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Genetic Mapping of *Fusarium oxysporum* f.sp. *tracheiphilum* Race 3 and Race 4, *Macrophomina phaseolina* Resistance and Other Traits in Cowpea (*Vigna unguiculata* [L.] Walp).

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

Plant Biology (Plant Genetics)

by

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The text of this dissertation, in part or in full, is a reprint of the material as it appears in Plos ONE, July 2012. The co-author, Timothy J. Close, listed in that publication directed and supervised the research which forms the basis for this dissertation. Co-authors Philip A. Roberts, Steve Wanamaker, Yaquin Q. Ma and Jeffrey D. Ehlers provided technical assistance.

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Dedication

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ABSTRACT OF THE DISSERTATION

Genetic Mapping of *Fusarium oxysporum* f.sp. *tracheiphilum* Race 3 and Race 4, *Macrophomina phaseolina* Resistance and Other Traits in Cowpea (*Vigna unguiculata* [L.] Walp).

by

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Cowpea (*Vigna unguiculata*) is a legume crop which is grown in many warm regions around the world. Genomic resources have been developed for cowpea which has enabled the identification of QTL and candidate genes which can be utilized in trait improvement.

Fungal diseases cause significant constraints to cowpea yield. *Fusarium oxysporum* f.sp. *tracheiphilum* (Fot) race 3 and race 4 cause vascular wilt disease and are problematic in California. Genetic mapping identified the *Fot3-1* locus which confers resistance to Fot race 3 in the CB27 x 24-125B-1 population. *Fot3-1* was identified on BAC clone CH093L18, which carries leucine-rich repeat serine/threonine protein kinases. QTLs were identified which confer resistance against Fot race 4. *Fot4-1* was identified in the IT93K-503-1 x CB46 population and *Fot4-2* was identified in the CB27 x 24-125B-1 and

CB27 x IT82E-18(Big Buff) populations. The syntenic loci for *Fot4-1* and *Fot4-2* were examined with *Glycine max*, where several disease resistance candidate genes were identified.

Macrophomina phaseolina is a fungal pathogen which causes diseases under high temperatures and drought-stress. QTLs, *Mac-10*, *Mac-11*, *Mac-12* and *Mac-13*, were identified in the Sanzi x Vita 7 population. The *Mac-11* locus was positioned within BAC clone CH038D17 where an auxin response factor was present. *Mac-13* was identified within BAC clones CH062O11 and CH069K06, where an auxin-responsive *GH3* family protein was present.

Leaf morphology was studied in the cowpea RIL population, Sanzi x Vita 7, in which a QTL was identified for leaf shape, *Hls*. High co-linearity was observed for the syntenic *Hls* region in *Medicago truncatula* and *Glycine max* where EZA1/SWINGER genes were present.

Heat-induced browning of seed coats is caused by high temperatures which discolors the seed coats of cowpea. Three QTL, *Hbs-1*, *Hbs*-2, and *Hbs-3*, were identified using cowpea RIL populations IT93K-503-1 x CB46 and IT84S-2246 x TVu14676. *Hbs-1* was identified in BAC clone CM018C23 where ethylene forming enzymes were present. *Hbs-3* was identified in BAC clones CH047M01 and CM014K16 where ACC synthase genes were present.

Practical outcomes from these studies are the identification of molecular markers which can be used in a Marker Assisted Selection breeding scheme, which should expedite variety development for cowpea.

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Chapter 1

Introduction

Cowpea classification

Cowpea [*Vigna unguiculata* (L.) Walp] is a warm-season annual legume which belongs to the Leguminosae or Fabaceae family. The legume family is further subdivided into three subfamilies, Caesalpinieae, Mimosoideae, and Papilionoideae, of which cowpea belongs to the latter (Doyle and Luckow 2003). The Papilionoideae subfamily is further divided into four clades, of which cowpea belongs to the phaseoloid/millettioid clade, which includes other warm-season and economically important legumes such as common bean, pigeon pea and soybean (Doyle and Luckow 2003).

The genus Vigna to which cowpea belongs contains other legumes which are important for human consumption such as *V. unguiculata* ssp. sesquipedalis (yard-long bean or asparagus bean), *V. radiata* (mungbean), *V. mungo* (blackgram), *V. angularis* (azuki bean) and *V. subterranea* (bambara groundnut). Some of the common names used for cowpea include "southern bean", "crowder bean" and "black-eye pea". "Black-eye pea" describes a popular variety of cowpea which has a white seed coat and black pigment around the hilum, or "eye", which is grown in the United States and countries in Africa.

Cowpea origin and distribution, worldwide production

Cowpeas origins are from Africa where it was cultivated and domesticated. A recent study of the genetic structure of cowpea landraces determined that there are two major gene pools; one located in western Africa and the other located in eastern Africa (Huynh et al. 2013a). However, cowpeas are a warm-season annual crop grown in tropical, sub-tropical and semi-arid regions around the world, which includes Africa, Asia, South

America, the Mediterranean Sea, the United States of America and the Caribbean region (Hall et al. 2011). The worldwide cowpea dry grain production in 2012 was estimated at 5,714,575 tons, with the majority being produced in West African countries which was estimated at 4,635,653 tons (http://faostat.fao.org). Following Africa, the major cowpea production regions are Haiti, the former Yugoslav Republic of Macedonia, Sri Lanka, Madagascar, Serbia, Egypt, Swaziland, Bosnia and Herzegovina, Iraq, Philippines, Jamaica, Cyprus, Guyana, Croatia, Occupied Palestinian Territory and Trinidad and Tobago (http://faostat.fao.org). Cowpea production in the United States of America was 231 tons total; dry cowpea production was 195 tons (http://faostat.fao.org).

Utilization of Cowpea

Cowpea is a multipurpose crop; the majority of the plant can be used for either human or livestock consumption. Cowpea is mainly grown in semi-arid regions of Sub-Saharan Africa by subsistence farmers. The fresh or dried seeds, fresh pods and leaves which are eaten as vegetables and the leftover parts of the plant, leaves and stems (haulms), can be used as fodder for livestock (Inaizumi et al. 1999). However, cowpea is mainly grown as a pulse crop, for the production of the dried mature seeds. Cowpea is also useful as a rotation crop because it increases the nitrogen content of the soil, due to its symbiotic relationship with soil *Bradyrhizobium* spp (Jordan 1984). Cowpea lines have been released and recommended for use as a cover crop since it grows vigorously, ensuring that weeds do not grow, and is able to survive in low-fertility soils (Harrison et al. 2014).

Cowpea genomic resources

Cowpea is a diploid species (2n=22) with a relatively small genome size of approximately 630 Mb (Arumuganathan and Earle 1991a)(http://data.kew.org/cvalues/). Molecular genetic 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, Africa, India, Brazil and Asia. These integrated genomic resources include a 1536 Illumina GoldenGate SNP genotyping platform which was used to create the cowpea consensus genetic maps vs. 2 (Muchero et al. 2009a) , vs.3 (Diop et al. 2012) and vs. 4 (Lucas et al. 2011) and partially bound with the cowpea physical map (http://phymap.ucdavis.edu/cowpea) as well as syntenic maps with *Glycine max, Phaseolus vulgaris* and *Medicago truncatula* (http://harvest.ucr.edu).

The 1536 Illumina GoldenGate SNP genotyping platform was designed from cDNA libraries, or Expressed Sequence Tags (ESTs), which are housed in the HarvEST: Cowpea database (http://harvest.ucr.edu). The HarvEST: Cowpea database contains approximately 183,000 cowpea EST sequences which were derived from 17 cDNA libraries (http://harvest.ucr.edu). The cDNA libraries were derived from 15 diverse cowpea genotypes and from a large range of tissues and under drought conditions (http://harvest.ucr.edu). The HarvEST:Cowpea database also contains the latest version of the cowpea consensus genetic map vs. 6 which was derived from 11 mapping populations, 1091 SNP markers and is 680 cM in length with an average of 0.6 cM distance between markers (Lucas and Huynh,2012, University of California Riverside, unpublished). A consensus genetic map, which is a combination of several genetic maps,

is an important resource enabling the study of inheritance of traits as well as the statistical associations of markers with traits. The genetic mapping of traits enables us to pinpoint where in the genome a particular trait resides, and the closely linked markers with the trait of interest are then used to introgress the locus or beneficial alleles into improved varieties.

The collection of cowpea RIL mapping populations is an important resource in itself. The majority have been advanced to the F_8 generation or beyond, providing the ability to map traits which segregate within the populations. Several abiotic stress traits have been mapped in cowpea RIL populations such as seedling-stage drought tolerance and maturity (Muchero et al. 2009b), heat-tolerance during reproductive development (Lucas et al. 2013a) and heat-induced browning of seed coats (Pottorff et al. 2014). The cowpea RIL populations have been especially important for identifying QTLs involved in disease resistance (Huynh et al. 2013b). Resistance against Macrophomina (ashy stem blight or charcoal rot) (Muchero et al. 2011), foliar damage due to thrips (Muchero et al. 2010), Fusarium race 3 (Pottorff et al. 2012b) , Fusarium race 4 (Pottorff et al. 2014), foliar thrips (Lucas et al. 2012) and bacterial blight (Agbicodo et al. 2010) are just a few of the disease resistance traits which have been studied. Traits involved in domestication are also very useful for cowpea improvement, in which cowpea RIL populations have enabled the study of leaf morphology (Pottorff et al. 2012a) and seed size (Lucas et al. 2013c).

Syntenic relationships amongst legume species

Another genomic resource that is available for cowpea research is the known syntenic relationships with the model legume species such as *P. vulgaris*, *G. max* and *M. truncatula*. Comparative analysis between the sequenced legume genomes with the sequenced-based cowpea genetic and physical maps may be a valuable way of transferring knowledge within the legume family. The EST-derived SNP markers from the cowpea consensus genetic map and sequenced BAC clones from the cowpea physical map have been aligned to *G. max, P. vulgaris* and *M. truncatula* sequenced genomes (http://harvest.ucr.edu). If a co-linear relationship exists for a mapped trait locus between cowpea and common bean, soybean or Medicago, the genetic content within the syntenic locus of the sequenced genome can be extrapolated to predict candidate genes for the trait in cowpea.

Gene prediction utilizing synteny has accelerated research in root nodulation in several crop legumes; a high co-linearity of *M. truncatula* with crop legumes led to the cloning and functional analysis of orthologous root nodulation candidate genes, confirming their roles within their traits of interest (Endre et al. 2002; Limpens et al. 2003; Stracke et al. 2004). Endre et al. (2002) cloned *NORK*, a nodulation receptor kinase involved in the Nod-factor perception/transduction system in *Medicago sativa* utilizing the high co-linearity between *M. sativa* and *M. truncatula*. Similarly, Limpens et al. (2003) observed that the SYM2 locus in pea, involved in the rhizobial nodulation process, was highly syntenic with the Medicago genome. Medicago BAC clones covering the SYM2 region

were sequenced, identifying several candidate genes of which two LysM-domain receptor kinases were shown to be involved in the infection process and confirming that SYM2 was in fact orthologous to these Medicago counterparts (Limpens et al. 2003). Stracke et al. (2004) utilized the co-linearity of *Pisum sativum*, *Arabidopsis thaliana* and *Lotus japonicus* to isolate and clone the *L. japonicus LjSYM2* gene and the pea ortholog *PsSYM19*, which are required for the nitrogen-fixing nodulation process. These studies show the accelerated process of extracting knowledge from the sequenced genomes and extrapolating to crop legume species.

Conserved gene order with cowpea and common bean, soybean and Medicago has been utilized in several QTL mapping studies, where several interesting candidate genes were identified for Macrophomina resistance (Muchero 2011), leaf morphology in cowpea (Pottorff et al. 2012a) , Fot race 3 (Pottorff et al. 2012b), Fot race 4 (Pottorff et al. 2014), heat-induced browning of cowpea seed coats (Pottorff et al. 2014) and heat-tolerance during reproductive development (Lucas et al. 2013a). Identification of candidate genes utilizing the syntenic relationships with sequenced legume genomes can enable "perfect markers' or molecular markers based on the underlying gene controlling the trait which can be utilized in MAS breeding schemes. Additionally, the identification of candidate genes opens up a more basic area of research for cowpea whereas the genes can be tested using different functional analysis methods.

The cowpea physical map is another genomic resource developed for cowpea and is integrated with the cowpea consensus genetic map via SNP markers and can also be

accessed via the Harvest: Cowpea database (http://phymap.ucdavis.edu/cowpea/) (http://harvest.ucr.edu). The cowpea physical map was assembled using *Hind*III and *Mbo*I genomic bacterial artificial chromosome (BAC) libraries from African breeding genotype IT97K-499-35 (http://phymap.ucdavis.edu/cowpea/). The cowpea physical map is made up of 790 contigs, 43,717 BACs, with a 10x depth of genome coverage (http://phymap.ucdavis.edu/cowpea/). Additionally, 4,300 BAC clones within the minimal tiling path (MTP) of BAC contigs have been sequenced and annotated. The cowpea physical map and associated BAC-end sequences (BES) and sequenced BAC clones of the MTP will enable the identification of candidate genes, fine mapping of traits, gene cloning and the discovery of additional markers. The integrated cowpea physical map has been utilized to identify cowpea candidate genes for traits such as resistance against Fusarium race 3 (Pottorff et al. 2012b) and heat-induced browning of seed coats (Pottorff et al. 2014). Additionally, sequences for the first drafts of the cowpea genome, vs.0.03, have been assembled and can be accessed and BLAST (www.harvest-blast.org).

Genetic linkage maps and physical maps as well as genome sequences are limited to the variation present in a small number of genotypes. A broader understanding of genetic diversity of cowpea and analysis of a wider range of traits can be studied utilizing large numbers of genetically unrelated germplasm. Of which, over 500 diverse cowpea accessions have been SNP-genotyped which can be utilized in association mapping studies. Association mapping studies have been used to identify loci for delayed senescence, biomass, and grain yield under drought stress (Muchero et al. 2013) and seed

size (Lucas et al. 2013b) in cowpea. SNP genotyping of cowpea accessions also enabled the study of the gene pool structure and the domestication of African cowpea landraces (Huynh et al. 2013a).

Other important cowpea resources include the worldwide germplasm collections. The International Institute of Tropical Agriculture (IITA) houses approximately 14,500 cowpea accessions from 65 different countries (http://genebank.iita.org), followed by the United States Department of Agriculture (USDA), which houses 6,8411 cowpea accessions from 50 countries (http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?188) and the University of California, Riverside, which houses over 5,000 cowpea accessions from over 40 countries.

These integrated cowpea genomic resources will greatly enhance cowpea breeding programs, enabling the efficient identification of trait loci and closely linked molecular markers which can be used in Marker Assisted Selection (MAS) breeding schemes which could halve the traditional breeding process. Recently, a 45,000 custom SNP genotyping assay was developed by the cowpea research group at the University of California, Riverside. The 45k SNP genotyping platform will be used to create higher density genetic maps and will be applied to cowpea breeding lines. These efforts will be used to develop cultivars adapted to production environments for the United States and West African countries with improved traits such as yield, seed quality, maturity, drought tolerance and resistance against abiotic and biotic stress.

Dissertation overview

The objectives of my dissertation studies were to map quantitative trait loci (QTL) for agronomically important traits in cowpea, identify molecular markers which could be used in a marker-assisted selection (MAS) breeding efforts as well as identify possible candidate genes for the selected traits. The integrated genomic resources that were developed for cowpea were utilized for this dissertation study. The focus of my genetic mapping efforts was to identify QTL conferring disease resistance against two soil-borne fungal pathogens of cowpea, *Fusarium oxysporum* f.sp. *tracheiphilum* race 3 (Pottorff et al. 2012b) and race 4 (Pottorff et al. 2014) and *Macrophomina phaseolina*. Two other traits were studied, which included the leaf morphology of hastate leaf shape vs. round leaf shape (Pottorff et al. 2012a) and the heat-induced browning of seed coats phenotype (Pottorff et al. 2014) in cowpea. Identification of agronomic traits in cowpea will contribute to the efficient development of improved cowpea cultivars for the United States and African production regions.

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Chapter 2

Genetic and Physical Mapping of Candidate Genes for Resistance to *Fusarium oxysporum* f.sp. *tracheiphilum* Race 3 in Cowpea [*Vigna unguiculata* (L.) Walp]

Abstract

Fusarium oxysporum f.sp. *tracheiphilum* (Fot) is a soil-borne fungal pathogen that causes vascular wilt disease in cowpea. Fot race 3 is one of the major pathogens affecting cowpea production in California. Identification of Fot race 3 resistance determinants will expedite delivery of improved cultivars by replacing time-consuming phenotypic screening with selection based on perfect markers, thereby generating successful cultivars in a shorter time period.

Resistance to Fot race 3 was studied in the RIL population California Blackeye 27 (resistant) x 24-125B-1 (susceptible). Bi-parental mapping identified a Fot race 3 resistance locus, *Fot3-1,* which spanned 3.56 cM on linkage group one of the CB27 x 24-125B-1 genetic map. A marker-trait association narrowed the resistance locus to a 1.2 cM region and identified SNP marker 1_1107 as co-segregating with *Fot3-1* resistance. Macro and microsynteny was observed for the *Fot3-1* locus region in *Glycine max* where six disease resistance genes were observed in the two syntenic regions of soybean chromosomes 9 and 15. *Fot3-1* was identified on the cowpea physical map on BAC clone CH093L18, spanning approximately 208,868 bp on BAC contig250. The *Fot3-1* locus was narrowed to 0.5 cM distance on the cowpea genetic map linkage group 6, flanked by SNP markers 1_0860 and 1_1107. BAC clone CH093L18 was sequenced and four cowpea sequences with similarity to leucine-rich repeat serine/threonine protein kinases were identified and are cowpea candidate genes for the *Fot3-1* locus. This study has shown how readily candidate genes can be identified for simply inherited agronomic

traits when appropriate genetic stocks and integrated genomic resources are available. High co-linearity between cowpea and soybean genomes illustrated that utilizing synteny can transfer knowledge from a reference legume to legumes with less complete genomic resources. Identification of Fot race 3 resistance genes will enable transfer into high yielding cowpea varieties using marker-assisted selection (MAS).

Introduction

Fusarium oxysporum f.sp. *tracheiphilum* (Fot) is a soil-borne fungal pathogen which causes vascular wilt disease in cowpea (Armstrong and Armstrong 1981). Fusarium wilt disease can be problematic wherever cowpea is grown. Incidents of Fusarium wilt have been reported in the North Western Territory of Australia, northeastern parts of Brazil as well as Nigeria (Summerell et al. 2011; Assunção et al. 2003; Armstrong and Armstrong 1980). Fusarium wilt is especially problematic in cowpea production regions within the United States including the southeastern United States and the Central Valley of California (Hare 1953). The pathogen invades the vascular tissue via the root system, causing wilting and chlorosis of the leaves and sometimes stunting of the entire plant. Broad patches of infected cowpea plants are observed in fields infested with this pathogen. The outward symptoms typically become evident at the seedling stage or during flowering and early pod development, resulting in high mortality in the affected areas with significant overall yield loss.

Breeding to develop Fusarium-resistant cowpea cultivars began in the 1930's in California after the disease was recognized (Patel 1985b). Several races of Fot have evolved, races 1, 2, 3, and 4, which are identified according to differential interactions on several cowpea genotypes (Hare 1953; Patel 1985; Smith et al. 1999a). Currently, Fot race 3 is the predominant and most widely distributed race (Smith et al. 1999a). Alternative disease management practices such as applications of fungicides are not economically feasible and there are possible health and environmental concerns with

such approaches. Host plant resistance is a proven strategy for managing Fusarium wilt disease in cowpea, and in infested production areas all new varieties must have resistance to race 3 and preferably to race 4 as well. Several successful cultivars have been bred specifically for their resistance to Fot race 3 combined with preferred agronomic traits, for example, California Blackeye 27, California Blackeye 46 and recently released California Blackeye 50 (Ehlers et al. 2000; Ehlers et al. 2009). These cultivars were developed using conventional breeding approaches that rely on phenotypic assessments as a basis for selection. For Fot race 3 resistance, several rounds of phenotypic selection are typically needed to identify and confirm putative resistant individuals during the breeding process. Marker-assisted selection (MAS) reduces the time and effort needed for the phenotypic evaluation portion of the breeding process, but may not be fully efficient due to recombination between the trait determinant and marker, proportional to their cM distance. Less than full linkage between the trait and marker will result in some individuals being misclassified during the selection process. Identification of the genetic determinants for Fot race 3 resistance will enable development of gene-based 'perfect markers' that will improve the efficiency of transferring resistance into elite varieties.

Molecular genetic and genomic resources have been developed for cowpea with an objective of enhancing breeding programs for improving 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 cowpea 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) (Muchero et al. 2009a). A cowpea physical map anchored partially to the cowpea consensus genetic map using the same SNP markers is also available (http://phymap.ucdavis.edu/cowpea). In addition, > 500 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 the underlying genetic component(s) of this trait, which will facilitate cultivar improvement using marker-assisted breeding.

The goal of this study was to identify and precisely map Fot race 3 resistance determinants in the cowpea genome. Outcomes of this study are to develop molecular markers closely linked to the *Fot3-1* resistance gene which will support breeding efforts to produce Fusarium-resistant cowpea varieties. In addition, candidate genes for the *Fot3-1* locus were identified, enabling opportunities for functional analysis which can benefit Fusarium studies in other crop plants.

Results

Interval mapping analysis of three experimental datasets from the CB27 x 24-125B-1 population identified one major locus for Fot race 3 resistance. The locus spanned 3.6 cM, from 49.4 cM to 53.0 cM on linkage group 1 of the CB27 x 24-125B-1 genetic map (Table 2.1, Figure 2.1). Of the two disease phenotypes, vascular discoloration symptoms resulted in higher LOD scores and explained a higher percent variation in phenotype than the wilting/stunting phenotype (Table 2.2). The wilting/stunting phenotype proved to be more sensitive to environmental variation than the vascular discoloration phenotype; however, it was still a good criterion for measuring disease resistance to Fusarium. SNP markers 1_1107, 1_0860, 1_1484 and 1_0911 were consistently the most significant linked markers over all three experiments based on six mapping results (Table 2.2). For two experiments, markers 1_0860 and 1_1484, which are in the same marker bin, accounted for the highest percent phenotypic variance for the vascular discoloration phenotype, 25.2% (LOD 4.91) and 27.3% (LOD 5.16), respectively (Table 2). Marker 1_1107 had the highest association with the vascular discoloration phenotype in the third experiment, accounting for 27.8% of the phenotypic variance (LOD 4.97) (Table 2.2). Henceforth, the Fot race 3 resistance locus will be referred to as *Fot3-1*.

The corresponding location of *Fot3-1* was positioned on the cowpea consensus genetic map using the highly significant markers from the bi-parental mapping study. *Fot3-1* spanned 15.4 cM to 18.3 cM on linkage group 6 of the cowpea consensus genetic map (Table 2.1).

A marker-trait association panel of known Fot race 3 resistant and susceptible genotypes was used to further narrow the *Fot3-1* locus on the cowpea consensus genetic map. Genotypic data comprised of SNPs, marker loci, cowpea varieties and lines were visualized using Flapjack software (Figure 2.2) (Milne et al. 2010). CB27, CB46, Iron Clay, SH49-10-4-1-1, SH50-17-9-1-1 (also known as California Blackeye No. 50), SH50-7-9-2 and West African genotype IT93K-503-1 are resistant to Fot race 3. Genotypes, 24-125B-1, CB5, Bambey 21, IT82E-18 (Big Buff), and IT84S-2049 are susceptible to Fot race 3. Markers in the *Fot3-1* locus on the cowpea consensus genetic map were examined with the twelve cowpea genotypes to associate an allele with the response to Fot race 3; resistance or susceptibility. SNP marker 1_1107, which was highly significant in the bi-parental mapping studies, was the only marker with alleles that co-segregated perfectly with a corresponding resistant or susceptible phenotype (Figure 2.2). The resistant genotype at this locus is associated with the adenine nucleotide which is color-coded green in Figure 2.2. The susceptible genotype was associated with the guanine nucleotide which is color-coded red in Figure 2.2. SNP marker 1_1107 was derived from the cowpea P12 assembly unigene 12265 position 693, which was annotated as a cysteine desulfurase and can be viewed in HarvEST: Cowpea (Figure 2.3) (http://harvest.ucr.edu). The marker-trait association narrowed the *Fot3-1* locus to a 1.2 cM region and was defined by flanking SNP markers 1_1484 and 1_0704 (Figure 2.2).

The cowpea region carrying the *Fot3-1* locus was compared with the soybean genome using HarvEST: Cowpea to determine if the gene order was conserved between species. High co-linearity with the *Fot3-1* region in any of the sequenced genomes may enable identification of candidate genes. The *Fot3-1* region was found to be highly co-linear with two regions of soybean, chromosome 9 and chromosome 15 (Figure 2.4). The syntenic region in soybean chromosome 9 extended from soybean locus Glyma09g02100 to Glyma09g02560 which corresponded to 17.14 cM to 19.04 cM of the *Fot3-1* locus on the cowpea consensus genetic map (Table 2.3). The syntenic region was scanned for known disease resistance genes on the soybean genome browser (http://www.phytozome.org) where two soybean disease resistance genes were observed. Soybean locus Glyma09g02210 was flanked by orthologous soybean genes to EST-derived SNP markers 1_1211 and 1_1484 and was annotated as a leucine-rich repeat (LRR) serine/threonine protein kinase (Table 2.3). Glyma09g02420 was flanked by SNP markers 1_0860 and 1_1107 and was annotated as a disease resistance protein of the NBS-LRR class (Table 2.3). The *Fot3-1* syntenic locus in soybean chromosome 15 extended from soybean locus Glyma15g12830 to Glyma15g13470 which corresponded to 17.14 cM to 19.04 cM of the *Fot3-1* locus on the cowpea consensus genetic map (Table 2.3). The syntenic region of soybean chromosome 15 was scanned and four LRR genes were observed, Glyma15g13100, Glyma15g13290, Glyma15g13300 and Glyma15g13310 (Table 2.3). Glyma15g13100 was flanked by orthologous soybean genes to SNP markers 1_1077 and 1_1484 and was annotated as a LRR serine/threonine protein kinase (Table 2.3). Soybean loci Glyma15g13290, Glyma15g13300 and

Glyma15g13310 were identified between orthologous soybean genes to markers 1_0860 and 1_1212 (Table 2.3). Glyma15g13290 and Glyma15g13300 were annotated as disease resistance proteins of the NBS-LRR class while Glyma15g13310 was annotated as an LRR protein (Table 2.3). Due to the high co-linearity of gene order between cowpea and soybean at the two syntenic loci, the observed soybean disease resistance genes were considered as orthologous candidate genes for the *Fot3-1* locus. Soybean is the closest related legume model species to cowpea and both are members of the economically important warm season Phaseoleae clade (Choi et al. 2004). The ability to use the sequenced soybean genome as a means to identify candidate genes within syntenic regions in cowpea exhibits the utility of these closely related legumes.

The cowpea physical map (http://phymap.ucdavis.edu/cowpea) which has been partially anchored to the cowpea consensus genetic map via EST-derived SNP markers was used to identify BAC clones that span the physical region of *Fot3-1*. The most significant markers identified in the bi-parental mapping study and closely linked markers from the cowpea consensus genetic map identified BAC contig250 as spanning the most significant region of *Fot3-1* (Table 2.1). The length of the contig is estimated at 885,600 bp (540 non-repeated fingerprint bands) and consists of 46 BAC clones, 21 of which have BAC-end sequences (BES) available. Nine BAC clones from contig 250 were identified as harboring SNP markers (Table 2.1, Figure 2.5). SNP marker 1_1107 was identified on two overlapping BAC clones, CH051M10 and CM001C09 (Table 2.1, Figure 2.5). Markers 1_0860 and 1_0704 which are closely flanking markers to 1_1107 on the

cowpea consensus genetic map also were found to flank 1_1107 on the physical map (Table 2.1, Figure 2.5). 1_0860 was identified on BAC clones CH093L18 and CH076D23 (Table 2.1, Figure 2.5). 1_0704 was identified sharing BAC clone CH051M10 with 1_1107, and was also identified on BAC clone CM054B04 (Table 2.1, Figure 2.5). Marker 1_1212 which could not be placed on the cowpea consensus genetic map was identified sharing two BAC clones with 1_1107, CH093L18 and CH051M10 (Figure 2.5). SNP marker 1_1212 was also identified on BAC clone CM001C09, which it shares with marker 1_0860 (Figure 2.5). Marker 1_1484, which flanks 1_1107 on the cowpea consensus genetic map, was not identified on the cowpea physical map (Table 2.1). The significant region of the *Fot3-1* locus spanned three overlapping BAC clones, CH093L18, CM001C09 and CH051M10 (Figure 2.5). However, since CH093L18 and CH051M10 overlap the total length of BAC clone CM001C09, *Fot3-1* was narrowed to two overlapping BAC clones which span an approximate total length of 375,560 bp (Figure 2.5).

The two BAC clones, CH093L18 and CH051M10, which overlap the significant region of the *Fot3-1* locus, were sequenced to identify cowpea candidate genes. The BAC clone sequences were assembled using Velvet software (Zerbino and Birney 2008). Cowpea BAC clone CH051M10 which harbored SNP markers 1_1212, 1_1107 and 1_0704, was assembled and resulted in seventy-four contigs with an approximate length of 188,000 to 203,000 bp, which matched the expected size of the BAC clone including the vector (http://harvest.web.org). BAC clone CH051M10 was BLASTed with EST sequences

from which SNP markers 1_1212, 1_1107 and 1_0704 were derived to confirm that the markers were present and to assure the quality of the sequence assembly; all three SNP sequences were identified (Table 2.4). The BES of CH051M10 was also identified after BLASTing to the assembled sequence (Table 2.4). The orthologous soybean candidate disease resistance genes were BLASTed to the BAC clone CH051M10 sequences, however, no orthologous cowpea genes were identified which eliminated the BAC as a candidate for harboring the *Fot3-1* gene (Table 2.5).

The candidate BAC clone CH093L18 which harbors SNP markers 1_0860 and 1_1212 was also sequenced and the assembly resulted in 127 contigs with an estimated length of 184,856 bp (http://harvest.web.org). The EST sequences from which SNP markers 1_0860 and 1_1212 were derived were BLASTed to the assembled BAC clone CH093L18 to confirm their presence and the quality of assembly; both SNPs were identified (Table 2.6). The six soybean candidate genes were BLASTed to CH093L18 to possibly identify orthologous cowpea candidate genes. Glyma09g02210 was the only soybean gene which returned a high similarity with several nodes of the cowpea BAC clone (Table 2.7). The assembled sequences of BAC clone CH093L18 were then BLASTed to the soybean genome to determine gene annotations for the entire clone. It appeared that there were twenty-five putative cowpea genes on BAC clone CH093L18 and that the only disease resistant-type genes were NODES 50, 57, 65 and 104 which were annotated as leucine-rich repeat serine/threonine protein kinases (Table 2.7). The

Fot3-1 resistance locus was narrowed to BAC clone CH093L18 and leucine-rich repeat serine/threonine protein kinases were identified as the cowpea candidate gene for *Fot3-1*.

