Leaf morphology in Cowpea \([Vigna\ unguiculata\ (L.)\ Walp]\): QTL analysis, physical mapping and identifying a candidate gene using synteny with model legume species

Marti Pottorff\(^1\), Jeffrey D Ehlers\(^1,2\), Christian Fatokun\(^3\), Philip A Roberts\(^4\)* and Timothy J Close\(^1\)*

Abstract

**Background:** Cowpea \([Vigna\ unguiculata\ (L.)\ Walp]\) exhibits a considerable variation in leaf shape. Although cowpea is mostly utilized as a dry grain and animal fodder crop, cowpea leaves are also used as a high-protein pot herb in many countries of Africa.

**Results:** Leaf morphology was studied in the cowpea RIL population, Sanzi (sub-globose leaf shape) x Vita 7 (hastate leaf shape). A QTL for leaf shape, \(Hls\) (hastate leaf shape), was identified on the Sanzi x Vita 7 genetic map spanning from 56.54 cM to 67.54 cM distance on linkage group 15. SNP marker 1\_0910 was the most significant over the two experiments, accounting for 74.7% phenotypic variance (LOD 33.82) in a greenhouse experiment and 71.5% phenotypic variance (LOD 30.89) in a field experiment. The corresponding \(Hls\) locus was positioned on the cowpea consensus genetic map on linkage group 4, spanning from 25.57 to 35.96 cM. A marker-trait association of the \(Hls\) region identified SNP marker 1\_0349 alleles co-segregating with either the hastate or sub-globose leaf phenotype. High co-linearity was observed for the syntenic \(Hls\) region in *Medicago truncatula* and *Glycine max*. One syntenic locus for \(Hls\) was identified on Medicago chromosome 7 while syntenic regions for \(Hls\) were identified on two soybean chromosomes, 3 and 19. In all three syntenic loci, an ortholog for the EZA1/SWINGER (AT4G02020.1) gene was observed and is the candidate gene for the \(Hls\) locus. The \(Hls\) locus was identified on the cowpea physical map via SNP markers 1\_0910, 1\_1013 and 1\_0992 which were identified in three BAC contigs; contig926, contig821 and contig25.

**Conclusions:** This study has demonstrated how integrated genomic resources can be utilized for a candidate gene approach. Identification of genes which control leaf morphology may be utilized to improve the quality of cowpea leaves for vegetable and or forage markets as well as contribute to more fundamental research understanding the control of leaf shape in legumes.

**Keywords:** QTL analysis, Leaf morphology, Genomics, Genetics, Physical map, Synteny, Candidate genes, Cowpea, Legumes, EZA1/SWINGER

* Correspondence: philip.roberts@ucr.edu; timothy.close@ucr.edu
\(^1\)Department of Botany & Plant Sciences, University of California Riverside, Riverside, CA, USA
\(^2\)Department of Nematology, University of California Riverside, Riverside, CA, USA
\(^4\)Full list of author information is available at the end of the article

© 2012 Pottorff et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Background
Cowpea \([Vigna\ unguiculata\ (L.\)\ Walp]\) exhibits a considerable variation in leaf shape. Cowpea leaves are compound, having two asymmetrical side leaflets and one central terminal leaflet which is symmetrical. Typically, the central leaflet of the trifoliate is used in classifying the leaf shape due to variability of the side leaflets. In cowpea, the leaf shape is important for taxonomic classification and also for distinguishing cowpea varieties. However, there isn’t a central naming convention for cowpea leaves nor detailed descriptions of the leaf shapes, thus, many researchers name the leaf shapes differently. The two largest cowpea germplasm agencies are the International Institute of Tropical Agriculture (IITA) and the United States Department of Agriculture (USDA). IITA, which houses 14,500 cowpea accessions from 65 different countries, classifies cowpea leaf shapes into four categories, sub-globose, sub-hastate, globose and hastate/lanceolate (http://genebank.iita.org). The USDA, which houses 6,8411 cowpea accessions from 50 countries, classifies cowpea leaf shapes into five categories; globose, hastate, sub-globose, sub-hastate, strip and ovate-lanceolate (http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?188).

Multipurpose cowpea
Cowpea is a multipurpose crop; the entire plant can be used for either human or livestock consumption. In 2009, cowpea dry grain production was estimated at 5,249,571 tons worldwide (http://faostat.fao.org). Although cowpea is not one of the highest production crops worldwide, nearly 90% of cowpea is produced in West Africa, which is estimated at 4,447,358 tons (http://faostat.fao.org). Cowpea is mainly grown in semi-arid regions by subsistence farmers, who sell the fresh or dried seeds, fresh pods and leaves as vegetables and the green or dried leftover parts of the plant, leaves and stems (haulms), can be used as fodder for livestock [1].

Young cowpea leaves are eaten as a pot herb and enjoyed in many parts of Africa. The freshly harvested leaves are sold in local markets in many parts of Ghana, Mali, Benin, Cameroon, Ethiopia, Uganda, Kenya, Tanzania and Malawi [2]. Cowpea shoots and leaves are rich sources of calcium, phosphorous and Vitamin B [3]. The young leaves are especially important in drought-prone regions of Sub-Saharan Africa to tide local populations over during the “hungry period” which occurs after planting but before the main harvest of fresh pods and dry grains. In Mozambique, dried cowpea seeds are mainly consumed by the poorer classes of people, whereas all social strata consume cowpea leaves eaten as a vegetable (personal communication, Rogerio Chiulele). Importantly, farmers can harvest and sell the young tender cowpea leaves while waiting for the cowpea grain crop to mature, which helps provide income to buy staple foods. Cowpea seedlings and tender young leaves are also a local delicacy and inherent to Zimbabwean cultures (personal communication, Wellington Muchero).

