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Research Article

Growing Conditions and Varietal Ecologies Differently Regulates the Growth-regulating-factor (GRFs) Gene Family in Rice

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Background: Growth-regulating factors (GRFs) are crucial in rice for controlling plant growth and development. Among the rice cultivation practices, aerobic methods are water efficient but result in significant yield reduction relative to non-aerobic cultivation. Therefore, mechanistic insights into aerobic rice cultivation are important for improving the aerobic performance of rice.

Objectives: This study aimed to examine the evolution of GRFs in different rice species, analyse the phenotypic differences between aerobic and non-aerobic conditions in three rice varieties, and assess the expression of GRFs in these varieties under both aerobic and non-aerobic conditions.

Materials and Methods: This study comprehensively examined the GRFs gene family in 11 rice species (*Oryza barthii, Oryza brachyantha, Oryza glaberrima, Oryza glumipatula, Oryza sativa* subsp. *indica, Oryza longistaminata, Oryza meridionalis, Oryza nivara, Oryza punctata, Oryza rufipogon, Oryza sativa* subsp. *japonica*) focusing on phylogenetic analysis. Additionally, the expression patterns of 12 GRFs were investigated in three distinct genotypes of *O. sativa* subsp. *indica* rice, under both non-aerobic and aerobic conditions.

Results: Three major phylogenetic clades were formed based on conserved motifs in the 123 GRFs proteins in eleven rice species. Further, novel motifs were identified especially in *O. longistaminata* indicative of the species level evolutionary differences in rice. Among the trait performance, the number of tillers was reduced by ~ 36% under aerobic conditions, but the reduction was found to be less in CR Dhan 201, an aerobic variety. Besides, three GRFs namely GRF3, GRF4, and GRF7 were found to be distinct in expression between aerobic and non-aerobic conditions.

Conclusion: Three GRF genes namely GRF3, GRF4, and GRF7 could be associated with the aerobic adaptation in rice

Keywords: Aerobic-cultivation, Growth-regulating-factor, Phylogeny, Rice

1. Background

Growth regulating factors (GRFs) are often found in plants as transcription factors (TFs) that play essential roles in several facets of plant growth and development, including the development of roots, stems, leaves, and flowers. Additionally, it possesses a plant growth-regulating role in various biotic and abiotic stress circumstances. (1-5). The GRF family in

plants is characterised by the presence of two domains, namely QLQ and WRC, which exhibit a high degree of conservation (6). The QLQ domain, comprising of glutamine, leucine, and glutamine amino acids, plays a crucial role in facilitating protein-protein interactions. Typically, this domain is seen in conjunction with bulky aromatic or hydrophobic amino acids (7). The QLQ motif is also present in SWI2/SNF2 protein of *Saccharomyces cerevisiae* which forms a complex with other proteins and involved in chromatin remodelling (8). The second conserved domain WRC which has an amino acid stretch of tryptophan, arginine, and cysteine, linked with a C3H-motif, is essential for DNA binding and nuclear localization (7, 9). The involvement of GRFs in the production of gibberellic acid (GA3) is well-documented, as they play a crucial role in promoting cell growth and elongation in tissues at different stages of development. The regulation of GRF expression is governed by miR396, and it has been observed that the GRF-miRNA396 regulatory module plays a pivotal role in several developmental and stress response mechanisms (10).

Rice (*Oryza sativa* L.) is a prominent cereal grain that holds significant global importance. It serves as a vital source of sustenance and economic prospects for numerous individuals. Within its genetic makeup, rice harbours a total of 12 GRF genes viz., GRF1 to GRF12. The GRF genes are known to have substantial implications for multiple dimensions of plant physiology, including growth, development, and reactions to environmental stresses. GRF1 helps gibberellic acid (GA) to elongate stems (7). GRF2 regulates leaf size and is highly expressed in leaves (11). The KNOX gene and GRF3 regulate meristems (12), GRF4 regulates grain size and yield (13). GRF5 aids floral organogenesis (14), while chilling stress is coordinated by GRF6 (15). Leaf development is regulated by GRF7 and GRF8 (16). Young inflorescences express GRF10 and GRF11, which regulate floral growth (14) and GRF12 is expressed during GA-mediated shoot development (11). Rice is grown in a variety of conditions, including anaerobic (flood-irrigated rice), rainfed, and aerobic (upland rice). Aerobic rice cultivation is a way of cultivating rice in fields that are not constantly non-aerobic with water. In traditional wetland rice agriculture, fields are non-aerobic during most of the growing season (17). Rice cultivation in aerobic conditions resulted in lower growth and yield compared to non-aerobic ecology (18-20). Multiple studies have shown that the genes associated with growth-regulating factors (GRF) exhibit responsive behaviour in the presence of drought or salt stress. Modulating the expression of these genes, either by upregulating or downregulating them, has been found to enhance the ability of crops to withstand adverse environmental conditions. In comparison to wild-type plants, the AtGRF7 mutant exhibits heightened resilience to salt and drought-induced stress (21, 22). GRF1 and GRF3 target the abscisic acid biosynthesis pathway and modulate the level of defence in stress responses (23). Earlier research compared rice varieties' agronomic performance in aerobic and non-aerobic settings, but GRF gene expression patterns have not been identified.