The soybean candidate disease resistance gene, Glyma09g02210, was BLASTed to the cowpea genome vs. 0.02 to identify candidate genomic sequences for *Fot3-1*. The BLASTn search for the genomic and cDNA sequence of Glyma09g02210 returned a high alignment with scaffold 17795 with e-score values of e-155 and e-147, respectively (Table 2.8). The sequences for scaffold 17795 were then BLASTed back to BAC clone CH093L18 to determine which NODE of the assembly had the highest similarity; NODE 50 returned a perfect alignment with e-score value of 0.0 (Table 2.9). We concluded that NODE 50 on BAC clone CH093L18 was the best candidate cowpea gene for the *Fot3-1* locus and that scaffold 17795 may be the cowpea ortholog to soybean Glyma09g02210.

After determining that *Fot3-1* was located on cowpea BAC clone CH093L18, the physical distance of *Fot3-1* was compared to the cowpea consensus genetic map. The marker-trait association analysis delimited *Fot3-1* to a 1.16 cM region as determined by flanking SNP markers 1_1484 and 1_0704 to 1_1107 (Table 2.1, Figure 2.2). Since *Fot3-1* was located on BAC clone CH093L18 which housed SNP markers 1_0860 and 1_1212 (Figure 2.5); correspondingly, *Fot3-1* was narrowed to a 0.5 cM region on the cowpea consensus genetic map, flanked by SNP markers 1_0860 (17.82 cM position) and 1_1107 (18.31 cM position) since 1_1212 was not positioned on the cowpea consensus genetic map (Table 2.1).

The cowpea genome size is estimated at 630 Mb (Arumuganathan and Earle 1991b). The cowpea consensus genetic map vs.3 (Diop et al. 2012) estimated the total genetic distance as 680 cM which provides an estimated mean genetic to physical distance ratio of 1.1 cM per Mb. The *Fot3-1* BAC clone CH093L18 is approximately 232,880 bp using the cowpea physical map estimates (http://phymap.ucdavis.edu/cowpea). Therefore, the BAC clone carrying the *Fot3-1* locus and flanking markers at a distance of 0.5 cM has at least two times the mean genetic to physical distance, suggesting that the *Fot3-1* gene resides in a relatively recombination-active region of the cowpea genome. This is fortuitous in the context of resistance gene introgression because the higher recombination rate means a decreased likelihood of deleterious genes being co-introgressed by linkage drag. It also highlights the value of eventually identifying the actual *Fot3-1* gene in order to have a "perfect marker" that will not segregate from the trait.

Conclusion

In this study, we report the identification of the *Fot3-1* locus which confers resistance to Fot race 3 in cowpea. By utilizing the integrated cowpea genomic resources, the *Fot3-1* locus was narrowed to a single BAC clone CH093L18, which identified four leucine-rich repeat serine/threonine protein kinases as candidate genes for *Fot3-1*.

Typically, resistance to Fusarium has been shown to be a dominant and monogenic trait (Zink and Thomas 1990; Rubio et al. 2003; McGrath et al. 1987; Scott and Jones 1989; Sarfatti et al. 1991) which fits the gene-for-gene hypothesis whereby pathogen and host express complementary dominant genes (Flor 1971). The alteration or loss to either the host's resistance gene or pathogen's avirulence gene leads to disease (Flor 1971). The majority of disease resistance genes are classified as having an NBS-LRR motif which has been further sub-divided by their difference at the N- terminus, either having homology with the TIR domain (TIR-NBS-LRR) (Meyers et al. 1999; Pan et al. 2000) or a coiled-coil motif (CC-NBS-LRR or non TIR-NBS-LRR) (Pan et al. 2000). Currently, two genes have been cloned which confer resistance to *F. oxysporum*, *I-2* and *Fom-2* (Simons et al. 1998; Joobeur et al. 2004). The *I-2* locus, which confers resistance to *F. oxysporum* f.sp. *lycopersici* (Fol) race 2 in tomato was determined to be a CC-NBS-LRR disease resistance gene (Simons et al. 1998). The *Fom-2* locus, which confers resistance to *F. oxysporum* f.sp *melonis* (Fom) in melon was also identified as a CC-NBS-LRR gene (Joobeur et al. 2004).

Although the majority of cloned R genes have the conserved NBS-LRR structure, there are several disease resistance genes identified as belonging to the receptor-like kinase (RLK) family. RLKs are proteins that span the plasma membrane, recognizing and responding to extracellular signals (Geer et al. 1994). The majority of RLK have serine/threonine kinases and LRR motifs (Becraft 1998). The receptor-like cytoplasmic kinase (RLCK) disease resistance genes include *PBS1*, *Pti* and *Pto* (Shiu and Bleecker 2001). *PBS1* confers resistance against *Pseudomonas syringae* pv *phaseolicola* in Arabidopsis (Swiderski and Innes 2001). *Pti* and *Pto* both confer resistance to the bacterium *Pseudomonas syringae* pv *tomato* (Zhou et al. 1995; Martin et al. 1993). *Xa21* is a LRR RLK and confers resistance against *Xanthomonas campestris* pv *oryzae* in rice (Song et al. 1995). *Lrk10* which confers resistance to the fungus, *Puccinia recondite* in wheat was also determined to be a serine/threonine protein kinase (Feuillet et al. 1997). The *I-3* locus which confers resistance to *F. oxysporum* f.sp. *lycopersi* race 3 in tomato, was determined to be positioned within a large cluster of S-locus receptor-like kinases (SRLK)(Hemming et al. 2004). Interestingly, we recently identified TIR-NBS-LRR proteins and leucine-rich repeat serine/threonine protein kinases in the *Fot4-1* and *Fot4-2* syntenic regions of soybean (unpublished data). *Fot4-1* and *Fot4-2* confer resistance to Fot race 4 in cowpea (unpublished data). It may be possible that leucine-rich repeat serine/threonine protein kinases are the R genes conferring resistance in the cowpea-Fusarium pathovar system.

A practical outcome of this study is the development of molecular markers closely linked to the *Fot3-1* locus. These markers can be used in marker-assisted breeding to optimize cowpea genetic improvement via different strategies including pedigree backcrossing and marker-assisted recurrent selection. These approaches should expedite variety development by at least halving the current traditional breeding selection process which relies on time-consuming and costly phenotyping. The identification of the Fot race 3 resistance gene would provide 'perfect markers' and further improve marker-assisted breeding efficiency.

Future goals include functional analysis of *Fot3-1* candidate genes to define the genetic resistance determinant. Identifying the *Fot3-1* gene will enhance our understanding of resistance to Fusarium as well as broaden our knowledge of resistance genes within the legume family.

Materials and methods

Resistance to Fot race 3 was tested on a RIL population which was developed by an intra-specific cross between cultivar California Blackeye 27 (CB27) and 'C93W-24-125B-1'. Each of the 90 lines was advanced by single seed descent to the F_{10} generation. CB27 is a cultivar which was bred for resistance to *F. oxysporum* f.sp. *tracheiphilum* races 3 and 4 (Ehlers et al. 2000). C93W-24-125B is a breeding line from Cameroon and is highly susceptible to Fot race 3 (Hall et al. 2003; Kitch et al. 2001). These materials were available from the University of California Riverside cowpea germplasm collection.

Two strains of Fot race 3, which were isolated previously from infected cowpea plants in the San Joaquin Valley, California, were used for inoculum cultures (unpublished data, Shirley Smith). Individual strains were developed from single spore lines. Isolates were dried and stored on sterile potato dextrose agar (PDA) plates at -80 °C. 1-cm^2 plugs were cut from frozen Fusarium-containing PDA plates and transferred aseptically to flasks containing 500ml of potato-dextrose broth, then incubated in a shaker at 27 °C and 30 rpm under lighted conditions for three days. The liquid culture was strained through four layers of cheesecloth to eliminate mycelium, followed by adjustment of the spore concentration to 1.0×10^6 microconidia per ml using a hemocytometer. Greenhouse experiments were conducted using a modified root-dip inoculation method as previously described (Rigert and Foster 1987). Ten greenhouse grown seeds per line were planted in seeding trays filled with vermiculite and watered daily for one week. After one week,

five seedlings per line were gently uprooted and half of the root system was clipped and then dipped for one minute into suspended inoculum. Inoculated seedlings were transplanted into one gallon pots, randomized on benches and watered daily. Greenhouse day temperatures were set to 28 °C and night temperatures set to 16 °C.

Plants were evaluated five weeks post inoculation for Fusarium disease symptoms. The wilting/stunting phenotype was evaluated by approximating the percentage of wilting or stunting on the entire plant. The vascular discoloration phenotype was evaluated by uprooting the entire plant, then slicing the stem vertically to evaluate the extent of the disease symptoms (Figure 2.6). The severity of the disease was evaluated on a zero to five rating scale for the wilting/stunting and vascular discoloration phenotypes. A score of zero indicated a healthy plant with no signs of disease, 1 = approximately 10% of the plant showing symptoms of disease, 2 = approximately 25% of the plant showing symptoms of disease, 3 = approximately 50% of the plant showing symptoms, 4 = approximately 75% of the plant showing symptoms and 5 = 100% of the plant showing disease symptoms. Five replicates per line were evaluated individually then averaged to determine the disease severity for each RIL.

The California Blackeye 27 x 24-125B-1 population and genotypes CB27, CB46, Iron Clay, SH49-10-4-1-1, SH50-17-9-1-1 (also known as California Blackeye No. 50), SH50-7-9-2, IT93K-503-1, 24-125B-1, CB5, Bambey 21, IT82E-18/ Big Buff and IT84S-2049 were genotyped at the F_8 generation or above using bi-allelic SNP markers

from the 1536 Illumina GoldenGate Assay as previously described in Muchero, et al. (2009).

A SNP genetic map for the California Blackeye 27 x 24-125B-1 population was created previously and is included in both cowpea consensus genetic map vs.2 (Muchero et al. 2009a) and vs. 3 (Diop et al. 2012). The map was generated using 339 SNP markers and 90 individuals and consisted of sixteen linkage groups and spans approximately 600 cM total distance (Diop et al. 2012). The cowpea consensus genetic map vs. 3 (Diop et al. 2012) was used for this study which is an updated version of the Muchero, et al. (2009) map. The vs. 3 map was developed using ten RIL populations and two breeding populations which increased the marker density and improved the marker order (Diop et al. 2012). The vs. 3 consensus genetic map is 680 cM in length and contains 1043 markers which is an addition of 115 markers and an average 0.65 cM between markers (Diop et al. 2012). The current SNP-based cowpea linkage map is included in a publicly available browser called HarvEST: Cowpea, which can be downloaded as a Windows software from http://harvest.ucr.edu or viewed online at www.harvest-web.org.

Resistance to Fot race 3 was mapped using the CB27 x 24-125B-1 genetic map and greenhouse inoculation datasets which were comprised of wilting/stunting and vascular discoloration phenotypes. Kruskal-Wallis and Interval Mapping analysis packages of MapQTL 5.0 software were used to conduct the bi-parental mapping (Van Ooijen 2004). A locus was considered significant if the same locus was identified using both phenotypic

ratings 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 determine the span of the locus using 1-LOD and 2-LOD to determine left and right margins. Results were visualized using MapChart 2.2 software (Voorrips 2002).

Synteny was examined between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes as described previously (Muchero et al. 2009a). Syntenic relationships between the cowpea, soybean, *M. truncatula* and *A. thaliana* can be examined in 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 13 lines drawn per linkage group. Due to limited resolution in the software images, not all markers are presented in the screenshot images output from Harvest: Cowpea. In order to view each individual marker, the linkage group must be magnified in the HarvEST: Cowpea database.
The cowpea physical map (http://phymap.ucdavis.edu/cowpea) was developed in work to be described elsewhere using an advanced African breeding line IT93K-399-35 and two BAC clone libraries developed with restriction enzymes *Hind*III and *Mbo*I (Amplicon Express, Pullman, WA). Contigs were assembled using the snapshot method of DNA fingerprinting (Luo et al. 2003a) and was completed at the University of California,

Davis by Ming Cheng Luo. The length of the BAC clones was estimated by multiplying the number of unique bands generated from the fingerprinting assay by 1640bp (personal communication, Ming Cheng Luo).

BAC clones CH051M10 and CH093L18 were sequenced using an Illumina GA_{II} or HiSeq 2000 sequencer, respectively, at the Institute of Integrative Genome Biology, University of California, Riverside. BAC clones were purified using a QIAGEN 96 prep kit following manufacturer's instructions (Valencia, CA). Purified BAC clones were sheared using a Diogenode Bioruptor UCD-200 (Liege, Belgium) for 14 minutes at the maximum setting, alternating on and off for 30 seconds. Fragments ranging from 300-500 bases in length were visualized and excised from a 1% precast E-gel® (Invitrogen, Carlsbad, CA). BAC clone fragments were prepared for sequencing using Illumina's Paired End DNA Sample Prep kit following manufacturer's instructions. A QIAquick PCR Purification kit was used in between amplification steps (QIAGEN, Valencia, CA). Sequences from CH051M10 were generated as 36-base single-end reads from a single sample on an Illumina GA_{II} instrument. CH093L18 sequences were generated as 100-base paired-end reads within a 14-sample multiplex in one lane on an Illumina HiSeq 2000 instrument. BAC clone sequences were first filtered to remove *E. coli* sequences then assembled using Velvet software (Zerbino and Birney 2008) using a range of k-mer lengths from 19 to 35 to identify an optimal assembly considering the estimated depth of coverage, number of nodes, N50 and maximum node length. The optimum assembly of CH051M10 was obtained using k-mer size 25 (N50 = 6,384). The optimum assembly of

CH093L18 was obtained using k-mer size 27 (N50 = 7,717). A NODE is defined as a sequence or contig which can be consistently reconstructed using the sequencing reads (Zerbino 2010a; Zerbino and Birney 2008). All sequence data is publicly available via the Harvest: Cowpea database (www.harvest.ucr.edu) and version 0.02 of the assembled cowpea genome (www.harvest-blast.org).

Cowpea genome version 0.02 which contained approximately 200 Mb of assembled scaffolds and contigs covered about 97% of previously identified cowpea genes (UCR cowpea group, unpublished) is available for BLAST searches and sequence retrieval (www.harvest-blast.org).

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Table 2.1 *Fot3-1* locus in the CB27 x 24-125B-1 genetic map, cowpea consensus genetic map and cowpea physical map.

CB27 x 24-125B-1 genetic map			Cowpea genetic map			Cowpea physical map	
LG	cM	SNP	LG	cM	SNP	Contig	BAC clone(s)
1	52.98	1_0911	6	15.43	1_0911	1117	CM012O18
		N/A	6	16.51	1_0830	N/A	
		N/A	6	16.88	1_1381	771	CH001O04
		N/A	6	17.14	1_0895	250	CH046G19
		N/A	6	17.14	1_1077	250	CM002B24, CM015O07
		N/A	6	17.14	1_1363	250	CM015O07, CH045I01
		N/A	6	17.40	1_0897	250	CH045I01, CM002B24, CM015O07
1	50.49	1_0860	6	17.82	1_0860	250	CH076D23, CH093L18
1	50.49	1_1484	6	17.88	1_1484	N/A	
1	49.42	1_1107	6	18.31	1_1107	250	CM001C09, CM051M10
		N/A	6	19.04	1_0704	250	CM054B04, CH051M10

Table 2.2 Bi-parental mapping of *Fot3-1* in the CB27 x 24-125B-1 population.						
Experiment	Statistical analysis	Phenotype	1_1107	1_0860	1_1484	1_0911
2007	IM LOD	Wilting/Stunting	1.83	1.95	1.95	1.57
	IM R^2	Wilting/Stunting	10.2	10.7	10.7	8.8
	IM LOD	Vascular Discoloration	4.49	4.91	4.91	3.52
	IM R^2	Vascular Discoloration	23.2	25.2	25.2	18.7
	Kruskal-Wallis test statistic	Wilting/Stunting	6.18	6.22	6.22	5.52
	Kruskal-Wallis p-value	Wilting/Stunting	0.05	0.05	0.05	0.05
	Kruskal-Wallis test statistic	Vascular Discoloration	29.09	32.42	32.42	23.08
	Kruskal-Wallis p-value	Vascular Discoloration	0.0001	0.0001	0.0001	0.0001
2009a	IM LOD	Wilting/Stunting	3.7	4.26	4.26	3.37
	IM R^2	Wilting/Stunting	20.2	22.7	22.7	18.4
	IM LOD	Vascular Discoloration	4.44	5.16	5.16	4.67
	IM R^2	Vascular Discoloration	24.2	27.3	27.3	24.9
	Kruskal-Wallis test statistic	Wilting/Stunting	10.23	12.87	12.87	9.24
	Kruskal-Wallis p-value	Wilting/Stunting	0.005	0.0005	0.0005	0.005
	Kruskal-Wallis test statistic	Vascular Discoloration	12.747	15.97	15.97	14.54
	Kruskal-Wallis p-value	Vascular Discoloration	0.0005	0.0001	0.0001	0.0005
2009b	IM LOD	Wilting/Stunting	3.09	2.98	2.98	2.06
	IM R^2	Wilting/Stunting	18.4	17.7	17.7	12.6
	IM LOD	Vascular Discoloration	4.97	4.85	4.85	3.23
	IM R^2	Vascular Discoloration	27.8	27	27	18.9
	Kruskal-Wallis test statistic	Wilting/Stunting	13.33	12.13	12.13	8.37
	Kruskal-Wallis p-value	Wilting/Stunting	0.0005	0.0005	0.0005	0.005
	Kruskal-Wallis test statistic	Vascular Discoloration	24.19	22.63	22.63	16.03
	Kruskal-Wallis p-value	Vascular Discoloration	0.0001	0.0001	0.0001	0.0001

IM= Interval Mapping analysis

Table 2.3 Synteny of *Fot3-1* with *G. max* chromosomes 9 and 15.					
G. max chromosome	*G. max* locus	Phytozome annotation	Cowpea locus	LG	cM
9	Glyma09g02100	Aspartyl protease	1_1363	6	17.14
9	Glyma09g02130	Sodium hydrogen exchanger	1_0897	6	17.40
9	Glyma09g02160	ENDO-1,4-BETA-GLUCANASE	1_1434	10	45.22
9	Glyma09g02210	Leucine-rich repeat serine/threonine protein kinase	N/A	N/A	N/A
9	Glyma09g02290	Protein of unknown function	1_1484	6	17.88
9	Glyma09g02310	Vesicle-associated membrane protein	1_0860	6	17.82
9	Glyma09g02420	Disease resistance protein (NBS-LRR)	N/A	N/A	N/A
9	Glyma09g02450	Cysteine desulfurylase	1_1107	6	18.31
9	Glyma09g02560	Glycolipid transfer	1_0704	6	19.04
15	Glyma15g12830	DNA-directed RNA polymerase	1_0895	6	17.14
15	Glyma15g13000	Aspartyl protease	1_1363	6	17.14
15	Glyma15g13030	Sodium/hydrogen exchanger	1_0897	6	17.40
15	Glyma15g13080	Glycosyl hydrolase family 9	1_1077	6	17.14
15	Glyma15g13100	Leucine-rich repeat serine/threonine protein kinase	N/A	N/A	N/A
15	Glyma15g13210	Protein of unknown function	1_1484	6	17.88
15	Glyma15g13220	Vesicle-associated membrane protein	1_0860	6	17.82
15	Glyma15g13290	Disease resistance protein (NBS-LRR)	N/A	N/A	N/A
15	Glyma15g13300	Disease resistance protein (NBS-LRR)	N/A	N/A	N/A
15	Glyma15g13310	Leucine-rich repeat protein	N/A	N/A	N/A
15	Glyma15g13330	No functional annotation	1_1212	Not mapped	Not mapped
15	Glyma15g13470	Glycolipid transporter activity	1_0704	6	19.04

Table 2.4 BLAST of cowpea SNP markers and BES to cowpea BAC clone CH051M10.			
Sequence of SNP or BES	Sequence position	Bits	e-score
1_0704	NODE_16	549	e-158
1_1212	NODE_30	460	3-131
1_1107	NODE_18	737	0.0
BES of CH051M10	NODE_3	1548	0.0

SNP = single nucleotide polymorphism, BES = Bacterial Artificial Chromosome-end sequence

Table 2.5 *G. max* candidate genes BLAST to cowpea BAC clone CH051M10.						
G. max locus	tBLASTn	Bits	e-score	BLASTn	Bits	e-score
Glyma09g02210	NODE_19	23	4.2	NODE_4	30	0.290
Glyma09g02420	NODE_22	23	4.6	NODE_16	30	0.400
Glyma15g13100	NODE_31	26	0.72	NODE_34	30	0.420
Glyma15g13290	NODE_21	23	7.4	NODE_50	32	0.098
Glyma15g13300	NODE_18	25	2	NODE_16	30	0.400
Glyma15g13310	NODE_11	23	1.9	NODE_12	30	0.180

Table 2.6 Cowpea SNP markers BLAST to cowpea BAC clone CH093L18.			
Cowpea locus	Sequence position	Bits	e-score
1_0860	NODE_24	827	0
1_1212	NODE_3	460	e-131

Table 2.7 Cowpea BAC clone CH093L18 sequences annotated using *Glycine max* BLAST results.			
Cowpea sequence	*G. max* locus	*G. max* e-score	Phytozome annotation
NODE_5	Glyma09g02350	3e-083	GDP-fucose protein O-fucosyltransferase
NODE_7	Glyma15g13210	1e-158	APOPTOSIS INHIBITOR 5-RELATED
NODE_10	Glyma09g02340	1e-129	RING/U-box superfamily protein
NODE_11	Glyma09g02310	9e-037	Vesicle-associated membrane protein 721
NODE_13	Glyma08g32320	3e-005	Reverse transcriptase
NODE_14	Glyma02g12430	1e-104	Translation initiation factor 2C
NODE_15	Glyma15g00440	1e-165	SWIM zinc finger
NODE_18	Glyma15g00440	1e-171	SWIM zinc finger
NODE_19	Glyma13g19430	4e-052	Actin depolymerizing factor 1
NODE_20	Glyma09g02280	4e-063	Magnesium transporter CorA-like family protein
NODE_22	Glyma09g02350	8e-099	GDP-fucose protein O-fucosyltransferase
NODE_24	Glyma09g02310	2e-020	Vesicle-associated membrane protein
NODE_25	Glyma15g13120	1e-155	NAD dependent epimerase/dehydratase
NODE_28	Glyma15g13220	5e-016	Vesicle-associated membrane protein 726
NODE_29	Glyma09g02310	3e-009	Synaptobrevin-related protein 1
NODE_32	Glyma02g42330	3e-078	Pleckstrin homology (PH) domain superfamily protein
NODE_33	Glyma09g02350	3e-023	GDP-fucose protein O-fucosyltransferase
NODE_41	Glyma09g02350	5e-009	GDP-fucose protein O-fucosyltransferase
NODE_50	Glyma09g02210	3e-032	Leucine-rich repeat serine/threonine protein kinase
NODE_52	Glyma15g13190	5e-060	SNARE-like superfamily protein
NODE_54	Glyma09g02350	5e-025	O-fucosyltransferase family protein
NODE_56	Glyma15g13250	1e-008	GDP-fucose protein O-fucosyltransferase
NODE_57	Glyma08g34790	7e-010	Leucine-rich repeat serine/threonine protein kinase
NODE_65	Glyma09g02210	3e-012	Leucine-rich repeat serine/threonine protein kinase
NODE_104	Glyma07g40100	9e-008	Leucine-rich repeat serine/threonine protein kinase

Table 2.8 *Glycine max* candidate gene BLAST to the cowpea genome.						
Glycine max locus	BLASTn (genomic)	Bits	e-score	BLASTn (cDNA)	Bits	e-score
Glyma09g02210	scaffold 17795	553	e-155	scaffold 17795	523	e-147

Table 2.9 Cowpea genomic sequences BLAST to BAC clone CH093L18.			
Cowpea genomic sequences	Bits	e-score	Sequence position
scaffold 17795	2343	0.0	NODE 50

Figure 2.1 Resistance to *Fusarium oxysporum* f.sp. *tracheiphilum* race 3 in the CB27 x 24-125B-1 population. The *Fot3-1* locus (Interval Mapping analysis shown) spanned approximately 12.5 cM on the CB27 x 24-125B-1 genetic map, linkage group 1. The 2007 experiment LOD scores are plotted in red; the 2009a experiment is plotted in green and 2009b experiment is plotted in blue. Solid colored lines indicate the vascular discoloration phenotype and the wilting/stunting phenotype which are depicted by broken colored lines. SNP markers 1_1107, 1_0860, 1_1484 and 1_0911 which were the most significant markers over the three experiments are highlighted in red on the linkage group. The LOD significance threshold of 2.0 is indicated by a dashed horizontal line on the graph.

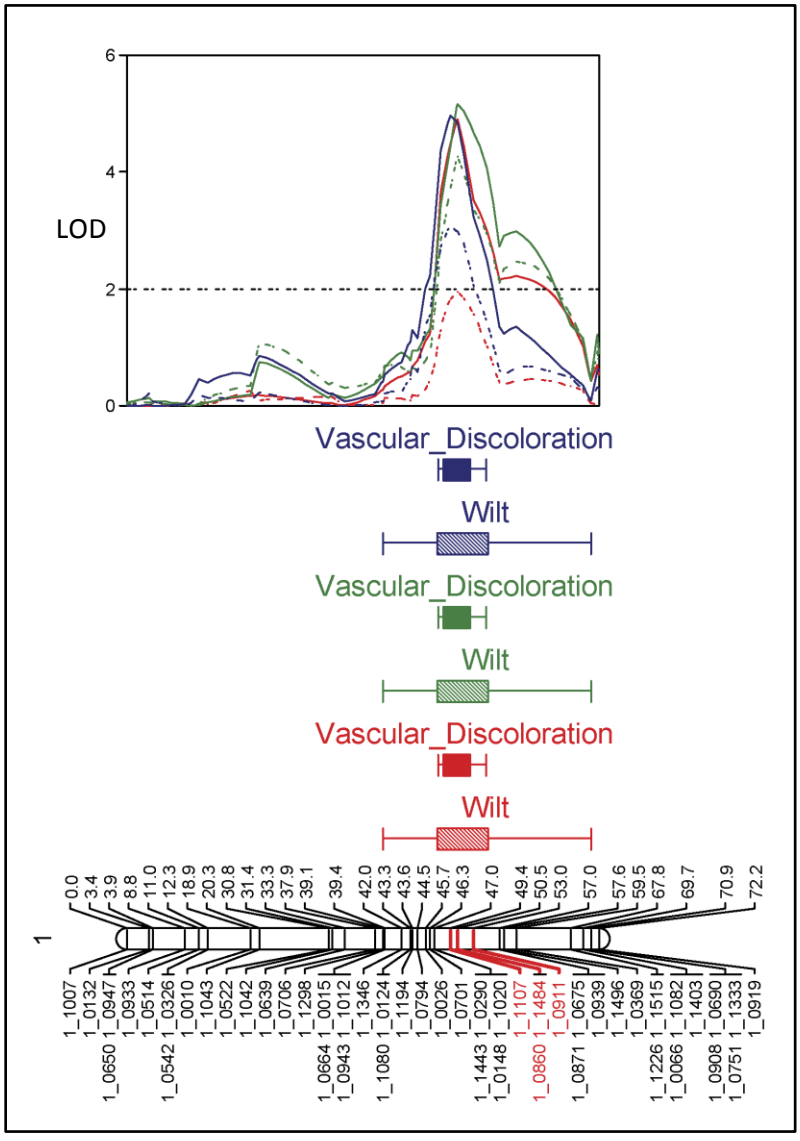


Figure 2.2 Marker-trait association in the *Fot3-1* locus. The *Fot3-1* locus on the cowpea consensus genetic map is depicted along with twelve cowpea genotypes which differ in their response to *Fusarium oxysporum* f.sp. *tracheiphilum* (Fot) race 3. "R" indicates a resistant genotype to Fot race 3 and "S" indicates a susceptible genotype to Fot race 3. SNP marker 1_1107 (18.3 cM) alleles co-segregated with the resistant and susceptible genotypes along with the corresponding disease phenotype. The adenine nucleotide is the resistant allele which is color-coded green while the susceptible allele is the guanine nucleotide which is color-coded red.

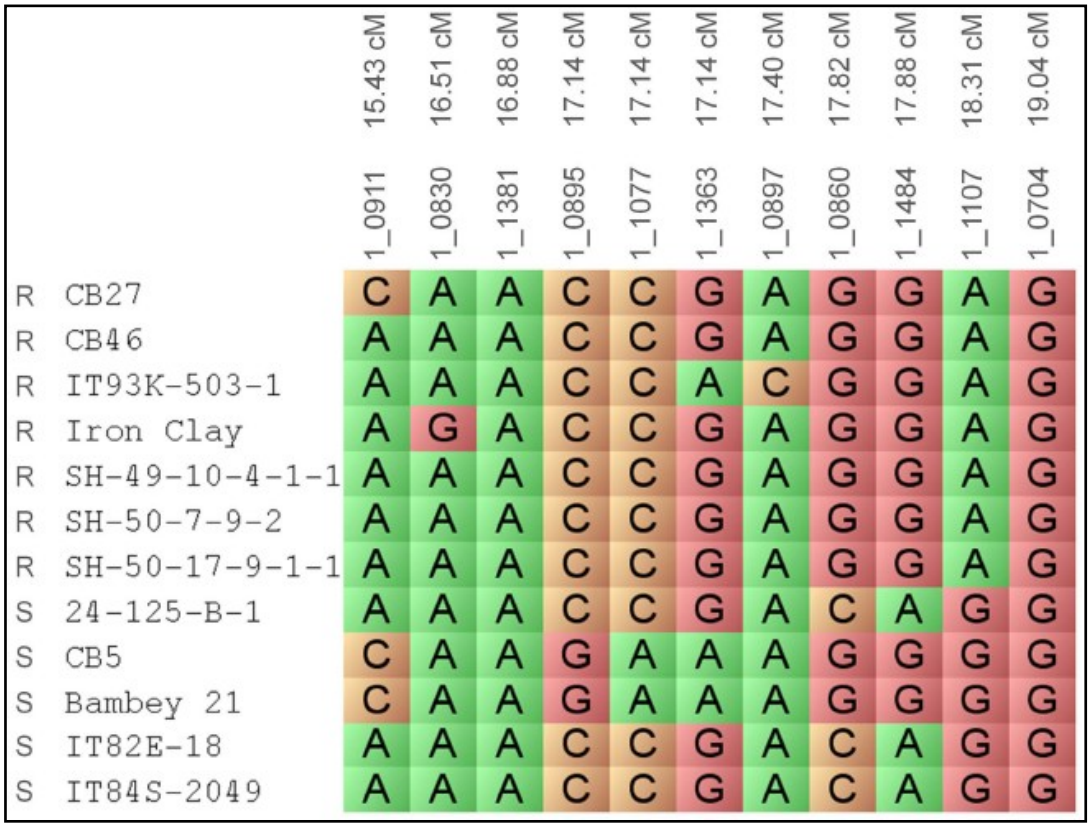


Figure 2.3 SNP marker 1_1107 EST sequence and SNP position.

