

**DISTRIBUTION OF MICRONUTRIENTS WITHIN
GRAINS OF RICE (*Oryza sativa* L.) GENOTYPES
UNDER AEROBIC CONDITION**

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**DEPARTMENT OF PLANT BIOTECHNOLOGY
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2015

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In partial fulfilment of the requirements

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MASTER OF SCIENCE (Agriculture)

IN

PLANT BIOTECHNOLOGY

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***Affectionately
Dedicated To
My Beloved Parents
and Sister***



**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
GKVK, BENGALURU-65**

CERTIFICATE

This is to certify that the thesis entitled, “**DISTRIBUTION OF MICRONUTRIENTS WITHIN GRAINS OF RICE (*Oryza sativa* L.) GENOTYPES UNDER AEROBIC CONDITION**” submitted in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** to the University of Agricultural Sciences, GKVK, Bengaluru is a record of *bona-fide* research work done by **Mr. KARTHIK P. BHAT, ID No. PALB-3239**, during the period of his 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.

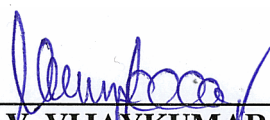
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(Karthik P. Bhat)

"Distribution of Micronutrients within Grains of Rice (*Oryza sativa* L.) Genotypes under Aerobic Condition"

Karthik, P. Bhat

ABSTRACT

In India, rice is the staple food and major component of diet, but lacks micronutrients in its polished form as major portion of micronutrients are lost during polishing. An effort was made to study the relative expression of micronutrient transporters within different grain parts of the grain before and after polishing. 12 genotypes contrasting for grain zinc and iron were selected and grown in field under aerobic condition at GKVK, following RCBD design, during *Khariiff*-2014. High GCV and PCV coupled with high heritability and GAM was observed for plant height, shoot dry weight, grain size, brown rice zinc content, and white rice iron content. Significant positive correlation was observed for iron content in brown rice with test weight and brown rice zinc content with white rice zinc content. Several transporter genes like *OsZIP4*, *OsFER1*, *OsNAS1*, *OsNAS2*, *OsYSL2*, *OsYSL15*, *OsYSL18* etc. were found responsible for micronutrient transport into rice grains. Gene specific primers were designed for these genes. Relatively higher expression of *OsZIP4a*, *OsNAS2a*, and *OsYSL15b* were found in embryo and endosperm of Moroberekan which had highest brown and white rice zinc content. Relatively higher expression of *OsYSL2b* and *OsYSL2c* was found in embryo and relatively lower expression of *OsZIP4a* was found in endosperm of AM 72 which suffered higher loss of zinc and iron due to polishing. The study suggested that among several genes studied *OsZIP4*, *OsYSL2*, *OsYSL15*, and *OsNAS2* were found to play a major role in zinc and iron accumulation within different parts of rice grain.

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UAS, GKVK, Bengaluru.



Signature of major advisor
(Dr. H. E. Shashidhar)

ಏರೋಬಿಕ್ ಪದ್ಧತಿಯಲ್ಲಿ ಭತ್ತದ (ಒರೈಜಾ ಸಟ್ಲೆವಾ ಎಲ್.) ವಂಶವಾಹಿಗಳ ಧಾನ್ಯದೊಳಗೆ

ಲಘುಪೋಷಕಾಂಶಗಳ ಹಂಚಿಕೆ

ಕಾರ್ತಿಕ್, ಪಿ. ಭಟ್

ಪ್ರಬಂಧ ಅಮೂರ್ತ

ಭಾರತದಲ್ಲಿ ಭತ್ತವು ಪ್ರಧಾನ ಆಹಾರವಾಗಿದ್ದು ಅಲ್ಲಿನ ಆಹಾರ ಕ್ರಮದ ಪ್ರಧಾನ ಅಂಶವಾಗಿದೆ. ಆದರೆ, ಪಾಲಿಶಿಂಗ್ ಮಾಡುವಾಗ ಬಹುಪಾಲು ಲಘುಪೋಷಕಾಂಶಗಳು ವ್ಯಯವಾಗುವುದರಿಂದ ಪಾಲಿಶ್ ಮಾಡಿದ ಭತ್ತದಲ್ಲಿ ಲಘುಪೋಷಕಾಂಶಗಳ ಕೊರತೆಯನ್ನು ಕಾಣಬಹುದಾಗಿದೆ. ಪಾಲಿಶ್ ಮಾಡುವ ಮೊದಲು ಹಾಗೂ ನಂತರ ಭತ್ತದ ಧಾನ್ಯದ ವಿವಿಧ ಭಾಗಗಳಲ್ಲಿ ಲಘುಪೋಷಕಾಂಶ ವಾಹಕಗಳ ತುಲನಾತ್ಮಕ ಪರಿವ್ಯಕ್ತತೆಯನ್ನು ಅಧ್ಯಯನ ಮಾಡಲಾಯಿತು. ವಿಭಿನ್ನ ಸತು ಮತ್ತು ಕಬ್ಬಿಣಾಂಶವಿರುವ ೧೨ ಭತ್ತದ ವಂಶವಾಹಿಗಳನ್ನು ಆರಿಸಿ ಜಿ.ಕೆ.ವಿ.ಕೆಯಲ್ಲಿ ಏರೋಬಿಕ್ ಪದ್ಧತಿಯಲ್ಲಿ ಬೆಳೆಯಲಾಯಿತು. ಗಿಡದ ಎತ್ತರ, ಕಾಂಡದ ತೂಕ, ಧಾನ್ಯದ ಗಾತ್ರ, ಕಂದು ಅಕ್ಕಿಯಲ್ಲಿನ ಸತು ಹಾಗೂ ಬಿಳಿ ಅಕ್ಕಿಯಲ್ಲಿನ ಕಬ್ಬಿಣಾಂಶಗಳು ಹೆಚ್ಚಿನ ಜಿ.ಸಿ.ವಿ, ಪಿ.ಸಿ.ವಿ, ಅನುವಂಶೀಯತೆ ಮತ್ತು ಜಿ.ವಿ.ಎಮ್.ಗಳನ್ನು ಹೊಂದಿದ್ದವು. ಕಂದು ಅಕ್ಕಿಯಲ್ಲಿನ ಕಬ್ಬಿಣಾಂಶವು ನೂರು ಧಾನ್ಯದ ತೂಕದ ಜೊತೆಯಲ್ಲಿ ಹಾಗೂ ಕಂದು ಅಕ್ಕಿಯಲ್ಲಿನ ಸತುವಿನಾಂಶವು ಬಿಳಿ ಅಕ್ಕಿಯ ಸತುವಿನಾಂಶದ ಜೊತೆಯಲ್ಲಿ ಹೆಚ್ಚಿನ ಧನಾತ್ಮಕ ಸಂಬಂಧವನ್ನು ಹೊಂದಿದ್ದವು. ಲೇಖನಗಳ ಸಮೀಕ್ಷೆ ನಡೆಸಿದಾಗ, ಓಎಸ್ ಜೆಡ್‌ಐಪಿಒ, ಓಎಸ್ ಎಫ್‌ಈಆರ್‌೧, ಓಎಸ್ ಎನ್‌ಐಎಸ್‌೧, ಓಎಸ್ ಎನ್‌ಐಎಸ್‌೨, ಓಎಸ್ ವೈಎಸ್‌ಎಲ್‌೧೫, ಓಎಸ್ ವೈಎಸ್‌ಎಲ್‌೧೬ರಂತಹ ವಾಹಕ ವಂಶವಾಹಿಗಳು ಭತ್ತದ ಧಾನ್ಯದೊಳಗೆ ಲಘುಪೋಷಕಾಂಶಗಳ ಸಾಗಣೆಯಲ್ಲಿ ತೊಡಗಿದ್ದವೆಂದು ಕಂಡುಬಂದಿತು. ಈ ವಂಶವಾಹಿಗಳಿಗೆ ನಿರ್ದಿಷ್ಟವಾದ ಪ್ರಾರಂಭಿಕಗಳನ್ನು ವಿನ್ಯಾಸ ಮಾಡಲಾಯಿತು. ಕಂದು ಅಕ್ಕಿ ಹಾಗೂ ಬಿಳಿ ಅಕ್ಕಿಯಲ್ಲಿ ಗರಿಷ್ಠ ಸತುವಿನ ಅಂಶ ಹೊಂದಿದ್ದ ಮೊರೋಬೆರೆಕ್ಕ್‌ನ್ ಧಾನ್ಯದ ಭೂಣ ಮತ್ತು ಎಂಡೋಸ್ಪರ್ಮ್‌ನಲ್ಲಿ ಓಎಸ್ ಜೆಡ್‌ಐಪಿಒ, ಓಎಸ್ ಎನ್‌ಐಎಸ್‌೨ಎ ಹಾಗೂ ಓಎಸ್ ವೈಎಸ್‌ಎಲ್‌೧೫ಬಿಗಳ ಹೆಚ್ಚಿನ ತುಲನಾತ್ಮಕ ಪರಿವ್ಯಕ್ತತೆ ಕಂಡುಬಂದಿತು. ಪಾಲಿಶಿಂಗ್‌ನಿಂದಾಗಿ ಹೆಚ್ಚಿನ ಸತು ಮತ್ತು ಕಬ್ಬಿಣದ ವ್ಯಯ ಕಂಡುಬಂದ ಎಎಮ್ ೭೨ನ ಧಾನ್ಯದ ಭೂಣದಲ್ಲಿ ಓಎಸ್ ವೈಎಸ್‌ಎಲ್‌೨ಬಿ ಮತ್ತು ಓಎಸ್ ವೈಎಸ್‌ಎಲ್‌೨ಸಿಗಳ ತುಲನಾತ್ಮಕ ಪರಿವ್ಯಕ್ತತೆ ಹೆಚ್ಚಾಗಿ ಕಂಡುಬಂದಿದೆ. ಓಎಸ್ ಜೆಡ್‌ಐಪಿಒಯ ಪರಿವ್ಯಕ್ತತೆ ಎಂಡೋಸ್ಪರ್ಮ್‌ನಲ್ಲಿ ತುಲನಾತ್ಮಕವಾಗಿ ಕಡಿಮೆ ಕಂಡುಬಂದಿತು. ಈ ಅಧ್ಯಯನದಿಂದ, ಪರೀಕ್ಷಿಸಲ್ಪಟ್ಟ ಅನೇಕ ವಂಶವಾಹಿಗಳ ಪೈಕಿ ಓಎಸ್ ಜೆಡ್‌ಐಪಿಒ, ಓಎಸ್ ವೈಎಸ್‌ಎಲ್‌೨, ಓಎಸ್ ವೈಎಸ್‌ಎಲ್‌೧೫ ಹಾಗೂ ಓಎಸ್ ಎನ್‌ಐಎಸ್‌೨ಗಳು ಭತ್ತದ ಧಾನ್ಯದ ವಿವಿಧ ಭಾಗಗಳಲ್ಲಿ ಸತು ಮತ್ತು ಕಬ್ಬಿಣದ ಕ್ರೋಢೀಕರಣದಲ್ಲಿ ಪ್ರಮುಖ ಪಾತ್ರ ವಹಿಸುತ್ತವೆಂದು ತಿಳಿದುಬಂದಿತು.

ಸೆಪ್ಟೆಂಬರ್ ೨೦೧೫

ಕೃಷಿ ವಿಶ್ವವಿದ್ಯಾನಿಲಯ, ಜಿ.ಕೆ.ವಿ.ಕೆ., ಬೆಂಗಳೂರು

ಶ್ರೀಧಿಶ್
ಪ್ರಧಾನ ಸಲಹೆಗಾರರು
(ಡಾ.ಎಚ್.ಈ.ಶಶಿಧರ್)



Distribution of Micronutrients within Grains of Rice (*Oryza sativa* L.) Genotypes under Aerobic Condition

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Introduction

- Rice is the staple food of India.
- India stands first in area, second in production and fourth in export of rice in the world.
- Rice is considered as 'Grain of Life' for Asians as it supplies majority of starch, protein and micronutrient requirements.
- It provide 50-85 % of daily energy source and is consumed in large amounts (300-900g/day/person).
- So, even a small increase in its nutritive value can be highly significant for human nutrition.
- Nearly one-third of world's population is affected by zinc deficiency and more than 5 billion people are affected by iron deficiency causing around 1-2 million deaths annually.
- Majority of zinc and iron present in rice grain was reported to be lost during milling and polishing. Bio-fortification of zinc and iron in rice is a difficult task because of several reasons like due to its unequal distribution within grains, presence of inhibitors like phytic acid etc.

Thus as a part of attempt to understand role of transporters in micronutrient bio-fortification, the present study was done to study the distribution of micronutrients within different parts of rice grain.

Objectives

- Phenotyping of rice genotypes for high zinc and iron content.
- Study the distribution of zinc and iron within different parts of rice grain.

Materials & Methods

- Plant Material and Area:** Twelve rice genotypes differing in their grain zinc and iron content were selected and grown under aerobic condition at Department of Plant Biotechnology, UAS, GKVK, Bengaluru.
- Readings taken:** Readings like plant height, No. of tillers, No. of productive tillers, grain yield per plant, days to maturity, panicle length, hundred grain weight, brown & white rice zinc and iron content etc., were taken after harvesting.
- Estimation of zinc and iron content in different parts of rice grain:** Grains were dehulled using Dehuller. Zinc and iron content of the cleaned brown rice (dehulled rice) was estimated using XRF.

Brown rice samples were polished in polisher (Mini Lab Rice Polisher, Model:K-710-Non Ferrous Series). Resulting white rice samples were cleaned for zinc and iron estimation using XRF (X-Ray Fluorescence).

- Statistical analysis:** The readings obtained from rice samples were subjected to statistical analyses. (Fig 1, Table 1 & 2).

- RNA isolation:** Total RNA was isolated from embryo and endosperm of the study genotypes using modified TRIzol method for further study (Fig. 2).

Results

- A significant difference was found between genotypes for plant height, NOPT, grain yield per plant, hundred grain weight, brown and white rice zinc content, and white rice iron content at both 5% and 1% LOS. Further, there was no significant difference between genotypes for number of tillers and brown rice iron content.
- A significant negative correlation was found between WZ and WF, WZ and GYP, BF and DFF. Also, a significant positive correlation was observed between BZ and BF.

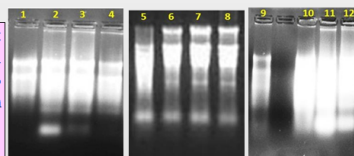


Fig 2: Total RNA from selected genotypes

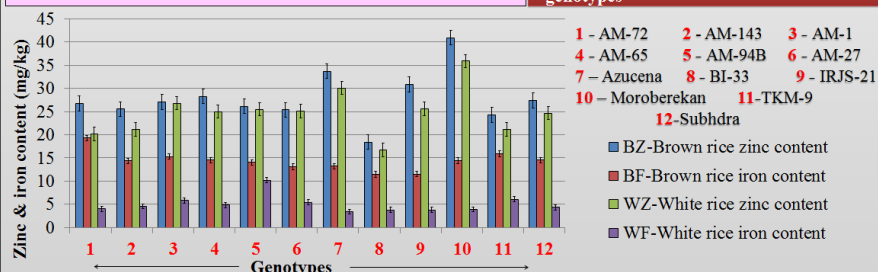


Fig 1: Zinc and iron content in brown and white rice of twelve genotypes under study.

Analysis	PH (cm) **	NOT (no.)	NOPT (no.) **	SDW(g) **	GYP (g) **	PL (cm) *	HGW (g) **	DFF (days) **	DFPF (days) **	DTM (days) **	BZ (mg/kg) **	BF (mg/kg) **	WZ (mg/kg) **	WF (mg/kg) **
Mean	75.03	13.94	8.13	40.83	12.33	17.70	2.00	98.25	113.2	135.3	27.50	14.30	25.28	5.03
S.D	23.00	2.67	2.11	18.16	7.46	2.22	0.32	7.99	5.28	6.76	5.77	2.08	4.84	1.84
Co.Var.	9.65	9.14	15.98	19.47	19.53	12.52	16.03	8.13	4.66	4.99	20.97	14.51	19.14	16.61
F-cal	20.32	2.05	1.77	6.15	7.72	3.06	9.21	17.39	4.87	8.46	22.71	1.51	208.90	169.2
S.E	6.64	0.77	0.61	5.24	2.15	0.64	0.09	2.31	1.52	1.95	1.66	0.60	1.40	0.53
p value	1E-09	0.07	0.12	1E-04	1E-05	0.01	3.4E-6	7.7E-9	5.8E-4	7.3E-6	5E-10	0.19	5E-21	6E-20
C.D @5%	14.89	5.44	4.64	21.38	7.84	3.70	0.31	5.59	6.98	6.79	3.53	4.93	0.56	0.41
C.D @1%	20.18	7.37	6.29	28.97	10.62	5.01	0.42	7.57	9.46	9.20	4.79	6.68	0.76	0.56

NOT - No. of tillers NOPT - No. of productive tillers HGW - Hundred grain weight DFF - Day for first flowering DFPF - Day for 50% flowering DTM - Days to maturity BZ - Brown rice zinc content BF - Brown rice iron content WZ - White rice zinc content WF - White rice iron content. * - Significant @ 5% LOS ** - Significant @ 1% LOS.

Table 1: Analysis Of Variance of different trait parameters considered and other statistical analyses

	PH	NOPT	SDW	GYP	PL	HGW	DFF	DFPF	DTM	BZ	BF	WZ
NOPT	-0.36	1.00										
SDW	0.76	-0.43	1.00									
GYP	0.36	0.33	-0.06	1.00								
PL	0.49	0.20	0.61	0.30	1.00							
HGW	0.28	0.09	0.46	0.26	0.69	1.00						
DFF	0.34	-0.76	0.47	-0.28	0.12	0.36	1.00					
DFPF	0.24	-0.65	0.48	-0.32	0.27	0.36	0.92	1.00				
DTM	0.41	-0.76	0.37	-0.07	-0.04*	0.14	0.79	0.58	1.00			
BZ	0.47	-0.52	0.74	-0.13	0.34	0.60	0.57	0.48	0.36	1.00		
BF	-0.04*	0.50	0.05	0.30	0.54	0.61	-0.02*	-0.02*	-0.17	0.04*	1.00	
WZ	0.36	-0.71	0.57	-0.02*	0.12	0.50	0.62	0.52	0.52	0.86	-0.11	1.00
WF	-0.42	0.20	-0.43	0.27	-0.08	0.23	-0.02*	0.02*	0.00*	-0.26	0.09	-0.02*

Table 2: Correlation analysis of different traits considered in the study with their correlation co-efficients.

Discussion

- Highest zinc content was found in Moroberekan both in brown and white rice (40.95 & 35.90 mg/kg respectively). Whereas, highest iron content in brown and white rice was observed in AM-72 and AM-94B (19.30 & 10.20 mg/kg) respectively.
- Lowest zinc and iron content was found in BI-33 for brown rice samples (18.40 & 11.45 mg/kg). For white rice samples, lowest zinc was found in BI-33 (16.70 mg/kg) and lowest iron was observed in Azucena (3.80 mg/kg).

Summary

- An approximate loss of about 8% and 65% of zinc and iron respectively was observed due to polishing.
- Total RNA from different parts of grain was isolated from genotypes studied to know relative expression of micronutrient transporters(Fig. 2).

Advisory Committee

Chairman : Dr. H. E. Shashidhar.
Members : Dr. H. V. Vijayakumar Swamy.
Dr. T. H. Ashok.
Dr. D. Vijayalakshmi.

CONTENTS

CHAPTER	TITLE	PAGE No.
I	INTRODUCTION	1-3
II	REVIEW OF LITERATURE	4-22
III	MATERIAL AND METHODS	23-34
IV	EXPERIMENTAL RESULTS	35-58
V	DISCUSSION	59-68
VI	SUMMARY	69-70
VII	REFERENCES	71-87

LIST OF TABLES

Table No.	Title	Page No.
1	List of genotypes selected for the study	24
2	Genetic parameters of yield and its associated traits among selected rice genotypes	36
3	Genetic parameters for grain morphological traits among selected rice genotypes	36
4	Genetic parameters for zinc and iron content in brown rice of selected genotypes	39
5	Analysis of variance for seven yield and its associated traits in selected rice accessions	40
6	Analysis of variance for six yield attributing traits among selected rice genotypes	41
7	Analysis of variance for grain morphological traits and zinc and iron content in brown rice among selected rice genotypes	41
8	Estimates of phenotypic correlation coefficients for yield and its associated traits among selected rice accessions	42
9	Estimates of phenotypic correlation coefficients for grain morphological traits and brown rice zinc and iron content in selected rice genotypes	43
10	Direct and indirect effects of yield associated traits on grain yield among selected genotypes	44
11	Direct and indirect effects of grain morphological traits on grain yield among selected rice genotypes	44
12	Mean zinc and iron content in different parts of rice grain among selected genotypes	46
13	Genetic parameters for zinc and iron content in different parts of rice grain among selected genotypes	46
14	Analysis of variance for distribution of zinc and iron content in different grain parts among selected genotypes	47

Table No.	Title	Page No.
15	Estimates of phenotypic correlation coefficients for distribution of zinc and iron in different grain parts among selected genotypes	48
16	Direct and indirect effects of distribution of zinc and iron in different grain parts among selected genotypes	48
17	Per cent loss of zinc and iron in selected genotypes due to polishing	49
18	Analysis of variance for per cent loss of zinc and iron due to polishing among selected genotypes	50
19	Estimates of phenotypic correlation coefficients for per cent loss of zinc and iron due to polishing in selected genotypes	50
20	List of micronutrient transporters explored for present study from literature	51
21	Details of transporters genes selected for the study	51
22	List of gene specific primers designed for molecular study	52
23	Nanodrop readings for total RNA isolated from embryo and endosperm of selected genotypes	53
24	Different combinations of primer concentrations used for PCR optimization	54

LIST OF FIGURES

Fig. No.	Title	Between Pages
1	Zinc content in brown and white rice samples of selected genotypes	46-47
2	Iron content in brown and white rice samples of selected genotypes	46-47
3	Zinc content in brown rice of genotypes selected for study	46-47
4	Zinc content in white rice of genotypes selected for study	46-47
5	Iron content in white rice samples of selected genotypes	48-49
6	Zinc and iron productivity of genotypes selected for study	48-49

LIST OF PLATES

Plate No.	Title	Between Pages
1a	A view of the experimental plot	24-25
1b	A view of a Moroberekan plant in the experimental plot	24-25
2	XRF instrument used to estimate Zn and Fe content in different parts of rice grain	26-27
3a	Total RNA isolated from embryo of selected genotypes	52-53
3b	Total RNA isolated from endosperm of selected genotypes	52-53
4	Separation of embryo and endosperm under aseptic and nuclease-free condition	54-55
5	Optimization of annealing temperature for OsZIP4b by gradient PCR	54-55
6	Optimization of MgCl ₂ concentration for SQ RT-PCR	54-55
7	Effect of BSA on amplification of desired cDNA	54-55
8	Effect of primer concentration on amplification of desired cDNA	54-55
9	Optimization of cycle number for OsZIP4a	54-55
10a	Relative expression of OsZIP4a in embryo of selected genotypes	56-57
10b	Relative expression of OsZIP4a in endosperm of selected genotypes	56-57
11a	Relative expression of OsZIP4b in embryo of selected genotypes	56-57
11b	Relative expression of OsZIP4b in endosperm of selected genotypes	56-57
12a	Relative expression of OsFER1c in embryo of selected genotypes	56-57
12b	Relative expression of OsFER1c in endosperm of selected genotypes	56-57
13a	Relative expression of OsNAS1a in embryo of selected genotypes	56-57
13b	Relative expression of OsNAS1a in endosperm of selected genotypes	56-57
14a	Relative expression of OsNAS2a in embryo of selected genotypes	56-57

Plate No.	Title	Between Pages
14b	Relative expression of OsNAS2a in endosperm of selected genotypes	56-57
15a	Relative expression of OsYSL2b in embryo of selected genotypes	56-57
15b	Relative expression of OsYSL2b in endosperm of selected genotypes	56-57
16a	Relative expression of OsYSL2c in embryo of selected genotypes	58-59
16b	Relative expression of OsYSL2c in endosperm of selected genotypes	58-59
17a	Relative expression of OsYSL15b in embryo of selected genotypes	58-59
17b	Relative expression of OsYSL15b in endosperm of selected genotypes	58-59
18a	Relative expression of OsYSL18b in embryo of selected genotypes	58-59
18b	Relative expression of OsYSL18b in endosperm of selected genotypes	58-59

I INTRODUCTION

Rice (*Oryza sativa* L.) is one of the important food crops in India in terms of area, production and consumer preference. India is the second largest producer of rice in the world after China. Indian share in global rice production has been hovering in the range of 19.5 per cent to 24.5 per cent (USDA, 2012).

Rice is one of the major cereal crops belonging to the genus *Oryza* and the tribe *Oryzeae* of the family *Gramineae* (*Poaceae*). The genus *Oryza* contains 25 recognized species, of which 23 are wild and two are cultivated (Khush and Brar, 2003). Asia is the leader in rice production accounting for about 90 per cent of the world's production. Over 75 per cent of the world supply is consumed by people in Asian countries and thus rice is of immense importance to food security of Asia (FAOSTAT, 2013).

Rice is now cultivated in as north as the banks of the Amur River (53° N) on the border between Russia and China, and in as south as central Argentina (40° S). It is grown in cool climates in the mountains of Nepal and India, and under irrigation in the hot deserts of Pakistan, Iran and Egypt. It is an upland crop in parts of Asia, Africa and Latin America. The other environmental extremes are floating rice, which thrive in deeply flooded areas such as river deltas of Mekong in Vietnam, the Chao Phraya in Thailand, the Irrawady in Myanmar, and Ganges-Brahmaputra in Bangladesh and Eastern India. Rice can also be grown in saline, alkali or acid-sulfate soils (OECD, 1999).

Rice growing ecosystems in India are classified into four distinct types, irrigated rice, rainfed upland rice, rainfed lowland rice, and flood prone rice ecosystems. New systems for rice cultivation are continuously evolved for increased productivity by introducing management practices in various ecosystems. Two such examples are aerobic rice and system of rice intensification (Huaqi *et al.*, 2002).

Aerobic rice is a renewed way of growing rice in non-submerged unpuddled condition in aerated soils. Aerobic rice can be grown like any other crops like maize or sorghum on dry soils with surface irrigations provided when necessary with intensive agronomic practices. The aerobic rice varieties developed for the purpose yield as much as irrigated rice varieties traditionally grown in puddled soil (<http://www.aerobicrice.org>).

Rice is a nutritious cereal crop, used mainly for human consumption. It is the main source of energy and is an important source of protein providing substantial amounts of the recommended nutrient intake of niacin. However, rice is very low in calcium, zinc (Zn), iron (Fe), thiamine and riboflavin and nearly devoid of β -carotene. Rice protein is biologically the richest by virtue of its high true digestibility (88 %) among cereal proteins and also provides minerals and fiber (Gopalan *et al.*, 2007).

In India, rice is generally not consumed as brown rice because of less consumer preference and poor longevity. Hence, rice is subjected to the process of milling and polishing to remove the parts of rice grain which would make rice rancid or unpalatable otherwise over long storage. Among the milling fractions of rice, the bran has the highest

energy and protein content and the hull has the lowest content. Abrasive or friction during milling to remove the pericarp, seed coat, testa, aleurone layer, and embryo to yield milled rice results in loss of fat, protein, crude fiber, thiamine, riboflavin, niacin, and α -tocopherol. Available carbohydrates, mainly starch, are higher in milled rice than in brown rice (FAO, 1994). The gradients for the various nutrients are not identical as evidenced from analysis of successive milling fractions of brown rice and milled rice (Barber and Barber, 1991).

Iron distribution in brown rice is 51 per cent in bran, 10 per cent in germ, 10 per cent in the polish, and 28 per cent in the milled rice fraction (Juliano *et al.*, 1993).

Zinc is an essential nutrient that plays an important role in numerous physiological processes in plants, serving as co-factor for many enzymes and key structural motifs in transcriptional regulatory proteins. A deficiency of Zn, therefore, decreases growth, but excess Zn has significant toxicity to biological systems through metal-based cytotoxic reactions. Therefore, the uptake and transport of Zn must be strictly regulated. Intracellular Zn homeostasis is achieved through the co-ordinated regulation of specific transporters engaged in Zn influx, efflux, and intracellular compartmentalization (Ishimaru *et al.*, 2005).

Iron is another important micronutrient which is involved in the biosynthesis of chlorophyll. Although Fe is the fourth most abundant element in Earth's crust, it is found mainly as stable, insoluble oxyhydroxide polymers that effectively limit free transport of Fe into plant for their proper growth. Iron also act as a chelator and co-factor in many enzymatic reactions in plants. As Fe makes a part of chlorophyll, plants that are deficient in Fe show chlorosis, yellowing and cupping of leaves. Iron deficiency is a limiting factor of plant growth. Iron is present at high quantities in soils, but its availability to plants is usually very low, and therefore Fe deficiency is a common problem (Lemanceau, 2009).

As both Zn and Fe are trace elements, their available forms are present in soil solutions at exceedingly low amounts. To utilize these trace elements, plants should use high-affinity transport systems for accumulation. A number of genes involved in metal transport in plants have already been identified. Some are identified by yeast complementation tests, and others based on the sequence similarity, using a variety of approaches including database mining, degenerate PCR, and heterologous hybridization techniques (Morrissey and Guerinot, 2009). One of the prominent transporter families in rice is ZIP (ZRT, IRT-like Protein) family of transporters.

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 transmembrane 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-stipe 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).

Apart from ZIP and YSL family of metal transporters, there are many other transporters reported to play an important role in transport and homeostasis of Zn and Fe. Some of the transporters are tissue specific which are expressed at high levels in specific tissues only. Likewise, there is a network of transporters that contribute to the transport of Zn and Fe from maternal tissues (husk, flag leaf) into endosperm and again from endosperm to embryo. It has been reported that nutrients coming inside the seeds reach endosperm and then enter embryo where it is utilized for several purposes like development, maturity, germination etc. (Waters and Sankaran, 2011).

There are several seed specific transporters reported in rice some of which are *OsZIP3*, *OsZIP4*, *OsYSL2*, *OsYSL15*, *OsYSL18*, *OsDMAS1*, *OsNAS1*, *OsNAS2*, *OsNAS3*, *OsFER1*, *OsFER2*, *OsFRO1*, etc. Study of these transporters at molecular level would help to explain the differential distribution of micronutrients within rice grain.

It is evident from central dogma of molecular biology that the coded genetic information hard-wired into DNA is transcribed into individual transportable cassettes, composed of mRNA; each mRNA cassette contains programs for synthesis of particular proteins. In the same way, transporter proteins will be produced from their mRNA counterparts, which in turn are transcribed from specific genomic DNA sequences. Hence, molecular study of transporters can be done at genomic DNA sequence level, transcriptome level or protein level (Ding and Cantor, 2004).

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 all points, an experiment was designed 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. Experiment was conducted using twelve genotypes like Azucena, Moroberekan, AM 1, AM 27, AM 65, AM 72, AM 94B, AM 143, ARB 6, IRJS 21, Subhadra, and TKM 9. The present study was undertaken with following objectives;

1. Phenotyping of rice genotypes for Zn and Fe content,
2. Study the distribution of Zn and Fe within different parts of rice grain and
3. Molecular characterization of micronutrient transporters within rice grain.

II REVIEW OF LITERATURE

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

- 2.1 Importance of Zn and Fe in plants and their transport into rice grains.
- 2.2 Aerobic rice cultivation.
- 2.3 Genetic variability for Zn and Fe content in rice.
- 2.4 Transporters linked with Zn and Fe distribution in rice grain.
- 2.5 Bioinformatics resources.
- 2.6 Gene expression studies in rice.

2.1 Importance of Zn and Fe in plants and their transport into rice grains

Zinc deficiency in plants cause decrease in membrane integrity, increased susceptibility to heat stress, decreased synthesis of carbohydrates, cytochromes, nucleotides, auxin and chlorophyll (Marschner, 1995). Typical symptoms of Zn deficiency in plants include high plant mortality, stuntedness, chlorosis and necrosis on the leaves, delay in flowering, and susceptibility to biotic and abiotic stresses (Broadley *et al.*, 2007).

High micronutrient content in the grain will certainly possess a significant advantage of rapid crop improvement, especially in nutrient deficient soil. Grain is the main mineral nutrient source for the seedlings and the grain Fe content is high in plants adapted to soils which are low in available Fe. Zinc in plants helps them to grow vigorously; to withstand weed competition and higher stress and also to tolerate pathogen attack (Gregorio *et al.*, 1999).

Zinc is one of the essential trace elements for plants, animals and microorganisms. In plants, it is involved in a large number of enzymes, carbohydrate and protein synthesis. The human body requires at least 49 nutrients that play a vital role as structural and functional components for a large number of proteins/enzymes (Welch, 2001; Welch and Graham, 2004).

Maser *et al.* (2001) reported that uptake and translocation of cationic nutrients (Zn^{2+} , Fe^{3+} etc.) play essential roles in physiological processes including plant growth, nutrition, signal transduction, and development.

Inoue *et al.* (2003; 2008) reported the genes involved in MA (Mugenic acid family of phytosiderophores) biosynthesis that were characterized already; *OsNAS1-3* that are responsible for trimerization of SAM, *OsNAAT1* that converts NA to a 3'-keto intermediate, and *OsDMAS1* that converts the intermediate into DMA. It was also reported that the expression of these MA family genes change in a diurnal fashion (Nozoye *et al.*, 2004; Inoue *et al.*, 2009).

Nishizawa *et al.* (2005) reported the involvement of Zn in many cellular functions, gene expression, protein metabolism, cellular and membrane integrity, chlorophyll synthesis, photosynthesis, respiration and seed maturation.

Metals have vital roles in a plant's life cycle, yet there are many impediments to proper metal homeostasis. Fe, Zn, Cu, Mn, Mo and Ni are the metal micronutrients considered essential for plants. Among all these, Fe is found in the center of Fe-S clusters, which act as electron acceptors and donors in a number of key cellular processes including photosynthesis, respiration, sulfate assimilation and ethylene biosynthesis (Balk and Labreaux, 2005).

Virk *et al.* (2006) reported that Zn concentration in the brown rice ranged from 15.3 to 58.4 ppm while Fe concentration ranged from 6.3 to 24.4 ppm. On an average 70 per cent and 24 per cent of Fe and Zn is lost during milling respectively.

