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ORIGINAL PAPER

Development of an Iron-enriched High-yieldings *Indica* Rice Cultivar by Introgression of A High-iron Trait from Transgenic Iron-biofortified Rice

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Abstract Low level of iron in staple food crops is one reason for the predominance of iron-deficiency anemia in developing countries. Most of the iron in rice grains accumulates in the outer aleurone layer and embryo, which are removed during milling, and the edible endosperm contains very low amounts of iron. In an effort to increase iron nutrition, we report here the transgene introgression of a high-iron trait into a high-yielding *indica* rice cultivar. The *ferritin* gene from soybean (*soyfer1*) was introduced into rice plants through interbreeding between soybean *ferritin*-overexpressing transgenic IR68144 and the high-yielding cultivar Swarna. The stable integration of the *soyfer1* gene was confirmed in the BC_2F_4 generation, and the hybrid seeds showed 2.6-fold soybean *ferritin* gene expression over the recurrent parent Swarna. The hybrid milled seeds revealed a 2.54-fold increase in iron and 1.54-fold increase in zinc compared to Swarna. Agronomic data and an SSR marker analysis of the hybrid rice plants were taken into account for NIL character identification.

Keywords Introgression · *Ferritin* · Overexpression · Iron · Rice · Near-isogenic lines

Abbreviations

IDA	Iron-deficiency Anemia
DW	Dry Weight
NIL	Near-isogenic Line
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
QTL	Quantitative Trait Locus
RIL	Recombinant Inbred Lines
SSR	Simple Sequence Repeat

Introduction

Iron is an essential nutritional mineral and its deficiency leads to the major nutritional disorder IDA along with several neurological disorders [1]. Dietary iron deficiency or poor iron bioavailability is the foremost reason for IDA [2] Cassava, a staple food in Africa, contains very low levels of iron and zinc [3]. Iron bioavailability can be increased by the ingestion of commercially available supplements, but most are too expensive for routine consumption in developing countries. Therefore, the development of iron-biofortified food is preferable to the use of iron supplementation [4].

Rice is a staple food crop for more than half of world's population yet provides very low amounts of nutritional minerals in the consumable endosperm [5, 6]. Genotypic variation in grain iron concentrations has been extensively studied among rice varieties [7, 8]. In brown rice, a wide range of both iron (6.3–24.4 μg/g seed dry weight) and zinc (13.5–58.4 μg/g seed dry weight) has been observed [7], but such rice varieties do not show considerable iron enrichment in milled rice grains [9]. Several biotechnological approaches have been successfully implemented in developing high-iron

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rice [10]; accordingly, transgene integration may emerge as an additive strategy for generating iron-biofortified, local high-yielding *indica* rice cultivars. In addition, the transgene introgression strategy has already been proven to be effective for improving the nutritional quality of *indica* rice grains exhibiting high carotenoid contents in the seeds [11].

Ferritin, a large protein molecule, can store approximately 4500 iron atoms in a bioavailable form. The overexpression of the *ferritin* gene in seeds increases the demand for iron in transgenic seeds, and the resulting signal is transmitted to the roots of the plants. It has been reported that some groups of iron transporters can also translocate zinc ions [10]. Therefore, it can be suggested that a large amount of iron and zinc are simultaneously transported to seeds via different groups of iron and zinc transporters to meet the requirements of iron storage proteins.

In the present study, the homozygous transgenic line IR68144 overexpressing the *ferritin* gene was selected for introgression of a transgene for a high-iron trait, the *soyfer1* gene, into a high-yielding local *indica* rice cultivar, Swarna. BC_2F_4 introgression lines were evaluated for transgene expression as well as iron accumulation in the endosperm. The milled rice grains of the BC_2F_4 plants showed 2.6-fold *ferritin* overexpression along with a 2.54-fold increase in iron and 1.54-fold increase in zinc accumulation compared to Swarna.

In addition, NIL characters of the hybridized plants were investigated by SSR marker analysis and agronomic evaluation.

