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Full Length Research Paper

Genetic diversity and linkage disequilibrium of two homologous genes to maize *D8*: Sorghum *SbD8* and pearl millet *PgD8*

Yongle Li[1], Sankalp Bhosale[2], Bettina I. G. Haussmann[3], Benjamin Stich[4], Albrecht E. Melchinger[2] and Heiko K. Parzies[2*]

[1]Plant Breeding, Technische Universität München/Centre of Life and Food Sciences Weihenstephan, 85350 Freising, Germany.
[2]Institute for Plant Breeding, Seed Science, and Population Genetics, University of Hohenheim, 70593 Stuttgart, Germany.
[3]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), BP 12404, Niamey, Niger.
[4]Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany.

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Yield and yield stability of sorghum [*Sorghum bicolor* (L.) Moench.] and pearl millet [*Pennisetum glaucum* (L.) R.Br.] are highly influenced by flowering time and photoperiodic sensitivity in the arid to semi-arid regions of West and Central Africa. Photoperiodic sensitivity is the key adaptation trait of local landraces because it assures flowering at the end of the rainy season, independent of variable dates of planting. Flowering time genes are mainly integrated into four pathways with close interaction among each other: Vernalization, autonomous, GA (gibberellic acid) and photoperiod. In the GA pathway, maize *D8*, wheat *RHT1* and rice *SLR* have been identified as homologous genes to the Arabidopsis *GAI,* which is a negative regulator of GA response. We have identified two homologous genes to *D8*: Sorghum *SbD8* and pearl millet *PgD8.* These genes were expressed in the root and leaves of sorghum and pearl millet as revealed by EST database search and reverse transcription PCR, respectively. The genetic diversity of *SbD8* was considerably lower than that of *PgD8*. The extent of linkage disequilibrium in *PgD8* is lower than that of maize *D8*. *SbD8* and *PgD8* polymorphisms might be appropriate for dissection of photoperiod sensitivity using association mapping approaches.

Key words: DELLA proteins, GA pathway, flowering time, photoperiod sensitivity, sorghum, pearl millet.

INTRODUCTION

Sorghum (*Sorghum bicolor* (L. Moench.) and pearl millet (*Pennisetum glaucum* (L.) R.Br.) are the major staple crops and sources of income for about 120 million people in the arid to semi-arid regions of West and Central Africa (WCA). WCA is characterized by high climate variability which results in a high variability with respect to sowing date (Niangado, 2001). Local Guinea-race sorghum and pearl millet landraces have developed mechanisms of adaptation to these unpredictably changing growing conditions. Photoperiod sensitive flowering, the response of the plant to length of day, is one of the mechanisms present in a large portion of the local WCA cereal landraces. It can enhance adaptation to variable planting dates that are due to a scattered beginning of the rainy season, as is typical for WCA. It enhances simultaneous flowering of the cultivar in the target region, independent

*Corresponding author. E-mail: parzies@uni-hohenheim.de. Tel: 0049711459-23488. Fax: 0049711 459-22343.

Abbreviations: GA, Gibberellic acid; **EST,** expressed sequence tag; **WCA,** West and Central Africa; **LD,** linkage disequilibrium; **InDels,** insertions or deletions; **SNP,** single nucleotide polymorphism.

of the individual planting date in different fields. This has particular advantages in terms of reducing bird damage and insect pressure, adjusting vegetative development. Therefore, sorghum and pearl millet cultivars with photoperiod sensitivity may have the potential to increase yield and yield stability (Haussmann et al., 2007). Most sorghum and pearl millet cultivars are considered as quantitative short day plants, but different cultivars differ in their responses to photoperiod. Some cultivars are day-neutral, whereas others show a high response to photoperiod. For sorghum and pearl millet, only little information is available on flowering time genes, which are candidate genes for photoperiodic sensitivity. In contrast, several models for the molecular mechanism of flowering time were proposed for the model plant *Arabidopsis thaliana* (Roux and Touzet, 2006; Bernier and Périlleux, 2005; Putterill et al., 2004; Simpson and Dean, 2002). In these models, flowering time genes are mainly integrated into four closely interacting pathways: vernalization, autonomous, gibberellic acid (GA) and photoperiod. In vernalization pathway, *VERNALIZATION 2* (*VRN*2) inhibits flowering via repressing *VERNALIZATION* 1 (*VRN*1). This inhibition is overcome by prolonged exposure of a long period of cold in winter (Amasino, 2005). In autonomous pathway, internal developmental signals are required instead of environmental factors. Both pathways regulate a strong repressor of the flowering gene *FLOWERING LOCUS C* (*FLC*) (Bernier and Périlleux, 2005; Putterill et al., 2004). Genes included in the GA pathway such as *GIBBERELLIC ACID INSENSITIVE* (*GAI*), *REPRESSOR OF GA1-3* (*RGA*), and *RGA-LIKE1-3* (*RGL*1-3), act as constitutive growth repressors whose growth-repressing activity is opposed by GA in modulating floral development of *A. thaliana* (Cheng et al., 2004; Tyler et al., 2004). The photoperiod pathway involves genes encoding the photoreceptors *PHYTOCHROME -A to -E* (PHYA to PHYE) and *CRYPTOCHROME 1-2* (CRY1-2). Furthermore, the circadian clock components are required for correct circadian time measurement, several clock-associated genes, such as *GIGANTEA* (*GI*), *F-BOX* 1 (FKF1) and *PSEUDO-RESPONSE REGULATORS7* (PRR7) are activators of *CONSTANS* (*CO*) (Bernier and Périlleux, 2005; Imaizumi and Kay, 2006).