2. Objective

The following objectives were studied in this work namely 1) investigation of GRFs evolution in different rice species, 2) phenotypic variations between aerobic and non-aerobic conditions in three rice varieties and 3) expression analysis of GRFs in three rice varieties under non-aerobic and aerobic conditions.

3. Materials and Methods

3.1. Retrieval of Sequence

The protein sequence of the eleven rice species (*O. barthii, O. brachyantha, O. galleria, O. glumipatula, O. sativa* subsp. *indica, O. longistaminata, O. meridionalis, O. nivara, O. punctata, O. rufipogon, O. sativa* subsp. *japonica*) was retrieved from the Ensembl plants database (https://plants.ensembl.org) (24). A local database has been created using BioEdit software (https://bioedit.software.informer.com), and BLASTP analysis was performed against the reference proteins sequences using the GRFs homologs having domain Pfam Ids namely PF08880 (QLQ Domain) and PF08879 (WRC Domain). Further, Hidden Markov Models (HMM) in the Pfam database were used to compare the functional domains in the GRF homologs retrieved from several *Oryza* genera (25).

3.2. Multiple Sequence Alignment, Phylogenetic Tree Construction and Orthologous Gene Analysis

A sum of 123 GRF homologs identified were aligned using the MUSCLE algorithm in Molecular Evolutionary Genetics Analysis (MEGA X) software

(https://www.megasoftware.net) (**Fig. 1**). Phylogenetic tree was built using the Neighbour-joining (NJ) algorithm in the MEGA X software. For NJ analysis, the Jone-Taylor- Thornton (JTT) substitution model, pairwise deletion data subset and bootstrapping (1000 replicates) were used. Further, the phylogenetic tree was visualized using the interactive tree of life (iTOL) (https://itol.embl.de/upload.cgi). Then, orthologous protein clustering was done using Othovein analysis (https://orthovenn2.bioinfotoolkits.net/home). Motif analysis was done using the motif search web tool (https://www.genome.jp/tools/motif/).

3.3. Plant Materials and Treatments

In this study, the popular varieties of *O. sativa* subsp. *indica* i.e **Swarna, IR-29 and CR Dhan 201** of different rice ecologies were selected. Varietal characteristics is in **S**upplementary **Table 1**. The experiment was conducted with three replications for each variety maintained in pots (15.6cm height and 10.5cm radius) containing three plants. The pots were maintained in aerobic and non-aerobic conditions. The soil composition was clay loam texture, comprising 32% clay, 38% silt, and 33% sand and medium bulk density of 1.45 g.cm^3. Each pot was filled with 2.1 Kg of soil and kept at Net house,

Table 1A: Analysis of variance (ANOVA) of different traits, genotypes, and its interactions between aerobic and non-aerobic condtions. PH-Plant Height, **LN**- Leaf Number**, NT**- Number of Tillers, **SPAD**-Soil plant analysis development, **LL**- Leaf length, **LW**- Leaf width, **RA**- Root Area, **SA**- Shoot Area, **FSW**-Fresh shoot weight, **FRW**- Fresh Root Weight, **DSW**- Dry Shoot Weight, **DRW**- Dry root weight. P values and significant codes: *: P < 0.05; **: P < 0.01; ***: P < 0.001.

Sl. No.	Traits	Genotype	Treatment	Genotype x Treatment
1	PH-25d	0.106	0.943	0.669
2	PH-36d	0.0418 *	0.0798	0.6405
3	PH-46d	0.000134 ***	0.364177	0.739204
4	PH-60d	0.00136 **	0.00264 **	0.84794
5	LN-25d	0.424	0.102	0.268
6	LN-36d	0.6966	0.0803	0.4854
7	LN-60d	0.7878	0.0558	0.4038
8	NT-36D	0.4689	0.0291 *	0.4689
9	NT-46d	0.289	0.299	0.289
10	NT-60d	0.976	0.923	0.859
11	SPAD-25d	0.000231 ***	0.161299	0.369123
12	SPAD-36d	0.121	0.193	0.321
13	SPAD-46d	0.00677 **	0.48850	0.52454
14	SPAD-60d	0.125	0.883	0.824
15	LL-60d	0.0013 **	0.0290 *	0.8284
16	LW-60d	0.226	0.772	0.859
17	RA-60d	0.0493 *	0.1496	0.0519
18	SA-60d	0.134	0.147	0.476
19	FSW-60d	0.0829	0.0464 *	0.5661
20	FRW-60d	0.73821	0.00159 **	0.68124
21	DSW-60d	0.1343	0.0629	0.6838
22	DRW-60d	0.0944	0.0636	0.6351