Materials and Methods

Plant Material and Growth Conditions

Rice (*Oryza sativa* L. subspecies *indica*) cultivar Swarna was used as the recurrent parent, and FR-19-7-6, a soybean *ferritin*-overexpressing transgenic homozygous IR68144 rice plant [12], was selected as the donor plant for introgression of a high-iron trait in the breeding program. The cross was advanced to the BC_2F_5 generation. All the hybridized plants along with control Swarna and transgenic FR-19-7 plants were grown in a greenhouse with a day/night temperature regime of 30/25 °C under natural illumination conditions and a relative humidity of 70–80 %.

Evaluation of Donor Plants

The presence of the soybean *ferritin* gene was confirmed in germinated seedlings by a *soyfer1* gene-specific PCR analysis. The iron and zinc concentrations of the milled seeds of donor plants and different *indica* rice cultivars including Swarna were analyzed to categorize their seed iron levels. The hybrid seeds were grown for backcrossing and selected using the transgene as a marker.

Screening and Expression Analysis of Hybridized Materials

The BC_2F_4 plants were studied for the presence of the soybean *ferritin* gene (*soyfer1*), and four randomly selected positive BC_2F_4 plants were chosen for *soyfer1* gene expression and metal (iron and zinc) concentration analyses. Genomic DNA was extracted from fresh leaves (2–3 cm) of the hybridized plants following a rapid DNA isolation protocol to screen for the presence of a 780-bp fragment of the *soyfer1* gene [12, 13]. Total RNA was extracted from mature milled seeds (100 mg) following a modified RNA extraction protocol [14]. cDNA (200 ng) from each material was used to amplify a 166-bp internal fragment of the *soyfer1* gene by quantitative RT-PCR using a SYBR Green reaction mixture (BIO-RAD, USA). The rice *β-tubulin* gene was used as a housekeeping gene to normalization in the gene expression analysis. To validate the results, each experiment was performed in three replicates.

Metal Concentration Analysis

Seeds were milled (each of 30 s) in a bench top rice miller (Satake, Japan). A 2-g sample of the powder was used to analyze iron and zinc concentrations using atomic absorption spectroscopy (AAS) (AAnalyst200, Perkin Elmer, USA) at their respective wavelengths of 248.3 and 213.9 nm. A modified protocol of dry ashing digestion was employed for the AAS analysis [15].

SSR Primer Screening and Data Analysis

Twelve RM SSR primers were used to study the polymorphic amplification and genetic distance map (dendrogram) of the hybridized (BC_2F_4) plants with recurrent Swarna and IR68144 transgenic plants. Each band was considered a single allele. The alleles were scored as the ascending number according to the position of the band (the top band considered as 1, and the next band as 2) in the gel. To calculate the PIC analysis of the three genotypes, the mathematical equation PIC=1- Square of frequency of each allele was applied.

For the genetic distance matrix or dendrogram analysis, the allele was scored as present (1) or absent (0) using Gene Profiler Software. The dendrogram for the three genotypes was calculated by NTSYS software using 1, 0 matrix data.

Agronomic Evaluation

The plant height and number of tillers and panicles of ten BC_2F_4 progeny were calculated and compared with control Swarna plants. After harvesting the grains, the length, breadth and length/breadth ratio were calculated for ten individual hybridized and control Swarna seeds. The DW of ten dehusked seeds of each plant was measured using three replicas, and the 100-seed DW was calculated accordingly.

Statistical Analysis

Analyses of the performance data were performed using GraphPad Prism5 software. The experimental data values were the mean value from three independent series, each performed with three replicates. The results are presented as the means± standard error, based on three replications, and differences among the means (ANOVA) were analyzed using Bonferroni post-tests. The statistical significance at $P<0.05$ was calculated.

