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Spatial distribution pattern analysis of Dof1 transcription factor in different tissues of three *Eleusine coracana* genotypes differing in their grain colour, yield and photosynthetic efficiency

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Abstract In the present study Dof1 gene of finger millet was cloned and sequenced. In silico analysis reveals 61% identity with the *Sorghum bicolor* and 57% identity with the *Oryza sativa* Dof1 sequence. A comparative analysis of gene sequences from different crops and three finger millet genotypes {Brown (PRM-1), Golden (PRM-701) and White (PRM-801)} differing in grain colour, yield and photosynthetic efficiency showed a high degree of sequence identity of Dof1 sequence gene ranging from 22 to 70% as evident from distance matrix of the built phylogenetic tree showing two major clusters. A total of five conserved motifs were observed in Dof1 sequences of different cereals. Motif 1 with multilevel consensus sequence CKNCRRYWTKGGAMRNVPVG contains zinc finger Dof domain. Motif 3 and motif 5 contains protein kinase phosphorylation site. Motif 2 contains Dof domain and zinc finger *N*-glycosylation site while motif 4 is involved in Zinc finger type profiling. Further, we studied the spatial distribution of Dof1 gene in three vegetative tissues (root, stem and flag leaf) as well as four stages of developing spikes (S1, S2, S3 and S4) of the three finger millet genotypes using qualitative and quantitative PCR based approaches. Physiological parameters (plant height, leaf area, chlorophyll content, SPAD value and photosynthetic efficiency) at the time of flowering was found to be highest in white (PRM-801) genotype followed by golden (PRM-701) and brown (PRM-1) genotype. Semi-quantitative RT-PCR and quantitative real-time PCR analysis revealed that the expression of Dof1 is highest in leaves and lowest in roots, which suggests its role in regulation of photosynthesis-related genes and carbon skeleton synthesis. Also at grain maturity stage, expression of Dof1 was higher in white (PRM-801) genotype followed by golden (PRM-701) and brown (PRM-1) genotype. The result is suggestive of Dof1 role in the accumulation of grain protein and yield attribute through regulation of key enzymes involved in source to sink relationship during grain filling stage.

Keywords *Eleusine coracana* · Dof1 · Motif · Reverse transcriptase PCR · Real time PCR

Abbreviations

Dof	DNA binding with one finger only
RT PCR	Reverse transcriptase polymerase chain reaction

Introduction

For all eukaryotic life, transcriptional activation or repression, as well as the processing of transcripts, are critical for the appropriate expression of relevant genes and the finely tuned control of development. A number of factors are required for regulation of gene expression, among which transcription factors (TFs) are especially notable. Because TFs, as opposed to most structural genes, tend to control multiple pathway steps, they have emerged as powerful tools for the manipulation of complex metabolic pathways in plants. Plant TF studies have contributed substantially in the elucidation of gene expression mechanisms in several

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physiological processes, such as development of seeds [1, 2] and vascular tissues [3], ABA and light responses [4], plant defence and stress tolerance [5]. They are largely responsible for the selectivity of gene regulation and are often expressed in a tissue-specific, developmental-stage-specific, or stimulus-dependent manner. Based on conserved sequences and similarities in their nucleic acid-binding domains, TFs can be classified into many different families.

Plant gene expression involves classes of TFs that have specifically evolved to regulate plant specific genes and/or to mediate a variety of plant-specific signals. The DOF (DNA binding with One Finger) family is one of the well characterized plant specific TF [6–8] having diverse roles. DOF proteins, which are typically composed of 200–400 amino acids, are defined as DNA-binding proteins that have a highly conserved Dof domain [7]. The first protein to be identified with a Dof domain was MNB1a of maize (*Zea mays*), which can interact with the cauliflower mosaic virus 35S promoter [9]. Since then, numerous Dof proteins have been reported to participate in the regulation of gene expression in diverse plant-specific biological processes such as seed germination [10, 11], plant defence mechanisms [12], secondary metabolism [13], gibberellin response [14], auxin response [15], salicylic acid-responsive [16], stomata guard cell specific gene regulation [17], light responses [18, 19], in both monocots and dicots.