Table 1B: Least significant difference (LSD) analysis of different traits between the aerobic and non-aerobic conditions. PH-Plant Height, LN- Leaf Number, NT- Number of Tillers, SPAD- Soil plant analysis development, LL- Leaf length, LW- Leaf width, RA- Root Area, SA- Shoot Area, FSW- Fresh shoot weight, FRW- Fresh Root Weight, DSW- Dry Shoot Weight, DRW- Dry root weight. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Sl. No.	Traits	Non-aerobic	Aerobic	P value
1	PH-25d	25.01 ± 4.02a	24.15 ± 4.78a	0.015*
2	PH-36d	40.22 ± 6.15a	33.86 ± 5.47a	0.194
3	PH-46d	47.93 ± 11.71a	42.41 ± 9.60a	0.033*
4	PH-60d	66.45 ± 13.76a	52.14 ± 11.17a	0.197
5	LN-25d	4.57 ± 0.33a	4.19 ± 0.55a	0.311
6	LN-36d	13.51 ± 1.08a	10.76 ± 0.28b	0.932
7	LN-60d	47.41 ± 9.70a	37.41 ± 9.93a	0.188
8	NT-36D	4.13 ± 0.29a	3.35 ± 0.20b	0.983
9	NT-46d	5.51 ± 0.63 a	4.98 ± 0.34a	0.890
10	NT-60d	15.44 ± 1.57a	9.30 ± 1.29b	0.862
11	SPAD-25d	28.02 ±2.82a	27.29 ± 2.10a	0.066
12	SPAD-36d	32.20 ± 1.87a	30.33 ± 0.67a	0.377
13	SPAD-46d	31.82 ± 3.58a	29.75 ± 3.76a	0.051
14	SPAD-60d	42.83 ± 1.34a	42.37 ± 1.78a	0.021*
15	LL-60d	35.06 ± 7.88a	33.82 ± 6.58a	0.030*
16	LW-60d	0.96 ± 0.15a	0.96 ± 0.20a	0.030*
17	RA-60d	2621.91 ± 447.86a	2268.32 ± 607.67a	0.413
18	SA-60d	5875.26 ± 438.37a	5119.28 ± 568.60a	0.341
19	FSW-60d	19.35 ± 4.40a	13.08 ± 2.26a	0.460
20	FRW-60d	8.35 ± 0.98a	2.79 ± 0.76b	0.978
21	DSW-60d	5.29 ± 0.94a	3.81 ± 0.57 a	0.465
22	DRW-60d	1.22 ± 0.42a	0.70 ± 0.12 a	0.504

Mean ± SD, Fisher's LSD – with same letters are not significant

ICAR-NRRI. Mature, healthy seeds were collected from previous seasons. Seeds were sun dried for three days and oven dried at 45 °C for five days for breakdown of dormancy. Then germinated on moist paper at room temperature and transferred to pots at 7 DAS (Days after sowing). Aerobic and non-aerobic conditions were maintained by irrigating the pots with field capacity or up to complete saturation and maintaining pots up to ~ 5 cm of standing water, respectively. The standard recommended dose (Nitrogen: Phosphate: Potash 60:40:40) has been applied during the Tiller initiation stage. Several parameters namely vegetative plant height, leaf number, vegetative tiller no., leaf length, leaf width, shoot area, root area and SPAD value were taken at 25^{th}, 36^{th},45^{th} and 60^{th} intervals to determine the varietal performance towards the non-aerobic and aerobic conditions. Image-based phenotyping methodologies were adopted from previous report (26). The captured photos were subjected to analysis of their geometric characteristics using the open-source programme Image J (https://imagej.nih.gov/ij/) previously reported by (27). Further, the fresh weight of collected plant samples was recorded and then allowed to dry at 42 °C for 7 days for dry weight measurements.

3.4. Expression Study of GRF Genes

Total RNA was isolated from three biological replicates of shoot samples collected on 60 days after sowing using the RNEasy isolation Kit, Qiagen, Germany. The replicated shoot samples comprise of three varieties with two conditions (Non-aerobic and Aerobic). First-strand cDNA was synthesized from 1 µg of DNase-treated RNA using qScript cDNA SuperMix (Quantabio, Germany). Quantitative real-time PCR was carried out using the StepOnePlusTM Real-Time PCR System with three biological and technical replicates. The Primers were designed using IDT primer quest tool (https://www.idtdna.com/pages/tools/primerquest) using the CDS sequence of different GRFs. A total volume of 10 µL reaction mixture consisting of 5 µL of 2xSYBR Master mix reagent (Agilent, U.S), 1µL of cDNA, and 1µL of gene-specific primers (0.5 pM each) and 3 µL of MilliQ water was used. Primer sequence details are given in **Supplementary Table 2**. A qPCR was carried out with the following steps: activation at 95 °C for 10min, followed by 40 cycles of 15s denaturation at 95 °C, 30s of annealing at 60 °C, followed by a final extension step of 72 °C for 30s Melt curve analysis for the PCR cycle was done with default conditions. Relative fold change in gene expression was measured using CT method (28), with rice 25S housekeeping gene used as an internal reference gene for normalization.