GAGCAAATTGAAAGATATGTTCTCACGGAAGACCAAAATAGTAGTCGCTCAT
CATGTTTCAAATGTGCTTGCTTCTGTCCTTCCTATTAGAGATATTGCACAATG
GGCACATGATGTTGGAGCAAAAGTTCTTGTATGCTTGTCAGAGTGTTCCACAC
ATGGTGGTTGATGTCCAGAGCCTTAATGTTGATTTTCTTGTTGCTTCTTCTCAC
AAGATGTGTGGGCCTACGGGAATTGGATTCTTATATGGTAAAATAGACCTCTT
GTCTTCCATGCCTCCATTTTTAGGTGGTGGTGAAATGATTTCTGATGTATATCT
TGATCATTCAACTTATGCCGAACCTCCTTCCAGATTTGAGGCTGGAACACCAG
CTATTGGGGAAGCAATTGGTTTAGGAGCAGCAATTGATTACTTATCTGGGATT
GGTATGCAAACTATACATGATTATGAGGTGGAGCTTGGTAGTTATCTGTACG
AAAGGCTTCTTTCAGTCCCAAATATTCGCATCTATGGGCCAGCACCTTCAGAA
AATGTTCAACGAGCAGCTCTTTGTTCTTTCAATGTTGAGAATTTGCATCCCAC
TGATCTTGCAACATTTCTGGACCAACAGCATGGAGTGGCTATCAGATCAGGT
CACCATTGTGCCCAACCCCTCCATCGCTTCTTAGGAGTCAGCTCAAGTGCACG
CGCC**(A/G)**GTCTCTACTTCTACAACACAAAGGAAGATGTGGACTACTTTATCC
ATGCCCTCAACGACACAGTCAACTTTTTCAACTCATTCAAGTAACCAGAATGT
ATTTTAATGTATATTAAATTTTGTTTATACGCCAATGAGAGGGTTGTCTTAGTT
GGTAGGAAAGCTGCGTCAATGAAATGTTCTTGAATTTCATTCCTTCTATTGAT
GTCAATGGTAGGAACTAGGCATCCATTAATTGCAGTATTGAAACCTATCTAC
AGCTGAACTTTTATGCATAAAAAGAATGCCCATAAGCATTTTAATTAAAAAA
AAAAAAAAAGTTGGAAGTTGAATGTTTTATCCATTTTTACTTTTTGATGGAAT
AAAAAAAAAAAAAAAA

Figure 2.4 Synteny of *Fot3-1* locus with *Glycine max*. Synteny was examined for the *Fot3-1* locus between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Fot3-1* locus on the cowpea consensus genetic map, linkage group 6 (17.88 cM to 19.04 cM), was determined to be syntenic with soybean chromosomes 9 and 15. The *Fot3-1* syntenic locus in soybean chromosome 9 extended from soybean locus Glyma09g02100 to Glyma09g02560, where two disease resistance genes, Glyma09g02210 and Glyma09g02420, were observed. The *Fot3-1* syntenic locus in soybean chromosome 15 extended from soybean locus Glyma15g12830 to Glyma15g13470 where four disease resistance genes were observed, Glyma15g13100, Glyma15g13290, Glyma15g13300 and Glyma15g13310. The syntenic map was drawn using HarvEST: Cowpea database (http://harvest.ucr.edu) using a cut-off e-score value of -10 and a minimum number of 13 lines drawn per linkage group.

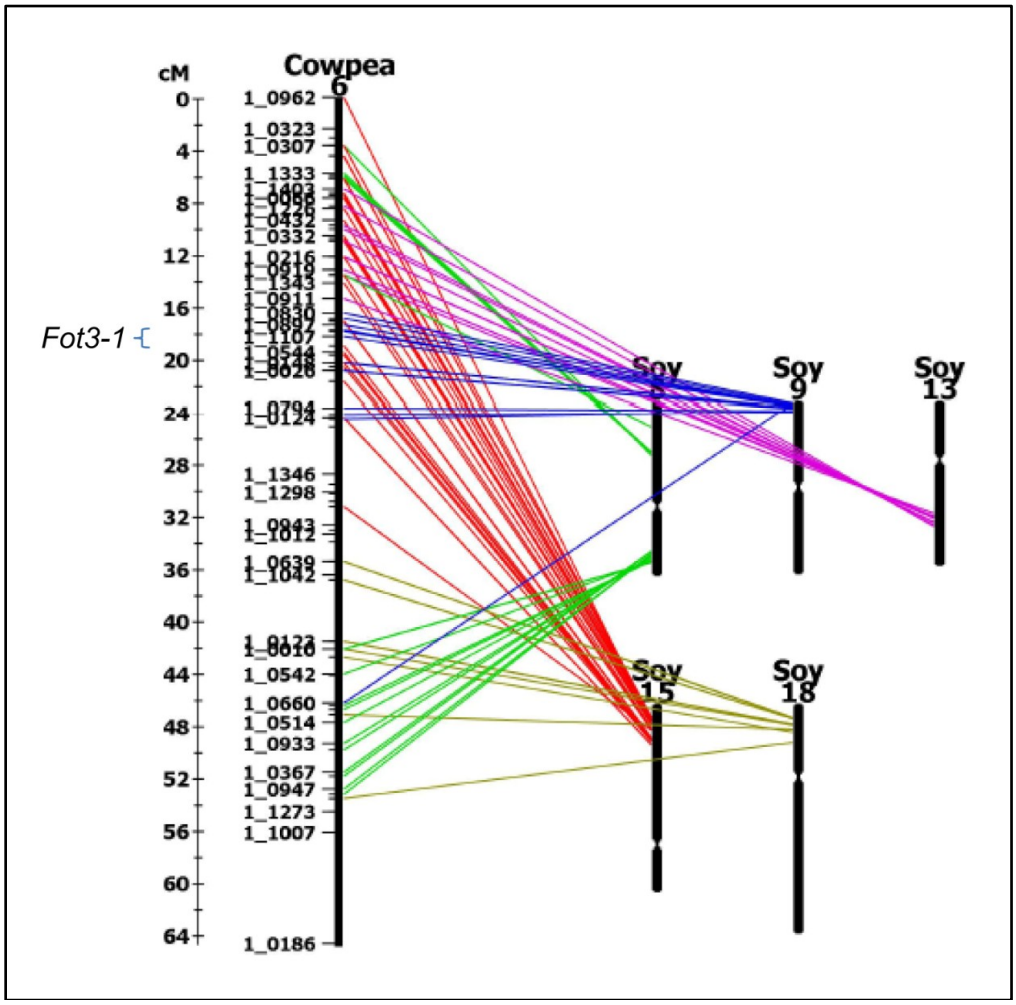


Figure 2.5 Cowpea BAC contig250 which harbors *Fot3-1*. BAC contig250 consists of 46 BAC clones. Nine BAC clones in the minimum tiling path (MTP) were previously identified as harboring SNP markers and are currently shown. The *Fot3-1* locus spans three overlapping BAC clones, CH093L18, CM001C09 and CH051M10. However, since CH093L18 and CH051M10 overlap the total length of BAC clone CM001C09, *Fot3-1* was narrowed to two overlapping BAC clones which span an approximate total length of 375,560 bp of the total contig length of 885,600 bp. The BAC clones which have been identified with SNP markers are labeled as such. The bar graph at the bottom of the figure represents number of fingerprinting bands.

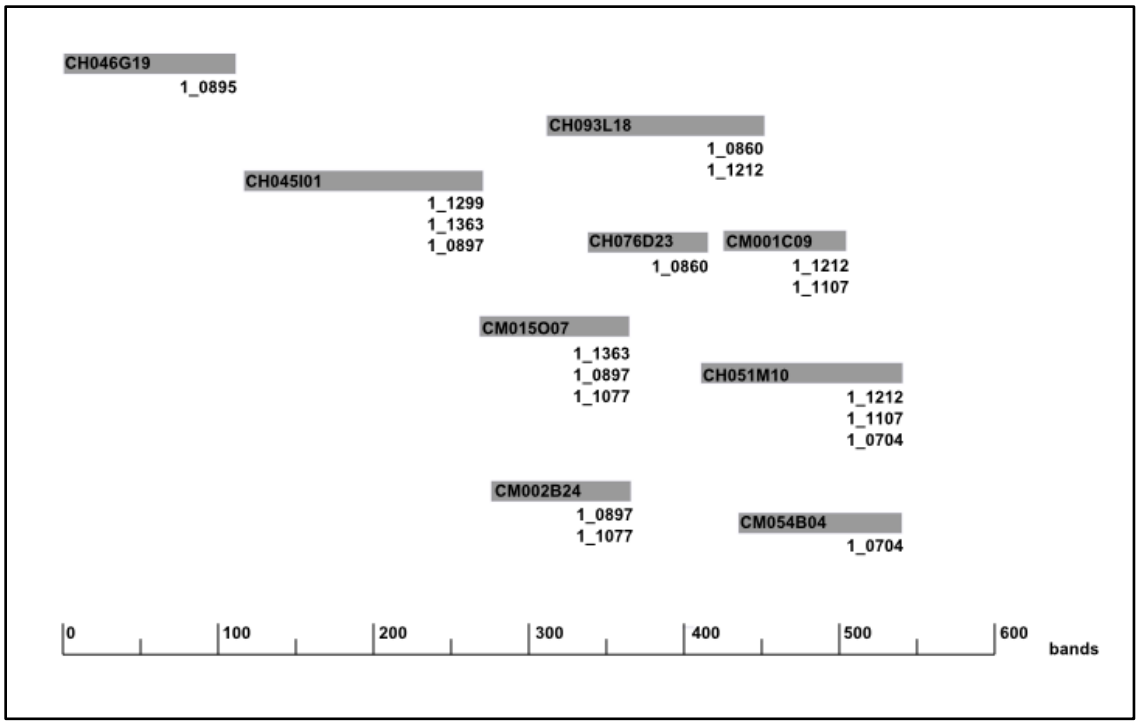
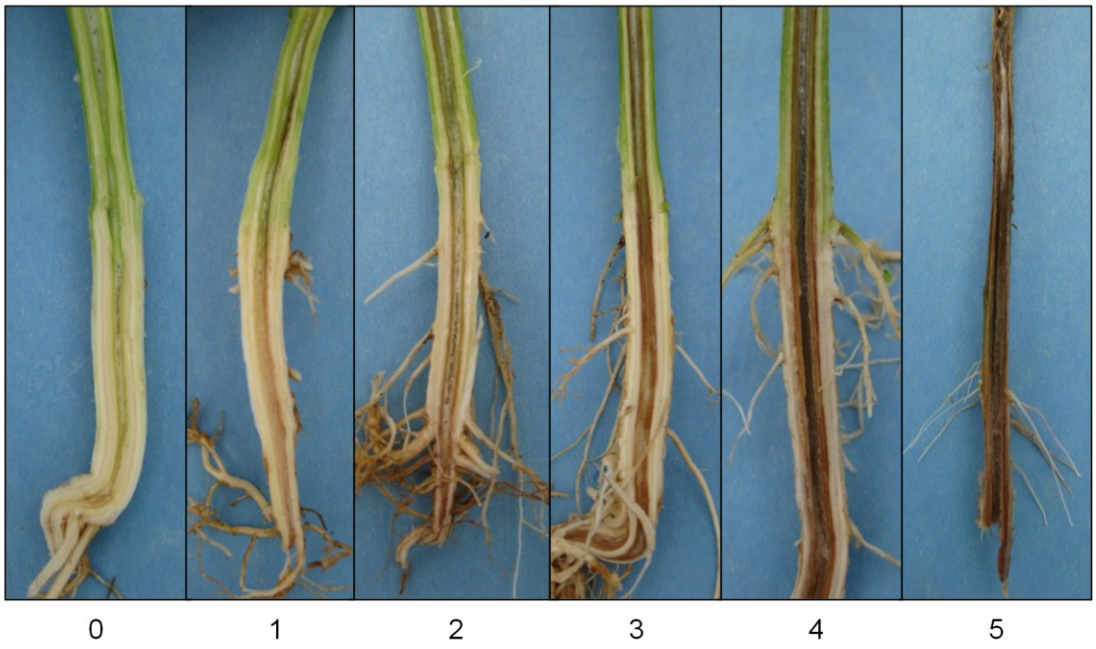


Figure 2.6 *Fusarium oxysporum* f.sp. *tracheiphilum* phenotyping for vascular discoloration symptoms. The severity of the vascular discoloration disease symptom was evaluated on a zero to five rating scale. A rating of zero indicated a healthy plant with no signs of disease, 1 indicated approximately 10% of the plant showed disease symptoms, 2 indicated approximately 25% of the plant showed disease symptoms, 3 indicated approximately 50% of the plant showed disease symptoms, 4 indicated approximately 75% of the plant showed symptoms and 5 indicated 100% of the plant showed disease symptoms. Five replicates per line were evaluated individually then averaged to determine the disease severity for each RIL.



Chapter 3

Genetic Mapping, Synteny and Physical Location of Two Loci for *Fusarium oxysporum* f.sp. *tracheiphilum* Race 4 Resistance in Cowpea [*Vigna unguiculata* (L.) Walp]

Abstract

Fusarium wilt is a vascular disease caused by the fungus *Fusarium oxysporum* f.sp. *tracheiphilum* (Fot) in cowpea. In this study, we mapped loci conferring resistance to Fot race 4 in three cowpea RIL populations: IT93K-503-1 x CB46, CB27 x 24-125B-1 and CB27 x IT82E-18/Big Buff. Two independent loci which confer resistance to Fot race 4 were identified, *Fot4-1* and *Fot4-2*. *Fot4-1* was identified in the IT93K-503-1 (resistant) x CB46 (susceptible) population and was positioned on the cowpea consensus genetic map, spanning 21.57 cM to 29.40 cM on linkage group 5. The *Fot4-2* locus was validated by identifying it in both the CB27 (resistant) x 24-125B-1 (susceptible) and CB27 (resistant) x IT82E-18(Big Buff) (susceptible) populations. *Fot4-2* was positioned on the cowpea consensus genetic map on linkage group 3; the minimum distance spanned 71.52 cM to 71.75 cM whereas the maximum distance spanned 64.44 cM to 80.23 cM. *Fot4-1* and *Fot4-2* were positioned on the cowpea consensus genetic map and it was determined that they were independent of each other and to *Fot3-1*, which was previously identified as the locus conferring resistance to Fot race 3. The *Fot4-1* and *Fot4-2* syntenic loci were examined in *Glycine max*, where several disease resistance candidate genes were identified for both loci. Additionally, *Fot4-1* and *Fot4-2* were coarsely positioned on the cowpea physical map. *Fot4-1* and *Fot4-2* will contribute to molecular marker development for future use in marker-assisted selection (MAS), thereby, expediting introgression of Fot race 4 resistance into future cowpea cultivars.

Introduction

Fusarium oxysporum f.sp. *tracheiphilum* (Fot) is a soil-borne fungal pathogen that causes vascular wilt disease in cowpea (Armstrong and Armstrong 1981). The pathogen enters the plant through the root system and invades vascular tissue, causing wilting and leaf chlorosis and often stunting the entire plant. Broad irregular patches of affected plants are visible in infested cowpea fields. The external symptoms typically become evident during the flowering and early pod development stages resulting in high mortality in the affected areas with severe overall yield loss. Worldwide, the occurrence of Fusarium infecting cowpeas has been reported in the Northern Territory of Australia, northeastern parts of Brazil and Nigeria (Summerell et al. 2011; Assunção et al. 2003; Armstrong and Armstrong 1980). Fusarium wilt of cowpea is a significant problem in the United States, especially in the southeastern states and California (Hare 1953).

In California, the prevalence of the disease stimulated breeding efforts to develop Fusarium resistance in cowpea from the 1930's onward (Patel 1985a). In conjunction with the use of resistance in commercial cowpea cultivars, several races of Fot have evolved (races 1, 2, 3, and 4) which are identified according to differential interactions on cowpea genotypes with different resistance backgrounds (Hare 1953; Patel 1985a; Smith et al. 1999a). Fot race 3 has been the most prevalent and wide-spread race within the state of California (Smith et al. 1999b) and several cultivars with resistance have been grown as a primary disease management tactic (Pottorff et al. 2012b). However, in recent years, widely grown cowpea cultivars which were resistant to Fot race 3, such as

California Blackeye 46, showed Fusarium disease symptoms in some fields, indicating that a new race had evolved which required a new focus in breeding for resistance (Davis and Frate 2007). Alternative disease management practices such as applications of fungicides are not feasible due to economic constraints as well as possible health and environmental concerns. Therefore, host resistance is an effective and preferred solution for managing the disease in cowpea and new cultivars for production in the United States must have resistance to both Fot race 3 and race 4. Several new cultivars have been bred specifically to incorporate resistance to Fot race 4, including California Blackeye 27 (Ehlers et al. 2000) and recently released California Blackeye 50 (Ehlers et al. 2009). These cultivars were developed using traditional breeding methods that involved screening and identifying appropriate resistant germplasm sources and then introgressing the resistance trait, often taking a decade or more to release a new cowpea cultivar. Precision breeding using marker-assisted selection (MAS) with trait-linked markers could reduce the length of breeding time to less than half. However, the efficiency will depend on the extent of recombination between the trait determinant and marker based on the genetic distance between them. To improve breeding efficiency, gene-based 'perfect markers' could be developed through the identity of the genetic determinants for Fot race 4 resistance, as we reported recently for resistance to Fot race 3 in cowpea (Pottorff et al. 2012b).

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

genomic resources have been integrated by using a 1536-SNP genotyping platform and include an EST-derived SNP cowpea consensus genetic map, known syntenic relationships between cowpea, *M. truncatula*, *G. max* and *A. thaliana*, and a cowpea EST sequence collection housed in HarvEST: Cowpea database (http://harvest.ucr.edu) (Muchero et al. 2009a) (Lucas et al. 2011). The cowpea physical map which has been anchored to the cowpea consensus genetic map using the same SNP genotyping platform is currently available (http://phymap.ucdavis.edu/cowpea). In addition, more than 500 diverse cowpea accessions have been SNP-genotyped and a first draft of the cowpea genome sequence 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 (AM). In this study, greenhouse inoculation experiments were used to identify QTLs conferring resistance against Fot race 4 in three cowpea RIL populations. Two loci which confer resistance to Fot race 4 were identified, *Fot4-1* and *Fot4-2*. The target outcome of this study will be to develop molecular markers closely linked to the *Fot4-1* and *Fot4-2* resistance genes for application in resistance breeding.

Results

The distribution of Fot race 4 phenotypes amongst the three cowpea populations was examined and is shown in Figure 3.1, 3.2 and 3.3. The mean disease value for the parental genotypes is labeled as such in the figures.

Fot race 4 QTL analysis in 3 cowpea populations

IT93K-503-1 x CB46: IM and rMQM mapping using three phenotyping datasets identified one major QTL conferring resistance to Fot race 4 (Figure 3.4). The length of the locus, which is designated here as *Fot4-1*, spanned from 28.86 cM to 40.67 cM on linkage group 8 and was identified by SNP markers 1_0557, 1_1492 and 1_0030 (Table 3.1, Table 3.2, Figure 3.4). SNP Marker 1_1492 was the most significant marker over all three experiments, accounting for 32.6% (LOD 6.77), 32.7% (LOD 7.48) and 32.7% (LOD 7.22) phenotypic variance for the wilting/stunting phenotype and 30.3% (LOD 6.74), 28.5% (LOD 6.33) and 46.5% (LOD 11.42) of the phenotypic variance for the vascular discoloration phenotype (Table 3.2).

The corresponding location of *Fot4-1* was positioned on the cowpea consensus genetic map using the significant markers identified in the QTL analysis. The *Fot4-1* locus spanned from 21.57 cM to 29.40 cM on the cowpea consensus genetic map linkage group 5 (Table 3.1, Figure 3.5). The length of the *Fot4-1* region on the cowpea consensus genetic map, 7.83 cM, was less than the estimated length of 11.81 cM identified on the IT93K-503-1 x CB46 individual map (Table 3.1). However, the 7.83 cM estimated

length of *Fot4-1* on the cowpea consensus map is most likely the more accurate estimate due to higher recombination utilizing the 12 constituent genetic maps (Lucas et al. 2011).

CB27 x 24-125B-1: Phenotyping datasets from two experiments were used to map Fot race 4 resistance which identified one locus, which we designated as *Fot4-2*. *Fot4-2* spanned 64.22 cM to 72.55 cM on linkage group 9 in the CB27 x 24-125B-1 population map (Table 3.3, Table 3.4 and Figure 3.6). Marker 1_0594 was the most significant in the first experiment for both disease phenotypes, accounting for 37.6% (LOD 7.69) variance for the wilt phenotype and 40.2% (LOD 8.49) variance for the vascular discoloration phenotype (Table 3.4). The second experiment identified SNP markers 1_0984, 1_0380 and 1_1162 as the most significant for both the wilting and the vascular discoloration phenotype (Table 3.4). SNP markers 1_0984, 1_0380 and 1_1162 were all in the same bin on the individual genetic map due to lack of recombination in the region (Table 3.3), thereby, each marker accounted for 32.3% (LOD 3.82) of the phenotypic variance for wilting and 35.6% (LOD 4.31) variance for the vascular discoloration phenotype (Table 3.4).

Using the highly significant markers from the QTL study, *Fot4-2* was positioned on the cowpea consensus genetic map where it spanned the region from 64.44 cM to 80.23 cM on linkage group 3 (Table 3.3, Figure 3.5). The estimated length of 15.79 cM for the *Fot4-2* locus on the cowpea consensus genetic map is probably more accurate than the estimated 8.33 cM length on the individual map; particularly since eight out of eleven markers shared the same marker bin in the *Fot4-2* locus in the CB27 x 24-125B-1

population (Table 3.3). Only 11 of the 26 markers in the *Fot4-2* locus on the cowpea consensus map were polymorphic in the CB27 x 24-125B-1 genetic map which also may account for the smaller QTL length on the individual map (Table 3.3).

CB27 x IT82E-18(Big Buff): Fot race 4 resistance was mapped using phenotyping datasets from two experiments. The QTL was identified on linkage group 1 of the individual map, spanning from 72.8 cM to 73.18 cM (total 0.38 cM) (Table 3.3, Figure 3.7). SNP marker 1_0352 was the most significant over the two experiments, accounting for 27.1% (LOD 10.66) and 19.6% (LOD 7.34) phenotypic variance for the wilting phenotype and 24% (LOD 9.45) and 18.9% (LOD 7.11) of the phenotypic variance for vascular discoloration (Table 3.5).

The QTL observed in the CB27 x IT82E-18(Big Buff) population was positioned on the cowpea consensus genetic map spanning from 71.52 cM to 71.75 cM (0.23 cM total distance) on linkage group 3 (Table 3.3, Table 3.5 and Figure 3.5). This locus overlapped with the position of *Fot4-2* identified in the CB27 x 24-125B-1 population (Table 3.3, Figure 3.5). The length of *Fot4-2,* 0.23 cM, on the cowpea consensus genetic map was similar to the length identified in the CB27 x IT82E-18(Big Buff) individual map, 0.38 cM (Table 3.3).

Subsequently, the *Fot4-2* locus was validated because it was identified in two different populations which share the same Fot race 4 resistance donor parent, CB27. Nevertheless, the *Fot4-2* locus identified in the two populations did not overlap perfectly on the cowpea consensus genetic map, because many of the markers that were significant

in the CB27 x 24-125B-1 population (1_0594, 1_1162, 1_0380 and 1_0984) were not polymorphic in the CB27 x IT82E-18/Big Buff population, and vice versa. SNP marker 1_1087 was the only marker identified as being highly significant in both populations (Table 3.3, Table 3.4 and Table 3.5). The maximum length of *Fot4-2* was defined by the QTL identified in the CB27 x 24-125B-1 population, which spanned from 64.44 cM to 80.23 cM on the cowpea consensus genetic map (Table 3.3). However, as stated previously, there was much less recombination within the *Fot4-2* locus in the CB27 x 24-125B-1 population, indicated by several of the markers having the same cM position (Table 3.3) which greatly limited the ability to narrow the QTL position. Considering that the *Fot4-2* locus identified in the CB27 x IT82E-18/Big Buff population was smaller due to rapid decrease of the significance threshold of the markers outside of the 2-LOD score (Table 3.5), the shorter length spanning from 71.52 cM to 71.75 cM (0.23 cM distance) on the cowpea consensus genetic map may be a more accurate estimation of *Fot4-2*.

The results from this study established that *Fot4-1* and *Fot4-2* are independent of each other as observed on the cowpea consensus genetic map (Figure 3.5). *Fot4-1* is positioned on linkage group 5, spanning 21.57 cM to 29.40 cM. The minimum distance of *Fot4-2* identified in CB27 x IT82E-18(Big Buff) spanned from 71.52 cM to 71.75 cM on linkage group 3, while the maximum distance determined by the resistance locus identified in the CB27 x 24-125B-1 spanned from 64.44 cM to 80.23 cM (Figure 3.5). *Fot3-1,* which was previously identified in the CB27 (resistant) x 24-125B-1 (susceptible) population spanning from 49.92 cM to 50.49 cM on linkage group one of

the individual genetic map and flanked by SNP markers 1_0860 and 1_1107 (Pottorff et al. 2012b), was positioned on vs. 4 cowpea consensus genetic map where it spanned 47.86 cM to 48.31 cM region on linkage group 6 (Figure 3.5). Therefore, we determined that both of the Fot race 4 resistance loci, *Fot4-1* and *Fot4-2,* are independent of the Fot race 3 locus, *Fot3-1* (Figure 3.5).

The *Fot4-1* and *Fot4-2* loci were examined for markers which might co-segregate an allele with an associated disease resistance phenotype using several cowpea genotypes with known reactions to Fot race 4. However, no such marker-trait associations were found for any of the markers in the *Fot4-1* or the *Fot4-2* loci. This suggests that the density of markers in the Fot race 4 resistance regions was not high enough to find a marker closely linked with resistance and neither *Fot4-1* nor *Fot4-2* could be narrowed further.

Synteny of Fot race 4 loci with *G. max*

The *Fot4-1* and *Fot4-2* loci in cowpea were compared with the soybean genome to determine if a syntenic relationship exists. A high co-linearity of the *Fot4-1* or *Fot4-2* loci with the sequenced soybean genome may enable identity of candidate disease resistance genes. The *Fot4-1* locus in cowpea was compared with the soybean genome, which was found to be highly co-linear with soybean chromosome 14 (Table 3.6, Figure 3.8). Soybean genes orthologous to cowpea SNP markers identified the syntenic locus spanning from soybean locus Glyma14g15370 to Glyma14g36620 which corresponded to the 21.57 cM to 29.40 cM region in the *Fot4-1* locus (Table 3.6). The orthologous

soybean genes were in the same order as the SNP markers in the cowpea consensus genetic map with the exception of the ortholog for SNP 1_ 1492, which was missing (Table 3.1, Table 3.6). The nearby cowpea SNP markers to the *Fot4-1* locus, 1_0557 and 1_0662, were examined on the soybean genome browser on the Phytozome webpage for known disease resistance genes (http://www.phytozome.net). Although, cowpea markers were not precisely positioned within soybean genes, three disease resistance soybean genes were observed in the syntenic *Fot4-1* locus Glyma14g17910, Glyma14g23930 and Glyma14g34880, and were considered as orthologous disease resistance candidate genes (Table 3.6). Soybean loci Glyma14g17910 and Glyma14g23930 were both annotated as Toll/interleukin1-like receptor nucleotide binding site leucine-rich repeat (TIR-NBS-LRR) genes (Table 3.6). Glyma14g34880 was annotated as a leucine-rich repeat protein kinase (Table 3.6).

The *Fot4-2* locus was examined for a possible syntenic relationship with the soybean genome, in which a co-linear relationship at the macro and micro-level was observed with soybean chromosomes 16 and 18 (Table 3.7, Figure 3.9). The syntenic region in soybean chromosome 16 spanned from soybean locus Glyma16g15790 to Glyma16g23710 corresponding to the 64.78 cM to 73.79 cM region of the *Fot4-2* locus on the cowpea consensus genetic map (Table 3.3, Table 3.7). The soybean genes that were orthologous to cowpea EST-derived SNP markers were in the same marker order as in the cowpea consensus genetic map with the exception of the soybean ortholog of SNP 1_0604 (64.78 cM) which preceded the corresponding 71.52 cM to 73.79 cM region (Table 3.3). The syntenic region spanning between orthologous soybean genes to cowpea

SNP markers 1_1087 and 1_0984 was examined on the soybean genome browser on the Phytozome webpage for known disease resistance genes (http://www.phytozome.net). Two disease resistance soybean loci were observed in the syntenic region, Glyma16g17380 which was annotated as a leucine-rich repeat protein kinase and Glyma16g22620 which was annotated as a TIR-NBS-LRR disease resistance gene (Table 3.7). Additionally, a cluster of five leucine-rich repeat protein kinases was observed flanked between soybean genes orthologous to SNP markers 1_0380 and 1_1162 which corresponded to 73.42 cM to 73.79 cM of the *Fot4-2* locus (Table 3.7). Due to the close proximity to the most significant region of *Fot4-2* (71.52 cM to 71.75 cM) all seven of the soybean genes were considered as orthologous candidate genes for the *Fot4-2* locus.

The syntenic *Fot4-2* region of soybean chromosome 18 spanned from soybean locus Glyma18g18980 to Glyma18g38670 where five out of six soybean orthologs for cowpea SNP markers corresponded to 65.16 cM to 66.99 cM of the *Fot4-2* region on cowpea linkage group 3 (Table 3.3, Table 3.7). The soybean genes orthologous to cowpea SNP markers were in the same linear order as on the cowpea genetic map, however, this syntenic locus preceded the most significant region of the *Fot4-2* locus, 64.78 cM to 73.79 cM, and no disease resistance candidate genes were observed or expected (Table 3.7).