Maize Dof1 has been suggested to be a regulator of C4PEPC gene expression. Studies by Yanagisawa and Sheen [18] revealed that the TF DOF1 is capable of activating transcription from the C4PEPC promoter in transiently transfected maize leaf protoplasts in a light dependent manner. DOF1 seems to be ubiquitously expressed and tissue-specificity is apparently brought about by the homologous transcriptional repressor DOF2 that is only expressed in shoots and roots and that competes for the same binding site [20, 21]. Further analysis showed that maize DNA binding with one finger (DOF) proteins are associated with expression of multiple genes involved in carbon metabolism in maize [22]. Moreover, over-expression of maize Dof1 in *Arabidopsis* induced genes involved in carbon metabolism and improved nitrogen fixation under nitrogen limited conditions [23].

Finger millet is a nitrogen efficient crop which capitalizes on low nitrogen inputs and grown mainly by subsistence farmers. Of all the major cereal crops, it is one of the most nutritious and is very rich in calcium, minerals and phosphorous as compared to other cereals. It contains on an average of 7.3% protein. Since, from both economical and ecological point of view, there is dire need of shifting agricultural practices towards extensive systems dependent on low inputs of N fertilizers. Hence, a better knowledge of physiological and molecular basis of nitrogen and carbon metabolism in economically important crop such as finger millet is essentially required. Although a number of regulatory proteins and genes have been identified, Dof1 appears to be a key regulator in the coordinated gene expression involved in carbon-skeleton production and there is an intimate link between carbon and nitrogen metabolism. In the present study, attempts were made to clone the Dof1 gene from finger millet and to investigate the role of spatial distribution of Dof1 gene in vegetative and developing spike tissues to relate its expression with photosynthetic efficiency and grain protein accumulation in three genotypes differing in colour in the finger millet crop, which is being considered as nutraceutical crop. Earlier studies [24] on these finger millet genotypes indicate that, the grain protein content of the white (PRM-801) genotype is highest (9.56%), followed by golden (PRM-701; 9.0%) and brown (PRM-1; 7.5%) genotypes.

Materials and methods

Plant material and selection of tissues and different stages of developing finger millet spikes

Three finger millet genotypes PRM-1, PRM-701 and PRM-801 (differ in seed coat colour and protein content) were grown in pot conditions under natural conditions. The tissues, including, root, stem and flag leaf at vegetative stage & S1 (spike just peeping out of the tip/from the centre), S2 (spike stage after anthesis and fertilization), S3 (slight grain formation) and S4 (mature seeds) stages of developing spikes of the three finger millet genotypes were collected for semi quantitative RT PCR and quantitative real time PCR analysis.

Growth parameters

The plant height and leaf area (LICOR-3000 leaf area meter) was measured at the flowering stage. Heading date was determined by counting the number of days from sowing to 50% of spikes fully emerged from the boot. Random sample of the grains from individual genotype were obtained at the time of harvest. These samples were used to detect 1,000 grain weight after drying the grains at room temperature (30°C) to minimize intrinsic moisture content uniformity (1,000 grains were counted from each plot and weight was recorded in grams).

Chlorophyll related parameters: total chlorophyll content, SPAD value and chlorophyll fluorescence

The chlorophyll content was estimated in the flag leaf at flowering stages by using dimethyl sulphoxide (DMSO) extraction procedure [25]. SPAD value gives the indication

of the amount of chlorophyll present in plant leaves. The chlorophyll content of plant leaves is related to the condition of the plant. The SPAD value is measured by chlorophyll meter SPAD-502. The time of measurement was kept constant and measurement was carried out on the flag leaf of the plant, the average of three values of leaf recorded was used for analysis. Chlorophyll 'a' fluorescence emitted by green plants reflects photosynthetic ability of PS-II. A handy plant efficiency analyzer (Handy PEA, Hansatech, UK) was used to monitor chlorophyll fluorescence (F_v/F_{max}). Measurements were taken at flowering stage. All the measurements were recorded in the forenoon hours (8.00–9.00 a.m.) to avoid photo-inhibition.