3.5. Statistical Analysis

Microsoft Excel's built-in data analysis feature was used for descriptive statistics analysis. Statistical measures such as mean, median, mode, kurtosis, skewness and range were determined using data analysis option. Analysis of variance (ANOVA) and Z test at 5% and 1% level of significance were used to determine the significant difference between the treatments. Principal component analysis (PCA) was done in XLSTAT software using correlation values with significance and factor loadings for the treatments (https://www.xlstat.com/en/). Correlation analysis and plot were drawn using corrplot package in Rstudio using coefficient number and test of significance values (29). The experiment used a completely randomised design (CRD) with three replications for testing of level of significance at 5% and 1%.

4. Results

4.1. Motif Analysis

A total of 123 GRFs proteins were retrieved from 11 different species of rice from Ensembl plants database after the removal of sequence redundancy. In motif analysis, QLQ and WRC domains are highly conserved among the identified GRF genes in rice. Additionally, GRF1 homologs in *O. nivara*, *O. sativa* subsp. *japonica* and *O. glabberima* share an extra novel domain of SUIM_assoc (unstructured C-terminal UIM of Ataxin3), which is rich in glutamine amino acid residues. Besides, *O. branchyantha* has an extra domain of prokaryotic E2 family B in both GRF1 and GRF2 homologs. Further, an additional WRC domain is present in the GRF2 homolog of *O. nivara*. Another wild rice, *O. longistaminata* has four extra domains in GRF3 and GRF4 viz., GPHR-N (The golgi pH regulator family N-terminal), ABA-GPCR (Abscisic acid- G protein-coupled receptor), LMBR1 (Limb development membrane protein 1)-like membrane protein, DUF4618 (Domain of unknown function 4618) and MCR-C (Methyl-coenzyme M reductase operon protein C). GRF5 homolog of *O. longistaminata*, shares all the extra domains with *O.punctata* except methyl-accepting chemotaxis protein (MCP) signalling domain. In GRF6, *O. sativa* subsp. *indica* has ancillary domain of RNA methyltransferase as compared to homologs in different rice species. The GRF8 homolog in *O. sativa* subsp. *japonica* does not have the characteristic WRC domain. Specifically, GRF9, GRF10, and GRF12 homologs have an extra overlapping zinc finger binding protein in the position of WRC domains except for GRF10 homolog of *O. longistaminata*. Further, mucin-like domain is present in most of the homologs of GRF9 except *O. branchyantha*, *O. glumaepatula* and *O. punctata*. In addition, GRF10, GRF12 homolog in *O. longistaminata* has multiple motifs namely QLQ, WRC, bVLRF1 (Bacteroidetes VLRF1 release factor), SLAC1(voltage-dependent anion channel), Ankyrin5(many copies repeat), Ankyrin2 (3 copy repeats), TIMELESS and VATC (Vms1-associating treble clef) domain and these motifs were not identified in other GRF homologs (**Supplementary Fig. 1**).

4.2. Phylogeny and Orthologs Analysis

The phylogenetic study of protein sequences in the *Oryza* GRFs revealed the formation of three significant clades among the eleven species. These clades were

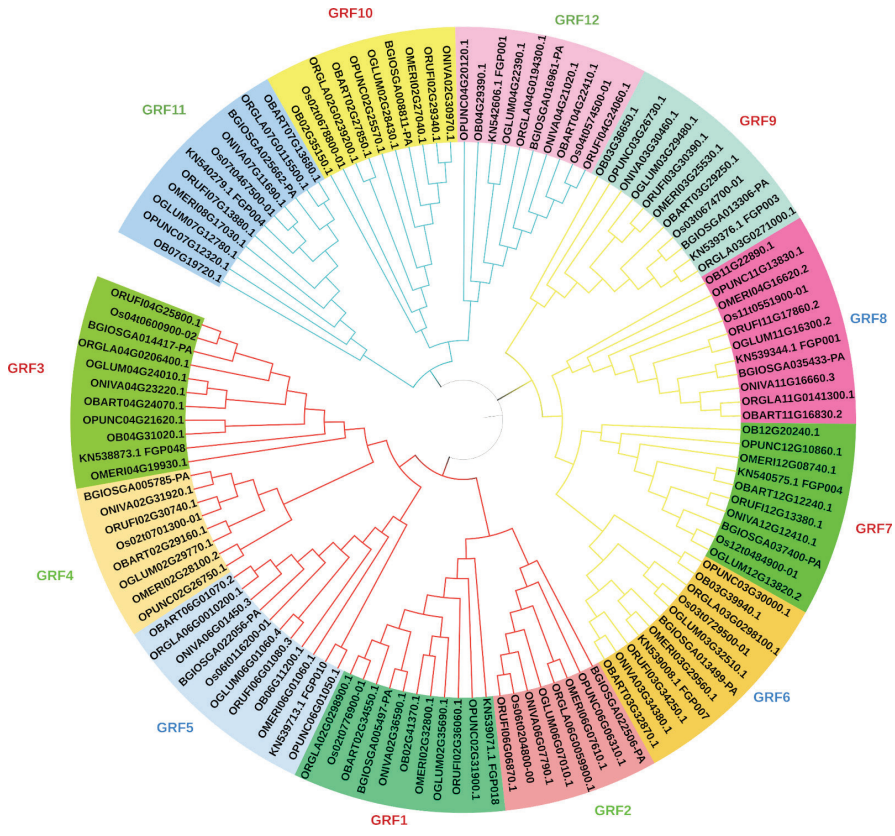

Figure 1. Phylogenetic tree of growth regulating factor (GRFs) gene family in different rice species. GRFs homologs are shaded in different colours.

