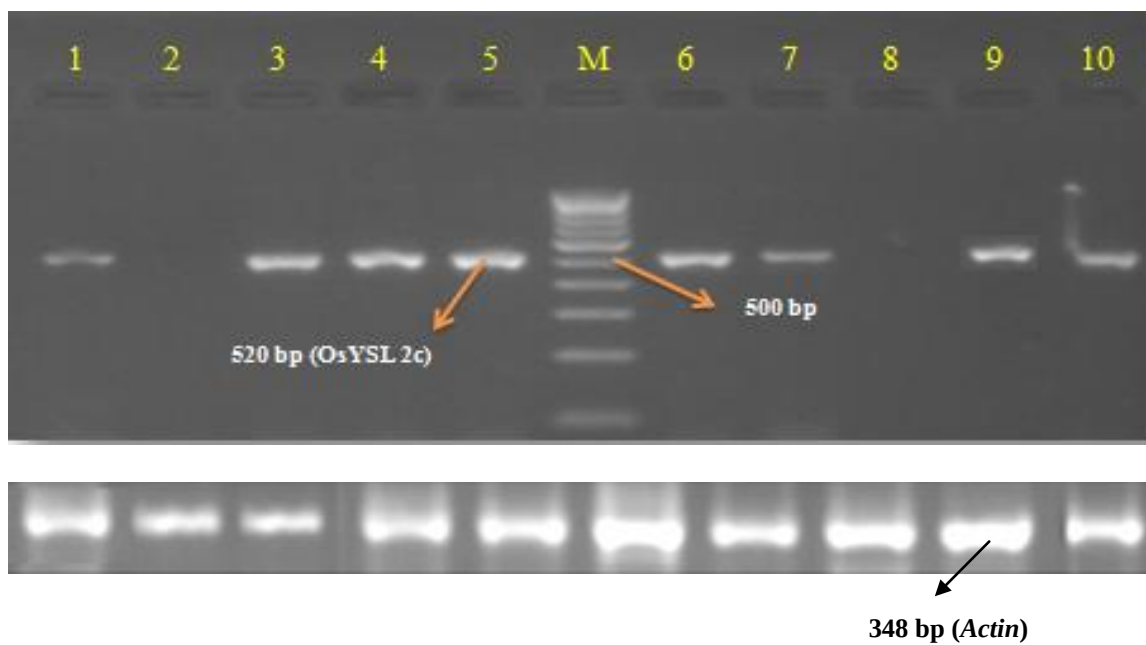


Plate 8: Relative expression of OsYSL2c in endosperm of selected genotypes

Legends:

1- ARB-6, 2- Black rice, 3- Chittimuthyalu, 4- AM-65, 5- Azucena,
6- AM-1 7- AM-27, 8- AM-143, 9- AM-94B, 10- AM-72