PCR amplification of Dof1 gene and its cloning

The genomic DNA of leaf of the three finger millet genotypes were isolated by standard method [26], quantified and analyzed on agarose gel electrophoresis. PCR was performed in 25 µl of 1× KCl buffer (Fermentas) containing 0.2 mM dNTPs, 30 ng of each primer, 1.5 mM $MgCl_2$, 0.8 U *Taq* DNA polymerase (Fermentas) and 100 ng of cDNA. Amplification was carried out according to the following temperature profile: 5 min initial denaturation at 95°C; 40 cycles of 94°C for 1 min, 60°C for 90 s, 72°C for 2 min; final extension of 10 min at 72°C; and final hold 4°C. Based on the consensus sequences, Dof1 internal primers were designed from sequences of *Sorghum bicolor* Dof1, *Triticum aestivum* Dof1 and *Oryza sativa* Dof1. Primer used for amplification of Dof1 gene was: Fwd 5′-CCCGAAAGAGAAGCTGAAGA-3′ and Rev 5′-CATGTCAGAGCCAAAGCTGA-3′. The amplicons obtained by PCR were eluted from gel with the help of gel elution kit as per manufacturer instructions (Qiagen). The PCR amplicons were ligated in p^{GEM} T-Easy cloning vector (Promega). The integrity of the cloned fragments was confirmed by analyzing the p^{GEM} T-BMDL plasmid through colony PCR and restriction digestion. Nucleotide sequence of independent clones were determined with the dye terminator kit (ABI Prism, Perkin Elmer, NJ) and analyzed on Applied Biosystems 370 at University of Delhi.

In silico sequence analysis

The sequenced DOF1 gene of three finger millet genotypes was subjected to homology search with Dof1 gene sequences of different crops using BLASTN, BLASTP. The Dof1 gene was aligned using ClustalW [27] and phylogenetic tree was constructed using UPGMA method and MEGA version 4.0.02 [28]. Each node was tested using the bootstrap approach by taking 1,000 replications and a random seeding of 64,238 to as certain the reliability of nodes. The number indicated in percentages against each node.

Motif analysis

The protein sequences of the 15 DOF1 genes of different plants were analyzed by means of the MEME (Multiple EM for Motif Elicitation) program software version 4.4.0 for motif analysis. To identify conserved motifs in these sequences, the selection of maximum number of motif was set to 5 with minimum and maximum width of 10 and 20 nucleotides respectively while other factors were of default selections.

Expression analysis of Dof1 gene

Total RNA isolation and cDNA synthesis

Total RNA was isolated from each tissue of three genotypes of finger millets by using the total RNA isolation *iRIS* system (developed by IHBT, Palampur) according to the manufacturer's instructions. RNA preparations were subjected to DNase digestion according to manufacturer's instruction (Fermentas). Total RNA (5 µg) of each sample was used to synthesize first strand cDNA by using oligo$(dT)_{18}$ primer with Revert Aid^{TM} H Minus M-MuLV reverse transcriptase (RT) (Fermentas) according to the manufacture's instruction. The efficiency of cDNA synthesis was assessed by reverse transcriptase PCR amplification of a control gene encoding tubulin.

Semi quantitative RT PCR analysis

A semi-quantitative RT-PCR was carried out to analyse the Dof1 transcript levels in different tissues using Dof1 specific primer. Dof1 specific primer was designed from *Eleusine coracana* Dof1 sequence available in Genbank database (Acc. No. GQ260456). The gene specific primer used was Dof1 Fwd CCCGAAAGAGAAGCTGAAGA and Rev CATGTCAGAGCCAAAGCTGA which yield the product size of 310 bp. Tubulin gene was selected as endogenous internal standard, because it is a house keeping gene and expressed at all stages and in all the tissues. The tubulin primer used as internal control was designed from Genbank database (Acc. No. CX265249). The internal control primer used was Tubulin Fwd CTCCAAGCTTTCTCCCTCCT and Rev GCATCATCACCTCCTCCAAT. Oligonucleotides were purchased from Sigma-Aldrich (Darmstadt, Germany). cDNAs were exponentially amplified using Fermentas Taq Polymerase. PCR was performed in 25 µl of 1× KCl buffer (Fermentas) containing 0.2 mM dNTPs, 30 ng of each primer, 1.5 mM MgCl2, 0.8 U *Taq* DNA polymerase