Most of the minerals are concentrated in the outer layer of the rice grain (pericarp, tegmen and aleurone). These parts are lost during the process of milling. As rice is consumed in the polished state, there is a great scope to study the genetic variation for Fe and Zn content in polished rice. (Virk *et al.*, 2007).

Intracellular Zn homeostasis is achieved through the co-ordinated regulation of specific transporters engaged in Zn influx, efflux, and intracellular compartmentalization thereby strictly regulating Zn uptake and transport (Nishizawa *et al.*, 2007).

Zinc, unlike Fe, is non-redox active. Pronounced Lewis acid characteristics of the Zn^{2+} ion and the flexibility of the co-ordination sphere with respect to geometry and the number of ligands, explain about diverse roles of Zn inside the cell (Lim *et al.*, 2005). It is required as a co-factor in over 300 enzymes, including RNA polymerase, superoxide dismutase (SOD), alcohol dehydrogenase and carbonic anhydrase (Broadley *et al.*, 2007). It also has key structural roles, such as its use in the Zinc finger family of transcription factors for formation of the DNA binding domain that interacts with the major groove of DNA during transcription (Yilmaz and Mittler, 2008).

Jiang *et al.* (2008); Ciftci and Mittler (2008) reported the free transport of Zn between roots and shoots, but the transport between stem and rachis and between bran and endosperm was limited due to barriers, while overall Zn density in the plant increased. They also reported that Zn density (mg/kg of grain dry matter) in polished rice was less than 25 mg/kg.

Kobayashi *et al.* (2009) studied the role of transcription factors (TFs) in controlling Fe homeostasis in rice. They found that expression of one of the TFs, *IDEF1*, was correlated with the expression of *OsIRO2*, *OsYSL15*, *OsIRT1*, *OsYSL2*, *OsNAS1*, *OsNAS2*, *OsNAS3* and *OsDMAS1* just after the onset of Fe deficiency, suggesting that *IDEF1* is essential for the early onset of the Fe deficiency response.

Bashir *et al.* (2010) opined that choosing the appropriate chelators and Fe transporters driven by appropriate promoters was the key in developing rice varieties that are tolerant to low Fe availability and which accumulates high grain levels of Fe.

Among the transporters involved in the Fe homeostasis, *YSL* genes, *IRT* genes and citrate transporters were characterized. Rice genome contains 18 putative *YSL* family genes, among which *OsYSL2*, *OsYSL15* and *OsYSL18* were characterized in detail (Ishimaru *et al.*, 2010). *OsYSL2* have been identified as an Fe-NA transporter, *OsYSL15* have been reported to transport Fe(III)-DMA from rhizosphere to roots and *OsYSL18* was found to encode protein that is involved in DMA-mediated Fe distribution in reproductive organs, lamina joints and the phloem cell at the base of the leaf sheath (Aoyama *et al.*, 2009; Inoue *et al.*, 2009; Koike *et al.*, 2004).

Yoneyama *et al.* (2010) found that accumulation of Fe in grain tissues occur through xylem and Fe from vegetative tissues would be partly transported to grain through phloem transport. They also reported that Zn in grains was mobilized Zn from leaf blades of flag leaf and upper leaves through phloem and also due to xylem-phloem transfer in the roots. Iron stored in the leaves may be transported to the grains via the phloem.

2.2 Aerobic rice cultivation

Rice is a semi-aquatic cereal crop grown in diverse climatic conditions. It is one of the most water consuming crops which receive an estimated 34-43 per cent of the total world's irrigation water, or 24-30 per cent of the total world's freshwater withdrawals. Asia is the major rice producer which accounts for an estimated production of 90 per cent of the world rice crop. ARB 6 is an aerobic adapted high yielding rice variety developed from a cross between Budha and IR 64 in University of Agricultural Sciences, GKVK, Bengaluru (www.aerobicrice.org).

Aerobic rice has been considered a promising rice cultivation system as water scarcity is increasing around the world. Compared to lowland rice, water input in aerobic rice is lower, and the water productivity is higher. Cultivation of flooded lowland rice requires about 1,300 mm of water, whereas aerobic rice requires only 470-644 mm.

Bouman and Tuong (2001) reported that the shift from irrigated to aerobic systems of rice cultivation will have major positive consequences with respect to weed, disease and insect pest management, nutrient and soil organic matter dynamics and green-house gases emission sequestration at experimental station, Changping, Beijing.

Bouman *et al.* (2002) compared aerobic rice varieties with the lowland rice. Highest recorded aerobic rice yields were 4.7-6.6 t/ha, compared to 8-8.8 t/ha of lowland rice, and reported aerobic rice variety Han Dao 502 as most promising because of its relatively high yield under both conditions and because of its good quality fetching a high market price. Their experiments showed that water inputs in aerobic rice were more than 50 per cent lower; water productivity is 64-88 per cent higher, gross returns 28-44 per

cent lower and labour use 55 per cent lower. Because of its low water use, aerobic rice can be produced in areas where lowland rice cannot be grown anymore.

Gani *et al.* (2002) reported that rice under aerobic method produced more tiller/hill (40.6), longer root length (32.5 cm) as compared to traditional methods of planting. Yang *et al.* (2002) revealed that the aerobic rice varieties out yielded when compared to lowland rice varieties and also consuming only 470 mm of water.

The cultivation of aerobic rice aims to minimize the water requirement of rice while maintaining yield levels close to the irrigated lowlands. Traditionally, aerobic rice is grown in fragile rainfed uplands with low or no inputs and uncertain water availability. As fresh water is becoming scarce, aerobic rice is expected to expand to the regions of more intense cropping with high external inputs and productivity (Lafitte *et al.*, 2002).

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

Atlin *et al.* (2004) noticed that both irrigated high yielding varieties and aerobic types yielded approximately 4 t/ha under lowland conditions, in the non-stress upland environment. Aerobic rice varieties significantly out-yielded all other types and recorded significantly more panicles than traditional upland varieties with higher water use efficiency compared to normal method of planting.

Breeding efforts specifically towards upland environments has also led to the development of aerobic rice which is high yielding than traditional upland varieties and combines input responsiveness with improved lodging resistance and harvest index (Atlin *et al.*, 2006).

In a study by Vijaykumar *et al.* (2006), over 2600 improved germplasm lines were screened in a puddled field (clay soil) under aerobic (non-flooded, irrigated) condition with Rasi and Vandana as check varieties. The checks, known for their water stress tolerance were planted at regular intervals to serve as control. In both the checks, within as well as between the strips, there was no variation for number of days for heading, but a significant variation was found for grain yield. Apart from visual selection, three different methods utilizing appropriate statistics were employed for identifying promising genotypes.

Forty rice cultivars and breeding lines used in the IRRI upland rice breeding program were evaluated in adjacent weed free and weedy trials in aerobic soil conditions during the wet seasons of 2001, 2002 and 2003. The objective of this study was to investigate genetic variability in weed suppression and yield and to identify traits that could be used as selection criteria for improved weed competitiveness. Correlation among heritability (H) of agronomic traits and early vigour were estimated in weedy and weed free trials. Cultivar yield, duration, biomass, harvest index, height and vegetative

vigour under weed free conditions were closely correlated with the same traits measured under weedy conditions (Zhao *et al.*, 2006).

Aerobic rice has efficient water use efficiency which saves about 45 per cent of water utilization, decrease methane production; reduce production cost and eco-friendly compared to conventional irrigated rice (Shashidhar, 2007).

Aerobic rice provides an effective use of rain that falls on the farmers' field, as there was no standing water at any time in the crop cycle. Irrigation using the farmer's source can be skipped if rain water is available thus there is better use of existing water crop. Aerobic rice production system 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 rainfall and thus helps in enhancing water productivity, concomitant loss of soil sediments, silt and fertility from the soil. Aerobic rice system can save about 45 per cent of water, and minimizes methane production (Shashidhar, 2007).

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).

Verulkar *et al.* (2010) identified breeding lines based on high yield potential with drought tolerance by employing direct selection for grain yield under drought stress, and testing their performance in rainy season managed-drought screenings of different stress severity levels at several locations during the main season in rainfed rice production areas in India. These breeding lines out-yielded widely grown controls by 0.5 t/ha under moderate stress condition and 0.7-1.0 t/ha under severe stress.

As a response option to the looming water-scarcity problem, a new way of growing rice has been developed the aerobic rice system, which uses drought tolerant, input responsive, lodging resistant and weed competitive varieties that can give acceptable yields using roughly half the water input of traditional lowland rice culture. The development of this technology started in China in the 1980s for breeding high yielding rice varieties grown in non-puddled, non-flooded aerobic soil (Templeton and Bayot, 2011).

As per the Indian classification, rice varieties are grouped into five categories as Long-slender (6 mm length, ≥ 3 mm length-breadth ratio), Long-bold (>6 mm length, <3 mm length-breadth ratio), Short-slender (<6 mm length, >3 mm length-breadth ratio), Short-bold (<6 mm length, <2.5 mm length-breadth ratio), Medium-slender (<6 mm length, 2.5-3 mm length-breadth ratio) (Department of Biotechnology, Ministry of Science and Technology, 2011).

Kadiyala *et al.* (2012) had conducted field experiments during 2009-10 and 2010-11 to compare crop yield, growth 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 37-45 per cent over flooded method of rice cultivation. Significant increase in yields in both systems with increased N rates up to 120 kg/ha. Significantly higher yields were observed in no-till maize grown subsequent to the aerobic rice than that grown after flooded rice which was possibly due to residual rice N and improved soil physical conditions.

Physiological attributes like soluble protein, chlorophyll content, fluorescence, relative water content, osmotic adjustment and scavenging enzymes play a major role in aerobic rice. Aerobic rice saved 73 per cent of irrigation water for land preparation and 56 per cent during the crop growth period. Aerobic rice with micro-irrigation practices leads sustainable rice production methodology for immediate future to address water scarcity. This technology is a better remedy for future climate change. Selection of good aerobic rice variety with desired physiological attributes give better performance (Parthasarathi *et al.*, 2012).

2.3 Genetic variability for Zn and Fe content in rice.

Qui *et al.* (1995) reported a higher variability in mineral contents in some rice cultivars and the level of Fe content varied from 15.41 mg kg⁻¹ to 162.37 mg kg⁻¹ and Zn content ranged from 23.92 mg kg⁻¹ to 145.78 mg kg⁻¹. Wang *et al.* (1997) reported the range for Zn content in grains of rice ranged from 0.79-5.89 mg/100 g with an average of 3.34 mg/100 g in a study done among 57 rice varieties. Ahmed *et al.* (1998) found a considerable variation in chemical composition of rice cultivars and Fe content that ranged from 1.5 to 5.0 mg/100 g with an average value of 3.2 mg/100 g.

Yang *et al.* (1998) examined the concentration of Zn, Fe, Cu and Mn in polished grains of 285 rice genotypes (through AAS) and the relationship between micronutrient concentrations and concentration of protein and lysine. Significant differences were found in micronutrient concentrations in polished grains. Also, no close relationship was found between micronutrient concentrations and protein or lysine content among the genotypes.

Graham *et al.* (1999) noticed higher Fe content trait expressed in all rice environments tested such as dry season in normal and saline soils, in acid and neutral soils. Grain iron content generally varied much more with genotype, than with environment and genotype × environment interactions.

Graham *et al.* (1999) reported the presence of genetic variation for grain Zn concentration among 1000 rice genotypes grown and screened in International Rice Research Institute (IRRI) farm, Los Banos, Philippines, where its concentration in the brown rice ranged from 15.30-58.40 ppm while Fe concentration ranged from 6.30-24.40 ppm.

Genotypic variation for the accumulation of micronutrient accumulation in grain have been reported in staple crops such as rice (Graham *et al.*, 1999; Gregorio *et al.*, 1999; Gregorio *et al.*, 2000; Zhang *et al.*, 2004), wheat (Ortiz-Monasterio and Graham,

2000; Balint *et al.*, 2001) and maize (Arnold and Bauman, 1976; Banziger and Long, 2000), soybean (Raboy *et al.*, 1984), and bean (Moraghan and Grafton, 1999).

Ortiz-Monasterio *et al.* (2000) at CIMMYT, Mexico reported a significant genotype $\times$ environment interaction for Fe and Zn grain concentrations in wheat, where there was a strong genetic component for Fe and Zn accumulation in the grain.

Venuprasad *et al.* (2002) reported high phenotypic and genotypic coefficient of variation for number of tillers per plant, grain yield per plant, total biomass per plant, harvest index. Genetic diversity studies for eight mineral concentrations of brown rice, using 653 accessions showed that there is greater average genetic diversity index for *japonica* accessions compared to *indica* accessions (Zeng *et al.*, 2005).

Nine genotypes of rice were evaluated for Fe and Zn content in rice grain at IRRI by Martinez *et al.* (2006) and found a range of 8.8-21.0 mg/kg, 14.0-40.0 mg/kg for Fe and Zn respectively. Gopalan *et al.* (2007) reported that average composition of Fe in edible portion of milled rice was about 7 mg/kg.

Jiang *et al.* (2007) opined that when rice plants were grown under sufficient or surplus Zn supply, most of the Zn accumulated in the grain originates from the uptake by roots after flowering and not from Zn remobilization from leaves.

Kibanda and Luzi (2007) reported absence of environmental influence on grain length and grain size (length/width) but observed higher genetic variance for these traits. Jiang *et al.* (2008) studied within-plant allocation and potential Zn accumulation in the rice grains of four aerobic rice genotypes. At higher uptake levels, the ZnMC (Zn mass concentration) in stem increased the most, while the ZnMC in hulled grains (brown rice) increased the least. Milling test on two of the four genotypes showed that when ZnMC in brown rice increased from 13-45 mg/kg, ZnMC in polished grains (endosperm) also increased from 9-37 mg/kg but remained 3-5 times lower than that in the bran. Also, there was a significant difference between ZnMC of bran and endosperm in two genotypes suggesting a barrier for Zn transport between the two tissues. They also proposed the presence of a second barrier between stem and rachis.

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.

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).

Nagarathna *et al.* (2010) reported the presence of wide genetic variability in rice Zn content from 0.84-5.00 mg/100 g dry weight.

Martinez *et al.* (2010) reported the difference in the Fe and Zn content for milled and brown rice in 11,400 rice samples collected from local stores and supermarkets at Colombia. They found 2-3 ppm of Fe and 16-17 ppm of Zn in milled rice, and 10-11 ppm of Fe and 20-25 ppm of Zn in brown rice.

Wu *et al.* (2011) had done hydroponics experiment to compare Zn uptake and distribution in two different Zn-density rice genotypes (IR-68144 and IR-64) using stable isotope technique. Both the genotypes showed similar patterns of ^{68}Zn accumulation in new leaves at seedling stage and in developing grains at ripening stage, whereas ^{68}Zn in new leaves and grains of IR-68144 was consistently higher. These results suggested that a rapid root-to-shoot translocation and enhanced xylem loading capacity may be the crucial processes for high Zn density in rice grains.

The contents of Fe and Zn in grains of traditional rice genotypes were significantly higher than those of improved cultivars (Anandan *et al.*, 2011). On the basis of grain Zn content, rice genotypes could be grouped into three categories, low (less than 14 ppm), moderate (14 ppm–24 ppm) and high (greater than 25 ppm).

2.4 Transporters linked with Zn and Fe in rice grain

McKenzie and Rutger (1983) reported that rice grain characteristics were either controlled by one to two major genes plus few minor genes. On the other hand Shi and Zhu (1993) found that grain characteristics are controlled by quantitative minor genes that could be described by additive dominance models.

It is reported that roots and grains are the large sources for translocation of Zn into the grain during the grain filling stage. It is also shown in soybean that grains can also accumulate remobilized Zn from leaves (Khan and Weaver, 1989).

The same qualities that make the transition metals (Zn, Fe etc.) as essential co-factors can also make them highly toxic within the cell. The main strategies that the plant uses to combat this toxicity are sequestration and chelation to carrier molecules (Hulliwall and Gotteridge, 1989).

In case of dicots Zn moves through phloem. In monocots like wheat, transport of Zn occurs from stems and leaves to grain (Pearson *et al.*, 1995; 1996) or from older leaves to younger leaves (Page and Feller, 2005), indicating the involvement of phloem in transport of Zn.

Plant ferritins combine both functions in their subunits, which share higher homology with animal ferritins than with bacterial ferritins, although they are localized in the plastids, a typical prokaryotic environment (Briat and Lobréaux, 1998).

The *ZIP* family has representatives in several plant species, including arabidopsis, pea, tomato, soybean, rice and alfalfa (Grotz *et al.*, 1998, Vert *et al.*, 2002, Cohen *et al.*, 1998, Eckhardt *et al.*, 2001, Moreau *et al.*, 2002, Bughio *et al.*, 2002; Ramesh *et al.*, 2003, Ishimaru *et al.*, 2005, Ishimaru *et al.*, 2006, Burleigh *et al.*, 2003), as well as in archaea, yeast, bacteria and animals (Gaither and Eide, 2001).

Some of the metal transporters have broad specificity, but the range of specificity of metal transporters is still unknown because the functional characterization is still lagging behind the gene discovery (Korshunova *et al.*, 1999).

Members of the gene family *ZIP* are identified in plants are capable of transporting Cd, Fe, Mn and Zn. The hallmark feature of *ZIP* gene family is that they consist of 309-476 amino acid residues with eight transmembrane domains. They have a similar membrane topology in which, the amino- and carboxyl-terminals of the proteins are located on the outside of the plasma membrane (Guerinot, 2000).

Members of the *ZIP* gene family, a novel metal transporter family first identified in plants, are capable of transporting a variety of cations, including Cd, Fe, Mn and Zn (Guerinot, 2000).

Plant ferritin genes do not contain IREs (Iron Regulatory Elements), conserved regulatory sequences responsible for the translational repression of animal ferritin mRNA in response to Fe overload (Petit *et al.*, 2001). Instead, in the promoters of maize and arabidopsis ferritin genes, the presence of Iron-Dependent Regulatory Sequences (IDRS) was responsible for repression of transcription under low Fe supply.

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).

Krishnan and Dayanandan (2003) found that in rice as there is no discontinuity between rachis and grain vascular bundle and due to symplastic continuity existing between the cells of the vascular trace, chalaza, nucellar projection and nucellar epidermis, inward transport of mineral nutrients occurs from the outer grain tissues to the endosperm through the nucellar epidermis that completely encircles the endosperm.

The search for sequences homologous to the maize ferritin (*ZmFER1*) yielded several related sequences in rice. These include 20 ESTs, 2 sequenced cDNAs, 2 genomic sequences from the IRGSP and 4 BGI scaffolds. A comparative analysis of ESTs showed two distinct patterns of expressed genes, which differ in the 5' region by point variations

and two small deletions. The full length cDNA information confirmed the two distinct gene patterns, which were named *OsFER1* and *OsFER2* (Gross *et al.*, 2003).

Gross *et al.* (2003) studied the difference at sequence level between *OsFER1* and *OsFER2*. They reported that the two genes were similar with 96 per cent identity. In addition to 15 SNPs along the complete cDNA, *OsFER1* had two deletions of 12 bp not seen in *OsFER2*. One of the deletions is located in the 5' untranslated region and the other immediately downstream from the start codon. They also found that the rice ferritins had more than 30 per cent identity in relation to the mammalian ferritins and between 58 and 82 per cent with other plant ferritins.

Sunita *et al.* (2003) studied the metal selectivity and gene expression of two Zn transporters from rice (*OsZIP1* and *OsZIP3*). 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.

Koike *et al.* (2004) suggested that in rice, *OsYSL2* as a transporter that functions in moving Fe-NA complexes into phloem. *OsYSL2* expression increases dramatically during periods of Fe deficiency, suggesting a role in the Fe-uptake process. While lowered Fe accumulation in grains of *OsYSL2i* (RNA interference of *OsYSL2*) plants could be the result of impaired phloem loading in leaves, it could also result from a more local effect, since *OsYSL2* is normally expressed both in developing ovaries and in the endosperm and embryo of developing seeds. The combination of localization and knock-down phenotype strongly suggests that *OsYSL2* plays an important role in the loading of Fe into rice seeds.

Over expression of the *AtNAS1* gene in tobacco only marginally increased the young leaf Fe (approximately 1.5 fold) and especially Zn content (approximately 1.2 fold), although in both cases, NA was overproduced (Douchkov *et al.*, 2005). Over expression of the *HvNAS1* gene in tobacco approximately doubled the seed Fe and Zn concentrations (Takahashi *et al.*, 2003).

Ishimaru *et al.* (2005) have conducted an experiment on ZIP family of metal 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.

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.

ZIP transporters generally contribute to the homeostasis by transporting cations into the cytoplasm (Colangelo and Guerinot, 2006). They are found at every phylogenetic level, including archaea, bacteria, fungi, plants, and mammals (Grotz and Guerinot, 2006).

For the successful maintenance of Zn homeostasis, plants greatly rely on the co-ordinated regulation of multiplicity by Zn transporters, including uptake transporters such as members of the *ZIP* family, natural resistance-associated macrophage protein (NRAMP), and plant specific *Yellow-Stripe1-Like* (YSL) families, as well as efflux transporters, such as those in the cation diffusion facilitator (CDF) and P1B-ATPase families (Colangelo and Guerinot, 2006).

Seeds contain filial tissues (embryo and endosperm, aleurone) surrounded by maternal tissues (seed coat). The developing seed is connected to the maternal plant by a single vascular trace. This vascular bundle ends at the seed coat and is not symplastically connected to the endosperm or embryo. Nutrients moving to the seed are unloaded from the phloem, while excess water is thought to move in the opposite direction in this vascular strand, through the xylem or suberized cells (Zhang *et al.*, 2007; Hintz and Theil, 2006).

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).

Michael *et al.* (2008) discussed molecular genetic approaches for improving Zn content and bioavailability, especially in cereals. They have overviewed the transport pathway of a metal ion from soil to grain, and demonstrate that the root–shoot distribution of Zn is controlled mainly by heavy metal transporting P1B-ATPases and the *metal tolerance protein* (*MTP*) family. *MTP3* is thought to be a key protein in restricting the movement of Zn from the root to the shoot under conditions of non-toxic excesses of Zn.

Chen *et al.* (2008) provided a complete overview of *ZIP* family in rice (*Oryza sativa* L.). They have identified total of 17 *ZIP*-coding genes by blast analysis. They studied expression patterns of *OsZIP1*, *OsZIP3*, and *OsZIP4*, which encode Zn²⁺ transporters in rice, in the Zn-efficient and Zn-inefficient rice genotypes by semi quantitative RT-PCR analysis of roots, shoots, and panicle from the plants grown under Zn deficiency and normal conditions. They have elucidated the relationship between these *ZIP* genes and Zn efficiency in rice.

ZIP family Zn and Fe transporter proteins are reported for their use in improving micronutrient density in grains. They are involved in uptake, transport, storage and homeostasis of cationic micronutrient (Chen *et al.*, 2008 and Cakmak, 2008).

Water and Grusak (2008) have shown that silique hulls of *Arabidopsis* are a likely source of metals that are being loaded into *Arabidopsis* seeds. Grusak (1994) demonstrated that Fe applied to the pod wall of peas was rapidly re-translocated into developing seeds. Together, both these studies suggested that Fe is translocated repeatedly from organ to organ prior to seed loading: from leaf to developing inflorescence, from inflorescence to hulls, and, finally, from hulls to developing grains.

Stacey *et al.* (2008) reported another group of oligo-peptide transporter, *OTP3* that might be involved in transporting Fe. They also showed that *otp3-2* mutants have reduced seed Fe content and abrogated seedling growth under Fe-deficient conditions, which suggested a role of *OTP3* in seed Fe loading. Tauris *et al.* (2009) reported *HMA*, *ZIP*, *MTP*, *Nramp*, *NAS* and *YSL* as the genes involved in transporting the Zn and other micro nutrients into seeds and storage tissues. They had also reported that there was no even distribution of these genes in all the cells.

Waters *et al.* (2009); Curie *et al.* (2009) reported that remobilization process is unregulated under nutrient limitations. They have observed that remobilization of Fe and Zn increased when these micronutrients were withheld from the hydroponic solution. Inoue *et al.* (2009) examined the expression of 18 YSL genes (*OsYSL1-18*) in the epidermis, cortex, and stele of rice roots. They have concluded that rice *OsYSL15* play a crucial role in Fe homeostasis during the early stages of growth. Wu *et al.* (2010) in their study on wheat reported that flag leaf and lower leaves as the major sources of remobilized micronutrients, in contrast to results from rice, where culms accounted for the major source of Zn remobilized to grain.

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* overexpressing genotype. In nutshell, this study revealed the enormous potential of *OsNAS* genes, *OsNAS2* in particular, in increasing Fe and Zn content in polished rice.

Water and Sankaran (2011) studied on the transporters that are involved in phloem unloading and filial tissues of barley seeds. The Laser capture microdissection followed by microarray analysis by them further revealed the expression of genes from *HMA*, *ZIP*, *MTP*, *Nramp*, *NAS* and *YSL* families. They also found that expression of these genes was not evenly distributed among the different cell types, suggesting that, certain transporters are important in specific cell types to move micronutrients through the plant.

Bashir *et al.* (2011) studied the expression of *OsZIP1*, *OsZIP3*, *OsZIP4*, *OsZIP5* and *OsZIP8* in leaf blade, root, stem, anther, ovary and embryo at different growth stages. They found that in embryo, expression of *OsZIP4* increased from 7 DAF to 10 DAF and steadily increased until 24 DAF showing that the expression of *ZIP4* was important for Zn translocation during seed development.

Phloem unloading in the developing seed of *Arabidopsis* was believed to be symplastic into a phloem-unloading domain, which have symplastic connections to the entire seed coat. Another apoplastic barrier would be present in the seed coat where the outer two cell layers lack symplastic connectivity with the three inner cell layers of the seed coat (Stadler *et al.*, 2005; Werner *et al.*, 2011).

Bashir *et al.* (2012) reviewed on molecular mechanisms of Zn transport in rice. They found the involvement of *OsZIP1* and *OsZIP3* in Zn uptake from soil, *OsZIP4*, *OsZIP5* and *OsZIP8* in root to shoot translocation and *OsZIP4* and *OsZIP8* in transport of Zn to seeds. They found that the expression of *OsZIP4* was significantly higher when compared to other *OsZIPs* in embryo and endosperm of rice seeds during seed development.

2.5 Bioinformatics resources.

Bioinformatics is the application of statistics and computer science in the field of molecular biology. Application of computers in the field of molecular biology had reduced lot of time required in performing some of the wet-lab works. There are many databases available for public use which contains enormous quantity of data which can be used in the field of molecular biology. Here, in this study bioinformatics resources were used to select the grain specific micronutrient transporters at a particular stage of reproductive cycle, select the internal reference gene, and to design gene specific primers. Some of such databases are NCBI, GenBank, RiceXPro and Gramene.

NCBI (National Centre for Biotechnology Information)

The National Centre for Biotechnology Information (NCBI) is a part of United States National Library of Medicine (NLM), a branch of National Institute of Health (NIH). The NCBI is located in Bethesda, Maryland established in 1988 through legislation sponsored by Senator Claude Pepper. Initially NCBI was established as a national information resource for molecular biology information. NCBI creates public databases, conducts research in computational biology, develops software tools for analyzing genome data and finally disseminates biomedical information – all for the better understanding of molecular processes affecting human health and disease. NCBI houses a series of databases which have relevance to biotechnology and also biomedicine. Some of the important databases of NCBI include GenBank for DNA sequences and a bibliographic database of the biomedical literature called PubMed. All these databases are available online to public use through Entrez search engine (www.wikipedia.com, www.ncbi.nlm.nih.gov/About).

GenBank

GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (Benson *et al.*, 2013). GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA Data Bank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at NCBI. These three organizations exchange data on a daily basis.

Since 1992, NCBI has grown to provide other databases in addition to GenBank. NCBI provides Online Mendelian Inheritance in Man, the Molecular Modelling Database (3D protein structures), dbSNP a database of single-nucleotide polymorphisms, the Unique Human Gene Sequence Collection, a Gene Map of the human genome, a Taxonomy Browser, and coordinates with the National Cancer Institute to provide the Cancer Genome Anatomy Project. The NCBI assigns a unique identifier (Taxonomy ID number) to each species of organism.

The GenBank database is designed to provide and encourage access within the scientific community to the most up to date and comprehensive DNA sequence information. GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100,000 distinct organisms. GenBank continues to grow at an exponential rate, doubling every 10 months. Release 134, produced in February, 2003 and contained over 29.3 billion nucleotide bases in more than 23.0 million sequences. GenBank is built by direct submissions from individual laboratories, as well as from bulk submissions from large-scale sequencing centers.

Gramene

Gramene (<http://www.gramene.org>) is a community source for rice and a comparative genome mapping database for grasses. Rice is a model monocot plant for understanding the other economically important crops. Gramene uses a relational database based on Oracle, replacing the existing AceDB database "Rice Genes". Gramene provides curated and integrative information about maps, sequence, genes, genetic markers, mutants, QTLs, controlled vocabularies and publications. Its aims are to use the rice genetic, physical and sequence maps as fundamental organizing units, to provide a common denominator for moving from one crop grass to another and are to serve as a portal for interconnecting with other web-based crop grass resources (Ware *et al.*, 2002).

It is a collaborative project of 'cold spring harbour laboratory', Cornell University and rice community. The main core base of Gramene is the MySQL database management systems, make it a user friendly front end system and it is a stable and well supported. Rice is used as a framework genome to organized information for other grass species due to its smaller genome to organized information for other grasses such as maize (2,400 MB) and wheat (16,000 MB) having considerable larger genome.

The goals of Gramene are to (i) establish a database utilizing the rice genome as a framework for identifying and characterizing genes in other grasses (ii) provide, comparative maps between rice and other grasses based upon orthologous sequence and a wealth of genetic and phenotypic information available among the grasses (iii) develop a pilot study to assign gene ontology (GO) functional classification of 4000 confirmed or predicted rice genes and (iv) integrated with other plant databases to allow comparisons of conserved syntenic relationship and mutant phenotypes (Ware *et al.*, 2002).

RiceXPro

The Rice Expression Profile Database (RiceXPro) is a repository of gene expression profiles derived from microarray analysis of tissues/organs encompassing the

entire growth of the rice plant under natural field conditions, rice seedlings treated with various phytohormones, and specific cell types/tissues isolated by laser microdissection (Sato *et al.*, 2011). This database is a part of a project on rice transcriptome analysis using microarray technology aimed at characterizing the expression profile of all predicted genes in rice and providing reference information that can be used in functional genomics. All expression profiles were generated using a single microarray platform with probes based on manually curated gene models in RAP-DB and rice full-length cDNA sequence information in KOME database. This project supported by a grant from Ministry of Agriculture, Forestry and Fisheries of Japan (Cao *et al.*, 2012).

A global expression profile of each gene can be viewed from two major dataset categories, namely, ‘field/development’ and ‘plant hormone’. The field/development datasets correspond to microarray data derived from tissues/organs at various stages of development under natural field conditions. The plant hormone datasets correspond to microarray data from roots and shoots of seedlings treated with various plant hormones. Search options provide an overview of the overall transcriptome of the rice plant under each category.

This database provide an overview of the expression pattern of all rice genes in specific tissues/organs at various stages of growth and development under normal field conditions, rice plants treated with various phytohormones, and cell/tissue types isolated by LMD. The gene expression profiles will be presented as data sets from specific samples, experiments, or treatment conditions. The data table includes a list of samples used for microarray analysis and relevant information that may be useful in interpretation of gene expression levels. For example, RXP_0012 Embryo and Endosperm gene expression profile at ripening stage.

Gene expression profile of throughout the ripening process can be known using this database. After the pollination, embryo and endosperm samples were collected during ripening stage based on number of days after flowering (DAF). A total of 36 samples were used for hybridization using the Agilent one-color (Cy3) microarray-based gene analysis system. The expression profile for each gene will be shown as raw data representing Cy3 signal intensity and normalized data (log2). Search options include keyword search for individual RAP locus ID and accession number, of chromosome search to get a list of all genes for each chromosome.

RiceFRIEND is a gene co-expression database in rice based on a large collection of microarray data derived from various tissues/organs at different stages of growth and development under natural field conditions, and rice plants treated with various phytohormones. RiceFRIEND aims at providing a platform for identification of functionally related genes in various biological pathways and/or metabolic processes. Using the web interface of RiceXpro the data regarding expression of different Zn transporters was acquired for different growth stages of rice (Bashir *et al.*, 2011).

2.6 Gene expression studies in rice

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method is used for the rapid analysis of differences in gene expression. mRNA differential display technique, Northern blot analysis have been commonly used to confirm that a differentially displayed cDNA fragment represents a true difference transcript levels between the samples tested. It is usually impossible to use northern blot analysis when working in developmental biology, since the amount of material is so small (Atlas, 2008). To confirm the differential expression of mRNAs which correspond to cDNAs isolated by the mRNA differential display, a semi-quantitative RT-PCR assay which is simple, fast and which requires a little RNA is feasible.

2.6.1 Current methods for the study of gene expression at the transcript level

A. Hybridization-based techniques

- Northern blotting
- S1-mapping/ RNase protection
- Differential plaque hybridization
- Subtraction cloning
- DNA microarrays

B. PCR-based techniques

- Differential display
- RDA (Representational Difference Analysis)
- cDNA- SRAP- Sequence- related Amplified Polymorphism

C. Sequence-based techniques

- ESTs (Expressed Sequence Tags)
- SAGE (Serial Analysis of Gene Expression)
- MPSS (Massively parallel Signature Sequencing)
- DNA-sequencing chip
- Mass-spectrometry sequencing

Reverse transcription-polymerase chain reaction (RT-PCR) is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts (Erlich, 1989; Carding *et al.*, 1992). In most cases, when RNA analysis is required, a qualitative study is not sufficient to deliver a satisfactory answer. A common question is the quantification of the specific RNA transcripts and the detection of any variation in their expression levels under different experimental conditions.

Plant gene expression is controlled at different levels and significant number of micronutrient transporter genes appeared to be controlled at transcriptional level (Shinozaki and Yamaguchi-Shinizaki, 2000).

Sakurai *et al.* (2005) studied the expression level of different 33 rice aquaporin genes in leaf blades, roots and anthers of rice using semi-quantitative reverse transcription-PCR (RT-PCR). At both early tillering (21 days after germination) and panicle formation (56 days after emergence) stages, six genes, including *OsPIP2;4* and *OsPIP2;5*, were expressed predominantly in roots, while 14 genes, including *OsPIP2;7* and *OsTIP1;2* were found in leaf blades. Eight genes, such as, *OsPIP1;1* and *OsPIP4;1*, were evenly expressed in leaf blades, roots and anthers. The mRNA levels of ten genes including *OsPIP2;4* and *OsPIP2;5* markedly decreased in roots during chilling treatment and recovered after warming. The changes in the mRNA levels during and after the chilling treatment were comparable with that of the bleeding sap volume. These results suggested the relationship between the root water uptake and mRNA levels of different aquaporin with high water channel activity, such as *OsPIP2;4* and *OsPIP2;5*.