A crucial feature of the flowering regulatory network is that all four pathways ultimately regulate a common set of key integrator genes, *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*), which act on the floral meristem identity genes *APETALA 1* (*AP1*) and *LEAFY* (*LFY*) to initiate flowering (Boss et al., 2004; Henderson and Dean, 2004; Roux and Touzet, 2006). GA is a large family of tetracyclic diterpenoid plant growth factors, regulating seed germination, stem elongation, flowering and fruit development. In the facultative long-day plant Arabidopsis, GA is required for promoting flowering under short days (Wilson et al., 1992). Studies of GA signal transduction using genetic approaches have led to the identification of positive and negative signaling components. *GAI* was identified via transposon insertional mutagenesis and was characterized as a transcription factor that negatively regulates GA responses in Arabidopsis (Peng et al., 1997). A 51-bp deletion in the highly conserved N-terminal DELLA domain of *GAI* was identified as a dominant gain-of-function mutant (*gai*) with a dwarf phenotype caused by a reduced GA response (Peng et al., 1997). Recently, it was proposed that GA overcomes the DELLA repressing function by binding to the GA receptors OsGID1 or AtGID1a,b,c (Nakajima et al., 2006; Ueguchi-Tanaka et al., 2007) which induces the degradation of DELLA-proteins (Fu et al., 2002; Harberd, 2003). The involvement of photoperiod in the GA pathway suggests that DELLA is a putative crucial factor integrating these two different flowering pathways (Thomas et al., 1999; Garcia-Martinez and Gil, 2001; Achard et al., 2007). In several plant species, genes homologous to *GAI* of Arabidopsis were identified: *D8* in maize (Harberd and Freeling, 1989; Winkler and Freeling, 1994), the "green revolution" genes *Rht* in wheat (Gale and Youssefian, 1985), *SLN1* in barley (Chandler et al., 2002), *SLR1* in rice (Ikeda et al., 2001), *VvGAI* in grape (Boss and Thomas, 2002), *Brrga1-d* in *Brassica rapa* L. (Muangprom et al., 2005) and *MdDELLAs* in apple (Foster et al., 2007). However, information on homologous genes to *GAI* in sorghum and pearl millet is missing. Geneticists and biochemists have identified many relevant genes of the flowering time pathways using artificially induced variations, such as mutants. However, the ability to modify photoperiodic sensitivity in plant breeding programs will depend on an increased level of understanding naturally occurring variation (Yano et al., 2001). Deciphering the genetic determinism of natural variation is of interest not only to evolutionary biologists studying the genetics of adaptation in wild species, but also to plant breeders because it could provide useful guidelines for quantitative trait loci (QTL) studies and identification of target genes for selection as well as marker assisted selection (Morgante and Salamini, 2003). A genetic variant of maize *D8* leads to an earlier flowering phenotype (Thornsberry et al., 2001). This allele is present at a high frequency in North America while being almost absent in tropical regions and thus believed to be involved in maize climatic adaptation through diversifying selection for flowering time (Camus-Kulandaivelu et al., 2006). Nevertheless, only for few species, information is available on the genetic diversity of genes homologous to *GAI.* The objectives of our study were to investigate in a diverse set of sorghum and pearl millet genotypes: (i) the presence, (ii) the expression and (iii) the molecular diversity of genes homologous to *D8*.

MATERIALS AND METHODS

Plant material

Twenty six inbred lines in selfing generation >S_6 of sorghum and 20 inbreds (S_4) of pearl millet were selected from ~200 lines of

Table 1. Accession name and photoperiodic reaction of sorghum and pearl millet.

Accession name of sorghum	Photoperiodic reaction	Accession name of pearl millet	Photoperiodic reaction
IER(8)-02-SB–FSDT-12B	Sensitive	PE00057-B-B-1*	Sensitive
IER(9)-02PR-3009KB	Sensitive	PE05433-B-B-1	Sensitive
IER(13)-99-CLO 634B	Sensitive	PE05460-B-B-1	Sensitive
IS 3534 B	Sensitive	PE00991-B-B-1*	Sensitive
Fara Fara-17	Sensitive	PE00194-B-B-1	Sensitive
CS05/06AF(Guinea Niger)	Sensitive	PE05371-B-B-1	Sensitive
90 SN 1	Sensitive	PE05327-B-B-1	Sensitive
CS 0 4, SK 5912	Sensitive	PE05943-B-B-1	Sensitive
Bahu Banza	Sensitive	PE05303-B-B-1	Sensitive
Nigeria(SVMD Sova2006)	Sensitive	PE11322-B-B-1	Sensitive
ESO3(Fambe B)	Insensitive	PE02790-B-B-1	Sensitive
S05 AF- IS6731bf(R 127)	Insensitive	PE08058-B-B-1	Sensitive
ISO/ 06 P10 a- CSM63 E	Insensitive	PE08039-B-B-1*	Insensitive
IER(7)-97SB-FSDT-150 B	Insensitive	PE05927-B-B-1	Insensitive
GRINKAN	Insensitive	PE05321-B-B-1	Insensitive
MALISOR-92-1	Insensitive	PE02943-B-B-1*	Insensitive
Lata 3 (Balla Berthe)	Insensitive	Souna3-B-B-1	Insensitive
IER(18)-02-SB-F5DT169	Insensitive	PE00307-B-B-1*	Insensitive
IER(10)-00-KO-F5DT19	Insensitive	PE03855-B-B-1	Insensitive
IER(11)-08-SB-F4FT298	Insensitive	PE05393-B-B-1	No data
ST 9007-5-3-1	Insensitive		
IER(12)08-SB-F4FT189	Insensitive		
ISO/06P11dGPN99271202	No data		
ISO/05P7C- L224/25	No data		
ISO/05 P 11 d- CSM 388	No data		
ISO/03-CGM19/9-1-1	No data		

*RNA was extracted for RT-PCR.

sorghum and pearl millet (Table 1). The sorghum and pearl millet inbreds with diverse photoperiod responses were obtained from the regional centers of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Mali and Niger, respectively, and represent the genetic diversity present in WCA.