(Fermentas) and 100 ng of cDNA. Amplification was carried out according to the following temperature profile: 5 min initial denaturation at 95°C; 40 cycles of 94°C for 1 min, 60°C for 90 s, 72°C for 2 min; final extension of 10 min at 72°C; and final hold 4°C.

Densitometric analysis of gel for semi-quantitative analysis of expressed genes (Dof1 and tubulin)

Densitometry analysis was done with the help of Gene Profiler software, Alpha Innotech Corporation USA. Briefly, individual gels were scored by placing the curser over individual band and recording the relative densitometry values of three independent gels used for expression analysis.

Quantitative real time PCR

Real-time PCR was done using the 5 prime Real Master Mix SYBR ROX (Eppendorf India Private Limited) according to manufacturer's instructions. The 5 prime uses the fluorescent dye, SYBR green, to detect PCR products. The thermocycler used was Eppendorf thermocycler ep realplex. Two step real time PCR was carried out using cDNA prepared as mentioned earlier from different developmental stages of finger millet. The primers for Dof1 and tubulin gene use were same as earlier. The reverse transcription efficiencies of Dof1 and tubulin gene were almost equal as analyzed by comparing the C_T values at different dilutions of cDNA [29]. Final concentrations, in a total volume of 20 µl, were: 2.5× Real Master Mix SYBR ROX/20× SYBR Solution, 100 nM of each forward and reverse primers and 100 ng of cDNA. The following amplification program was used: 95°C for 2 min, 40 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; 60°C for 15 s and 95°C for 15 s. All samples were amplified in triplicate and the mean value was considered. CRD were used for analyzing the gel data and real time data. The cycle threshold (C_t) value is the number of cycles required to accumulate enough SYBR green fluorescent signal to exceed the threshold (background) level. The C_t value is proportional to the amount of RT-PCR product and was used for quantification. The relative value obtained for quantitation was expressed at $2^{-\Delta\Delta C_T}$ where ΔC_T represents the difference between the C_T value of the sample and that of tubulin (endogenous control) in the same sample and $\Delta\Delta C_T$ is difference between the ΔC_T value of a sample and that of its respective control.

Results and discussion

There was variation in the heading dates within these finger millet genotypes i.e. the heading date for brown (PRM-1) genotype ranged from 77 to 85 days whereas for golden (PRM-701) and white (PRM-801) genotypes ranged from 119 to 130 days. This indicates that brown (PRM-1) genotype is early flowering and golden (PRM-701) and white (PRM-801) are late flowering genotypes. The various growth and photosynthetic efficiency parameters were noted at the flowering stage.

Plant growth parameters

The plant height was found to be highest in white genotype followed by golden and brown genotypes at the flowering stage. Similar trend was observed in leaf area (Table 1).

1,000 grain weight was found to be highest in white (PRM-801) followed by golden (PRM-701) and brown (PRM-1) genotypes. Similar trend was observed in grain crude protein content, where white (PRM-801) genotype have 9.56% grain crude protein followed by golden (PRM-701) (9.00%) and brown (PRM-1) genotypes (7.56%) [24].

Photosynthesis and chlorophyll content

The SPAD value and chlorophyll content was found to be highest in white genotype followed by golden and brown genotypes at the flowering stage. Similar trend was observed in photosynthetic efficiency (Table 1).

