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Research Paper

Expression Analysis of C_4 Photosynthetic Pathway Related Genes in Seven Rice Genotypes During Grain Filling Stage

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Rice is a C_3 plant with relatively less photosynthetic efficiency and is a potential target for C_4 photosynthetic pathway engineering. Interestingly, rice genome encodes all the C_4 photosynthetic pathway genes. Fewer efforts have been made to study the expression of these genes in rice at different growth stages. We report here the expression analysis of six C_4 pathway related genes namely carbonic anhydrase (*CA*), phosphoenolpyruvate carboxylase (*PEPC*), NADP-dependent malic enzyme (*NADP-ME)*, malate dehydrogenase (*MDH*), pyruvate, orthophosphate dikinase (*PPDK*) and pyruvate, orthophosphate dikinase regulatory protein (*PPDK-RP*) during grain filling stage in seven different rice genotypes including three pure line varieties namely, Pusa Basmati-1 (PB-1), Pusa Basmati-6 (PB-6) and Pusa Sugandh-1121 (Pusa-1121), and four hybrids, *viz*. PA6444, PA6129, Karnataka Rice Hybrid-2 (KRH2), and Pusa Rice Hybrid-10 (PRH-10). All the cultivars showed expression of the six C_4 related photosynthetic pathway genes in the flag leaf during grain filling stage with considerable differences among the genotypes.

Key Words: C_4 Rice; C_4 pathway; Carbonic Anhydrase (CA); Phosphoenolpyruvate Carboxylase (PEPC); NADP-dependent Malic Enzyme (NADP-ME); Malate Dehydrogenase (MDH); Pyruvate; Orthophosphate Dikinase (PPDK)

Introduction

Rice is an important food crop and its cultivation is of immense importance for the global food security. It is the dominant source of calories for Asians [1]. Rice is a C_3 plant and lacks efficient CO_2 concentrating mechanism that is operative in C_4 plants such as maize and sorghum [2]. C_4 plants have double the water-use efficiency (WUE) as compared with C_3 plants, and use about 40% less nitrogen to achieve 50% higher yield [2-4]. Globally, efforts are being made to understand and engineer C_4 photosynthetic pathway genes in rice [5-6]. The five C_4 pathway enzymes, namely carbonic anhydrase (CA), phosphoenolpyruvate carboxylase (PEPC), NADP-dependent malic enzyme (NADP-ME), malate dehydrogenase (MDH), and pyruvate, orthophosphate dikinase (PPDK) plays an important role in concentrating CO_2 around the enzyme Ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco) in C_4 plants [7]. Phosphoenolpyruvate carboxylase kinase (PEPCK) and Pyruvate, orthophosphate dikinase regulatory protein (PPDK-RP) regulate the activity of PEPC and PPDK, respectively [8].

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Rice has large sink strength. However due to limiting source upto 20% spikelets remain unfilled. This suggests that another 10-20% of the juvenile spikelets could be converted into grains by increasing the source strength [9]. Wang and his co-workers [10] showed higher activity of the C_4 photosynthetic enzymes in a high yielding rice hybrid. It is documented well that C_4 related photosynthetic pathway genes are present in C_3 plants [11-12], including rice [13]. However, the exact role of C_4 photosynthetic pathway genes in C_3 plants has not been established [8, 14]. Expression of C_4 type PEPC in rice has been reported to improve photosynthetic efficiency with enhanced tolerance to photo-oxidation [15-17]. However, such attempts to engineer C_4 photosynthetic pathway genes failed to increase photosynthetic efficiency of the transgenic rice to the level of the C_4 plants [6, 8, 18]. Here we studied the expression of C_4 photosynthesis pathway related genes in seven rice genotypes comprising different maturity groups.

Materials and Methods

Plant Materials and Growth Conditions

The experiment was conducted at the research farm of the Indian Agricultural Research Institute, New Delhi, India (28.4°N, 77.1°E, 228.2 m a.s.l.) during *kharif* 2009. Soil of the experiment station is characterized as sandy loam with a pH of 7.8. Seeds of seven different rice genotypes including three pure line varieties namely, PB-1, PB-6 and Pusa-1121, and four hybrids, *viz*. PA6444, PA6129, KRH2, and PRH-10 were presoaked in distilled water at 30ºC over night and then coated with fully wet gauze cloth for germination at 25ºC in an illuminated incubator. The partial germinated seeds were sown in the nursery. 25 days old seedlings were transplanted in natural field condition with a 20 x 15 cm spacing geometry. The transplanting was done in randomized block design with recommended dose of fertilizers and experiments were kept free from insect, weeds and diseases and managed under standard agronomic practices. All the physiological parameters were measured in flag leaves during the grain filling stage with at least five replications. The flag leaves were used for molecular analysis.