Photoperiod response assessment

We employed a simple non-destructive method to assess photoperiod response. We measured the vegetative phase of each inbred line by sowing on two different planting dates. The vegetative phase is the duration between sowing and initiation of flowering. The difference between vegetative phase duration at June and July plantings is used as an index to determine the photoperiod sensitivity of these inbred lines (White and Laing, 1989).

DNA and RNA extraction

From all sorghum and pear millet inbreds, DNA was isolated from fresh leaves of 3 - 4 week old plants, according to the protocol of Saghai-Maroof et al. (1984) with some modifications. From a random set of five pearl millet inbreds, RNA was extracted from fresh leaves of 5 - 6 week old plants using the RNeasy Plant Mini Kit of Qiagen Company (Hilden, Germany).

Genomic PCR and reverse transcription PCR

Primer pair *D8*-3 was obtained from the literature (Andersen et al., 2005). The other five primer pairs were designed based on the sequence of the *D8* gene of *Zea mays* (GeneBank accession no. AF413114) to amplify conserved domains of *D8* (Table 2) using Primer Premier Software (Premier Biosoft International, Palo Alto, CA, USA). All primer pairs were synthesized by Biomers (Ulm, Germany).

Touchdown PCR for genomic PCR amplification was carried out as follows: After an initial denaturation at 96℃ for 10 min, 20 cycles were conducted of 96℃ for 1 min, 60 – 50℃ (65 – 55℃ for *D8*-3) for 1min (decreasing with 0.5℃ per cycle) and 72℃ for 1 min, followed by 15 cycles of 96℃ for 1min , 50℃ (60℃ for *D8*-3) for 1 min, and 72℃ for 1 min, with a final extension step at 72℃ for 15 min. Reverse transcription (RT) PCR was carried out as described above plus a reverse transcription process where RNA and reagents were placed at 50℃ water bath for 30 min before touchdown PCR.

Processing DNA sequence

All genomic PCR and RT-PCR products were sequenced by QIAGEN (Hilden, Germany). Consensus sequence contigs were generated using SeqMan (DNAstar, Madison, WI, USA). Three fragments amplified by DELLA-3, VHVVD, and *D8*-3 were

Table 2. Names, amplified regions, melting temperature (Tm), sequences and results of primer pairs for amplified sequences.

Primers	Amplified regions and expected size (bp)	Tm (°C)	Forward sequences(5´→3´) Reverse sequences(5´→3´)	Amplification product in
DELLA-3	DELLA	51	GCTCCTCCAAGGACAAGATG	Pearl millet
	450-500	52	TAGTGCGACCGCCATCC	
VHVVD	NLS	57	TGGATGGCGGTCGCACTAG	Pearl millet
	300-350	56	TGGGCGAACTTCAGGTAGGG	
D8-3	SH2	50	CGATGACACGGATGACGA	Pearl millet
	350-400	54	AGGCATTGGAGCCCAGGT	
D8-S	5´UTR and DELLA	57	GCTATCCCAGAACCGAAACCG	Sorghum
	600-650	57	CGACGAGGAAGACGAAGACGA	
D8-1614	NLS	54	TCCACATCGTCCACCGTCAC	Sorghum
	550-600	50	GGGCGAACTTCAGGTAGG	

combined to an incomplete pearl millet *PgD8* gene. Two fragments amplified by *D8*-1614 and *D8*-S were combined to an incomplete sorghum *SbD8* gene. Consensus sequence for all inbreds were aligned using CLUSTAL alignment implemented in MegAlign (DNAstar, Madison, WI, USA) and Genebee multiple alignment program (Brodsky et al., 1992). Polymorphisms appearing in less than three inbreds were rechecked on chromatogram files to avoid PCR or scoring errors. The putative amino acid sequences were deduced from nucleotide sequence by EMBL-EBI Transeq tool. From six reading frames of putative amino acid sequences, the one with the most similarity to *D8* amino acid sequence and not including a stop codon was chosen.

Phylogenetic and molecular genetic diversity analyses

Cluster analysis was performed on deduced amino acid sequences of the DELLA and VHYNP regions of *PgD8*, *SbD8*, *D8* (*Z. mays*, GeneBank accession no. Q9ST48), *SLN1* (*Hordeum vulgare,* Q8W127), *SLR1* (*Oryza sativa*, Q7G7J6), *RHT1* (*Triticum aestivum,* Q9ST59) and *GAI* (*A. thaliana,* Q9LQT8) using Genebee (Brodsky et al., 1992). We chose the amino acid sequences of AP2 DNA binding domain and the two CBF subfamily signature motifs as reference and performed the same analysis for *SbCBF5 (S. bicolor,* AY785898*)*, *ZmCBF5* (*Z. mays,* DV523865), *HvCBF5* (*H. vulgare,* AY785855), *OsCBF5* (*O. sativa,* AY327040), *TaCBF5* (*T. aestivum,* EF028752) and *AtCBF5* (*A. thaliana,* CAA18178).

We calculated π which is the average number of nucleotide differences per site between two sequences (Nei, 1987). Tajima´s test for selection was applied which is based on the differences between the number of segregating sites and the average number of nucleotide differences (Tajima, 1989). The squared allele frequency correlation r^2 was estimated for all pairs of polymorphic sites with allele frequencies ≥ 0.10. Chi-square test was used to determine significance of linkage disequilibrium (LD) between pairs of polymorphic sites. Sites containing alignment gaps and single nucleotide polymorphisms with more than two alleles were excluded from the molecular genetic analyses. All molecular genetic analyses were performed using DNASP software package (Rozas et al., 2003).