Dual purpose cowpea varieties which are bred for quality seeds, vegetables and fodder may add to a farmer’s revenue. For example, in Nigeria, farmers who sold dried cowpea fodder during the peak of the drought season saw a 25% increase to their annual income [4]. Although there is no emphasis in breeding cowpeas for the shape of their leaves, leaf shape is important for classifying and distinguishing cowpea varieties. The shape of the leaves may also be potentially useful as a morphological or physical marker used during the selection process if it is closely linked with an agronomic trait of interest. Interestingly, many wild cowpea relatives have the narrow or hastate leaf shape whereas most cultivated varieties of cowpea have the more common ovate or sub-globose leaf shape. However, any possible adaptive advantage for narrow leaves in wild cowpea has not been investigated. The hastate leaf shape was reported to be dominant to the ovate leaf shape in several studies [5–10]. This may indicate that the hastate shape is ancestral to the ovate leaf shape and the preponderance of the latter in most cultivated cowpea is due to direct or indirect selection by humans over time.

Molecular genetic tools and genomic resources have been developed for cowpea with an objective of enhancing breeding programs for the improvement of cowpea varieties for the United States, India, Brazil, and numerous countries in Africa and Asia. These integrated genomic resources include a 1536 SNP genotyping platform, an EST-derived SNP consensus genetic map, known syntenic relationships between cowpea, Medicago truncatula, Glycine max and Arabidopsis thaliana, and a cowpea EST sequence collection housed in HarvEST: Cowpea database (http://harvest.ucr.edu) [11,12]. A cowpea physical map has been partially anchored to the cowpea consensus genetic map using the same SNP markers (UCR cowpea group, unpublished) and is available publically (http://phymap.ucdavis.edu/cowpea). In addition, about 500 diverse cowpea accessions have been SNP-genotyped (UCR cowpea group, unpublished data) and a first draft of the cowpea genome, vs.0.02, has been assembled (www.harvest-blast.org). These resources will enable dissection of underlying genetic components of target agronomic traits using Quantitative Trait Locus (QTL) analysis and Association Mapping. The identified and confirmed QTLs will facilitate cultivar improvement using marker-assisted breeding.

In this study, we analyzed the genetics of leaf morphology in a segregating cowpea RIL population, Sanzi (sub-globose) x Vita7 (hastate). A QTL was identified for the “hastate leaf shape” locus, Hls, which was positioned on
the cowpea consensus genetic map and cowpea physical map. A candidate gene was identified using syntenic relationships between cowpea, soybean and Medicago. In addition, a SNP marker was found which co-segregated with the leaf morphology genotypes and phenotype, which could be used as a molecular marker for breeding purposes. Future perspectives for this study are to fine map the Hls locus and identify cowpea candidate genes which would be utilized for more basic studies on leaf morphology in cowpea.

Results and discussion
Inheritance of leaf morphology

The inheritance of leaf morphology was studied using phenotypic data from one greenhouse experiment and one field experiment on the cowpea RIL population, Sanzi (sub-globose) x Vita 7 (hastate). The hastate and sub-globose leaf shape segregated 58:60 in the greenhouse experiment and 59:57 in the field experiment ($x^2_{1:1} = 0.03$, p-value $= 0.85$) which fit the proposed model that the leaf shape is a qualitative trait (Table 1).

Several other researchers have studied the inheritance of the leaf shape in cowpea (hastate x ovate leaf shape) and reported that it was a qualitative trait [7,8,10,13]. Although the F1 generation was not assessed in the current study, the majority of researchers studying cowpea leaf shape have concluded that the hastate leaf shape is dominant to the more common ovate or sub-globose leaf shape [5–10]. However, Saunders et al. (1960b) reported that the hastate leaf shape was incompletely dominant to the ovate leaf shape.

QTL analysis

QTL analysis of the two phenotypic datasets identified one major QTL with a large effect for leaf shape morphology. The leaf morphology QTL spanned 11 cM distance on the Sanzi x Vita 7 individual genetic map from 56.54 cM to 67.54 cM on linkage group 15 (Figure 1, Tables 2, 3). SNP marker 1_0910 was the most significant marker in both of the datasets, accounting for 74.7% of the phenotypic variance (LOD 33.82) in the greenhouse experiment and 71.5% phenotypic variance (LOD 30.89) in the field experiment (Table 3). We propose the designation Hls (hastate leaf shape) for the QTL identified.

Other researchers studying the inheritance of the hastate leaf shape in cowpea have reported a single dominant gene controlling the hastate leaf shape over the ovate or sub-globose leaf shape. Several gene symbols have been proposed, the first being $L$, which is a dominant gene controlling lanceolate leaf shape [14]. Ogomo et al. (1977) proposed the gene symbol $Ha$ for the hastate leaf shape and Kolhe et al. (1970) proposed $Nlf$ for narrow leaf shape. Fery (1980) proposed the gene symbol, $La$, for the narrow leaf shape. However, all of the studies investigating the narrow leaf shape used different cowpea accessions to make their populations. Whether these many studies are describing the same leaf shape locus or whether they are describing multiple independent loci remains inconclusive. Interestingly, Ogundin et al. (2005) identified one major QTL for the hastate leaf shape, designated $La$, in *Vigna unguiculata* ssp. *textilus*. Subspecies *textilus* is closely related to cultivated cowpea (*V. unguiculata* ssp. *unguiculata*); however, it does not easily hybridize. $La$ could possibly be the syntenic locus of $Hls$ in *V. textilus*.