Physiological Analysis

The rice genotypes used in this study were categorized into three maturity groups namely early (PA6129 and PRH-10), medium (PB-1, Pusa-1121 and KRH2) and late (PB-6 and PA6444) maturity groups. For physiological analysis, five plants of each genotype were tagged and all the physiological parameters were analyzed in flag leaf of mother shoot. Chlorophyll content, net photosynthetic rate (P_N) and stomatal conductance (g_s) were measured on same day for all genotypes between 10.00 am to 11.00 am with air temperature ranging between for 31.2°C to 32.8°C (measured with hand held 8718 Thermo-Hygrometer, Spectrum Technologies, Inc., USA) during the time of measurements on different days. The chlorophyll was estimated by using hand held chlorophyll meter SPAD-502, Minolta Co., Japan and expressed as SPAD values. The net photosynthetic rate (P_N) and stomatal conductance (g_s) were measured by using a portable photosynthesis system (*LI 6400*, *LI-COR* Inc., Lincoln, NE, USA) at a fixed 1500 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD).

C_4 Specific Photosynthetic Pathway Genes in C_3 Rice

Sequences of the six rice C_4 specific photosynthetic genes *viz*., carbonic anhydrase (LOC_Os01g45274), phosphoenolpyruvate carboxylase (LOC_Os01 g11054), NADP-dependent malic enzyme (LOC_Os01g09320), malate dehydrogenase (LOC_Os01g46070), pyruvate, orthophosphate dikinase (LOC_Os05g33570) and pyruvate, orthophosphate dikinase regulatory protein (LOC_Os07g34640) were downloaded from MSU Rice Genome Annotation Project Database (http:// rice.plantbiology.msu.edu/index.shtml). Gene specific primers were designed (Table 1) for expression analysis by RT-PCR. Ubiquitin (LOC_Os01g22490) was used as internal control.

Digital Expression Analysis

The tissue specific and developmental stage specific expression of the selected C_4 pathway related genes was analyzed by using the publically available rice ESTs expression datasets from NCBI dbEST database

Table 1. List of genes and corresponding primers used for expression analysis by RT-PCR

Gene	Forward primer	Reverse primer
CA	ATGGTCCCAGCTTACTGCAAGA	ATTGGTCATCGAAAGGCAGCGA
PEPC	AAGCATTAGGAGCTGCCTGACA	TCGTCGATGCCGATGTTCTTGA
NADP-ME	TGAGGCTGGAACTGGTATTGCAGA	TCGCCAGTCCAAGTTCATAGGCTT
MDH	AGTACTGCCCGAATGCTCTTGTCA	TAGTTGCAGGAGTTGCCTGTGAGA
PPDK	AGGATGCAACAACAGCCAGACA	AGCTGCTCCAGTTTCAGAGCAT
PPDK-RP	TGAGGCAACTAAGAAGGCCTGTGA	GCATGGCGCACTTCTGTTTCCTAT
UBIQUITIN	AGCGCAAGAAGAAGACGTACACCA	TAAGCCTGCTGGTTGTAGACGTAG

and microarray datasets from Rice Oligonucleotide Array Database (ROAD) [19]. These databases provide the normalized expression data for a selected gene in different tissues and developmental stage [19].

