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Differential Expression of *PBF Dof* Transcription Factor in Different Tissues of Three Finger Millet Genotypes Differing in Seed Protein Content and Color

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Abstract Finger millet is one of the most nutritious cereal crops, as it is rich in calcium, minerals, and phosphorus. It also contains high protein, with prolamins being the major seed storage proteins. Genes encoding prolamins are coordinately expressed in developing endosperm wherein they are under spatial and temporal transcription control, involving *cis*-acting and *trans*-acting motifs in their promoters and *trans*-acting transcription factors. The *PBF Dof* (prolamin-binding factor DNA binding with one finger only) transcription factor can be an important regulator for seed storage protein gene expression. In this study, the spatial distribution of the *PBF Dof* gene has been investigated in different tissues, including root, stem, and flag leaf during vegetative growth as well as S1, S2, S3, and S4 stages of developing spikes in three finger millet genotypes, including brown (PRM-1), golden (PRM-701), and white (PRM-801) differing in grain protein content and color using reverse transcriptase polymerase chain reaction (PCR) and real-time PCR. Semi- quantitative and quantitative PCR results revealed that PBF Dof is expressed in all tissues analyzed; however, expression of PBF Dof is relatively higher in developing stages of spikes than in other tissues in all three genotypes. Spatial expression of PBF Dof genes in all tissues analyzed is similar to that reported for wheat PBF genes but in contrast to that of barley and maize PBFs wherein expression is restricted to the endosperm. Moreover, a negative correlation is observed between prolamin content and each of grain yield and grain protein in all three finger millet genotypes. 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, and it is related to higher expression of PBF Dof in S2, S3, and S4 stages, respectively. This indicates that early induction of seed protein genes in the white genotype provides a longer period for accumulation of seed storage proteins in the endosperm when compared with those in other genotypes.

Keywords Finger millet · *PBF Dof* · Seed storage proteins · Reverse transcriptase PCR · Real-time PCR

Abbreviations

PBF	Prolamin-binding box factor
Dof	DNA binding with one finger only
RT PCR	Reverse transcriptase polymerase chain reaction

Introduction

Seed proteins are broadly classified into two major classes, namely, "house keeping proteins" and "storage proteins". House-keeping proteins are important in metabolic and structural development of seeds (Lumer and de Benito 1990), while storage proteins, presented at high levels, serve to store nitrogen for seed germination and seedling growth. These seed storage proteins are of particular importance as they not only determine total protein content

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but also are influence seed quality for various end uses. Due to their abundance and economic importance, seed storage proteins are among the earliest proteins that have been characterized. However, an early detailed study of seed storage proteins (Osborne 1924) have classified into four groups based on their extraction and solubility in water (albumins), dilute saline (globulins), alcohol/water mixtures (prolamins), and dilute acid or alkali (glutelins).

The major seed storage proteins include albumins, globulins, and prolamins. Seeds contain large amounts of storage proteins that are used after germination as a source of nitrogen during the early stages of embryo development. In cereals, the major seed storage proteins are represented by a class of alcohol soluble proteins known as the prolamins (Shewry and Halford 2002**)**. Prolamins are characterized by a high glutamine, proline, and hydrophobic amino acid content (Bietz 1982; Shewry and Tatham 1990), and they have been extensively studied in cereals (Pelger and Bothmer 1992; Shewry 1995, 1999).