To investigate the expression status of SbD8, the nucleotide sequence of *SbD8* was “blasted” against the S. bicolor EST database of NCBI by BLASTN.

RESULTS

Amplification and alignment analysis of *SbD8* and *PgD8*

Sorghum DNA was successfully amplified by two primer pairs, *D8*-S and *D8*-1614 (Table 2). Twenty two of the 26 sorghum genotypes were amplified by *D8*-S; 24 of the 26 sorghum genotypes were amplified by *D8*-1614. Pearl millet DNA from 16 of the 20 genotypes were successfully amplified by three primer pairs VHVVD, DELLA-3 and *D8*-3. Alignment of the deduced amino acid sequences of *PgD8, SbD8* and *D8* (Figure 1) showed 51.5% homology. If non-amplified regions were neglected, homology was much higher (84.3%). Eight conserved regions were found. The first two domains (VHYNP and DELLA) are highly conserved within the DELLA protein subfamily (Figure 2), showing 80.9% homology.

Gene expression of *SbD8* and *PgD8*

The nucleotide sequences of *SbD8* and *PgD8* were used as queries to search the *Sorghum bicolor* and *Pennisetum glaucum* EST database in NCBI by BLASTN (http://www.ncbi.mln.nih.gov/). Six ESTs were found with significant homology to *SbD8* (Table 3).

The highest individual query coverage was only 61%, however, the total query coverage was 91.6% (data not shown). This high total query coverage together with a low E-value, high individual ESTs total score, and maximum identity suggested that *SbD8* was expressed in *S. bicolor*.

No EST was found with significant homology to *PgD8* in NCBI *P. glaucum* EST database.

Fifteen ESTs were found in Gramene

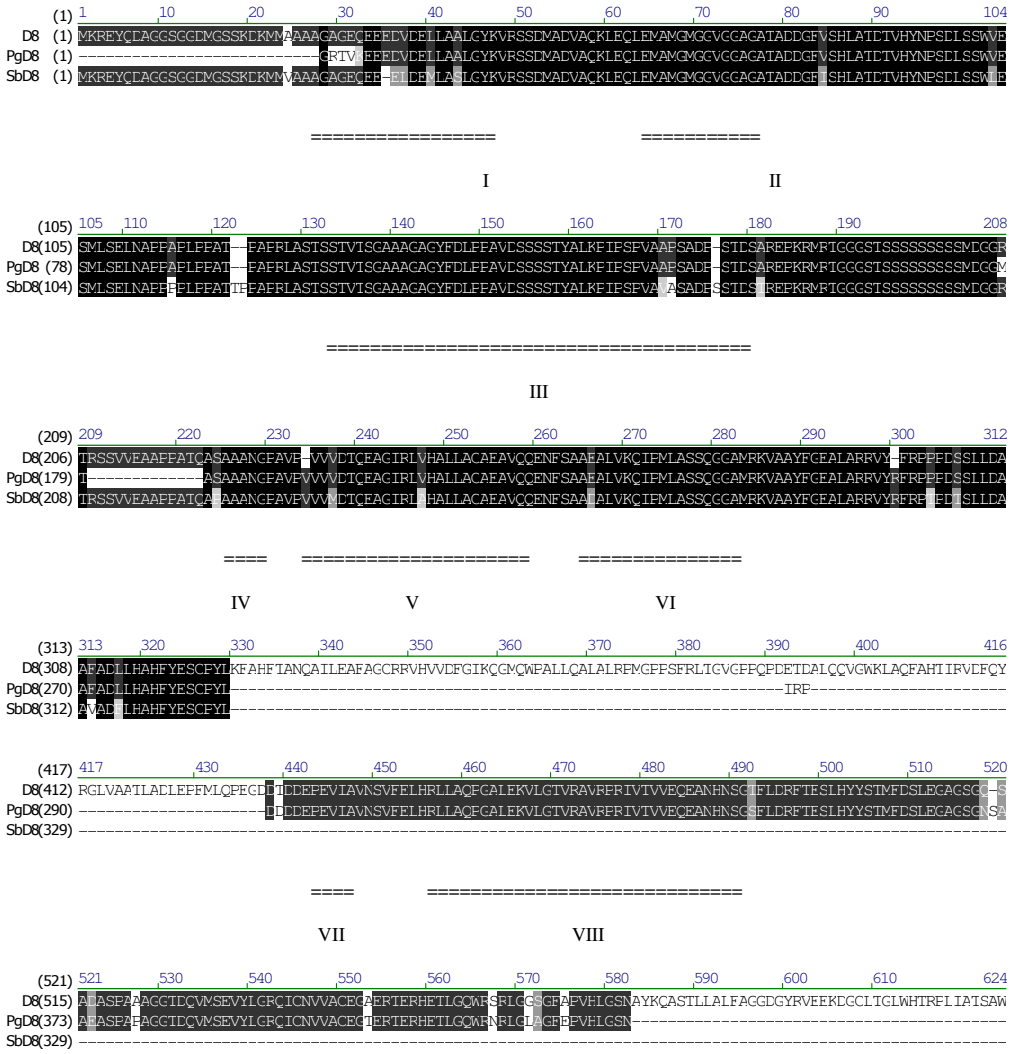

Figure 1. Alignment of the deduced amino acid sequences of *PgD8*, *SbD8* and *D8* (*Z. mays*, GenBank accession no. Q9ST48). Gaps in position 122, 123, 176, 234 and 300 indicated by single line (-) are introduced to maximize alignment. Other gaps indicated by single line (-) are the regions which have not been amplified. Identical amino acid residues are highlighted in black. Similar amino acid residues are highlighted in dark grey. Eight conserved sequence regions are indicated by double lines: Region I is the DELLA motif; Region II is VHYNP; Region III is PolyS/T; Region IV is valine-rich; Region V is LHR1; Region VI is a nuclear-localization signal (NLS); Region VII is the LXXLL motif; Region VIII is the putative Src homology 2 (SH2).