The corresponding location of $Hls$ was identified on the cowpea consensus genetic map. SNP markers which identified the $Hls$ locus in the Sanzi x Vita 7 genetic map were aligned with the cowpea consensus genetic map (Table 3). The $Hls$ locus spans from 25.57 cM to 35.96 cM on the cowpea consensus genetic map linkage group 4 (Table 3). The length of $Hls$ on the individual genetic map, 11 cM, is nearly the same as on the cowpea consensus genetic map, 10.39 cM which may reflect accuracy of marker order (Table 3). The $Hls$ locus on the cowpea consensus genetic map has several SNP markers which were not present in the Sanzi x Vita 7 population because of lack of polymorphism in the individual population (Table 3). In addition, there was a slight difference in the order of the SNP markers in the Sanzi x Vita 7 population versus the cowpea consensus genetic map due to the merging of twelve individual genetic maps.

Marker-trait association analysis

Seventeen diverse cowpea genotypes which have either the hastate or sub-globose leaf shape were used in a marker-trait association study to identify a SNP marker in the $Hls$ region linked with the leaf shape phenotype. The hastate genotypes used for the analysis were selected from the USDA GRIN cowpea accession database and under their naming convention were classified as “strip” leaved. Vita 7, PI 632869, PI 632870, PI 632871, PI 632900, PI 632876, PI 632901, PI 632899 and PI 598341 were chosen for the hastate leaf shape phenotype (Additional file 1). PI 632882, CB27, Bambe 21, PI 418979, PI 448337 and PI 448682 were chosen from the USDA GRIN database and under their naming convention were classified as “sub-globose” leaf shape (Additional file 1). Accessions designated “TVNu” are wild cowpeas, many of which have the hastate leaf shape.
The alleles of SNP marker 1_0349 (35.9 cM position) co-segregated perfectly with the hastate or sub-globose leaf phenotype (boxed in green in Figure 2). The allele for the hastate genotype at this locus was the thymine nucleotide (color coded blue in Figure 2). The allele for the sub-globose genotype was the cytosine nucleotide (color coded red in Figure 2). The thymine/cytosine SNP for 1_0349 is at position 2122 in the cowpea P12 assembly unigene 8605 and can be viewed in HarvEST:Cowpea (http://harvest.ucr.edu) (Additional file 2). The marker-trait association narrowed the Hls QTL to a 0.3 cM region and was defined by flanking SNP markers 1_0083 and 1_0417 (Figure 2).

Candidate gene analysis using synteny with *M. truncatula* and *G. max*

The Hls locus was compared with the soybean, Medicago and Arabidopsis genomes to determine if a syntenic

![Figure 1](link-to-figure)

Figure 1: Hls locus on the Sanzi x Vita 7 genetic map. Using Interval Mapping and Kruskal-Wallis analysis (only Interval Mapping analysis shown), Hls mapped to linkage group 15 on the Sanzi x Vita 7 genetic map, spanning from 56.54 cM to 67.54 cM. The greenhouse experiment data are plotted in blue and the field experiment data in green. SNP markers 1_0992 and 1_0910 are highlighted in red on the linkage group. The LOD significance threshold of 2.0 is indicated by a horizontal dotted line on the graph.

Table 2: QTL analysis of the Hls locus in the Sanzi x Vita 7 population

| Experiment | Analysis | 1_0106 LOD | 1_1316 LOD | 1_0417 LOD | 1_0349 LOD | 1_0992 LOD | 1_0910 LOD |
|------------|----------|------------|------------|------------|------------|------------|------------|
| Greenhouse | IM LOD   | 27.27      | 27.18      | 27.21      | 31.21      | 33.82      |
|           | IM R²    | 66.2       | 69.1       | 62.7       | 62.7       | 71.9       | 74.7       |
|           | KW test statistic | 76.12 | 78.68 | 71.38 | 71.38 | 81.74 | 84.91 |
|           | KW p-value | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Field     | IM LOD   | 27.29      | 27.18      | 27.21      | 31.21      | 33.82      |
|           | IM R²    | 66.2       | 69.1       | 59.9       | 59.9       | 68.7       | 71.5       |
|           | KW test statistic | 76.08 | 78.62 | 68.30 | 68.30 | 78.15 | 81.31 |
|           | KW p-value | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |

IM = Interval Mapping analysis, KW = Kruskal-Wallis analysis.
relationship exists. A high co-linearity or a conservation of gene order utilizing the EST-derived SNP markers with any of the sequenced genomes might reveal candidate genes. Synteny was examined using EST-derived SNP markers previously BLASTed and aligned to the soybean, Medicago and Arabidopsis genomes which are housed in the HarvEST:Cowpea database and are publicly available (http://harvest.ucr.edu). Due to limited resolution in the software images, not all markers are presented in the screenshot images output from Harvest:Cowpea. However, the cowpea consensus genetic map vs. 4 [12] has been used in fidelity. In order to view each individual

| Table 3 The *Hls* locus in the Sanzi x Vita 7 genetic map, cowpea consensus genetic map and cowpea physical map |
|------------------------------------------------------------------------------------------------------------------|
| Sanzi x Vita 7 genetic map | Cowpea consensus genetic map | Cowpea physical map |
|----------------------------|-----------------------------|---------------------|
| LG | cM | Locus | LOD | LG | cM | Locus | Contig | BAC clone(s) |
| 15 | 56.55 | 1_0106 | 27.32 | 4 | 25.57 | 1_0106 | 383 | CM056F01, CM067G06, CM007L11 |
| N/A | 27.60 | 1_0678 | 1014 | CH021P21 |
| N/A | 27.90 | 1_1209 | N/A |
| N/A | 29.30 | 1_0117 | N/A |
| N/A | 29.51 | 1_0128 | N/A |
| 15 | 63.65 | 1_1316 | 28.80 | 4 | 31.88 | 1_1316 | N/A |
| N/A | 32.21 | 1_0157 | N/A |
| N/A | 33.57 | 1_0038 | 926 | CM002I07, CM052G13 |
| N/A | 34.09 | 1_1013 | 926 | CM050B03, CH004H23, CH046B08 |
| 15 | 67.54 | 1_0910 | 33.82 | 4 | 34.09 | 1_0910 | 821 | CH050F07 |
| 15 | 67.20 | 1_0992 | 31.21 | 4 | 34.69 | 1_0992 | 25 | CM041C03 |
| N/A | 35.66 | 1_0083 | N/A |
| 15 | 66.46 | 1_0349 | 24.18 | 4 | 35.87 | 1_0349 | N/A |
| 15 | 66.46 | 1_0417 | 24.18 | 4 | 35.96 | 1_0417 | N/A |

SNP markers are aligned in the order defined by the cowpea consensus genetic map.