The spatial and temporal expression of storage protein genes is primarily regulated at the transcriptional level. The genes encoding prolamins are coordinately expressed in the developing endosperm where they are under spatial and temporal transcription control, involving *cis*-acting and *trans*-acting motifs in their promoters and *trans*-acting transcription factors. A number of consensus sequences such as the prolamin box (P box), GCN4, AACA, and ACGT motifs, among others, have been identified as elements that are critical in determining the endosperm specificity of cereal seed storage protein genes (Zheng et al. 1993; Takaiwa et al. 1996; Washida et al. 1999; Wu et al. 2000). A conserved element, referred to as the endosperm box, located 300 bp upstream of the transcriptional start site, has been found in many cereal prolamin genes (Forde et al. 1985). The endosperm box has a bifactorial motif that is composed of the P box (TGTAAAG) and the GCN4 motif [TGA(G/C)TCA], which are separated by less than 10 nucleotides (Hammond-Kosack et al. 1993; Muller and Knudsen 1993). The GCN4 motif is recognized by endosperm specific basic Leu zipper (bZIP) *trans*-activators such as maize (*Zea mays*) opaque 2 (O2), wheat (*Triticum aestivum*) SPA1, barley (*Hordeum vulgare*) BLZ2, and rice RISBZ1 (Albani et al. 1997; Vicente-Carbajosa et al. 1998; Conlan et al. 1999; Onate et al. 1999; Onodera et al. 2001; Isabel-LaMoneda et al. 2003; Yanagisawa 2004). The P box has been reported to be recognized by a Dof (DNA binding with one finger) type transcription factor (Vicente-Carbajosa et al. 1997). This conserved P-box is found in all the prolamin gene promoters of cereals like maize, sorghum, barley, wheat, rice, oat, and rye, suggesting that *PBF Dof* activates the transcription of prolamins by binding with P-box.

Dof proteins are a group of plant-specific transcription factors that share a single highly conserved zinc finger motif named the Dof domain and have been associated with many biological processes (Yanagisawa 2002, 2004; Lopez et al. 2008; Dejun et al. 2009). It has been reported that the Dof protein P-box binding factor (PBF) is implicated in *trans*-activation of cereal seed storage protein genes (Vicente-Carbajosa et al. 1997; Mena et al. 1998). Deletion and point mutation experiments revealed that PB is important for the regulation of expression of the endosperm-specific genes (Mena et al. 1998; Díaz et al. 2005). Several *trans*-acting factors, including bZIP and DOF family members, jointly function in regulating expression of storage proteins during seed development (Isabel-LaMoneda et al. 2003; Mena, et al. 1998, 2002; Vicente-Carbajosa et al. 1997). The interaction between maize PBF and O2 (opaque 2) was involved in the zein protein expression during the seed development (Vicente-Carbajosa et al. 1997), and it is conserved in many promoters of cereal storage-protein genes (Yanagisawa 2002). Recent study shows that *BPBF* (Barley PBF) interacts with HvGAMYB protein by its C-terminal to activate the endosperm-specific genes during seed development (Diaz et al. 2002). Taken together, these studies demonstrated that the Dof proteins not only bind the *cis* element but also mediate protein–protein interaction through its Dof domain or C-terminal.

Maize *PBF* also regulates γ-zein gene expression in maize endosperms. PBF is an efficient activator of γ-zein gene, most likely by binding to AAAG motifs (prolamin boxes) present in the γ-zein regulatory sequence (Marzabal et al. 2008). Wheat DOF transcription factor *WPBF* interacts with TaQM and activates transcription of an alpha-gliadin gene during wheat seed development (Dong et al. 2007). *RPBF* from rice are involved in regulation of rice seed storage protein gene expression (Yamamoto et al. 2006). *BPBF* from barley is also involved in control of Hordein genes in barley endosperm (Mena et al. 1998). The *PBF Dof* transcription factor can be an important tool for seed storage protein manipulation for nutritional quality improvement based on its interactions with promoters of prolamins genes (Diaz et al. 2002; Vicente-Carbajosa et al. 1997).

Finger millet is a neutraceutical crop, i.e., very rich in calcium, minerals, and phosphorus as compared with other cereals. Finger millet contains on an average 7.3% protein. It is a good source of limiting amino acids like lysine and methionine and rich in vitamins like thiamine, riboflavin, and niacin. Thus, finger millet is a natural gift to the poor for their nutritional security, as they cannot afford to buy expensive fruits and livestock products. However, it is still a neglected crop and considered as low-status food (Barbeau and Hilu 1993; FAOSTAT Data 2006), and not much work has been done for its improvement in the

concerted fashion. Attempts were made to investigate the molecular mechanism associated with seed storage protein gene expression in the finger millet, which is being rich in protein content, and in essential amino acids is therefore considered in the present study. The *PBF Dof* gene of *Eleusine coracana* (Kushwaha et al. 2008) is first reported based on *in silico* studies, though it has already been reported in *T. aestivum*, *H. vulgare*, and *Z. mays*. Motif analysis reveals that there are motifs present in *PBF Dof* gene that are related with the expression of seed storage protein genes. To further confirm their function related with regulation of seed storage protein genes, spatial expression analysis of *PBF Dof* gene was done in three finger millet genotypes differing in seed protein content and color.