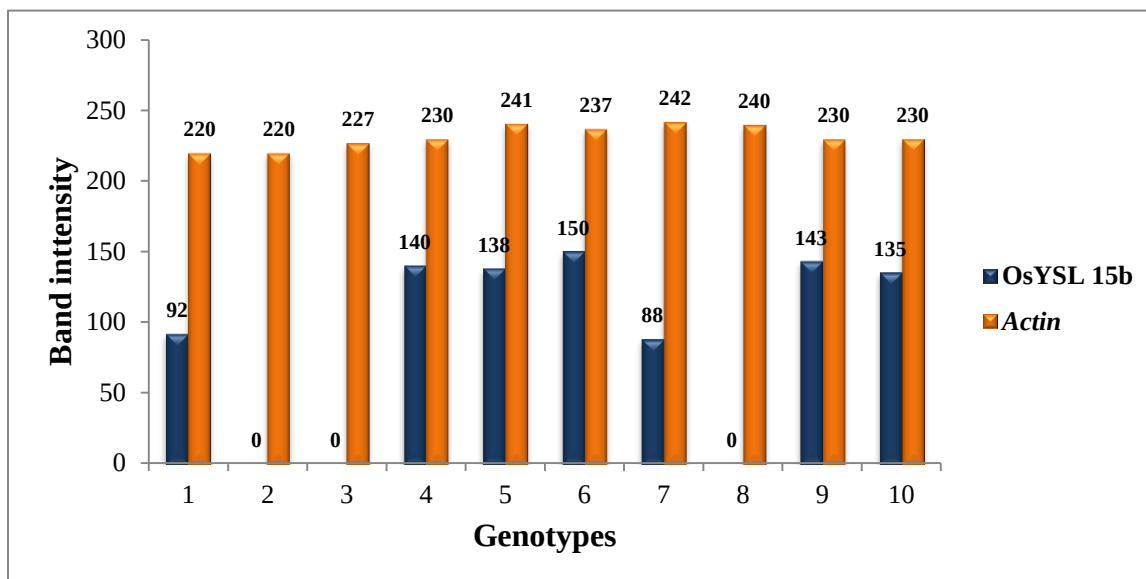
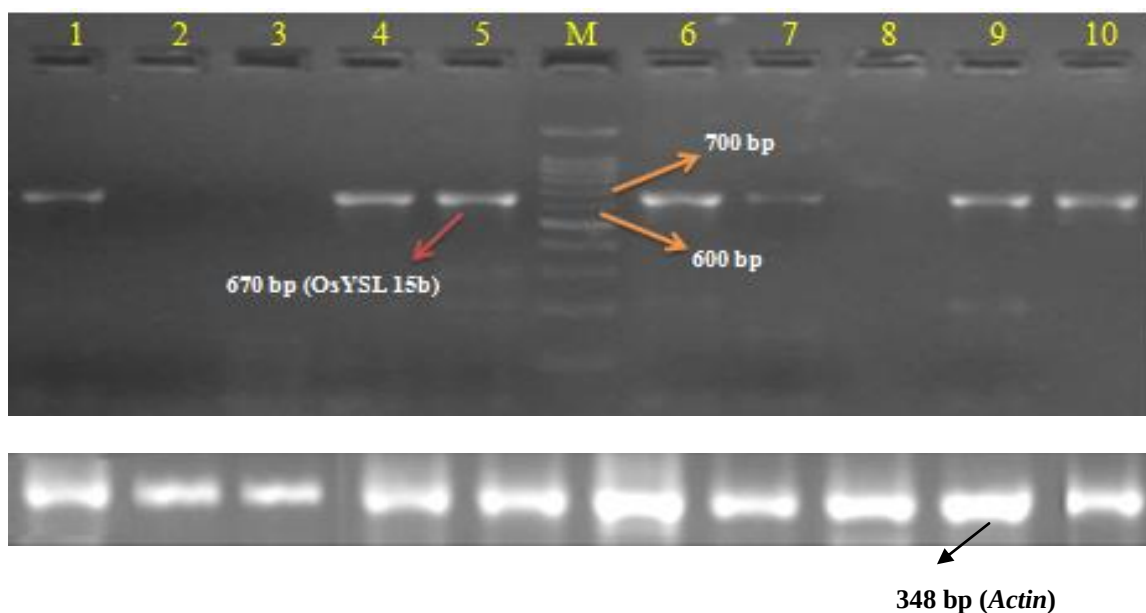


Plate 9a: Relative expression of OsYSL15b in endosperm of selected genotypes

Legends:

1- ARB-6, 2- Black rice, 3- Chittimuthyalu, 4- AM-65, 5- Azucena,
6- AM-1 7- AM-27, 8- AM-143, 9- AM-94B, 10- AM-72