RNA Sampling and RT-PCR Analysis

Flag Leaf samples were collected from the healthy plants during their grain filling stage. Leaf samples were collected 35DAA, 25DAA and 18DAA for early, medium and late maturity groups respectively. The collected leaf samples were stored at –80ºC for further use. High quality RNA was extracted using TRI REAGENT® RT reagent (Molecular Research Center, Inc.) following manufacturer's instructions. 1 µg of total RNA was used to synthesis first strand cDNA by using Superscript –III reverse transcriptase (Invitrogen, USA), oligo$(dT)_{20}$ primer, following the manufacturer's instructions. Two µl of cDNA was used in 25 µl of reaction volume with the following PCR conditions to study the gene expression: 30 cycles of 94°C for 1 min, annealing temperature according to melting temperature of primers for 1 min, and 72°C for 1 min, and then final extension at 72°C for 10 min. List of gene specific primers used in the study are given in the Table 1. The amplified product was analyzed by electrophoresis on a 1.5% agarose gel. Quantitative estimation of RT-PCR amplicon on the gel was calculated as integrated density value (IDV) using AlphaEase®FC software. The gene expression was normalized using the internal control ubiquitin by using the following formula.

$$\frac{\text{IDV of a specific gene of specific genotype}}{\text{IDV of internal control ubiquitin of specific genotype}} \times$$

Average IDV of internal control ubiquitin of all the genotypes.

Results and Discussion

Engineering C_4 photosynthetic pathway in C_3 rice is imperative to meet out the future food grain demand [20]. Rice genome encodes all genes that code for the C_4 photosynthetic pathway. Fewer efforts have been made to understand the expression of these genes in different rice genotypes. In this study, we studied the expression pattern of C_4 photosynthetic pathway related genes in different genotypes comprising different maturity groups under similar environmental conditions during grain filling stage. Our study showed the expression of C_4 photosynthetic pathway related genes, *viz*. *CA, PEPC, NADP-ME, MDH, PPDK* and the regulatory protein *PPDK-RP* in the flag leaf during grain filling stage in different rice genotypes. Although all the cultivars showed expression of the six C_4 photosynthetic related pathway genes, wide variations in the expression levels were observed between the genotypes (Fig. 1).

Digital Expression Analysis

Table 2 shows the abundance of ESTs of selected C_4 related genes. These genes showed expression in various rice tissues, *viz* callus, leaf, panicle, stem, root and seed. *CA*, *PEPC*, *NADP-ME* and *PPDK*