Fot4-1 and *Fot4-2* loci on the cowpea physical map

The cowpea physical map (http://phymap.ucdavis.edu/cowpea) which has been anchored to the cowpea consensus genetic map via SNP markers was used to identify contigs which span the physical regions of *Fot4-1 and Fot4-2*. Significant markers from the *Fot4-1* locus and closely linked markers from the cowpea consensus genetic map vs.4 identified two cowpea BAC contigs, contig77 and contig417, which incompletely span the *Fot4-1* region (Table 3.1). The only significant SNP marker, 1_0030, identified in the *Fot4-1* locus was identified in contig417 within BAC clones CH027H24 and CH035P21 on the cowpea physical map (Table 3.1). SNP 1_0662, which is linked with marker 1_0030 on the cowpea consensus genetic map was identified in BAC contig 77 within BAC clone CH095K15 (Table 3.1). The other SNP markers within the *Fot4-1* locus, 1_0557 and 1_1492, were not observed in the cowpea physical map and are probably not present in the African breeding line IT97K-499-35 which was used to create the cowpea physical map.

SNP markers from the *Fot4-2* locus on the cowpea consensus genetic map identified seven contigs and nine BAC clones which partially span the locus on the cowpea physical map. The significant markers for the *Fot4-2* region resulting from the QTL analysis identified four contigs and five BAC clones in CB27 x 24-125B-1 and three contigs and four BACs in CB27 x IT82E-18(Big Buff) (Table 3.3). The most significant marker identified in the CB27 x IT82E-18(Big Buff) population, 1_0352, was identified in contig1094, BAC clone CM052M22 (Table 3.3). Since the *Fot4-1* and *Fot4-2* loci could

not be narrowed further and the physical map spanning both regions was incomplete, the physical to genetic map distance was not analyzed.

Conclusion

This study has identified two independent loci, *Fot4-1* and *Fot4-2*, which confer resistance against *F. oxysporum* f.sp. *tracheiphilum* race 4 in cowpea. These two resistance loci were inherited from two different cowpea genotypes which differ in origin; Fot4-1 is derived from African breeding line, IT93K-503-1 and *Fot4-2* is derived from a U.S. blackeye dry grain cultivar, CB27. In addition, *Fot4-1*, *Fot4-2* and the previously identified *Fot3-1* were positioned on the cowpea consensus genetic map, confirming that these loci which confer race-specific resistance are independent of each other. The *Fot4-2* QTL was validated since it was identified in two independent populations, whose resistance locus was derived from the same CB27 resistant parent. The physical locations of *Fot4-1* and *Fot4-2* were roughly identified on the cowpea physical map which will enable generating tightly linked markers with segregate with Fot race 4 resistance. Identification of the two independent Fot race 4 loci will enable gene pyramiding which may promote the durability of Fot race 4 resistance in future cowpea cultivars.

The candidate gene discovery utilizing synteny between cowpea and soybean identified TIR-NBS-LRR proteins and leucine-rich repeat serine/threonine protein kinases in the soybean syntenic regions of the *Fot4-1* and *Fot4-2* loci. Previous reports of resistance to Fusarium have indicated that the resistance is a monogenic trait with dominant expression (Zink and Thomas 1990; Rubio et al. 2003; McGrath et al. 1987; Scott and Jones 1989; Sarfatti et al. 1991). This profile conforms to the classic gene-for-gene model of Flor

(1971) in which the pathogen and host express complementary dominant genes, avirulence and resistance genes, with the alteration or loss of either resulting in a compatible interaction and disease. Most disease resistance genes fitting this profile have an NBS-LRR motif which depending on the N- terminus design, have homology with the TIR domain (TIR-NBS-LRR) (Meyers et al. 1999; Pan et al. 2000) or a coiled-coil motif (CC-NBS-LRR or non TIR-NBS-LRR) (Pan et al. 2000). Of the two cloned genes which confer resistance to Fusarium wilt, both the *I-2* locus for resistance to *F. oxysporum* f.sp. *lycopersici* (Fol) race 2 in tomato (Simons et al. 1998) and the *Fom-2* locus for resistance to *F. oxysporum* f.sp *melonis* (Fom) in melon (Joobeur et al. 2004) were found to be CC-NBS-LRR genes.

Beyond the conserved NBS-LRR structure, other disease resistance genes belonging to the receptor-like kinase (RLK) family, whose proteins span the plasma membrane, respond to extracellular signals (Geer et al. 1994). The majorities of RLKs have serine/threonine kinases and LRR motifs (Becraft 1998) and include genes *PBS1*, *Pti*, *Pto* and *Xa21* which confer resistance to bacterial pathogens in Arabidopsis, tomato and rice (Shiu and Bleecker 2001; Song et al. 1995) and *Lrk10* which confers resistance to the fungus, *Puccinia recondite* in wheat (Feuillet et al. 1997). Furthermore, the *I-3* locus which confers resistance to *F. oxysporum* f.sp. *lycopersici* race 3 in tomato was determined to be positioned within a large cluster of S-locus receptor-like kinases (SRLK)(Hemming et al. 2004) and recently we sequenced a BAC clone in the *Fot3-1* locus, which had four cowpea sequences with homology to leucine-rich repeat serine/threonine kinase receptors (LRR-STKR) (Pottorff et al. 2012). Based on these

reports and our current findings, it is a good possibility that LRR-STKRs are the resistance genes responsible in the cowpea-Fusarium pathovar system.

Currently, the sequencing of BAC clones within the MTP of each BAC contig of the cowpea physical map is underway. This combined with identification of markers more closely linked with the *Fot4-1* and *Fot4-2* loci will enable the direct identification of cowpea disease resistance candidate genes. A more immediate practical outcome of this study is the development of molecular markers closely linked to the *Fot4-1*and *Fot4-2* loci. These markers can be used for indirect selection of resistance in breeding schemes such as pedigree backcrossing and marker-assisted recurrent selection.

Materials and methods

Plant materials

Three cowpea RIL populations which segregate for Fot race 4 resistance were used for QTL mapping studies. The IT93K-503-1 (resistant) x CB46 (susceptible) population consisted of 113 lines advanced to the F_{10} generation using single seed decent. IT93K-503-1 is an advanced breeding line developed by the International Institute for Tropical Agriculture (IITA) with strong resistance to Fot race 4. CB46 was bred for resistance to Fot race 3 but is highly susceptible to Fot race 4 (Davis and Frate 2007).

The CB27 (resistant) x 24-125B-1 (susceptible) population consisted of 90 lines that were advanced to the F_9 generation using single seed descent. CB27 was bred for resistance to several pathogens including root-knot nematodes and Fot race 4 and also for heat tolerance (Ehlers et al. 2000). 24-125B-1 is an advanced breeding line from the Institute of Agricultural Research for Development (IRAD) and is susceptible to Fot race 4 (Kitch et al. 2001).

The CB27 (resistant) x IT82E-18 (Big Buff) (susceptible) population consisted of 162 RILs and was advanced to the F_8 generation by single seed decent. IT82E-18 is an advanced breeding line developed at IITA which was released as cultivar 'Big Buff' in Australia (Imrie 1995). IT82E-18 is highly susceptible to Fot race 4. All cowpea RIL populations were obtained from the University of California Riverside cowpea germplasm collection.

Inoculum preparation

Two strains of Fot race 4, which originated from infected cowpea plants in Bakersfield, California, were used for inoculation cultures. Individual isolates were developed from single spore lines. Isolates were dried and stored on sterile potato dextrose agar (PDA) plates at -80°C. 1-cm^2 plugs were cut from frozen Fusarium-containing PDA plates and transferred aseptically to flasks containing 500ml of potato-dextrose broth, then incubated in a shaker at 27°C, 30 rpm under lighted conditions for three days. The liquid culture was strained through four layers of cheesecloth to eliminate mycelia and the spore concentration was adjusted to 1.0 x 10^6 microconidia per ml using a hemocytometer.

Plants were inoculated using a modified root-dip inoculation method described by Rigert and Foster (1987). Modification to the root-dip method was as follows: 10 greenhouse grown seeds per RIL were planted in seeding trays filled with vermiculite and watered daily for one week. After one week, five replicate seedlings per RIL were gently uprooted, the distal half of the root system was clipped and the remaining root system dipped for one minute in suspended inoculum with a concentration of 1x10^6 spores/ml. Inoculated seedlings were transplanted into 3.8 L pots, watered daily with greenhouse day temperatures set to 28 °C and night temperatures to 16°C.

Phenotyping

Plants were evaluated 35 days post-inoculation for Fusarium disease symptoms. The wilting/stunting phenotype was evaluated by approximating the percentage of wilting or stunting to the entire plant similar to the disease severity index (DSI) utilized by the

Centro International de Agricultura Tropical (CIAT)(Pastor-Corrales and Abawi 1987; Fall et al. 2001). The reddish-brown vascular discoloration which is the necrosis caused by the fungus as it moves both vertically and horizontally throughout the vascular system was evaluated by uprooting the entire plant, slicing the stem vertically to evaluate the extent of the disease symptoms internally (Figure 3.10). The severity of the Fusarium symptoms was evaluated on a zero to five rating scale for the wilting/stunting and vascular discoloration phenotypes. A score of zero indicated a healthy plant with no signs of disease, 1 = approximately 10% of the plant showing symptoms of disease, 2 = approximately 25% of the plant showing symptoms of disease, 3 = approximately 50% of the plant showing symptoms, 4 = approximately 75% of the plant showing symptoms and 5 = 100% of the plant showing disease symptoms. Five replicates per RIL were evaluated individually then averaged to determine the disease severity for each RIL.

Genetic maps

All populations and parental lines were genotyped at the F_8 generation with bi-allelic SNP markers using the 1536 Illumina GoldenGate Assay previously described in Muchero, et al. (2009).

A SNP-based genetic map for the IT93K503-1 x CB46 population was developed previously and was included in the cowpea consensus genetic map (Lucas et al. 2011). The IT93K503-1 x CB46 genetic map consisted of eleven linkage groups and approximately 734 cM length and was generated using 113 RILs and 423 SNP markers (Lucas et al. 2011).

The SNP-based genetic map for the CB27 x 24-125B-1 population was also developed previously and included in the cowpea consensus genetic map (Lucas et al. 2011). The CB27 x 24-125B-1 genetic map was generated using 339 SNP markers and 90 RILs and consisted of sixteen linkage groups and approximately 600 cM in length (Lucas et al. 2011).

The CB27(resistant) x IT82E-18(Big Buff) genetic map was generated using 162 RILs and 419 polymorphic SNP markers and consisted of 14 linkage groups and was approximately 728 cM in length (Lucas et al. 2011).

The Lucas et al. (2011) cowpea consensus genetic map vs. 4 is the most recent cowpea consensus genetic map, succeeding the vs. 2 (Muchero et al. 2009a) and vs. 3 (Diop et al. 2012) maps. The vs. 4 cowpea consensus genetic map increased the marker density and improved the marker order using ten RIL populations and two F_4 breeding populations (Lucas et al. 2011). The map is 680 cM in length and contains 1107 markers with an average of 0.65 cM between markers (Lucas et al. 2011). The current SNP-based cowpea linkage map is included in the publicly available database HarvEST: Cowpea (http://harvest.ucr.edu) (www.harvest-web.org).

Statistical analysis

MapQTL 5.0 software was used to conduct the QTL analyses (Van ooijen 2004). QTLs were first analyzed using the Interval Mapping (IM) package to approximate putative QTLs; the closest marker to the putative QTL was used as a cofactor as a genetic background control for the MQM package of MapQTL5.0 (Van Ooijen 2004). The

restricted MQM (rMQM) package was then used to determine the percentage of variance (R^2) explained by the QTL. A one-way analysis of variance (ANOVA) using the Kruskal-Wallis (KW) package from MapQTL5.0 was used to confirm QTL loci (Van Ooijen 2004). Logarithm of the odds (LOD) thresholds were calculated using 1000 permutations, resulting in a 95% LOD threshold of approximately 2.1. 1-LOD and 2-LOD of the maximum peak were used to determine the left and right margins and the entire span of the QTL (Van ooijen 2004). QTLs were visualized using MapChart 2.2 (Voorips 2002).

Synteny

Synteny was examined using EST-derived SNP markers from the vs. 4 cowpea consensus genetic map which were and aligned to the soybean genome and functionally annotated using the most significant similarity using BLAST (Lucas et al. 2011). The cowpea consensus genetic map and syntenic relationships with model species can be viewed in the HarvEST: Cowpea database (http://harvest.ucr.edu) (www.harvest-web.org). Syntenic maps were drawn using HarvEST: Cowpea using a cut-off e-score value of -10. A minimum of 5 lines per linkage group was chosen to enable better viewing of syntenic relationships within the trait loci. Due to a limitation in the resolution, not all markers are presented in the screenshot images output from Harvest: Cowpea. In order to view each individual marker, the linkage group must be magnified in the HarvEST: Cowpea database (http://harvest.ucr.edu) (www.harvest-web.org).

Cowpea physical map

The cowpea physical map was developed using an advanced African breeding line IT93K-399-35 (http://phymap.ucdavis.edu/cowpea). Two BAC clone libraries were developed using restriction enzymes *Hind*III and *Mbo*I (Amplicon Express, Pullman, WA). Contigs were assembled using the snapshot method of DNA fingerprinting by Ming Cheng Luo at the University of California, Davis (Luo et al. 2003b). The final physical map is an assembly of 43,717 BACs with an 11x genome depth of coverage (http://phymap.ucdavis.edu/cowpea).

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Author contributions

MP and GL performed the Fusarium experiments. MP performed the QTL analyses, synteny analyses, comparison of the cowpea consensus genetic map and cowpea physical map and drafted the manuscript. MP, JDE, PAR and TJC participated in the design, interpretation of data and writing of the manuscript.

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Table 3.1 *Fot4-1* in the IT93K-503-1 x CB46 population, the cowpea consensus genetic map and the cowpea physical map.									
IT93K-503-1 x CB46 genetic map					Cowpea consensus genetic map			Cowpea physical map	
LG	cM	SNP	LOD	R^2	LG	cM	SNP	contig	BAC clone(s)
8	28.86	1_0557	6.42	28.8	5	21.57	1_0557	N/A	
8	35.21	1_1492	7.48	32.7	5	25.13	1_1492	N/A	
		N/A			5	25.70	1_0662	77	CH095K15
		N/A			5	25.70	1_0986	N/A	
8	40.67	1_0030	5.98	27.1	5	29.40	1_0030	417	CH027H24, CH035P21

Table 3.2 QTL analysis of *Fot4-1* in the IT93K-503-1 x CB46 population.					
Experiment	Phenotype	QTL analysis	1_0557	1_1492	1_0030
2007	Wilt/stunting	MQM LOD	5.28	6.77	5.29
	Wilt/stunting	R^2	26.5	32.6	26.5
	Wilt/stunting	KW	19.558	24.413	19.849
	Wilt/stunting	p-value	0.0001	0.0001	0.0001
	Vascular discoloration	MQM LOD	4.96	6.74	5.06
	Vascular discoloration	R^2	23.3	30.3	23.7
	Vascular discoloration	KW	18.881	25.013	19.554
	Vascular discoloration	p-value	0.0001	0.0001	0.0001
2010a	Wilt/stunting	MQM LOD	6.42	7.48	5.98
	Wilt/stunting	R^2	28.8	32.7	27.1
	Wilt/stunting	KW	24.599	30.152	26.053
	Wilt/stunting	p-value	0.0001	0.0001	0.0001
	Vascular discoloration	MQM LOD	5.71	6.33	4.36
	Vascular discoloration	R^2	26.1	28.5	20.6
	Vascular discoloration	KW	34.299	40.254	29.653
	Vascular discoloration	p-value	0.0001	0.0001	0.0001
2010b	Wilt/stunting	MQM LOD	5.91	7.22	5.01
	Wilt/stunting	R^2	27.7	32.7	24
	Wilt/stunting	KW	12.861	13.733	24.068
	Wilt/stunting	p-value	0.0005	0.0005	0.0001
	Vascular discoloration	MQM LOD	8.1	11.42	7.65
	Vascular discoloration	R^2	35.9	46.5	34.3
	Vascular discoloration	KW	12.61	13.074	30.602
	Vascular discoloration	p-value	0.0005	0.0005	0.0001

Table 3.3 *Fot4-2* locus in the CB27 x 24-125B-1 and CB27 x IT82E-18/Big Buff populations, the cowpea consensus genetic map and the cowpea physical map.

CB27 x 24-125B-1 genetic map					CB27 x IT82E-18/Big Buff genetic map					Cowpea consensus genetic map			Cowpea physical map	
LG	cM	SNP	LOD	R^2	LG	cM	SNP	LOD	R^2	LG	cM	SNP	contig	BAC(s)
9	64.48	1_0953	8.18	39.1	1	85.13	1_0953	2.6	7.4	3	64.44	1_0953	398	CH042B12
9	64.48	1_0604	8.18	39.1			N/A			3	64.78	1_0604	N/A	
		N/A					N/A			3	65.16	1_0444	N/A	
		N/A					N/A			3	65.16	1_1027	N/A	
9	64.22	1_0400	8.18	39.1			N/A			3	65.51	1_0400	N/A	
9	64.48	1_0139	8.18	39.1	1	77.54	1_0139	7.7	20.5	3	66.99	1_0139	736	CH080L05, CM027B20
		N/A					N/A			3	66.99	1_0207	N/A	
9	64.48	1_1369	8.18	39.1	1	77.54	1_1369	7.7	20.5	3	66.99	1_1369	N/A	
		N/A			1	77.23	1_0938	7.56	20.1	3	67.15	1_0938	N/A	
9	64.48	1_0831	8.18	39.1	1	76.95	1_0831	8.16	21.5	3	67.55	1_0831	N/A	
		N/A			1	76.95	1_1075	8.16	21.5	3	68.17	1_1075	1081	CM008G11
		N/A					N/A			3	68.49	1_1109	1004	CM005N24, CM029M15
		N/A					N/A			3	70.89	1_1085	N/A	
9	64.48	1_1087	8.18	39.1	1	73.18	1_1087	9.92	25.5	3	71.52	1_1087	N/A	
		N/A			1	72.80	1_0352	10.66	27.1	3	71.75	1_0352	1094	CM052M22
9	65.07	1_0380	8.18	39.1			N/A			3	73.42	1_0380	1045	CM045O05
9	65.07	1_0984	8.18	39.1			N/A			3	73.42	1_0984	1045	CM045O05
9	66.27	1_1162	8.18	39.1			N/A			3	73.79	1_1162	756	CH093M08
		N/A			1	57.68	1_0345	4.33	12.1	3	77.55	1_0345	N/A	
		N/A			1	57.68	1_0964	4.33	12.1	3	77.55	1_0964	N/A	
		N/A			1	57.34	1_0718	4.8	13.3	3	77.55	1_0718	N/A	
		N/A			1	57.34	1_1452	4.8	13.3	3	77.55	1_1452	N/A	
		N/A			1	55.65	1_1015	3.33	9.4	3	78.71	1_1015	N/A	
		N/A					N/A			3	79.20	1_1513	N/A	
		N/A					N/A			3	79.86	1_1134	N/A	
9	72.55	1_0594	8.49	40.2			N/A			3	80.23	1_0594	N/A	

Table 3.4 QTL analysis of *Fot4-2* in the CB27 x 24-125B-1 population.

Experiment	Phenotype	QTL analysis	1_0400	1_0604	1_0139	1_0953	1_0831	1_1087	1_1369	1_0984	1_0380	1_1162	1_0594
2007	Wilt/stunting	MQM LOD	7.61	7.65	7.65	7.65	7.65	7.65	7.65	7.65	7.65	7.65	7.69
	Wilt/stunting	R^2	37.3	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.6
	Wilt/stunting	KW	22.58	26.13	26.13	26.13	26.13	26.13	26.13	26.13	26.13	26.13	26.40
	Wilt/stunting	p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
	Vascular discoloration	MQM LOD	8.31	8.18	8.18	8.18	8.18	8.18	8.18	8.18	8.18	8.18	8.49
	Vascular discoloration	R^2	39.5	39.1	39.1	39.1	39.1	39.1	39.1	39.1	39.1	39.1	40.2
	Vascular discoloration	KW	23.75	27.99	27.99	27.99	27.99	27.999	27.99	27.99	27.99	27.99	28.51
	Vascular discoloration	p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
2009	Wilt/stunting	MQM LOD	3.56	3.56	3.56	3.56	3.56	3.56	3.56	3.82	3.82	3.82	3.31
	Wilt/stunting	R^2	30.5	30.5	30.5	30.5	30.5	30.5	30.5	32.3	32.3	32.3	28.7
	Wilt/stunting	KW	14.10	14.32	14.32	14.32	14.32	14.32	14.32	14.90	14.90	14.90	14.03
	Wilt/stunting	p-value	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
	Vascular discoloration	MQM LOD	3.73	3.73	3.73	3.73	3.73	3.73	3.73	4.31	4.31	4.31	3.44
	Vascular discoloration	R^2	31.7	31.7	31.7	31.7	31.7	31.7	31.7	35.6	35.6	35.6	29.7
	Vascular discoloration	KW	13.40	13.31	13.31	13.31	13.31	13.31	13.31	15.25	15.25	15.25	13.09
	Vascular discoloration	p-value	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0001	0.0001	0.0001	0.0005

Table 3.5 QTL analysis of *Fot4-2* in the CB27 x IT82E-18/Big Buff population.				
Experiment	Phenotype	QTL analysis	1_0352	1_1087
2009a	Wilt/stunting	MQM LOD	10.66	9.92
	Wilt/stunting	R^2	27.1	25.5
	Wilt/stunting	KW	39.42	36.57
	Wilt/stunting	p-value	0.0001	0.0001
	Vascular discoloration	MQM LOD	9.45	8.96
	Vascular discoloration	R^2	24.5	23.4
	Vascular discoloration	KW	32.81	30.74
	Vascular discoloration	p-value	0.0001	0.0001
2009b	Wilt/stunting	MQM LOD	7.39	6.82
	Wilt/stunting	R^2	19.6	18.2
	Wilt/stunting	KW	30.31	27.84
	Wilt/stunting	p-value	0.0001	0.0001
	Vascular discoloration	MQM LOD	7.11	6.72
	Vascular discoloration	R^2	18.9	18
	Vascular discoloration	KW	26.00	24.16
	Vascular discoloration	p-value	0.0001	0.0001

Table 3.6 Synteny of *Fot4-1* with *G. max* chromosome 14.					
G. max locus	*G. max* location	Phytozome annotation	Cowpea locus	LG	cM
Glyma14g15370	Gm14: 16294823 - 16294996	Ribosomal protein	1_0557	5	21.57
Glyma14g17910	Gm14: 19987489 - 19988368	TIR-NBS-LRR disease resistance protein	N/A	N/A	N/A
Glyma14g23930	Gm14: 28439271 - 28446522	TIR-NBS-LRR disease resistance protein	N/A	N/A	N/A
Glyma14g34880	Gm14: 43590997 - 43594201	Leucine-rich repeat serine/threonine protein kinase	N/A	N/A	N/A
Glyma14g35330	Gm14: 44224418 - 44225596	Phosphate-responsive protein	1_0662	5	25.70
Glyma14g35340	Gm14: 44234374 - 44235568	Phosphate-responsive protein	1_0986	5	25.70
Glyma14g36620	Gm14: 45983440 - 45985244	60S ribosomal protein	1_0030	5	29.40

Table 3.7 Synteny of *Fot4-2* with *G. max* chromosomes 16 and 18.						
G. max chromosome	*G. max* locus	*G. max* location	Phytozome annotation	Cowpea locus	LG	cM
16	Glyma16g15790	Gm16: 16709092 - 16712991	Unknown function	1_1087	3	71.52
16	Glyma16g17190	Gm16: 18531838 - 18537592	Pectinacetylesterase	1_0604	3	64.78
16	Glyma16g17380	Gm16: 18846672 - 18849661	Leucine-rich repeat serine/threonine protein kinase	N/A		
16	Glyma16g17680	Gm16: 19294324 - 19298758	NmrA-like family	1_0352	3	71.75
16	Glyma16g22620	Gm16: 26094883 - 26102980	TIR-NBS-LRR disease resistance protein	N/A		
16	Glyma16g23120	Gm16: 26788171 - 26791817	Aspartyl protease	1_0984	3	73.42
16	Glyma16g23230	Gm16: 26958715 - 26960456	Skp1 family protein	1_0380	3	73.42
16	Glyma16g23430	Gm16: 27190882 - 27193074	Leucine-rich repeat serine/threonine protein kinase	N/A		
16	Glyma16g23450	Gm16: 27214661 - 27216604	Leucine-rich repeat serine/threonine protein kinase	N/A		
16	Glyma16g23500	Gm16: 27258637 - 27261832	Leucine-rich repeat serine/threonine protein kinase	N/A		
16	Glyma16g23530	Gm16: 27327094 - 27329549	Leucine-rich repeat serine/threonine protein kinase	N/A		
16	Glyma16g23560	Gm16: 27364956 - 27367998	Leucine-rich repeat serine/threonine protein kinase	N/A		
16	Glyma16g23710	Gm16: 27560220 - 27563581	Oxidoreductase	1_1162	3	73.79
18	Glyma18g18980	Gm18: 20554229 - 20556614	BURP domain	1_0400	3	65.51
18	Glyma18g19050	Gm18: 20735387 - 20738374	Alcohol dehydrogenase	1_0444	3	65.16
18	Glyma18g24740	Gm18: 28509583 - 28511103	No functional annotation	1_1369	3	66.99
18	Glyma18g27710	Gm18: 31737329 - 31742252	Serine hydroxymethyltransferase	1_0139	3	66.99
18	Glyma18g38670	Gm18: 46319160 - 46324669	Alcohol dehydrogenase	1_0207	3	66.99

Figure 3.1 Frequency distribution of the Fot race 4 phenotype in the IT93K-503-1xCB46 population. The mean resistance values for the parents are indicated by the arrows. Figures 1a and 1b belong to experiment 1; Figures 2a and 2b to experiment 2 and Figures 3a and 3b to the third experiment.

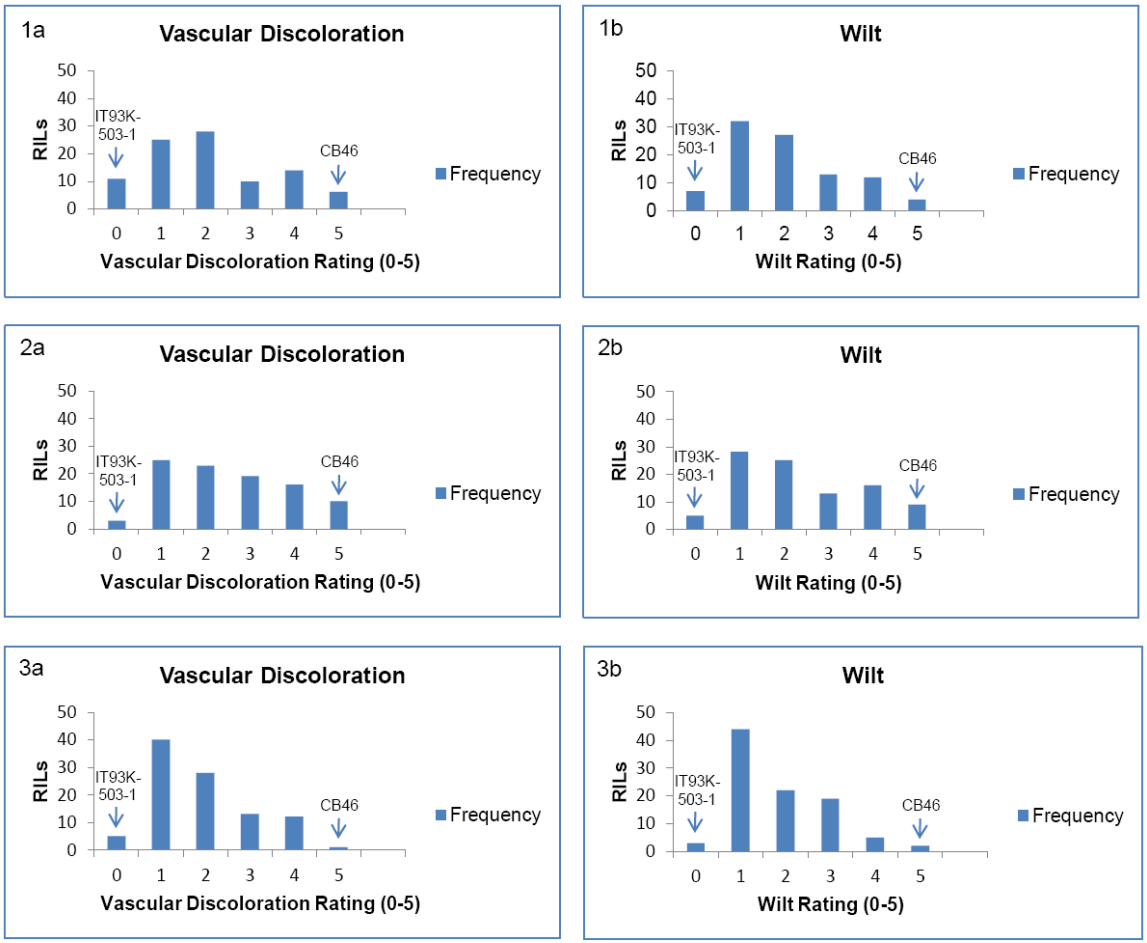


Figure 3.2 Frequency distribution of the Fot race 4 phenotypes in the CB27x24-125B-1 population. The mean resistance values for the parents are indicated by the arrows. Figures 1a and 1b belong to the first experiment and Figures 2a and 2b belong to the second experiment.