Figure 2 Marker-trait association in the *Hls* locus. The *Hls* locus on the cowpea consensus genetic map linkage group 4 is depicted vertically along with cowpea genotypes which differ in hastate or sub-globose leaf shape. Red colored blocks indicate the “AA” allele, blue colored blocks indicate the “BB” allele and grey colored blocks indicate that the locus has no detected SNP. Leaf shapes for cowpea accessions are labeled below: “S” indicates a sub-globose leaf shape and “H” indicates the hastate leaf shape. A marker-trait association was found for SNP marker 1_0349 (35.90 cM position) which is boxed in green. SNP marker 1_0349 co-segregated with the hastate and sub-globose leaf genotypes and the corresponding leaf phenotype. The allele for the hastate leaf genotype at this locus is the thymine nucleotide, color coded blue. The allele for the sub-globose genotype is the cytosine nucleotide, color coded red. The thymine/cytosine SNP for 1_0349 is at position 2122 in the cowpea P12 assembly unigene 8605 and can be viewed in HarvEST:Cowpea (http://harvest.ucr.edu).
marker, the linkage group must be magnified in the HarvEST:Cowpea database.

The Hls locus was examined for synteny with the Arabidopsis genome; however very low synteny was displayed at the macro level between cowpea and Arabidopsis so no further examination was pursued (Additional file 3).

A high co-linearity was observed for the Hls locus with Medicago chromosome 7 (Figure 3, Table 4). Eight Medicago genes orthologous to cowpea SNP markers were identified in the syntenic region of Medicago chromosome 7 (Table 4). The syntenic region spanned from Medtr7g084010 to Medtr7g134530 which corresponded to 29.30 cM to 35.96 cM of the Hls locus on the cowpea consensus genetic map (Tables 3, 4). The region which spanned from Medicago genes orthologous to cowpea SNP markers 1_0349 to 1_0349 were in the same linear order as on the cowpea consensus genetic map (Tables 3, 4). The region spanning between Medicago chromosomes 3 and 19.

![Figure 3 Synteny of the Hls locus with Medicago truncatula and Glycine max](http://www.biomedcentral.com/1471-2164/13/1/234)
genes orthologous to cowpea SNP markers 1_0910 (most significant marker in the QTL analysis) and 1_0349 (co-segregated with leaf genotype and phenotype) was examined for genes known to be associated with the molecular control of leaf morphology in other plant species [15] on the Medicago genome browser on the Phytozome webpage (http://www.phytozome.net). The Medicago locus Medtr7g133020 was observed between Medicago genes orthologous to cowpea SNP markers 1_0992 and 1_0083 and was annotated as an ortholog of the Arabidopsis gene AT4G02020.1 aka EZA1 or SWINGER (SWN) (Table 4). Medtr7g133020 has a SET domain (protein lysine methyltransferase enzyme) with two copies of a cysteine rich motif and is annotated as KOG: 1079; transcriptional repressor EZA1 (http://www.phytozome.net) (accessed April 2012).

The Hls region was examined for synteny with the soybean genome and was found to be highly co-linear with soybean chromosomes 3 and 19 (Figure 3, Table 5). Eight Medicago genes orthologous to cowpea SNP markers identified the region from locus Glyma03g34240 to Glyma03g38550 as the Hls syntenic locus in soybean chromosome 3 (Table 5). The soybean syntenic locus corresponded to 27.60 cM to 35.96 cM region in the Hls locus and was also in the same general marker order as the cowpea consensus genetic map (Table 5). The region spanning between orthologous soybean genes to cowpea SNP markers 1_1013 and 1_0349 was examined for leaf morphology candidate genes on the soybean genome browser on the Phytozome webpage (http://www.phytozome.net). Soybean locus Glyma03g38320 was observed flanked by orthologous genes for cowpea SNP markers 1_1013 and 1_0349 and was annotated as an ortholog of EZA1/SWINGER (SWN) gene. Glyma03g38320 has a SET domain (protein lysine methyltransferase enzyme) and two copies of a cysteine rich motif and is annotated as KOG: 1079; transcriptional repressor EZA1 (http://www.phytozome.net) (accessed April 2012).

The Hls syntenic region in soybean chromosome 19 was identified by thirteen out of fourteen SNP markers, spanning from Glyma19g36180 to Glyma19g41150 which corresponded to 24.10 cM to 39.80 cM on the cowpea consensus genetic map (Table 5). The syntenic region in soybean between orthologous cowpea SNP markers 1_0910 and 1_0349 was examined for known leaf development genes using the soybean genome browser on the