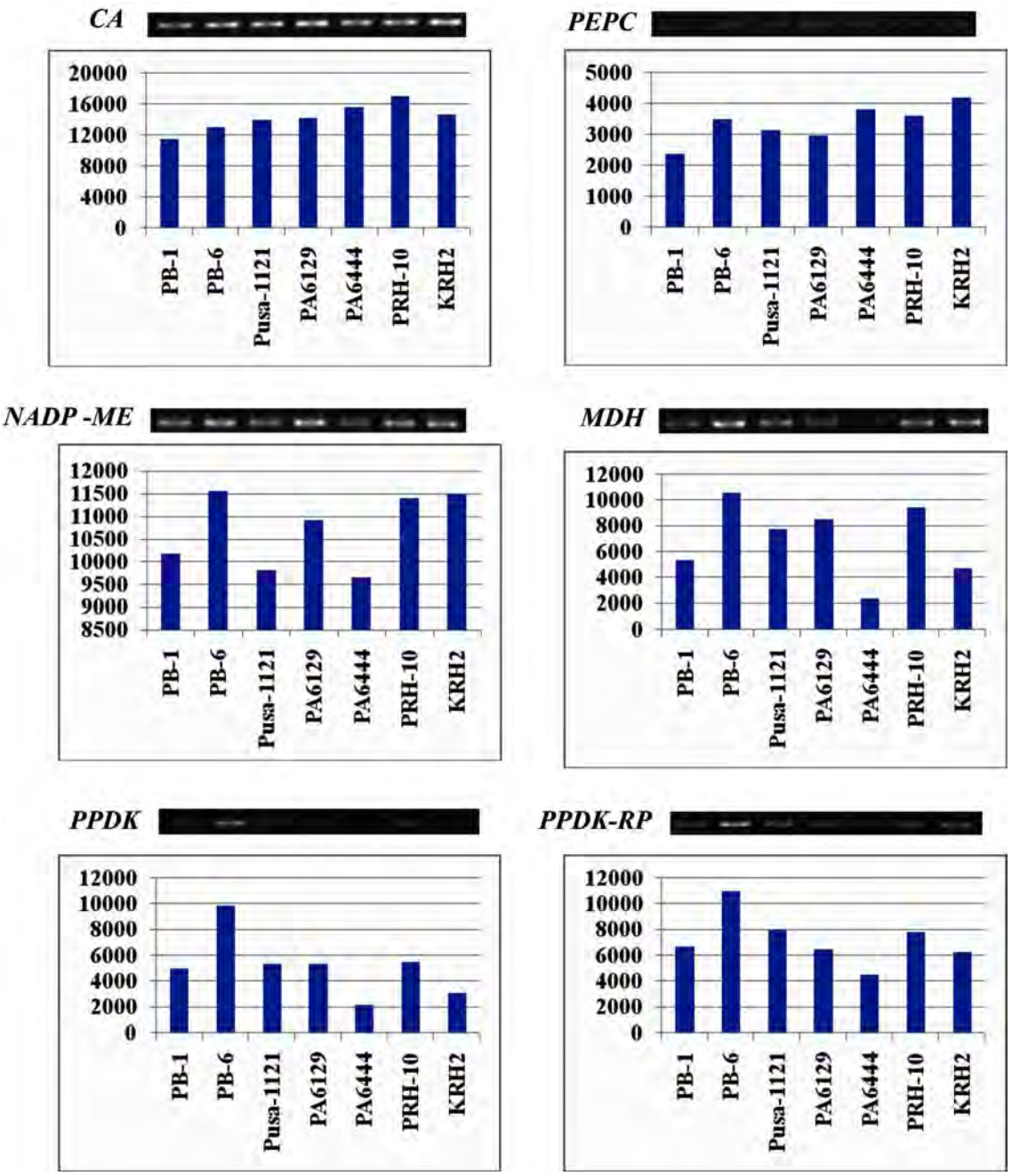

Fig. 1: Expression level of C_4 related photosynthetic genes in different rice genotypes during grain filling stage in the form of Integrated Density Value (IDV). PB-1: Pusa Basmati-1; PB-6: Pusa Basmati-6; Pusa-1121: Pusa Sugandh-4; PRH-10: Pusa Rice Hybrid-10; KRH2: Karnataka Rice Hybrid-2

genes showed high levels of expression in leaf tissue (Table 2). Microarray data analysis also showed that all the selected C_4 related genes were expressed in all the rice tissues and in all developmental stages with significant differences in their expression level (Fig. 2a & 2b). The expression level of *PEPC* is relatively low as compared to other C_4 genes in different tissues and developmental stages (Fig. 2a & 2b).

Physiological Analysis

Hybrids PRH-10 and PA6129 flowered earlier and had a long grain filling duration. At 33 DAA both hybrids showed less P_N which decreased steadily,

Table 2: Digital expression analysis of C_4 specific photosynthetic genes

Genes	Tissue and organ specific EST profiles from NCBI dbEST database (value represents no. of transcripts per million of transcripts)								No. of ESTs in dbEST	No. of FL-cDNA in NCBI
	Callus	Flower	Leaf	Panicle	Root	Seed	Stem	Vegetative meristem		
CA	48	43	594	72	87	216	505	436	376	10
PEPC	2	22	176	88	59	30	120	0	92	4
MDH	98	290	74	168	505	61	216	661	220	4
NADP-ME	104	22	199	256	223	30	64	0	152	7
PPDK	154	364	273	102	119	1450	64	440	239	6
PPDK-RP	12	29	56	7	14	0	8	0	27	3

while there was a sharp decline in chlorophyll after 33 DAA. g_s showed a sharp decline after 37 DAA (Fig. 3). Mid-late maturing genotypes (KRH2, PB-1 and Pusa-1121) recorded a gradual decline in P_N, g_s and chlorophyll after 25 DAA (Fig. 3). Variety PB-1 had initially low P_N but showed less decline after 25 DAA as compared to KRH2 and Pusa-1121 (Fig. 3). In late maturing genotypes, hybrid PA6444 had higher P_N and g_s than variety PB-6 at 18 DAA but these declined sharply by 22 DAA (Fig. 3). A sharp decline in chlorophyll content was observed after 22 DAA in both the genotypes (Fig. 3).