Narayanan *et al.* (2007) profiled the expression of 36 metal-related genes from rice both in flag leaves as well as in non-flag leaves of four rice cultivars during mid-grain filling stage. 24 out of 36 genes exhibited low to non-detectable signals in macroarray, while 12 genes were found highly expressed in both flag and non-flag leaf of all four cultivars. Additional semi-quantitative and q-PCR results were consistent with the macroarray, but semi-quantitative PCR showed the additional expression of nine more genes in the leaves. This study helped them to short-list the candidate genes, expressed in leaves, which might contribute to the process of metal transport to distant sinks, such as seeds.

Chen *et al.* (2008) studied the expression pattern of *OsZIP1*, *OsZIP3* and *OsZIP4* in Zn-efficient and Zn-inefficient rice genotypes by semi-quantitative RT-PCR analysis of roots, shoots and panicle from plants grown under Zn deficient and normal conditions. They found that *OsZIP1* were expressed only in roots and very weakly if at all, in panicles, while other two genes were expressed in all parts of the plants under study.

Micronutrient dynamics and distribution measured as micronutrient content in the grain and several studies done at molecular level are useful in locating the genetic regions for identifying candidate genes. The study of differences in gene expression patterns is one of the most promising approaches for understanding mechanisms of differentiation and development (Farooq *et al.*, 2009).

Sperotto *et al.* (2009) conducted suppression subtractive hybridization analysis in flag leaves of two rice cultivars. They found that the flag leaves are the major source of remobilized metals for developing seeds. They also isolated 78 sequences that were up-regulated in flag leaves during grain filling stage relative to the panicle exertion stage. Differential expression of target genes was confirmed by quantitative RT-PCR. Further, using four different rice cultivars they showed that *OsNAC5* up-regulation is higher and earlier in flag leaves and panicles of genotypes with higher seed Fe, Zn and protein. So,

they suggested *OsNAC5* as a novel senescence-associated ABA-dependent NAC transcription factor and its function could be related to Fe and Zn remobilization from green tissues to seeds.

It was reported that constitutive overexpression of *NAS* genes in rice was able to increase the Zn concentration in the rice grain by in average twofold, 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). It was reported that overexpression of *OsNAS* in combination with ferritin affects Fe accumulation in the rice endosperm (Wirth *et al.*, 2009; Ravet *et al.*, 2009; Olsen and Palmgren, 2014).

Park *et al.* (2010) studied the molecular mechanisms of floral development and associated traits (floral morphology, colour and fragrance production), flower buds of two typical Korean *Calanthe* species, *C. discolor* and *C. sieboldii*, were subjected to gene expression analysis by differential display RT-PCR (DDRT-PCR). A total of 66 non-redundant differentially expressed genes (DEGs) were isolated and sequenced. Of these, 26 and 40 DEGs were found to be highly expressed in *C. discolor* and *C. sieboldii*, respectively. Moreover, the expression patterns of a subset of genes presumably implicated in signal transduction, metabolic pathways and hormonal signaling differed between the two species.

Banerjee and Chandel (2010) studied the expression of 25 metal transport related genes in roots and leaves of 12 diverse rice genotypes at maximum tillering stage (MTS) and mid-grain fill (MGF) stage to understand the role of transporters in Fe/Zn uptake, transport and remobilization. Differential expression of metal homeostasis related genes was observed among the genotypes, tissue types and developmental stages. Root transcriptome profile revealed the expression of nine genes at both developmental stages, three genes at MTS only and seven genes at MGF only. Rest of the five genes showed either poor or negligible expression in root tissue. In the flag leaf transcriptome, five genes were expressed at uniformly high levels in all genotypes among which *OsFER2* expressed only in flag leaf tissue. Study of expression pattern of metal related candidate genes in reference to grain Fe/Zn contents of 12 rice genotypes indicated that largely the differential expression of genes at MTS was correlated to difference in grain Zn contents whereas differential expression of transporter genes at MGF stage, especially in root tissues, was correlated to grain Fe contents.

Banerjee and Chandel (2010) reported that in rice flag leaf, expression of nine genes (*OsFER1*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsYSL6*, *OsYSL12*, *OsYSL4*, *OsZIP8* and *OsZIP8*) was correlated to high grain Fe content, seven genes (*OsNAC*, *OsYSL2*, *OsYSL9*, *OsZIP4*, *OsVIT1*, *OsNAAT1* and *OsNRAMP7*) correlated to high grain Zn content and two genes (*OsFRO2* and *OsZIP7*) correlated to both high grain Fe/Zn contents.

Sperotto *et al.* (2010) conducted a study to identify the molecular players that might contribute to the process of metal transport from flag leaf to the seeds. They analyzed the expression of 25 metal-related genes in flag leaves of eight rice cultivars with contrasting levels of grain Fe/Zn content during panicle emergence and grain filling stage. Expression level of nine of these genes in flag leaf (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*, *OsNAS1*, *OsFRO1* and *OsNAC5*) exhibited significant correlations with Fe and/or Zn concentrations in the seeds.

III MATERIAL AND METHODS

This chapter deals with the details of materials used, methods adapted, protocols and procedures followed and statistical tools employed for the analysis of the experiment conducted in the Aerobic rice laboratory of the Department of Plant Biotechnology, GKVK, Bengaluru.

EXPERIMENT I

3.1 Phenotyping of rice genotypes for high Zn and Fe content

3.1.1 Selection of genotypes

Twelve contrasting rice genotypes for Zn and Fe content were selected based on earlier studies of Narayanrao (2012) and Bekele (2012) using more than 1200 rice accessions. The studies were based on the X-Ray Fluorescence (XRF) readings for Zn and Fe content analysis. The list of genotypes selected for present study is given in Table 1.

3.1.2 Layout of the experiment

The layout of the experiment is Randomized Complete Block Design (RCBD) with three replications. Each genotype was grown in the aerobic rice plot of Department of Plant Biotechnology, GKVK, Bengaluru located at the latitude of 12° 58' North; longitude 77° 35' East and altitude of 930 meters above mean sea level in three replication with 10 plants per row in each block. A view of the experimental plot is given in Plate 1a and 1b. The plants were grown by direct hand sowing maintaining a spacing of 20 × 10 cm. Basal dose of 50:50:50 kg NPK/ha was met by applying 1.170 kg Urea, 3.375 kg SSP and 900 g MOP for a whole plot area of 36 m². Then the plot was top dressed with 1.170 kg Urea at 45 DAS. Irrigation was done on a regular basis on alternate days. All the necessary measures were taken to control pests and diseases. The soil at the experimental site had 0.74 ppm of Zn, a p^H 6.1 and 7.4 g/kg of organic carbon.

3.1.3 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.

3.1.3.1 Plant height at maturity (cm)

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.

3.1.3.2 Number of tillers per plant at maturity

Total number of tillers per plant was counted at the time of harvest and recorded.

Table 1: List of 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.	Azucena**	Local accession
8.	Moroberekan**	Local accession
9.	ARB 6**	Budha × IR-64
10.	IRJS 21	IRRI-38 × Jeerigesanna
11.	Subhadra**	Local accession
12.	TKM 9**	Local accession

** = Genotypes used for molecular study of micronutrient transporters.



Plate 1a: A view of the experimental plot



Plate 1b: View of a Moroberekan plant in the experimental plot

3.1.3.3 Number of productive tillers at maturity

Total number of productive tillers (excluding the non-productive tillers) per plant was counted and recorded.

3.1.3.4 Shoot dry weight at maturity (g)

The shoots were sun dried and shoot weight (without grains) was recorded and measured in terms of grams.

3.1.3.5 Grain yield per plant (g)

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

3.1.3.6 Length of the panicle (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.

3.1.3.7 Hundred grain weight (g)

Weight of hundred randomly selected healthy grains from a plant was measured in terms of grams and recorded.

3.1.3.8 Productivity per day per plant (g/day/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.

3.1.3.9 Length of the grain (mm)

Length of the five randomly selected grains was measured using digital Vernier caliper and readings were recorded.

3.1.3.10 Breadth of the grain (mm)

Breadth of the five randomly selected grains was measured using digital Vernier caliper and readings were recorded.

3.1.3.11 Width of the grain (mm)

Width of the five randomly selected grains was measured using digital Vernier caliper and readings were recorded.

3.1.3.12 Grain size (mm³)

On the basis of length (L), Breadth (B) and Width (W) of five grains, grain size was calculated by using the following formula.

$$\text{Grain size (mm}^3\text{)} = L \times B \times W$$

3.1.3.13 Harvest Index

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

3.1.3.14 Brown rice Zn and Fe content (ppm)

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

Healthy grains were dehusked in 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 ICRISAT, Hyderabad. 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 (mg/kg). Three replications were averaged to get the final value (in ppm). A picture of the XRF instrument used to estimate Zn and Fe in different parts of rice grain is given in Plate 2.

3.1.3.15 White rice Zn and Fe content (ppm)

Brown rice samples subjected to XRF analysis were polished for 2 minutes in non-ferrous polisher at DRR, Hyderabad (Mini Lab Rice Polisher. Model: K-710-Non Ferrous Series). Deformed and half cut grains were excluded.

About five grams of white 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 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.

3.1.5 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		



Plate 2: X- Ray Fluorescence instrument used to estimate Zn and Fe in different parts of rice grain

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.

3.1.6. 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.

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

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

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.

3.1.7 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 per cent was considered as low, 30-60 per cent as moderate and more than 60 per cent as high.

3.1.8 Genetic advance (GA)

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

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

Wherein,

h^2 = Heritability (Broad sense)

δ_p =Phenotypic standard deviation

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

3.1.9 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.

3.1.10 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).

$$r_p = \frac{COV(X, Y)}{[V(X) \cdot V(Y)]^{1/2}} \times 100$$

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.

3.1.11 Path-coefficient analysis

Path-coefficient analysis was carried out using phenotypic correlation values of different characters as suggested by Wright (1921) and as illustrated by Dewey and Lu (1959) was carried out to know the direct and indirect effect of the morphological traits and grain Zn content on yield. Standard path-coefficients which are the standardized partial regression coefficients were obtained using statistical software package Genres 1. These values were obtained by solving the following set of 'P' simultaneous equations by using the above package.

$$P_{01} + P_{02} r_{12} + \text{-----} + P_{0P} r_{1P} = r_{01}$$

$$P_{01} + P_{12} r_{02} + \text{-----} + P_{0P} r_{2P} = r_{02}$$

$$P_{01} + r_{1P} + P_{02} r_{2P} \text{-----} + P_{0P} = r_{0P}$$

Wherein, P_{01} , P_{02} , ----- P_{0P} are the direct effects of variables 1, 2,-----p on the dependent variable 0 and r_{12} , r_{13} , ----- r_{1P} ----- $r_{P (P-1)}$ are the possible correlation coefficients between various independent variables and r_{01} , r_{02} , r_{03} ----- r_{0P} are the correlations between dependent and independent variables. The indirect effect of the i^{th} variable via j^{th} variable is attained as $(P_{0j} \times r_{ij})$. The contribution of remaining unknown factor is measured as the residual factor, which is calculated and given below.

$$P_{0X}^2 = 1 - [P_{01}^2 + 2P_{01} P_{02} r_{12} + 2P_{01} P_{03} r_{13} + \text{-----} + P_{02}^2 + 2P_{02} P_{03} r_{13} + \text{----} + P_{0P}^2]$$

$$\text{Residual factor} = (P_{0X}^2)^{1/2}.$$

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 3.1.1.

3.2.2 Layout of the experiment

Same as mentioned in 3.1.2.

3.2.3 Zn and Fe content of brown rice (ppm)

Same as mentioned in 3.1.3.14.

3.2.4 Zn and Fe content of white rice (ppm)

Same as mentioned in 3.1.3.15.

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. White rice is typically a part of endosperm. By comparing the Zn and Fe content values of brown and white 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.

White rice = Endosperm (Gunstone, 2011)

By deducing the Zn and Fe content values of white 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,

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

3.3 Molecular characterization of Zn and Fe transporters in rice grains

3.3.1 Selection of genotypes

As mentioned in 3.1.1.

3.3.2 Layout of the experiment

Same as mentioned in 3.1.2.

3.3.3 Selecting differentially expressed transporter genes in rice grain

Differentially expressed genes at dough stage of grain development were scanned in the RiceXPro database (Rice expression profile database) (<http://ricexpro.dna.affrc.go.jp/>). From this analysis both up-regulated and down-regulated genes specifically expressed in rice grains were selected based on literature cited. The selected genes were cross checked in Rice Oligonucleotide Array Database (ROAD) (Cao *et al.*, 2012) which supports meta-analysis of genes expressed in different tissues and at different developmental stages. Primers were designed to the selected genes using NCBI online primer designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and Primer 3 software. And all the selected primers were checked for their quality and precision using the online tool sequence manipulation suit (SMS) (Stothard, 2000).

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

Since RNA is highly prone to RNase contamination, extreme care was taken during RNA isolation and further steps to avoid RNase contamination. Total RNA was isolated from different parts of rice grains using modified TRIzol protocol. Total RNA isolated was re-suspended in nuclease free water (0.1 % DEPC treated water).

3.3.5.1 Requirements for total RNA isolation

- Seed samples were collected from all the twelve study genotypes at early dough stage. Grains were separated into embryo and endosperm under aseptic condition in Laminar Air Flow (LAF) and were stored at -80 °C.

- Extraction buffer-I

TrisHCl (p ^H -8)	100 mM
Lithium Chloride	150 mM
EDTA	50 mM
SDS	1.5 per cent

(Tris, Lithium chloride and EDTA were autoclaved and 1.5 per cent SDS was added after autoclaving)

1.5 % β-mercaptaethanol (added freshly).

- Phenol: Chloroform (1:1) p^H.

- Extraction buffer-II

Guanidium sulfate	70 per cent
Sodium citrate	0.75 M
Sodium acetate (p ^H - 4.0)	2 M

TRIzol was used instead of Guanidium Sulfate

- Chloroform: Isoamylalcohol (24:1).
- 2-Propanol.
- 1.2 M Sodium Chloride.
- 70 per cent Ethanol.
- RNase free water (DEPC treated).

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 pre-chilled with liquid nitrogen and then finely ground to powder form in pre-chilled mortar.
- The samples were homogenized with 800 μ L Extraction buffer-I and 400 μ L TRIzol. The homogenized samples were then transferred to a 2 ml microfuge tube.
- These homogenized samples were kept overnight at -20 °C.
- The next day, the samples were thawed completely and centrifuged at 13,000 rpm for 15 minutes at 4 °C.
- Take the supernatant to a new microfuge tube and add 250 μ L of Extraction buffer-II, mixed by inversion and kept for incubation at room temperature for 10 minutes.
- After incubation, add 200 μ L of Chloroform: Isoamylalcohol (24:1) to the mixture and centrifuge at 13,000 rpm for 15 minutes at 4 °C.
- Take the aqueous layer to a new 1.5 mL microfuge tube and add 300 μ L 2-Propanol and 250 μ L of 1.5 M NaCl. Mix by inversion and incubate on ice for 15 minutes. Then centrifuge at 13,000 rpm for 15 minutes at 4 °C.
- Discard the aqueous layer and add 400 μ L of 70 per cent ethanol. Centrifuge at 12,000 rpm for 5 minutes at 4 °C. Then discard the ethanol, dry the pellet and re-suspend the pellet in 20-40 μ L DEPC water or TE buffer and stored at -80 °C for using further downstream processes.

3.3.6 Optimization of PCR conditions and normalization of reference gene

3.3.6.1 Primer selection and determination of T_m

The gene specific primers were designed using NCBI online primer designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and synthesized at Indus BioSolutions, Bengaluru. Designing conditions were chosen according to the following parameters:

Length of primer set between 18-25, optimal of 23 bases,

T_m comprised between 50 °C and 56 °C, optimal T_m 54-55 °C,

Length of the amplification product was set between 50 and 450 base pairs.

PCR primers selected in such a way that the 5' and the 3' primers span different exons, so that the amplification product obtained from the cDNA would be of different length from that which was obtained from any contaminant genomic DNA comprising intronic sequences.

3.3.6.2 Primer concentration

Each primer was used in a concentration range of 0.5 μ L to 1 μ L.

3.3.6.3 Determination of optimal MgCl₂ concentration

Determination of the optimal concentration for each primer set was performed using different MgCl₂ concentrations (1.5, 2, 2.5 and 3 mM) in the standard PCR reaction conditions and by adjusting the water volume consequently. The products were run on an agarose gel to choose the condition that gave the highest yield and specificity.

3.3.6.4 Determination of cycling parameters

The standard program comprised a cycle of 30 minutes at 45 °C, hold at 94 °C, followed by 1 minute at 95 °C, 45 seconds at annealing temperature specific to primer and 45 seconds at 72 °C for different number of cycles to decide optimum number of cycles where amplification is visible and have not yet reached stationary phase. Appropriate number of cycles was selected based on the visibility on agarose gel.

3.3.7 Reverse transcription and semi quantitative RT-PCR

Reverse transcription and semi quantitative RT-PCR will be done in a single tube. It is called one-step RT-PCR which reduces contamination to a greater extent and imparts high sensitivity to the RT process.

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). 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,

***M-MuLV* Reverse transcription kit components**

1. 5X Reaction mixture.
2. Oligo(dT) primer.
3. Random hexamer.
4. *M-MuLV* Reverse transcriptase.
5. DEPC water.
6. PCRgrade water.

Protocol for one-step RT-PCR

In this process, synthesis of first strand of cDNA from RT enzyme and further amplification of target region of cDNA from gene specific primer and *Taq* Polymerase occurs in a single tube in a sequential manner. To avoid the formation of primer dimer, HotStart PCR was followed where primers were added to PCR tubes after first strand cDNA synthesis at 94 °C hold.

The PCR mix was made fresh in bulk depending on the number of samples each time. Each 15 μ L mix contained;

Taq buffer	2.0 μ L
dNTPs	2.0 μ L
5X RT reaction mixture	1.5 μ L
Oligo(dT) primer	0.25 μ L
Random hexamer	0.25 μ L
Forward gene specific primer	0.5 μ L
Reverse gene specific primer	1.0 μ L
<i>Taq</i> DNA polymerase	0.5 μ L
RNA template	5.0 μ L
DEPC water	2.0 μ L
TOTAL	15.0 μL

PCR amplification conditions

I-Step	II-Step	III-Step			IV-Step	V-Step
42 °C	94 °C	94 °C	T °C**	72 °C	72 °C	4 °C
30 minutes	Hold	1 minute	45 seconds	45 seconds	3 minutes	Final hold

N cycles**

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

0.5 μ L of forward gene specific primer and 1 μ L of reverse gene specific primer were added in the second step of PCR process at 94 °C hold to minimize primer dimer formation and high sensitivity in target specific amplification of cDNA. Semi-quantitative RT-PCR can quantify specific mRNAs in extremely small amounts of starting material (counting only few molecules of a specific mRNA). The principle of quantitative RT-PCR is to measure the amount of a specific mRNA by quantifying the corresponding PCR product from cDNA.

3.3.8. 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 *khariff*-2014 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 Phenotyping of rice genotypes for high Zn and Fe content

4.1.1 Performance of genotypes with respect to morphological traits.

4.1.2 Performance of genotypes with respect to Zn and Fe content in brown rice.

4.1.3 Statistical analysis of data.

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

4.2.1 Fe and Zn content in different parts of rice grain.

4.2.2 Statistical analysis of data.

4.3 Molecular characterization of Fe and Zn transporters in rice grain

4.3.1 Zn and Fe transporters.

4.3.2 Primer designing.

4.3.3 Total RNA isolation from different parts of rice grain.

4.3.4 Reverse transcription of RNA.

4.3.5 Semi-quantitative PCR.

4.3.6 Relative expression of Zn and Fe transporters in different parts of grain.

4.1 Phenotyping of rice genotypes for high Zn and Fe content

4.1.1 Performance of selected genotypes with respect to morphological traits

Various shoot morphological characters and grain morphological characters were recorded. 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 shoot and grain morphological parameters are presented in Table 2 and 3 respectively.

4.1.1.1 Plant height (PH)

Genetic parameters estimated for plant height were tabulated. Minimum and maximum plant height was found to be 47.87 cm (TKM 9) and 108.13 cm (AM 65) respectively with a range of 60.26 and average of 75.03 cm. The trait also possessed high GCV of 73.34, PCV of 76.56, heritability of 85.83 per cent and Genetic advance as per mean (GAM) of 56.95 per cent.

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

Trait	Mean±SE	Min.	Max.	Range	PCV (%)	GCV (%)	h ² (%)	GAM (%)
PH	75.03±1.64	47.87	108.13	60.26	76.56	73.34	85.83	56.95
NOT	13.94±0.77	9.97	17.33	7.36	14.51	3.61	25.51	14.18
NOPT	8.13±0.61	4.27	11.2	6.93	9.01	2.19	24.33	18.49
SDW	40.83±5.24	20.61	83.09	62.48	70.24	66.45	61.42	65.29
GYP	10.27±1.11	6.44	19.65	13.21	32.12	9.53	29.67	34.58
HI	0.31±0.05	0.12	0.63	0.51	0.052	0.01	27.45	41.95
PL	17.7±0.64	14.31	21.47	7.16	8.21	3.26	39.72	13.24
DFP	98.25±2.31	88.33	115.67	27.34	63.49	0.001	19.93	5.85
DFPF	113.25±1.52	105	123	18.00	38.17	22.68	59.41	6.68
DTM	135.59±1.95	124	150.67	26.67	56.11	40.56	72.28	8.23
ZPD	0.203±0.01	0.14	0.3	0.16	0.03	0.001	0.76	1.21
FPD	0.11±0.01	0.09	0.14	0.05	0.01	0.001	23.08	11.19
PDP	0.08±0.01	0.05	0.14	0.09	0.002	0.003	27.86	30.14

Table 3: Genetic parameters for grain morphological characters among selected rice accessions

Trait	Mean±SE	Min.	Max.	Range	PCV (%)	GCV (%)	h ₂ broad sense	GAM (%)
GL	8.32±0.16	7.54	9.06	1.52	0.53	0.18	34.27	6.15
GB	2.38±0.08	1.94	2.75	0.81	0.09	0.07	77.29	19.42
GW	1.86±0.04	1.62	2.14	0.52	0.04	0.01	15.71	3.53
LBR	3.59±0.14	2.74	4.27	1.53	0.32	0.2	63.36	20.53
GS	37.23±2.05	23.61	52.4	28.79	76.03	37.51	49.34	23.81
HGW	2.003±0.09	1.33	2.48	1.15	0.13	0.09	72.07	26.37

GCV= Genotypic coefficient of variation **PCV**= Phenotypic coefficient of variation **h²**= heritability in broad sense **GAM**= Genetic advance as per mean **PH**= Plant height **NOT**= Number of tillers **NOPT**= Number of productive tillers **SDW**= Shoot dry weight **GYP**= Grain yield per plant **HI**= Harvest index **PL**= Panicle length **DFP**= Days for first flowering **DFPF**= Days for 50 per cent flowering **DTM**= Days to maturity **ZPD**= Zinc productivity per day **FPD**= Iron productivity per day **PDP**= Productivity per day per plant **GL**= Grain length **GB**= Grain breadth **GW**= Grain width **LBR**= Length-breadth ratio **GS**= Grain size **HGW**= Hundred grain weight

4.1.1.2 Number of tillers (NOT)

Minimum number of tillers was recorded in Azucena (9.97), whereas maximum number of tillers was observed in Subhadra (17.33) with a range value of 7.36 and mean value of 13.94. The trait had low GCV (3.61), moderate PCV (14.15), low heritability (25.51 %) and moderate GAM (14.18 %).

4.1.1.3 Number of productive tillers (NOPT)

Maximum number of productive tillers was observed in AM 72 (17.33) and minimum NOPT was observed in Azucena (4.27) with an average of 8.13, exhibiting high variability among genotypes with a range of 6.93. Low GCV (2.19), low PCV (9.01), low heritability (24.33 %) and moderate GAM (18.49 %) was recorded for this trait.

4.1.1.4 Shoot dry weight (SDW)

Minimum and maximum shoot dry weight was found to be 20.61 g (Subhadra) and 83.09 g (Moroberekan) respectively with a range of 62.48 and average of 40.83. The trait possessed high GCV of 66.45, high PCV of 70.24, heritability of 61.42 per cent, and high GAM of 65.29 per cent.

4.1.1.5 Harvest index (HI)

The harvest index values for genotypes studied ranged from 0.12 g/kg (Moroberekan) to 0.63 g/kg (TKM 9) with an average of 0.31 g/kg. Among the genotypes studied, low GCV of 0.014, low PCV of 0.052, low heritability of 27.45 per cent and high GAM of 41.95 per cent was recorded for the trait.

4.1.1.6 Grain yield per plant (GYP)

The range value for grain yield/plant was found to be 13.21 with maximum of 19.65 g/plant (AM 65) and minimum of 6.44 g/plant (IRJS 21) and average GYP of 10.27 g/plant. The trait had low GCV, high PCV, low heritability and high GAM of 9.53, 32.12, 29.67 per cent and 34.58 per cent respectively.

4.1.1.7 Panicle length (PL)

Maximum panicle length of 21.47 cm (Moroberekan), minimum panicle length of 14.31 cm (Subhadra) with average of 17.7 cm was recorded among the genotypes. The trait possessed a low GCV of 3.26, low PCV of 8.21, moderate heritability of 39.72 per cent and moderate GAM of 13.24 per cent.

4.1.1.8 Days for first flowering (DFF)

The range value for the trait was estimated to be 27.34 with minimum of 88.33 days (Subhadra), maximum of 115.67 days (Azucena) and average days for first flowering being 98.25 days among the genotypes. Indeed, a low GCV of 0.001, high PCV of 63.49, low heritability of 19.93 per cent and low GAM of 5.85 per cent was recorded for this trait among the genotypes under study.

4.1.1.9 Days for fifty percent flowering (DFPF)

Days for fifty percent flowering ranged from 105 days (Subhadra) to 123 days (Moroberekan) with a mean of 113.25 days and range of 27.34. The trait exhibited high GCV of 22.68, high PCV of 38.17, moderate heritability of 59.41 per cent, and low GAM of 6.68 per cent among the genotypes selected.

4.1.1.10 Days to maturity (DTM)

The trait ranged from 124 days (Subhadra) to 150.67 days (Azucena) with an average of 135.59 days and range of 26.67. A high GCV of 40.56, high PCV of 56.11, high heritability of 72.28 per cent and low GAM of 8.23 per cent was observed for the trait among the selected rice accessions under study.

4.1.1.11. Productivity per day per plant (PDP)

The values for PDP ranged from 0.05 g/day/plant (Azucena) to 0.14 g/day/plant (AM 65) with an average of 0.08 g/day/plant and range of 0.09. The trait were shown to have a low GCV of 0.003, low PCV of 0.002, low heritability of 27.86 per cent and high GAM of 30.14 per cent when analyzed the genotypes under study.

4.1.1.12 Grain length (GL)

The average length of the grain between the genotypes under study was found to be 8.32 mm ranging from 7.54 mm (Subhadra) to 9.06 mm (AM 27). The trait has recorded low GCV of 0.18, low PCV of 0.53, moderate heritability of 34.27 per cent, and low GAM of 6.15 per cent.

4.1.1.13 Grain breadth (GB)

The maximum grain breath was found to be 2.75 mm (TKM 9), minimum was found to be 1.94 mm (ARB 6), with a mean of 2.38 mm range of 0.81. The trait exhibited low GCV of 0.07, low PCV of 0.09, high heritability of 77.29 per cent, and moderate GAM of 19.42 per cent.

4.1.1.14 Grain width (GW)

The average grain width of rice grain was measured to be 1.86 mm, ranging from 1.62 mm (ARB 6) to 2.14 mm (Moroberekan), with a range value of 0.52. The trait possessed low GCV of 0.01, low PCV of 0.04, low heritability of 15.71 per cent, and low GAM of 3.53 per cent.

4.1.1.15 Length-Breadth ratio (LBR)

The length-breadth ratio of rice seeds ranged from 2.74 (TKM 9) to 4.27 (ARB 6) with an average of 3.59 and difference between maximum and minimum values (range) of 1.53. The trait was observed to have low GCV and PCV of 0.20 and 0.32 respectively; high heritability and GAM of 63.36 per cent and 20.53 per cent respectively.

4.1.1.16 Grain size (GS)

The values of grain size among the selected accessions ranged from 23.61 mm³ (ARB 6) to 52.40 mm³ (Moroberekan), with a mean, range of 37.23 mm³ and 28.79 respectively. The trait was found to have high GCV, PCV and GAM of 37.51, 76.03 and 23.81 per cent respectively with moderate heritability of 49.34 per cent.

4.1.1.17 Hundred Grain weight or Test weight (HGW or TW)

Weight of hundred grains of rice accessions was found to be ranging between 1.33 g (ARB 6) to 2.48 g (Moroberekan), with a range value of 1.15 and mean of 2.003 g. Adding to these parameters, the trait was found to be having low GCV and PCV of 0.09 and 0.13 respectively with high heritability and GAM of 72.07 per cent and 26.37 per cent respectively.

4.1.1.18 Zinc productivity per day (ZPD)

Minimum Zn productivity was found in ARB 6 (0.14 mg/kg/day) and maximum Zn productivity found in Moroberekan (0.30 mg/kg/day). The average Zn productivity among the genotypes was calculated to be 0.203 mg/kg/day with range of 0.16. The trait exhibited low GCV, PCV, heritability and GAM of 0, 0.03, 0.76 per cent, and 1.21 per cent respectively.

4.1.1.19 Iron productivity per day (FPD)

The Fe productivity of the genotypes under study ranged from 0.09 mg/kg/day (Azucena, ARB 6, and IRJS 21) to 0.14 mg/kg/day (AM 72) with an average of 0.11 mg/kg/day, range of 0.05. The trait possessed low GCV, PCV and heritability of 0, 0.01, and 23.08 per cent respectively, moderate GAM of 11.19 per cent.

4.1.2 Performance of genotypes with respect to Zn and Fe content in brown rice

Similar to morphological traits, phenotyping of rice genotypes was also analyzed by comparing the Zn and Fe content in the brown rice of selected accessions, subjecting statistical analysis over genotypes. The genetic parameters like minimum, maximum, mean, range, coefficient of variation, standard deviation, GCV, PCV, heritability percentage (h^2 %) and GAM were estimated and tabulated in Table 4.

Table 4: Genetic parameters for zinc and iron content in brown rice among selected genotypes

Trait	Mean±SE	Min.	Max.	Range	PCV (%)	GCV (%)	h^2 broad sense	GAM (%)
BZC	27.5±1.67	18.4	40.95	22.55	35.89	31.95	89.01	39.95
BFC	14.304±0.6	11.45	19.30	7.85	9.78	1.57	16.07	7.24

GCV= Genotypic coefficient of variation PCV= Phenotypic coefficient of variation h^2 = heritability in broad sense GAM= Genetic advance as per mean BZC= Brown rice zinc content BFC= Brown rice iron content

4.1.2.1 Brown rice Zn content (BZC)

Minimum Zn content in brown rice was found to be 18.40 ppm (ARB 6) and maximum brown rice Zn content found to be 40.95 ppm (Moroberekan). The trait has the range of 22.55, mean of 27.5 ppm. The trait was also found to have a high GCV, PCV, heritability and GAM of 31.95, 35.89, 89.01 per cent and 39.95 per cent respectively.

4.1.2.2 Brown rice iron content (BFC)

The Fe content in rice grain was found to have a mean value of 14.30 ppm, minimum of 11.45 ppm (ARB 6), maximum of 19.30 ppm (AM 72), range of 7.85. It was also found that the trait exhibited low GCV, PCV, h^2 %, and GAM of 1.57, 9.78, 16.07 per cent, and 7.24 per cent respectively.

4.1.3 Statistical analysis of data

4.1.3.1 Analysis of variance

Sum of squares due to various sources of variation for yield and its associated traits, grain morphological traits and 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. Highly significant differences among the genotypes under study were found for four traits viz., plant height, shoot dry weight, days for 50 per cent flowering, and days to maturity at 5 per cent and 1 per cent level of significance (LOS) indicating a wide variance among the genotypes for these traits. There was no significant difference among the genotypes for seven traits viz., number of tillers, number of productive tillers, productivity per day per plant, Zn productivity per day, Fe productivity per day, days for first flowering, and harvest index. For remaining two traits viz., panicle length and grain yield per plant, significant difference was found among the genotypes at 5 per cent LOS, however showed non-significance at 1 per cent LOS.

Table 5: ANOVA for 7 yield and its associated traits in selected rice accessions

Source of variation	df	Mean Sum of Squares						
		PH	NOT	NOPT	SDW	GYP	ZPD	FPD
Genotype	11	1586.33**	21.36 ^{NS}	13.39 ^{NS}	989.14**	51.17*	0.03 ^{NS}	0.001 ^{NS}
Error	22	82.72	10.54	6.82	171.23	22.59	0.03	0.01
CD @ 5 %		15.4	5.49	4.42	22.16	8.05	0.3	0.04
CD @ 1 %		20.93	-	-	30.12	-	-	-
CV		30.65	19.13	25.96	44.47	37.54	19.59	15.13
SD		22.99	2.67	2.11	18.16	18.85	0.04	0.02

* Significant at 5 % ** Significant at 1 % ^{NS}= Non-significant **PH**= Plant height **NOT**= Number of tillers **NOPT**= Number of productive tillers **SDW**= Shoot dry weight **GYP**= Grain yield per plant **ZPD**= Zinc productivity per day **FPD**= Iron productivity per day

Table 6: ANOVA for 6 yield attributing traits among selected genotypes

Source of variation	df	Mean Sum of Squares					
		HI	PL	DFF	DFPF	DTM	PDP
Genotype	11	0.08 ^{NS}	14.73*	152.45 ^{NS}	83.52**	137.22**	0.003 ^{NS}
Error	22	0.04	4.95	304.01	15.49	15.55	0.001
CD @ 5 %		0.33	3.77	29.53	6.67	6.68	0.06
CD @ 1 %		-		-	9.06	9.08	-
CV		52.49	12.51	18.26	3.48	2.91	44.61
SD		0.16	2.22	7.99	5.28	6.76	0.03

* Significant at 5 % ** Significant at 1 % NS= Non-significant HI= Harvest index PL= Panicle length DFF= Days for first flowering DFPF= Days for 50 per cent flowering DTM= Days to maturity PDP= Productivity per day per plant

4.1.3.1.2 Analysis of variance for grain morphological traits and grain Zn and Fe content

Highly significant differences were found for all the grain morphological characters except grain length and grain width at both 5 per cent and 1 per cent LOS. Grain length showed significant differences among the genotypes only at 5 per cent LOS and, grain width showed non-significance at both 5 per cent and 1 per cent LOS. Analysis of variance of grain morphological traits and brown rice zinc and iron content among selected genotypes is tabulated in Table 7.