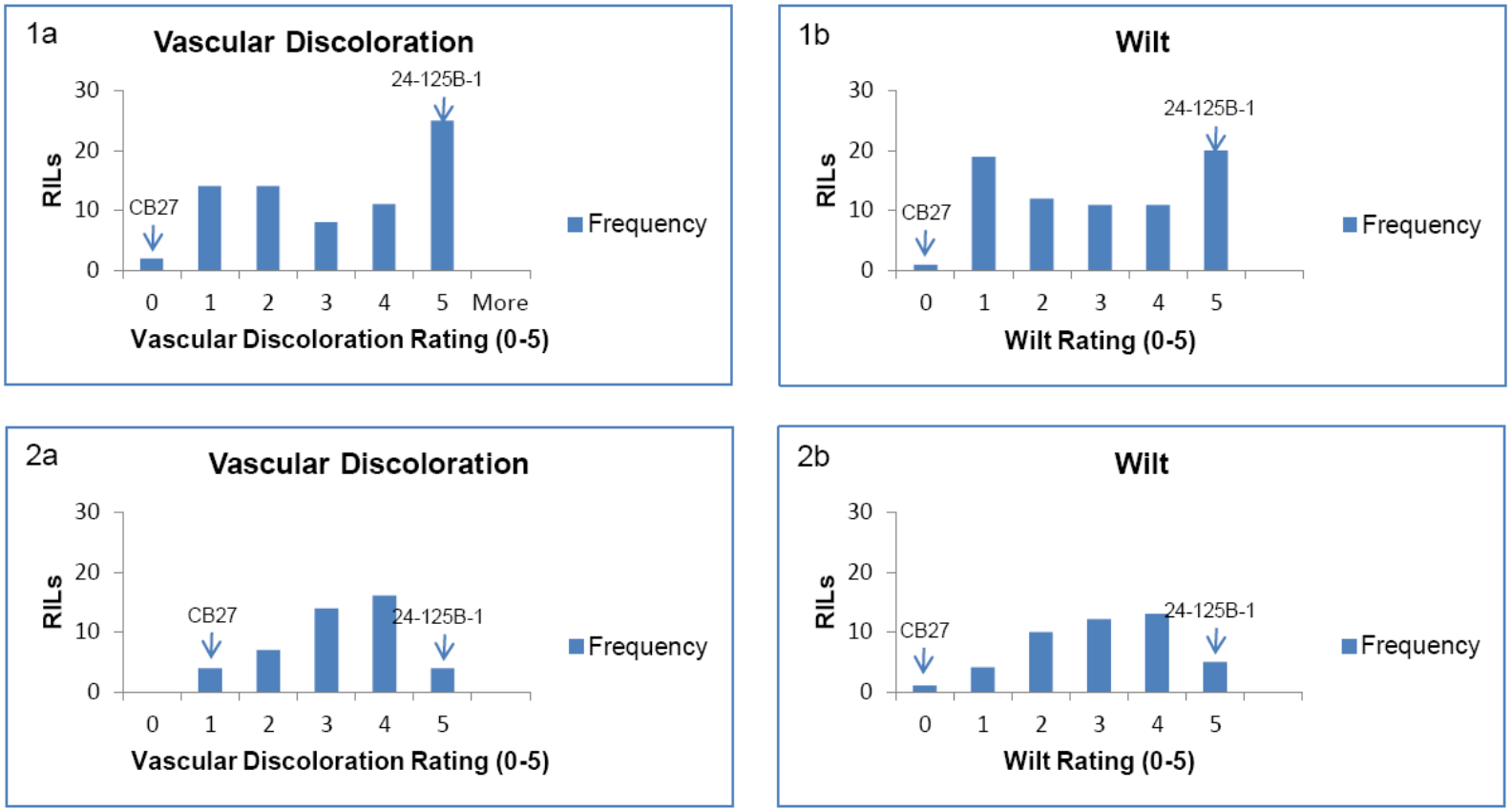


Figure 3.3 Frequency distribution of the Fot race 4 phenotypes in the CB27xIT82E-18 population. The mean resistance values for the parents are indicated by the arrows. Figures 1a and 1b belong to the first experiment and Figures 2a and 2b belong to the second experiment.

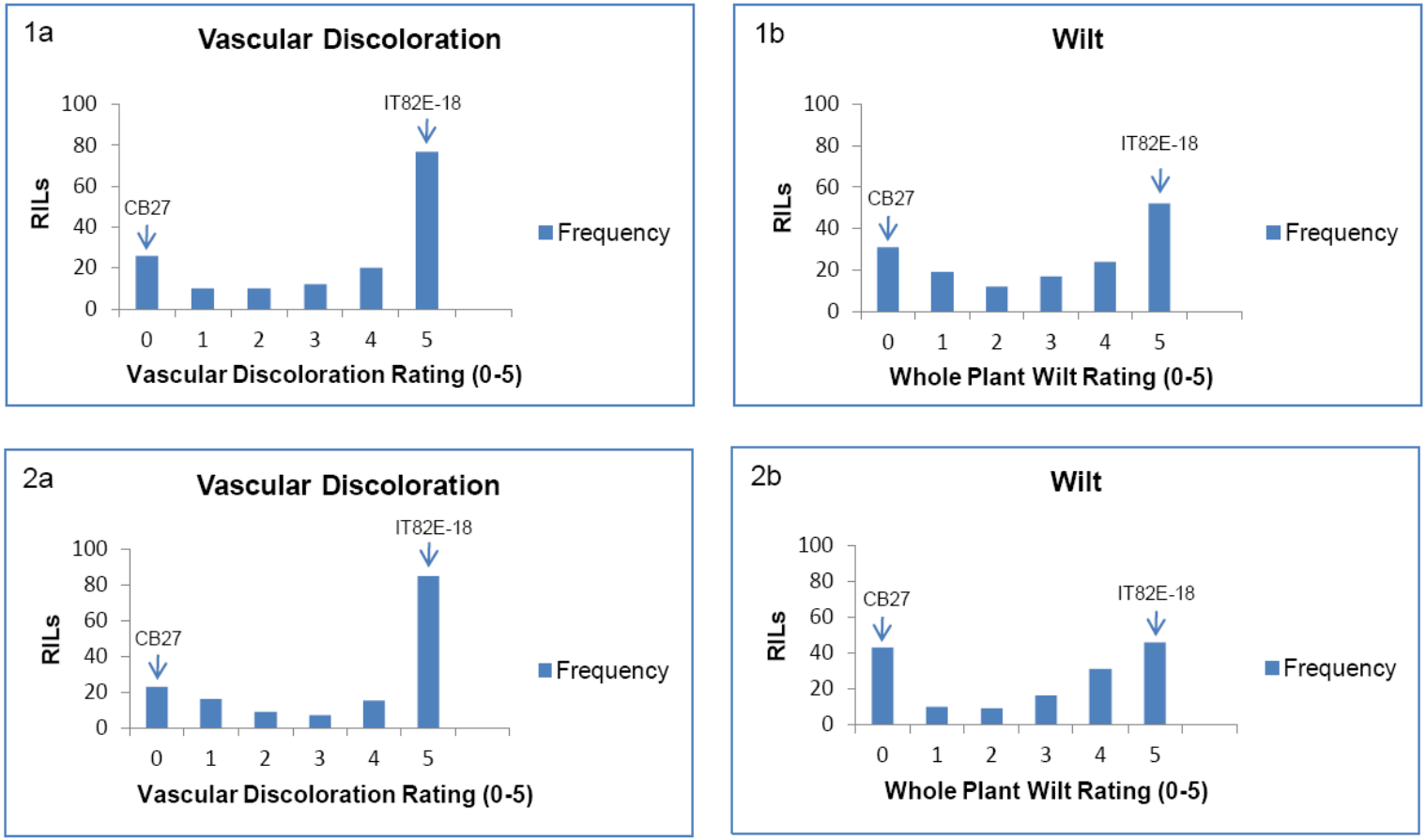


Figure 3.4 Resistance to *Fusarium oxysporum* f.sp. *tracheiphilum* race 4: *Fot4-1* QTL in the IT93K-503-1 x CB46 population. The *Fot4-1* QTL mapped to linkage group 8. LOD scores for the first (2007), second (2010a) and third (2010b) experiments are plotted in red, green and blue, respectively. Solid colored lines indicate the wilting/stunting phenotype and broken colored lines indicate the vascular discoloration phenotype. SNP marker 1_1492 which is highlighted in red showed the most significant association with Fot race 4 over the three experiments. The LOD significance threshold of 2.0 is indicated by a horizontal broken line.

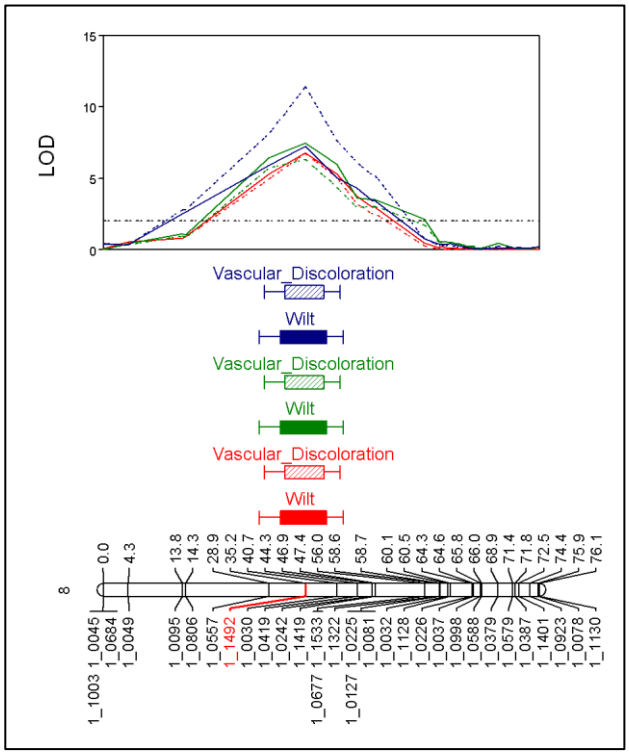


Figure 3.5 *Fusarium oxysporum* f.sp. *tracheiphilum* race 3 and race 4 resistance (*Fot3-1, Fot4-1 and Fot4-2)* on the cowpea consensus genetic map. QTLs which confer resistance to Fot race 3 and race 4 were positioned on the cowpea consensus genetic map vs. 4. *Fot3-1*, which confers resistance to Fot race 3in the CB27 x 24-125B-1 population was positioned on linkage group 6, spanning from 47.86 cM to 48.31 cM. *Fot4-1*, which confers resistance to Fot race 4 in the IT93K-503-1 x CB46 population, spanned from 21.57 cM to 29.40 cM on linkage group 5. The *Fot4-2* locus, which confers resistance to Fot race 4 in the CB27 x 24-125B-1 and CB27 x IT82E-18/Big Buff populations, was positioned on linkage group 3. Using the locus identified in the CB27 x IT82E-18/Big Buff population, the minimum distance of *Fot4-2* spanned from 71.52 cM to 71.75 cM. The maximum distance of *Fot4-2* identified in the CB27 x 24-125B-1 population spanned from 64.44 cM to 80.23 cM.

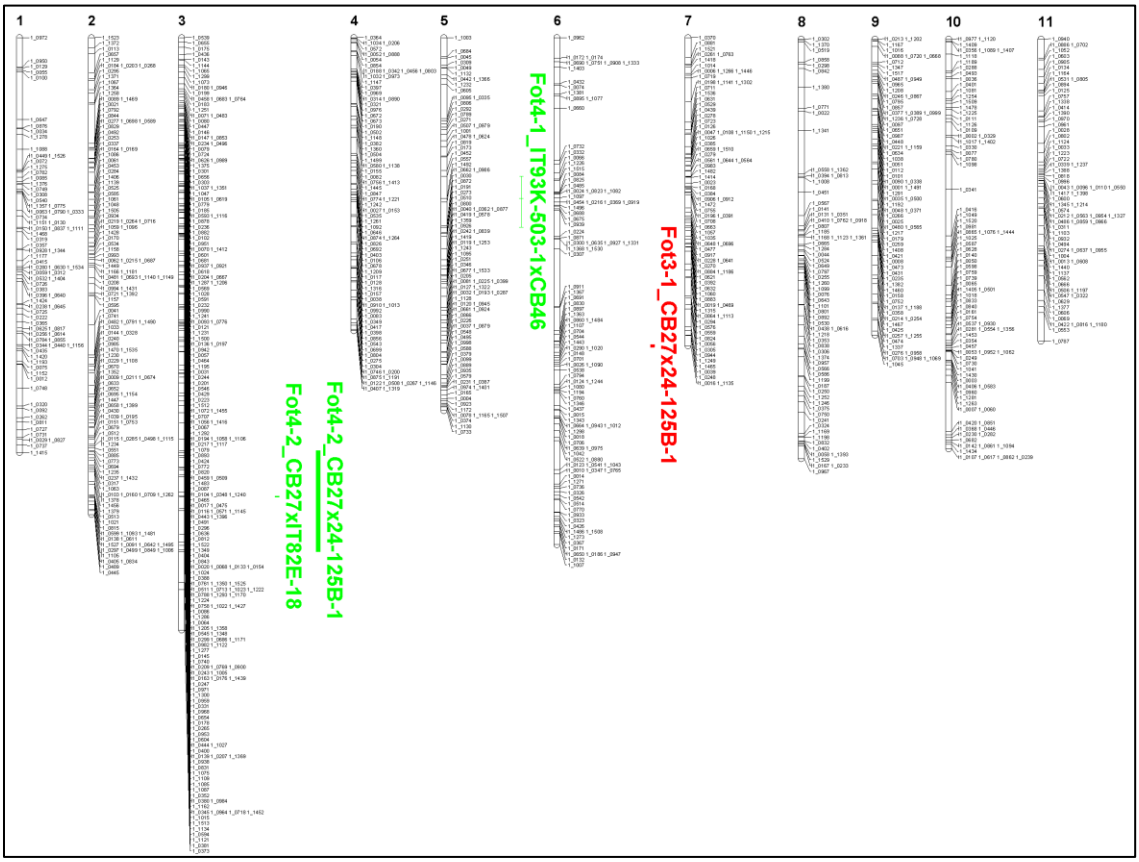


Figure 3.6 Resistance to *Fusarium oxysporum* f.sp. *tracheiphilum* race 4: *Fot4-2* QTL in the CB27 x 24-125B-1 population. The *Fot4-2* QTL mapped to linkage group 9. LOD scores are plotted in blue and pink for the first and second experiments, respectively. Over the two experiments, SNP markers 1_0594, 1_0984, 1_0380 and 1_1162 showed the most significant association with Fot race 4 resistance and are highlighted in red on the linkage group. The LOD significance threshold of 2.0 is indicated by a horizontal broken line.

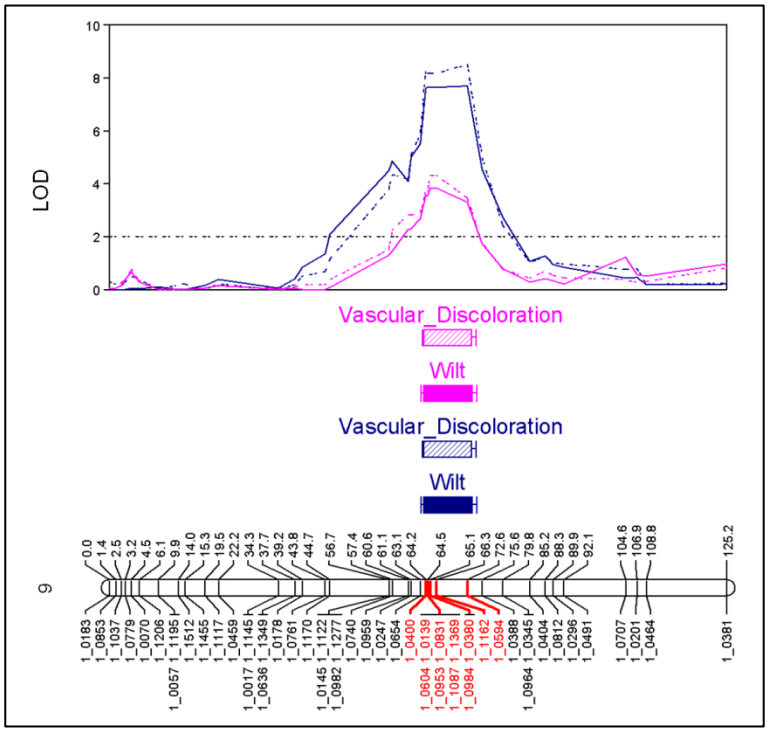


Figure 3.7 Resistance to *Fusarium oxysporum* f.sp. *tracheiphilum* race 4: *Fot4-2* QTL in the CB27 x IT82E-18/Big Buff population. The *Fot4-2* mapped to linkage group 1. LOD scores for the two experiments are plotted in green and pink. SNP marker 1_0352 was the most significant marker over both experiments and is highlighted in red. The LOD significance threshold of 2.0 is indicated by a horizontal broken line.

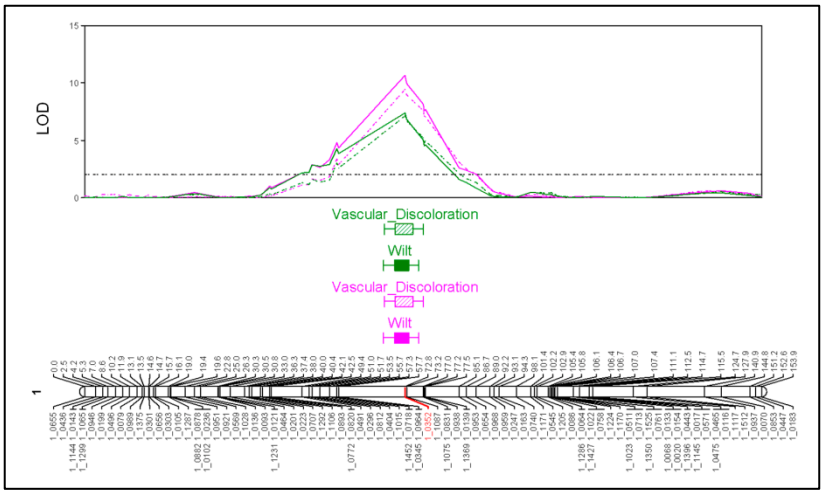


Figure 3.8 Synteny of *Fot4-1* with *G. max* chromosome 14. Synteny was examined for the *Fot4-1* locus between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes. *Fot4-1* spans from 21.57 to 29.40 cM on the cowpea consensus genetic map linkage group 5 and was syntenic at a macro and micro scale with soybean chromosome 14. The *Fot4-1* syntenic locus in soybean was identified by soybean orthologs to cowpea SNP markers 1_0557, 1_0662, 1_0986 and 1_0030 and spanned from soybean locus Glma14g15370 to Glyma14g36620. Three soybean disease resistance genes, Glyma14g17910, Glyma14g23930 and Glyma14g34880, were observed in the syntenic locus and were considered as orthologous disease resistance candidate genes for the *Fot4-1* locus. Glyma14g17910 and Glyma14g23930 were both annotated as TIR-NBS-LRR genes and Glyma14g34880 was annotated as a leucine-rich repeat protein kinase.

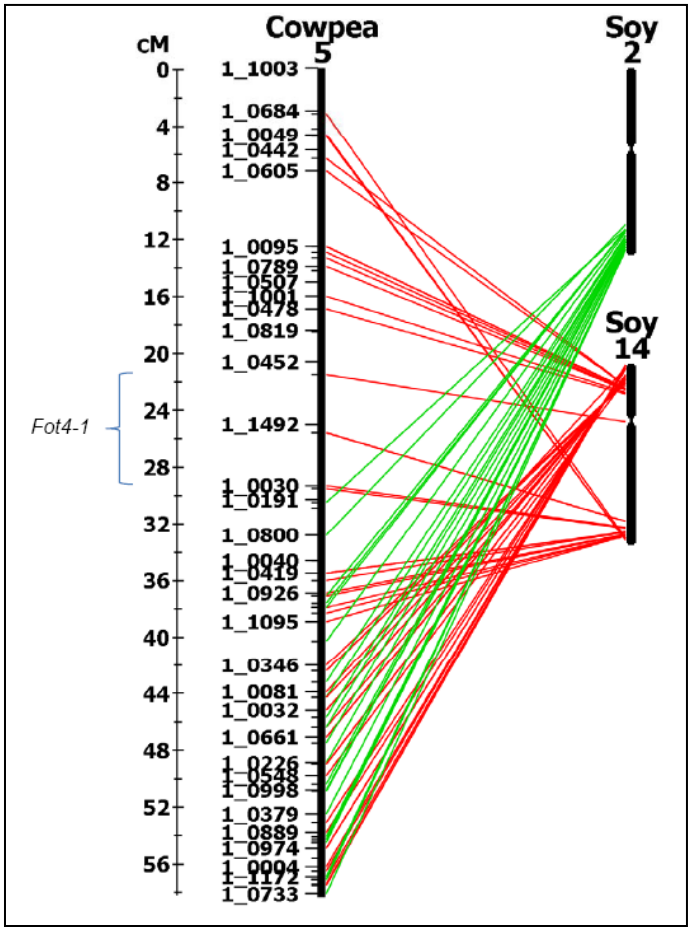


Figure 3.9 Synteny of *Fot4-2* locus with *G. max* chromosome 16 and 18. Synteny was examined for the *Fot4-2* locus between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes. The *Fot4-2* locus which spanned 64.44 cM to 80.23 cM on cowpea consensus genetic map linkage group 3 was determined to be co-linear with soybean chromosomes 16 and 18. The syntenic region in soybean chromosome 16 spanned from soybean locus Glyma16g15790 to Glyma16g23710; where two soybean disease resistance genes, Glyma16g17380 and Glyma16g22620, were observed. Glyma16g17380 was annotated as a leucine-rich repeat protein kinase and Glyma16g22620 was annotated as a TIR-NBS-LRR disease resistance gene. The syntenic *Fot4-2* region of soybean chromosome 18 spanned from soybean locus Glyma18g18980 to Glyma18g38670 which corresponded to 65.16 cM to 66.99 cM of the *Fot4-2* locus. However, the syntenic region preceded the most significant region of the *Fot4-2* locus, and no candidate genes were observed.

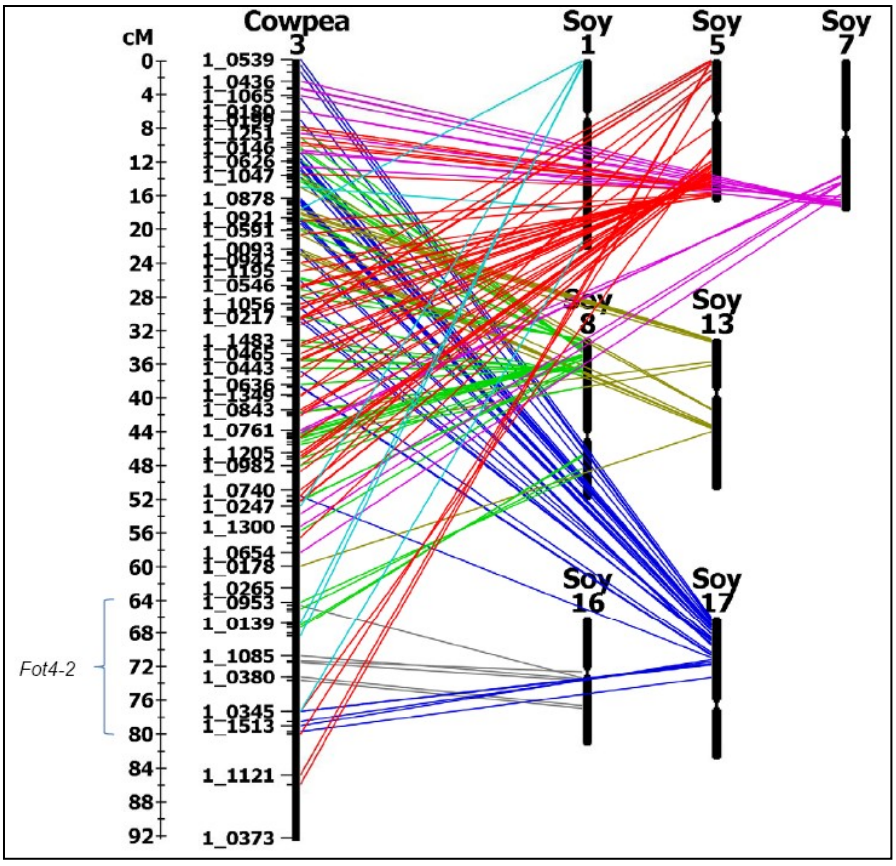
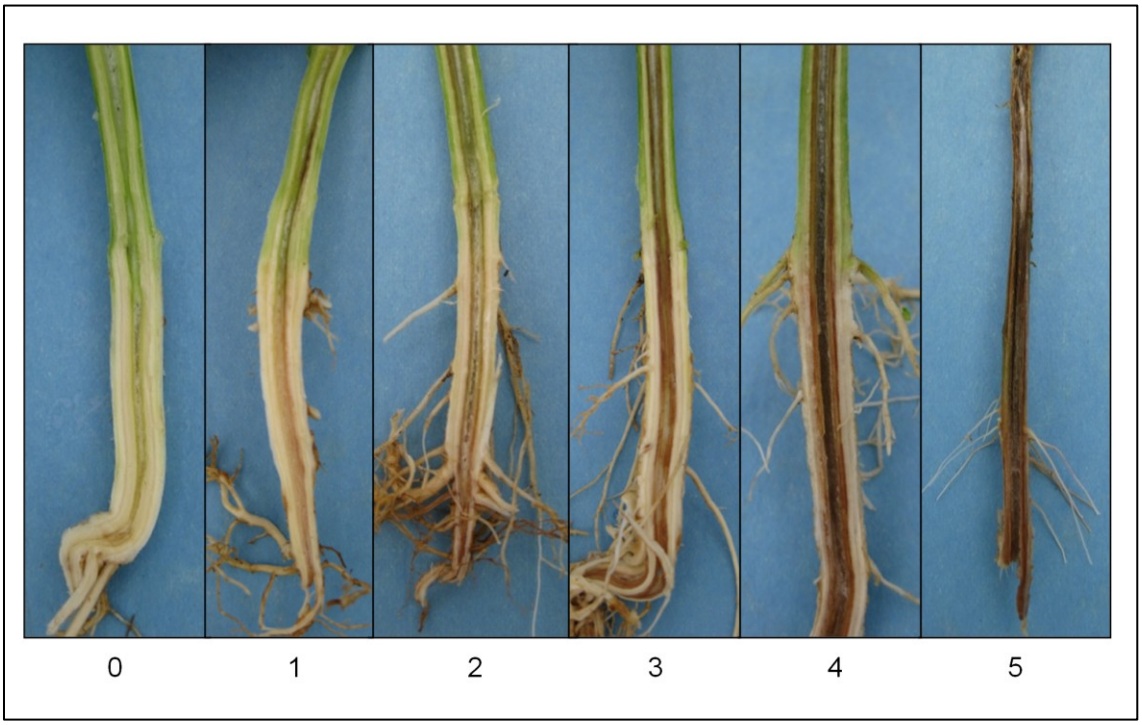


Figure 3.10 *Fusarium oxysporum* f.sp. *tracheiphilum* race 4 phenotyping for vascular discoloration symptoms. The severity of the vascular discoloration disease symptom was evaluated on a zero to five rating score. A rating of zero indicated a healthy plant with no signs of disease, 1 indicated approximately 10% of the plant with disease symptoms, 2 indicated 25% , 3 indicated 50%, 4 indicated 75% and 5 indicated 100% of the plant with disease symptoms.



Chapter 4

Macrophomina phaseolina resistance in cowpea [*Vigna unguiculata* (L.) Walp]: Utilizing integrated genomic resources to identify candidate genes underlying quantitative disease resistance.

Abstract

Macrophomina phaseolina (Tassi) `Goid is a soil-borne necrotrophic fungal pathogen which is problematic worldwide, especially in regions prone to high temperatures and drought conditions. Four Macrophomina resistance QTL, *Mac-10*, *Mac-11*, *Mac-12* and *Mac-13*, were identified in the cowpea RIL population Sanzi x Vita 7 under a two-year field experiment in Riverside, California. The QTL were positioned on the cowpea consensus genetic map and cowpea physical map. Syntenic relationships were examined with the genomes of *Glycine max, Medicago truncatula* and *Phaseolus vulgaris* to identify candidate genes. *Mac-10* accounted for 9.9% of the phenotypic variance, spanned 27.24 - 86.07 cM on LG3, and within syntenic loci in soybean chromosomes 5 and 17, WRKY72 transcription factors were present. *Mac-11* accounted for 10 – 16.3% of the phenotypic variance and spanned 37.04 - 50.85 cM on LG5 in which SNP 1_1419 co-segregated with short-day photoperiod-sensitive late-maturing Macrophomina-resistant genotypes. *Mac-11* was positioned within BAC clone CH038D17 on the cowpea physical map, in which annotations revealed the presence of a photosystem II reaction center protein B and an auxin response factor as candidate genes for short-day photoperiod-sensitivity induced late-maturity and Macrophomina-resistance. *Mac-12* spanned 4.09 - 31.04 cM on LG7 and accounted for 8.5 – 15.1% of the phenotypic variance. *Mac-13* accounted for 10.8% of the phenotypic variance and spanned 20.72 - 25.57 cM on LG4. A photosystem II reaction center W gene was present in the *Mac-13* locus and was considered a candidate gene for the short-day photoperiod-sensitive induced late-maturity trait. A marker-trait association in the *Mac-13* locus identified

SNP 1_1242 co-segregating with short-day photoperiod-sensitive late-maturing Macrophomina-resistant genotypes. *Mac-13* was positioned within BAC clones CH062O11 and CH069K06 on the cowpea physical map, where auxin-responsive *GH3* family proteins were present and were considered as candidate genes. This study identified new QTL for field-effective protection against *Macrophomina* infection in the later stages of cowpea growth, complementing previously discovered seedling-stage Macrophomina resistance traits. Molecular markers closely linked with the *Mac-10* and *Mac-12* loci will be targeted for future MAS breeding schemes which will contribute to drought and Macrophomina tolerant cowpea cultivars.

Keywords: cowpea, *Macrophomina phaseolina*, quantitative disease resistance, genomics, synteny, candidate genes, auxin response factor, auxin responsive *GH3* family proteins, WRKY72 transcription factor, photosystem II reaction center genes

Introduction

Macrophomina phaseolina (Tassi)`Goid is a soil-borne necrotrophic fungal pathogen which has a broad host range, causing disease in over 500 plant species (Dhingra and Sinclair 1978; Su et al. 2001). Macrophomina is problematic worldwide, especially in regions prone to both high temperatures and drought conditions. This causes particular difficulty for cowpea production since cowpeas are often grown to withstand these very same conditions, since it is a more drought and heat tolerant legume. Diseases caused by Macrophomina on cowpea have been reported in Brazil (Almeida et al. 2003), several African countries (Songa and Hillocks 1996; Adandonon et al. 2004; Ndiaye 2007; Singh et al. 1990; Emechebe and McDonald 1979; de Mooy et al. 1986 ; Adam 1990; Gray et al. 1990), India (Lodha and Singh 1984) and the United States of America (Muchero et al. 2011).

Among legume species, Macrophomina-induced diseases have been studied in many important crops including groundnut (Arora et al. 2001; Shanmugam and Govindaswamy 1973; Shweta et al. 2008), soybean (Gupta et al. 2012; Smith and Carvil 1997; Songa et al. 1997; Talukdar et al. 2009; Chakraborty and Purkayastha 1984), common bean (Echavez-Badel and Beaver 1987; Hernández-Delgado et al. 2008, 2009; Mayek-Pérez et al. 1997; Miklas and Beaver 1994; Miklas et al. 1998; You et al. 2011), cowpea (Muchero et al. 2011), lentil (Anver et al. 1991; Kaiser 1992; Tiyagi et al. 2001; Singh and Azam 2010; Ahmad 1996) chickpea (Singhl and Gogoiz 2006; Srivastava et al. 2001; Westerlund Jr et al. 1974; Selvarajan and Jeyarajan 1996) and mung bean (Zhang et al. 2011; Hussain et al. 1990; Raguchander et al. 1993; Mahakhant et al. 1998; Ehteshamul-

Haque et al. 1995; Saravanakumar et al. 2007), plus forage legumes such as alfalfa (Pratt et al. 1998; Guiñazú et al. 2012; Doerksen 2011; Eken and Demirci 2001) and clover (Wong et al. 1985; Quesenberry et al. 1993).