Expression Analysis of C_4 Genes in Rice

All the C_4 related genes analyzed in this study showed expression in the flag leaf, in all the genotypes tested during grain filling stage. The *PEPC* expression level was relatively low as compared to the other C_4 genes (Fig. 1). In early maturating genotypes, the high yielding short duration hybrids PRH-10 and PA6129 showed expression of all C_4 pathway genes even 33 days after anthesis. Among the early maturity group, the expression levels of all C_4 pathway genes were higher in PRH-10 as compared to PA6129 (Fig. 1). In Mid-late maturing genotypes, KRH2 displayed higher level of expression of all C_4 pathway genes expect *PPDK* gene as compared to PB-1and Pusa-1121.

In late maturing genotypes PA6444 displayed highest expression of *CA* and *PEPC*, whereas, PB-6 showed highest expression of *NADP-ME*, *MDH*, *PPDK* and *PPDK-RP*. Previously it was shown that *PPDK* gene is up-regulated in naturally senescing leaves and plays a role in nitrogen remobilization during leaf senescence [21]. However, transgenic rice overexpressing four C_4 pathway enzyme encoding genes, *PEPC, NADP-ME, PPDK* genes from maize and sorghum *MDH* gene displayed stunted growth, which could possibly be due to the generation of futile cycles and a lack of correct circadian regulation of enzyme activity [5, 18]. Thus, for generating a two-celled C_4 system in C_3 leaves, additional genetic components that are required for kranz type anatomy and regulatory elements that regulates the C_4 pathway need to be engineered in C_3 plants [5-6, 22].

Conclusion

Our studies showed that the C_4 photosynthetic pathway related genes express in C_3 rice leaves with a wide genotypic variation. Further studies on the role and contribution of C_4 photosynthetic pathway related genes of rice in carbon metabolism will help to enhance the photosynthetic efficiency of rice.

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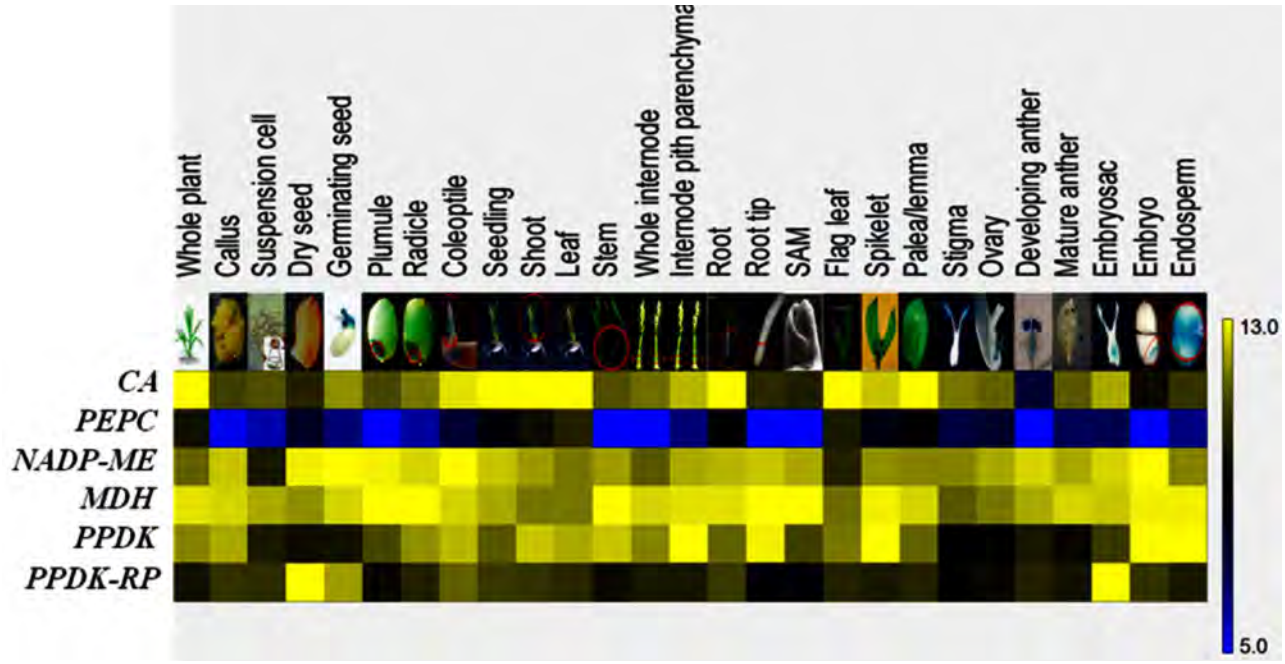