Table 7: ANOVA for grain morphological traits and brown rice zinc and iron content among selected genotypes

Source of variation	df	Mean sum of Squares							
		GL	GB	GW	LBR	GS	HGW	BZC	BFC
Genotype	11	0.89*	0.22**	0.05 ^{NS}	0.72**	151.05**	0.31**	99.79**	12.92 ^{NS}
Error	22	0.35	0.02	0.04	0.12	38.51	0.04	3.94	8.21
CD @ 5 %		0.99	0.24	0.32	0.58	10.51	0.32	3.36	4.85
CD @ 1 %		-	0.32	-	0.79	14.28	0.43	4.57	-
CV		6.53	11.22	7.19	13.68	16.67	9.39	7.22	20.03
SD		0.16	0.27	0.13	0.49	7.09	0.32	5.77	2.08

* Significant at 5 % ** Significant at 1 % NS= Non-significant GL= Grain length GB= Grain breadth GW= Grain width LBR= Length-breadth ratio GS= Grain size HGW= Hundred grain weight BZC= Brown rice zinc content BFC= Brown rice iron content

Zn content in brown rice showed a significant difference among the genotypes at both 5 per cent and 1 per cent LOS. There was no significant difference among the genotypes for brown rice Fe content either at 5 per cent or at 1 per cent LOS.

4.1.3.2 Correlation studies

The results of correlation studies for yield and its associated traits and grain morphological and grain Zn-Fe content are presented in the Table 8 and 9 respectively.

Table 8: Estimates of phenotypic correlation coefficients for yield and its associated traits among selected genotypes

	PH	NOT	NOPT	SDW	GYP	HI	PL	DFE	DFPF	DTM	PDP	ZPD
NOT	-0.56	1.00										
NOPT	-0.36	0.86**	1.00									
SDW	0.76**	-0.37	-0.43	1.00								
GYP	0.36	0.01	0.33	-0.06	1.00							
HI	-0.62*	0.49	0.58*	-0.65*	0.12	1.00						
PL	0.49	-0.02	0.20	0.61*	0.30	-0.14	1.00					
DFE	0.34	-0.67*	-0.76**	0.47	-0.28	-0.24	0.12	1.00				
DFPF	0.24	-0.6*	-0.65*	0.48	-0.32	-0.11	0.27	0.92**	1.00			
DTM	0.41	-0.7*	-0.76**	0.37	-0.07	-0.46	-0.04	0.79**	0.58*	1.00		
PDP	0.15	0.41	0.56	0.09	0.34	0.52	0.36	-0.13	-0.01	-0.23	1.00	
ZPD	0.40	-0.13	-0.35	0.71**	-0.09	-0.39	0.39	0.38	0.34	0.11	-0.14	1.00
FPD	-0.22	0.68*	0.76**	-0.15	0.27	0.45	0.41	-0.31	-0.24	-0.53	0.47	0.01

* Significant at 5 % ** Significant at 1 % NS= Non-significant PH= Plant height NOT= Number of tillers NOPT= Number of productive tillers SDW= Shoot dry weight GYP= Grain yield per plant HI= Harvest index DFE= Days for first flowering DFPF= Days for 50 per cent flowering DTM= Days to maturity PDP= Productivity per day per plant ZPD= Zinc productivity per day FPD= Iron productivity per day

4.1.3.2.1 Correlation analysis for yield and its associated traits

Plant height had a significant positive correlation with shoot dry weight (0.76) and a significant negative correlation with harvest index (-0.62). Number of tillers showed a significant positive correlation with number of productive tillers (0.86). Number of productive tillers in turn showed significant positive correlation with harvest index (0.58). Number of tillers and number of productive tillers revealed a significant negative correlation with days for first flowering (0.67 and 0.76 respectively), days for 50 per cent flowering (0.60 and 0.65 respectively) and days to maturity (0.70 and 0.76 respectively).

Table 9: Estimates of phenotypic correlation coefficients for grain morphological traits and brown rice zinc and iron content among selected genotypes

	GL	GB	GW	LBR	GS	HGW	BZC
GB	-0.06	1					
GW	0.40	0.75**	1				
LBR	0.39	-0.92**	-0.54	1			
GS	0.47	0.81**	0.95**	-0.57	1		
HGW	0.50	0.69*	0.71**	-0.48	0.82**	1	
BZC	0.62*	0.45	0.83**	-0.19	0.80**	0.57	1
BFC	-0.06	0.42	0.41	-0.50	0.36	0.61*	0.04

* Significant at 5 % ** Significant at 1 % NS= Non-significant GL= Grain length GB= Grain breadth GW= Grain width LBR= Length-breadth ratio GS= Grain size HGW= Hundred grain weight BZC= Brown rice zinc content BFC= Brown rice iron content

Days for first flowering had a significant positive correlation with days for 50 percent flowering (0.92) and days to maturity (0.79). Shoot dry weight revealed a significant positive correlation with panicle length (0.61) and in contrary, showed a significant negative correlation with harvest index (-0.65). Days for 50 per cent flowering was observed to have a significant positive correlation with days to maturity (0.58).

Zn productivity per day disclosed a significant positive correlation with shoot dry weight (0.71). Iron productivity per day revealed a significant positive correlation with number of tillers (0.68), and number of productive tillers (0.76).

4.1.3.2.2 Correlation analysis for grain morphological traits and grain Zn and Fe content among selected genotypes

A significant positive correlation was observed for grain breadth with grain width (0.75), grain size (0.81) and hundred grain weight (0.69). On contrary, grain breadth showed a high significant negative correlation with grain length to breadth ratio (-0.92). Grain width revealed a significantly high positive correlation with grain size (0.95) and hundred grain weight (0.71). A significantly high positive correlation was observed for the trait grain size with hundred grain weight (0.82).

Brown rice Zn content revealed a significant positive correlation with grain length (0.62), grain width (0.83) and, grain size (0.80). Brown rice Fe content showed a significant positive correlation with hundred grain weight (0.61).

4.1.3.3 Path coefficient analysis

4.1.3.3.1 Path coefficient analysis for yield attributing traits on grain yield

Path-coefficient analysis was carried out to discern the direct and indirect effects of yield attributing traits and grain morphological traits on grain yield. The results obtained are presented in Table 10 and 11 respectively.

Table 10: Direct and indirect effects of yield associated traits on grain yield among selected genotypes

	PH	NOT	NOPT	SDW	PL	HI	DFE	DFPF	DTM	PDP	ZPD	FPD
PH	-0.261	0.103	-0.016	0.319	-0.099	0.295	-0.001	-0.104	-0.100	0.034	0.009	0.107
NOT	0.238	-0.113	0.019	-0.292	0.032	-0.327	-0.001	0.287	0.226	0.201	0.032	-0.864
NOPT	0.176	-0.096	0.023	-0.324	-0.028	-0.339	-0.001	0.283	0.254	0.203	0.021	-1.201
SDW	-0.219	0.087	-0.019	0.379	-0.141	0.279	-0.002	-0.194	-0.089	0.041	0.026	-0.114
PL	-0.137	0.019	0.003	0.284	-0.188	0.082	-0.005	-0.143	0.029	0.130	0.062	-0.731
HI	0.227	-0.109	0.023	-0.312	0.046	-0.338	-0.002	0.033	0.141	0.139	-0.017	-0.419
DFE	-0.044	-0.029	0.005	0.252	-0.218	-0.141	0.004	-0.165	0.017	0.299	0.008	-0.174
DFPF	-0.084	0.099	-0.020	0.225	-0.082	0.034	-0.002	-0.326	-0.149	0.071	-0.001	0.285
DTM	-0.117	0.114	-0.026	0.152	0.024	0.213	0.003	-0.218	-0.224	-0.102	-0.004	0.495
PDP	-0.026	-0.067	0.014	0.045	-0.072	-0.137	-0.003	-0.068	0.067	0.342	0.002	-0.708
ZPD	-0.194	-0.308	0.041	0.822	-0.985	0.492	-0.003	0.016	0.081	0.044	0.012	-1.302
FPD	0.036	-0.129	0.037	0.057	-0.182	-0.188	-0.001	0.123	0.147	0.320	0.129	-0.756

RESIDUAL EFFECT= 0.085

PH= Plant height NOT= Number of tillers NOPT= Number of productive tillers SDW= Shoot dry weight PL= Panicle length HI= Harvest index DFE= Days for first flowering DFPF= Days for 50 per cent flowering DTM= Days to maturity PDP= Productivity per day per plant ZPD= Zinc productivity per day FPD= Iron productivity per day

Table 11: Direct and indirect effects of grain morphological traits on grain yield among selected genotypes

	GL	GB	GW	LBR	GS	HGW
GL	5.734	0.179	0.66	-5.153	-0.462	-0.434
GB	-0.546	-1.883	-0.338	7.03	-1.658	-0.989
GW	5.151	0.866	-0.734	-1.933	-1.134	-1.028
LBR	4.363	1.954	0.21	-6.771	0.353	0.078
GS	2.18	-2.57	0.686	1.97	-1.215	-0.735
HGW	3.419	-2.559	1.038	0.728	-1.226	-0.728

RESIDUAL EFFECT= 1.522

GL= Grain length GB= Grain breadth GW= Grain width LBR= Length-breadth ratio GS= Grain size HGW= Hundred grain weight

Shoot dry weight showed the highest direct positive effect on grain yield (0.379) followed by productivity per day per plant (0.342), whereas lowest direct positive effect was shown by days for first flowering (0.004).

Highest direct negative effect was observed for Fe productivity per day (-0.756), and lowest direct negative effect was observed for number of tillers (-0.113).

Highest indirect positive effect exhibited by Fe productivity per day (0.495) *via.*, days to maturity towards grain yield per plant. Lowest indirect positive effect was observed for Zn productivity per day (0.002) *via.*, productivity per day per plant.

Highest indirect negative effect was observed for Fe productivity per day (-1.302) *via.*, Zn productivity per day, whereas lowest indirect negative effect was observed in days for first flowering (-0.001) *via.*, plant height, number of tillers, number of productive tillers, and Fe productivity per day towards grain yield.

4.1.3.3.2 Path coefficient analysis for grain morphological traits on grain yield

Among the grain characters studied, only grain length showed the direct positive effect on grain yield (5.734), whereas all other traits showed negative effect. Length-breadth ratio showed the highest direct negative effect (-6.771), whereas hundred grain weight was observed to have the lowest direct negative effect (-0.728) on grain yield.

Highest indirect positive effect was observed for length-breadth ratio (7.030) *via.* grain breadth, and lowest indirect positive effect was shown for hundred grain weight (0.078) *via.* length-breadth ratio.

Among the characters studied among the genotypes, length-breadth ratio was seen to have highest indirect negative effect (-5.153) *via.* grain length and lowest indirect negative effect was observed for grain width (-0.338) *via.*, grain breadth.

4.2 Study the distribution of Zn and Fe within different parts of rice grain

4.2.1 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 12. 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 13.

4.2.1.1 Brown rice zinc content (BZC)

The minimum BZC was found to be 18.40 ppm (ARB 6) and maximum was found to be 40.95 ppm (Moroberekan) with an average of 27.90 ppm and range value of 22.55. The trait was also found to have high GCV, high PCV, high heritability and high genetic advance as per mean (20.55, 21.78, 89.01 %, and 39.95 % respectively) (Fig. 1 and 3).

4.2.1.2 White rice zinc content (WZC)

The trait had a minimum value of 16.7 ppm (ARB 6) and a maximum value of 35.9 ppm (Moroberekan) with an average of 24.82 ppm and a range of 19.2. A moderate

GCV, moderate PCV, high heritability and high GAM (19.09, 19.23, 98.53 % and 39.04 % respectively) was found for this trait (Fig. 1 and 4).

Table 12: Mean zinc and iron content in different parts of rice grain among selected genotypes

Genotype	BZC	BFC	WZC	WFC
AM 72	26.75	19.30	20.15	4.00
AM 143	25.55	14.35	21.15	4.60
AM 1	27.05	15.30	26.80	5.80
AM 65	28.30	14.55	25.00	4.85
AM 94B	26.15	14.05	25.50	10.20
AM 27	25.40	13.10	25.10	5.45
Azucena	33.70	13.20	30.10	3.40
ARB 6	18.40	11.45	16.70	3.80
IRJS 21	30.85	11.50	25.60	3.80
Moroberekan	40.95	14.40	35.90	3.95
TKM 9	24.30	15.90	21.15	6.10
Subhadra	27.40	14.55	24.65	4.35

BZC- Brown rice zinc content **BFC**- Brown rice iron content **WZC**- White rice zinc content
WFC- White rice iron content

Table 13: Genetic parameters for zinc and iron content in different parts of rice grain among selected genotypes

Trait	Mean±SE	Min.	Max.	Range	PCV (%)	GCV (%)	h ² (%)	GAM (%)
BZC	27.9±1.59	18.4	40.95	22.55	21.78	20.55	89.01	39.95
WZC	24.82±1.43	16.7	35.9	19.20	19.23	19.09	98.53	39.04
BFC	14.3±0.59	19.3	19.3	7.85	21.86	8.76	16.07	7.24
WFC	5.03±0.53	10.2	6.8	6.80	36.85	36.49	98.07	74.46

GCV= Genotypic coefficient of variation **PCV**= Phenotypic coefficient of variation **h²**= heritability in broad sense **GAM**= Genetic advance as per mean **BZC**= Brown rice zinc content **WZC**= White rice zinc content **BFC**= Brown rice iron content **WFC**= White rice iron content

4.2.1.3 Brown rice iron content (BFC)

Minimum Fe content in brown rice was 11.45 ppm (ARB 6); maximum was 19.30 ppm (AM-72) with an average of 14.304 ppm and a range of 7.85. A low GCV, high

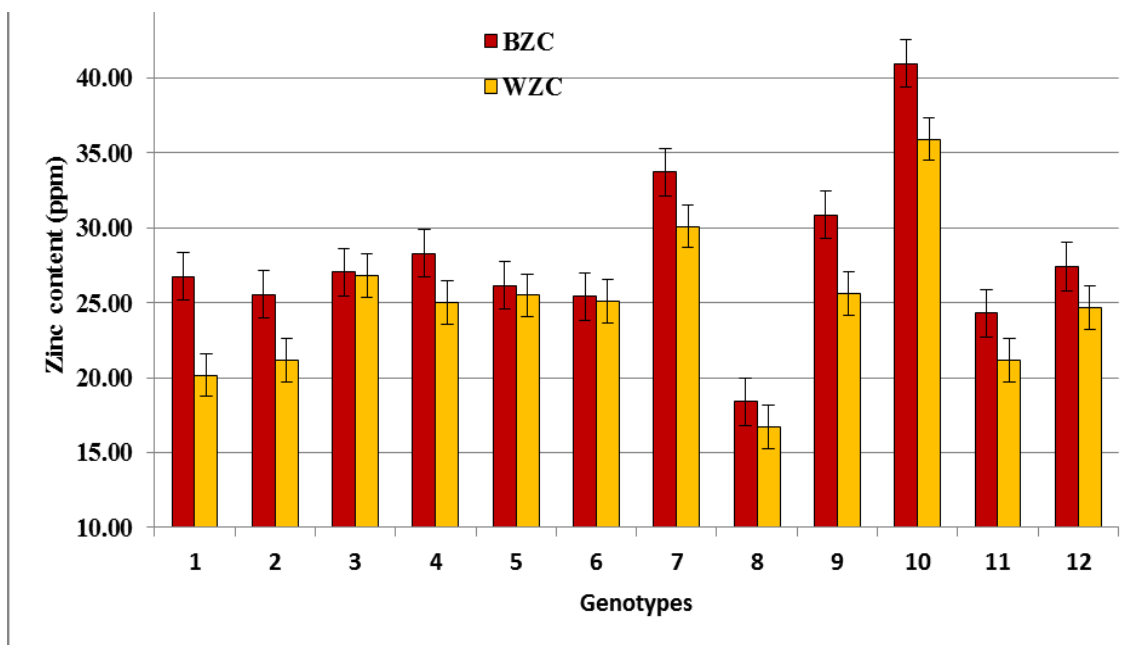


Fig. 1: Zinc content in brown and white rice samples of selected genotypes

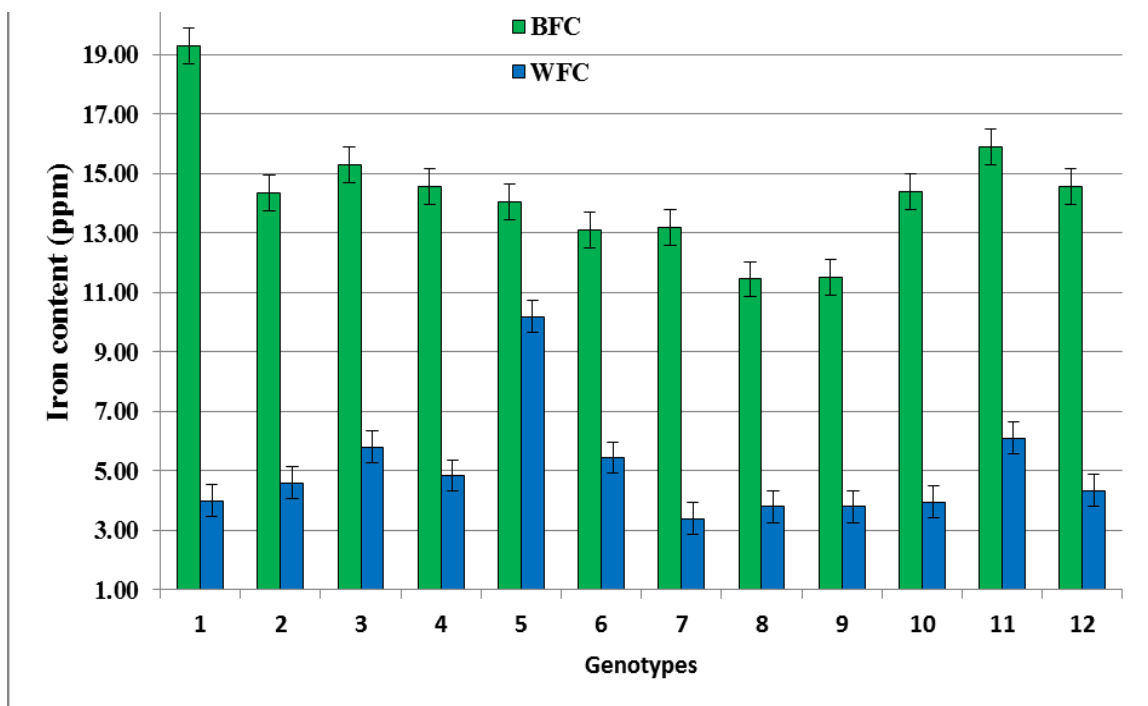


Fig. 2: Iron content in brown and white rice samples of selected genotypes

Legends:

1- AM 72, 2- AM 143 3- AM 1 4- AM 65 5- AM 94B 6- AM 27 7- Azucena
 8- ARB 6 9- IRJS 21 10- Moroberekan 11- TKM 9 12- Subhadra

BZC-Brown rice zinc content **BFC**- Brown rice iron content

WZC- White rice zinc content **WFC**- White rice iron content

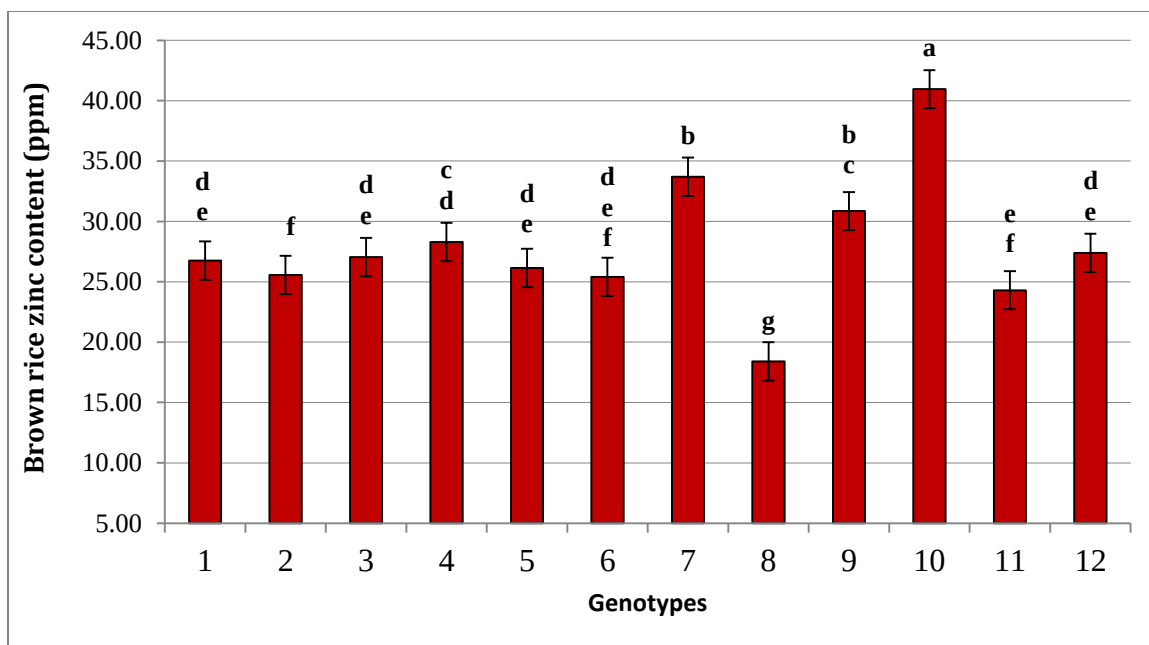


Fig. 3: Zinc content (ppm) in brown rice of genotypes selected for study

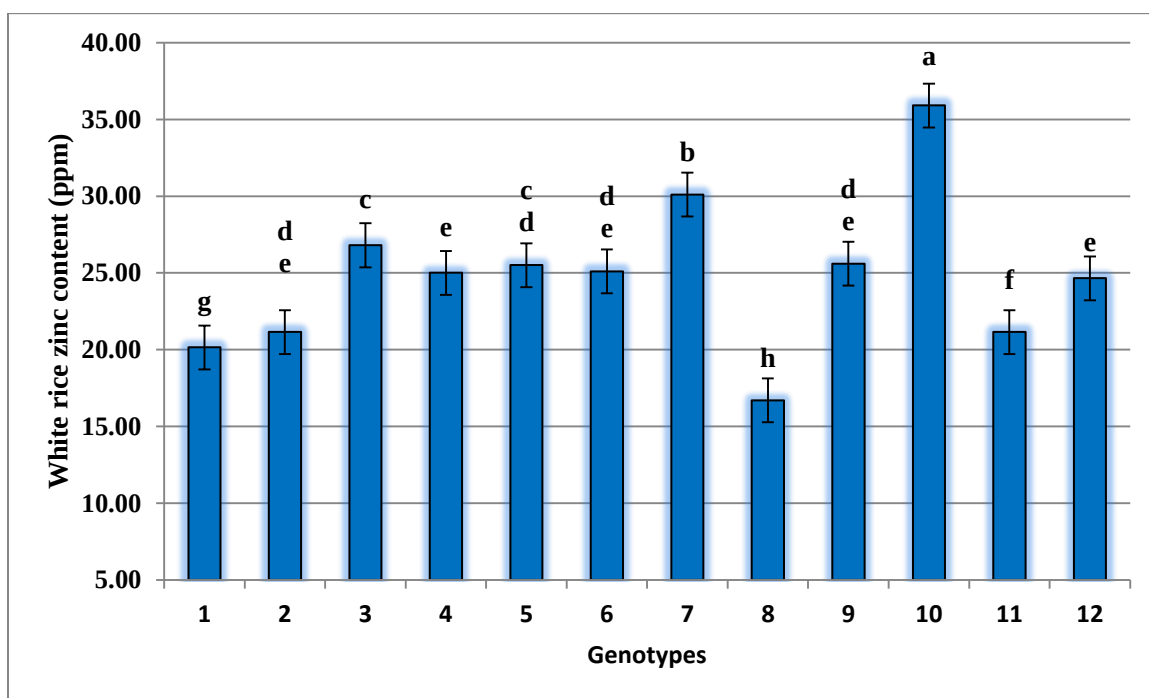


Fig. 4: Zinc content (ppm) in white rice of genotypes selected for study

Legends:

1- AM 72, 2- AM 143 3- AM 1 4- AM 65 5- AM 94B 6- AM 27 7- Azucena
8- ARB 6 9- IRJS 21 10- Moroberekan 11- TKM 9 12- Subhadra

PCV, low heritability, and low GAM (8.76, 21.86, 16.07 % and 7.24 % respectively) was observed for this trait (Fig. 2).

4.2.1.4 White rice Fe content (WFC)

For this trait, minimum reading obtained was 3.4 ppm (Azucena), and maximum readings recorded was 10.2 ppm (AM 94B) with a mean value of 5.03 ppm and range of 6.8. A high GCV, high PCV, high heritability, and high GAM (36.49, 36.85, 98.07 % and 74.46 % respectively) were recorded for this trait when selected genotypes were compared (Fig. 2 and 5).

4.2.1.5 Correlation analysis for loss of Zn and Fe due to polishing among selected genotypes

A significant negative correlation was found for percent loss of Fe with harvest index (-0.65) and white rice Fe content (-0.96).

4.2.2 Statistical analysis of data

4.2.2.1 Analysis of variance (ANOVA) 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 14.

Table 14: ANOVA for distribution of zinc and iron content in different grain parts among selected genotypes

Source of variation	df	Mean sum of squares			
		BZC	BFC	WZC	WFC
Genotype	11	99.79**	12.92 ^{NS}	70.24**	10.15**
Error	22	3.94	8.21	0.35	0.07
CD @ 5 %		3.36	-	0.99	0.43
CD @ 1 %		4.57	-	1.36	0.59
CV		7.22	20.03	2.33	5.09
SD		5.50	2.08	4.96	2.25

** Significant at 1 % NS= Non-significant **BZC**= Brown rice zinc content **WZC**= White rice zinc content **BFC**= Brown rice iron content **WFC**= White rice iron content

There was a significant difference among the study genotypes for all the traits except for brown rice Fe content. That is, significant difference was found among the genotypes for brown rice Zn content, white rice Zn and Fe content at both 1 per cent and 5 per cent LOS.

The difference among the genotypes for brown rice Fe content was found to be statistically insignificant.

4.2.2.2 Correlation studies

The results of correlation coefficients for Zn and Fe content in different parts of rice grain are presented in Table 15.

Table 15: Estimates of correlation coefficients for distribution of zinc and iron among selected genotypes

	BZC	BFC	WZC
BFC	-0.037	1	
WZC	0.811**	-0.067	1
WFC	-0.235	0.073	-0.017

** Significant at 1% **BZC**= Brown rice zinc content **WZC**= White rice zinc content **BFC**= Brown rice iron content **WFC**= White rice iron content

A significant positive correlation was found between brown rice Zn content and white rice Zn content (0.811) while correlation between other trait combinations were found to be statistically insignificant.

4.2.2.3 Path coefficient analysis

The results of direct and indirect effects of Zn and Fe content in different rice grain parts on grain yield are presented in Table 16.

Table 16: Direct and indirect effects of distribution of zinc and iron in different grain parts on grain yield among selected genotypes

	BZC	BFC	WZC	WFC
BZC	8.398	-0.048	-8.149	-0.944
BFC	0.664	0.611	1.989	0.42
WZC	7.83	0.139	-8.741	0.157
WFC	-2.081	-0.067	-0.359	3.81

RESIDUAL EFFECT= 3.456

BZC= Brown rice zinc content **WZC**= White rice zinc content **BFC**= Brown rice iron content **WFC**= White rice iron content

Highest direct positive effect on grain yield was observed for brown rice Zn content (8.39), whereas lowest direct positive effect was found to be for brown rice Fe content (0.611). Negative direct effect on grain yield was revealed by only white rice Zn content (-8.741). All other traits showed direct positive effects.

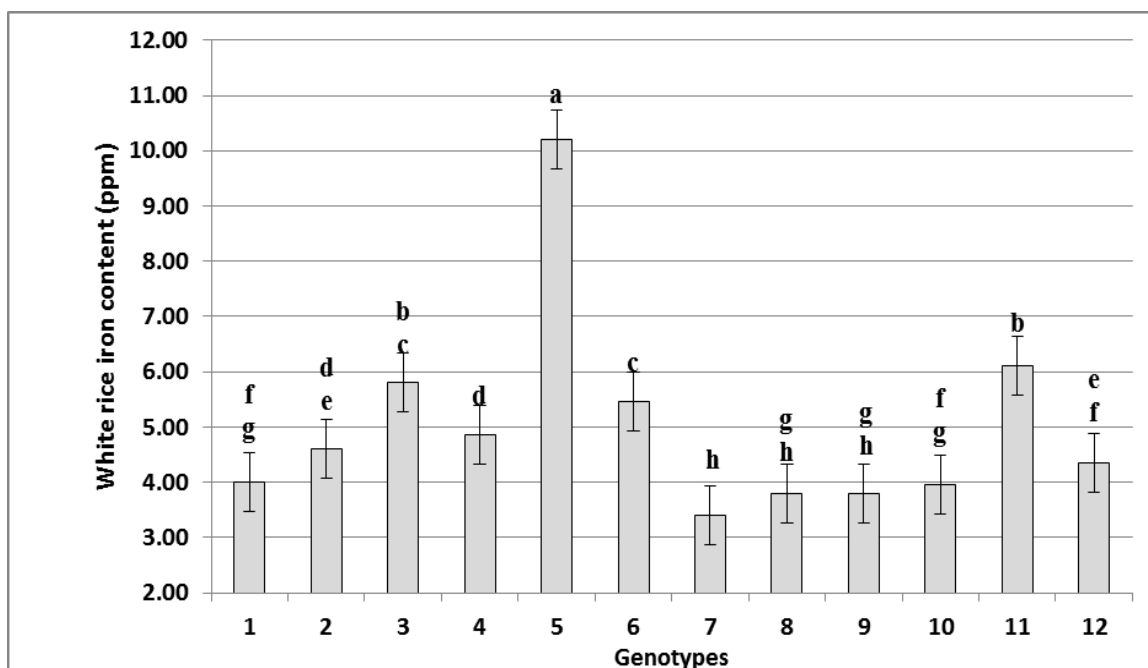


Fig. 5: Iron content (ppm) in white rice samples of selected genotypes

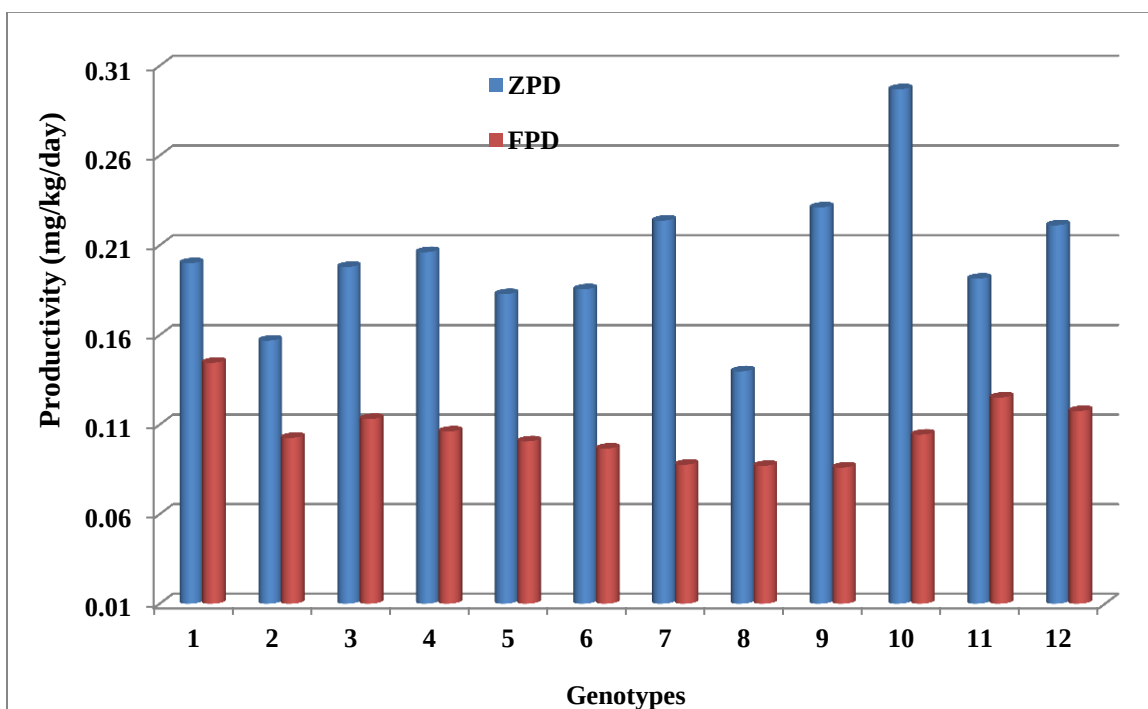


Fig. 6: Zinc and iron productivity (mg/kg/day) of genotypes selected for study

Legends:

1- AM 72, 2- AM 143 3- AM 1 4- AM 65 5- AM 94B 6- AM 27 7- Azucena
 8- ARB 6 9- IRJS 21 10- Moroberekan 11- TKM 9 12- Subhadra

ZPD- Zinc productivity per day **FPD**- Iron productivity per day

Among the traits studied, brown rice Zn content showed highest indirect positive effect on grain yield *via* white rice Zn content (7.83), whereas lowest indirect positive effect on grain yield was of brown rice Fe content *via* white rice Zn content (0.139).

Highest negative indirect effect on grain yield was revealed by white rice Zn content *via* brown rice Zn content (-8.149), whereas lowest negative indirect effect was observed for brown rice Fe content *via* brown rice Zn content (-0.048).