Macrophomina phaseolina infects the roots and lower stems of cowpea, sometimes causing pre- and post-emergence seedling damping-off and reddish-brown to black lesions near the soil line which can extend up the stem (Figure 4.1). Stunted growth, wilting, non-abscission and chlorosis of leaves (Figure 4.1) can occur in mature cowpea plants, and the disease is typically referred to as "charcoal rot" or "ashy stem blight" (Ratnoo and Bhatnagar 1993; Luttrell and Weimer 1952), which refers to the stem discoloration caused by visible black microsclerotia formed on the surface of the stem (Figure 4.1).

Several Macrophomina disease management approaches have been investigated including solarization (Israel et al. 2005), compost amendments (Ndiaye et al. 2010), chemical applications to seeds (Sinha and Khare 1977), alternative cropping systems (Singh et al. 1990) and biocontrol using antagonistic pathogens (Pal et al. 2001; Gupta et al. 2002; Arora et al. 2001; Gupta et al. 1999; Sendhilvel et al. 2005). However, under the limited-resource, rain-fed farming systems typical of cowpea production, these strategies have not been effective.

Identifying host resistance within cowpea germplasm is necessary in order to introgress Macrophomina resistance into drought-tolerant, early-maturing varieties of cowpea as a disease management strategy. However, there are limited reported sources of resistance

in the cowpea germplasm (Singh and Lodha 1986; Muchero et al. 2011) and this may be due in part to disease symptoms in the field, such as wilting and stunting, often being misdiagnosed as abiotic symptoms of drought stress. Also, the epidemiology of Macrophomina disease is complex, since it is synergistic with heat, drought and salinity stress as well as an early maturity-susceptibility in cowpea.

Resistance to *M. phaseolina* has been demonstrated to be quantitative in most crop species, including cowpea, (Muchero et al. 2011), sorghum (Reddy et al. 2008; Patil 2011) and common bean (Hernández-Delgado et al. 2009; Miklas et al. 1998). However, the genetic components underlying quantitative resistance to *Macrophomina* within cowpea and other crops are unknown. Quantitative plant disease resistance relies on several genes or QTL which have a small and partial but often durable effect on resistance and is generally pathogen species non-specific or race non-specific (Poland et al. 2009; Kou and Wang 2010).

Plant water stress increases the incidence of *Macrophomina*-related diseases (Dhingra and Sinclair 1978; Diourte et al. 1995; Edmunds 1964). The genetic mechanisms underlying the synergistic nature of drought stress-induced *Macrophomina* disease are not well understood. Muchero et al. (2011) showed that the genetic components for Macrophomina resistance and seedling-stage drought tolerance were independent in the cowpea RIL population, IT93K-503-1 x CB46 (Muchero et al. 2011). Currently, the overlap of drought-tolerance with *Macrophomina* resistance has been reported only for sorghum and the stay-green trait (Tenkouano et al. 1993; Diourte et al. 1995).

Combining drought tolerance with Macrophomina resistance is a breeding goal in cowpea and other crops.

Early-maturing varieties within some crops have been found to be more susceptible to disease than late-maturing varieties. Macrophomina resistance has been shown to be positively correlated with late-maturing varieties in some crop plants (Smith and Carvil 1997; Songa et al. 1997; Edmunds 1964; Cloud and Rupe 1994). In independent field studies of seedling-stage drought with early-maturity-senescence and Macrophomina resistance in cowpea, Muchero et al. (2009b; 2011) observed that a maturity related leaf-senescence QTL *Mat-1* co-located with Macrophomina resistance QTL *Mac-7*. The *Mac-7* locus was also associated with late-maturity (Muchero et al. 2011).

Molecular genetic tools and genomic resources developed recently for cowpea include a 1536 SNP genotyping platform, an EST-derived SNP consensus genetic map (Muchero et al. 2009a; Lucas et al. 2011; Diop et al. 2012) and known syntenic relationships between cowpea, *Medicago truncatula*, *Glycine max*, *Phaseolus vulgaris*, and *Arabidopsis thaliana*, and a cowpea EST sequence collection housed in HarvEST:Cowpea database (http://harvest.ucr.edu). A cowpea physical map (http://phymap.ucdavis.edu/cowpea) anchored to the cowpea consensus genetic map with SNP markers is also available, together with a first draft of the cowpea genome sequence, vs. 0.03 (http://harvest-blast.org). These resources will enable dissection of underlying genetic components of target agronomic traits using quantitative trait locus (QTL) and association mapping

analysis. The identified and confirmed QTLs will facilitate cultivar improvement using marker-assisted breeding.

In this study, we identified four major Macrophomina resistance QTLs, *Mac-10*, *Mac-11*, *Mac-12* and *Mac-13*, in cowpea using the Sanzi (susceptible) x Vita7 (resistant) RIL population. Comparative analysis between cowpea, soybean, common bean and Medicago revealed candidate genes for the *Mac-10* and *Mac-12* loci and cowpea-specific candidate genes for the *Mac-11* and *Mac-13* loci. In addition, two SNP markers were identified which co-located with obligate short-day photoperiod-induced late-maturing Macrophomina-resistant genotypes. These could be used in a marker-assisted breeding strategy to combine drought-tolerance and Macrophomina-resistance in cowpea cultivars.

Results

Macrophomina QTL analysis

Four QTLs associated with *M. phaseolina* resistance were identified over two years of field experiments and were named chronologically as they were observed, *Mac-10*, *Mac-11, Mac-12* and *Mac-13*. The 2009 field experiment yielded three QTLs, *Mac-10*, *Mac-11* and *Mac-12*. The 2010 field experiment yielded three QTLs, *Mac-11*, *Mac-12* and *Mac-13*. *Mac-11* and *Mac-12* were the only QTLs observed in both years.

Macrophomina resistance QTL, *Mac-10*

Mac-10 was observed only in the 2009 field experiment during the 1 week post germination (wpg) stage, spanning from 35.44 cM to 40.08 cM on linkage group 2 of the Sanzi x Vita 7 genetic map (Table 4.1, Table 4.2 and Figure 4.2). SNP marker 1_ 0381 was the most significant linked marker accounting for 9.9% of the phenotypic variance (LOD score 2.9) (Table 4.1, Table 4.2). The *Mac-10* locus was positioned on the cowpea consensus genetic map using markers identified in the QTL analysis, where it spanned 27.24 cM to 86.07 cM on LG3 (Figure 4.3). However, the difference between the length of *Mac-10* on the individual genetic map (4.64 cM distance) vs. the cowpea genetic map vs.4 (58.83 cM distance) was quite significant (Table 4.2). We examined the *Mac-10* locus on the cowpea consensus genetic map vs. 6 (http://harvest.ucr.edu) where it spanned 74.80 cM to 78.61cM (3.81 cM distance) on LG3, which is most likely the accurate length (Table 4.4). The *Mac-10* locus overlapped two previously identified Macrophomina resistance QTLs on the cowpea consensus genetic map vs.4, *Mac-3* and

Mac-4, which were previously identified in a Macrophomina field study using cowpea RIL population IT93K-503-1 (resistant) x CB46 (susceptible) (Muchero et al. 2011) (Table 4.5, Figure 4.3). *Mac-3* spanned 44.50 cM to 77.55 cM on LG3, while *Mac-4* encompassed the 24.22 cM to 36.98 cM region (Table 4.5, Figure 4.3). The most significant markers for *Mac-3* (1_0604, 64.78 cM), *Mac-4* (1_0201, 26.29 cM) and *Mac-10* (1_0381, 86.07 cM) were not in close proximity to each other, indicating these loci may be independent (Table 4.5, Figure 4.3).

Mac-10 synteny with *G. max*

Synteny was observed for the *Mac-10* locus on the cowpea consensus genetic map vs.6 with soybean chromosomes 5 and 17 (Table 4.6, Figure 4.4). On soybean chromosome 5, the syntenic region from Glyma05g00400 to Glyma05g02190 corresponded to 75.16 cM to 78.18 cM of the *Mac-10* locus (Table 4.4). Soybean locus Glyma05g01280 which was annotated as a WRKY72 transcription factor (TF) was positioned close to the soybean homolog for the most significant marker 1_0381 and was the only disease-related gene in the region (Table 4.6). On soybean chromosome 17 the *Mac-10* region extended from Glyma17g08630 to Glyma17g11060 where another WRKY72 TF was observed and was considered as a candidate gene for the *Mac-10* locus (Table 4.6). The WRKY72-type transcription factor has been reported to be instrumental to both basal defense and gene-for-gene resistance mediated by the *Mi-1* gene in tomato against root-knot nematodes (RKN) and potato aphids (Bhattarai et al. 2010). Additionally, the

Arabidopsis ortholog of WRKY72 was found to be required for basal immunity against RKN and the oomycete *Hyaloperonospora arabidopsidis* (Bhattarai et al. 2010).

Macrophomina resistance QTL, *Mac-11*

Mac-11 was observed in the 2009 experiment from 1 wpg to 8 wpg stage, spanning 19.31 cM to 38.38 cM on LG5 of the individual genetic map (Table 4.1, Figure 4.5). SNP marker 1_0251 was the most significant marker for the percent surviving plants during the 1 wpg stage, accounting for 12.4% phenotypic variance (LOD 3.66) (Table 4.1). The percent of seedling damping-off symptoms for weeks 1 through 3 wpg mapped to the same locus, where SNP marker 1_0251 accounted for 10% of the phenotypic variance (LOD 2.93) (Table 4.1). In the 2010 field experiment, *Mac-11* was observed from the 2 wpg stage to the 9 wpg stage, the most significant region spanned from 19.31 cM to 27.45 cM on LG5 (Table 4.7, Figure 4.5). SNP 1_0495 (20.25 cM) was the most significant marker, peaking at the 9 wpg and explaining 16.3% of the phenotypic variance (LOD 4.91) (Table 4.7).

***Mac-11* locus on the cowpea consensus genetic map**

Mac-11 was positioned on the cowpea consensus genetic map where it spanned 37.04 to 50.85 cM on LG5 (Table 4.8, Figure 4.3). Cowpea genotypes differing in their resistance to Macrophomina, photoperiod-sensitivity and maturity were chosen for a marker-trait association to narrow the *Mac-11* locus. Macrophomina-resistant, short-day photoperiod-sensitive and late-maturing cowpea genotypes which flower after 70 days under long-day photoperiods in Riverside, California, included Vita 7, IT98K-503-1, Apagbaala,

IT97K-499-39, Suvita-2, Moussa Local and Iron Clay. Macrophomina-susceptible, day-neutral (photoperiod insensitive) and early-maturing genotypes which flower 40 to 60 days under long-day photoperiods in Riverside, CA, included Sanzi, IT82E-18(Big Buff), CB46, CB27, UCR 24, 524-B and Bambey 21. Within the *Mac-11* locus, SNP marker 1_1419 (37.73 cM position) was observed co-segregating with late-maturing (short-day photoperiod sensitive) Macrophomina-resistant and early-maturing (day neutral) Macrophomina-susceptible genotypes (Figure 4.6). The late-maturing Macrophomina-resistant genotypes contained the cytosine allele, while the early-maturing Macrophomina-susceptible genotypes contained the guanine allele (Figure 4.6). The guanine/cytosine SNP is position 560 of cowpea unigene 3720 and was annotated as 2-succinylbenzoate-CoA ligase (http://harvest.ucr.edu). Since SNP 1_1419 co-segregated with resistant and susceptible genotypes, it could be used as a molecular marker to screen against late-maturity Macrophomina resistance.

Interestingly, nearby to SNP 1_1419 on the cowpea consensus genetic map, were two markers annotated as genes involved in photosynthesis or light-response. SNP 1_0926 was annotated as a cytochrome b-c1 complex, subunit 8 protein and SNP 1_1253 was annotated as a phototropic-responsive NPH3 family protein, both of which were considered as candidate genes for the short-day photoperiod sensitivity-induced late maturity trait (Table 4.8).

Mac-11 was observed overlapping two previously identified Macrophomina resistance QTL on the cowpea consensus genetic map. QTL *Mac-8* (Muchero et al. 2011) preceded

the *Mac-11* locus (37.04 cM to 50.85 cM), spanning 21.57 cM to 37.73 cM on LG 5 (Table 4.5, Figure 4.3). The *Mac-8* and *Mac-11* region of overlap included the late-maturity Macrophomina-resistance co-segregating marker 1_1419 (37.73 cM position) (Table 4.5, Figure 4.3). *Mac-11* also overlapped previously identified QTL *Mac*-9 (Muchero et al. 2011), which spanned 42.51 cM to 57.58 cM on LG5 (Table 4.5, Figure 4.3). The most significant markers in the *Mac-9* locus, at 42.51 cM and 45.28 cM, were in close proximity with the co-segregating SNP marker for *Mac-11* at 37.73 cM, which suggested that they could be the same QTL (Table 4.5, Figure 4.3). However, currently we are considering them as independent loci.

Mac-11 and synteny with *G. max*, *M. truncatula* and *P. vulgaris*

The *Mac-11* locus had high co-linearity with soybean chromosomes 2 and 14 (Table 4.9, Figure 4.7). Soybean orthologs for eleven out of twenty-three SNP markers were identified in the co-linear region of soybean chromosome 2, from Glyma02g40530 to Glyma02g43640 which corresponded to 37.23 cM to 46.51 cM of the *Mac-11* locus (Table 4.9). Soybean locus Glyma02g40650, which was annotated as an auxin response factor (ARF), was closely linked with the soybean ortholog for SNP 1_1419 and was considered a candidate gene for the *Mac-11* locus (Table 4.9). The syntenic locus on soybean chromosome 14, from Glyma14g39280 to Glyma14g04890, corresponded to 37.23 cM to 47.18 cM of the *Mac-11* locus on the cowpea consensus genetic map (Table 4.9). In the soybean region corresponding to 37.23 cM to 38.09 cM of *Mac-11*, soybean

locus Glyma14g38940, annotated as an auxin response factor, was present and was also considered as a candidate gene for the *Mac-11* locus (Table 4.9).

Medicago chromosome 5 was observed to be highly co-linear with the *Mac-11* locus at the macro- and micro-levels (Table 4.10, Figure 4.8). The syntenic locus was identified by eight out of eight SNP markers, spanning from Medtr5g083440 to Medtr5g085190, which corresponded to 37.04 cM to 39.04 cM of the *Mac-11* locus (Table 4.10, Figure 4.8). Medicago genes orthologous to SNP markers 1_0242, 1_1419, 1_0839 and 1_0119 were clustered together but in slightly different order than on the cowpea consensus genetic map (Table 4.10). The region surrounding the Medicago ortholog of cowpea SNP marker 1_1419 was examined in Phytozome (Goodstein et al. 2012) and contained Medtr5g084140 locus, which was annotated as an auxin response factor with a B3 DNA binding domain (Table 4.10).

The *Mac-11* locus was highly co-linear with common bean chromosome 8, extending from locus Phvul.008G203600 to Phvul.008G243400 (Table 4.11, Figure 4.9). The marker-order between cowpea and common bean was highly conserved, however, the gene order was inverted (Table 4.11). Phvul.008G242400 was slightly upstream of marker 1_1419, which was annotated as an auxin response factor (Table 4.11).

Mac-11 on the cowpea physical map

Seven cowpea BAC contigs were identified which incompletely spanned the *Mac-11* locus (Table 4.8). SNP marker 1_1419 which co-segregated with late-maturity Macrophomina resistance was identified in BAC clone CH038D17 of contig426 (Table

4.8). Contig426 has 23 overlapping BAC clones and 244 non-repeating bands with an estimated size of 400,160 bp (http://phymap.ucdavis.edu/cowpea). BAC clone CH038D17 has 99 non-repeating bands with an estimated size of 162,360 bp (http://phymap.ucdavis.edu/cowpea). In addition to marker 1_1419, BAC clone CH038D17 contained cowpea SNP markers 1_0242, 1_0745 and 1_0839 which corresponded to 37.23 cM to 37.73 cM on the cowpea consensus genetic map (Table 4.8). BAC clone CH038D17 had fourteen nodes/genes (Table 4.12). The sequence of CH038D17_VU1.3_NODE_0029 had the highest similarity with Phvul.008G242400 which was annotated as an auxin response factor (Table 4.12). The sequence had 96.8% similarity with soybean Glyma02g40650.1 and 78.4% similarity with At5g37020.1 which was annotated as an auxin response factor 8 (Goodstein et al. 2012). In addition, BAC clone CH038D17 also contained a photosystem II (PSII) reaction center protein B gene which was considered a candidate gene for the short-day photoperiod sensitive late-maturity phenotype (Table 4.12).

Macrophomina resistance QTL, *Mac-12*

The *Mac-12* locus was observed throughout the entire 2009 experiment and SNP marker 1_1302 (7.61 cM) was the most significant marker during the entire experiment. At the 8 wpg stage, SNP 1_1302 accounted for 14.6% of the phenotypic variance, LOD score 4.38 (Table 4.1, Figure 4.10). In the 2010 field experiment, *Mac-12* was observed intermittently. At 1 wpg, *Mac-12* spanned 31.48 cM to 45.16 cM on LG 6 in which SNP 1_0559 was the most significant marker (11.6%; LOD 3.36) (Table 4.7) and again at 8

wpg, where it spanned from 7.61 cM to 30.03 cM on LG 6 containing SNP marker 1_0708 (13.3% variance; LOD 3.95) (Table 4.7). At 9 wpg, *Mac-12* spanned 0 cM to 28.56 cM on LG 6 in which SNP marker 1_0278 accounted for 15.1% phenotypic variance (LOD 4.54) (Table 4.7). The inconsistency in genome location and time of appearance of *Mac-12* between the 2009 and 2010 experiments may be due to lower average temperatures in 2010 causing a G x E interaction (Additional file 1).

Mac-12 on the cowpea genetic and physical maps

Mac-12 spanned 4.09 cM to 31.04 cM on the cowpea consensus genetic map linkage group 7 (Table 4.13, Figure 4.3). *Mac-12* overlapped a previously identified seeding-stage drought tolerance QTL, *Dro-1*, positioned at 13.15 cM to 32.69 cM on LG7, SNP marker 1_0983 (21.60 cM) (Table 4.5, Figure 4.3)(Muchero et al. 2009b). SNP marker 1_1302 identified in the 2009 experiment shared the same marker bin as markers 1_0198 and 1_1141 on the consensus genetic map, which were identified in BAC clone CH074C16 of contig196 (Table 4.13). Annotations for clone CH074C16 revealed genes that are involved in plant growth and development including fructokinase-like 2 and protein kinase superfamily proteins and genes involved in both plant growth and development and plant defense including copper amine oxidase family protein and 6-phosphogluconolactonase 1 (Table 4.14). SNP marker 1_0708 identified in the 2010 experiment was identified on BAC clones CH068C24 and CH061D01 of contig337 (Table 4.14). Annotations for CH068C24 and CH061D01 revealed a large block of disease resistance genes including DZC (Disease resistance/zinc finger/chromosome

condensation-like region) domain containing protein, LRR and NB-ARC domain-containing disease resistance proteins and LRR protein kinase family proteins (Table 4.15).

Macrophomina resistance QTL, *Mac-13*

The *Mac-13* locus was observed in the 2010 experiment from 2 to 9 wpg positioned at 41.89 cM to 55.18 cM on LG 10 of the individual map (Table 4.7, Figure 4.11), in which SNP 1_0826 at 9 wpg accounted for 10.8% (LOD 3.16) of the phenotypic variance (Table 4.7).

***Mac-13* locus in the cowpea consensus genetic map**

A marker-trait association of the *Mac-13* locus identified SNP marker 1_1242 alleles co-segregated with late-maturing Macrophomina-resistant and early-maturing Macrophomina-susceptible genotypes (Figure 4.12). The late-maturing, short-day photoperiod-sensitive, Macrophomina-resistant genotypes had the cytosine allele, while the early-maturing, day-neutral, Macrophomina-susceptible genotypes had the thymine allele (Figure 4.12). The cytosine/thymine allele at position 685 in cowpea unigene 4217 is annotated as an N-terminal acetyltransferase (http://harvest.ucr.edu). SNP 1_1242 could be used as a molecular marker to screen for late-maturity Macrophomina resistance since it co-segregated with Macrophomina-resistant and susceptible genotypes. In addition, SNP marker 1_0027 was annotated as a photosystem II reaction center W protein was nearby and was considered a candidate gene for the short-day photoperiod-sensitive induced late-maturity trait (Table 4.16).

Mac-13 at 20.20 cM to 25.57 cM on the cowpea consensus genetic map LG4, overlapped with previously identified Macrophomina resistance QTL *Mac-7* (Muchero et al. 2011), which spanned 20.72 to 35.96 cM (Table 4.5, Figure 4.3). The most significant marker for the *Mac-7* locus, SNP 1_0153 (21.49 cM position), was 0.77 cM away from SNP 1_1242 (20.72 cM position) which co-segregated with late-maturity Macrophomina resistance in the *Mac-13* locus (Figure 4.12). *Mac-13* also overlapped with maturity-related leaf senescence locus *Mat-1* (Muchero et al. 2009b, 2011), which spanned 21.49 cM to 27.60 cM with SNP 1_0678 at 27.60 cM the most significant marker (Table 4.5, Figure 4.3). Another maturity-related leaf senescence locus, *Mat-2* (Muchero et al. 2009b, 2011), and Macrophomina resistance QTL *Mac-6* (Muchero et al. 2011), were close to *Mac-13*, both spanning 37.46 cM to 45.67 cM on consensus map LG4 (Table 4.5, Figure 4.3).

Mac-13 synteny with *G. max* and *P. vulgaris*

The *Mac-13* locus showed a high co-linearity with soybean chromosomes 3 and 19 (Figure 4.13). Eight of twelve SNP markers identified the syntenic locus in soybean chromosome 3 from Glyma03g31080 to Glyma03g33270, and corresponding to 20.72 cM to 25.31 cM of the *Mac-13* locus (Figure 4.13). Glyma03g31100, Glyma03g31080 and Glyma03g31110 were identified upstream of *Mac-13* and were considered candidate genes; Glyma03g31100 was annotated as a chalcone-flavanone isomerase and Glyma03g31080 and Glyma03g31110 were annotated as terpene synthase related genes (Table 4.17). In addition, Glyma03g31520 and Glyma03g31530, which were annotated

as AUX/IAA family genes were both identified downstream of SNP 1_1242 and considered candidate genes (Table 4.17).

Eight out of twelve SNP markers spanning from Glyma19g34380 to Glyma19g36180 on soybean chromosome 19, corresponded to 21.49 cM to 25.57 cM of *Mac-13* (Table 4.17). Glyma19g34370 and Glyma19g34380, both annotated as AUX/IAA family genes, were observed near the *Mac-13* locus (Table 4.17).

A syntenic relationship was observed for the *Mac-13* locus with *P. vulgaris* chromosome 1, extending from Phvul.001G147300 to Phvul.001G174300 (Table 4.18, Figure 4.14). Several interesting genes were observed in the syntenic region and were considered candidate genes for the *Mac-13* locus; auxin-responsive *GH3* family protein, AUX/IAA transcriptional regulator family protein, auxin-induced protein 13, terpenoid cyclases, chalcone-flavanone isomerase and an ethylene response factor 1 (Table 4.18).

Mac-13 on the cowpea physical map

Four BAC contigs incompletely spanned the *Mac-13* locus (Table 4.16). Although SNP 1_1242, which co-segregated with late-maturity Macrophomina-resistance was not identified on the physical map, its closest marker, SNP 1_1221, was identified in BAC clones CH022D17, CH062O11 and CH069K06 of contig445 on the physical map (Table 4.16). Contig445 has 16 overlapping BAC clones and 183 non-repeating bands which estimated the contig size at 300,120 bp (http://phymap.ucdavis.edu/cowpea). BAC clone CH06211 has 56 non-repeating bands and is estimated at 91,840 bp (http://phymap.ucdavis.edu/cowpea). BAC clone CH069K06 has 66 non-repeating bands

and is estimated at 108,240 bp (http://phymap.ucdavis.edu/cowpea). BAC clones CH062O11 NODE_0025 and CH069K06 NODE_0028 had a high similarity with common bean locus Phvul.001G147300 which was annotated as an auxin-responsive *GH3* family protein (Table 4.19). The sequence had 90.1% similarity with soybean Glyma03g30590 and 53.5% similarity with Arabidopsis At5g13320 which was annotated as an auxin-responsive *GH3* family protein (Goodstein et al. 2012).

Discussion

Short-day photoperiod-sensitivity induced late-maturity and Macrophomina resistance

The *Mac-11* and *Mac-13* loci displayed several similar features regarding short-day photoperiod sensitivity influencing late-maturity and Macrophomina resistance. Within both loci, SNP markers were identified which co-segregated with short-day photoperiod-sensitive late-maturing Macrophomina-resistant genotypes. Additionally, *Mac-11* co-located with *Mac-9* and *Mac-13* co-located with *Mac-7*, which were previously identified Macrophomina resistance QTLs in the IT93K-503-1 (resistant) x CB46 (susceptible) population (Muchero et al. 2011). The Macrophomina-resistant parents from the two populations, Vita 7 and IT93K-503-1, are both obligate short-day photoperiod sensitive genotypes which do not flower until after 70 to 100 days under long-day summers in Riverside, CA or until the photoperiod returns to short day. The Macrophomina-susceptible parents of the two populations, Sanzi and CB46 are both day-neutral and are considered early-maturing genotypes, flowering around 40 to 50 days under long-day photoperiods in Riverside, CA. In addition, *Mac-13* was found to overlap the maturity-related leaf senescence QTL, *Mat-1*, from the IT93K-503-1 x CB46 population further indicating that the locus is associated with maturity.

Photosystem II reaction center genes in both the *Mac-11* and *Mac-13* loci could be candidate genes contributing to the short-day photoperiod sensitive late-maturing phenotype. In the *Mac-11* locus on the cowpea physical map, a PSII reaction center

protein B gene was present, and in *Mac-13* SNP 1_0027 was annotated as a PSII reaction center W gene. Muchero et al. (2011) also identified a PSII reaction center W gene in the *Mac-7* syntenic locus in Medicago and considered it a candidate gene for the Macrophomina resistance QTL which was also associated with late-maturity.

The PSII reaction center is located in the core of PSII, which is involved in the harvesting of light energy for photosynthesis and regulates the excess excitation energy which can be used (Horton and Ruban 2005). The PSII reaction center is made up of several polypeptides which bind a few chromophores, pigments and co-factors and is where sunlight-induced charge separation occurs (van Amerongen and Croce 2013). Mechanisms regarding light harvesting and photosynthesis CO_2 fixation has been found to be controlled by circadian rhythms (Dodd et al. 2014). A circadian oscillation for the rate of electron transport in chloroplasts has been reported for peas, soybean and common bean (Lonergan 1981). Functional analyses of genes underlying the *E* loci which are associated with flowering and maturity and display differences in photoperiod and sensitivity to light quality in soybean have revealed genes involved in light-dependent photoperiodic pathways. The *E2* locus encodes a soybean homolog of GIGANTEA (GI) which is circadian clock controlled gene in Arabidopsis (Watanabe et al. 2011). The maturity and flowering loci, *E3* (Watanabe 2009) and *E4* (Liu 2008), encode GmPhyA3 and GmPhyA2 and which are phytochrome A genes (Watanabe et al. 2009; Liu et al. 2008).

Increasingly, cross-talk between the photosynthesis, metabolism and defense response pathways to pathogens is being revealed (Roden and Ingle 2009; Kangasjärvi et al. 2012; Kangasjärvi et al. 2013; Trotta et al. 2014). In the incompatible interaction between cowpea and *Colletotrichum gloeosporioides*, significant proteome changes in the leaves were found associated with photosynthetic proteins as well as metabolism, response to stress, oxidative burst, defensive signaling and pathogenesis-related proteins (Moura et al. 2014). Photosystem II specific protein changes included PSII 10kDA polypeptide isoform 1 which underwent a 648.3 fold change 24 hours post inoculation (hpi), a 23 kDa polypeptide of the oxygen evolving complex of PSII underwent a 233 fold change 72 hpi and a PSII oxygen-evolving complex protein 3 underwent 1.9 fold change 72 hpi (Moura et al. 2014). The fact that PSII reaction center genes have been specifically identified as being up-regulated in another cowpea-fungal pathogen relationship and that several QTLs identified for flowering and maturity in soybean are circadian rhythm controlled elements of photosynthesis, makes PSII reaction center genes plausible candidates for contributing to short-day photoperiod sensitive-induced late maturity in cowpea. However, more work is needed to determine whether the genetic mechanisms underlying *Mac-11* and *Mac-13* are due to closely linked genes as we are suggesting rather than being a pleotrophic trait. In this arrangement, PSII reaction center genes would contribute to short-day photoperiod sensitivity whereby a late-maturing phenotype occurs under a long-day photoperiod, and auxin-responsive genes contribute to quantitative Macrophomina resistance.

Auxin candidate genes in the *Mac-11* and *Mac-13* loci

Auxin (indole-3-acetic acid or IAA) is an important plant hormone which is involved in many aspects of plant growth and development, but more recently has been found to be involved in disease resistance interactions (Kazan and Manners 2009; Fu and Wang 2011). Interestingly, an auxin response factor 8 (ARF8) was observed in *Mac-11* and two auxin-responsive *GH3* family proteins were observed in *Mac-13*. ARFs are transcription factors which bind to the Auxin-Responsive Elements (AuxRE) within promoters of some early/primary auxin responsive genes (Ulmasov et al. 1997; Woodward and Bartel 2005) which include Aux/IAA, the small auxin-up RNA (SAUR) and some members of the *GH3* gene family. ARF7, ARF8 and ARF17 can regulate the transcription of some auxin-induced *GH3* family proteins which in turn regulate auxin levels in plants (Stowe-Evans et al. 1998; Takase et al. 2004; Tian et al. 2004; Mallory et al. 2005). Interestingly, several *GH3* proteins contribute to minor disease resistance QTLs in rice (Wen et al. 2003). For example, the *OsGH3-2* gene encodes an IAA-amido synthetase and contributes broad-spectrum and partial disease resistance against bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), bacterial streak caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), and fungal blast caused by *Magnaporthe grisea* (Fu et al. 2011; Terol et al. 2006). A second gene, *OsGH3-8*, contributed to a minor disease resistance QTL against rice bacterial blight (Hu et al. 2008; Ding et al. 2008), and *GH3* protein *OsGH3-1* enhanced resistance to *M. oryzae* (Domingo et al. 2009). Thus, evidence for auxin-induced gene action in other plant-pathovar systems makes auxin-related genes good candidates for the *Mac-11* and *Mac-13* loci.