| G. max chromosome | G. max locus | Location (bp) | Phytozome annotation | Cowpea SNP | LG | cM |
|------------------|-------------|---------------|----------------------|------------|----|----|
| 3                | Glyma03g34240 | Gm03: 41726178–41732134 | Protein phosphatase type 2A | 1_1209 | 4 | 27.90 cM |
| 3                | Glyma03g34420 | Gm03: 41865023–41866819 | UDP glycosyl transferase | 1_0678 | 4 | 27.60 cM |
| 3                | Glyma03g35490 | Gm03: 42670842–42672212 | Small nuclear ribonucleoprotein G | 1_0117 | 4 | 29.30 cM |
| 3                | Glyma03g36050 | Gm03: 43046482–43052190 | Glycosyl transferase | 1_1316 | 4 | 31.88 cM |
| 3                | Glyma03g36560 | Gm03: 43503702–43504835 | 60S ribosomal protein L21 | 1_0157 | 4 | 32.21 cM |
| 3                | Glyma03g37080 | Gm03: 43844395–43846869 | Tetrahydrofolate dehydrogenase | 1_1013 | 4 | 34.09 cM |
| 3                | Glyma03g38320 | Gm03: 44664969–44672254 | EZA1 (SWINGER); transcription factor | N/A | N/A | N/A |
| 3                | Glyma03g38520 | Gm03: 44857426–44863787 | Cysteine proteinase | 1_0417 | 4 | 35.96 cM |
| 3                | Glyma03g38550 | Gm03: 44884051–44889833 | ATP-dependent RNA helicase | 1_0349 | 4 | 35.87 cM |
| 19               | Glyma19g36180 | Gm19: 43520883–43522581 | 40S ribosomal protein S23 | 1_0106 | 4 | 25.57 cM |
| 19               | Glyma19g36250 | Gm19: 43594256–43596114 | 60S ribosomal protein L19 | 1_0061 | 2 | 24.10 cM |
| 19               | Glyma19g38130 | Gm19: 45131688–45132559 | Small nuclear ribonucleoprotein G | 1_0117 | 4 | 29.30 cM |
| 19               | Glyma19g39170 | Gm19: 45946131–45951841 | Protein phosphatase | 1_1349 | 3 | 39.80 cM |
| 19               | Glyma19g39240 | Gm19: 45993099–45993972 | 60S ribosomal protein L21 | 1_0157 | 4 | 32.21 cM |
| 19               | Glyma19g39570 | Gm19: 46201543–46203746 | 60S ribosomal protein L19 | 1_0038 | 4 | 33.57 cM |
| 19               | Glyma19g39710 | Gm19: 46301684–46304736 | Tetrahydrofolate dehydrogenase | 1_1013 | 4 | 34.09 cM |
| 19               | Glyma19g40080 | Gm19: 46544712–46546719 | 60S ribosomal protein L19 | 1_0038 | 4 | 33.57 cM |
| 19               | Glyma19g40090 | Gm19: 46547961–46552179 | Histidine kinase | 1_0910 | 4 | 34.09 cM |
| 19               | Glyma19g40300 | Gm19: 46736094–46743350 | Glycosyl hydrolase family | 1_0992 | 4 | 34.69 cM |
| 19               | Glyma19g40430 | Gm19: 46838345–46844721 | EZA1 (SWINGER); transcription factor | N/A | N/A | N/A |
| 19               | Glyma19g41120 | Gm19: 47437575–47443343 | Cysteine proteinase | 1_0417 | 4 | 35.96 cM |
| 19               | Glyma19g41150 | Gm19: 47465990–47471582 | ATP-dependent RNA helicase | 1_0349 | 4 | 35.87 cM |
Phytozome webpage (http://www.phytozome.net). Glyma19g40430 locus was observed flanked by soybean genes orthologous to SNP markers 1_0992 and 1_0417 and was annotated as an ortholog of the Arabidopsis EZA1/SWINGER (SWN) gene (Table 5). Glyma19g40430 has a SET domain (protein lysine methyltransferase enzyme) and two copies of a cysteine rich motif and is annotated as KOG: 1079; transcriptional repressor EZA1 (http://www.phytozome.net) (accessed April 2012).

The candidate gene approach using syntenic relationships between cowpea, soybean and Medicago for the Hls locus identified orthologous candidate genes for the Arabidopsis gene AT4G02020.1 or EZA1/SWINGER (SWN). EZA1/SWINGER (SWN) is one of three Arabidopsis E(Z) orthologs of the Drosophila melanogaster gene ENHANCER OF ZESTE [E(Z)], which includes CURLY LEAF (CLF) and MEDEA (MEA) [16]. EZA1/SWINGER (SWN) is an H3K27 methyltransferase transcription factor and belongs to the Polycomb group proteins (Pc-G). Pc-Gs are involved in epigenetic regulation of developmental processes and are highly conserved in plants, animals and humans. In plants, Pc-G proteins are essential in regulating processes such as seed development [17], flower organ development [18–20] and leaf development [18,21].

CLF and SWN are expressed throughout many phases of plant development and have been shown to be involved in regulating leaf development. CLF is expressed during leaf and flower development [18] and EZA1/SWINGER is expressed in regions of dividing cells and meristems during vegetative and reproductive development [19]. CLF has been shown to directly target and repress the floral homeotic gene, AGAMOUS (AG), and a homeobox gene, SHOOTMERISTEMLESS (STM) [20,21]. SWN has been shown to have partially redundant functions with CLF and therefore may also be involved in regulating leaf development [19]. A clf swn double mutant produced narrow cotyledons, hypocotyls and roots and as it matured, the cotyledons developed finger-like growth on the margins as well as other abnormalities such as the shoot apex not developing leaves but a disorganized mass of undifferentiated tissue [19]. The fact that EZA1/SWINGER has been associated with leaf development in Arabidopsis makes it a plausible candidate gene for regulating leaf morphology in cowpea.