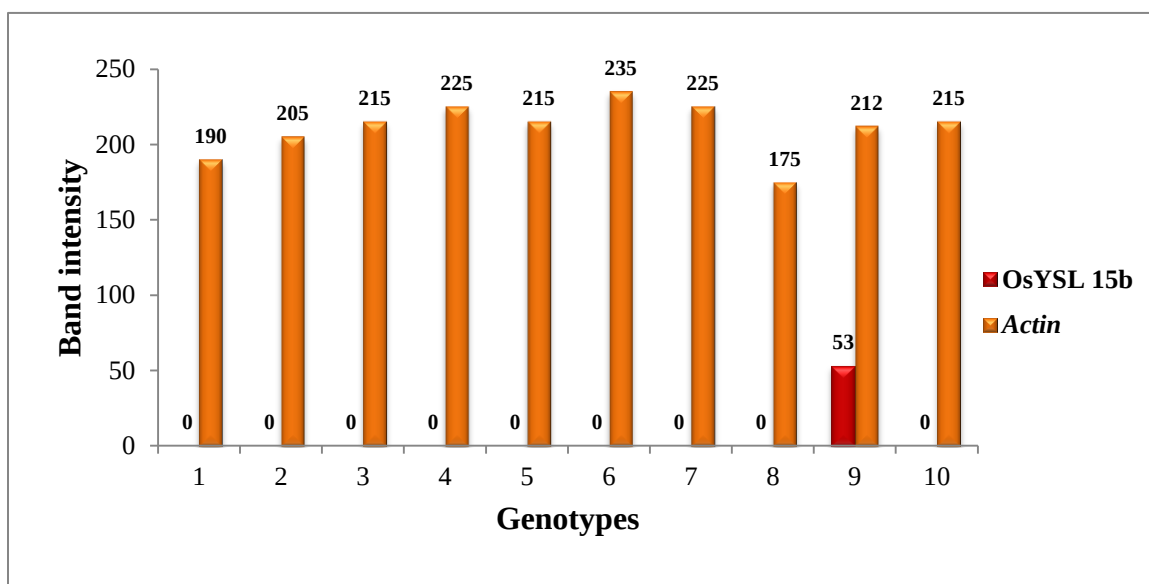
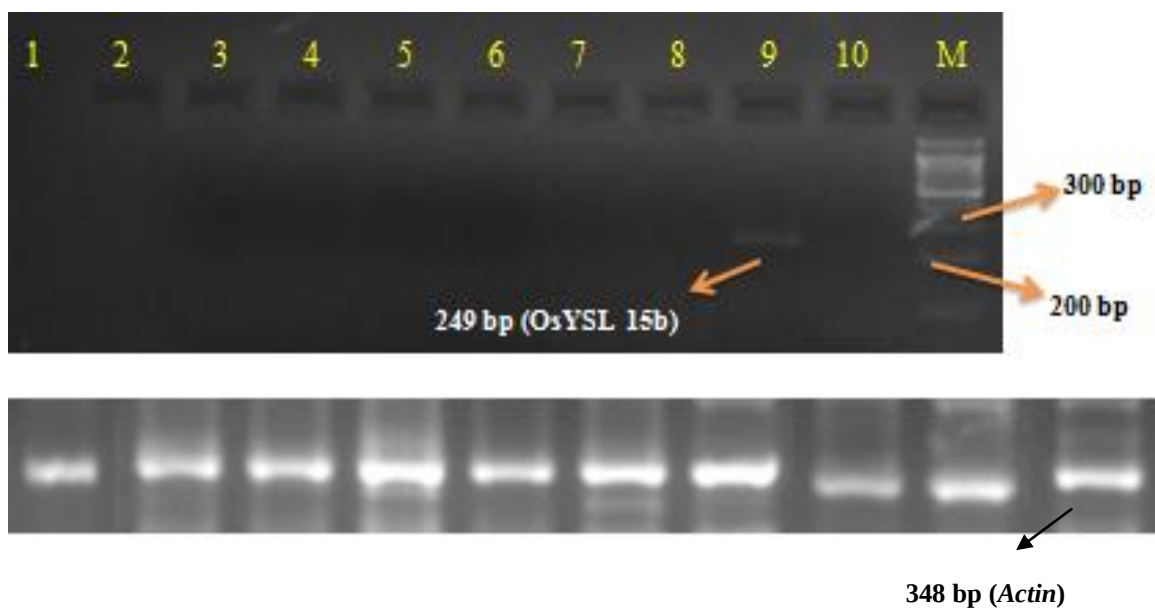


Plate 9b: Relative expression of OsYSL15b in embryo of selected genotypes

Legends:

1- ARB-6, 2- Black rice, 3- Chittimuthyalu, 4- AM-65, 5- Azucena,
6- AM-1 7- AM-27, 8- AM-143, 9- AM-94B, 10- AM-72

4.2.4.4 OsYSL2c

The result obtained for OsYSL 2c from RT-PCR analysis for endosperm and of selected genotypes are displayed in Plate 8a and 8b respectively.