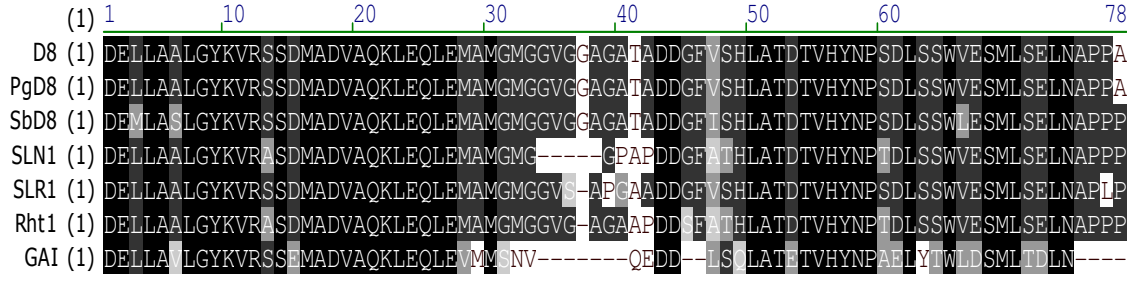

Figure 2. Deduced amino acid sequences alignment in the DELLA and VHYNP regions of *PgD8*, *SbD8* and another DELLA subfamilies: *D8* (*Z. mays*, GenBank accession no. Q9ST48), SLN1 (*H. vulgare* no. Q8W127), SLR1 (*O. sativa* no. Q7G7J6), RHT1 (*T. aestivum* no. Q9ST59) and GAI (*A. thaliana* no. Q9LQT8). Identical amino acid residues are highlighted in black. Gaps indicated by single line (-) are introduced to maximize alignment. Similar amino acid residues are highlighted in dark grey or grey.

Table 3. S.bicolor EST that hit *SbD8* by BLASTN.

Accession number	Description	Total score	Query coverage	E-value	Maximum identity
CN131369.1	Acid- and alkaline-treated roots *S. bicolor* cDNA, mRNA	1346	61%	0.0	99%
CD219739.1	Callus culture cell suspension *S. bicolor* cDNA, mRNA	1113	51%	0.0	99%
BE595338.1	Pathogen induced 1 (PI1) *S. bicolor* cDNA, mRNA	1219	55%	0.0	99%
BM318611.1	Pathogen induced 1 (PI1) *S. bicolor* cDNA, mRNA	1023	46%	1e-170	99%
BG411689.1	Embryo 1 (EM1) *S. bicolor* cDNA, mRNA	446	21%	6e-124	98%
CN137035.1	Oxdatively-stressed leaves and roots *S. bicolor* cDNA , mRNA	339	16%	1e-91	97%

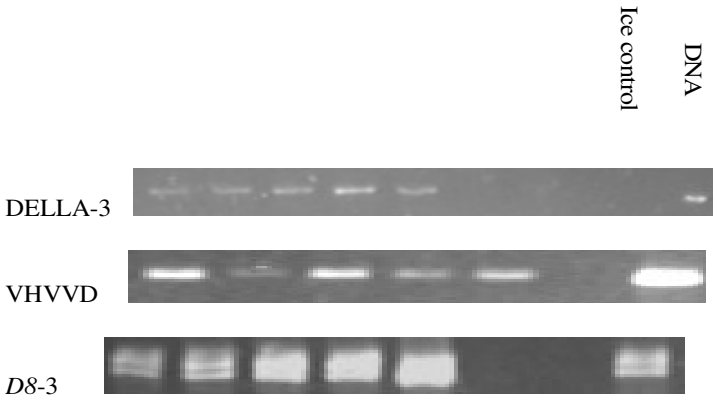

Figure 3. Gel Electrophoresis of reverse transcriptase PCR (RT-PCR) products. All 5 Pearl millet genotypes were amplified by three different primer pairs (DELLA-3, VHVVD, and *D8*-3). Ice control was a negative control for DNA contamination. DNA was a positive control.

(http://www.gramene.org) but high E-value (>0.5) and low coverage (<0.5%) prevented us from confirming that *PgD8* is expressed in pearl millet. Therefore, RT-PCR was performed to investigate the expression of *PgD8* (Figure 3). Pearl millet mRNA, extracted from 4-week old leaves of five inbreds, was amplified by RT-PCR with three primer pairs (VHVVD, DELLA-3, *D8*-3). The sequences of the RT-PCR product confirmed that *PgD8* is expressed.

Molecular genetic diversity and LD

Out of 1124 sites investigated, five were polymorphic in *SbD8* and out of 1228 sites, 32 were polymorphic in *PgD8* (Table 4). One 3-bp insertion or deletion (InDel) was observed near the SH2-like domain in the C-terminal of the open reading frame of *PgD8.* By contrast, no InDel were found in *SbD8*. All other polymorphism were SNPs. According to the value of π/bp, *PgD8* showed higher nucleotide diversity than *SbD8*. For sorghum as well as pearl millet, the nucleotide diversity for non-synonymous polymorphic sites was higher than for synonymous polymorphic sites. Tajima's D test was significant ($P<0.05$) for *PgD8*, but not for *SbD8.*

The number of pair-wise comparisons versus significant ($P<0.05$) pair-wise comparisons by Chi-square test in *SbD8* and *PgD8* were 15 versus 3 and 496 versus 381,

Table 4. *SbD8* and *PgD8* nucleotide diversity.