The combination of the marker-trait association and the identity of candidate genes in the syntenic loci enabled us to narrow the Hls region on the consensus genetic map, from 10.39 cM to approximately 1.87 cM distance. The narrowest distance between flanking markers to an orthologous candidate gene was in the Medicago locus, where Medtr7g133020 was flanked by SNP markers 1_0992 (34.69 cM position) and 1_0083 (35.66 cM position) which narrowed it to a 0.97 cM region. In soybean chromosome 19, the EZA1/SWINGER ortholog Glyma19g40430 was flanked by SNP markers 1_0992 (34.69 cM position) and 1_0417 (35.96 cM position) which narrowed the region to 1.37 cM. The furthest distance between flanking markers to orthologous candidate genes was in the syntenic locus in soybean chromosome 3, where Glyma03g38320 was flanked by SNP marker 1_1013 (34.09 cM position) and 1_0417 (35.96 cM position) with an approximate distance of 1.87 cM. On average, the most significant region in the Hls locus was narrowed to a 1.4 cM distance using the position of the candidate genes to narrow the QTL region. Assuming that the co-linearity of these three syntenous regions is upheld when extrapolated back to cowpea; the cowpea ortholog of EZA1/SWINGER should be present in this narrowed region.

Differences in marker significance under different analyses may be of interest. For example, SNP marker 1_0910 was the most significant in the QTL analysis while SNP marker 1_0349 co-segregated with the genotype and phenotype for leaf shape. QTL analysis often identifies large confidence intervals depending on the heritability of the trait and because all genes on a chromosome will show some linkage amongst themselves, a QTL will be associated with several markers [22]. This was the case for SNP markers 1_0349 and 1_0910, which are 1.08 cM distance apart on the individual genetic map and 1.78 cM on the cowpea consensus genetic map (Table 3). We have found that small pheno-typing differences between experiments may move the most significant marker by 1 cM or more. The marker-trait association in which SNP marker 1_0349 co-segregated with the genotype and phenotype for leaf shape utilized a simplified haplotype analysis, where unrelated individuals were examined for inheritance of alleles within a specific region. The synteny study revealed that Medicago and soybean orthologs to cowpea SNP markers 1_0083, 1_0092, 1_1013 and 1_0417 were flanking the EZA1 candidate genes (Tables 4, 5, Additional file 4). These four markers flank the most significant marker from the QTL analysis, 1_0910, and 1_0349 which co-segregated with the genotype and phenotype for leaf shape (Additional file 4). By utilizing QTL analysis, marker-trait association and candidate gene analysis using synteny, validation was provided that the genetic determinant is most likely located within a 1.37 cM region of closely linked markers.

Leaf morphology candidate genes BLAST to cowpea genomic resources
The genomic sequences for Medtr7g133020, Glyma03g38320, Glyma19g40430 and the Arabidopsis EZA1 gene (AT4G02020.1) were BLASTed to the cowpea genome vs. 02 (www.harvest-blast.org) and HarvEST:Cowpea
database (http://harvest.ucr.edu) to identify orthologous cowpea sequences. The Medtr7g133020 and AT4G02020.1 genomic sequences returned a high BLAST alignment with contig C27495629 (Table 6). The genomic sequences for Glyma03g38320 and Glyma19g40430 returned a high alignment with contig C27664167 and scaffold28398 (Table 6). All genomic sequences when BLASTed to Harvest:Cowpea database returned the best alignment with cowpea unigene 21752 which was annotated as an EZA1 ortholog (Table 6). Interestingly, unigene 21752 was obtained from leaf and shoot meristems used for a mature pre-flowering developmental stage cDNA library from cowpea varieties Danilla, Tvu11986, Tvu7778 and 12008D (http://harvest.ucr.edu). The genomic and unigene sequences identified for the cowpea ortholog for EZA1 will enable future studies to clone and confirm the candidate gene.

Hls in the cowpea physical map

The cowpea physical map (http://phymap.ucdavis.edu/cowpea) which has been partially anchored to the cowpea consensus genetic map via the same SNP markers was used to identify BAC contigs which span the Hls region. Significant markers from the QTL study and closely linked markers from the cowpea consensus map identified several BAC contigs which incompletely span the Hls region (Table 3). The most significant SNP marker from the QTL analysis, 1_0910, was identified in BAC clone CH050F07 of contig821 (Table 3). Contig821 has four overlapping BAC clones and 128 non-repeating bands which estimated the contig size at 209,920 bp (http://phymap.ucdavis.edu/cowpea). SNP marker 1_0992 which was closely linked with the EZA1 candidate gene in two out of three of the syntenic loci, was identified in BAC clone CM041C03 of contig25 (Table 3). Contig25 has 731 overlapping BAC clones and 1843 non-repeated bands which estimated the length as 3,022,520 bp (http://phymap.ucdavis.edu/cowpea) (Table 3). The combined length of the two BAC contigs which span the most significant region of the Hls QTL is 3,232,440 bp. Since SNP marker 1_0992 was closely linked to the EZA1/SWINGER candidate gene in the Hls syntenic locus in Medicago chromosome 7 and soybean chromosome 19, the cowpea EZA1 gene may be located on BAC contig25. Currently, there are BAC-end sequences (BES) of approximately 700 bp for clones in the minimum tiling path (MTP) of BAC contigs in the cowpea physical map. However, none of the BESs of clones in either contig25 or contig821 yielded cowpea EZA1 genes when BLASTed to the Harvest:Cowpea database. Future perspectives for enhancing the cowpea physical map may include sequencing BAC clones within the MTP of each BAC contig which would enable the direct identification of genes of interest.

To test the candidacy of the cowpea EZA1 gene for the Hls locus, a molecular marker could be developed and mapped to ensure it co-locates in the Hls locus in the Sanzi x Vita 7 population. Additionally, the cowpea EZA1 gene would need to be cloned and sequenced from both parents to determine the allelic variation for phenotype followed by complementation tests to validate gene function.

Conclusion

This study has identified one major QTL, Hls, which is associated with the hastate and sub-globose leaf shape in the cowpea RIL population Sanzi x Vita 7. Our candidate gene approach utilized mapping the locus and a marker-trait association to narrow the QTL locus of 11 cM to one marker which co-segregated with the trait. The conserved gene order amongst closely related species, cowpea and soybean, and members within the same legume family, cowpea, Medicago and soybean, enabled the identification of a candidate gene for the Hls locus. Future goals will be to utilize the molecular marker which co-segregated with leaf shape in MAS breeding efforts. A more fundamental study could also be undertaken to determine if the candidate gene EZA1/SWINGER is the genetic determinant governing leaf morphology in cowpea.