Molecular cloning of Dof1 gene

Dof1 gene was amplified using the same PCR conditions in three genotypes. The primer sets designed from highly conserved domains were efficient in reproducible amplification of expected sizes of amplicons. Amplification of Dof1 gene gave product size of 250 bp in the three genotypes. The sequences were submitted to NCBI databank and are available online at NCBI website. Dof1 gene (HM623862, HM623863, HM623864) were subjected to homology search using BLASTN and BLASTP and multiple sequence alignment by CLUSTAL W. Multiple sequence alignment of all dof1 protein of different plant shows conserved cysteine residues which are highlighted by different colours (Fig. S1—Supplementary material). All the plants show four conserved cysteine residues except some genotype of *Eleusine coracana* and *Oryza sativa* which show only two cysteine residues due to the partial sequence.

The phylogenetic tree was constructed of all 15 dof1 protein sequences using UPGMA method of MEGA version 4.0.02. The phylogenetic tree differentiated into two major clusters A and B respectively. Cluster A and B further differentiated into two subgroups. The three genotype i.e. brown, golden and white of *Eleusine coracana* are present in a same cluster B with rice which shows more

Table 1 Physiological parameters (except 1,000 grain weight and crude grain protein) recorded in the three finger millet genotypes at flowering stages

Finger millet genotype/ physiological parameters	Plant height (cm)	Leaf area (cm^2)	1,000 grain weight (g)	SPAD value	Chlorophyll (mg/g fresh weight)	Photosynthetic efficiency
Brown (PRM-1)	41.70	24.00	2.33	19.53	0.46	0.44
Golden (PRM-701)	60.00	25.00	3.02	22.00	1.00	0.56
White (PRM-801)	67.00	26.00	3.25	25.00	1.60	0.89

similarity with them while mostly other cereals like *Triticum aestivum*, *Hordeum vulgare*, *Zea mays* and *Glycine max* are present in cluster A.

In silico analysis reveals that there are 61% identity with the *Sorghum bicolor* and 57% identity with the *Oryza sativa* Dof1 sequence. A comparative analysis of gene sequences from different crops and finger millet genotypes showed a high degree of sequence identity of Dof1 sequence gene ranging from 22 to 70% as evident from distance matrix of the built phylogenetic tree (Fig. S2—Supplementary material).

A total of five conserved motifs were observed in Dof1 sequences of different cereals. The distribution of conserved motifs in different accessions is provided in Fig. 1. The overall multilevel consensus sequences associated with each of the five motifs is provided in Table 2. The multilevel consensus sequence corresponding to the motif is an aid in remembering and understanding the motif. It is calculated from the motif position-specific probability matrix. MEME motifs are represented by position-specific probability matrices that specify the probability of each possible letter appearing at each possible position in an occurrence of the motif. All the five motifs are present only in white genotype of *E. coracana* and brassica while Golden and Brown genotype of *E. coracana* contain motif 1, 3 and motif 5. Motif 1 and motif 3 are the uniformly observed in the all cereals except sorghum which contain only motif 2 might be due to its partial sequence. Motif 1 with multilevel consensus sequence CKNCRRYWTKGGAMRNVPVG contains zinc finger Dof domain. Motif 3 and motif 5 contains protein kinase phosphorylation site. Motif 2 contains Dof domain and zinc finger *N*-glycosylation site while motif 4 is involved in zinc finger type profiling.

Table 2 Multilevel consensus sequences for the MEME defined motifs

Motif	Width	*E* value	Sequence
1	20	3.3e−177	CKNCRRYWTKGGAMRNVPVG
2	20	5.5e−130	STNTKFCYYNNYNLSQPRHF
3	15	1.3e−027	GGCRKNKRSSSSSHY
4	14	1.1e−017	KIPKEEEILKCPRC
5	20	2.6e−013	LQRVRAALPVDPLCVSAKTN