Fig. 2a: Tissue specific expression of C_4 related photosynthetic genes

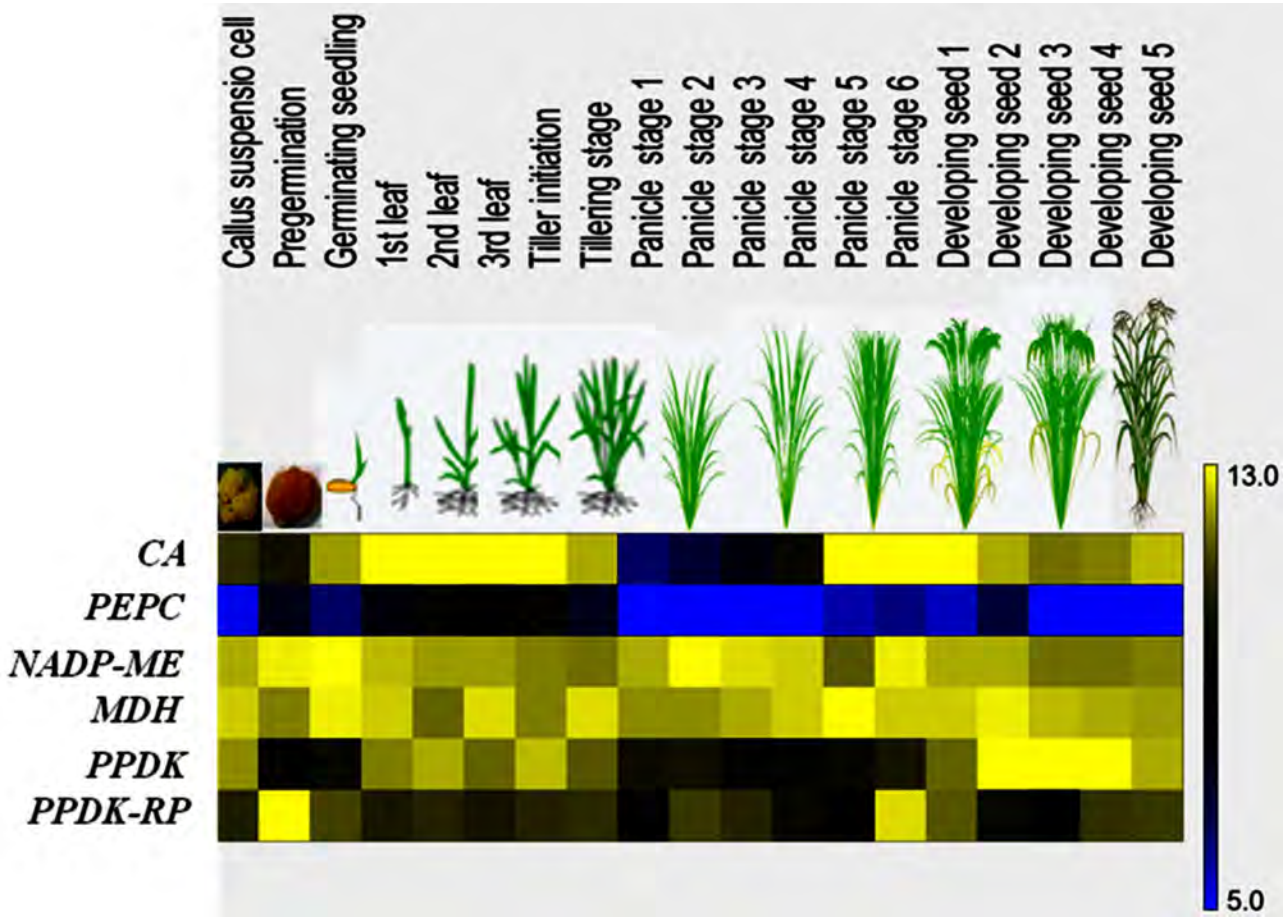

Fig. 2b: Developmental stage specific expression of C_4 related photosynthetic genes