determined based on the examination of motifs and amino acids similarity. Clade I is the largest clade which comprises 49 GRF proteins and five GRF orthologs (GRF1-GRF5) shares a common TQL domain at C terminal end, Clade II (GRF6-GRF9) and Clade III (GRF10-GRF12) include 43 and 31 GRF proteins shares a very short C terminal region from the end of the WRC domain, respectively (11). Further, indica and japonica GRF1 homolog were clustered with *O. nivara* and *O. glaberrima*, respectively whereas japonica GRF2 was clustered with *O. rufipogon* and indica was present as a single branch in the subcluster. In GRF3, both the indica and japonica protein were clustered with *O. rufipogon*. Alternatively, indica and japonica homologs of GRF4 were present within both the *O. nivara* and *O. rufipogan* subclusters. The next major cluster II and III are also grouped into two subclusters each, consisting of four and three GRF orthologs of rice, respectively. Furthermore, among the 11 rice species, 12 orthologous clusters were found, and most of them share six common orthologous clusters containing 66 GRF proteins represented by all eleven species of rice (**Supplementary Fig. 2**). The rest are single-copy gene clusters having 10, 10, 10, 10, 8, 8 each of GRF proteins with an absence of at least one species of rice (**Supplementary Table 3**). Functional analysis of these clusters showed a common function i.e., GA induced stem elongation and regulatory role in the downstream of different genes (**Supplementary Table 4**).

4.3 Morphological Analysis

The descriptive statistics of non-aerobic and aerobic conditions for 22 different traits among the three genotypes are given in **Supplementary Tables 5 and 6**. Among the various traits, mean vegetative plant height 24.58 cm (P=0.015) and 37.04 cm (P=0.033) on 25th day and 36th day respectively, mean SPAD value 42.6 (P=0.020), mean leaf length 34.44 cm (P=0.029) and mean leaf width 0.96 cm (P=0.030) on 60th day were found to be highly significant between

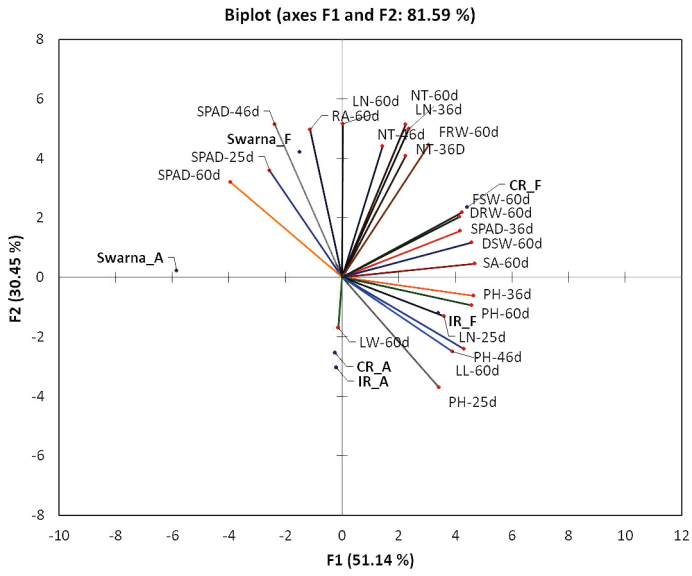

Figure 2. Principal component analysis of morphological traits and condtions in three different rice varieties. A-Aerobic, F-Non-aerobic. . **PH**-Plant Height, **LN**- Leaf Number**, NT**- Number of Tillers, **SPAD**- Soil plant analysis development, **LL**- Leaf length, **LW**- Leaf width, **RA**- Root Area, **SA**- Shoot Area, **FSW**- Fresh shoot weight, **FRW**- Fresh Root Weight, **DSW**- Dry Shoot Weight, **DRW**- Dry root weight

the varieties. Further, mean vegetative plant height 59.29 cm (P=0.00264) and mean leaf length 0.96 cm (P=0.0290) on 60 DAS, mean number of tillers ~4 (P=0.0291) on 36 DAS, mean fresh shoot weight 16.21 mg (P=0.0464) and mean root weight 5.57 mg (P=0.0015) were found to be significantly different for the aerobic and non-aerobic treatments (**Table 1A and 1B**). To validate the G×E interaction in two-way a nova analysis, there is significance between varieties and traits interaction (**Supplementary Table 7**). Further, the average difference between the vegetative plant height among the varieties in both conditions are minimal in initial days but there was ~14% difference between mean vegetative plant height of genotypes maintained in aerobic and non-aerobic conditions during active tillering stage, i.e., 46th days to 60th days. Similarly, there was 31-45% difference observed in number of tillers between aerobic and non-aerobic conditions at 60 DAS. Among all the traits, SPAD value range was almost similar in both the non-aerobic and aerobic conditions. Also, fresh shoot and root weight between aerobic and non-aerobic conditions were found to be 1.49 and ~3-fold different, respectively. Similarly, the fold difference for dry shoot and root weight was 1.38 and 1.74, respectively between the conditions.