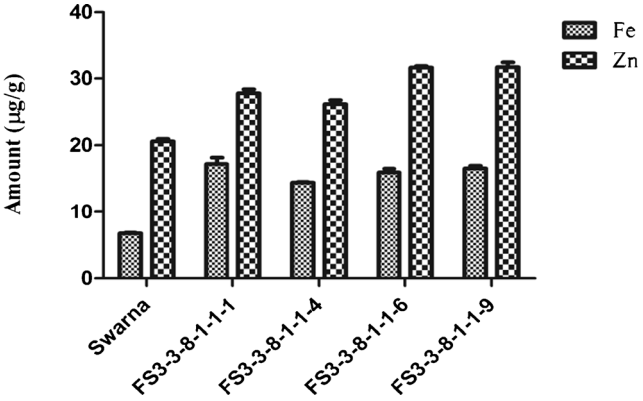

Fig. 2 Amount of iron and zinc in the hybridized (BC_2F_5) milled seeds. Double asterix indicate the level of significance at ($P<0.01$) level

Results and Discussion

Performance of Parental Materials

The introgression of the high-iron trait into Swarna was primarily studied by the integration of the *soyfer1* gene in the hybridized progeny. The presence of a 780-bp fragment of the *soyfer1* gene in all ten progeny indicated the stable integration of the transgene in the donor plant (Online Resource 1a). Hence, FR-19-7-6, one of the obtained progeny, was randomly selected based on the PCR result. The role of the soybean *ferritin* gene has been established with regard to the development of iron-biofortified rice grains in transgenic experiments [12]. The milled seeds of transgenic FR-19-7 showed higher iron (16.02±0.009 µg/g of seed DW) and zinc (27.5± 0.029 µ g/g of seed DW) accumulation over the non-transgenic control IR68144 (6.5±0.005 µg/g of seed DW for iron and 23.4±0.056 µg/g of seed DW for zinc) (Online Resource 1b). Among the five local high-yielding varieties screened, the milled seeds of recurrent parent Swarna contained the least amount of iron (6.75±0.1153 µg/g of seed DW) and zinc (20.57±0.3696 µg/g of seed DW) and were considered as the recurrent parent in the breeding program (Online Resource 2).

Expression Analysis of the *soyfer1* Gene in Hybridized Seeds

The development of the BC_2F_4 generation was clearly demonstrated in Online Resource 3. In the BC_2F_4 generation, the presence of the transgene in all seventeen plants signified the stable integration of *soyfer1* (Fig. 1a). The expression of the soybean *ferritin* gene was restricted to the hybridized milled seeds (BC_2F_5) but was not found in Swarna based on a quantitative PCR analysis (Fig. 1b). The hybridized seeds from four BC_2F_4 plants exhibited 0.6–to 2.6-fold increases in the *soyfer1* gene expression level, as analyzed by qRT-PCR.