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

Loss of Zn and Fe in terms of percent is tabulated in Table 17. Average percent loss of Zn for all the genotypes studied was found to be 11.29 per cent, and it was 63.92 per cent for Fe. Highest loss of Zn after polishing was found for AM 72 (23.09 %) and lowest loss was found to be for AM 27 (2.23 %). In case of Fe, highest percent loss of Fe was found to be for AM 72 (76.50 %) and lowest for AM 94B (27.38 %). 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 18. Correlation coefficients for per cent loss of Zn and Fe are tabulated in Table 19.

Table 17: Per cent loss of zinc and iron from selected genotypes due polishing

Genotype	PLZ	PLF
AM 72	23.09	76.50
AM 143	14.19	66.83
AM 1	2.89	62.08
AM 65	11.67	65.23
AM 94B	9.49	27.38
AM 27	2.23	58.39
Azucena	10.41	74.18
ARB 6	9.22	66.81
IRJS 21	16.93	66.85
Moroberekkann	12.25	72.57
TKM 9	12.99	60.13
Subhadra	10.06	70.09

PLZ= Per cent loss of zinc PLF= Per cent loss of iron

Table 18: ANOVA for per cent loss of zinc and iron due to polishing among selected genotypes

Source of variation	Df	Mean sum of Squares	
		PLZ	PLF
Genotype	11	94.23 ^{NS}	487.39**
Error	22	45.45	28.29
CD @ 5 %		-	9.01
CD @ 1 %		-	12.24
CV		59.75	8.32
SD		5.61	12.75
Mean ± SE		11.29±1.62	63.92±3.68
Min.		2.23	27.38
Max.		23.09	76.5

** Significant at 1 % NS= Non-significant **PLZ**= Per cent loss of zinc **PLF**= Per cent loss of iron

Table 19: Correlation coefficients indicating correlation of per cent zinc and iron lost due to polishing with grain yield

	GYP	HI	WFC	PLZ
HI	0.48	1		
WFC	0.27	0.72**	1	
PLZ	-0.01	-0.18	-0.30	1
PLF	-0.21	-0.65*	-0.96**	0.36

* Significant at 5 % ** Significant at 1 % **GYP**= Grain yield per plant **HI**= Harvest index **WFC**= White rice iron content **PLZ**= Per cent loss of zinc due to polishing **PLF**= Per cent loss of iron due to polishing

4.3 Molecular characterization of Zn and Fe transporters in rice grain

Out of 12 genotypes studied, only ten were selected for molecular characterization. AM 27 and IRJS 21 are the two genotypes that are excluded in this objective.

4.3.1 Zn and Fe transporters

A list of all the rice micronutrient transporters explored from several literatures is shown in Table 20.

Table 20: List of micronutrient transporters explored for the present study from literature

Transporter gene	Reference
<i>OsZIP4</i>	Chen <i>et al.</i> , 2008
<i>OsFER1</i>	Chandel and Banerjee, 2011
<i>OsNAS1</i>	Chandel and Banerjee, 2011
<i>OsNAS2</i>	Inoue <i>et al.</i> , 2003
<i>OsYSL2</i>	Conte and Walker, 2011
<i>OsYSL15</i>	Inoue <i>et al.</i> , 2009
<i>OsYSL18</i>	Aoyama <i>et al.</i> , 2009
<i>UBQ5</i>	Jain <i>et al.</i> , 2006

4.3.2 Primer designing

4.3.2.1 Sequences of transporter genes selected for study

Details of the transporter genes used in the study are presented in Table 21. cDNA sequences of all the mentioned transporters are downloaded in FASTA format, which was further used to design gene specific primers.

Table 21: Details of transporter genes selected for the study

Sl. No.	Transporter	Gene	Gene Id	Chrm.	NCBI ref seq.
1	<i>OsZIP4</i>	Os08g0207500	47169686	8	NC_008401.2
2	<i>OsFER1</i>	Os11g0106700	21686525	11	NC_008404.2
3	<i>OsNAS1</i>	Os03g0307300	11176989	3	NM_001056430.1
4	<i>OsNAS2</i>	Os03g0307200	11176985	3	NC_008396.2
5	<i>OsYSL2</i>	Os02g0649900	297599682	2	NM_001054121.2
6	<i>OsYSL15</i>	Os02g0650300	297721466	2	NM_001186167.1
7	<i>OsYSL18</i>	Os01g0829900	115440824	1	NM_001051227.1
8	<i>UBQ5</i>	Os01g0328400	115436313	1	NM_001049450.1

4.3.2.2 Primer designing

Primers specific to the nucleotide sequence of cDNA of transporters are given in Table 22. The length of the primers ranged from 18 bp to 25 bp, expected product size ranged from 69 bp to 449 bp, and annealing temperature ranged from 48.9 °C to 55.8 °C.

Out of 25 primers designed, only nine primers were selected for relative expression studies in selected genotypes by semi-quantitative RT-PCR analysis.

Table 22: List of primers used in the present research for molecular study

Primer	Forward and reverse primer sequence	T (°C)	Product size (bp)
<i>OsZIP4a</i>	F-CAGGCAAATTTTAAGGCGAAAGC (23) R-GACCAGGGACATGTAGATCAGTAT (24)	54.6	186
<i>OsZIP4b</i>	F-AATCTCATCCAGCTATAGCAAGCAC (25) R-GCTGAAGCTTTGTGTTTGTGTTGAAG (25)	55.2	158
<i>OsFER1c</i>	F-CGTTCTCTCACTTAATCCG (20) R-TCATCGCTGGATTCTTTGAA (20)	49.8	429
<i>OsNAS1a</i>	F-GAGGCTCAGAACCAAGAGGTC (21) R-CTTGCTCAGGTTGACGTAGTTG (22)	55.6	327
<i>OsNAS2a</i>	F-GAGGCTCAGAACCAAGAGGTC (21) R-CTTGCTCAGGTTGACGTAGTTG (22)	55.6	327
<i>OsYSL2b</i>	F-GTAATTGCGATACCCATAATGTTCC (25) R-CTTGTACTAGCTGCTTCACTATTC (23)	54.2	235
<i>OsYSL2c</i>	F-TTCTTATAAACGGATTCCATACCCC (25) R-GAACATTTCCAGGATAACCACTTAC (24)	54.2	336
<i>OsYSL15b</i>	F-GATGATTTGCTCACACCTTG (20) R-CGTAGGCTATGATCACGTAG (20)	50.8	449
<i>OsYSL18b</i>	F-CCTTCTTCTTCCTCGTTAGCTTC (23) R-GTTCCGTTCAAACGTATGTAGAAAC (25)	54.9	313
<i>UBQ5</i>	F-ACCACTTCGACCGCCACTACT (21) R-ACGCCTAAGCCTGCTGGTT (19)	54.8	69

T= Calculated annealing temperature of the primer set

4.3.3 Total RNA isolation from different parts of rice grain

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

Sampling of rice seeds was done during late dough stage of seed development and stored immediately at -80 °C. Just before RNA isolation, seeds are separated into embryo and endosperm under aseptic and nuclease free condition as shown in Plate 4. Total RNA was isolated from embryo and endosperm using modified TRIzol method recommended by Li and Trick (2005).

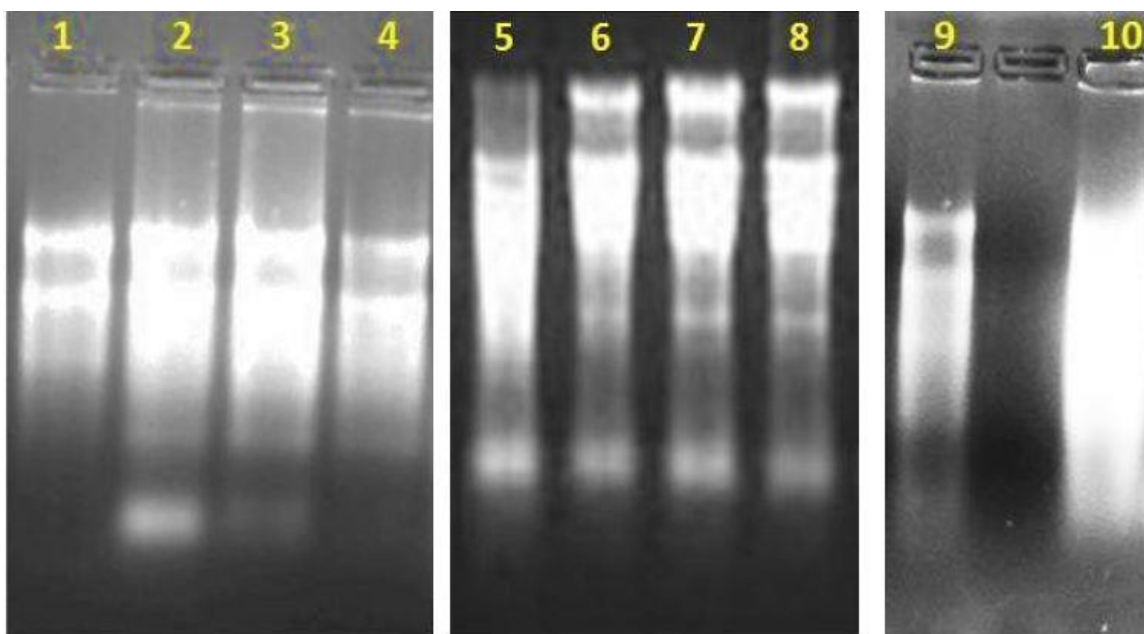


Plate 3a: Total RNA isolated from embryo of selected rice genotypes

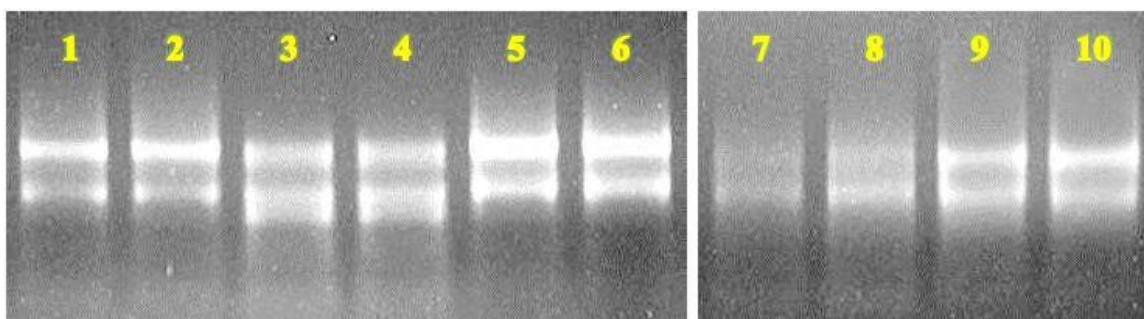


Plate 3b: Total RNA isolated from endosperm of selected rice genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72,
7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

Total RNA isolated were quantified using nanodrop (Eppendorf Biospectrometer). Nanodrop readings obtained for RNA isolated from embryo and endosperm are tabulated in Table 23.

Table 23: 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
Azucena	63.9	98.8	1.59	2.38	2.01	1.89
Moroberekan	110.1	36.5	2.75	1.28	2.03	1.49
AM 1	99.2	10.8	2.48	0.26	2.00	1.55
AM 65	88.7	109.8	2.22	2.23	2.04	1.79
AM 72	84.8	73.2	2.12	1.88	2.01	1.88
AM 94B	85.3	11.2	2.13	0.39	2.01	1.61
AM 143	118.3	95.4	2.96	2.24	2.00	1.99
ARB 6	90.6	45.3	2.26	1.19	1.97	1.89
Subhadra	104.4	25.4	2.61	0.69	2.00	1.50
TKM 9	73.8	18.2	1.85	1.23	1.97	1.88

4.3.4 PCR optimization and cDNA normalization

PCR optimization was done for annealing temperature for primers used, optimum MgCl₂ concentration, use of BSA in PCR, optimum primer concentrations of forward and reverse primer, optimization of cycle numbers.

Optimum annealing temperature for maximum amplification to each of the primer used in the study was assessed by gradient PCR analysis. Lower temperature level used in gradient PCR was calculated by subtracting 5 °C from the annealing temperature of forward or reverse primer, which ever had lower annealing temperature. The interval of temperature was set to 0.5 °C. Optimum annealing temperature was found out for each of the primers by observing the temperature at which amplification was more when all other PCR conditions were kept constant. Plate 5 represents the gradient PCR done for OsZIP4b (158 bp). The optimum annealing temperature for OsZIP4b was found to be 53.5 °C. The same was performed for all the primers that were studied.

Use of MgCl₂ was reported to increase *Taq* polymerase activity and also improves specificity of *Taq* polymerase. Optimum MgCl₂ concentration was assessed by using 50 mM MgCl₂ at different concentrations like 1.5 mM, 2 mM, 2.5 mM and 3 mM. Optimum amplification was observed at MgCl₂ concentration of 2.5 mM. Plate 6 reveals the effect of MgCl₂ on PCR amplification.

It is evident that use of BSA (Bovine Serum Albumin) in PCR enhance PCR efficiency, enzyme stabilization (*Taq* polymerase) and to perform PCR with low purity templates. In the present study, effect of BSA on PCR efficiency was found to be significant. Result obtained by PCR from treatment with BSA and control is given in Plate 7.

It is evident that high primer concentration in PCR will lead to primer dimer formation which comes in the way of amplification of target sequences. So, both forward and reverse primers were used at four different concentrations and the effect was observed to be significant. Different combinations of primer concentrations used are given in Table 24 and the effect of different combinations of primer concentration is given in Plate 8. Optimum primer concentration was found to be 0.5 μ L of forward primer and 1 μ L of reverse primer.

Table 24: Different combinations of primer concentrations used for PCR optimization

Reverse primer (μ L)	Forward primer (μ L)	
0.5	0.5	1
1	1	0.5

For semi quantitative RT-PCR, optimum cycles that PCR is to be performed should be standardized. Optimum cycle number is that number cycles where bands are clearly visible on an agarose gel and amplification is in exponential phase and not yet reached the plateau (stationary) phase. The optimum cycle number varies from one RNA sample to another. Hence, optimum cycle number for each of the RNA samples studied was found out by manually pipetting out about 5 μ l of amplified product from PCR tubes after 25, 30, 35, 40, 45, and 50 cycles and loading them on to agarose gel for visualization. Optimization of cycle number for OsZIP4a is displayed in Plate 9. Optimum cycle number for OsZIP4a was found to be 40. Same procedure was followed for all the remaining primers to optimize cycle number.

4.3.5 Semi Quantitative RT-PCR (SQ RT-PCR)

A single step RT-PCR was done where reverse transcription of RNA and amplification of resulting cDNA using specific primers occur in the same tube. Relative expression of seven genes (*OsZIP4*, *OsFER1*, *OsNAS1*, *OsNAS2*, *OsYSL2*, *OsYSL15*, and *OsYSL18*) was assessed by performing semiquantitative RT-PCR using nine primers viz., *OsZIP4a*, *OsZIP4b*, *OsFER1c*, *OsNAS1a*, *OsNAS2a*, *OsYSL2b*, *OsYSL2c*, *OsYSL15b*, and *OsYSL18b*. UBQ5 is used to amplify Ubiquitin gene which act as internal control for relative quantification.

4.3.5.1 Normalization of reference gene

Internal control gene (reference gene) used in this study is *UBQ5*. Normalization was done by amplifying the reference gene in all the RNA samples of test genotypes and visualization of amplified product on agarose gel. Since, reference genes are expressed in

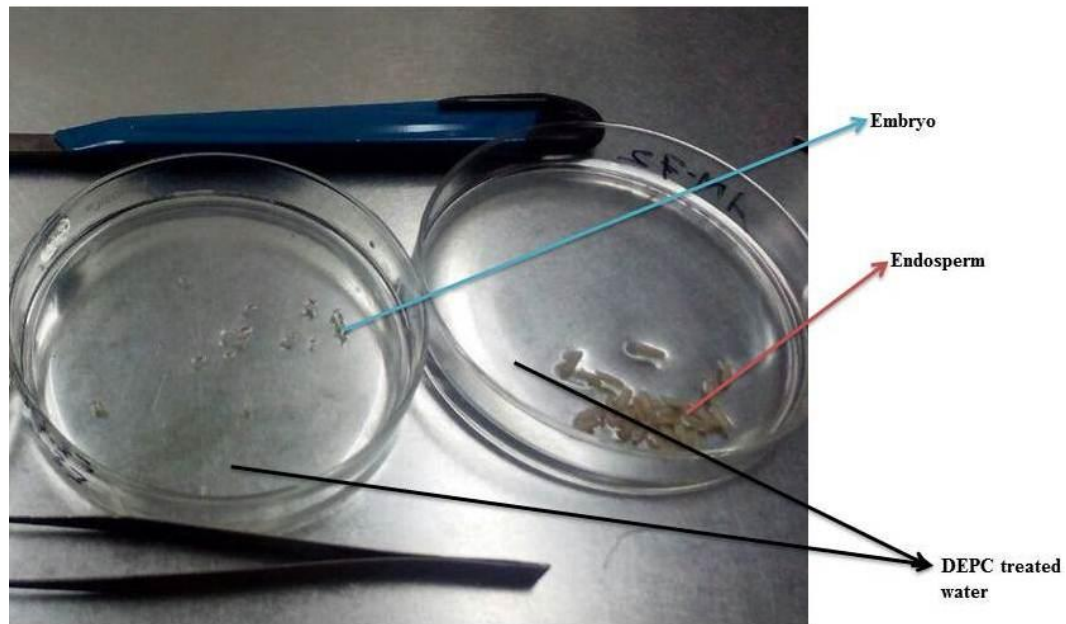


Plate 4: Separation of embryo and endosperm under aseptic and nuclease-free condition

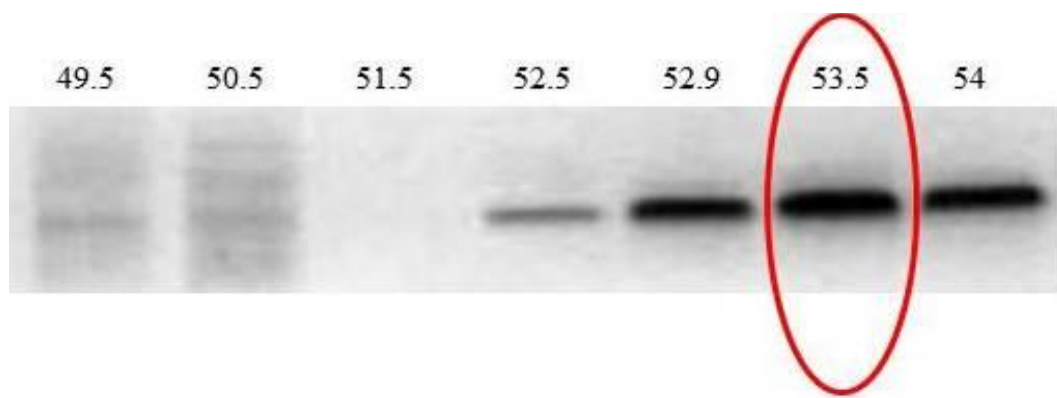


Plate 5: Optimization of annealing temperature ($^{\circ}\text{C}$) for OsZIP4b by gradient PCR

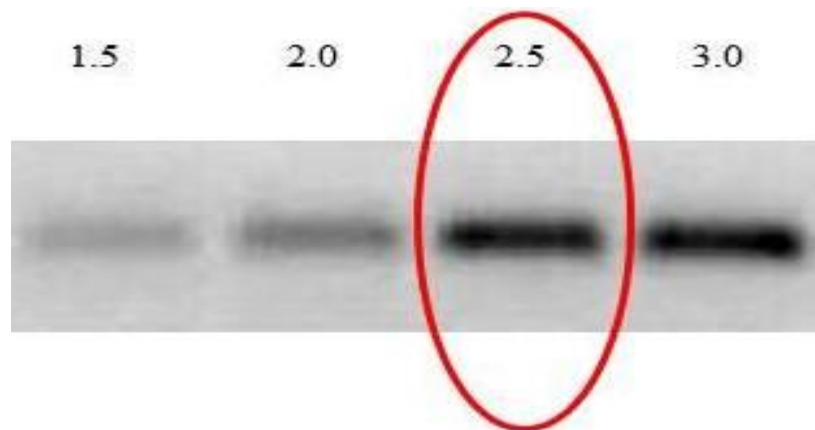


Plate 6: Optimization of MgCl_2 concentration (mM) for SQ RT-PCR

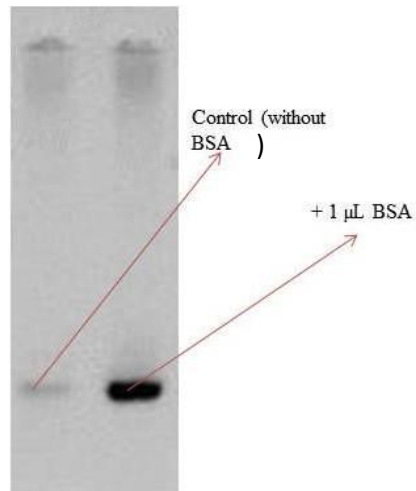


Plate 7: Effect of BSA on amplification of desired cDNA

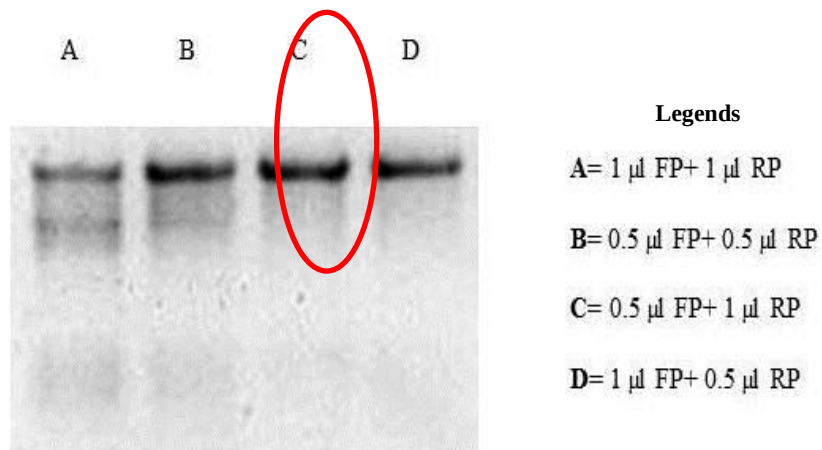


Plate 8: Effect of different primer concentrations on amplification of desired cDNA

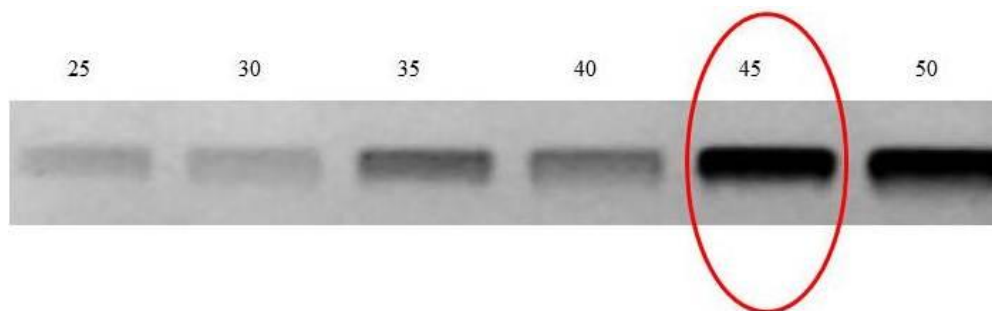


Plate 9: Optimization of cycle number for OsZIP4a

same levels constitutively, the intensity of the bands are expected to be same in all samples. Any change in the band intensity could be due pipetting error. Such difference in band intensity should be considered while discerning the relative expression of genes under study. There was no significant difference in the band intensities for both the tissues (embryo and endosperm) among the genotypes under study. Hence, confirms the expression of reference gene in same level in both the tissues in all the genotypes under study.

4.3.6 Relative expression of transporter genes under study

Totally nine primers were studied to know the relative expression of seven genes. Results obtained for nine primers are presented below as subheadings. For each primer, the band intensity values were generated by using GelAnalyzer 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.

4.3.6.1 OsZIP4a

Relative expression of OsZIP4a (a segment of cDNA of *OsZIP4* gene) obtained from semi quantitative RT-PCR analysis for embryo and endosperm are displayed in Plate 10a and 10b respectively.

Expression of OsZIP4a was found to be relatively higher in embryo than in endosperm in all the genotypes under study.

Both in embryo and endosperm, the band intensity values of genotypes under study for OsZIP4a generated using Gel Analyzer software revealed relatively higher expression of OsZIP4a in Moroberekan, lower expression in AM 94B for embryo and Subhadra for endosperm when compared to other genotypes under study.

4.3.6.2 OsZIP4b

The results obtained from semi quantitative RT-PCR analysis for OsZIP4b in embryo and endosperm of selected genotypes is displayed in Plate 11a and 11b respectively.

Among embryo and endosperm, expression of OsZIP4b was found to be relatively higher in endosperm than in embryo.

In embryo, expression of OsZIP4b was found to be relatively higher in Moroberekan lower in Subhadra than in other genotypes under study. In endosperm, expression was found to be relatively higher in Azucena and lower in TKM 9 than in other study genotypes. It was also found that the expression of OsZIP4b was very less or nil in the endosperm of Moroberekan, and AM 1.

4.3.6.3 OsFER1c

The results obtained from semi quantitative RT-PCR analysis for embryo and endosperm among selected genotypes are displayed in Plate 12a and 12b respectively.

Among embryo and endosperm, expression of OsFER1c was found to be relatively higher in endosperm than in embryo in all the genotypes under study.

In embryo, expression of OsFER1c was found to be relatively higher in Azucena and lower in AM 143 when compared with other genotypes under study. It was also observed that expression was very less or nil in embryo of AM 1, AM 65, AM 94B, ARB 6, and Subhadra. In endosperm, expression of OsFER1c was found to be relatively higher in AM 65 and lower in TKM 9 than in other genotypes under study. There was no or little expression in endosperm of Azucena, ARB 6 and Subhadra.

4.3.6.4 OsNAS1a

The results obtained for OsNAS1a from semi quantitative RT-PCR analysis for embryo and endosperm of selected genotypes are displayed in Plate 13a and 13b respectively.

The expression of OsNAS1a was found to be relatively higher in embryo than in endosperm in all the selected genotypes except in AM 94B where expression was higher in endosperm than in embryo.

In embryo, expression of OsNAS1a was found to be relatively higher in Moroberekan and lower in AM 94B when compared with other genotypes under study. Negligible or no expression was found in embryo of AM 65. In endosperm, expression was found to be relatively higher in Azucena and relatively lower in AM 1 when compared with other selected genotypes.

4.3.6.5 OsNAS2a

The results obtained for OsNAS2a from semi quantitative RT-PCR analysis for embryo and endosperm among genotypes under study is shown in Plate 14a and 14b respectively.

The expression of OsNAS2a was found to be relatively higher in embryo than in endosperm in all the genotypes selected for the study.

In both embryo and endosperm, expression of OsNAS2a was relatively higher in Moroberekan than in other genotypes under study and lower relative expression was observed in AM 65 for both embryo and endosperm. Expression of OsNAS2a in embryo of TKM 9 was almost same as that in embryo of Moroberekan.

4.3.6.6 OsYSL2b

The results for relative expression study done for OsYSL2b in embryo and endosperm of selected genotypes is displayed in Plate 15a and 15b respectively.

Expression of OsYSL2b was observed to be relatively higher in endosperm than in embryo among the genotypes selected for the study except for AM 94B and AM 143.

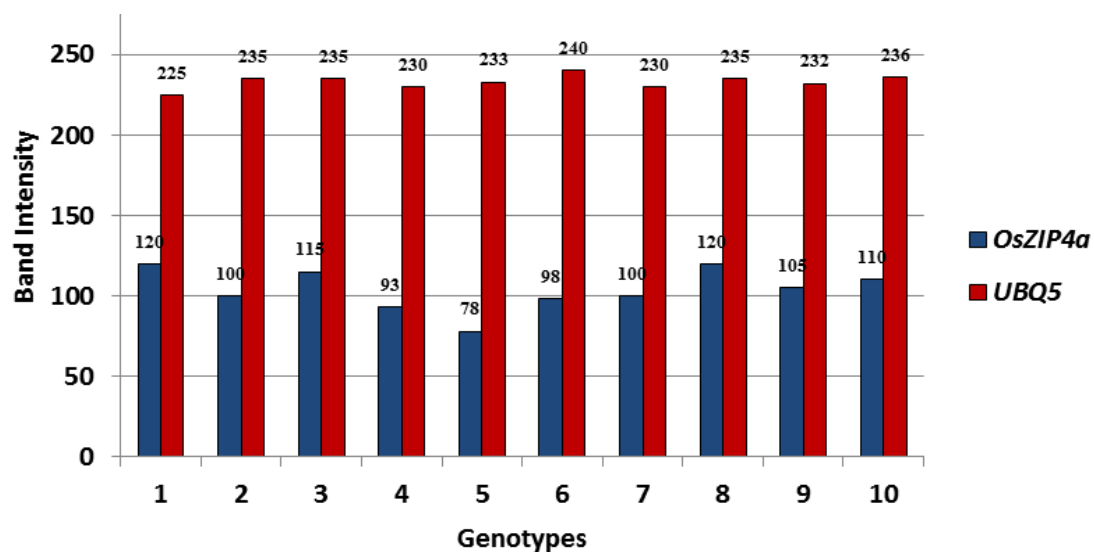
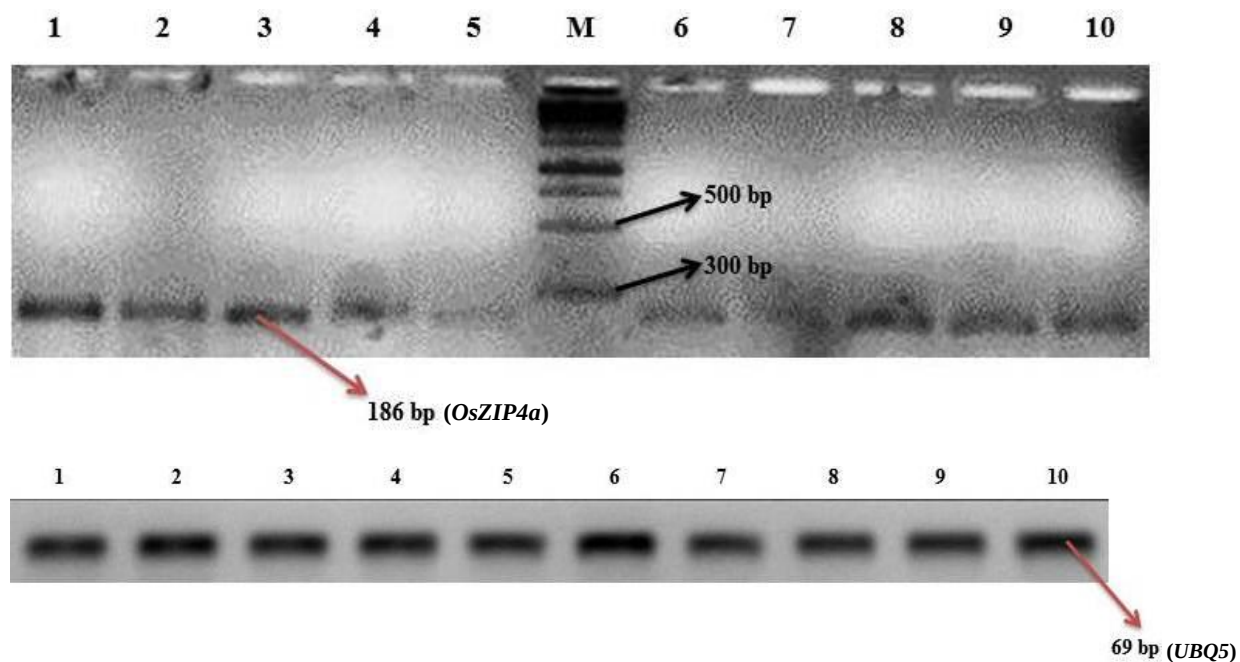


Plate 10a: Relative expression of *OsZIP4a* in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

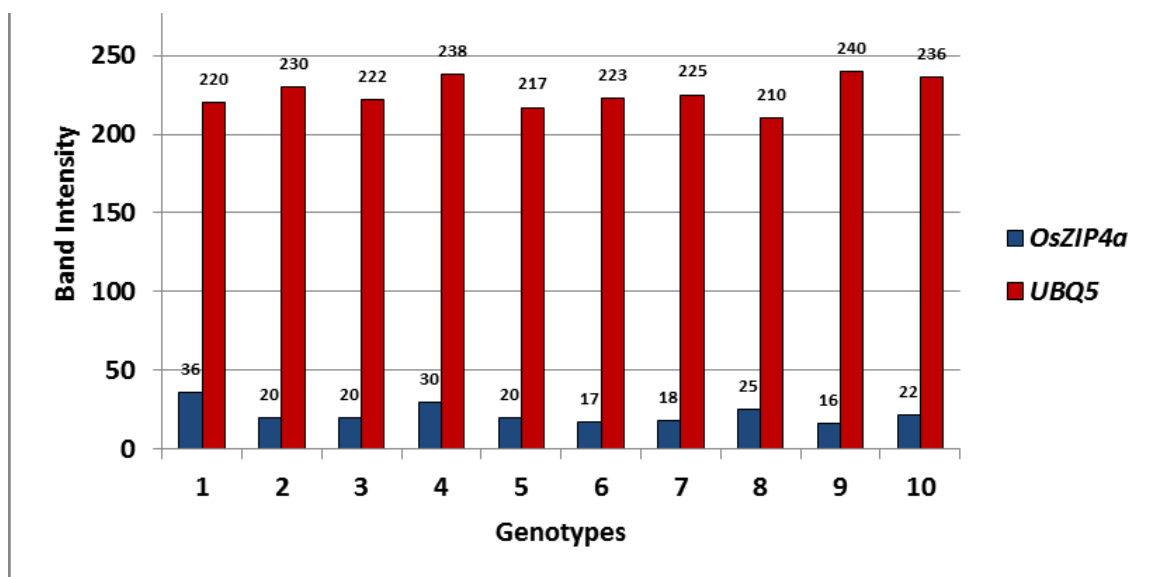
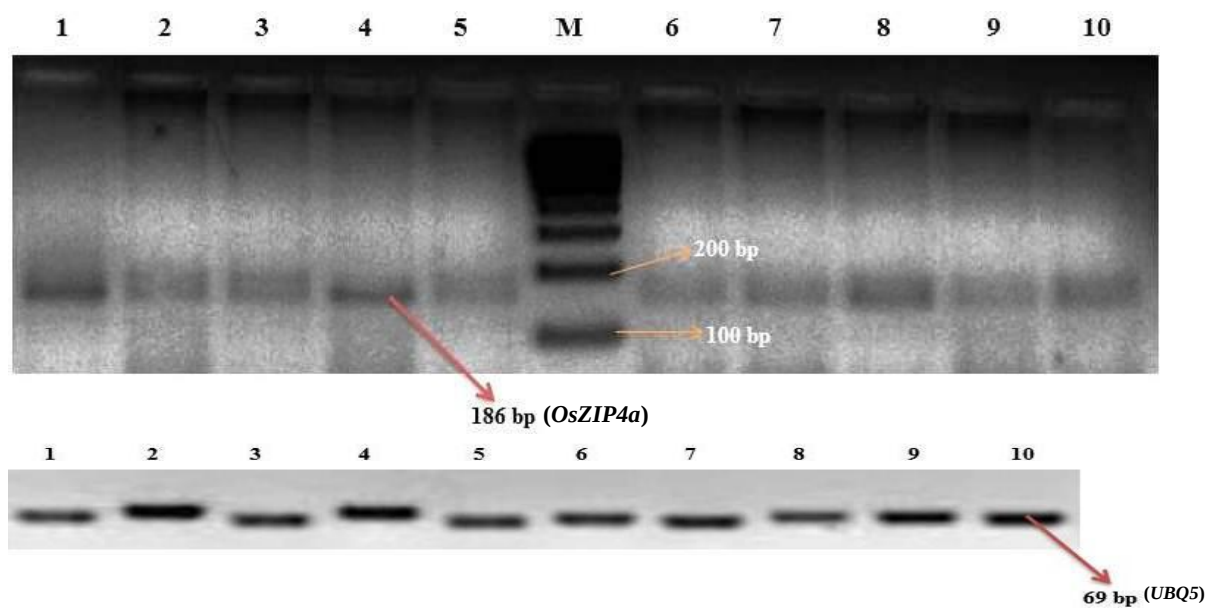