Region	No. of polymorphic sites	% sites with polymorphism	Nucleotide diversity π/bp
SbD8			
Synonymous	0	0	0
Non-synonymous	5	0.62	0.00204
Total	5	0.45	0.00163
PgD8			
Synonymous	6	0.67	0.00532
Non-synonymous	26	2.89	0.00792
Total	32	3.56	0.00704

respectively. LD between pairs of sites against nucleotide distance remained almost the same in *SbD8*, whereas in *PgD8* it declined exponentially

DISCUSSION

Photoperiod reaction and flowering time

The ability to recognize and respond to changes in day length is known as photoperiodism. For cultivars with photoperiodic sensitive reaction, that is, photoperiod-dependent flowering, flowering time is regulated by the daily duration of light. Photoperiod sensitivity is a key agronomical trait of local landraces of sorghum and pearl millet in WCA. This is due to the fact that photoperiod sensitivity assures flowering at the end of the rainy season, independent of the date of sowing, which is extremely important because of the high variability in the start of the rainy season (Vaksman et al., 1996; Niangado, 2001).

Amplification and alignment analysis of homologous genes to *D8*: *SbD8* and *PgD8* in sorghum and pearl millet

The successful genomic amplification of *SbD8* and *PgD8* suggested that the homologous genes to *D8* are also present in sorghum and pearl millet. This finding was further confirmed by alignment analysis of the deduced amino acid sequences of *PgD8, SbD8* and *D8*. Eight conserved regions were found (Figure 1). Region I: the highly conserved N-terminal DELLA motif. This region is absent in Arabidopsis mutant with dwarf phenotype caused by a reduced GA response (Peng et al., 1997 and 1999). Region II: a conserved domain that acts like the DELLA motif (Sun and Gubler, 2004; Gubler et al., 2002) and both of them contain putative phosphorylation sites for GA signal perception (Itoh et al., 2005). Region III: PolyS/T, a putative enhancer for suppressive activation of the DELLA proteins (Silverstone et al., 1998; Itoh et al., 2002). Region IV: Leucine heptad repeats, which are found in transcription factors such as bZIP proteins and are important for protein–protein interaction (Bolle, 2004). Region V: a Valine-rich region characteristic for transcription factors. Region VI: a nuclear localization signal (NLS) Nakai and Kanehisa, 1992). Region VII: a LXXLL region (where X stands for any amino acid), that was identified in a number of steroid receptor co-activators (SRCs) and is responsible for SRC binding to steroid receptors in the nucleus (Heery et al., 1997; Torchia et al., 1997). Region VIII: a putative Src homology 2 (SH2) phosphotyrosine binding domain (Peng et al., 1999) which is present in a family of transcription factors called signal transducers and activators of transcription (STATs) in animals (Darnell, 1997). The function of SH2 is to mediate the binding of STATs to various receptor tyrosine kinases by which the STATs are then activated and translocated from the cytoplasm to the nucleus (Ikeda et al., 2001). In maize, a 6bp deletion flanking the SH2-like domain of *D8* was significantly associated with flowering (Thornsberry et al., 2001). This finding suggested that SH2 might play a role in controlling flowering time.

Molecular genetic diversity and LD

In our investigation, the nucleotide diversity π/bp of *SbD8* was 0.00163. In contrast, the nucleotide diversity π/bp of *PgD8* was higher (0.00704). This finding might be explained by (1) the lower inbreeding generation (S_4 vs. S_6) of pearl millet compared with sorghum and (2) the higher rate of polymorphism in allogamous species such as pearl millet compared to autogamous species such as sorghum (Rafalski, 2002). Thornsberry et al. (2001) reported a nucleotide diversity π/bp for *D8* in maize of 0.0018 which is similar to *SbD8* but lower than that of *PgD8*. The opposite result was expected as maize, is an allogamous species. This discrepancy might be explained by the fact that different gene fragments were examined in our study and that of Thornsberry et al. (2001).

The Tajima's D test values of *SbD8* and *PgD8* were 0.34062 and -1.95606, respectively. The value for *SbD8* was non-significantly (P=0.05) different from 0, indicating