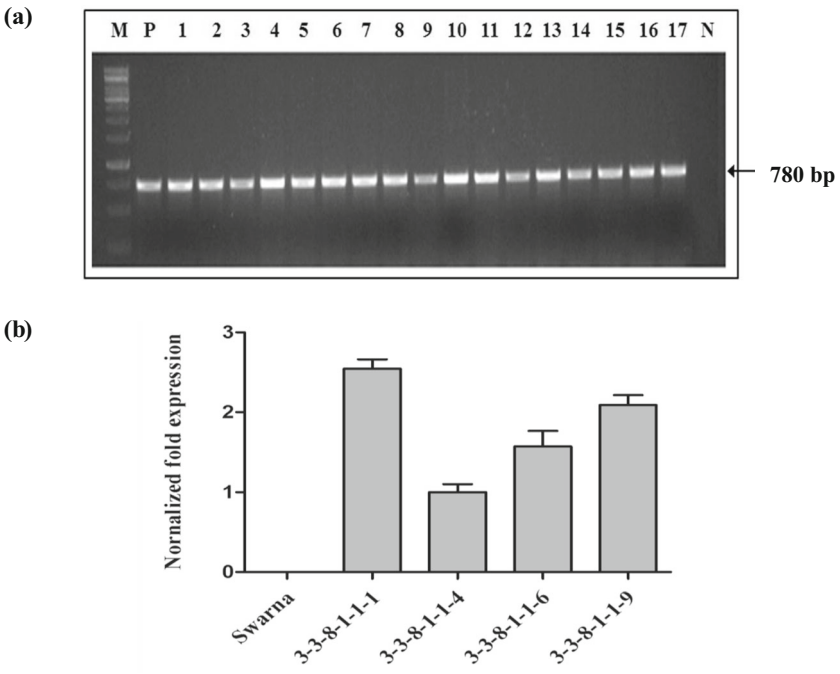

Fig. 1 **a** Gel picture showing PCR confirmation of *soyfer1* in hybridized plants of BC_2F_4 generation.(M=1 Kb gene ruler, P=positive control, N=negative control, lane 1–17=plants positive for *ferritin* gene) **b** Quantitatve RT-PCR analysis of soybean *ferritin* gene of milled BC_2F_5 seeds showing the highest overexpression in FS3-3-8-1-1-1

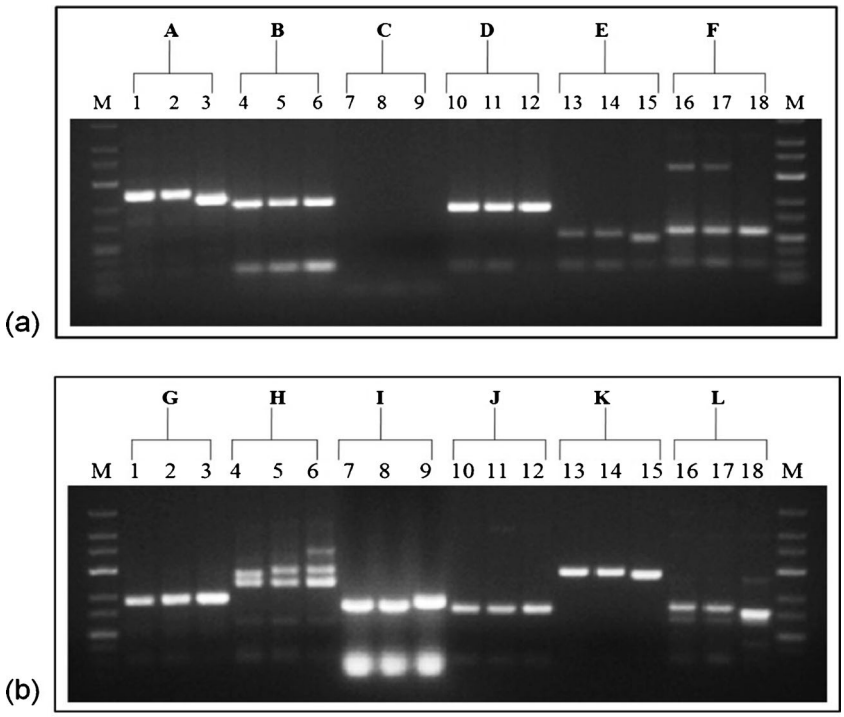

Fig. 3 Amplification of PCR products of swarna, BC_2F_4 plants and IR68144 line by different SSR primers (**a**) RM19 (A), RM 316 (B), RM 206 (C), RM 552 (D), RM 271 (E), RM 510 (F) and (**b**) RM 320 (G), RM 20 (H), RM 264 (I), RM 215 (J), RM 566 (K), RM 152 (L) (Lane M=25 bp ladder, Fermentas, lane 1,4,7,10,13,16=Swarna, lane 2,5,8,11,14,17=BC_2F_4, and lane 3,6,9,12,15,18=IR68144)

The BC_2FS3-3-8-1-1-1 plant was selected as the highest over-expressing line due to its maximum 2.6-fold overexpression of the soybean *ferritin* gene compared to the milled seeds of the recurrent Swarna parent. The presence of the *Osglub1* promoter in the upstream region of the soybean *ferritin* gene (transgenic FR 19-7-6) confined gene expression to the seed endosperm and facilitated reaching the up to 1.6-fold expression level [12].