Plate 10b: Relative expression of *OsZIP4a* endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

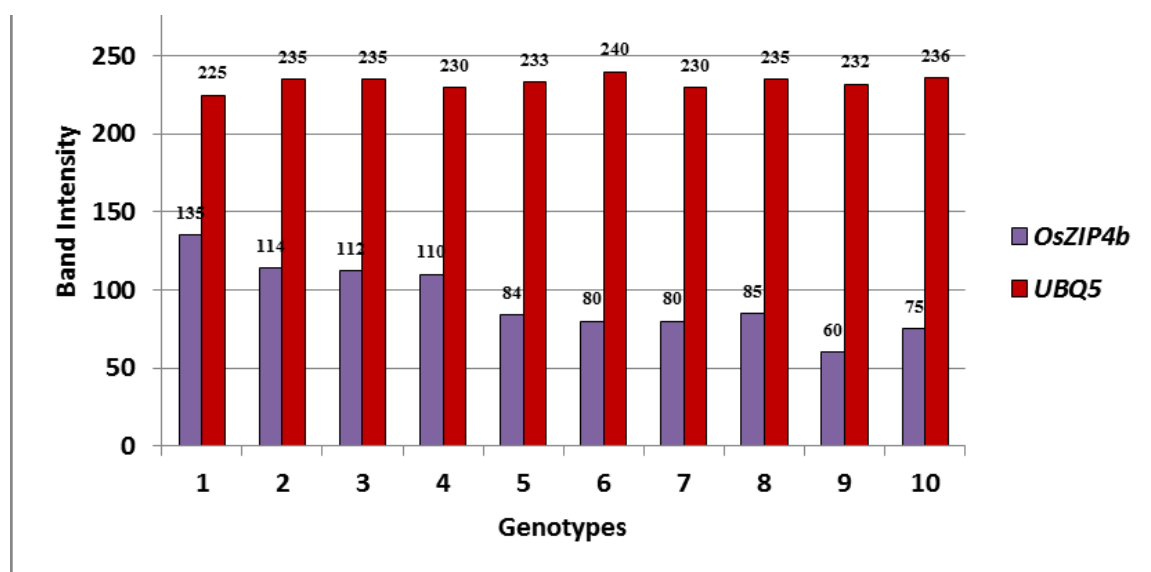
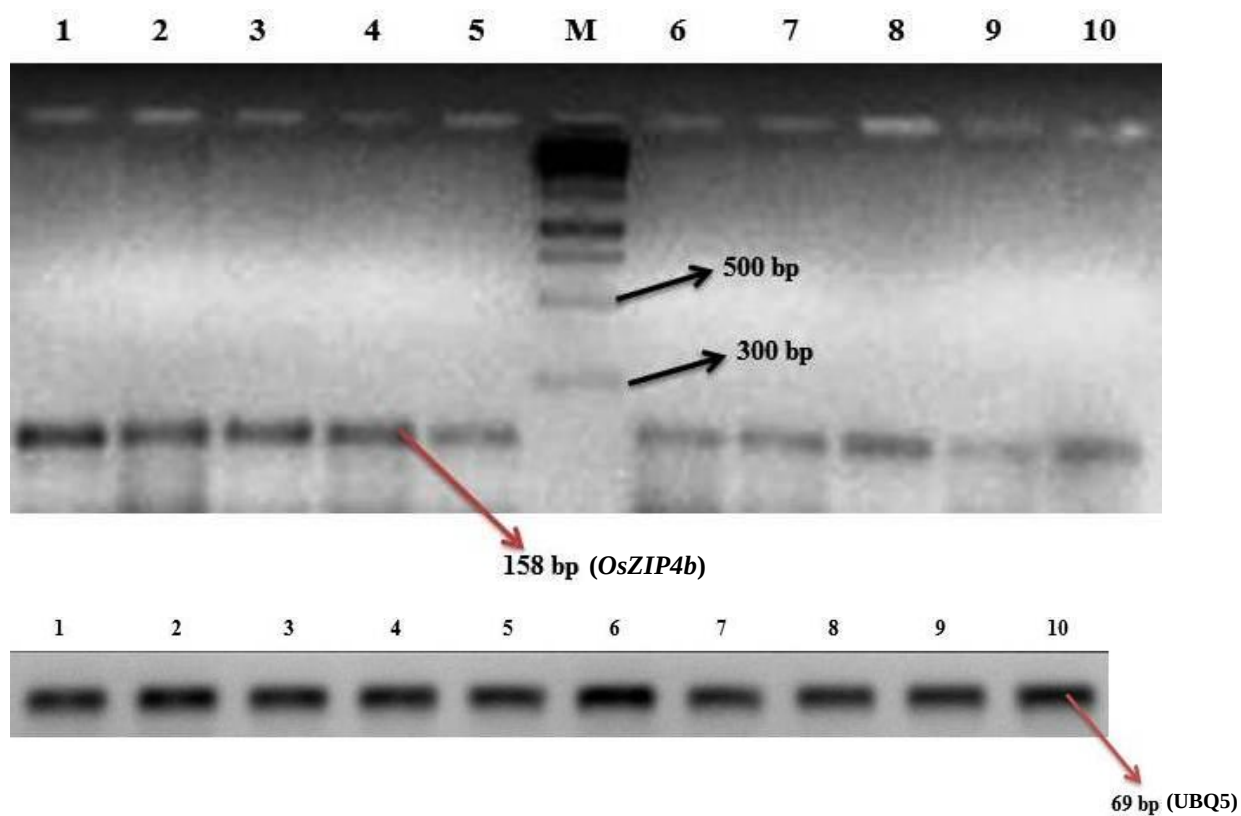


Plate 11a: Relative expression of OsZIP4b in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

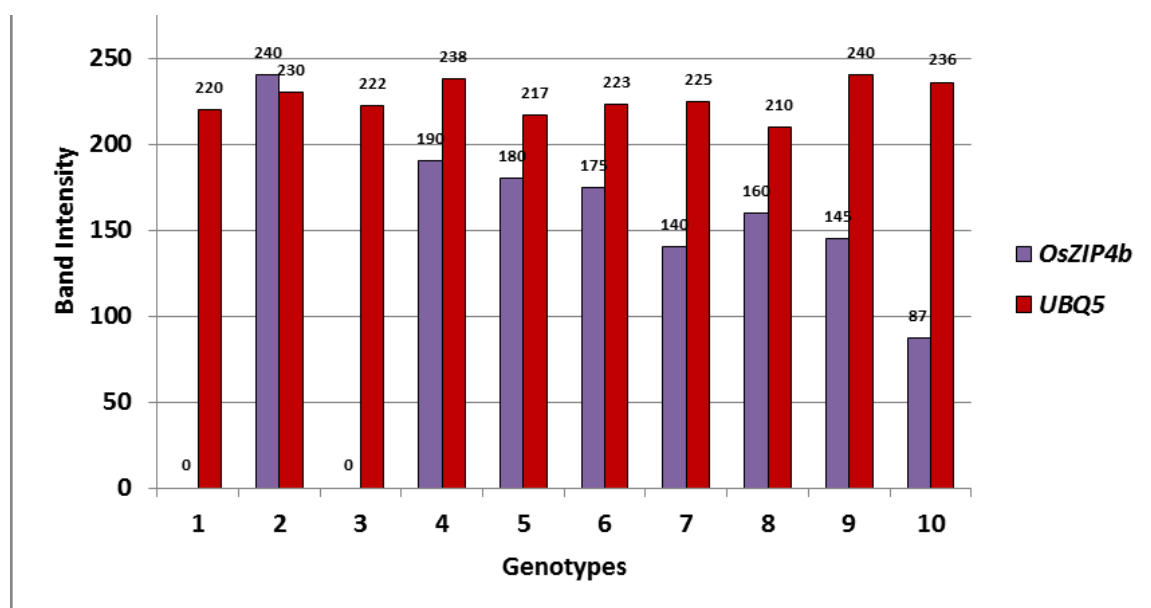
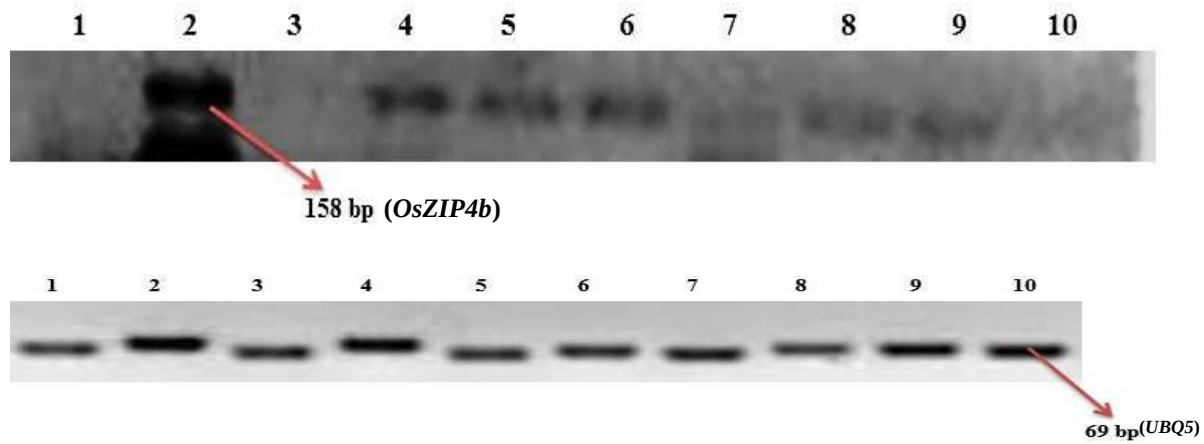


Plate 11b: Relative expression of *OsZIP4b* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

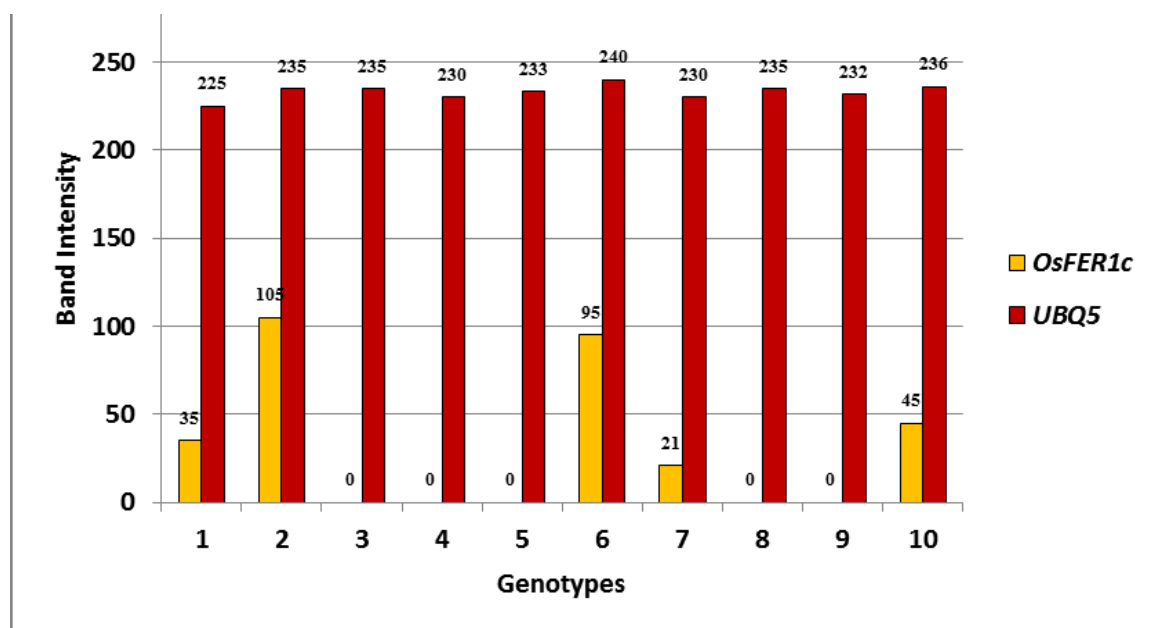
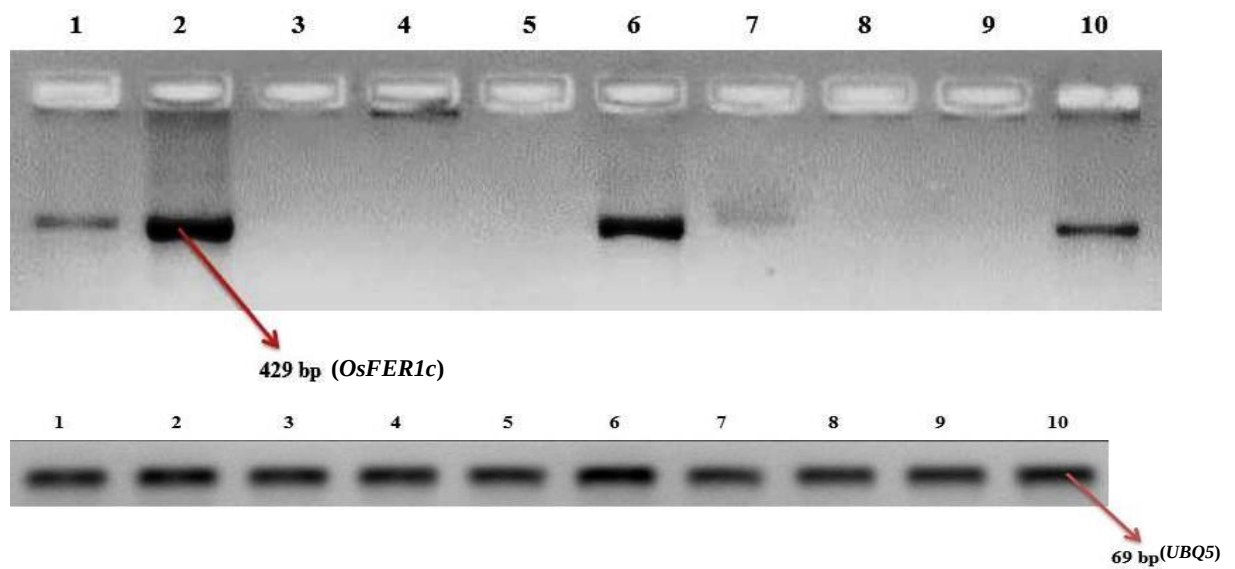


Plate 12a: Relative expression of *OsFER1c* in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

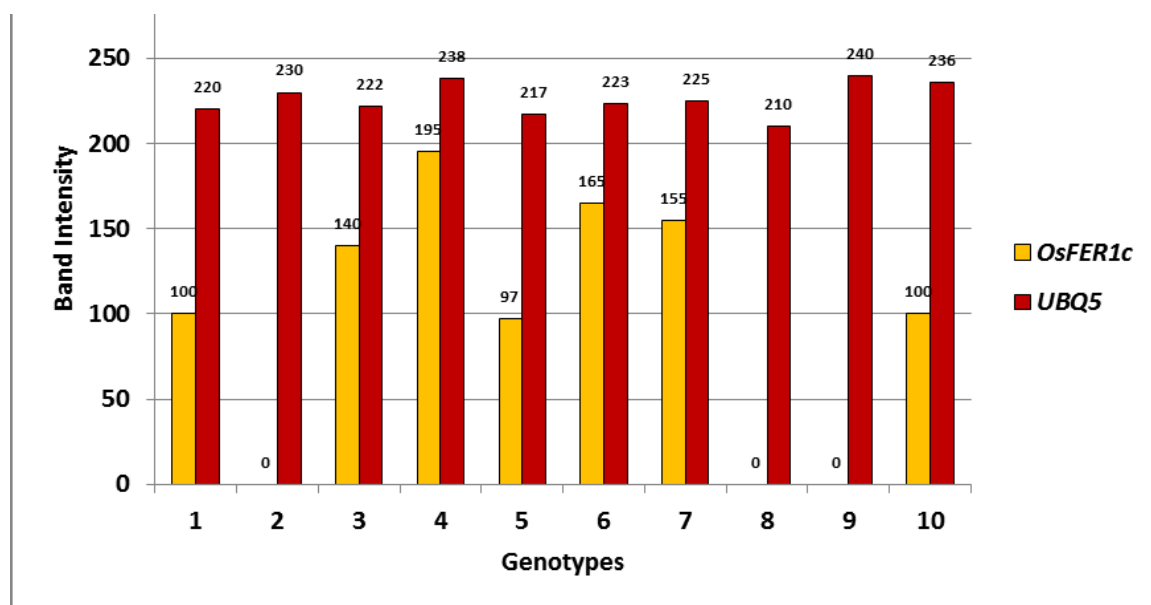
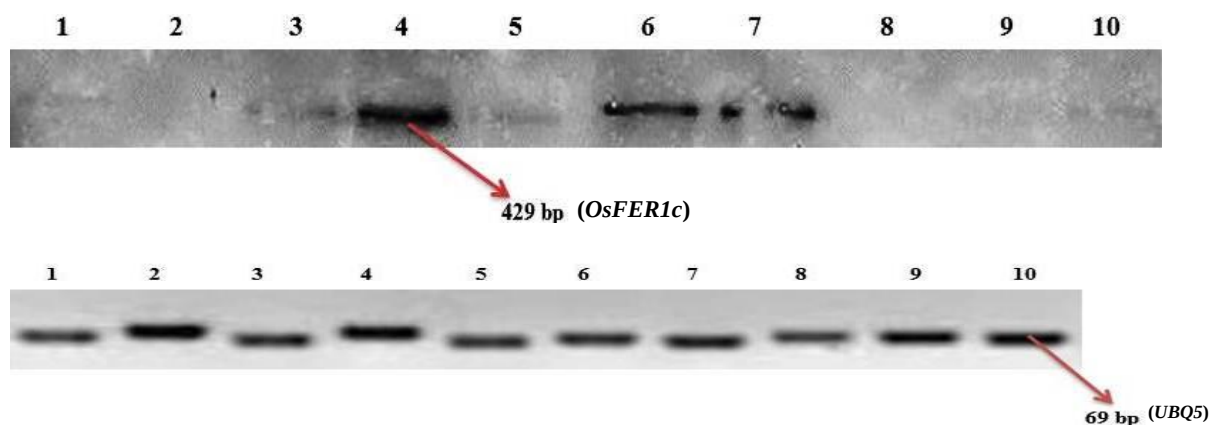


Plate 12b: Relative expression of *OsFER1c* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

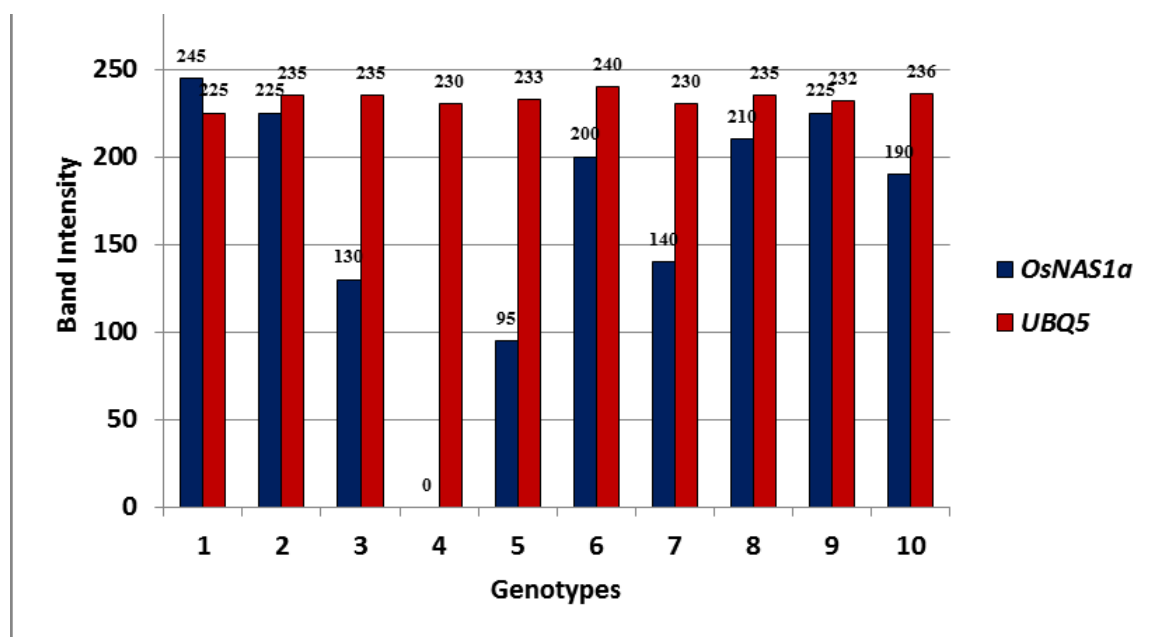
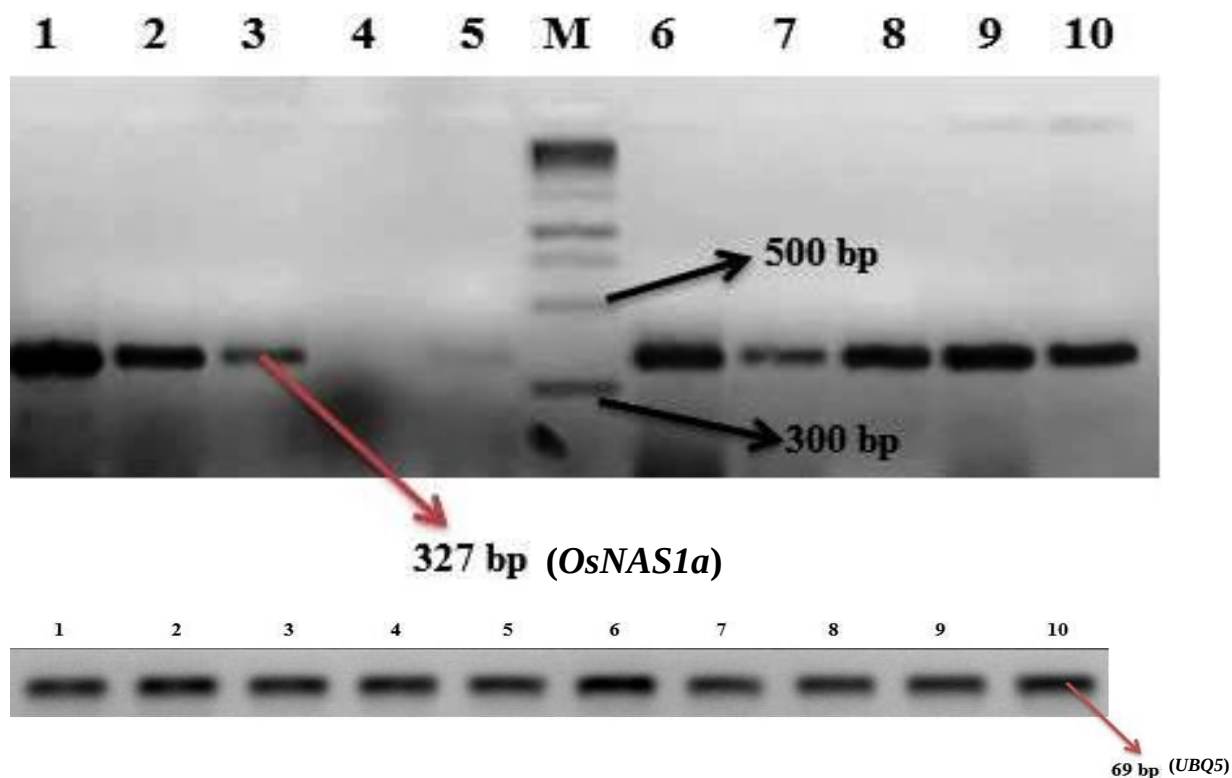


Plate 13a: Relative expression of *OsNAS1a* in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

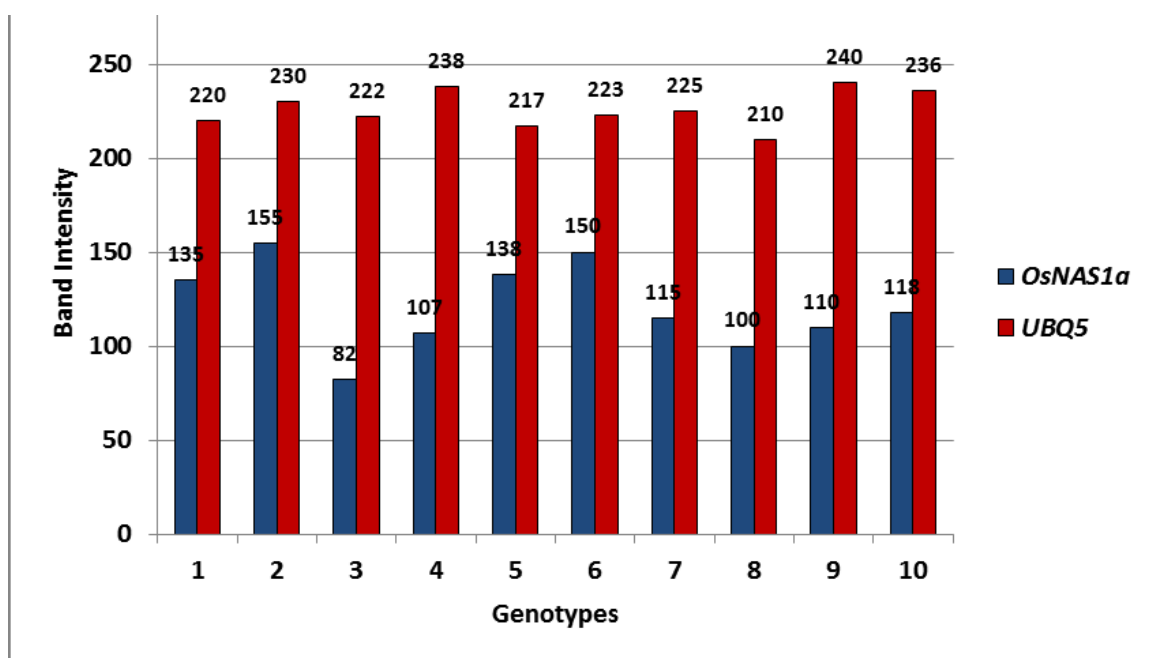
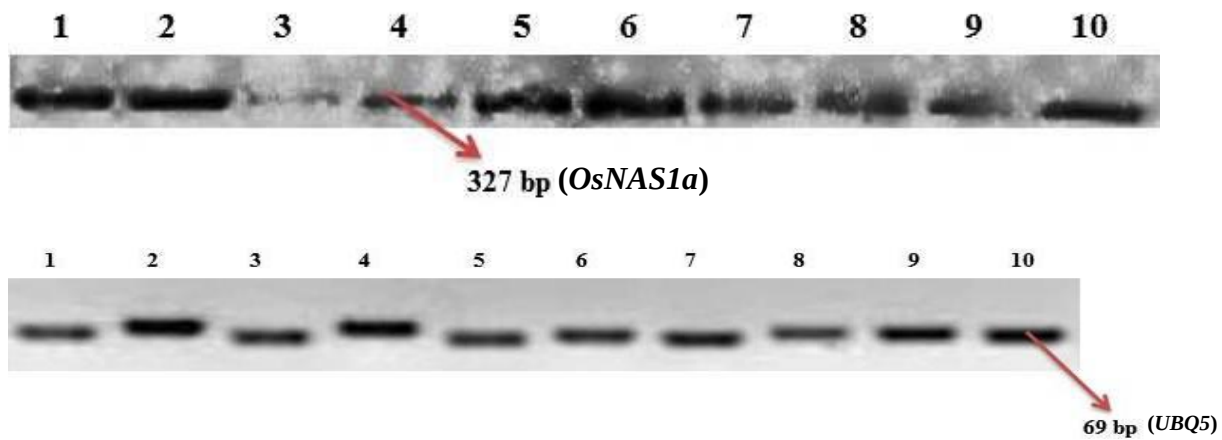


Plate 13b: Relative expression of *OsNAS1a* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

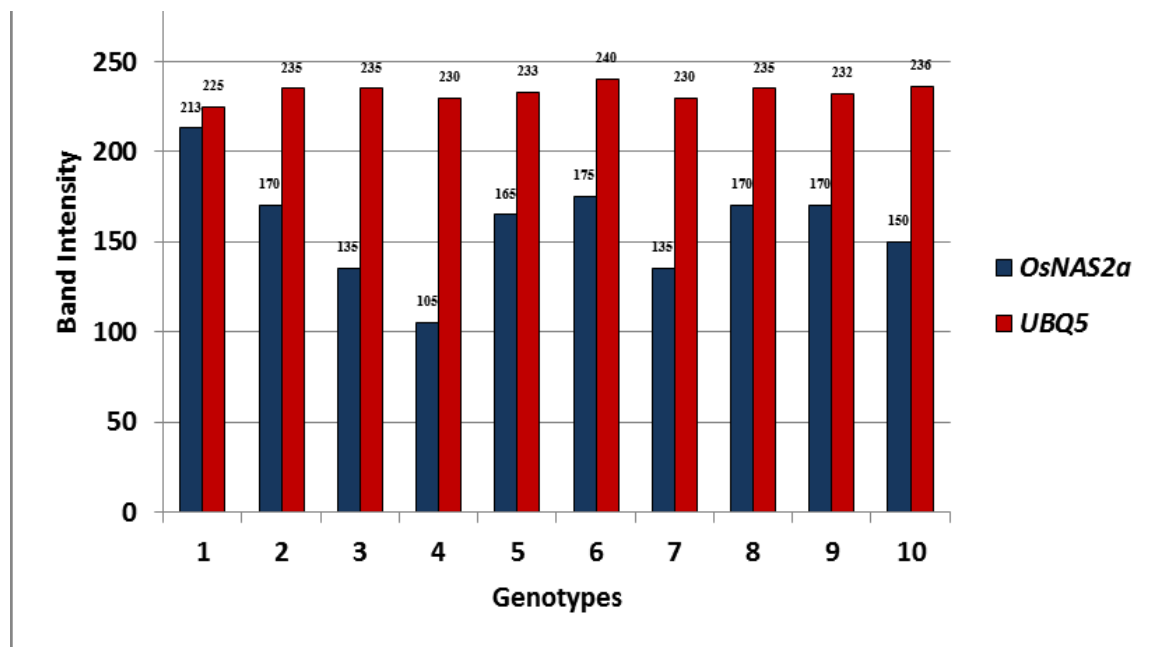
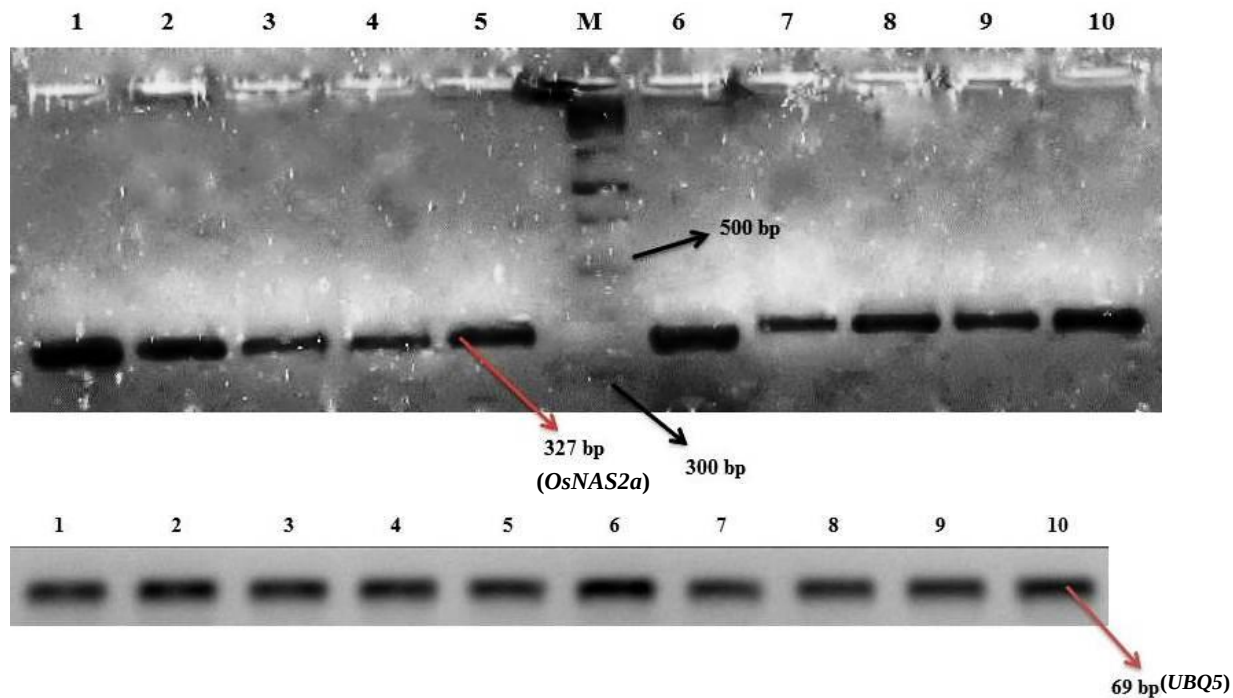