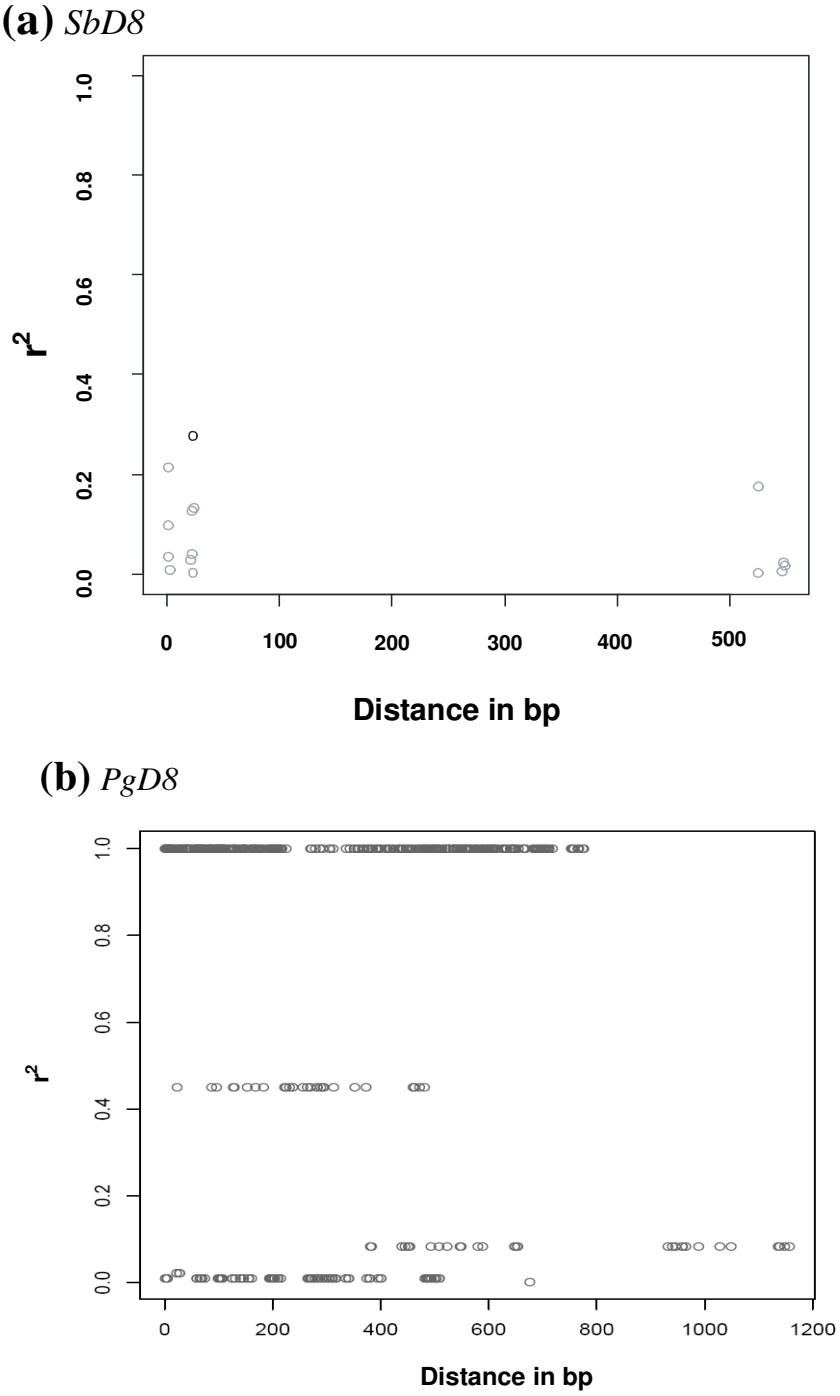

Figure 4. Plots of squared correlation of allele frequencies (r^2) against nucleotide distance between polymorphic sites in *SbD8* and *PgD8*.

that polymorhisms are selectively neutral, whereas the value for *PgD8* was significant, suggesting that it has been a target of selection.

The r^2 values for *PgD8* declined to 0.1 or less within 1000 bp (Figure 4). In contrast, Remington et al. (2001) reported for *D8* that in a diverse set of 102 maize inbred lines LD decays within 2400 bp to r^2 values <0.1. This can be explained by the bottleneck effect since selection in maize was more intensive and consequently only few allelic combinations were passed on to future generations. Since we have only investigated one gene in 20 inbred lines, a better understanding of LD and consequently mapping resolution in pearl millet would require a larger number of loci and genotypes. In sorghum, only

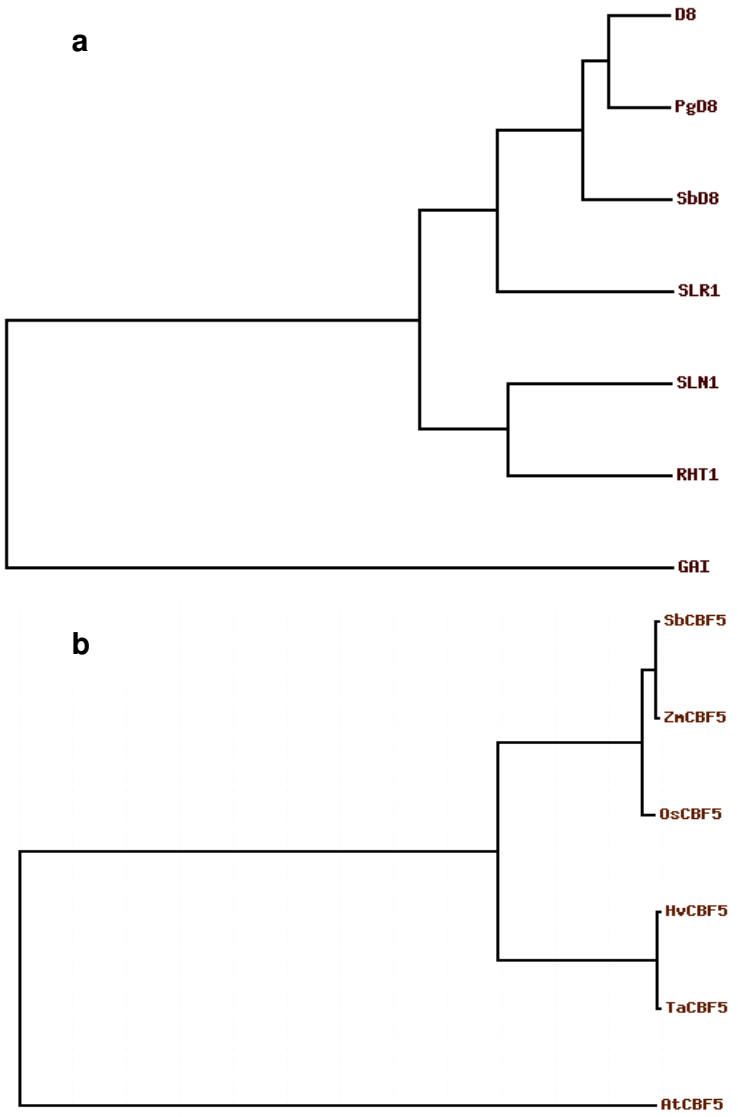

Figure 5. a) Cluster analysis of *PgD8*, *SbD8*, *D8* (*Z. mays*), *SLN1* (*H. vulgare*), *SLR1* (*O. sativa*), *RHT1* (*T. aestivum*) and *GAI* (*A. thaliana*) based on deduced amino acid sequences of DELLA and VHYNP region. Species that are connected by a single branch point (node) are sister taxa. The more nodes separating two species, the more distantly related they are. b) Cluster analysis of *SbCBF5 (S. bicolor)*, *ZmCBF5* (*Z. mays*), *HvCBF5* (*H. vulgare*), *OsCBF5* (*O. sativa*), *TaCBF5* (*T. aestivum*) and *AtCBF5* (*A. thaliana*) based on amino acid sequences of AP2 DNA binding domain and two CBF subfamily signature motifs.

few polymorphic sites were observed. Thus, no clear conclusions can be made regarding the decay of LD.