Table 1 SSR Markers tested on Swarna, hybrid (BC_2F_4), IR68144 transgenic lines (Max. alleles: Maximum alleles)

Microsatellite (SSR) primers	Sequence	Amplification temperature (°C)	PIC	Max. alleles
RM 19 (A)	F: 5′ CAAAAACAGAGCAGATGAC 3′ R: 5′ CTCAAGATGGACGCCAAGA 3′	56	0.444	2
RM 316 (B)	F: 5′ CTAGTTGGGCATACGATGGC 3′ R: 5′ ACGCTTATATGTTACGTCAAC 3′	57	0	1
RM 206 (C)	F: 5′ CCCATGCGTTTAACTATTCT 3′ R: 5′ CGTTCCATCGATCCGTATGG 3′	57	Nil (No Band)	-
RM 552 (D)	F: 5′ CGCAGTTGTGGATTTCAGCG 3′ R: 5′ TGCTCAACGTTTGACTGTCC 3′	57	0	1
RM 271 (E)	F: 5′ TCAGATCTACAATTCCATCC 3′ R: 5′ TCGGTGAGACCTAGAGAGCC 3′	57	0.444	2
RM 510 (F)	F: 5′ AACCGGATTAGTTTCTCGCC3′ R: 5′TGAGGACGACGAGCAGATTC3′	58	0.480	2
RM 320 (G)	F: 5′ CAACGTGATCGAGGATAGATC 3′ R: 5′ GGATTTGCTTACCACAGCTC 3′	58	0	1
RM 20 (H)	F: 5′ ATCTTGTCCCTGCAGGTCAT 3′ R: 5′GAAACAGAGGCACATTTCATTG3′	58	0.612	3
RM 264 (I)	F: 5′ GTTGCGTCCTACTGCTACTTC 3′ R: 5′ GATCCGTGTCGATGATTAGC 3′	59	0.444	2
RM 215 (J)	F: 5′ CAAAATGGAGCAGCAAGAGC 3′ R: 5′ TGAGCACCTCCTTCTCTGTAG 3′	59	0	1
RM 566 (K)	F: 5′ ACCCAACTACGATCAGCTCG 3′ R; 5′ CTCCAGGAACACGCTCTTTC 3′	59	0.444	2
RM 152 (L)	F: 5′GAAACCACCACACCTCACCG3′ R: 5′CCGTAGACCTTCTTGAAGTAG3′	59	0.625	3