4.4. Correlations Analysis

Correlations between different traits under non-aerobic and aerobic conditions across genotypes were analyzed by Pearson correlation coefficient analysis. The highest correlation coefficient (r =0.99) *** was found interestingly between 36th day SPAD and 60th-day root dry weight. However, SPAD was found to be negatively correlated with vegetative plant height at all four different time points after sowing (25 DAS (r =-0.85) ***, 36 DAS (r =0.99) ***, 46 DAS (r =-0.93) ***, and 60 DAS (r = -0.87) ***). Further, vegetative plant height and shoot area were also positively correlated for all the four-time intervals. Similarly, vegetative plant height and dry biomass were also found to be positively correlated. Shoot area was found to be significantly correlated with vegetative plant height (r= 0.8728) ***, fresh shoot weight (r=0.8828) **, dry shoot (r=0.9508) *** and root weight (r=0.8621). Also, dry root weight was found to be positively correlated with vegetative plant height (r= 0.833) ***, shoot area at 60 DAS (r=0.8621) ***, fresh shoot weight (r=0.9897) ***, and dry shoot weight (r=0.9511) *** (**Supplementary Fig. 3**). A similar correlation relationship was also found between dry shoot weight and the above mentioned four traits.

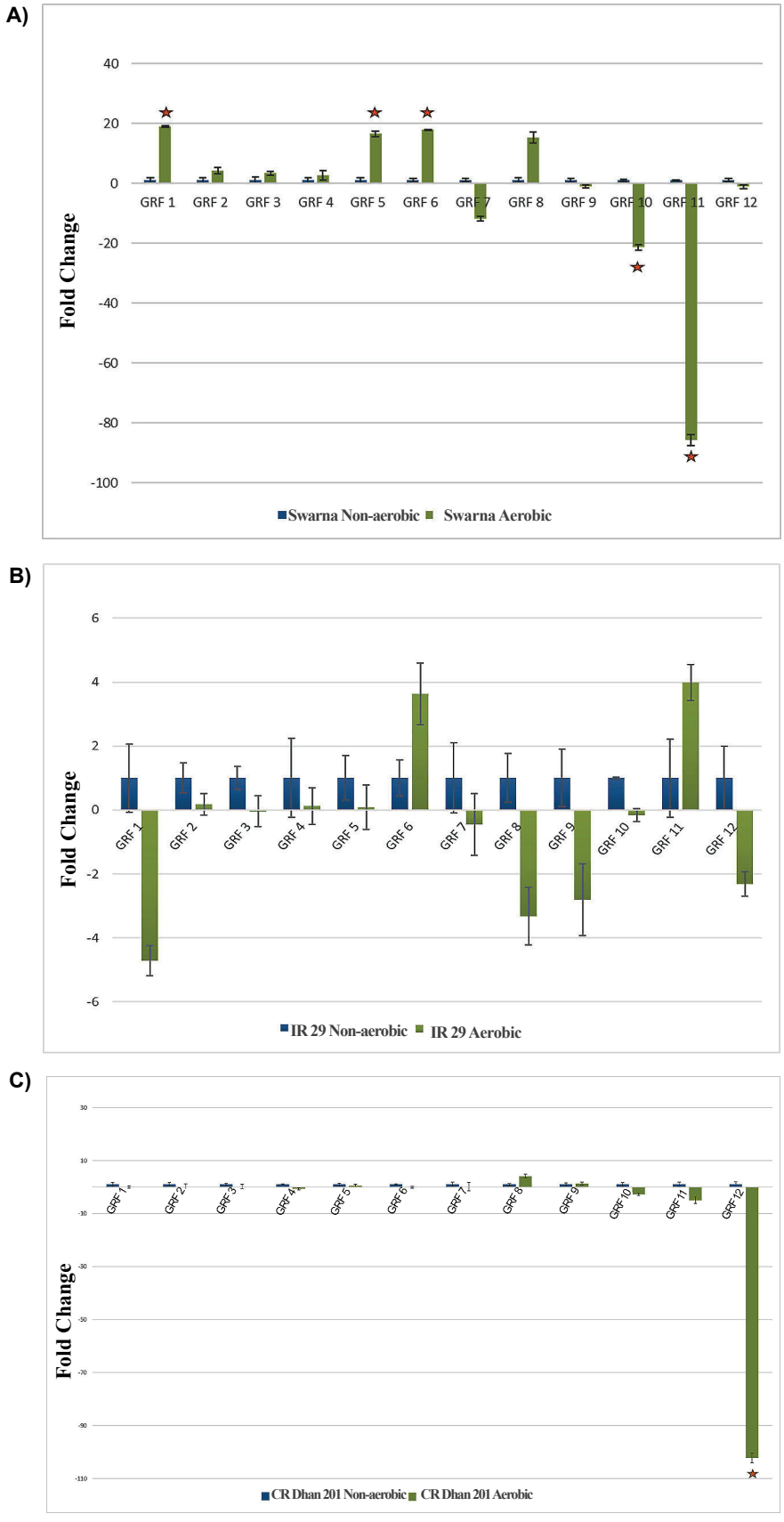

Figure 3. Relative changes in the gene expression of Growth Regulating Factors (GRFs) between aerobic and non-aerobic conditions. A) in Swarna, **B)** in IR29, **C)** in GRFs. Asterisk sign represents 5% level of significance ($P<0.05$).

The level of significance was marked as ***' 0.001 '**' 0.01 '*' 0.05 respectively. Regression analysis identified only four traits namely Vegetative plant height 36 days, 46 days, 60 days, Leaf width 60 days showed high co-efficient of determination of > 0.8 between non-aerobic and aerobic conditions (**Supplementary Table 8**).

4.5. Principal Component Analysis

PCA analysis showed a total cumulative variance of 81.59% in the first (51.14%) and second principal components (30.45%). Further, the first component separated the aerobic condition of three varieties (Swarna_A, IR29_A, and CR Dhan 201_A) to that of the non-aerobic conditions except for Swarna_F (**Figure 2**). Besides, SPAD value and root area were highly correlated and grouped in the same component (F1) along with the Swarna-F condition. Also, dry shoot and root weight were highly correlated and grouped along with the CR Dhan 201 non-aerobic condition. Further, traits like vegetative plant height and leaf number, were highly correlated and grouped along with the IR29 non-aerobic conditions. Interestingly, none of the traits was found to be grouped along with the aerobic condition of all three varieties.

4.6. qRT-PCR