Recently, auxin has been implicated as a key player in the compatible interaction between Medicago and Macrophomina in a microarray study (Mah et al. 2012). Auxin signaling pathway genes had a significant change in expression after inoculation with Macrophomina. ARF5 expression increased at 24 hours post inoculation (hpi) then decreased at 36 and 48 hpi while two Medicago orthologs of *Nt-GH3* family proteins steadily increased in expression from 24 to 48 hpi (Mah et al. 2012). Also, external application of IAA to young Medicago plants increased tolerance to Macrophomina (Mah et al. 2012).

Auxin also has been implicated in resistance responses in plant host interactions with necrotrophic pathogens including *Botrytis cinerea*, *Plectosphaerella cucumerina* and *Alternaria brassicicola* infecting Arabidopsis (Llorente et al. 2008; Qi et al. 2012). In another example of a necrotrophic pathogen, researchers studying Arabidopsis infection by *Alternaria brassicicola* induced auxin biosynthesis and enhanced the auxin response (Qi et al. 2012). Robert-Seilaniantz et al. (2007), Bari and Jones (2009) and Fu and Wang (2011) proposed a model that auxin accumulation enhances resistance against necrotrophic pathogens; whereas in the interactions between biotrophic pathogens and plant hosts, auxin negatively affects resistance. Interestingly, the candidate genes that we identified, a WRKY72 transcription factor in *Mac-10*, an *ARF8* in *Mac-11* and an auxin responsive *GH3* family protein in *Mac-13*, are key components in the signaling pathway for necrotrophic pathogen resistance.

Conclusion

In this study we report the identification of four QTLs, *Mac-10*, *Mac-11*, *Mac-12* and *Mac-13*, associated with resistance to *Macrophomina phaseolina* in the cowpea RIL population Sanzi x Vita 7. Integrated cowpea genomic resources which include a cowpea consensus genetic map, cowpea physical map and known syntenic relationships with soybean, Medicago and common bean enabled the identification of loci and significantly linked molecular markers as well as several candidate genes for all of the Macrophomina resistance loci (Table 4.20). *Mac-10*, *Mac-11* and *Mac-13* co-located in the vicinity of previously mapped Macrophomina QTLs; however, it remains to be determined which of these are independent. *Mac-12* was the only new independent locus. *Mac-11* and *Mac-13* were found to be associated with short-day photoperiod induced late-maturity Macrophomina resistance and were determined to not be useful in pyramiding disease resistance; although, markers were identified for both *Mac-11* and *Mac-13* which could be used to screen against the late-maturity associated Macrophomina resistance. The *Mac-10* and *Mac-12* loci appear to be the best options for pyramiding Macrophomina resistance QTL to provide both seedling-stage and season-long protection.

Future perspectives for this research could include the functional analysis of the candidate genes identified for short-day photoperiod sensitivity induced late-maturity and Macrophomina resistance in the *Mac-11* and *Mac-13* loci to determine if the phenotypic traits are tightly linked or if these are examples of a pleotrophic trait. This would enhance our understanding of the phenomenon of late-maturity associated with disease

resistance which has been recognized in other plant-pathovar systems (Visker et al. 2003; Bormann et al. 2004).

A practical outcome of this study was the identification of molecular markers associated with Macrophomina resistance which could be useful for pyramiding disease resistance into cowpea cultivars towards the breeding objectives of developing early-maturing, drought and Macrophomina tolerant cowpea cultivars.

Materials and methods

Plant population

Macrophomina resistance was studied in the cowpea RIL population Sanzi (susceptible) x Vita 7 (resistant) which was developed from an intra-specific cross. Sanzi is a local landrace from Ghana which has a prostrate-sprawling architecture, grayish-purple seeds, and sub-globose leaf shape (Pottorff et al. 2012a). Vita 7(PI 580806 or TVu-8461) is an advanced breeding line from IITA (International Institute of Tropical Agriculture), Nigeria and has an upright bush-type architecture, beige seeds and hastate leaf shape (Pottorff et al. 2012a). The Sanzi x Vita 7 population was advanced by single seed descent to F_{10} and was received from Christian Fatokun, IITA, Ibadan, Nigeria. All other cowpea materials were available from the University of California Riverside cowpea germplasm collection.

Experiments

Phenotyping for *Macrophomina* resistance consisted of two consecutive year field experiments conducted at the University of California Citrus Research Center-Agricultural Experiment Station (CRC-AES) in Riverside, CA. The field site has a history of Macrophomina infestation and was used previously to phenotype for Macrophomina resistance (Muchero et al. 2011). Both field experiments were replicated four times using a randomized complete block design. The 2009 field experiment was conducted from June 23 to September 1, 2009. Seeds were hand-planted into 1-m plots

on dry raised beds, ten seeds per plot spaced 10 cm apart. The raised beds were spaced 76 cm apart. The field experiment was irrigated once after planting and then water was withheld for the remainder of the experiment. The 2010 field experiment was conducted from June 16 to August 30, 2010. Twenty-five seeds per plot, on average 10 cm apart, were machine-planted into pre-irrigated raised beds. The beds were spaced 76 cm apart and water was withheld for the remainder of the experiment.

Phenotyping

Plants were evaluated for Macrophomina disease symptoms every seven days beginning two weeks after planting (about one week post-emergence). RILs were analyzed individually for each of the four replicated blocks per experiment and then averaged to determine the mean value of response to Macrophomina infection. The percent surviving plants (number of healthy surviving plants divided by number of germinated seeds) was used to evaluate Macrophomina resistance. The percent of surviving plants was used rather than percent mortality due to the disintegration of dead plants which made it difficult to keep accurate records over the course of the experiment. The percent of seedling damping-off (number of seedling damping-off divided by number of emerged seedlings) was also taken and mapped for the 2009 field experiment. In the 2010 field experiment, seedling damping-off symptoms did not occur, so only the percent of surviving individuals phenotype was used to map Macrophomina resistance. Due to differences in the average daily temperature, the 2009 field experiment exhibited more Macrophomina disease symptoms and mortality than the 2010 field experiment. The

2009 average daily maximum temperature was 34°C compared to31°C in 2010 (Table 4.3) (http://www.ipm.ucdavis.edu/WEATHER/index.html). There was no significant rainfall during the 2009 and 2010 field experiments (Table 4.3). In the 2010 field experiment, percentage surviving individuals (mortality rate) for weeks 2 to 7 post-germination did not changed significantly, which was reflected in the QTL analysis.

To validate that the damping-off symptoms were caused by Macrophomina, the diseased tissue of seedlings (lower stem and some of the root system) was dissected and aseptically transferred to sterile dionized 1% water-agar plates and incubated at 33 ºC for 3 to 5 days. The typical mycelium patterns and macrosclerotia of *M. phaseolina* were observed. Koch's postulate was partially confirmed by inoculating the root systems of one-week-old cowpea seedlings using a 1500 macrosclerotia/ml inoculum culture and a modified root-clip method (Rigert and Foster 1987). Macrophomina disease symptoms which consisted of brownish red lesions at the soil line accompanied with internal vascular stem discoloration were observed and on some older plants, ashy stem blight could be observed. However, seedling damping-off symptoms could not be reproduced using this inoculation method.

Genetic maps

The Sanzi x Vita 7 population was genotyped at the F_8 generation with bi-allelic SNP markers using the 1536 Illumina Golden Gate Assay as previously described (Muchero et al. 2009). The cowpea cultivars used for the marker-trait association study were SNP genotyped at the F_8 or later generation.

A SNP genetic map for the Sanzi x Vita 7 RIL population was previously created and is included in the cowpea consensus genetic map vs.2 (Muchero et al. 2009a), vs.3 (Diop et al. 2012) and vs.4 (Lucas et al. 2011). The individual map was generated using 122 RILs and 416 SNP markers and consists of 19 linkage groups which span approximately 753 cM (Lucas et al. 2011).

The cowpea consensus genetic map vs. 4 (Lucas et al. 2011) was the main consensus genetic map used for the present study. The cowpea consensus vs. 4 map consists of eleven RIL populations and two breeding populations which increased the marker density and improved the marker order (Lucas et al. 2011). The map is 680 cM in length and contains 1107 markers with an average of 0.65 cM between markers (Lucas et al. 2011). The cowpea consensus genetic map vs. 6 is a subsequent version of the cowpea consensus genetic map, which consists of 1091 SNP markers and is 680 cM in length. The version 6 genetic map is available via HarvEST: Cowpea (http://harvest.ucr.edu).

Statistical analysis

The Kruskal-Wallis and Interval Mapping analysis packages of MapQTL 5.0 were used to conduct the QTL analysis (Van Ooijen 2004). A QTL was considered significant if the same QTL was identified using both phenotypic ratings 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 determine the span of

QTLs using 1-LOD and 2-LOD to determine left and right margins. QTLs were visualized using MapChart 2.2 software (Voorrips 2002).

Synteny

Synteny was examined between cowpea and *G. max* and cowpea and *M. truncatula* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes (Lucas et al. 2011). Syntenic relationships amongst the different legume genomes can be examined in HarvEST: Cowpea (http://harvest.ucr.edu/). The soybean and Medicago annotations were taken from the Phytozome webpage (Goodstein et al. 2012). 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. Due to limited resolution in the software images, not all markers are presented in the screenshot images output from Harvest: Cowpea. The linkage group must be magnified using the HarvEST: Cowpea software in order to view each individual marker.

Marker-trait associations

Genotypic data comprised of cowpea varieties and SNP markers from the cowpea consensus genetic map for the *Mac-11* and *Mac-13* loci were visualized using Flapjack software (Milne et al. 2010).

Cowpea physical map

The physical map was developed using an advanced African breeding line IT97K-499-35 (http://phymap.ucdavis.edu/cowpea/). It consists of two BAC clone libraries developed

using restriction enzymes *Hind*III and *Mbo*I (Amplicon Express, Pullman, WA). Contigs were assembled using the snapshot method of DNA fingerprinting (Luo et al. 2003b) and was 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 (http://phymap.ucdavis.edu/cowpea/). The size of the BAC clones was estimated by multiplying the number of unique bands generated from the fingerprinting assay by 1640bp (personal communication, Ming Chen Luo).

Raw data sequences were generated for cowpea BACs using an Illumina HiSeq 2000 sequencer by John Weger at the Institute of Integrative Genome Biology, University of California, Riverside from DNA samples prepared by Yaqin Ma (UCR). Sequences of each BAC clone were generated from paired-end 100 base reads using the combinatorial pooling method described previously (Lonardi et al. 2013). A NODE is defined as a sequence or contig which can be consistently reconstructed using the sequencing reads (Zerbino and Birney 2008; Zerbino 2010b). All sequence data is publicly available via the Harvest: Cowpea database (http://harvest.ucr.edu/) and version 0.03 of the assembled cowpea genome (http://harvest-blast.org/). Cowpea genome version 0.03 which contained approximately 200 Mb of assembled scaffolds and contigs covered about 97% of previously identified cowpea genes is available for BLAST searches and sequence retrieval (http://harvest-blast.org/).

List of abbreviations

ARF: auxin response factor; AUX: auxin; BAC: bacterial artificial chromosome; bp: base pairs; cM: centiMorgan; EST: expressed sequence tags; G x E: genotype x environment; hpi: hours post inoculation; IAA: indole-3-acetic acid; LG: linkage group; LOD: logarithm (base 10) of odds; MAS; marker-assisted selection; Mb: megabases; PSII: photosystem II; QTL: quantitative trait loci; RKN; root-knot nematodes; RIL: recombinant inbred line; SNP: single nucleotide polymorphic sequence; TF: transcription factor; wpg: weeks post germination

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Authors' contributions

MP, JDE, TJC and PAR conceived and designed the experiment. MP conducted the experiments, statistical analyses and drafted the manuscript. SL led the BAC clone

sequencing. MP, JDE, TJC, and PAR wrote the paper. All authors read and approved the final manuscript.

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Table 4.1 QTL analysis of *M. phaseolina* resistance in Sanzi x Vita7 population, field experiment 2009.									
Date	Phenotype	QTL	LG	cM	Locus	Interval Mapping LOD	Interval Mapping R^2	Kruskal-Wallis test statistic	Kruskal-Wallis p-value
1 wpg	% Surviving	*Mac-10*	2	35.44	1_0223	2.50	8.6	9.66	0.0005
	% Surviving	*Mac-10*	2	35.92	1_1292	2.74	9.4	10.82	0.0005
	% Surviving	*Mac-10*	2	35.92	1_1121	2.74	9.4	10.82	0.0005
	% Surviving	*Mac-10*	2	35.92	1_1058	2.74	9.4	10.82	0.0005
	% Surviving	*Mac-10*	2	36.51	1_0381	2.90	9.9	11.47	0.001
	% Surviving	*Mac-10*	2	38.22	1_1079	2.49	8.6	9.90	0.0005
	% Surviving	*Mac-10*	2	40.08	1_0820	2.30	8.0	8.60	0.0005
	% Surviving	*Mac-10*	2	40.08	1_0772	2.30	8.0	8.60	0.0005
	% Surviving	*Mac-11*	5	19.31	1_0998	2.40	8.3	9.67	0.0005
	% Surviving	*Mac-11*	5	20.25	1_0495	2.59	8.9	10.47	0.0005
	% Surviving	*Mac-11*	5	20.96	1_0037	2.49	8.6	9.89	0.0005
	% Surviving	*Mac-11*	5	20.96	1_0879	2.49	8.6	9.89	0.0005
	% Surviving	*Mac-11*	5	22.23	1_0661	3.37	11.4	13.23	0.0005
	% Surviving	*Mac-11*	5	22.59	1_1128	3.16	10.7	12.27	0.0005
	% Surviving	*Mac-11*	5	26.44	1_1322	2.86	9.8	11.29	0.001
	% Surviving	*Mac-11*	5	27.45	1_0081	2.61	9.0	10.49	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0399	2.61	9.0	10.49	0.0005
	% Surviving	*Mac-11*	5	35.71	1_0251	3.66	12.4	15.45	0.0001
	% Surviving	*Mac-11*	5	38.38	1_1095	3.33	11.3	13.85	0.0005
	% Surviving	*Mac-12*	6	7.61	1_1302	2.74	9.4	10.00	0.0005
	% Damping	*Mac-11*	5	22.23	1_0661	2.49	8.6	9.31	0.005
	% Damping	*Mac-11*	5	22.593	1_1128	2.36	8.1	8.68	0.005
	% Damping	*Mac-11*	5	26.443	1_1322	2.58	8.9	9.98	0.005
	% Damping	*Mac-11*	5	27.45	1_0081	2.33	8.1	8.60	0.005
	% Damping	*Mac-11*	5	27.45	1_0399	2.33	8.1	8.60	0.005
	% Damping	*Mac-11*	5	35.71	1_0251	2.93	10	12.67	0.0001
	% Damping	*Mac-11*	5	38.376	1_1095	2.15	7.4	9.06	0.005
2 wpg	% Surviving	*Mac-11*	5	22.23	1_0661	2.30	7.9	9.54	0.0005
	% Surviving	*Mac-11*	5	22.59	1_1128	2.04	7.1	8.47	0.0005
	% Surviving	*Mac-11*	5	26.44	1_1322	2.17	7.5	8.93	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0081	1.99	6.9	8.05	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0399	1.99	6.9	8.05	0.0005
	% Surviving	*Mac-11*	5	35.71	1_0251	3.17	10.8	12.56	0.0005
	% Surviving	*Mac-11*	5	38.38	1_1095	2.93	10.0	11.83	0.001
	% Surviving	*Mac-12*	6	7.614	1_1302	2.50	8.6	10.26	0.005
	% Damping	*Mac-11*	5	35.71	1_0251	2.90	9.9	11.67	0.001

	% Damping	*Mac-11*	5	38.376	1_1095	2.32	8.0	9.30	0.005
3 wpg	% Surviving	*Mac-11*	5	35.71	1_0251	2.80	9.6	11.38	0.001
	% Surviving	*Mac-11*	5	38.38	1_1095	2.62	9.0	10.72	0.005
	% Surviving	*Mac-12*	6	7.61	1_1302	2.12	7.3	8.78	0.005
	% Damping	*Mac-11*	5	35.71	1_0251	3.01	10.3	12.09	0.001
	% Damping	*Mac-11*	5	38.376	1_1095	2.85	9.7	11.45	0.001
	% Damping	*Mac-12*	6	7.614	1_1302	2.46	8.5	10.21	0.005
4 wpg	% Surviving	*Mac-11*	5	35.71	1_0251	2.61	9.0	11.17	0.001
	% Surviving	*Mac-11*	5	38.38	1_1095	2.57	8.8	10.67	0.0005
	% Surviving	*Mac-12*	6	7.61	1_1302	2.24	7.7	10.00	0.0005
5 wpg	% Surviving	*Mac-11*	5	35.71	1_0251	2.93	10.0	12.46	0.0005
	% Surviving	*Mac-11*	5	38.38	1_1095	3.10	10.6	12.90	0.0005
	% Surviving	*Mac-12*	6	7.61	1_1302	2.24	7.7	9.65	0.005
6 wpg	% Surviving	*Mac-11*	5	22.23	1_0661	2.46	8.5	11.04	0.001
	% Surviving	*Mac-11*	5	22.59	1_1128	2.2	7.6	9.83	0.0005
	% Surviving	*Mac-11*	5	26.44	1_1322	1.94	6.8	8.53	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0081	1.86	6.5	8.19	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0399	1.86	6.5	8.19	0.0005
	% Surviving	*Mac-11*	5	35.71	1_0251	2.97	10.1	12.79	0.0005
	% Surviving	*Mac-11*	5	38.38	1_1095	3.10	10.5	13.21	0.0005
	% Surviving	*Mac-11*	5	39.54	1_0119	2.06	7.2	8.92	0.0005
	% Surviving	*Mac-11*	5	39.54	1_0242	2.06	7.2	8.92	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1243	2.06	7.2	8.92	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1253	2.06	7.2	8.92	0.0005
	% Surviving	*Mac-11*	5	39.54	1_0839	2.06	7.2	8.92	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1419	2.06	7.2	8.92	0.0005
	% Surviving	*Mac-11*	5	39.88	1_0926	2.06	7.2	9.78	0.0005
	% Surviving	*Mac-12*	6	7.61	1_1302	2.49	8.6	10.66	0.0005
	% Surviving	*Mac-12*	6	21.83	1_0023	2.06	7.1	8.70	0.0005
	% Surviving	*Mac-12*	6	21.83	1_0906	2.06	7.1	8.70	0.0005
	% Surviving	*Mac-12*	6	28.56	1_0270	2.02	7.0	7.89	0.0005
7 wpg	% Surviving	*Mac-11*	5	22.23	1_0661	2.67	9.1	12.28	0.0005
	% Surviving	*Mac-11*	5	22.59	1_1128	2.46	8.5	11.26	0.001
	% Surviving	*Mac-11*	5	26.44	1_1322	2.31	8.0	10.40	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0081	2.21	7.7	9.79	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0399	2.21	7.7	9.79	0.0005
	% Surviving	*Mac-11*	5	35.71	1_0251	3.26	11.1	14.06	0.0005
	% Surviving	*Mac-11*	5	38.38	1_1095	3.30	11.2	14.00	0.0005
	% Surviving	*Mac-11*	5	39.54	1_0119	2.20	7.6	9.70	0.0005

	% Surviving	*Mac-11*	5	39.54	1_0242	2.20	7.6	9.70	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1243	2.20	7.6	9.70	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1253	2.20	7.6	9.70	0.0005
	% Surviving	*Mac-11*	5	39.54	1_0839	2.20	7.6	9.70	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1419	2.20	7.6	9.70	0.0005
	% Surviving	*Mac-11*	5	39.88	1_0926	2.20	7.6	10.79	0.0005
	% Surviving	*Mac-12*	6	7.61	1_1302	3.14	10.7	14.90	0.0005
	% Surviving	*Mac-12*	6	17.81	1_0278	2.51	8.6	12.90	0.0005
	% Surviving	*Mac-12*	6	19.00	1_0385	2.64	9.1	12.70	0.0005
	% Surviving	*Mac-12*	6	19.98	1_0279	2.90	9.9	13.67	0.0005
	% Surviving	*Mac-12*	6	21.83	1_0023	3.06	10.4	14.27	0.0005
	% Surviving	*Mac-12*	6	21.83	1_0906	3.06	10.4	14.27	0.0005
	% Surviving	*Mac-12*	6	22.33	1_0708	2.63	9.0	12.34	0.0005
	% Surviving	*Mac-12*	6	27.37	1_0477	2.19	7.6	9.58	0.0005
	% Surviving	*Mac-12*	6	27.37	1_1057	2.19	7.6	9.58	0.0005
	% Surviving	*Mac-12*	6	27.37	1_1035	2.19	7.6	9.58	0.0005
	% Surviving	*Mac-12*	6	28.56	1_0270	2.43	8.4	10.69	0.0005
8 wpg	% Surviving	*Mac-11*	5	19.31	1_0998	2.26	7.8	10.86	0.001
	% Surviving	*Mac-11*	5	20.25	1_0495	2.44	8.4	12.01	0.001
	% Surviving	*Mac-11*	5	20.96	1_0037	2.47	8.5	12.15	0.0005
	% Surviving	*Mac-11*	5	20.96	1_0879	2.47	8.5	12.15	0.0005
	% Surviving	*Mac-11*	5	22.23	1_0661	3.42	11.6	15.99	0.0001
	% Surviving	*Mac-11*	5	22.59	1_1128	3.35	11.4	15.68	0.0001
	% Surviving	*Mac-11*	5	26.44	1_1322	2.78	9.5	13.07	0.0005
	% Surviving	*Mac-11*	5	27.45	1_0081	2.60	8.9	11.78	0.001
	% Surviving	*Mac-11*	5	27.45	1_0399	2.60	8.9	11.78	0.001
	% Surviving	*Mac-11*	5	35.71	1_0251	3.35	11.4	16.06	0.0001
	% Surviving	*Mac-11*	5	38.38	1_1095	3.60	12.1	16.45	0.0001
	% Surviving	*Mac-11*	5	39.54	1_0119	2.57	8.8	12.25	0.0005
	% Surviving	*Mac-11*	5	39.54	1_0242	2.57	8.8	12.25	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1243	2.57	8.8	12.25	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1253	2.57	8.8	12.25	0.0005
	% Surviving	*Mac-11*	5	39.54	1_0839	2.57	8.8	12.25	0.0005
	% Surviving	*Mac-11*	5	39.54	1_1419	2.57	8.8	12.25	0.0005
	% Surviving	*Mac-11*	5	39.88	1_0926	2.57	8.8	13.01	0.0005
	% Surviving	*Mac-12*	6	0.00	1_0006	3.16	10.8	12.52	0.0005
	% Surviving	*Mac-12*	6	0.00	1_0261	3.16	10.8	12.52	0.0005
	% Surviving	*Mac-12*	6	0.00	1_1266	3.16	10.8	12.52	0.0005
	% Surviving	*Mac-12*	6	0.00	1_1014	3.16	10.8	12.52	0.0005

	% Surviving	*Mac-12*	6	0.00	1_1446	3.16	10.8	12.52	0.0005
	% Surviving	*Mac-12*	6	0.00	1_1521	3.16	10.8	12.52	0.0005
	% Surviving	*Mac-12*	6	0.00	1_0763	3.16	10.8	12.52	0.0005
	% Surviving	*Mac-12*	6	0.00	1_1418	3.16	10.8	12.52	0.0005
	% Surviving	*Mac-12*	6	0.37	1_0719	2.99	10.3	11.78	0.001
	% Surviving	*Mac-12*	6	7.61	1_1302	4.38	14.6	18.41	0.0001
	% Surviving	*Mac-12*	6	17.81	1_0278	3.50	11.8	15.50	0.0001
	% Surviving	*Mac-12*	6	19.00	1_0385	3.38	11.4	14.35	0.0005
	% Surviving	*Mac-12*	6	19.98	1_0279	3.86	13.0	16.25	0.0001
	% Surviving	*Mac-12*	6	21.83	1_0023	4.09	13.7	17.10	0.0001
	% Surviving	*Mac-12*	6	21.83	1_0906	4.09	13.7	17.10	0.0001
	% Surviving	*Mac-12*	6	22.33	1_0708	3.70	12.5	15.61	0.0001
	% Surviving	*Mac-12*	6	27.37	1_0477	2.92	10.0	11.13	0.001
	% Surviving	*Mac-12*	6	27.37	1_1057	2.92	10.0	11.13	0.001
	% Surviving	*Mac-12*	6	27.37	1_1035	2.92	10.0	11.13	0.001
	% Surviving	*Mac-12*	6	28.56	1_0270	3.37	11.4	13.17	0.0005
	% Surviving	*Mac-12*	6	30.03	1_0621	2.33	8.0	9.73	0.0005

wpg- weeks post germination

Table 4.2 *Mac-10* on the Sanzi x Vita7 genetic map, the cowpea consensus genetic map and the cowpea physical map.								
Sanzi x Vita7 genetic map				Cowpea genetic map vs.4			Cowpea physical map	
LG	cM	Locus	LOD	LG	cM	Locus	Contig	BAC(s)
2	35.44	1_0223	2.5	3	27.24	1_0223	N/A	
		N/A		3	27.32	1_1512	365	CH049P18, CH061N02
		N/A		3	28.12	1_1072	N/A	
		N/A		3	28.12	1_1455	365	CM012F02
		N/A		3	28.21	1_0707	N/A	
		N/A		3	28.99	1_1056	N/A	
		N/A		3	28.99	1_1416	N/A	
		N/A		3	29.42	1_0067	N/A	
2	35.92	1_1292	2.74	3	29.52	1_1292	N/A	
		N/A		3	29.75	1_0194	N/A	
2	35.92	1_1058	2.74	3	29.75	1_1058	N/A	
		N/A		3	29.75	1_1106	N/A	
		N/A		3	30.54	1_0217	424	CM052A18, CH048L07
		N/A		3	30.54	1_1117	1107	CM060B15
2	38.22	1_1079	2.49	3	30.57	1_1079	N/A	
				3	30.70	1_0893	N/A	
		N/A		3	30.84	1_0424	1107	CM060B15
2	40.08	1_0772	2.3	3	30.90	1_0772	567	CM056D20
2	40.08	1_0820	2.3	3	31.12	1_0820	567	CM056D20, CM061J08, CM004K02
		N/A		3	31.40	1_0459	N/A	CH024M20, CH071B16
		N/A		3	31.41	1_0509	N/A	
		N/A		3	33.34	1_1483	N/A	
		N/A		3	33.71	1_0087	N/A	
		N/A		3	33.96	1_0104	N/A	
		N/A		3	33.96	1_0348	790	CM040H12
		N/A		3	34.01	1_1240	N/A	
		N/A		3	34.93	1_0465	357	CM041M09, CM034C13
		N/A		3	35.46	1_0017	N/A	
		N/A		3	35.46	1_0475	357	CM046A07, CM054A17
		N/A		3	35.65	1_0116	357	CM054A17, CH052E15
		N/A		3	35.65	1_0571	N/A	
		N/A		3	35.65	1_1145	N/A	
		N/A		3	36.61	1_0443	N/A	

		N/A		3	36.62	1_1396	216	CH096G20, CM065I11
		N/A		3	36.98	1_0491	N/A	
		N/A		3	37.72	1_0296	N/A	
		N/A		3	38.59	1_0636	428	CM063I18, CH048L02
		N/A		3	38.77	1_0812	N/A	
		N/A		3	38.90	1_1522	N/A	
		N/A		3	39.83	1_1349	N/A	
		N/A		3	39.96	1_0404	N/A	
		N/A		3	41.63	1_0843	110	CM043A17
		N/A		3	41.87	1_0020	N/A	
		N/A		3	41.87	1_0068	N/A	
		N/A		3	41.87	1_0133	N/A	
		N/A		3	41.87	1_0154	N/A	
		N/A		3	42.05	1_1024	428	CM054I20
		N/A		3	42.28	1_0388	N/A	
		N/A		3	43.97	1_0761	299	CH029F21, CM026C07
		N/A		3	43.97	1_1350	197	CM053A15, CM062N15, CH029F21
		N/A		3	43.97	1_1525	299	CH029F21, CM062N15
		N/A		3	44.29	1_0511	N/A	
		N/A		3	44.29	1_0713	820	CM043B21
		N/A		3	44.29	1_1023	N/A	
		N/A		3	44.29	1_1222	N/A	
		N/A		3	44.50	1_0788	118	CH075M09
		N/A		3	44.50	1_1293	N/A	
		N/A		3	44.50	1_1170	118	CM042L06, CH012G06
		N/A		3	44.70	1_1224	118	CH012G06, CH074E12, CH094H11
		N/A		3	44.93	1_0758	124	CM064M01
		N/A		3	44.93	1_1022	N/A	
		N/A		3	44.93	1_1427	124	CM064M01, CH001E06
		N/A		3	45.02	1_0086	124	CH001E06
		N/A		3	45.43	1_1286	305	CH079I11
		N/A		3	45.73	1_0064	305	CH079I11
		N/A		3	46.74	1_1205	N/A	
		N/A		3	46.74	1_1358	91	CM035L09, CM052K03
		N/A		3	47.13	1_0545	N/A	

		N/A		3	47.13	1_1348	690	CH003F13, CM050I08
		N/A		3	47.45	1_0299	181	CH054B14, CM031L09
		N/A		3	47.45	1_0686	181	CM031L09, CH054B14
		N/A		3	47.45	1_1171	181	CH054B14, CM022I20
		N/A		3	48.22	1_0982	68	CM040B23
		N/A		3	48.22	1_1122	339	CH002P16, CH068D20
		N/A		3	48.87	1_1277	339	CH068D20, CH095E08, CH002P16
		N/A		3	49.00	1_0145	339	CH030E02
		N/A		3	51.07	1_0740	86	CH031G07, CH093P22
		N/A		3	51.76	1_0209	N/A	
		N/A		3	51.76	1_0769	N/A	
		N/A		3	51.76	1_0900	N/A	
		N/A		3	52.26	1_0243	212	CM055F03, CH075L16
		N/A		3	52.26	1_1005	142	CM001M16, CH009B04
		N/A		3	52.43	1_0163	N/A	
		N/A		3	52.43	1_0176	917	CH057K24, CH077E17
		N/A		3	52.43	1_1439	N/A	
		N/A		3	53.03	1_0247	63	CH092M17, CH062H10
		N/A		3	53.88	1_0971	63	CM036K13
		N/A		3	55.36	1_1300	72	CH013B05, CH096P15, CM016F05
		N/A		3	55.92	1_0959	63	CM006D11
		N/A		3	56.74	1_0331	873	CM029O09
		N/A		3	57.38	1_0968	57	CM052B21, CH055P07
		N/A		3	58.49	1_0654	179	CM052M15
		N/A		3	60.08	1_0178	N/A	
		N/A		3	62.72	1_0265	N/A	
		N/A		3	64.44	1_0953	398	CH042B12
		N/A		3	64.78	1_0604	N/A	
		N/A		3	65.16	1_0444	N/A	
		N/A		3	65.16	1_1027	N/A	
		N/A		3	65.51	1_0400	N/A	
		N/A		3	66.99	1_0139	736	CH080L05, CM027B20
		N/A		3	66.99	1_0207	N/A	
		N/A		3	66.99	1_1369	N/A	
		N/A		3	67.15	1_0938	N/A	

		N/A		3	67.55	1_0831	N/A	
		N/A		3	68.17	1_1075	1081	CM008G11
		N/A		3	68.49	1_1109	1004	CM029M15, CM005N24
		N/A		3	70.89	1_1085	N/A	
		N/A		3	71.52	1_1087	N/A	
		N/A		3	71.75	1_0352	1094	CM052M22
		N/A		3	73.42	1_0380	1045	CM045O05
		N/A		3	73.42	1_0984	1045	CM045O05
		N/A		3	73.79	1_1162	756	CH093M08
		N/A		3	77.55	1_0345	N/A	
		N/A		3	77.55	1_0964	N/A	
		N/A		3	77.55	1_0718	N/A	
		N/A		3	77.55	1_1452	N/A	
		N/A		3	78.71	1_1015	N/A	
		N/A		3	79.20	1_1513	N/A	
		N/A		3	79.86	1_1134	N/A	
		N/A		3	80.23	1_0594	N/A	
2	35.92	1_1121	2.74	3	85.05	1_1121	N/A	
2	36.51	1_0381	2.9	3	86.07	1_0381	N/A	

Table 4.3 Weather data for 2009 and 2010 in Riverside, California at the Citrus Research Experiment Station.