Plate 14a: Relative expression of OsNAS2a in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

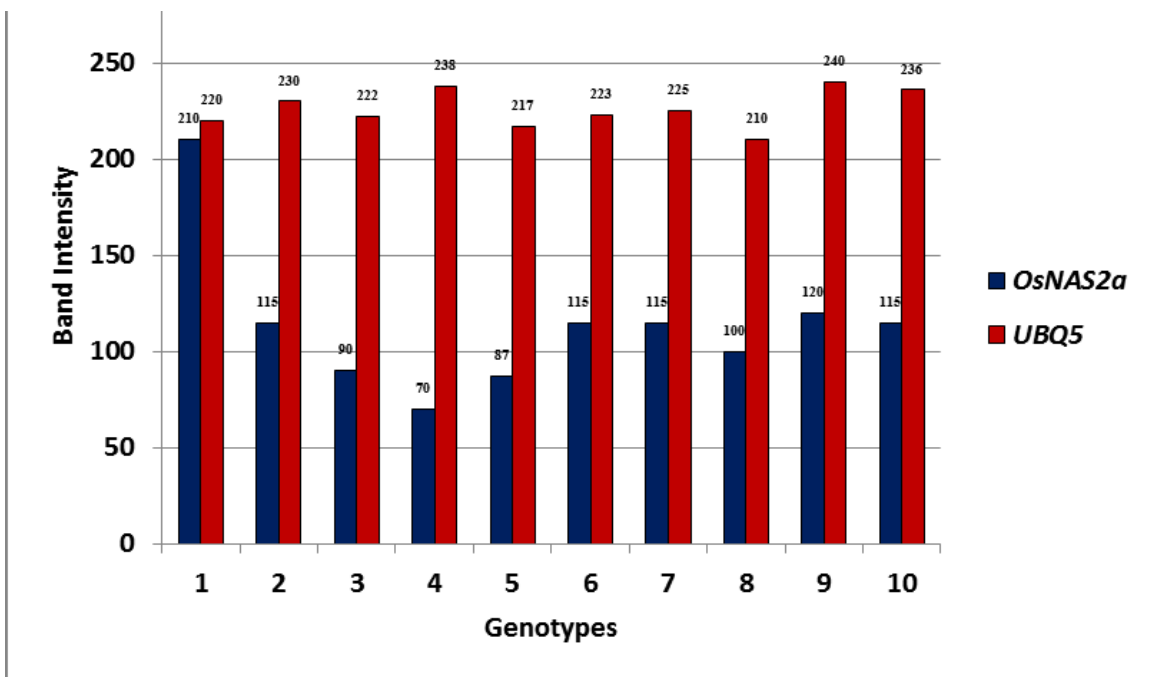
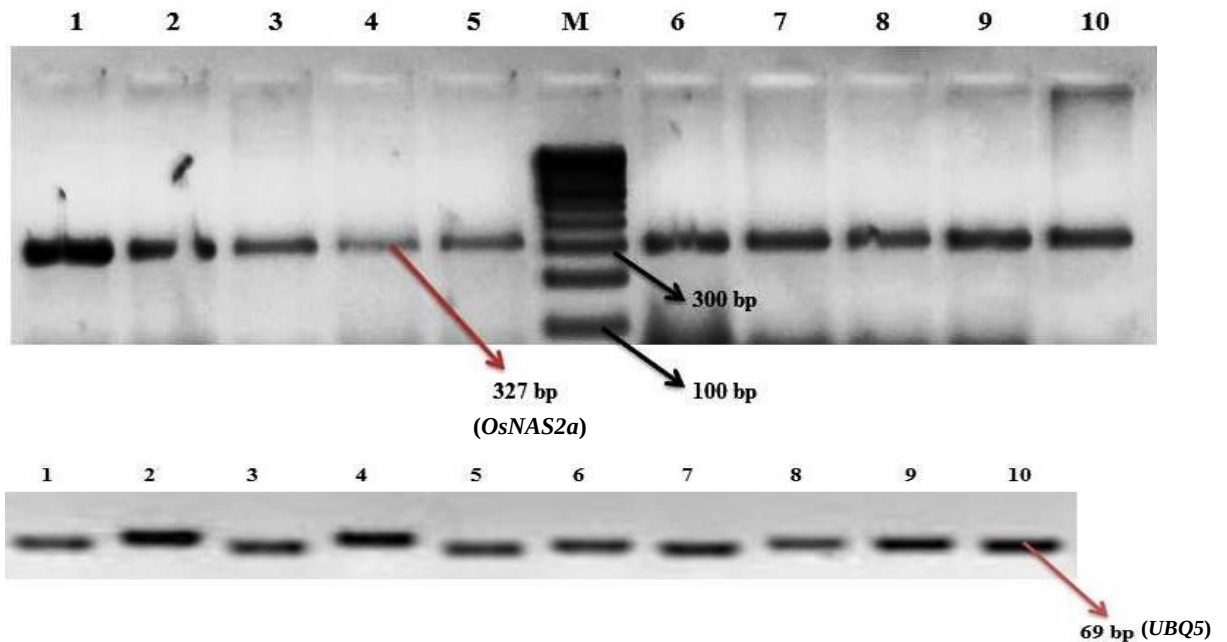


Plate 14b: Relative expression of *OsNAS2a* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

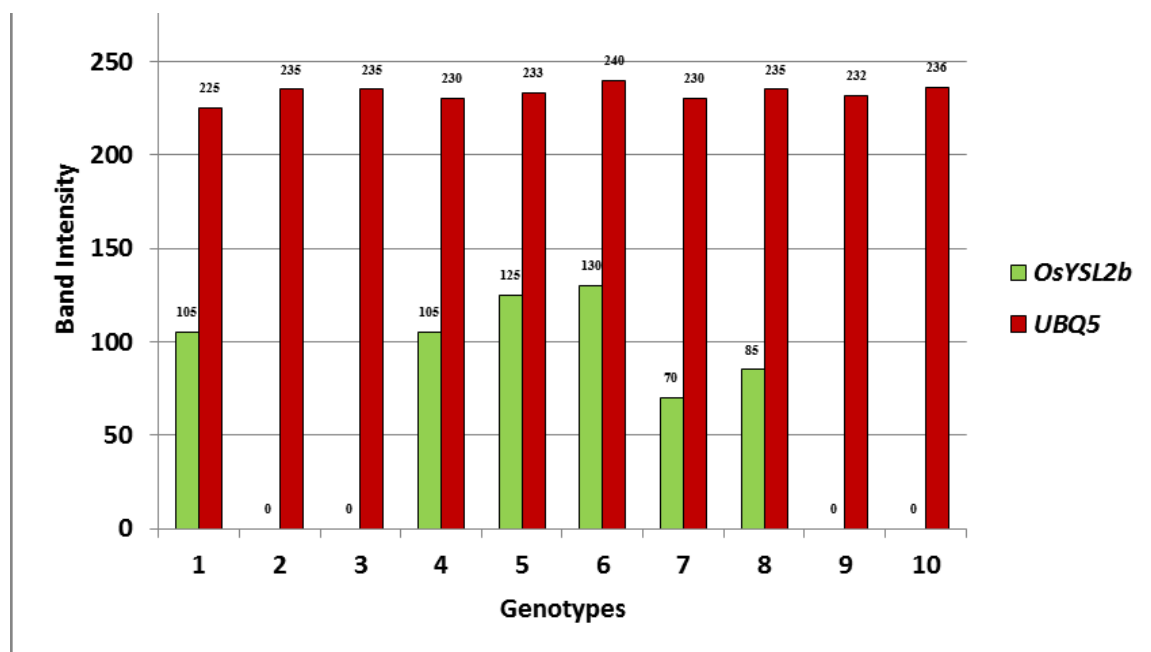
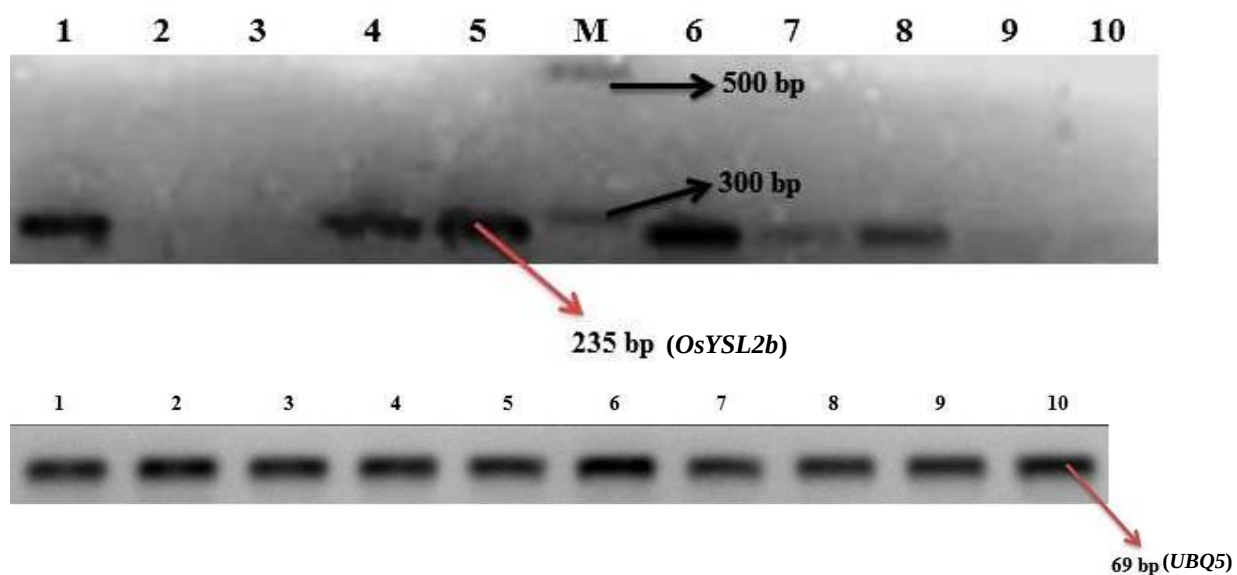


Plate 15a: Relative expression of *OsYSL2b* in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

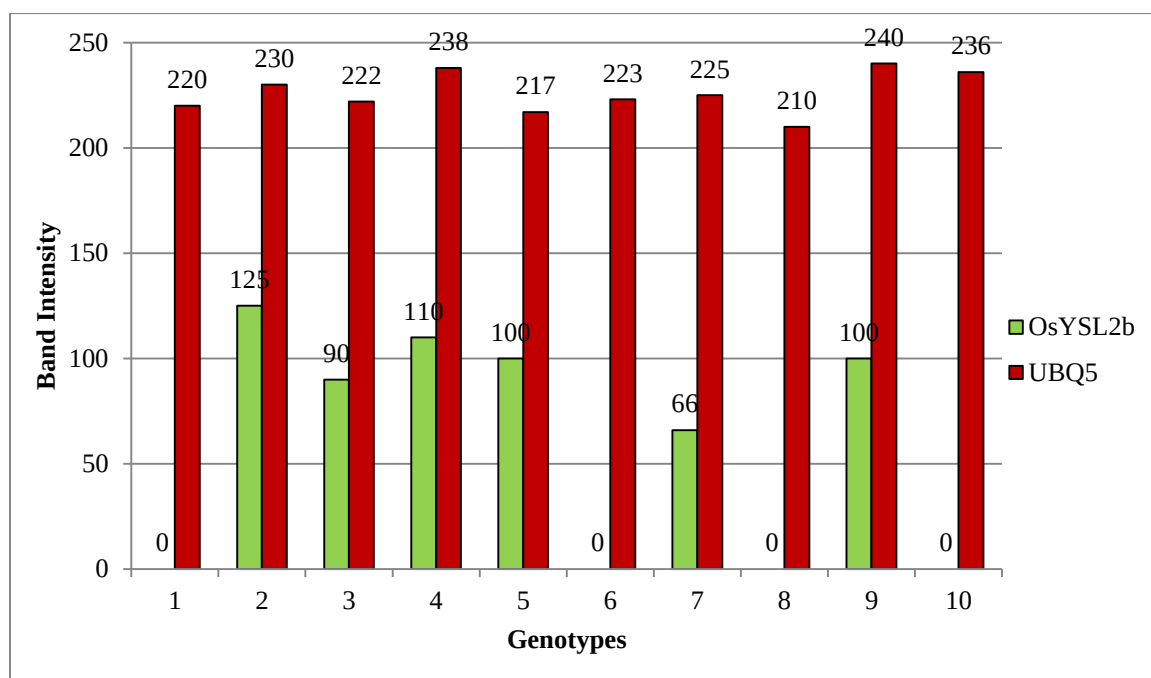
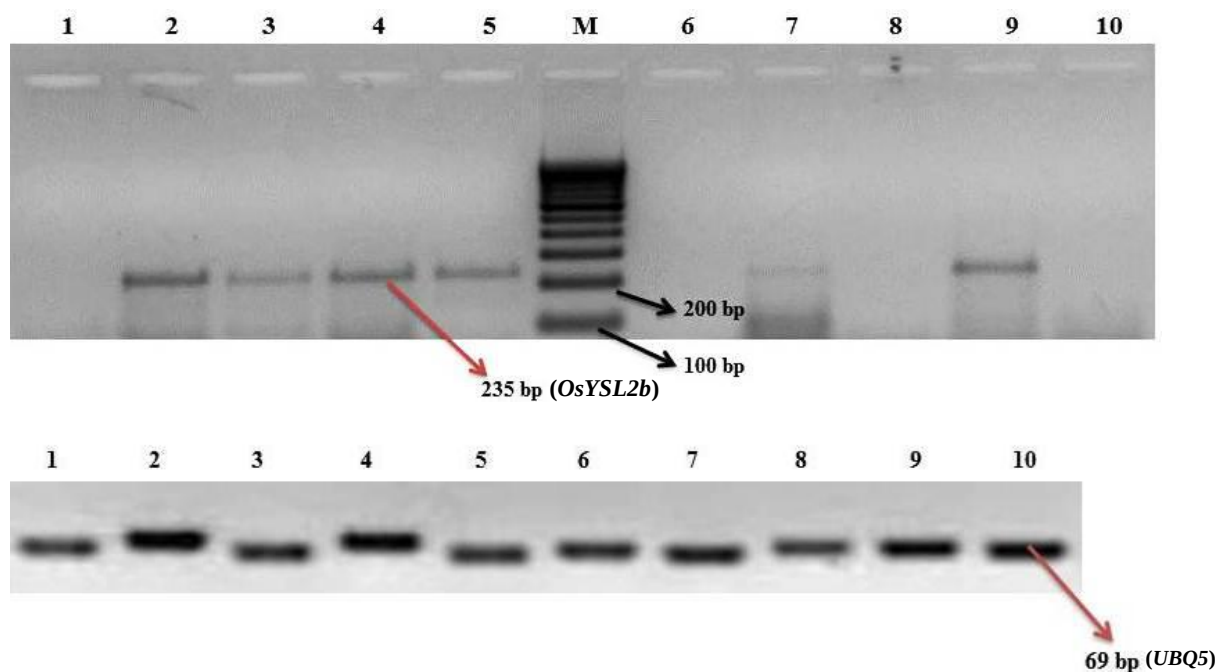


Plate 15b: Relative expression of *OsYSL2b* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

In embryo, expression was found relatively at higher levels in AM 72 when compared with other genotypes under study. Lower relative expression was observed in AM 143 and negligibly low or no expression was observed in embryo of Azucena, AM 1, Subhadra and TKM 9. In endosperm, expression of OsYSL2b was found to be relatively higher in Moroberekan than in other genotypes under study lower expression was found in AM 143. Very little or no expression was observed for OsYSL2b in endosperm of Moroberekan, AM 72, ARB 6 and TKM 9.

4.3.6.7 OsYSL2c

The results obtained for semi quantitative RT-PCR analysis for OsYSL2c in embryo and endosperm of selected genotypes is displayed in Plate 16a and 16b respectively.

Among embryo and endosperm, expression of OsYSL2c was found to be relatively higher in embryo than in endosperm for all the genotypes under study.

In embryo, expression of OsYSL2c was found to be relatively higher in AM 72 than in other genotypes selected for study whereas, lower relative expression was found in Moroberekan and negligible or no expression was observed in embryo of Azucena, ARB 6 and Subhadra. In endosperm, expression of OsYSL2c was found to be relatively higher in AM 1 than in other selected genotypes, lower relative expression was found in TKM 9 and very little or no expression was observed for OsYSL2c in endosperm of AM 72, AM 143, ARB 6 and Subhadra.

4.3.6.8 OsYSL15b

The results obtained from semi quantitative RT-PCR analysis for OsYSL15b in embryo and endosperm in selected genotypes are displayed in Plate 17a and 17b respectively.

The expression of OsYSL15b was found to be relatively higher in endosperm than in embryo among the selected genotypes except Azucena and AM 72 where OsYSL15b expression was relatively higher in embryo than in endosperm.

In embryo, relative expression of OsYSL15b was found to be higher for Moroberekan than for other genotypes under study. Lower relative expression for the gene was observed in AM 143 and negligible or no expression of OsYSL15b was observed in embryo of ARB 6. In endosperm, the expression of OsYSL15b was relatively higher in ARB 6 than in other genotypes selected and lower relative expression was observed in AM 72.

4.3.6.9 OsYSL18b

Results from relative expression study carried out through semi quantitative RT-PCR analysis for OsYSL18b in embryo and endosperm among selected genotypes is displayed in Plate 18a and 18b respectively.

Relative expression of OsYSL18b was found to be higher in embryo than in endosperm in all the selected genotypes except Moroberekan in which expression was found to be relatively higher in endosperm than that in the embryo.

In embryo, relative expression of OsYSL18b was found to be equally higher in Moroberekan and ARB 6 and negligible or no expression was found in AM 72. In endosperm, higher relative expression was found in Moroberekan. Lower relative expression of OsYSL18b was observed in TKM 9 for both embryo and endosperm.

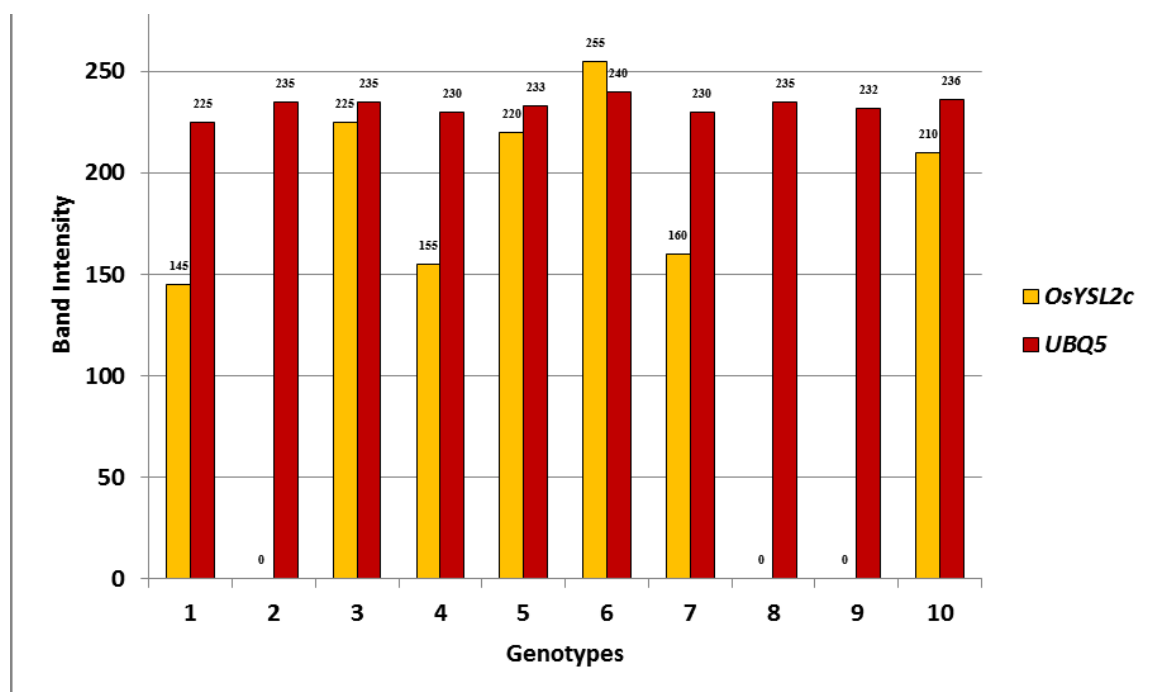
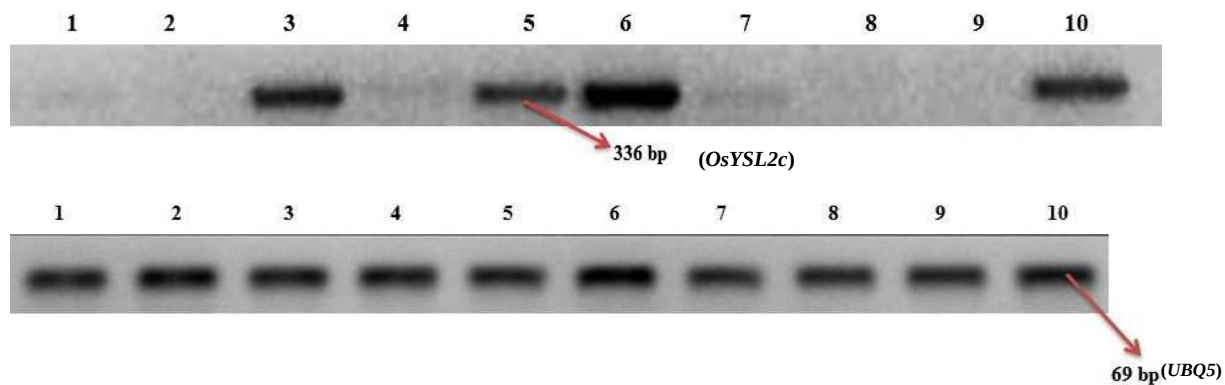


Plate 16a: Relative expression of *OsYSL2c* in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

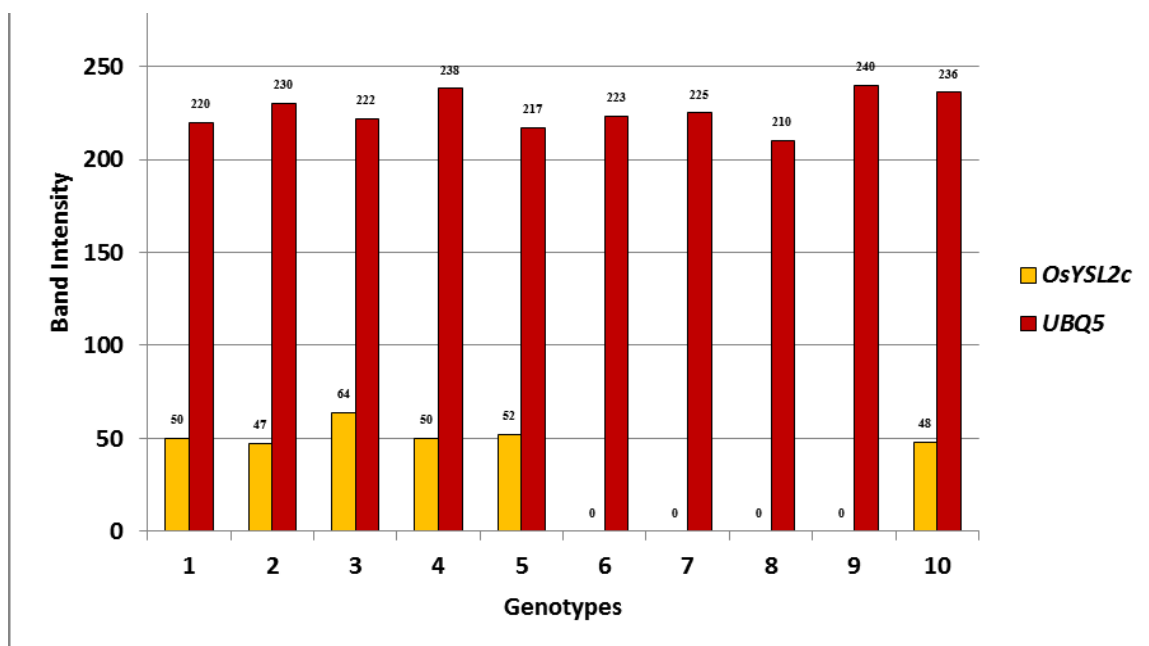
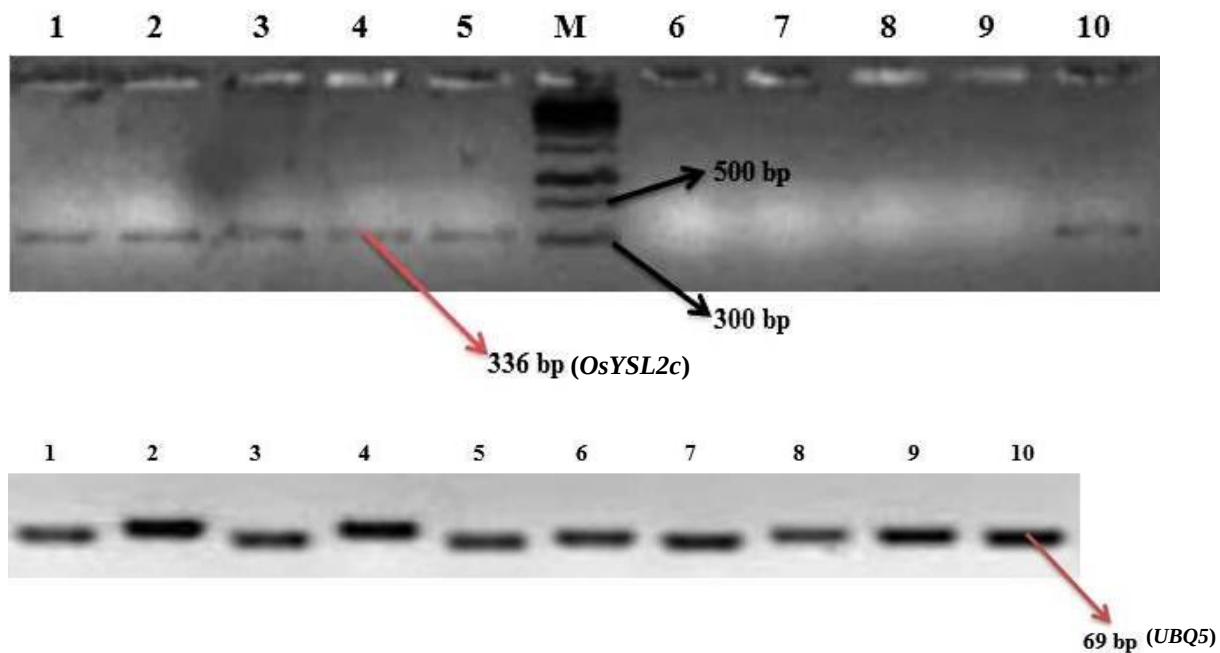


Plate 16b: Relative expression of *OsYSL2c* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

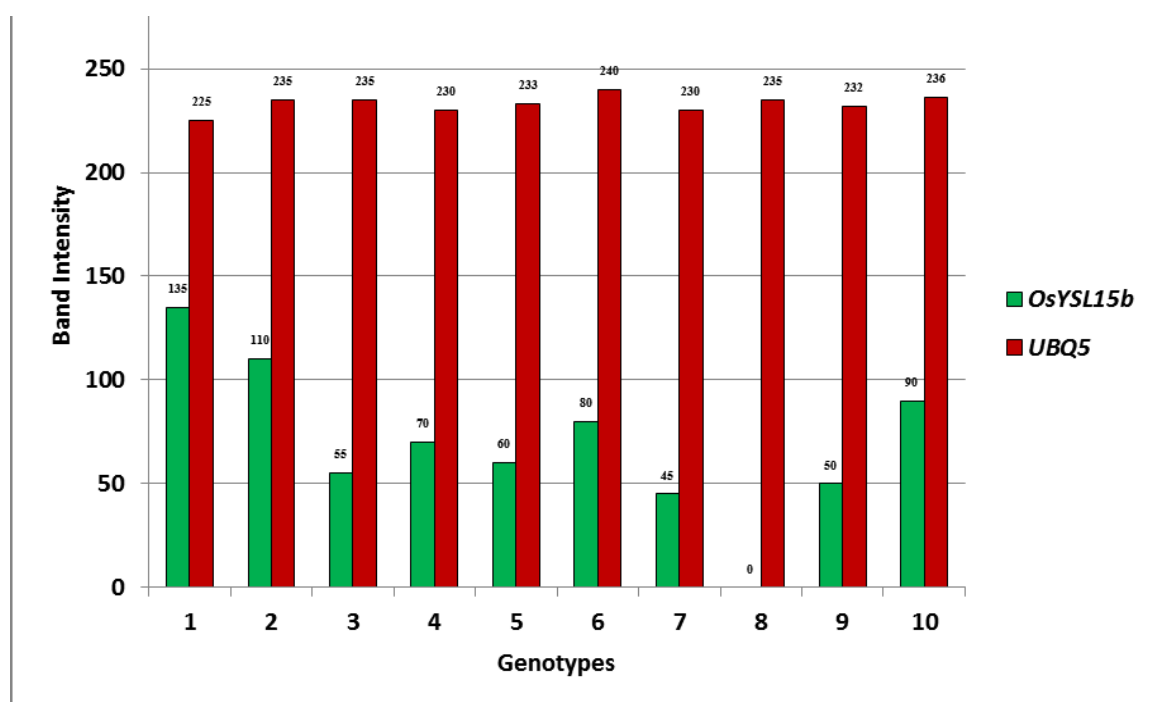
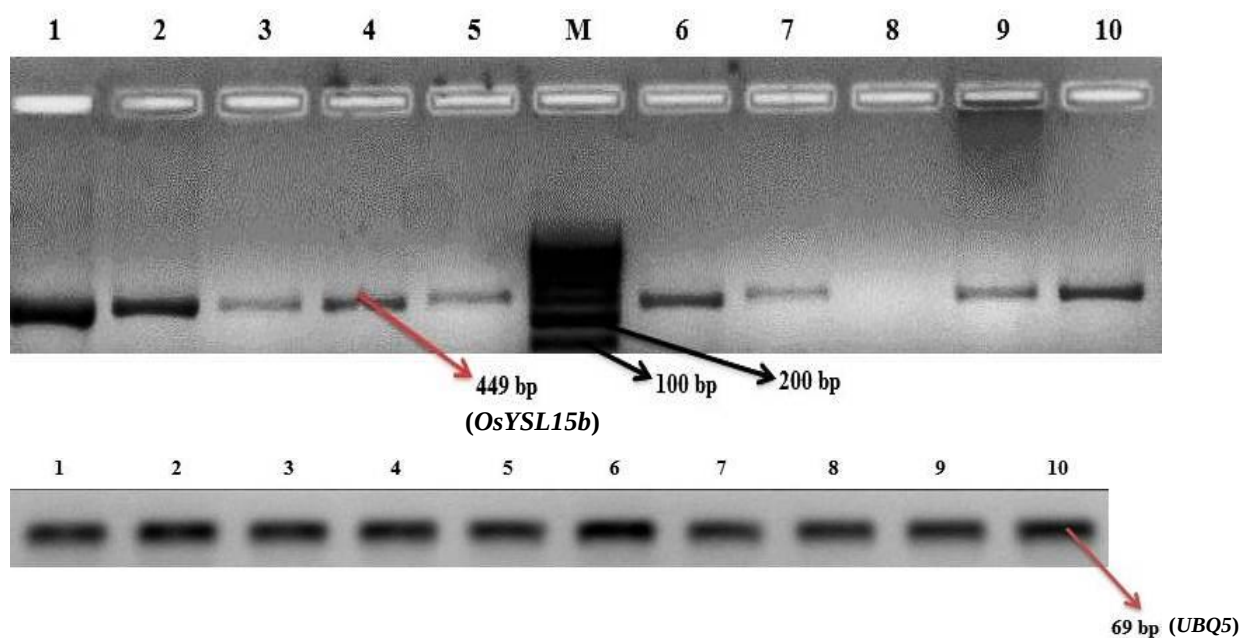


Plate 17a: Relative expression of *OsYSL15b* in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