The expression of the twelve GRF genes in shoots of 60-day old seedling was analyzed through qPCR and given in **Figure 3A**, **3B** and **3C**. The analysis showed unique expressions of the GRF gene family in all three varieties between aerobic and non-aerobic conditions. Among the three varieties, the expression pattern was almost similar in CR Dhan 201 in both aerobic and non-aerobic conditions for most of the GRF genes except for GRF8, GRF10, GRF11, and GRF12. Further, GRF12 was highly downregulated in CR Dhan 201 under aerobic relative to non-aerobic conditions. Similarly, GRF10 and GRF11 were also downregulated by 2.73 and 4.9-fold in aerobic conditions. In contrast, GRF8 was the only GRF upregulated (4.12-fold difference) under aerobic conditions in CR Dhan 201. In IR29, GRF6 (3.62-fold) and GRF11 (3.98-fold) were upregulated and GRF1 (4.7-fold), GRF8 (3.33-fold), GRF9 (2.8-fold) and GRF12 (2.31-fold) were downregulated in aerobic conditions as compared to the non-aerobic condition. In Swarna, three GRF genes namely GRF1, GRF5, and GRF8 were upregulated by 5.5, 4.2, and 5.01-fold, respectively in aerobic compared to non-aerobic conditions. Alternatively, five GRF genes (GRF7, GRF9, GRF10, GRF11, and GRF12) were downregulated. In comparison of all three varieties, GRF10 and GRF12 were found to be uniformly downregulated in all three varieties under aerobic conditions and GRF8 was found to be upregulated both in IR29 and Swarna but not CR Dhan 201 under aerobic conditions. Further, three GRF genes (GRF2, GRF3, and GRF4) showed uniform expression in both aerobic and non-aerobic conditions in all three varieties.

5. Discussion

In this study, the response of varieties to aerobic and non-aerobic conditions and expression of the GRF gene family was analyzed up to the active tillering stage to understand the ecology-specific differences in varietal response. Specifically, the number of tillers and biomass showed significant differences in condition (aerobic vs non-aerobic) and few GRF genes (GRF10, GRF12) were downregulated under aerobic conditions in all three varieties of different ecology. The significance of these findings in terms of aerobic cultivation in rice and adaptation are discussed below.

A significant reduction (31-45%) in the number of tillers at the active tillering stage was observed between aerobic and non-aerobic conditions in all three varieties. Both the irrigated (IR29) and rainfed shallow lowland (Swarna) varieties were highly sensitive than the aerobic variety (CR Dhan 201). Previously, also reported a significant reduction in tiller number between non-aerobic and aerobic rice conditions in active tillering stage in IR64, UPLRi7, and Apo rice varieties (20). This indicates the ability to maintain the tiller number under water limitation conditions is an important trait and breeding for stable tiller number QTLs (30) could be a viable strategy.