Expression of OsYSL2c was found only in endosperm. There is no or nil expression in embryo.

In endosperm relative expressions of OsYSL2c was found higher in Azucena and relatively lower in ARB-6. There is no expression was found in black rice and AM-143.

4.2.4.5 OsYSL15b

Results obtained from relative expression carried out through RT-PCR analysis for OsYSL 15b in endosperm and embryo among selected genotypes is displayed in Plate 9a and 9b respectively.

Relative expression of OsYSL 15b was found to be higher in endosperm than in embryo among all selected genotypes for study.

In endosperm, expression of OsYSL 15b found higher in AM-1 and lower in AM-27 when compared with other genotypes under study. In embryo expression of OsYSL 15b was seen in only AM-94B and no expression was found in all other selected genotypes under study.

V DISCUSSION

Micronutrient deficiencies or hidden hunger has become a major nutritional problem affecting more than two billion people in the developing countries of Asia, Africa, and Latin America. Countries where rice is the major source of energy are prone to hidden under the micronutrient malnutrition associated health risks have become a major hindrance in achieving the Millennium Development Goals (MDG) such as reducing poverty and hunger, improved maternal health status, and less child mortality. Rice map and deficiency map of the world overlap (World Health Organization, 2003).

Milling and polishing of rice grain is an inevitable for long term storage, embryo part of rice is rich in oil content hence polishing which removes the aleurone layer prevents rancidity. Hence, polishing could be considered as the necessary evil.

It is known that large quantity of micronutrients is present in the aleurone layer. Polishing removes this layer, contributing to the loss of nutritive value of the grain. It is known that consuming brown rice is healthier than polished rice.

5.1 Discerning zinc and iron distribution in different parts of the rice grain

5.1.1 Performance of genotypes with respect to morphological traits

The magnitude of phenotypic variability is reflected by range and deviation from the mean values. High range was noticed in plant height, total number of tillers, number of productive tillers, panicle length, days to maturity, days to fifty percent flowering, days to maturity, biomass, and grain yield per plant. Similar results were obtained by Hemareddy (1993); and Bisne *et al.*, (2009) and also the range in the means of brown rice Zn content was also high than the polished grain. Similar results obtained by Jiang *et al.*, (2008) and Gregorio *et al.*, (2000).

Improvement of any trait depends on the existence of the genetic variability. Thus, information on phenotypic and genotypic co-efficient of variation and heritability would be advantageous. Hence, PCV and GCV are used to compare the variability observed among the studied characters. The heritability estimates aid in determining the

relative amount of heritable portion of variation. However, heritability values itself provides no indication of the amount of genetic progress that would result from selecting the best individuals. Heritability estimates in broad sense would be reliable when accompanied by high genetic advance (Burton and De Vane, 1953; Johnson *et al.*, 1955).

Traits having high PCV and GCV exhibit the high variability among different rice genotypes. High PCV and GCV was observed for plant height, total number of tillers, number of productive tillers, grain yield per plant, biomass, harvest index, productivity per day per plant, zinc and iron productivity per day. Similar results were reported by Naveen *et al.*, (2013). Low PCV and GCV are obtained for, days to maturity, days for fifty percent flowering, and days for first flowering. Moderate PCV and GCV was obtained for panicle length Bekele, (2012).

High heritability was observed in plant height, panicle length, days to maturity, days for fifty per cent flowering, days for flowering, and grain yield per plant. High genetic advance as per mean was observed for plant height. Moderate heritability coupled with high genetic advance as per mean was observed for total number of tillers, number of productive tillers, biomass, grain yield per plant, harvest index, productivity per day per plant, zinc and iron productivity per day which indicated that these traits can be improved efficiently than other traits in the future generations through selection process.

Traits like days to flowering, days to 50 per cent flowering, days to maturity, shows low PCV and GCV coupled low genetic advance as per mean with which indicated that the variation that was observed for these traits is non-heritable because of high environment effect and other factors.