Methods

Plant population

Leaf morphology was studied in a cowpea RIL population which was developed from an intraspecific cross of Sanzi x Vita 7. The population consisted of 122 RILs which were advanced by single seed descent to the F10 generation. Sanzi is a local landrace from Ghana which has a prostrate sprawling architecture, grayish-purple seeds, and a sub-globose leaf shape. Vita 7 (PI 580806/TVu-8461) is an IITA advanced breeding line from Nigeria with an upright bush type architecture, beige seeds

Table 6 Medicago, soybean and Arabidopsis EZA1/SWINGER genes BLAST to cowpea genomic resources

| EZA1(SWINGER) ortholog | Cowpea genome | e-score | Cowpea unigene | e-score |
|------------------------|---------------|---------|----------------|---------|
| Medtr7g133020         | C27495629     | 1.00E-15| 21752          | 4.00E-11|
| Glyma03g38320         | C27664167     | 7.00E-30| 21752          | 1.00E-17|
| Glyma19g40430         | scaffold28398| 6.00E-36| 21752          | 6.00E-10|
| AT4G02020.1           | C27495629     | 3.00E-22| 21752          | 9.00E-21|
and hastate leaf shape (IITA germplasm database online; http://genebank.iita.org). The Sanzi x Vita 7 population was received from Christian Fatokun, IITA, Ibadan, Nigeria. All cowpea accessions were available from the University of California Riverside cowpea germplasm collection.

**Phenotyping**

The terminal central leaflet was observed and classified as “hastate” or “sub-globose” (Figure 4) five weeks after germination for each of the RILs. Two sets of phenotypic data were obtained; one dataset during a greenhouse experiment and the second dataset during a field experiment. The greenhouse study, which phenotyped 118 out of 122 RILs, was conducted from February to April 2010 in Riverside, California. Seedlings were transplanted into 3785 cm³ pots and watered daily, with day and night temperatures set to 28°C and 16°C, respectively. The field experiment, which phenotyped 116 out of 122 RILs, was conducted at the Citrus Research Center-Agricultural Experiment Station (CRC-AES) in Riverside CA, from July to September 2010. Twenty-five seeds per replicate were planted for each RIL in a randomized complete block design using four replicates. Seeds were machine-planted in single rows on pre-irrigated raised beds spaced 76 cm apart with 10 cm spacing between seeds.

**SNP genotyping**

The Sanzi x Vita 7 population was genotyped at the F₈ generation using bi-allelic SNP markers from the 1536 Illumina GoldenGate Assay as previously described [11]. All genotypes used for the marker-trait association study were SNP genotyped at the F₈ generation or above as previously described [11].

**Genetic map**

A SNP genetic map was developed previously for the Sanzi x Vita 7 RIL population and is included in the cowpea consensus genetic map vs.4 [12]. The individual map was generated using 122 RILs and 416 SNP markers. The map consists of nineteen linkage groups and spans approximately 753 cM total distance.

**Cowpea consensus genetic map**

The cowpea consensus genetic map vs. 4, which is an updated version of the Muchero et al. 2009 map, was used for this study [12]. The consensus version 4 map consists of ten RIL populations and two F₄ breeding populations, which has increased the marker density and improved the marker order. The map is 680 cM in length and contains 1107 markers with an average of 0.65 cM between markers. The current SNP-based cowpea linkage map is included in a publicly available browser called HarvEST:Cowpea, which can be downloaded from http://harvest.ucr.edu or viewed online at www.harvest-web.org.

**Statistical analysis**

The Kruskal-Wallis and Interval Mapping analysis packages of MapQTL 5.0 software were used to conduct the QTL analysis [23]. A QTL was considered significant if the same QTL was identified using both phenotypic datasets and if the statistical tests for the markers met significance thresholds for both Kruskal-Wallis and Interval Mapping analyses. A significance threshold was set to 0.05 for Kruskal-Wallis analysis and LOD thresholds for the Interval Mapping analysis were calculated using 1000 permutations at the 0.05 significance level. A 95% confidence interval was used to estimate the left and right margins of the QTL using 1-LOD and 2-LOD of the most likely position. QTLs were visualized using MapChart 2.2 software [24].

**Synteny**

Synteny was examined for cowpea with *G. max*, *M. truncatula* and *A. thaliana* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes. Annotations for the soybean and Medicago loci were taken directly from the Phytozome website (www.phytozome.org). Syntenic relationships amongst the different genomes can be examined in the HarvEST:
Cowpea database (http://harvest.ucr.edu). Syntenic maps were drawn using HarvEST:Cowpea using a cut-off e-score value of -10, with a minimum number of 10 lines drawn per linkage group.

Marker-trait association
Genotypic data comprised of cowpea varieties and SNP marker information in the Hs locus were visualized using GGT 2.0 software [25]. The cowpea consensus genetic map vs.4 [12] was loaded into the software to visualize linkage groups.

Cowpea physical map
The physical map was developed using an advanced African breeding line IT97K-499-35 (http://phyimap.ucdavis.edu/cowpea). It consists of two BAC clone libraries developed using restriction enzymes HindIII and Mbol (Amplicon Express, Pullman, WA). Contigs were assembled using the snapshot method of DNA fingerprinting [26] and completed at University of California Davis by Ming Cheng Luo. The final physical map is an assembly of 43,717 BACs with an 11x genome depth of coverage. The size of the BAC clones was estimated by multiplying the number of unique bands generated from the fingerprinting assay by 1640 bp (personal communication, Ming Cheng Luo).