A 63% and 53% percent reduction in fresh and dry root weight, respectively was observed between varieties in aerobic and non-aerobic treatments. This indicates uniform maintenance of water potential in non-aerobic conditions is required for optimum rice growth. This finding of low root biomass under aerobic conditions than non-aerobic was supported by previous studies from (31, 32). However, aerobic roots were vigorous in the findings of (33). In support of our observation, solution water potential reduction from -0.04 to -0.07 MPa reduced root biomass up to 81 percent in a low-

land rice variety, IRGA 424 (34). Also, reduced leaf water potential significantly affects leaf growth, photosynthetic rate, and dry matter production (35). Thus, maintenance of the uniform water potential under different water regimes in rice genotypes could be explored under aerobic cultivation. This observation is also supported by Kato and Katsura (18) and Joshi *et al*. (19) for reducing the yield penalty in aerobic conditions.

Vegetative plant height showed 11.2% and 14.35% reduction under aerobic condition up to 46 DAS, and 60 days of sowing respectively. This indicates active tillering and later stages of the crop are more sensitive to aerobic condition induced moisture alterations as previously reported by Joshi *et al*. (19). Besides, intermediate vegetative plant height for varieties is most preferred rather than semi-dwarf stature for aerobic rice cultivation (18). Thus, this study suggests the maintenance or less reduction in vegetative plant height under aerobic relative to the non-aerobic condition needs to be rationalized in breeding programs for optimum yield in rice. Furthermore, gibberellic acid treatment of seeds was reported to improve the seedling establishment in dry direct seeded and drought conditions in rice (36, 37). Additionally, well-recognized gibberellic acid pathways for cell elongation would be impaired under aerobic rice cultivation. Therefore, targeting the GA pathway for aerobic adaptation would provide valuable insights for higher yield of rice varieties.

QLQ and WRC domains are highly conserved and C-terminal portions of GRF proteins were diverged in different *Oryza* species. In many wild species, additional motifs were identified, which may be characteristic for specialized functions. For example, an additional WRC domain found in *O. nivara* GRF2 may facilitate the robust interaction between DNA and transcription factors. Furthermore, the presence of GPHR-N and ABA-GPCR domains in GRF3 and 4, which are found in the COLD1 (chilling tolerance divergence 1) genes may be responsible for *O. longistaminata*'s ability to resist low temperatures (38). Additionally, MCP and bVLRF1 motifs found in *O. longistaminata* might have transferred from the bacterial genomes (*Methylobacterium* and Bacteroides) and possibly support the host by promoting CH4-dependent N2 fixation in low nitrogen conditions (39). Therefore, as compared to the other species of rice, GRF motifs diversity is relatively higher in *O. longitaminata* indicating the GRF gene family evolutionary differences.

The expression of GRF3 and GRF4 were associated with the Swarna in aerobic conditions. Previously, GRF4 expression was reported to be negatively regulated by miR396 (1) and miR356 expression was downregulated in drought stress (40). The downregulation of miR396 would result in the higher expression of its GRFs targets and could assist in plant development under moisture limitation conditions. Thus, upregulation in GRF3 and GRF4 in Swarna aerobic conditions could be one of the adaptive strategies to enhance plant growth under aerobic ecology in rice. Further, KNOX genes regulate the plant meristem development under drought stress through ABA mediated pathway (41). KNOX is regulated by GRF3 through GA mediated regulation (12). Therefore, upregulation of the GRF3 could be associated with the regulation of the meristem activity in aerobic conditions. Therefore, the function of GRF3 and GRF4 in growth adaptation under aerobic conditions needs to be further validated.

GRF7 was found to be highly downregulated in Swarna (Rainfed shallow lowland) and IR29 (Irrigated) and was slightly downregulated in CR Dhan 201 (Aerobic). It was reported that GRF7 plays a crucial role in leaf development through regulation of a wide variety of osmotic stress-responsive genes (22). Since aerobic conditions would be inducing the osmotic stress-like conditions in rice, GRF7 downregulation in Swarna and IR29 could be associated with the reduced growth traits. However, aerobic variety CR Dhan 201 which showed the least reduction in the many morphological traits under aerobic conditions could sustain the growth through one of the mechanisms of maintenance in the expression of GRF7. Therefore, GRF7 under aerobic conditions could be a potential breeding target for reducing yield penalty in aerobic cultivation.

6. Conclusion

Aerobic conditions differently affect the response of rice varieties in irrigated, rainfed, and aerobic ecologies. Especially, the mean number of tillers in all three varieties at the active tillering stage was significantly reduced by ~ 35 percent in aerobic conditions. GRF gene family which regulates plant development also showed unique patterns of expression in Swarna, CR

Dhan 201, and IR29 varieties between non-aerobic and aerobic conditions. Specifically, GRF3, GRF4, and GRF7 could be associated with the aerobic adaptation in rice which needs to be explored. Additionally, domain novelties of GRFs present in *O. longistaminata* would be related to the evolutionary adaptation in rice.

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Conflict of Interests

No conflict of interests

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Consent for Submission

All the authors agree and approve the submission of the manuscript for reviewing.

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