5.1.2 Zn and Fe content in different parts of rice grain

High GCV and high PCV was observed for polished rice zinc content whereas in contrast, low GCV and low PCV was observed for brown rice iron content. This indicate that, highly variable brown rice zinc content could be improved more easily than brown rice iron content in future generations through selection.

High genetic advance as per mean was observed for polished rice iron content which indicated majority of variation observed in this trait was due to additive gene effects. In contrast, low genetic advance as per mean was observed for brown rice iron content which showed to less contribution of additive genes for this trait.

Difference between GCV and PCV was found to be comparatively less for brown rice zinc content than that for brown rice iron content which indicates that environment effect was more for brown rice iron content than for brown rice zinc content.

High heritability coupled with high genetic advance as per mean was observed for brown rice zinc content which indicated that improvement of this trait is comparatively easy through selection in future generations.

5.1.3 Statistical analyses of data

5.1.3.1 Analysis of variance

5.1.3.1.1 Analysis of variance for yield and its associated traits

The phenotypic variation manifested by the genotype has two components namely genotypic and environmental. Analysis of variance of all the 13 characters revealed that significant differences among the means of all genotypes for plant height, total number of tillers, number of productive tillers, grain yield, harvest index, panicle length, days for first flowering, days for fifty percent flowering, days to maturity, and productivity per day per plant. This indicated more scope for selection for these genotypes in future generations. The differences observed in these traits could be attributed to variations present in genotypes as well as the environmental factors.

5.1.3.1.2 Analysis of variance for Zn and Fe content in different parts of rice grain among selected genotypes

Variance analysis revealed significant differences from mean values in all genotypes studied for Zn and Fe content in brown and polished rice. The results suggested that, there is a wide scope for improvement of BZC, PZC, BFC and PFC through selection based breeding programmes.

Significant difference was found for percent loss of iron due to polishing among selected genotypes which indicates the difference in the behavior of genotypes towards polishing and difference in micronutrient accumulation capacity of different genotypes used.

5.1.3.2 Correlation studies

5.1.3.2.1 Correlation analysis for yield and its associated traits

Correlation coefficient analysis helps to determine the nature and degree of relationship between any two characters. The study of relationship of different traits has great importance in the indirect selection of a complex trait associated with a simple trait.

Correlation between plant morphological traits indicated a positive association between yield and its components with micronutrient bio-fortification Zn and Fe.

Iron and zinc productivity per day revealed a significant positive correlation with productivity per day per plant. Productivity per day per plant, and iron productivity per day had a significant positive correlation with zinc productivity per day.

5.1.3.2.2 Correlation analysis for grain Zn and Fe content

Correlation analysis of all four characters revealed significant positive correlation of brown rice zinc content with polished rice zinc content which indicated that rice genotypes exhibiting high zinc content in brown rice contained high zinc content in polished rice (Bhat., 2015). Brown rice iron content showed positive correlation with polished rice iron content and polished rice zinc content, it indicates that least amount of Zn and Fe loss during polishing process.

A significant negative correlation was observed for brown rice zinc and iron content with grain yield. Brown rice zinc content had a significant positive correlation with brown rice iron content and polished rice iron content. Brown rice iron content revealed significant positive correlation with polished rice zinc content.

5.1.4 Estimation of zinc and iron in bran, brown rice and polished rice by using AAS method

Micronutrients are acquired to plant from the soil. They cannot be synthesized or created in order to bio-fortified crop it is imperative that a plant acquires, transports and assimilates nutrients in different tissues as per the efficiency of transporters involved. Estimation of micronutrient in different tissues is very difficult. Different methods are adopted for estimation namely, AAS, XRF and ICPMS. Each one of this method is different. In order to check the efficiency different method, estimation was used for Zn and Fe quantification was done in each elite genotypes. Both destructive and non destructive methods were used where XRF was found to be better than AAS method. Estimated values of Fe and Zn from brown and polished rice and bran are shown in Table 16 & 17.

