Full Length Research Paper

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

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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...
ört 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 Périlleux, 2005; 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 (VRN2) inhibits flowering via repressing VERNALIZATION 1 (VRN1). 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 (RLG1-3), 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; Imazumi 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. GA1 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 GA1 was identified as a dominant gain-of-function mutant (ga1) 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 GA1 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), SLF1 in rice (Ikedo 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 GA1 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 GA1. 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 >S6 of sorghum and 20 inbreds (S6) of pearl millet were selected from ~200 lines of
Table 1. Accession name and photoperiodic reaction of sorghum and pearl millet.

<table>
<thead>
<tr>
<th>Accession name of sorghum</th>
<th>Photoperiodic reaction</th>
<th>Accession name of pearl millet</th>
<th>Photoperiodic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>IER(8)-02-SB–FSDT-12B</td>
<td>Sensitive</td>
<td>PE00057-B-B-1*</td>
<td>Sensitive</td>
</tr>
<tr>
<td>IER(9)-02PR-3009KB</td>
<td>Sensitive</td>
<td>PE05433-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>IER(13)-99-CLO 634B</td>
<td>Sensitive</td>
<td>PE05460-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>IS 3534 B</td>
<td>Sensitive</td>
<td>PE09991-B-B-1*</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Fara Fara-17</td>
<td>Sensitive</td>
<td>PE00194-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>CS05/06AF(Guinea Niger)</td>
<td>Sensitive</td>
<td>PE05371-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>90 SN 1</td>
<td>Sensitive</td>
<td>PE05327-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>CS 0 4, SK 5912</td>
<td>Sensitive</td>
<td>PE05943-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Bahu Banza</td>
<td>Sensitive</td>
<td>PE05303-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Nigeria(SVMD Sova2006)</td>
<td>Sensitive</td>
<td>PE11322-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>ESO3(Fambe B)</td>
<td>Insensitive</td>
<td>PE02790-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>S05 AF- IS6731bf(R 127)</td>
<td>Insensitive</td>
<td>PE08058-B-B-1</td>
<td>Sensitive</td>
</tr>
<tr>
<td>ISO/ 06 P10 a- CSM63 E</td>
<td>Insensitive</td>
<td>PE08039-B-B-1*</td>
<td>Insensitive</td>
</tr>
<tr>
<td>IER(7)-97SB-FSDT-150 B</td>
<td>Insensitive</td>
<td>PE05927-B-B-1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>GRINKAN</td>
<td>Insensitive</td>
<td>PE05321-B-B-1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>MALISOR-92-1</td>
<td>Insensitive</td>
<td>PE02943-B-B-1*</td>
<td>Insensitive</td>
</tr>
<tr>
<td>Lata 3 (Balla Berthe)</td>
<td>Insensitive</td>
<td>Souma3-B-B-1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>IER(18)-02-SB-F5DT169</td>
<td>Insensitive</td>
<td>PE00307-B-B-1*</td>
<td>Insensitive</td>
</tr>
<tr>
<td>IER(10)-00-KO-F5DT19</td>
<td>Insensitive</td>
<td>PE03855-B-B-1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>IER(11)-08-SB-F4FT298</td>
<td>Insensitive</td>
<td>PE05393-B-B-1</td>
<td>No data</td>
</tr>
<tr>
<td>ST 9007-5-3-1</td>
<td>Insensitive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IER(12)08-SB-F4FT189</td>
<td>Insensitive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISO/06P11dGP99271202</td>
<td>No data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISO/05P7C- L224/25</td>
<td>No data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISO/05 P 11 d- CSM 388</td>
<td>No data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISO/03-CGM19/9-1-1</td>
<td>No data</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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 pearl 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°C for 10 min, 20 cycles were conducted of 96°C for 1 min, 60 – 50°C (65 – 55°C for D8-3) for 1 min (decreasing with 0.5°C per cycle) and 72°C for 1 min, followed by 15 cycles of 96°C for 1 min, 50°C (60°C for D8-3) for 1 min, and 72°C for 1 min, with a final extension step at 72°C 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°C 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
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, CA059187).

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 r² 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 VHVD, 
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.nlm.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

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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Amplified regions and expected size (bp)</th>
<th>Tm (°C)</th>
<th>Forward sequences(5'→3')</th>
<th>Reverse sequences(5'→3')</th>
<th>Amplification product in</th>
</tr>
</thead>
<tbody>
<tr>
<td>DELLA-3</td>
<td>DELLA</td>
<td>51</td>
<td>GCTCTCTCAAGGAGAACAGATG</td>
<td>TATGCGGACCACCATCC</td>
<td>Pearl millet</td>
</tr>
<tr>
<td></td>
<td>450-500</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VHVD</td>
<td>NLS</td>
<td>57</td>
<td>TGGATGGCGGTCGCAGTACTAG</td>
<td>TGGGGCGAATTCAGGTAAGGG</td>
<td>Pearl millet</td>
</tr>
<tr>
<td></td>
<td>300-350</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D8-3</td>
<td>SH2</td>
<td>50</td>
<td>CGATGACACGGATAGCGA</td>
<td>AGGCATTGGAGCCAGGT</td>
<td>Pearl millet</td>
</tr>
<tr>
<td></td>
<td>350-400</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D8-S</td>
<td>5’UTR and DELLA</td>
<td>57</td>
<td>GCTATCCGAGAACCAGAACCG</td>
<td>CGACAGGAAGAGAAGACGA</td>
<td>Sorghum</td>
</tr>
<tr>
<td></td>
<td>600-650</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D8-1614</td>
<td>NLS</td>
<td>54</td>
<td>TCCACATCGTCACCCGTAC</td>
<td>GGGCGAACTCCAGGTAAGG</td>
<td>Sorghum</td>
</tr>
<tr>
<td></td>
<td>550-600</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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. Q9LOT8). 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.

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

![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)

(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 \( \pi \)/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,
respectively. LD between pairs of sites against nucleotide distance remained almost the same in \textit{SbD8}, whereas in \textit{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 \textit{D8}: \textit{SbD8} and \textit{PgD8} in sorghum and pearl millet**

The successful genomic amplification of \textit{SbD8} and \textit{PgD8} suggested that the homologous genes to \textit{D8} are also present in sorghum and pearl millet. This finding was further confirmed by alignment analysis of the deduced amino acid sequences of \textit{PgD8}, \textit{SbD8} and \textit{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 \textit{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 \(\pi/bp\) of \textit{SbD8} was 0.00163. In contrast, the nucleotide diversity \(\pi/bp\) of \textit{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 \(\pi/bp\) for \textit{D8} in maize of 0.0018 which is similar to \textit{SbD8} but lower than that of \textit{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 \textit{SbD8} and \textit{PgD8} were 0.34062 and -1.95606, respectively. The value for \textit{SbD8} was non-significantly (\(P=0.05\)) different from 0, indicating...
that polymorphisms 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 4. Plots of squared correlation of allele frequencies ($r^2$) against nucleotide distance between polymorphic sites in *SbD8* and *PgD8*. 
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), Pooidae (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 rbcL and ndhF. 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; Schönh 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 polymorphism (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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