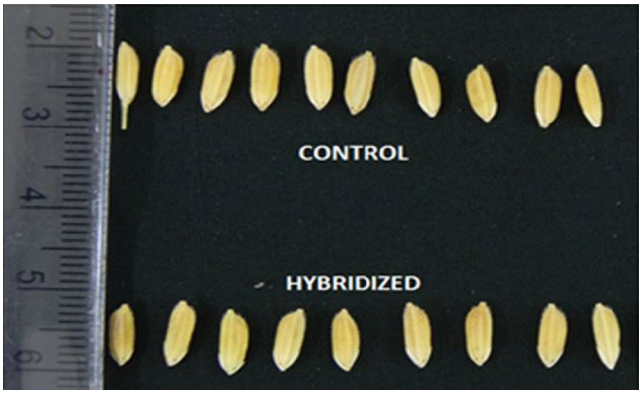

Fig. 4 Seed morphology of swarna and mature hybridized plants

Determination of Iron and Zinc Contents in Hybridized Seeds

Based on the AAS data, the hybridized milled seeds of the four BC_2F_4 plants accumulated from 14.38±0.09244 to 17.18 ±0.9648 μg/g seed DW iron and from 25.8±0.3812 to 31.65± 0.7390 μg/g seed DW zinc (Fig. 2). The hybrid milled seeds accumulated 2–to 2.54-fold higher amounts of iron and 1.54-fold higher amounts of zinc in comparison to the control Swarna seeds due to the overexpression of the soybean *ferritin* gene. Among the four BC_2F_4 plants analyzed, the milled seeds of BC_2-FS3-3-8-1-1-1 exhibited the highest level of iron accumulation (2.54-fold) over Swarna. Intriguingly, the 0.6–to 2.6-fold *soyfer1* overexpression (based on qRT-PCR data) ultimately led to 2–to 2.54-fold increases in iron in the hybridized seeds compared to the control, Swarna. This is likely because the overexpression of soybean *ferritin* is not directly related to the amount of iron accumulation, which is in accordance with previous reports [16, 17]. The positive correlation between iron and zinc was also shown in this study, as previously reported in transgenic soybean *ferritin*-overexpressing plants [11]. FS3-3-8-1-1-1, one of the BC_2F_4 plants, exhibited a maximum 2.54-fold enhancement in iron contents but lower zinc content compared to the 1.54-fold of FS3-3-8-1-1-9.

PIC Value and Dendrogram Analysis of SSR Markers

In breeding programs, microsatellite markers are widely used for the identification of genetic variability and heterotic groups of inbred genotypes in maize based on the genetic distance of polymorphic bands [18]. The amplification profiles of the three lines (recurrent Swarna, BC_2F_4 and IR68144) with 12 markers showed polymorphic alleles for 7 markers (Fig. 3). No band was visualized for Marker C. A total of 20 alleles were detected among the three lines. The number of alleles *per* locus was ranged from 1 (B, D, G, J) to 3 (H, L). The PIC value recorded for the allelic diversity and frequency were not uniform at all the loci tested and indicated the non-informativeness of markers B, D, G and J (Table 1). Among the seven informative markers, marker L showed the highest PIC value (0.625), whereas A, E, I and K were considered the lowest (0.444); the highest PIC value indicated the maximum genetic polymorphism, and the lowest showed the minimum. The PIC values from the seven informative markers clearly demonstrated polymorphism between the hybridized BC_2F_4 plants and IR68144 transgenic line. However, no polymorphism was detected between Swarna and the hybridized materials. The distance matrix results implied that the hybridized BC_2F_4 line is closely related to recurrent Swarna, whereas transgenic IR68144 was considered to be distantly related line (Online Resource 4). Unnecessary chromosome regions with microsatellite polymorphism were eliminated due to backcrossing, thus favoring the close relatedness of the hybridized plants to recurrent Swarna, as found in the dendrogram analysis.

Phenotypic Characters of Backcrossing of Progeny

The plant height, panicle length, number of functional tillers and grains/panicles and plant DW were equivalent ($P>0.05$) to the recurrent parent Swarna (Online Resource 5). The size of the BC_2F_5 grains insignificantly differed ($P>0.05$) from the harvested Swarna seeds (Fig. 4), as calculated using the length/breadth (L/B) ratio of dry seeds and 100-seed DW (Online Resource 6). Furthermore, all the hybridized seeds showed 99.33 to 100 % germination rates. However, no alterations in seed viability, seedling growth and delay in germination were observed. Due to the backcrossing of the hybridized materials (F_1), the insignificant variations in the agronomic parameters ($P>0.05$) of the BC_2F_4 plants and BC_2F_5 seeds could be attributed to NIL characters.

Conclusion

In summary, introgression of a high-iron trait (soybean *ferritin* gene) into a high-yielding *indica* rice cultivar, Swarna, resulted in approximately 2.54-fold and 1.54-fold increases in iron and zinc, respectively, in the milled hybridized seeds. The hybrid seeds (FS3-3-8-1-1-1) revealed a maximum 1.6-fold increase in soybean *ferritin* gene expression and 2.54-fold increase in iron accumulation in the milled condition. The BC_2F_4 plants exhibited very insignificant morphological differences from the parental Swarna plants, and NIL characters were confirmed by SSR marker studies. All the molecular, biochemical and morphological data clearly reveal that the *soyfer1* transgene introgression strategy played a pivotal role in significantly enhancing the amount of iron and zinc in high-yielding *indica* rice grains.

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Conflict of Interest Each author of the article does not have any conflict of interest. The article does not contain any studies with human or animal subjects.

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