Table 16: Iron content in bran, brown rice, and polished rice

Genotypes	Brown rice	Polished rice	Bran
AM-1	47.67	25.75	15.32
AM-27	24.64	4.00	19.16
AM-65	48.25	32.75	20.15
AM-72	55.60	53.60	21.25
AM-94B	47.10	37.75	15.82
AM-143	38.80	8.75	18.36
ARB-6	27.75	17.62	6.32
Azucena	45.83	31.93	0.61
Black rice	27.70	22.96	12.35
Chittimuthyalu	35.00	34.06	5.91

Table 17: Zinc content in brown rice, polished rice, and bran

Genotypes	Brown rice	Polished rice	bran
AM-1	11.33	3.05	51.4
AM-27	2.65	2.59	51.4
AM-65	7.38	3.50	48.5
AM-72	5.60	5.40	45.9
AM-94B	6.10	4.50	50.1
AM-143	4.50	3.67	55.4
ARB-6	4.23	2.90	58.5
Azucena	4.83	4.07	49.2
Black rice	6.20	2.68	45.8
Chittimuthyalu	6.89	3.69	53

5.2 Expression profiling of transporter across bio-fortified elite lines

5.2.1 Zn and Fe transporters

Transporter genes belonging to family of *OsZIP*, *OsNAS*, *OsFER* and *OsYSL* have been reported to play an important role in transportation and homeostasis of zinc and iron within rice grains. In this study expression of three genes (*OsZIP4*, *OsYSL2* and *OsYSL15*) was carried out at the transcript level to correlate the relative expression of these transporter genes in embryo and endosperm with the relative amounts of zinc and iron in embryo and endosperm among selected rice genotypes.

5.2.2 Total RNA isolation from different parts of rice grain

Isolation of RNA was one of the critical part of the research as the quality and quantity of RNA isolated would decide the efficiency of downstream processes like reverse transcription and semi-quantitative PCR analysis. Reverse transcription of poor quality RNA at subnormal quantity would be very difficult as such RNA would affect the efficiency of RT enzyme and thereby affecting the cDNA synthesis.

5.2.3 Reverse transcription of RNA and PCR

Reverse transcription can be done broadly in two ways. Namely, one step RT-PCR; and two step RT-PCR.

In two step RT-PCR, reverse transcription and amplification of target cDNA is carried out in different tubes. This kind of RT-PCR was recommended when chances of contamination are less and when there is a need for high sensitivity are more. In the present study, since high sensitivity was required and chances of contamination are more, a two-step RT-PCR was followed.

5.2.4 Relative expression of Zn and Fe transporters in different parts of grain

Relative expression of seven transporters genes was done in semi-quantitative manner and image analysis to know relative band intensity was done by Gel Analyzer software ,version 2010a (Lazar and Lazar, 2010).

The results suggest that, in embryo and endosperm, expression of *actin* was at same level in all the genotypes studied. Band intensity and Zn and Fe content of each genotype is tabulated in Table 18, to understand the relationship of relative expression of gene in each genotype with their zinc and iron content.

Candidate genes known to have profound influence on micronutrient transport into the grain were used for the study. Intra-genic variabilities in expression profile of these gene products based on RT-PCR is presented in Table18 .

Results of the study can be viewed from the point of view transporter genes being studied to understand their influence on a genotype bio-fortification potential, alternatively data can be viewed from the point of view of a genotype and their relative activity of those transporters.

It was observed that endosperm genes expression profiles matched with zinc and iron content of the grain. Higher expression was observed in genotype containing higher iron and zinc. It was interesting to know that AM-94B had moderate expression of *OsYSL15b* in embryo. AM-94B was also unique as all transporters show positive

expression. AM-72 and AM-65, two genotypes displayed high level of expression for all the transporters except *OsYSL15b*.

Expression of *OsZIP4a* was found in endosperm. AM-72 shows higher expression and ARB-6 found lower expression compared to all other genotypes under study. AM-72 is also containing relatively higher amount of Zn and Fe, percent loss of zinc was less and percent loss of iron was more. Results suggest that relatively more amount of zinc was localized in endosperm and expression of *OsZIP4a* was found to be negligibly low or nil in embryo of all genotypes under study.