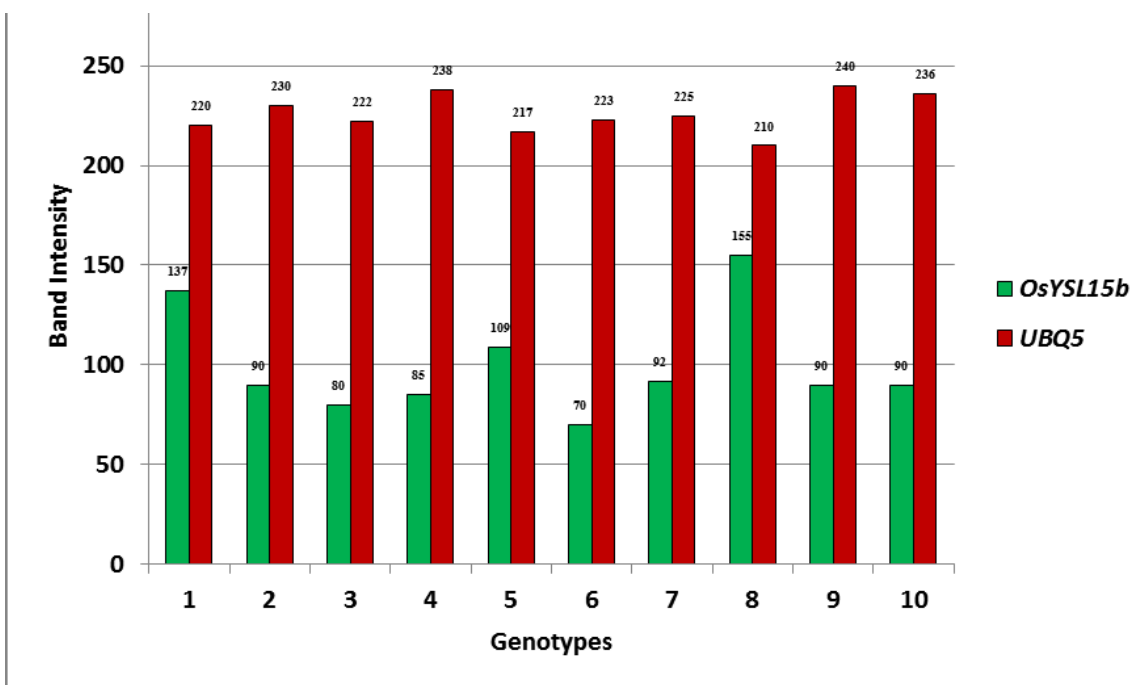
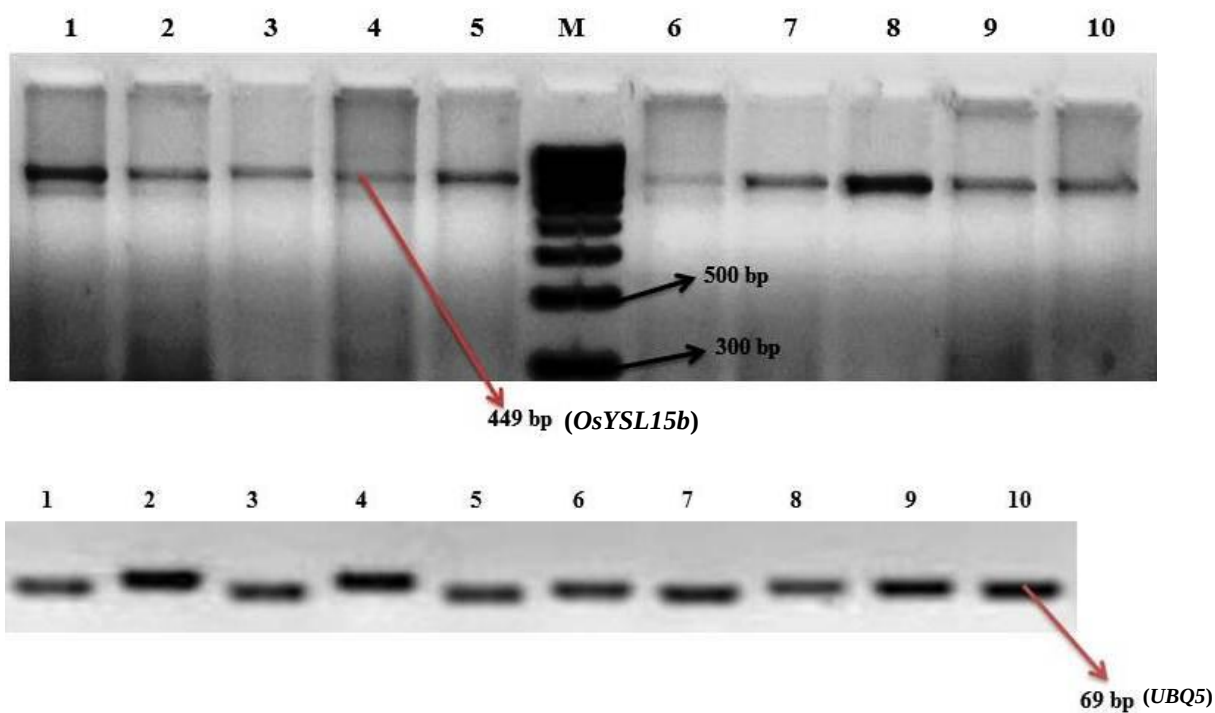


Plate 17b: Relative expression of *OsYSL15b* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

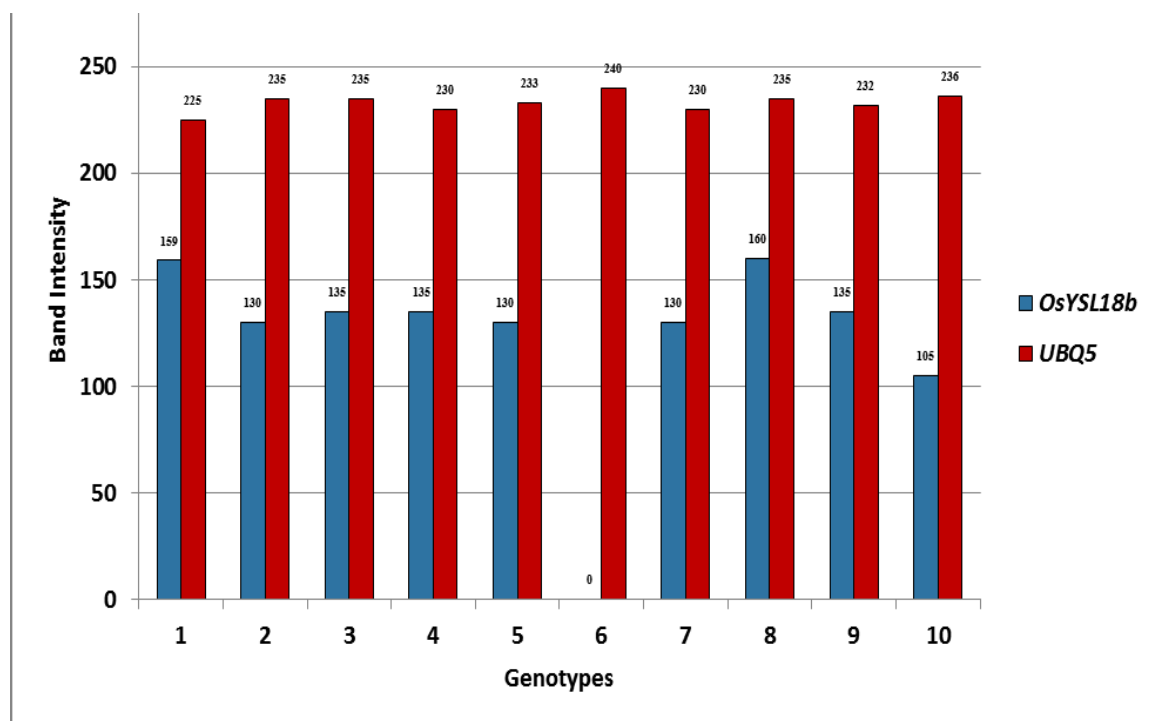
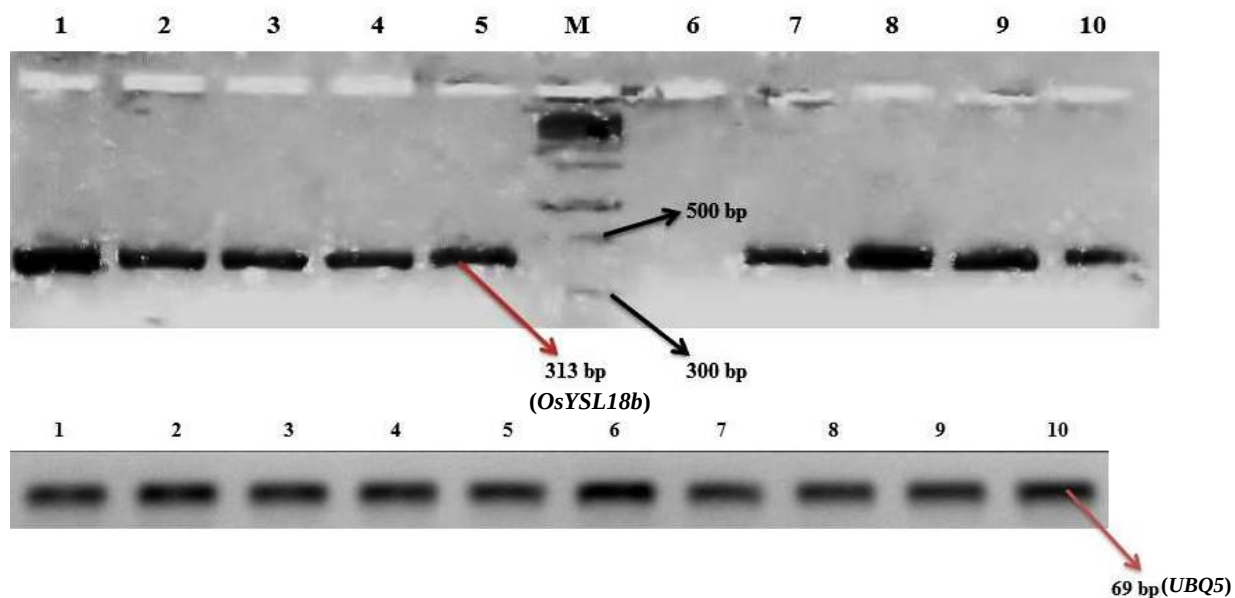


Plate 18a: Relative expression of *OsYSL18b* in embryo of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

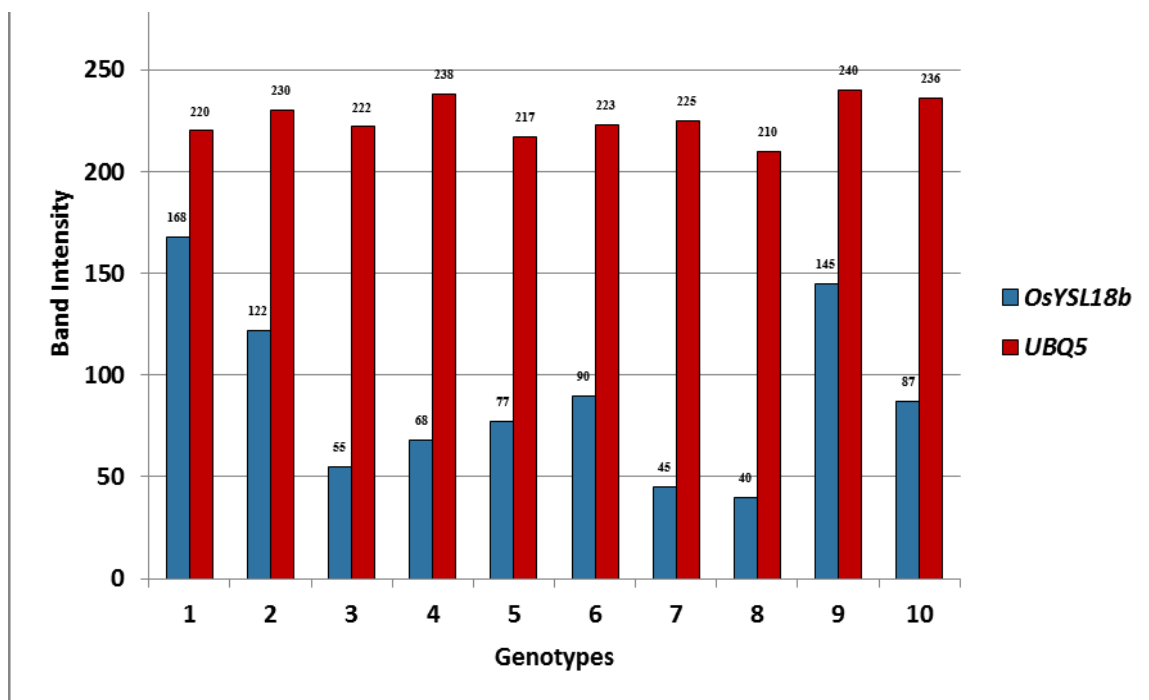
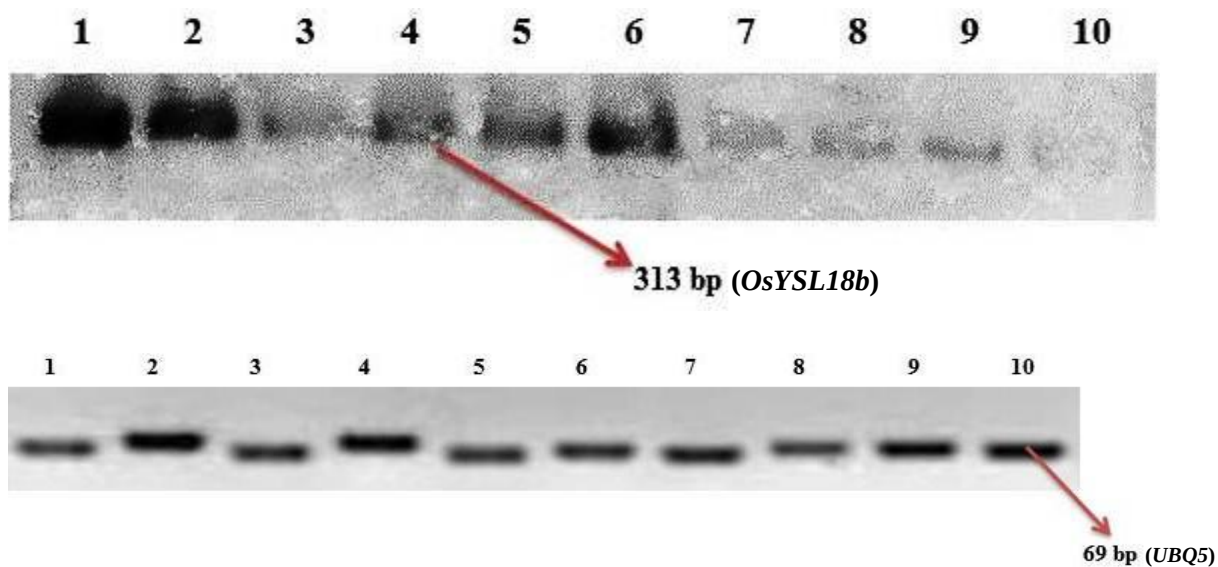


Plate 18b: Relative expression of *OsYSL18b* in endosperm of selected genotypes

Legend:

1- Moroberekan, 2- Azucena, 3- AM 1, 4-AM 65, 5- AM 94B, 6- AM 72, 7- AM 143, 8-ARB 6, 9- Subhadra, 10- TKM 9

V DISCUSSION

Rice is one of the staple food crops for the people living in South-Asian region. It forms a major part of diet in India. But, when it comes to contribution of this staple food in coping up the micronutrient requirements of the population, a poor contribution was reported.

Relative distribution of micronutrients within different parts of rice grain suggests the localization of major part of seed micronutrients within embryo, husk, and aleurone layer. These parts were reported to be lost during the process of milling and polishing.

Milling and polishing of rice grains is an inevitable process that has to be done to preserve the grains over long term. Because, embryo part of rice is rich in oil content. This would render the whole seed to become rancid over long term storage. So, polishing is done to remove the part which causes rancidity thereby providing scope for long term storage of rice grain. Hence, polishing could be considered as the necessary evil.

It has been reported that major part of micronutrients are concentrated in embryo, husk and aleurone part. However, some genotypes show high micronutrient content in seeds even after polishing due to remobilization of seed micronutrient into endosperm.

There are several seed specific transporters reported in rice which take part in localization and homeostasis of micronutrient within grain. Relative expression of such seed specific transporter within different parts of rice grain would therefore help in discerning the importance of transporters in localization of micronutrients in specific tissues.

Accumulation of Zn and Fe is desirable in endosperm rather than in embryo from consumer point of view. So, such genotypes are said to be desirable in which the transporters responsible for Zn and Fe accumulation are unregulated in endosperm and simultaneously downregulated in embryo, particularly, transporters that play a significant role in Zn and Fe accumulation in seed tissues.

The results obtained from the present study are discussed below.

5.1 Phenotyping of rice genotypes for high zinc and iron content

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. The range and standard deviation values calculated from different study traits reveals high variability in plant height, number of tillers, shoot dry weight, grain size, days to first flowering, grain yield per plant, and harvest index. Similar results were obtained from Hemareddy (1993); Mehetre *et al.* (1994); Zhu *et al.* (1998); and Prasad *et al.* (2001).

Improvement of any trait depends on the existence of the genetic variability and also the heritability. Any attempt to improve traits without the knowledge of heritability would be of no use. 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).

High coefficient of variation, high heritability and high genetic advance as per mean was observed for number of tillers, days to 50 per cent flowering, harvest index and grain yield per plant.

High GCV as well as high PCV was observed for plant height, shoot dry weight, days to 50 per cent flowering, days to maturity, and grain size. These traits with high GCV and PCV exhibit the high variability available for these traits among different rice genotypes and scope for improvement of the traits through breeding programme. Similar results were reported by Prasad *et al.* (2001); Vanaja and Babu (2006); Sabesan *et al.* (2009); and Seyoum *et al.* (2012).

High GCV was observed for plant height, shoot dry weight, days to 50 per cent flowering, days to maturity and grain size which indicates that improvement of these traits is possible through continuous selection process. Low GCV coupled with low genetic advance as per mean was observed for days to first flowering, zinc productivity per day, iron productivity per day, grain length, grain breadth and grain width which indicated that the variation that was observed for these traits is non-heritable because of high environment effect and other factors.

High genetic advance as per mean was observed for plant height, shoot dry weight, grain yield per plant, harvest index, productivity per day per plant, length-breadth ratio, grain size and hundred grain weight which indicated that majority of variation observed for these traits is attributed through additive gene effects. Also, difference between GCV and PCV was found to be low for plant height, iron productivity per day, zinc productivity per day, productivity per day per plant, grain breadth, grain width, length-breadth ratio, and hundred grain weight which revealed less or low environmental effect on these traits (majority of variation attributed to genotypic and phenotypic effect).

High heritability coupled with high genetic advance as per mean was observed for plant height, shoot dry weight, length-breadth ratio, and hundred grain weight, whereas moderate heritability and high genetic advance as per mean was observed for grain size in rice which indicated that these traits can be improved efficiently than other traits in the future generations through selection process.

Traits like panicle length, days to flowering, days to 50 per cent flowering, days to maturity, zinc and iron productivity per day, grain length and width showed extremely low heritability (<10 %) which indicates that improvement of these traits through selection in future generations is considerably difficult and virtually impractical due to masking effect of environment. Hence, special selection strategies have to be tried for improving these traits.

5.1.2 Performance of genotypes with respect to zinc and iron content in brown rice

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

High genetic advance as per mean was observed for brown rice zinc 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 analysis of data

5.1.3.1 Analysis of variance

The phenotypic variation manifested by the genotype has two components namely genotypic and environmental. Analysis of variance of all the 23 characters revealed that significant differences among the means of all genotypes for plant height shoot dry weight, grain yield per plant, panicle length, days to 50 per cent flowering, days to maturity, grain length, grain breadth, length-breadth ratio, grain size, hundred grain weight and brown rice zinc content. 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.

5.1.3.2 Correlation studies

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.

Significant negative correlation was found between plant height and harvest index which is in turn significantly correlated in a positive manner with number of productive

tillers. These results reveal that, high harvest index bearing rice plants could be obtained by indirectly selecting for high number of productive tillers with short stature.

Number of tillers and number of productive tillers were found to be significantly correlated in a negative manner to days to first flowering, days to 50 per cent flowering and days to maturity. These results suggest that, the rice plants with less number of tillers and number of productive tillers would mature lately. Or in other words, late maturing genotypes would have less number of tillers and number of productive tillers. Similar kinds of results were obtained by Zhu *et al.* (1998); Li *et al.* (2003); and Tao *et al.* (2006).

Significant positive correlation was found for zinc productivity per day and iron productivity per day with shoot dry weight and number of productive tillers respectively. Hence, selection of genotypes with high number of productive tillers and high biomass would result in selection of genotypes with high zinc and iron productivity per day but with late maturity as number of productive tillers is significantly negatively correlated with days to maturity.

No significant positive or negative correlation was observed for grain yield with any of the traits that were studied. In such cases, division of correlation into direct and indirect effect would help in discerning the hidden correlation of grain yield with other traits.

5.1.3.3 Path-coefficient analysis

The relationship between grain yield and its components may be negative or positive but it is the net result of direct effect of that particular trait and indirect effects *via* other traits. Hence, it is necessary to determine the path-coefficients which partition the observed correlation into direct and indirect effects and also reveals the cause and effect relationship between grain yield and their related traits.

Path coefficient analysis indicated highest direct positive effect of shoot dry weight on grain yield per plant, lowest direct positive effect of days to first flowering. Highest direct negative effect was observed for iron productivity per day on grain yield, and lowest direct negative effect for number of tillers.

If indirect effects were considered, highest indirect positive effect was observed for iron productivity per day *via* days to maturity on grain yield, lowest indirect positive effect observed for zinc productivity per day *via* productivity per day per plant.

Highest indirect negative effect was recorded for iron productivity per day *via* zinc productivity per day while lowest indirect negative effect was observed for days to 50 per cent flowering *via* plant height, number of tillers, number of productive tillers, and iron productivity per day.

Hence, from the above obtained results, it could be suggested that selection based on shoot dry weight would be most effective strategy in order to obtain higher yield as

shoot dry weight had highest direct positive effect on grain yield per plant. In contrast, iron productivity per day revealed highest direct negative effect and indirect negative effect on grain yield *via* zinc productivity per day. From above revealing, it could be summarized that, although iron productivity per day had highest positive indirect effect *via* days to maturity on grain yield per plant, selection based on iron productivity per day would not be feasible in order to obtain higher yields as it exhibited highest direct negative effect on grain yield.

5.2 Study the distribution of zinc and iron in different parts of rice grain

5.2.1 Zinc and iron content in different parts of rice grain

Statistical parameters that indicate variability of a trait, range and standard deviation were found to be high for white rice iron content which revealed high amount of variability for that trait. The trait also exhibited high range value which confirmed the extreme variability for white rice iron content among the study genotypes.

High GCV and high PCV was observed for brown rice zinc content and white rice iron content which revealed major part of variability due to genetic effects. Moderate GCV and PCV were observed for white rice zinc content.

High genetic advance as per mean was observed for brown rice zinc content, white rice zinc content, and white rice iron content which revealed that majority of variation exhibited by these traits were attributed by additive gene effects.

High heritability coupled with high genetic advance as per mean was observed for brown rice zinc content, white rice zinc content and white rice iron content which suggested the scope of including these characters in crop improvement and breeding programme.

Low GCV and low PCV was observed for brown rice iron content which suggested that any variation that was exhibited by this trait was non-heritable and hence could not be improved through selection based breeding programme. On the other hand, low difference between GCV and PCV was observed for white rice zinc content, brown rice zinc content and white rice iron content which indicated that environment effect in causing variability in these three traits is subtle.

5.2.2 Statistical analysis of data recorded

5.2.2.1 Analysis of variance

Results from variance analysis revealed significant differences from mean values in all genotypes studied for brown rice zinc content, white rice zinc content and white rice iron content. But, no significance was found for variability in brown rice iron content. The results suggested that, there is a wide scope for improvement of BZC, WZC and WFC through selection based breeding programmes. Also, 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.

5.2.2.2 Correlation analysis

Correlation analysis of all four characters revealed significant positive correlation of brown rice zinc content with white rice zinc content which indicated that rice genotypes exhibiting high zinc content in brown rice contained high zinc content in white rice (after polishing) with least loss during polishing process. Significant negative correlation was found for percent loss of iron due to polishing with harvest index and white rice iron content.

No significant correlation was found for other traits which indicate difficulty in prediction of zinc and iron content in brown rice and white rice and in predicting the loss due to polishing.

5.2.2.3 Path-coefficient analysis

Division of correlation coefficients into direct and indirect coefficients revealed highest direct positive effect of brown rice zinc content and lowest direct positive effect of brown rice iron content on grain yield per plant. Direct negative effect on grain yield per plant was exhibited by white rice zinc content.

Among indirect effects, highest indirect positive effect was observed for brown rice zinc content *via*. white rice zinc content. Lowest indirect positive effect was observed for white rice iron content *via*. white rice zinc content.

Highest indirect negative effect was exhibited by white rice zinc content *via* brown rice zinc content whereas lowest indirect negative effect was observed for brown rice iron content *via* brown rice zinc content.

From the above obtained results, it could be summarized that selection based on high brown rice zinc content directly result in obtaining high grain yielding genotype as brown rice zinc content has direct positive effect on grain yield. It should be noted that, although brown rice zinc content exhibited indirect negative effect on grain yield *via*. white rice zinc content, its direct positive effect is more in magnitude than its indirect negative effect. As a result, selection of rice genotypes with high zinc content in brown rice would indirectly select for genotypes with high yield and low zinc content in white rice.

5.3 Molecular characterization of zinc and iron transporters in rice grain

5.3.1 Zinc and iron transporters

Several 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. Hence, study of these genes at the transcript level would help in correlating 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.

5.3.2 Primer designing

Advances in the field of bioinformatics have become a boon for researchers working at the molecular level. The bioinformatics tools available in NCBI, Primer 3, RiceXPro, Gramene are helping the researchers to annotate, discern the variation at sequence level for same genes in different crops, design gene specific primers. NCBI is a repository of sequence information where several bioinformatics tools like 'BLAST' search tool, 'refseq mRNA' tool to get mRNA sequence of a gene, 'pick primers' tool to design primers specific to input nucleotide sequence.

Some of the primers designed in NCBI database using several bio-informatics tools mentioned above resulted in amplification, where some other gave no amplification. It might be due to reason that primers designed in NCBI was based on sequences of *japonica* rice available in the database and using such primers to amplify sequences in *indica* rice could not give amplification due to genomic variation at sequence level between *japonica* and *indica* rice.

5.3.3 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.

RNA isolated from different parts of rice grain like embryo and endosperm were quantified and checked for quality through Nanodrop (Eppendorf Biospectrometer). Results suggested that both quality and quantity of RNA isolated was high in embryo than in the endosperm. This might be because; presence of RNase and other impurities that come in the way of RNA isolation would be higher in endosperm than in the embryo. Hence, isolation of good quality RNA at optimum quantity from endosperm was challenging. The similar problem was reported by Li and Trick (2005) for which they designed a rapid RNA isolation protocol (see 3.3.5.2. for detailed protocol). The same protocol was followed in the present study also for total RNA isolation from embryo and endosperm.

5.3.4 PCR optimization and cDNA normalization

PCR optimization and cDNA normalization is one of the important steps in semi-quantitative RT-PCR. cDNA normalization was done by using reference genes. Reference genes are also called as housekeeping genes which express in all the tissues at the same levels for a given organism. In the present study, *UBQ5* (Ubiquitin5) gene was used as reference gene. As the expression of housekeeping genes should be same in all genotypes, any change in expression level reflects pipetting error. The results from cDNA normalization in present study suggest that the expression of reference gene is at the same level in all the genotypes for both the tissue types.

PCR optimization was done for optimum $MgCl_2$ concentration, optimum annealing temperature for all sets of primers used, optimum cycle number for a given set of primer, effect of addition of BSA on amplification, optimum primer concentration.

Optimum $MgCl_2$ concentration was found to be 2.5 mM for all the sets of primers in all the genotypes. Actual optimum annealing temperature differed from the calculated annealing temperature by about 2 °C for all primers used in the study. Optimum cycle number ranged from 35-40 cycles for all the sets of primers used. Cycle number at which stationary phase had reached was found to be least for OsNAS1a in embryo and OsZIP4b in endosperm which indicates maximum copy number of these genes when compared to others genes in the respective tissues.

1 μ L of BSA was used for all PCR reactions as the addition of BSA was found to improve the amplification significantly. The reason for improved amplification might be due to reasons that BSA stabilizes *Taq* polymerase thereby increasing its activity, and also it prevents the adhesion of enzymes and PCR components to the reaction tubes and tip surfaces. Similar positive effects for BSA on PCR was found by Kreader (1996); Nagai *et al.* (1997); Bessetti (2007) and Farell and Alexandre (2012). Different concentrations of forward and reverse primers were found to effect the amplification significantly. As the primer concentration decreased, amplification of target sequence was also decreased and higher concentrations of primers were found to form primer dimers which come in the way of amplification of target sequence. So, optimum concentration of primers is that concentration where concentration is high enough to amplify the target sequence, but low enough to form primer dimers that come in the way of relative quantification. Optimum concentration of primers in the present study ranged from 0.5 μ L-0.75 μ L for forward primer and 0.5 μ L-1 μ L for reverse primer.

5.3.5 SQ RT-PCR

Reverse transcription can be done broadly in two ways. One is one step RT-PCR, another is two step RT-PCR. In one step RT-PCR, reverse transcription and amplification of target cDNA would occur in single tube in an automatic sequential manner. This kind of RT-PCR was recommended when chances of contamination and need for high sensitivity are more. 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 flexibility in choosing primers and enzymes. In the present study, since high sensitivity was required and chances of contamination are more, a two-step RT-PCR was followed.

5.3.6 Relative expression of transporter genes under study

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

The results suggest that, in embryo and endosperm, expression of *UBQ5* was at same level in all the genotypes studied.

Expression of *OsZIP4a* was found to be relatively higher in embryo than in endosperm in all the genotypes studied where contrasting results were obtained for *OsZIP4b*. This variation in the transcript level of two different regions of same gene might be due to differences in complementarity between primes designed and target sequence. Among different genotypes studied, expression of *OsZIP4a* and *OsZIP4b* was found to be higher in embryo of Moroberekan. Moroberekan also showed higher percentage loss of zinc due to polishing, which suggested localization of zinc in bran or embryo part of seeds. Higher expression of *OsZIP4a* and *OsZIP4b* in embryo and localization of major part of seed zinc in bran reveals that *OsZIP4* plays an important role in localization of zinc in embryo or transporting zinc from other parts to embryo. Similar kind of results were observed by Bashir *et al.* (2011) where expression of *OsZIP4* within embryo than in endosperm increased consistently from 5 days after flowering up to 28 days after flowering.

Expression of *OsFER1c* was found to be relatively higher in endosperm than in embryo which suggests that *OsFER1* transports more iron from maternal tissues to endosperm than that from endosperm to embryo. This means that major part of iron would be localized in endosperm of genotypes which show relatively high expression of *OsFER1* in endosperm. In present study, expression of *OsFER1* was found to be relatively higher in endosperm than in embryo for AM 94B. In fact, expression of *OsFER1* was negligibly low or nil in embryo of AM 94B. On the other hand, localization of iron was found to be more in endosperm than in embryo as percent loss of iron after polishing was found to be least for AM 94B than any other genotypes. By correlating both results, it can be concluded that, *OsFER1* plays an important role in localization of most of the seed iron in endosperm thereby preventing the much loss of iron during the process of polishing.

Expression of *OsNAS1* was found to be relatively higher in embryo than that in endosperm in almost all the genotypes that were studied. Moreover, expression of *OsNAS1* was found to be relatively higher in embryo of Moroberekan and relatively low in AM 94B embryo. On the other hand, percent loss of iron due to polishing was found to be relatively higher for Moroberekan than for AM 94B which indicates higher fraction of seed iron localized in embryo of Moroberekan, and lower fraction of seed iron was localized in embryo of AM 94B. By correlating the above results, it could be hypothesized that *OsNAS1* plays an important role in localizing the seed iron in the tissues where it is upregulated.

Expression of *OsNAS2* was found to be relatively higher in embryo than in endosperm in all the genotypes that were studied. Relatively higher expression of *OsNAS2* was found in Moroberekan embryo and relatively lower expression was observed in AM 65. On the other hand, loss of iron due to polishing was relatively higher for Moroberekan than that for AM 65. Above two outcomes suggests that, relatively more amount of iron was localized in embryo of such genotypes where the expression of *OsNAS2* was found to be relatively higher in embryo.

Expression of *OsYSL2b* and *OsYSL2c* was found to be relatively high in embryo of AM 72. Relatively low expression was found in embryo of AM 143. On the other hand, loss of iron due to polishing was also relatively high for AM 72 than for AM 143. This suggests that the role of *OsYSL2* is to localize iron in tissues where it is upregulated or overexpressed.

Expression of *OsYSL15b* was found to be relatively higher in endosperm than in the embryo in all the genotypes that were studied. For embryo, relative higher and lower expression was found in Moroberekan and AM 143 respectively. For endosperm, relatively higher and lower expression was found to be in ARB 6 and AM 72 respectively. Accordingly, percent loss of iron due to polishing was found to be higher for Moroberekan and ARB 6 than for AM 143 and AM 72 respectively. These results suggest that, relatively higher amount of iron would be localized in the tissues where expression of *OsYSL15* is relatively high.

Expression of *OsYSL18b* was found to be negligibly low or nil in embryo of AM 72, but the gene was expressed in endosperm of AM 72. On the other hand, loss of iron due to polishing in AM 72 was found to be highest than any other genotypes that were studied, which suggest localization of major part of seed iron in embryo part of AM 72. But, expression studies suggest the inverse context. Hence, it indicates that loss of iron due to polishing could not be explained by the relatively high expression of *OsYSL18* in endosperm. So, there was no correlation found between the relative expression of *OsYSL18* and localization of iron in different rice grain parts.

FUTURE LINE OF WORK

- Validation of results obtained in this study could be done.
- Relative expression studies of some more genes like *OsNRAMP*, *OsDMAS*, *OsNAAT*, *OsZIP3* 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).

VI SUMMARY

In the present study, 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.

Significant difference was found among the genotypes for grain yield per plant, days to 50 per cent flowering, days to maturity, test weight, zinc content in brown rice and percent loss of iron due to polishing. Moroberekan had the highest zinc content before and after polishing, and highest iron content before polishing. AM 94B had the highest iron content even after polishing.

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.

It is notable that, extent of loss of iron due to polishing depended on harvest index of the genotype and brown rice iron content.

High GCV and PCV coupled with high heritability and genetic advance as per mean was observed for plant height, shoot dry weight, grain size, brown rice zinc content, and white rice iron content. High PCV, heritability and GAM was observed for grain yield per plant. High heritability and GAM was observed for white rice zinc content.

Significant positive correlation was obtained for plant height, and zinc productivity per day with shoot dry weight. Significant positive correlation was also observed for number of productive tillers with harvest index and iron productivity per day. Significant positive correlation was also observed for brown rice iron content with test weight and brown rice zinc content with white rice zinc content.

Direct positive effect on grain yield was observed for shoot dry weight, productivity per day per plant, grain length, brown rice zinc content, and white rice iron content.

Molecular analysis of relative expression of seven seed specific transporters was done for ten genotypes using ten primers including reference gene (housekeeping gene). Almost all the genes except OsZIP4b and OsFER1c, the expression was found to be relatively higher in embryo than in endosperm.

Among all genotypes studied, 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.

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. AM 1 that showed less percent loss of zinc due to polishing had relatively higher expression of *OsYSL15b* in its endosperm and relatively lower or negligible expression of *OsYSL15b* and *OsZIP4b* in its embryo. AM 94B that showed less percent loss of iron due to polishing had relatively higher expression of *OsZIP4b*, *OsNAS1a* and *OsYSL15b* in its endosperm than that in embryo.

Results suggest that, out of seven seed specific genes, *OsZIP4*, *OsYSL2*, *OsYSL15* and *OsNAS2* play an important role in accumulation of zinc and iron in different parts of rice grain.

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