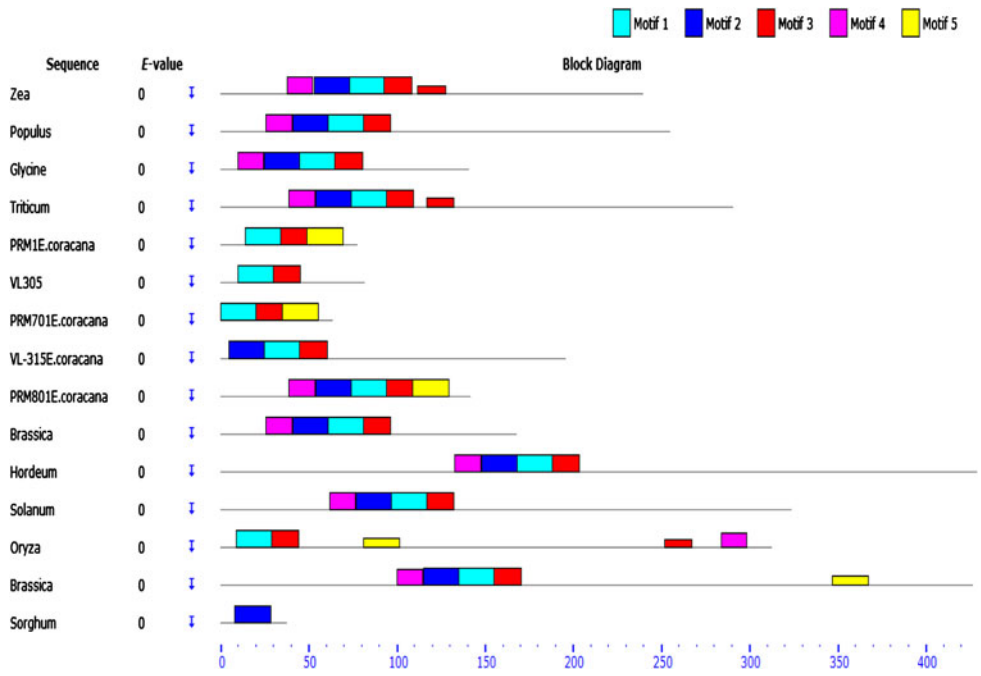

Fig. 1 Motif analysis through multiple EM for motif elicitation

Tissue specific expression pattern analysis of Dof1 gene in finger millet genotypes

The Dof1 transcription factor is found to be an activator for multiple gene expression associated with the organic acid metabolism including phosphoenolpyruvate carboxylase (PEPC)/photosynthetic gene expression [18, 22] thus it has very important role in maintaining plant growth and biomass. The expression profiling of Dof1 gene in different tissues of finger millet genotypes are given in Fig. 2a, b. Expression profiling studies at tissue level indicates that Dof1 gene was found to be expressed in all the developmental tissues studied viz, root, stem, leaf, S1, S2, S3 and S4 stages of developing spikes in all the three finger millet genotypes. Similarly maize DOF1 seems to be ubiquitously expressed and tissue-specificity is apparently brought about by the homologous transcriptional repressor DOF2 that is only expressed in shoots and roots and that competes for the same binding site [20, 21]. However there was variation in expression in all the three finger millet genotypes. The results of reverse transcription and real time PCR was almost parallel to each other. In all the three genotypes, the expression was found to be highest in leaf and least in root as Dof1 is mainly associated with photosynthetic gene regulation [18, 22]. Semi quantitative reverse transcriptase PCR (Fig. 2a) indicates that the expression of Dof1 was found to be highest in white genotype as compared to other two genotypes. For reverse transcription efficiency in real time PCR, reaction conditions were optimized with endogenous control (tubulin) and Dof1 gene(s). Different dilutions of cDNA were used and based on C_T values, these efficiencies were almost equal. This showed that tubulin gene can be used as endogenous control to analyze the relative expression of Dof transcription factor gene(s) in different tissues. Quantitative analysis (Fig. 2b) of the Dof1 transcript was performed in root, stem, leaf, S1, S2, S3 and S4 stages of developing spikes. Relative expression of Dof1 was calculated in brown (PRM-1) and it was expressed at significantly higher level in leaves (3.25 fold) followed by S4 (2.75), S1 (2.60), S3 (1.47) and S2 (1.21) as compared to root and lower Dof1 expression was observed in stem (0.82). Similarly, relative expression of Dof1 in golden (PRM-701) was calculated and it was expressed at significantly higher level in leaves (2.82 fold) followed by stem (2.26), S2 (2.12) and S3 (1.63) as compared to root. Comparatively lower expression was observed in S1 and S4. Similarly, Relative expression of Dof1 in white (PRM-801) was calculated and it was expressed at higher level in leaf (6.82) followed by stem (5.39), S4 (4.80), S3 (4.22), S2 (3.50), S1 (1.57) as compared to root.