Additional files

**Additional file 1:** Cowpea accessions with a hastate or sub-globose leaf phenotype.

**Additional file 2:** SNP marker 1,0349 sequence. cDNA sequence of P12 assembly unigene 8605 which is housed in Harvest:Cowpea database (http://harvest.ucr.edu). The SNP (thymine/cytosine) is located at position 2122, parenthesized, underlined and in bold.

**Additional file 3:** Synteny of the Hs locus with A. thaliana. Synteny was examined for the EST-derived SNP markers previously BLASTed and aligned to the syntenic map was drawn per linkage group.

**Additional file 4:** Summary of significant markers in the Hs locus.

**References**
1. Inaizumi H, Singh BB, Sanginga PC, Manyong VM, Adesina AA, Tarawali S: Adoption and Impact of Dry-season Dual-purpose Cowpea in the Semiarid Zone of Nigeria. Ibadan: International Institute of Tropical Agriculture (IITA); 1999.
2. Barrett RP: Integrating Leaf and Seed Production Strategies for Cowpea (Vigna unguiculata (L.) Walp.) in West Africa. Madison: Michigan State University, 1987.
3. Maynard DN: Underutilized and unexploited horticultural crops. Horticience 2008, 43:279.
4. Dugje FY, Omoigui LO, Beileme F, Kamara AY, Ajeigbe H: Farmers' Guide to Cowpea Production in West Africa. Ibadan: International Institute of Tropical Agriculture (IITA); 2009.
5. Khrihasnawarney N, Nambiar KK, Mariakulandai A: Studies on cowpea [Vigna unguiculata (L.) Walp.]. Madras Agric J 1945, 33:145–160.
6. Jindla L, Singh BB: Inheritance of flower color leaf shape and pod length in cowpea (Vigna sinensis L.). Indian J Hved 1970, 59:126–137.
7. Ojomo CO: Morphology and genetics of two gene markers, ‘Swollen stem base’ and ‘Hastate leaf’ in cowpea, Vigna unguiculata (L.) Walp. J Agric Sci 1971, 88:227–231.
8. Kohle AK: Genetic studies in Vigna sp. Poona Agric Coll Mag 1970, 59:126–137.
9. Fery RL: The genetics of cowpea: a review of the world literature. In Cowpea Research; Production and Utilization. Edited by Singh SR, Nichol KO. Chichester: John Wiley and Sons; 1985:25–62.
10. Oluwasotun OB: Inheritance of genes for leaflet shape and leaflet shape modifier in cowpea. Afr Crop Sci J 2002, 10:133–137.
11. Muchero W, Diop NN, Bhat PR, Wanamaker S, Pottorff M, Hearne D, Roberts PA, Close TJ: A consensus genetic map of cowpea (Vigna unguiculata (L.) Walp.) and synteny based on EST-derived SNPs. Proc Natl Acad Sci 2009, 106:18159–18164.
12. Lucas MR, Diop NN, Wanamaker S, Ehlers JD, Roberts PA, Close TJ: Cowpea–soybean synteny clarified through an improved genetic map. Plant Genome J 2011, 4:218–225.
13. Saunders AR: Inheritance in the cowpea III: mutations and linkages. S Afr J Agric Sci 1960, 3:327–348.
14. Harland SC: Inheritance of certain characters in the cowpea (Vigna sinensis). J Genet 1919, 18:101–132.
15. Barkoulas M, Galinha C, Grigg SP, Tsiantis M: From genes to shape: regulatory interactions in leaf development. Curr Opin Plant Biol 2007, 10:650–666.
16. Guttman A, Berger F: Control of reproduction by Polycorn Group complexes in animals and plants. Int J Dev Biol 2005, 49:707–716.
17. Wang D, Tyson MD, Jackson SS, Yadeegar R: Partially redundant functions of two SET-domain polycorn-group proteins in controlling initiation of seed development in Arabidopsis. Proc Natl Acad Sci 2006, 103:13244–13249.
18. Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G: A polycorn-group gene regulates homeotic gene expression in Arabidopsis. Nature 1997, 386:44–51.
19. Chanvivattana Y, Bishopp A, Schubert D, Stock C, Moon YH, Sung ZR, Goodrich J. Interaction of polycomb-group proteins controlling flowering in Arabidopsis. Development 2004, 131:5263–5276.

20. Schubert D, Primavesi L, Bishopp A, Roberts G, Doonan J, Jenuwein T, Goodrich J. Silencing by plant polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. EMBO J 2006, 25:4638–4649.

21. Katz A, Oliva M, Mosquera A, Hakim O, Ohad N. FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. Plant J 2004, 37:707–719.

22. Kearsey MJ, Farquhar AGJ. QTL analysis in plants: where are we now? Heredity 1998, 80:137–142.

23. Van Ooijen JW. MapQTL: S. Software for the Mapping of Quantitative Trait Loci in Experimental Populations. Wageningen: Kyazma BV; 2004.

24. Voorrips RE. MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 2002, 93:77–78.

25. Van Berloo R. GGT 2.0: versatile software for visualization and analysis of genetic data. J Hered 2008, 99:232–236.

26. Luo MC, Thomas C, You FM, Hisao J, Ouyang S, Buell CR, Malandro M, McGuire PE, Anderson OD, Dvorsk J. High-throughput fingerprinting of bacterial artificial chromosomes using the snapshot labeling kit and sizing of restriction fragments by capillary electrophoresis. Genomics 2003, 82:378–389.

doi:10.1186/1471-2164-13-234

Cite this article as: Pottorff et al.: Leaf morphology in Cowpea (Vigna unguiculata (L.) Walp): QTL analysis, physical mapping and identifying a candidate gene using synteny with model legume species. BMC Genomics 2012 13:234.