Materials and Methods

Plant Material

Three finger millet varieties PRM-1, PRM-701, and PRM-801 (differ in seed coat color) were selected. The tissues, including, root, stem, and flag leaf at vegetative stage and S1, S2, S3, and S4 stages of developing spikes of the three finger millet genotypes, were collected for semiquantitative reverse transcriptase polymerase chain reaction (RT PCR) and quantitative real-time PCR analysis.

Preparation of Total RNA and cDNA Synthesis

Total RNA was isolated from each sample 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 International. Canada). Total RNA (5 μg) of each sample was used to synthesize first-strand cDNA by using oligo$(dT)_{18}$ primer with RevertAid H Minus M-MuLV RT (Fermentas International Inc.) according to the manufacture's instruction. The efficiency of cDNA synthesis was assessed by reverse transcriptase PCR amplification of a control gene encoding tubulin.

Semiquantitative RT PCR Analysis

To analyze the *PBF Dof* transcript levels in different tissues, *PBF*- specific primers were designed from cloned *E. coracana PBF Dof* sequence available in Genbank database (Acc. No. EU760631). The gene-specific primer used was PBF Fwd CGTCCTCCCGACGTTTATGTCT and Rev CGGAGTCATCAACATCCCAATG, which yield the product size of 250 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 CTCCAAGCTTT CTCCCTCCT and Rev GCATCATCACCTCCTCCAAT. Oligonucleotides were purchased from Sigma ARK (Darmstadt, Germany). cDNAs were exponentially amplified using (Fermentas International Inc.) Taq Polymerase. PCR was performed in 25 μL of 1× KCl buffer (Fermentas International Inc.) containing 0.2 mM dNTPs, 30 ng of each primer, 1.5 mM $MgCl_2$, 0.8VU Taq DNA polymerase (Fermentas International Inc), 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 at 4°C.

Densitometric Analysis of Gel for Semiquantitative Analysis of Expressed Genes (PBF Dof and Tubulin)

Densitometry analysis was done with the help of Gene Profiler software, Alpha Innotech Corporation (San Leandro, CA). 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 Limited, Chennai, India) 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 *PBF Dof* and *Tubulin* gene used were same as earlier. The reverse transcription efficiencies of PBF Dof and tubulin gene were almost equal as analyzed by comparing the C_T values at different dilutions of cDNA (Livak and Schmittgen 2001). 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. Completely randomized design (CRD) was used for analyzing the gel data and real time data. The cycle threshold (Ct) value is the number of cycles required to accumulate enough SYBR green fluorescent signal to exceed the threshold (background) level. The Ct value is proportional to the amount of RT-PCR product and was used for quantification.

The relative value obtained for quantitation was expressed at $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ 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 $\Delta\Delta C_{\mathrm{T}}$ is difference between the ΔC_{T} value of a sample and that of its respective control.

Grain Weight Per Plant

Random sample of the grains from individual genotypes was obtained. These grain samples were dried at room temperature (30°C) to minimize intrinsic moisture content uniformity. Then these dried grain samples were weighed by electronic weighing balance to detect grain weight per plant.

Grain Protein Content

Nitrogen content in grains was determined by microkjeldahl method (Humphries 1956). The nitrogen content of grains was then multiplied by the factor 6.25 to obtain crude grain protein content and expressed in grams per 100 g of grain on a moisture-free basis.

Grain Prolamin Content

The sequential extraction method as described by Tatham et al. (1996) was used for determination of prolamin content of grains in finger millet genotypes.