Expression of *OsZIP4b* was found to be relatively more in embryo than in endosperm. In both the tissues Black rice shows higher expression and AM-72 shows lower expression. Percent loss zinc was more in black rice compared to AM-72. This suggests that the role of *OsZIP4b* is to localize zinc in tissues where it is upregulated or over expressed.

Expression of *OsYSL2b* and *OsYSL2c* was found to be higher in AM-72 and AM-94B lower expression showed in AM-94B and ARB-6 respectively. Expression of *OsYSL2b* & *2c* was found to be negligibly low or nil in embryo of all genotypes under study.

Expression of *OsYSL15b* was found to be relatively higher in endosperm than in embryo. Expression was higher in AM-1 and lower in AM-65. percent loss of zinc was less and iron was more for AM-65 than AM-1. The result suggests that, relatively higher amount of iron would be localized in the tissues where expression of *OsYSL15b* is relatively high. In embryo expression was seen in only AM-94B.

In the present study grain samples were selected at late dough stage. At the dough stage the endosperm could be developing faster than embryo and hence the out of five embryo specific transporters, only two were expressed in embryo. So the micronutrients will be transported to endosperm, later it transport into embryo and other parts of the grain reported by Li *et al.*, (2013). Such studies should carry out in mature grain to understand the transporting ability of tissue specific transporters in embryo and endosperm of rice grain.

Table 18: RNA based expression profile in grains of accessions for key transporters of Zinc and Iron

Genotypes	Fe	Zn	Endopserm					Embryo	
			OsZIP4a	OsZIP4b	OsYSL2b	OsYSL2c	OsYSL15b	OsZIP4b	OsYSL15b
ARB-6	16.7	22.5	135	122	156	121	92	149	0
Black rice	20.5	32.3	163	137	165	0	0	177	0
Chittimuthyalu	21	35.6	150	126	198	150	0	128	0
AM-65	16.5	25.2	185	128	189	163	140	120	0
Azucena	18.2	27.3	190	129	210	181	138	150	0
AM-1	17.4	28.6	215	128	198	150	150	133	0
AM-27	21.8	23.5	180	95	184	126	88	142	0
AM-143	19.1	23.3	173	120	173	0	0	160	0
AM-94B	17.5	24.1	177	75	125	170	143	115	53
AM-72	17.3	24.6	220	70	204	107	135	107	0

B.I= Banding intensity, Fe= Iron in ppm, Zn= Zinc in ppm.

VI SUMMARY

In the present study, ten genotypes were evaluated for distribution of zinc and iron within different parts of rice grain and molecular characterization of seed specific micronutrient transporters was done for ten genotypes.

Significant differences was found among the genotypes for plant height, total number of tillers, number of productive tillers, grain yield, harvest index, panicle length, days for first flowering, days for fifty percent flowering, days to maturity, and productivity per day per plant. Chittimuthyalu had the highest zinc and iron content before and after polishing. AM 94B had the highest iron content even after polishing.

High GCV and high PCV was observed for polished rice zinc content whereas, low GCV and low PCV was observed for brown rice iron content. Also high heritability coupled with high genetic advance as per mean was observed. Difference between GCV and PCV was found to be less for brown rice zinc content which indicates that environment effect was less for brown rice zinc content. This indicates that, brown rice zinc content could be improved in future generations through selection.

Polishing was done at normal level that is followed by commercial rice milling factories. It was found that there was a significant difference in loss of iron in percentage due to polishing among the selected genotypes and contrast was the result observed for percent loss of zinc due to polishing. So, it suggested large variation in extent of loss of iron due to polishing and negligible variation in extent of loss of zinc due to polishing among the genotypes selected. From above results, it is evident that, some genotypes resisted the loss of iron due to polishing while others were susceptible for the extensive loss of iron due to polishing. Response of all the genotypes was almost statistically on par for loss of zinc due to polishing.

Significant and positive correlation was observed for total number of tillers with productivity, iron and zinc productivity per day. Productivity, iron and zinc productivity per day disclosed a significant positive correlation with biomass and harvest index. Iron

and zinc productivity per day revealed a significant positive correlation with productivity per day per plant.