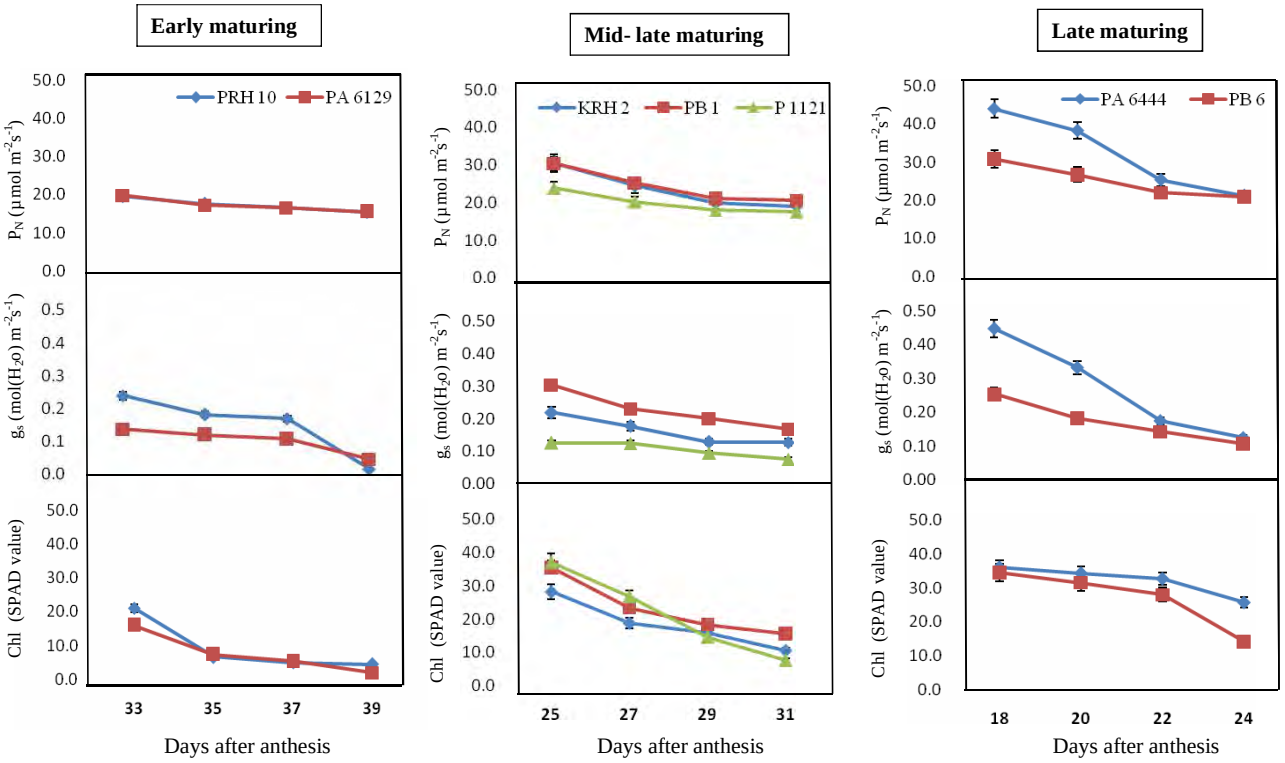

Fig. 3: Physiological parameters measured during grain filling stage in seven rice genotypes P_N: Net photosynthetic rate; g_s: Stomatal conductance; Chl: Chlorophyll content; PB 1: Pusa Basmati-1; PB 6: Pusa Basmati-6; P 1121: Pusa Sugandh 4; PRH 10: Pusa Rice Hybrid-10; KRH 2: Karnataka Rice Hybrid-2. (The values are means of five replicates ± SD)

Abbreviations

CA-Carbonic Anhydrase; DDA-Days After Anthesis; g_s-Stomatal conductance; IDV-Integrated Density Value; KRH2-Karnataka Rice Hybrid-2; MDH-Malate Dehydrogenase; NADP-ME- NADP-dependent Malic Enzyme; P_N-Net photosynthetic rate; PB-1-Pusa Basmati-1; PB-6-Pusa Basmati-6; PEPC-Phosphoenolpyruvate Carboxylase; PPDK-Pyruvate, orthophosphate dikinase; PPDK-RP-Pyruvate, orthophosphate dikinase regulatory protein; PRH-10-Pusa Rice Hybrid-10; Pusa-1121-Pusa Sugandh-4

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