Results and Discussion

The *PBF Dof* gene was cloned, and in silico analysis was performed by Kushwaha et al. (2008). This sequence was submitted to National Center for Biotechnology Information database and assigned the accession number EU760631 and protein ID ACF06717. In the present investigation, the primer was designed from this sequence to study spatial distribution of *PBF Dof* transcription factor in three-finger millet genotypes. *In silico* analysis of *PBF Dof* gene revealed 98% homology with *PBF* of *H. vulgare* sequence available in the database. The presence of motifs showing function related with regulation of endosperm-specific seed storage protein genes during seed development further confirms the identity of *PBF Dof* gene (Acc. no. EU760631) of *E. coracana* as *PBF Dof* (Kushwaha et al. 2008). Furthermore, the *PBF Dof* gene had the conserved DOF domain with four cysteine residues similar to what has been observed as typical feature of DOF families of proteins available from different plants.

The prolamin-box binding factor (PBF), a Dof transcription factor, is involved in gene regulation of seed storage proteins during seed development (Dong et al. 2007; Diaz et al. 2002; Vicente-Carbajosa et al. 1997). The expression profiling and densitometry analysis of *PBF Dof* gene in different tissues of finger millet genotypes are given in Figs. 1a, b; 2a, b; and 3a, b. Expression profiling studies at tissue level indicates that *PBF Dof* 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 genotype. However, there was variation in expression in all the three-finger millet genotypes. In the previous studies, it was observed that the expression of *PBF* was constrained to the endosperm in barley (Mena et al. 1998), and the expression of maize PBF was also limited to the endosperm (Vicente-Carbajosa et al. 1997). Dong et al. (2007) and Wang et al. (2006) reported that WPBF was constitutively expressed in all the tissues studied including root, leaf, and seeds except stem, which is quite different from that of the *PBFs* in barley and maize, where the expression was constrained to the endosperm. In the phylogenetic tree constructed based on DOF sequences of PBF of wheat, barley, maize, rice, and other crops using unweighted pair-group method with arithmetic mean and schematic distribution of respective conserved motifs

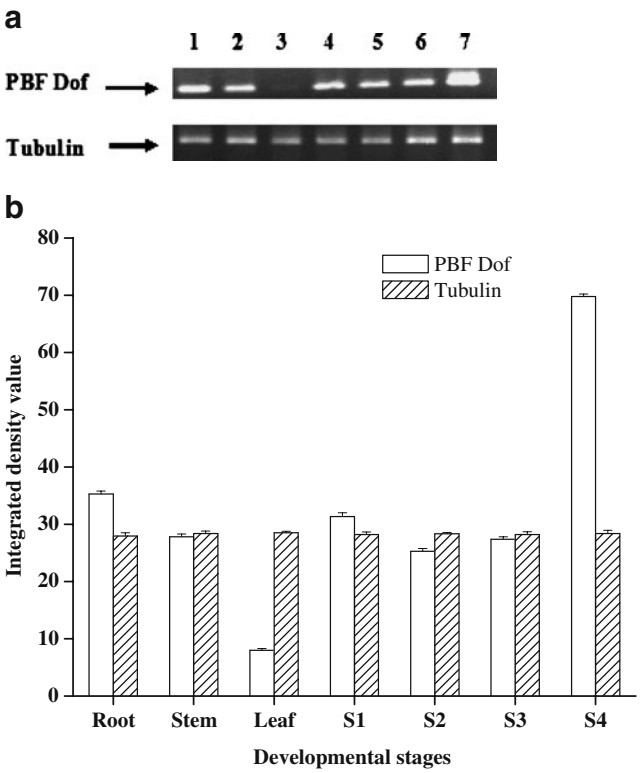

Fig. 1 **a** Expression pattern of *PBF Dof* in brown (PRM-1) genotype. Lane 1, root; lane 2, stem; lane 3, leaf; lane 4, S1 stage; lane 5, S2 stage; lane 6, S3 stage; lane 7, S4 stage. **b** Expression profiling of *PBF Dof* in different developmental stages of brown (PRM-1) genotype