Cluster analysis of *SbD8* and *PgD8*

Full justification on deduced amino acid sequences of the DELLA and VHYNP regions, the cluster analysis of *PgD8*, *SbD8*, maize *D8*, barley *SLN1*, rice *SLR1*, wheat *RHT1* and Arabidopsis *GAI* revealed four clusters: Panicoideae (sorghum, maize and pearl millet), Oryzoideae (rice), Pooideae (wheat and barley) and Brassicaceae (Arabidopsis). In order to compare our result with the cluster analysis of another gene, we performed the same analysis on the amino acid sequence of CBF5, which is a transcriptional factor specifically bound to *cis*- elements of cold responsive gene (COR) under cold stress. The cluster analysis of the *D8* homologous and CBF5 were in good accordance with each other (Figure 5).

Furthermore, the result of our cluster analysis was in good accordance with that expected on the basis of the grass phylogeny (Grass Phylogeny Working Group, 2001). However, one difference is that in our study, maize is more closely related to pearl millet than sorghum, whereas the opposite has been reported on the basis of the grass phylogeny. This difference might be due to the fact that different parts of the genome were examined. We examined the nuclear genes *Sbd8* and *PgD8* whereas the Grass Phylogeny Working Group (2001) based their analyses on chloroplast genes *rbc*L and *ndh*F. This explanation was supported by findings of Gaut and Doebley (1997) who reported a divergence time between sorghum and maize of 16.5 million years by using nuclear genes *mdh* and *waxy*, whereas 9 million years was believed to be the divergence time of these two crops in the studies of the Grass Phylogeny Working Group using chloroplast genes.

IMPLICATIONS AND PERSPECTIVES OF THIS STUDY FOR SORGHUM AND PEARL MILLET BREEDING

Sorghum and pearl millet are crops with high drought tolerance and economic importance in arid to semi-arid regions of WCA. However, it is difficult and time consuming to select cultivars with photoperiod sensitivity in the field. Based on this study, LD decays within 1000 bp in *PgD8* that is very useful information for identification of functional nucleotide polymorphisms using a LD-approach. The results of this study open up possibilities to develop advanced plant breeding techniques such as marker-assisted selection. Photoperiod sensitivity is of particular importance for local landraces of sorghum and pearl millet in WCA. Two parallel studies uncovered that Phytochrome Interacting Factors (PIFs) and DELLA are two crucial integration nodes in two previous distinguish flowering time pathways: photoperiod and GA pathway. (de Lucas et al., 2008; Feng et al., 2008). Maize *D8* seems to affect the quantitative variation of flowering time and plant height according to mutagenesis and quantitative trait locus studies (Peng et al., 1999; Koester et al., 1993; Schön et al., 1994). Furthermore, Thornsberry et al. (2001) identified a set of intragenic polymorphisms associated with differences in flowering time using association approaches, nine (four SNPs, four InDels and one miniature transposable element) of which were validated by Andersen et al. (2005). When population structure was ignored, six of the nine *D8* polymorphisms were significantly associated with flowering time. However, when population structure was taken into account, only a single polymorphisms (one InDel in the promoter region) was associated with flowering time. It is noteworthy that Thornsberry et al. (2001) used a worldwide collection of maize, whereas Andersen et al. (2005) used only European materials. Besides a 6-bp InDel near the SH2-like domain (position 3472) in the C-terminal of the open reading frame (ORF) had strong association with flowering time under long-day conditions, after correcting for population structure (Camus-Kulandaivelu et al., 2006). Interestingly, a 3-pb InDel near the SH2-like domain of *PgD8* has been found in our study. Therefore, it may be possible to select sorghum and pearl millet lines with photoperiod sensitivity using SNPs or InDels as indirect (statistical) functional markers (IFMs). IFMs are derived from polymorphic sites within genes causally affecting phenotypic trait variation by association studies (Andersen and Luebberstedt, 2003).

In order to develop IFMs, association studies need to be conducted to confirm a significant correlation between SNPs and photoperiod sensitivity. In this regard, population structure must be taken into account since it can lead to spurious associations between SNP-marker and the phenotype (Yu et al., 2006; Zhao et al., 2007; Stich et al., 2008). Additionally, IFMs need to be verified by using a mapping population. Association studies by using the candidate gene approach have been proven to be a powerful tool in medical genetics. As more advanced statistical methods are now available to overcome problems caused by population structure, association studies can be applied to practical cereal breeding approaches in the near future.

In this study, two homologous genes to *D8*: Sorghum *SbD8* and pearl millet *PgD8* have been found and characterised. EST database search and reverse transcription PCR suggested that *SbD8* and *PgD8* were expressed in the root and leaves of sorghum and pearl millet, respectively. The genetic diversity of *SbD8* was considerably lower than that of *PgD8*. The extent of linkage disequilibrium in *PgD8* is lower than that of maize *D8*. The implications of discovering *SbD8* and *PgD8* for sorghum and pearl millet breeding have been discussed.

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