Molecular analysis of relative expression of three seed specific transporters was done for ten genotypes using five primers including reference gene (housekeeping gene). Among all genotypes studied, AM-72 endosperm had relatively higher expression of *OsZIP4a* and *OsYSL2b* than in any other genotypes.

It is noticeable that, Black rice suffered highest percent loss of zinc and iron due to polishing and the genotype had relatively higher expression of *OsZIP4b* in its embryo and endosperm when compared to other genotypes. ARB-6 that showed less percent loss of zinc due to polishing had relatively higher expression of *OsYSL2b* and *OsYSL15b* in its endosperm and relatively lower or negligible expression of *OsYSL15b* in its embryo. AM-143 that showed less percent loss of iron due to polishing had relatively higher expression of *OsZIP4a*, and *OsYSL2b* in its endosperm than that in embryo.

FUTURE LINE OF WORK

- Expression studies in bran also could be done. It also part of grain and also contain relatively higher amount of micronutrient.
- Relative expression studies of some more genes like *OsNRAMP*, *OsDMAS*, *OsNAAT*, *OsZIP3*, *OsYSL18b*, *OsNAS*, *OsFER* etc. could be taken up to discern the importance of more seed specific transporter genes in localization of micronutrients in different grain parts.
- Absolute quantification of transporter genes that were studied in different grain parts could be done to discern the extent of contribution of each of the transporter genes in increasing zinc and iron content in polished grain (endosperm).

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Appendix 1: Mean value of yield associated and nutritional parameters in the selected genotypes obtained during *Kharif* 2016

Genotypes	PH	TNT	NPT	PL	BM	GY	DFPF	DTF	DTM	HI	BFC	PFC	BZC	WZ
ARB-6	66.10	25.87	25.20	17.63	32.36	22.37	99.33	108.33	145.67	0.65	16.73	2.73	22.56	20.30
BLACK RICE	91.47	16.47	16.00	18.16	23.29	3.86	89.00	96.00	144.00	0.18	20.53	3.53	32.3	25.47
CHITTIMUTHYALU	53.03	24.73	22.53	15.57	18.07	9.81	96.00	117.33	145.00	0.54	21.00	11.97	35.6	27.27
AM-65	109.45	11.47	10.93	21.64	29.50	16.17	107.33	118.00	153.33	0.57	16.53	5.33	25.2	20.43
AZUCENA	114.67	7.20	9.53	22.11	20.19	12.58	117.00	126.67	171.67	0.63	18.20	4.97	27.33	23.97
AM-1	89.87	13.33	12.73	21.81	32.16	9.61	95.33	112.67	143.33	0.30	17.47	3.13	28.63	24.20
AM-27	68.27	17.80	20.00	18.69	18.03	7.77	97.67	113.00	145.33	0.51	21.80	6.57	23.56	19.80
AM-143	50.06	19.53	17.93	14.27	13.01	9.34	96.00	105.67	145.00	0.78	19.17	8.93	23.3	18.90
AM-94B	53.60	18.53	16.07	15.54	18.41	11.85	107.00	116.67	153.33	0.62	17.50	5.37	24.1	19.10
AM-72	67.90	21.07	16.93	20.71	23.90	11.02	96.00	111.33	145.00	0.48	17.30	5.70	24.6	19.80

PH= Plant height **TNT**= Total number of tillers **NPT**= Number of productive tillers **GY**= Grain yield **HI**= Harvest index
PL= Plant height **DFP**=Days for first flowering **DFPF**= Days for fifty percent flowering **DTM**=Days to maturity
BM= Biomass **BFC**= Brown rice iron content **PFC**= Polished rice iron content **BZC**= Brown rice zinc content
PZC= polished rice zinc content.

Appendix 2: Physical-chemical properties of soil recorded in the experimental site

	Soil Analysis	
	Before sowing	After harvest
Organic Carbon	1.31	1.23
Organic matter	2.26	2.13
Fe (ppm)	21.968	7.256
Zn (ppm)	1.62	1.612
pH	6.104	7.56
Conductivity(micro seimens)	464.7	473.8