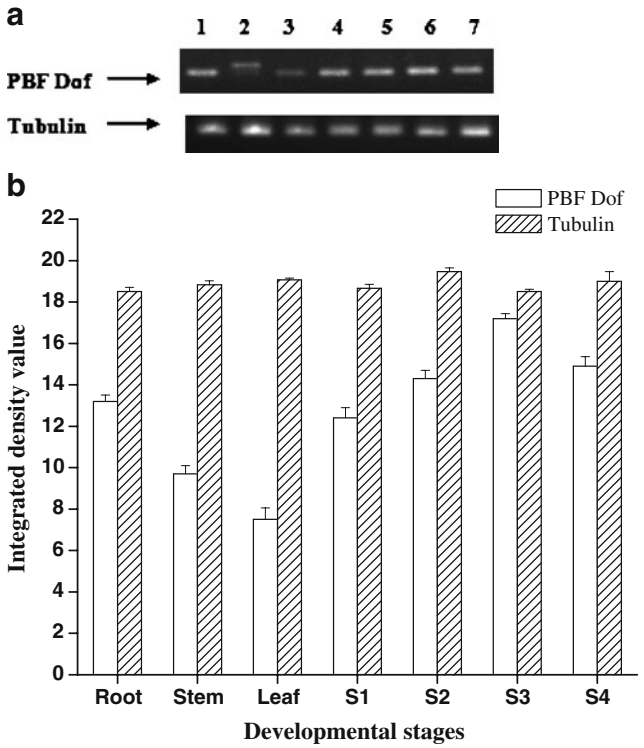

Fig. 2 **a** Expression pattern of *PBF Dof* in golden (PRM-701) genotype. Lane 1, root; lane 2, stem; lane 3, leaf; lane 4, S1 stage; lane 5, S2 stage; lane 6, S3 stage; lane 7, S4 stage. **b** Expression profiling of *PBF Dof* in different developmental stages of golden (PRM-701) genotype

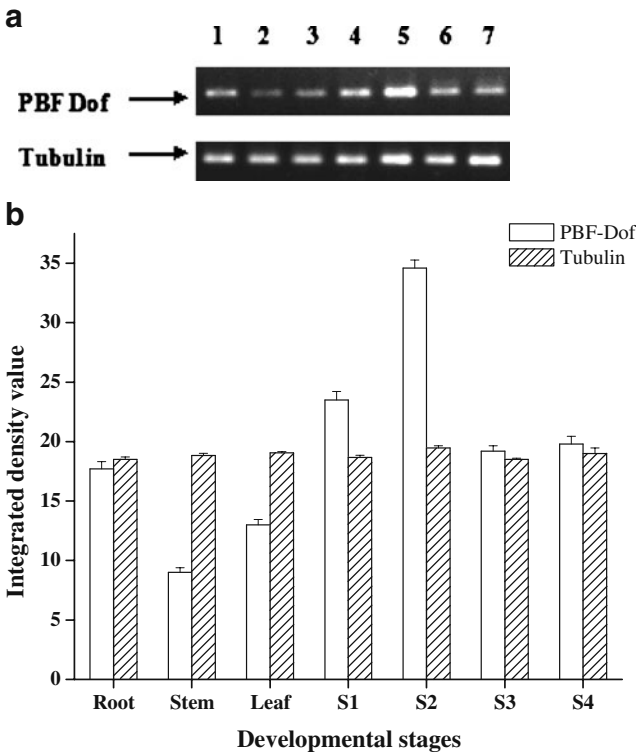

Fig. 3 **a** Expression pattern of *PBF Dof* in white (PRM-801) genotype. Lane 1, root; lane 2, stem; lane 3, leaf; lane 4, S1 stage; lane 5, S2 stage; lane 6, S3 stage; lane 7, S4 stage. **b** Expression profiling of *PBF Dof* in different developmental stages of white (PRM-801) genotype

identified by means of MEME software (Kushwaha et al. 2008), it was observed that the motifs arranged in the *PBF Dof* gene sequence were more aligned with wheat as compared with other crops. It could be said that finger millet *PBF* is more closed to wheat *PBF*. In our study also, the *PBF* gene was expressed in all the tissues including stem, which was related to wheat *PBF* but different from that of barley and maize *PBF* where expression was restricted to endosperm only.