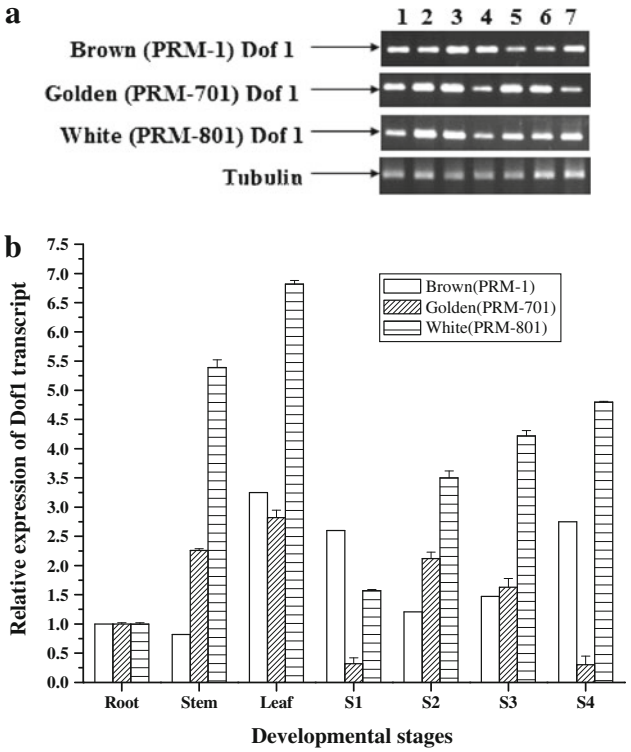

Fig. 2 **a** Expression profiling of Dof1 in brown (PRM-1), golden (PRM-701) and white (PRM-801) genotypes. *Lane 1* root, *2* stem, *3* leaf, *4* S1 stage, *5* S2 stage, *6* S3 stage, *7* S4 stage. **b** Determination of relative expression of Dof1 transcript in different tissues of brown (PRM-1), golden (PRM-701) and white (PRM-801) genotypes using real time PCR presentation of real time data as final graph

Dof1 gene was found to be expressed mainly in leaves as it is the main site for photosynthesis and leaves serve as source for nitrogen that is then translocated to grain at the time of grain filling. Also it was observed that Dof1 expression at grain maturity stage was higher in white genotype followed by golden (PRM-701) and brown (PRM-1). This indicates that Dof1 in white (PRM-801) is also active during maturity of grain which might be responsible for higher protein content in it. Interestingly, this relationship of Dof1 expression and high grain protein content of white genotype suggests the role of Dof1 also in grain protein accumulation.

The results suggest that the Dof1 gene plays a major role in photosynthetic tissues as the expression of Dof1 was found to be highest in leaves, the main photosynthetic part of the plants. Among three genotypes, it was observed that white (PRM-801) has better growth, yield and crude grain protein content. Also it was found that during grain maturity (S2, S3, S4 stages) the expression of Dof1 in white (PRM-801) was comparable to that of leaves. However, in other two brown and golden genotypes, the expression of Dof1 are variable though expression in leaves and also in developing spikes are relatively lower when compared with white genotype having high grain protein content. This is suggestive of the fact that Dof1 may have

some role in grain protein accumulation too beside the role of other *PBF Dof* transcription factor [24].

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