The results of reverse transcription and real-time PCR were almost parallel to each other. The expression profiling with reverse transcriptase PCR indicates that in brown (PRM-1) genotype (Fig. 1a), the expression of *PBF Dof* was found to be least in leaf and highest in S4. In golden (PRM-701) genotype (Fig. 2a), the *PBF Dof* expression was least in leaf followed by root. In all the developing stages of spikes, the expression of *PBF Dof* was observed to be similar and slightly higher than vegetative stage tissues. In white (PRM-801) genotype (Fig. 3a), the *PBF Dof* expression was least in stem followed by leaf and highest in S2 stage of developing spike. In white (PRM-801) genotype, PBF played important role in activating prolamin genes of finger millet after anthesis (S2), which ultimately helped in the accumulation of higher level of grain protein in this genotype, and at grain filling and in mature seeds, the expression therefore remained constant. For reverse transcription efficiency in real-time PCR, reaction conditions were optimized with endogenous

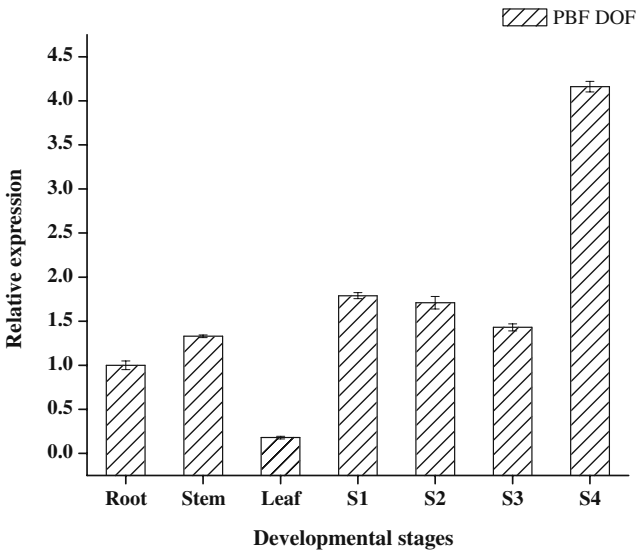

Fig. 4 Determination of relative expression of *PBF Dof* transcript in different tissues of brown (PRM-1) genotype using real-time PCR presentation of real*time data as final graph

control (*Tubulin*) and *PBF Dof* 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. Quantitation of the *PBF Dof* transcript was analyzed in root, stem, leaf, S1, S2, S3, and S4 stages of developing spikes. Relative expression of *PBF Dof* was calculated in brown (PRM-1) genotype (Fig. 4), and it was expressed at significantly higher level in S4 stage (4.16-fold) of developing spikes followed by S1 (1.79), S2 (1.71), S3 (1.43), stem (1.33), and leaf (1.18) as compared with root. Similarly, relative expression of *PBF Dof* in golden (PRM-701) genotype (Fig. 5) was calculated, and it was expressed at significantly higher level in S3 (1-fold) followed by S4 (0.68), S2 (0.54), and S1 (0.50) as compared with root (0.32), stem (0.12), and leaf (0.11). Similarly, relative expression of *PBF Dof* in white (PRM-801) genotype (Fig. 6) was calculated, and it was expressed at higher level in S2 (1.00) followed by S4 (0.48), S1 (0.46), and S3 (0.35) as compared with stem (0.30), leaf (0.30), and root (0.26). In brown (PRM-1) genotype, the expression of PBF Dof was higher at S4 stage of developing spike, which could not contribute for higher protein accumulation; however, in white (PRM-801) genotype, expression of *PBF Dof* was highest at S2 stage of developing spike, which gives more time for grain protein accumulation.

The grain protein content, grain prolamin content, and grain yield were calculated for the three-finger millet genotypes (Table 1). The grain protein content of brown (PRM-1) genotype was observed to be 7.5%, whereas of golden (PRM-701) and white (PRM-801) genotypes, it was found to be 9.0% and 9.56%, respectively. Similarly, grain prolamin content of brown (PRM-1) genotype was found to be lower (1.18%) as compared with golden (PRM-701) genotype (1.40%) and white (PRM-801) genotype (1.60%). However, grain yield of brown (PRM-1), golden (PRM-701), and white (PRM-801) was recorded to be 3.49 g per plant, 2.82 g per plant, and 1.66 g per plant, respectively. Thus, there was observed to be negative correlation between grain yield and grain protein/prolamin content. Negative associations between seed protein concentration and yield have been related to the higher energy cost of accumulating the extra protein (Hanson et al. 1961; Sinclair and de Wit 1975, 1976) since the synthesis of protein requires more glucose (energy) per gram than complex carbohydrates (Penning de Vries et al. 1974). Others have argued that increasing the amount of N in the seed would accelerate senescence and remobilization of N from leaves, shortening the seed-filling period and reducing yield (Sinclair and de Wit 1975, 1976; Frederick and Hesketh 1994; Triboi and Triboi-Blondel 2002).

The results are suggestive of the facts that the higher expression of *PBF Dof* is associated with developing stages of spikes as compared with vegetative tissues. The stages of

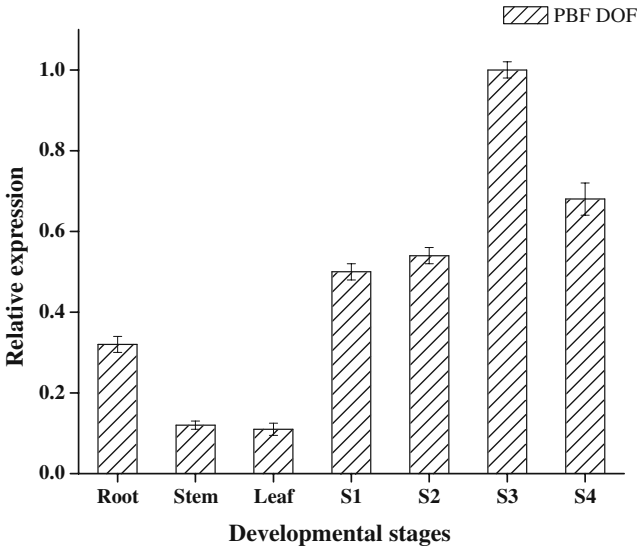

Fig. 5 Determination of relative expression of *PBF Dof* transcript in different tissues of golden (PRM-701) genotype using real-time PCR presentation of real-time data as final graph

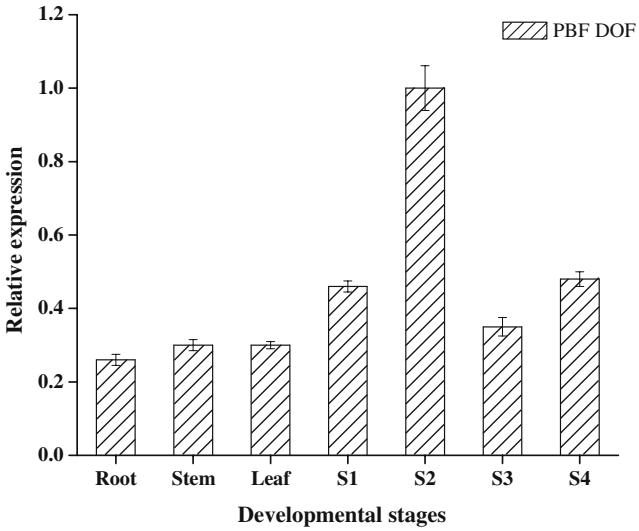

Fig. 6 Determination of relative expression of *PBF Dof* transcript in different tissues of white (PRM-801) genotype using real-time PCR presentation of real-time data as final graph

Table 1 Grain protein content, grain prolamin content, and grain yield of the three-finger millet genotypes

Genotype	Grain protein content (g%)	Grain yield (grain weight per plant)	Grain prolamin content (g%)
PRM-1	7.56	3.49	1.18
PRM-701	9.00	2.82	1.40
PRM-801	9.56	1.66	1.60

higher expression are also deciding the grain protein content in the finger millet genotypes depending on the higher expression of early or late stages of developing spikes.

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