2009	Max. temp (°C)	Min. temp (°C)	Rainfall (mm)	2010	Max. temp (°C)	Min. temp (°C)	Rainfall (mm)
6/1/2009	28	15	0	6/1/2010	24	12	0
6/2/2009	32	15	0	6/2/2010	27	13	0
6/3/2009	29	14	0.254	6/3/2010	28	13	0
6/4/2009	25	15	0	6/4/2010	29	14	0
6/5/2009	23	13	0	6/5/2010	33	16	0
6/6/2009	23	13	0	6/6/2010	32	15	0
6/7/2009	25	15	0	6/7/2010	31	16	0
6/8/2009	26	13	0	6/8/2010	28	16	0
6/9/2009	22	14	0	6/9/2010	26	16	0
6/10/2009	23	14	0	6/10/2010	24	15	0
6/11/2009	23	14	0	6/11/2010	22	14	0
6/12/2009	23	15	0	6/12/2010	22	14	0
6/13/2009	24	15	0	6/13/2010	28	12	0
6/14/2009	27	13	0	6/14/2010	32	14	0
6/15/2009	25	13	0	6/15/2010	31	15	0
6/16/2009	27	14	0	6/16/2010	26	13	0
6/17/2009	28	14	0	6/17/2010	28	12	0
6/18/2009	32	14	0	6/18/2010	27	13	0
6/19/2009	33	16	0	6/19/2010	28	12	0
6/20/2009	25	17	0	6/20/2010	28	12	0
6/21/2009	28	15	0	6/21/2010	26	13	0
6/22/2009	30	12	0	6/22/2010	28	12	0
6/23/2009	32	13	0	6/23/2010	30	13	0
6/24/2009	30	15	0	6/24/2010	31	14	0
6/25/2009	31	14	0	6/25/2010	29	14	0
6/26/2009	34	13	0	6/26/2010	27	14	0
6/27/2009	37	15	0	6/27/2010	29	14	0
6/28/2009	39	18	0	6/28/2010	27	14	0
6/29/2009	34	18	0	6/29/2010	28	16	0
6/30/2009	33	17	0	6/30/2010	32	16	0
7/1/2009	36	18	0	7/1/2010	32	16	0
7/2/2009	36	17	0	7/2/2010	31	15	0

7/3/2009	34	16	0	7/3/2010	27	14	0
7/4/2009	37	15	0	7/4/2010	27	14	0
7/5/2009	36	17	0	7/5/2010	27	14	0
7/6/2009	35	15	0	7/6/2010	24	14	0
7/7/2009	34	15	0	7/7/2010	26	14	0
7/8/2009	34	14	0	7/8/2010	26	14	0
7/9/2009	35	13	0	7/9/2010	31	13	0
7/10/2009	38	15	0	7/10/2010	29	16	0
7/11/2009	38	17	0	7/11/2010	29	16	0
7/12/2009	38	17	0	7/12/2010	31	16	0
7/13/2009	38	18	0	7/13/2010	35	16	0
7/14/2009	37	17	0	7/14/2010	37	19	0
7/15/2009	37	18	0	7/15/2010	38	22	0
7/16/2009	38	19	0	7/16/2010	38	22	0
7/17/2009	38	19	0	7/17/2010	38	22	0
7/18/2009	41	20	0	7/18/2010	35	20	0
7/19/2009	41	23	0	7/19/2010	33	18	0
7/20/2009	39	22	0	7/20/2010	31	17	0
7/21/2009	39	20	0	7/21/2010	29	16	0
7/22/2009	38	19	0	7/22/2010	30	16	0
7/23/2009	37	19	0	7/23/2010	31	16	0
7/24/2009	35	19	0	7/24/2010	32	17	0
7/25/2009	36	19	0	7/25/2010	29	16	0
7/26/2009	37	20	0	7/26/2010	28	15	0
7/27/2009	37	19	0	7/27/2010	27	14	0
7/28/2009	36	18	0	7/28/2010	29	13	0
7/29/2009	33	18	0	7/29/2010	31	14	0
7/30/2009	34	18	0	7/30/2010	30	15	0
7/31/2009	35	19	0	7/31/2010	31	15	0
8/1/2009	34	18	0	8/1/2010	31	14	0
8/2/2009	34	17	0	8/2/2010	34	16	0
8/3/2009	38	18	0	8/3/2010	33	16	0
8/4/2009	39	19	0	8/4/2010	32	16	0
8/5/2009	37	19	0	8/5/2010	31	14	0
8/6/2009	32	16	0	8/6/2010	31	13	0

8/7/2009	29	16	0	8/7/2010	28	14	0
8/8/2009	32	15	0	8/8/2010	28	14	0
8/9/2009	33	14	0	8/9/2010	29	13	0
8/10/2009	35	16	0	8/10/2010	30	13	0
8/11/2009	35	16	0	8/11/2010	30	13	0
8/12/2009	35	16	0	8/12/2010	31	13	0
8/13/2009	35	15	0	8/13/2010	32	14	0
8/14/2009	33	17	0	8/14/2010	33	13	0
8/15/2009	31	16	0	8/15/2010	34	14	0
8/16/2009	31	16	0	8/16/2010	36	18	0
8/17/2009	33	14	0	8/17/2010	36	19	0
8/18/2009	33	14	0	8/18/2010	36	21	0
8/19/2009	32	14	0	8/19/2010	34	21	0
8/20/2009	34	15	0	8/20/2010	35	18	0
8/21/2009	31	17	0	8/21/2010	35	18	0
8/22/2009	37	21	0	8/22/2010	36	18	0
8/23/2009	34	18	0	8/23/2010	38	18	0
8/24/2009	36	17	0	8/24/2010	39	19	0
8/25/2009	38	16	0	8/25/2010	41	21	0
8/26/2009	40	16	0	8/26/2010	39	22	0
8/27/2009	42	18	0	8/27/2010	33	16	0
8/28/2009	41	19	0	8/28/2010	26	14	0
8/29/2009	42	22	0	8/29/2010	24	14	0
8/30/2009	41	20	0	8/30/2010	26	14	0
8/31/2009	39	19	0	8/31/2010	31	13	0
9/1/2009	38	22	0	9/1/2010	36	14	0

Table 4.4 *Mac-10* on the Sanzi x Vita7 genetic map, the cowpea consensus genetic map vs. 6 and the cowpea physical map.

Sanzi x Vita7 genetic map				Cowpea genetic map vs.6				Cowpea physical map	
LG	cM	Locus	LOD	LG	cM	Locus	Annotation	Contig	BAC(s)
2	40.08	1_0820	2.3	3	74.80	1_0820	SufE/NifU family protein	567	CM056D20 CM061J08 CM004K02
2	40.08	1_0772	2.3	3	75.16	1_0772	1-deoxy-D- xylulose 5-phosphate reductoisomerase	567	CM056D20
2	38.22	1_1079	2.49	3	76.00	1_1079	Gibberellin- regulated family protein	N/A	
		N/A		3	76.17	1_0893	Protein kinase superfamily protein	N/A	
2	36.51	1_0381	2.9	3	76.68	1_0381	Ribosomal protein S4	N/A	
		N/A		3	77.07	1_0194	Pyridine nucleotide-disulphide oxidoreductase family	N/A	
2	35.92	1_1058	2.74	3	77.07	1_1058	Protein kinase superfamily protein	N/A	
		N/A		3	77.07	1_1106	Fucosyltransferase 1	N/A	
		N/A		3	77.08	1_0619	Thioredoxin superfamily protein	N/A	
2	35.92	1_1292	2.74	3	77.24	1_1292	D-ribulose-5- phosphate-3-epimerase	N/A	
2	35.92	1_1121	2.74	3	77.51	1_1121	Galactosyl transferase GMA12/MNN10 family protein	N/A	
		N/A		3	78.18	1_0707	Glycine-rich RNA-binding protein 3	N/A	
2	35.44	1_0223	2.5	3	78.61	1_0223	Plasmodesmata callose-binding protein 3	N/A	

Table 4.5 QTLs for *M. phaseolina* resistance, drought tolerance and maturity on the cowpea consensus genetic map.				
QTL	Reference	Cowpea consensus genetic map vs.4		
		LG	QTL interval (cM)	Most significant marker (cM position)
Dro-7	Muchero et al. 2009b	1	5.20 - 63.48	1_0029 (62.70)
Mac-1	Muchero et al. 2011	2	55.60 - 67.32	1_0709 (67.32)
Mac-2	Muchero et al. 2011	3	10.20 - 24.10	1_0853 (10.82)
Mac-5	Muchero et al. 2011	3	11.06 - 17.27	1_0496 (11.06), 1_0079 (11.85)
Dro-10	Muchero et al. 2009b	3	14.00 - 92.43	1_0464 (24.20)
Mac-4	Muchero et al. 2011	3	24.22 - 36.98	1_0464 (24.22), 1_0201 (26.29)
Mac-10	This study	3	27.04 – 86.07	1_0381 (86.07)
Mac-3	Muchero et al. 2011	3	44.50 - 77.55	1_0604 (64.78)
Dro-8	Muchero et al. 2009b	4	0.30 - 40.51	1_1209 (27.90), 1_0910 (34.10), 1_1013 (34.10)
Mac-13	This study	4	20.72 - 25.57	*1_1242 (20.72), 1_0826 (24.55)
Mac-7	Muchero et al. 2011	4	20.72 - 35.96	1_0153 (21.49), 1_0678 (27.60)
Mat-1	Muchero et al. 2009b, 2011	4	21.49 - 27.60	1_0678 (27.60)
Mat-2	Muchero et al. 2009b, 2011	4	37.46 - 45.67	1_0804 (40.51)
Mac-6	Muchero et al. 2011	4	37.46 - 45.67	1_0699 (39.44), 1_0804 (40.51)
Mac-8	Muchero et al. 2011	5	21.57 - 37.73	1_0030 (29.40)
Mac-11	This study	5	37.04 - 50.85	*1_1419 (37.73), 1_1095 (39.04), 1_0251 (40.44), 1_0495 (50.52)
Mac-9	Muchero et al. 2011	5	42.51 - 57.58	1_0032 (45.28), 1_1533 (42.51)
Mac-12	This study	7	4.09 - 31.04	1_1302 (9.96), 1_0278 (17.98), 1_0708 (24.53)
Dro-1	Muchero et al. 2009b	7	13.15 - 32.69	1_0983 (21.60)
Dro-3	Muchero et al. 2009b	8	40.41- 61.70	1_1198 (60.60)
Dro-3	Muchero et al. 2009b	11	2.93 - 41.74	1_0562 (32.20)

Table 4.6 Synteny of the *Mac-10* locus on the cowpea consensus genetic map vs.6 with soybean chromosomes 5 and 17.

G. max chromosome	*G. max* locus	Phytozome annotation	Cowpea genetic map vs.6		
			Cowpea SNP	LG	cM
Gm05	Glyma05g00400	GR-RBP3 (Glycine-rich RNA-binding protein 3); RNA binding	1_0707	3	78.18
Gm05	Glyma05g00860	Uncharacterized protein	1_1121	3	77.51
Gm05	Glyma05g00910	RPE (EMBRYO DEFECTIVE 2728); ribulose-phosphate 3-epimerase	1_1292	3	77.24
Gm05	Glyma05g00920	Uncharacterized protein	1_1106	3	77.07
Gm05	Glyma05g01180	40S ribosomal protein S9 (RPS9C)	1_0381	3	76.68
Gm05	Glyma05g01280	WRKY72 (WRKY DNA-binding protein 72); transcription factor	N/A		
Gm05	Glyma05g02190	Uncharacterized protein	1_0772	3	75.16
Gm17	Glyma17g08630	GR-RBP3 (Glycine-rich RNA-binding protein 3); RNA binding	1_0707	3	78.18
Gm17	Glyma17g08910	GAUT10/LGT4 (Galacturonosyltransferase 10)	1_1362	8	11.53
Gm17	Glyma17g09070	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein	1_0014	6	50.11
Gm17	Glyma17g09100	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein	1_0014	6	50.11
Gm17	Glyma17g09160	CDF1 (CELL GROWTH DEFECT FACTOR 1); heat shock protein binding	1_0201	3	79.04
Gm17	Glyma17g09670	ISU1 (Iron-sulfur cluster assembly complex protein)	1_0820	3	74.80
Gm17	Glyma17g09740	Uncharacterized protein	1_0772	3	75.16
Gm17	Glyma17g10630	WRKY72 (WRKY DNA-binding protein 72); transcription factor	N/A		
Gm17	Glyma17g10990	RPE (EMBRYO DEFECTIVE 2728); ribulose-phosphate 3-epimerase	1_1292	3	77.24
Gm17	Glyma17g11060	Uncharacterized protein	1_1121	3	77.51

Table 4.7 QTL analysis of *Macrophomina phaseolina* resistance in Sanzi x Vita7 population, field experiment 2010.								
Date	QTL	LG	cM	Locus	Interval Mapping LOD	Interval Mapping R^2	Kruskal-Wallis test statistic	Kruskal-Wallis p-value
1wpg	*Mac-12*	6	31.48	1_0392	2.36	8.3	12.95	0.0005
	Mac-12	6	32.15	1_0019	2.12	7.5	11.74	0.001
	Mac-12	6	32.15	1_0632	2.12	7.5	11.74	0.001
	Mac-12	6	32.15	1_0883	2.12	7.5	11.74	0.001
	Mac-12	6	33.17	1_1315	2.36	8.3	11.03	0.001
	Mac-12	6	40.00	1_0559	3.36	11.6	16.26	0.0001
	Mac-12	6	44.77	1_0056	3.1	10.8	13.09	0.0005
	Mac-12	6	44.77	1_0824	3.1	10.8	13.09	0.0005
	Mac-12	6	45.16	1_0305	2.12	7.5	9.84	0.005
2 wpg	*Mac-11*	5	19.31	1_0998	2.25	7.8	9.33	0.005
	Mac-11	5	20.25	1_0495	2.66	9.2	11.12	0.001
	Mac-11	5	20.96	1_0037	2.55	8.8	10.59	0.005
	Mac-11	5	20.96	1_0879	2.55	8.8	10.59	0.005
	Mac-11	5	22.23	1_0661	2.73	9.4	11.48	0.001
	Mac-11	5	22.59	1_1128	2.74	9.4	11.84	0.001
	Mac-13	10	41.89	1_1242	2.05	7.1	6.52	0.05
	Mac-13	10	48.25	1_0535	2.29	7.9	7.75	0.01
3 wpg	*Mac-11*	5	19.31	1_0998	2.25	7.8	9.33	0.005
	Mac-11	5	20.25	1_0495	2.66	9.2	11.12	0.001
	Mac-11	5	20.96	1_0037	2.55	8.8	10.59	0.005
	Mac-11	5	20.96	1_0879	2.55	8.8	10.59	0.005
	Mac-11	5	22.23	1_0661	2.73	9.4	11.48	0.001
	Mac-11	5	22.59	1_1128	2.74	9.4	11.84	0.001
	Mac-13	10	41.89	1_1242	2.05	7.1	6.52	0.05
	Mac-13	10	48.25	1_0535	2.29	7.9	7.75	0.01
4-7 wpg	*Mac-11*	5	19.31	1_0998	2.68	9.3	10.77	0.005
	Mac-11	5	20.25	1_0495	3.17	10.8	12.81	0.0005
	Mac-11	5	20.96	1_0037	3.05	10.4	12.26	0.0005
	Mac-11	5	20.96	1_0879	3.05	10.4	12.26	0.0005
	Mac-11	5	22.23	1_0661	3.26	11.1	13.22	0.0005
	Mac-11	5	22.59	1_1128	3.24	11.1	13.51	0.0005
	Mac-11	5	26.44	1_1322	1.91	6.6	7.97	0.005
	Mac-11	5	27.45	1_0081	1.76	6.1	6.94	0.01
	Mac-11	5	27.45	1_0399	1.76	6.1	6.94	0.01
	Mac-11	5	35.71	1_0251	2.29	7.9	9.75	0.005
	Mac-11	5	38.38	1_1095	2.01	7	8.90	0.005
	Mac-11	5	39.54	1_0119	2.16	7.5	9.75	0.005

	Mac-11	5	39.54	1_0242	2.16	7.5	9.75	0.005
	Mac-11	5	39.54	1_1243	2.16	7.5	9.75	0.005
	Mac-11	5	39.54	1_1253	2.16	7.5	9.75	0.005
	Mac-11	5	39.54	1_0839	2.16	7.5	9.75	0.005
	Mac-11	5	39.54	1_1419	2.16	7.5	9.75	0.005
	Mac-11	5	39.88	1_0926	2.16	7.5	6.00	0.05
	Mac-11	5	41.68	1_0578	1.5	5.3	7.72	0.01
	Mac-11	5	41.68	1_1359	1.5	5.3	7.72	0.01
	Mac-11	5	49.01	1_0030	2.23	7.7	12.06	0.001
	Mac-11	5	56.17	1_0662	2.21	7.6	11.73	0.001
	Mac-11	5	57.16	1_1492	2.33	8	12.09	0.001
	Mac-13	10	41.89	1_1242	2.15	7.4	7.35	0.01
8 wpg	*Mac-11*	5	19.31	1_0998	2.53	8.7	13.03	0.0005
	Mac-11	5	20.25	1_0495	2.72	9.4	13.76	0.0005
	Mac-11	5	20.96	1_0037	2.31	8	11.96	0.001
	Mac-11	5	20.96	1_0879	2.31	8	11.96	0.001
	Mac-11	5	22.23	1_0661	2.1	7.3	10.80	0.005
	Mac-11	5	22.59	1_1128	2.63	9.1	12.64	0.0005
	Mac-12	6	7.61	1_1302	2.22	7.7	9.86	0.005
	Mac-12	6	17.81	1_0278	3.48	11.8	14.09	0.0005
	Mac-12	6	19.00	1_0385	2.25	7.8	8.98	0.005
	Mac-12	6	19.98	1_0279	2.66	9.1	11.20	0.001
	Mac-12	6	21.83	1_0023	3.76	12.6	15.33	0.0001
	Mac-12	6	21.83	1_0906	3.76	12.6	15.33	0.0001
	Mac-12	6	22.33	1_0708	3.95	13.3	16.12	0.0001
	Mac-12	6	27.37	1_0477	2.87	9.8	13.50	0.0005
	Mac-12	6	27.37	1_1057	2.87	9.8	13.50	0.0005
	Mac-12	6	27.37	1_1035	2.87	9.8	13.50	0.0005
	Mac-12	6	28.56	1_0270	3.33	11.3	15.03	0.0005
	Mac-12	6	30.03	1_0621	2.27	7.8	9.79	0.005
	Mac-13	10	51.21	1_0874	2.04	7.1	6.65	0.01
	Mac-13	10	51.21	1_0646	2.04	7.1	6.65	0.01
	Mac-13	10	51.65	1_0826	2.4	8.3	8.19	0.005
	Mac-13	10	55.18	1_0106	2.12	7.3	6.40	0.05
9 wpg	*Mac-11*	5	6.64	1_0889	2.36	8.1	10.02	0.005
	Mac-11	5	7.62	1_0099	2	6.9	7.62	0.01
	Mac-11	5	19.31	1_0998	4.41	14.8	19.65	0.0001
	Mac-11	5	20.25	1_0495	4.91	16.3	21.87	0.0001
	Mac-11	5	20.96	1_0037	4.56	15.3	19.76	0.0001

	Mac-11	5	20.96	1_0879	4.56	15.3	19.76	0.0001
	Mac-11	5	22.23	1_0661	4.6	15.4	19.68	0.0001
	Mac-11	5	22.59	1_1128	4.67	15.6	20.41	0.0001
	Mac-11	5	26.44	1_1322	3.1	10.6	15.70	0.0001
	Mac-11	5	27.45	1_0081	2.91	10	13.87	0.0005
	Mac-11	5	27.45	1_0399	2.91	10	13.87	0.0005
	Mac-12	6	0.00	1_0006	2.47	8.5	7.80	0.01
	Mac-12	6	0.00	1_0261	2.47	8.5	7.80	0.01
	Mac-12	6	0.00	1_1266	2.47	8.5	7.80	0.01
	Mac-12	6	0.00	1_1014	2.47	8.5	7.80	0.01
	Mac-12	6	0.00	1_1446	2.47	8.5	7.80	0.01
	Mac-12	6	0.00	1_1521	2.47	8.5	7.80	0.01
	Mac-12	6	0.00	1_0763	2.47	8.5	7.80	0.01
	Mac-12	6	0.00	1_1418	2.47	8.5	7.80	0.01
	Mac-12	6	0.37	1_0719	2.46	8.5	7.98	0.005
	Mac-12	6	7.61	1_1302	3.03	10.3	11.52	0.001
	Mac-12	6	17.81	1_0278	4.54	15.1	16.28	0.0001
	Mac-12	6	19.00	1_0385	3.11	10.6	11.50	0.001
	Mac-12	6	19.98	1_0279	3.29	11.2	13.08	0.0005
	Mac-12	6	21.83	1_0023	3.92	13.1	16.51	0.0001
	Mac-12	6	21.83	1_0906	3.92	13.1	16.51	0.0001
	Mac-12	6	22.33	1_0708	3.97	13.3	16.45	0.0001
	Mac-12	6	27.37	1_0477	2.16	7.5	9.52	0.005
	Mac-12	6	27.37	1_1057	2.16	7.5	9.52	0.005
	Mac-12	6	27.37	1_1035	2.16	7.5	9.52	0.005
	Mac-12	6	28.56	1_0270	2.74	9.4	11.62	0.001
	Mac-13	10	48.25	1_0535	2.82	9.6	9.92	0.005
	Mac-13	10	49.57	1_1092	2.65	9.1	8.82	0.005
	Mac-13	10	51.21	1_0874	2.88	9.8	9.49	0.005
	Mac-13	10	51.21	1_0646	2.88	9.8	9.49	0.005
	Mac-13	10	51.65	1_0826	3.16	10.8	10.79	0.005
	Mac-13	10	55.18	1_0106	2.45	8.4	8.83	0.005

Table 4.8 *Mac-11* on Sanzi x Vita7 individual map, cowpea consensus genetic map and the cowpea physical map

Sanzi x Vita 7 genetic map				Cowpea genetic map vs.4			Cowpea physical map	
LG	cM	Locus	LOD	LG	Locus	cM	contig	BAC(s)
5	39.88	1_0926	2.06	5	1_0926	37.04	N/A	
5	39.54	1_0242	2.06	5	1_0242	37.23	426	CH086N04, CH038D17
5	39.54	1_0839	2.06	5	1_0839	37.23	426	CH038D17
5	39.54	1_1419	2.06	5	1_1419	37.73	426	CH038D17
5	39.54	1_0119	2.06	5	1_0119	38.09	N/A	
5	39.54	1_1253	2.06	5	1_1253	38.09	N/A	
5	39.54	1_1243	2.06	5	1_1243	38.39	488	CM002F20, CH032A08
5	38.38	1_1095	3.10	5	1_1095	39.04	N/A	
5	35.71	1_0251	2.97	5	1_0251	40.44	N/A	
		N/A		5	1_0346	42.05	N/A	
		N/A		5	1_0677	42.51	623	CM001D10, CM057F03
		N/A		5	1_1533	42.51	623	CM057F03, CM001D10
		N/A		5	1_0205	43.29	380	CM047E12
5	27.45	1_0081	1.86	5	1_0081	43.99	N/A	
		N/A		5	1_0225	43.99	N/A	
5	27.45	1_0399	1.86	5	1_0399	43.99	N/A	
		N/A		5	1_0127	44.42	N/A	
5	26.44	1_1322	1.94	5	1_1322	44.42	N/A	
		N/A		5	1_0032	45.27	N/A	
		N/A		5	1_0193	45.27	N/A	
		N/A		5	1_0287	45.27	N/A	
5	22.59	1_1128	2.20	5	1_1128	45.76	217	CM018C23
		N/A		5	1_0120	46.51	217	CM018C23
		N/A		5	1_0945	46.51	N/A	
5	22.23	1_0661	2.46	5	1_0661	47.18	N/A	

		N/A		5	1_0924	47.18	93	CM028D11
		N/A		5	1_0866	47.57	N/A	
		N/A		5	1_0226	48.96	822	CH057B24
5	20.96	1_0037	2.49	5	1_0037	49.10	N/A	
5	20.96	1_0879	2.49	5	1_0879	49.10	N/A	
		N/A		5	1_0548	49.89	N/A	
5	20.25	1_0495	2.59	5	1_0495	50.52	822	CH057B24, CM014M10
5	19.31	1_0998	2.40	5	1_0998	50.85	822	CH013J17, CH076M20

Table 4.9 Synteny of *Mac-11* locus with *G. max* chromosomes 2 and 14.					
G. max Chromosome	*G. max* locus	Phytozome annotation	Cowpea SNP	LG	cM
2	Glyma02g40530	Inosine-5-monophophate dehydrogenase	1_0242	5	37.23
2	Glyma02g40640	AMP dependent ligase/synthase	1_1419	5	37.73
2	Glyma02g40650	Auxin response factor	N/A		
2	Glyma02g40820	NADP-dependent isocitrate dehydrogenase	1_0119	5	38.09
2	Glyma02g40900	Putative RNA binding protein	1_1243	5	38.39
2	Glyma02g41220	Histone H1/H5	1_0251	5	40.44
2	Glyma02g41840	MAM33, mitochondrial matrix glycoprotein	1_0677	5	42.51
2	Glyma02g42260	60s Acidic ribosomal protein	1_0205	5	43.29
2	Glyma02g42560	Vesicle coat protein clathrin, heavy chain	1_0127	5	44.42
2	Glyma02g43550	Tyrosine phosphatase family	1_1128	5	45.76
2	Glyma02g43560	Iron/ascorbate family oxidoreductases	1_0120	5	46.51
2	Glyma02g43640	Glycosyl hydrolases family 17	1_0945	5	46.51
14	Glyma14g04890	Ubiquitin carboxyl-terminal hydrolase	1_0924	5	47.18
14	Glyma14g05250	Subtilisin/ kexin-related	1_0661	5	47.18
14	Glyma14g05270	Subtilisin/ kexin-related	1_0661	5	47.18
14	Glyma14g05300	Glycosyl hydrolases family 17	1_0945	5	46.51
14	Glyma14g05390	Iron/ascorbate family oxidoreductases	1_0120	5	46.51
14	Glyma14g05400	Tyrosine phosphatase family	1_1128	5	45.76
14	Glyma14g05800	SEC61 gamma subunit	1_0032	5	45.27
14	Glyma14g06330	Circadian protein clock/ARNT/BMAL/PAS	1_1322	5	44.42
14	Glyma14g06630	60S ribosomal protein family member	1_0205	5	43.29
14	Glyma14g06910	Small heat-shocked protein (HSP20)	1_0346	5	42.05
14	Glyma14g07110	Uncharacterized	1_1533	5	42.51
14	Glyma14g07130	MAM33, mitochondrial matrix glycoprotein	1_0677	5	42.51
14	Glyma14g38930	NC domain	1_0839	5	37.23
14	Glyma14g38940	Auxin response factor	N/A		
14	Glyma14g38950	40S Ribosomal protein S26	1_0081	5	43.99
14	Glyma14g39160	NADP-dependent isocitrate dehydrogenase	1_0119	5	38.09
14	Glyma14g39220	Putative RNA binding protein	1_1243	5	38.39
14	Glyma14g39280	Zinc ion binding	1_1095	5	39.04

Table 4.10 Synteny of *Mac-11* locus with *M. truncatula* chromosome 5.					
M. truncatula chromosome	*M. truncatula* locus	Phytozome annotation	Cowpea SNP	LG	cM
5	Medtr5g083440	Protein of unknown function	1_1253	5	38.09
5	Medtr5g083510	Protein of unknown function	1_0926	5	37.04
5	Medtr5g083950	Inosine-5-monophosphate dehydrogenase	1_0242	5	37.23
5	Medtr5g084090	AMP dependent ligase/synthetase	1_1419	5	37.73
5	Medtr5g084100	NC domain	1_0839	5	37.23
5	Medtr5g084140	Auxin response factor	N/A		
5	Medtr5g084930	Isocitrate/isopropylmalate dehydrogenase	1_0119	5	38.09
5	Medtr5g085030	PRP38 family	1_1243	5	38.39
5	Medtr5g085190	Zinc finger, C2H2 type	1_1095	5	39.04

Table 4.11 Synteny of *Mac-11* locus with *P. vulgaris* chromosome 8.					
P.vulgaris chromosome	*P. vulgaris* locus	Phytozome annotation	Cowpea SNP	LG	cM
8	Phvul.008G203600	Late embryogenesis abundant protein, group 2	1_0495	5	50.52
8	Phvul.008G204800	Translation elongation factor EF1B/ribosomal protein S6	1_0226	5	48.96
8	Phvul.008G208900	Hydroxy methylglutaryl CoA reductase 1	1_0866	5	47.57
8	Phvul.008G210300	Ubiquitin-specific protease 12	1_0924	5	47.18
8	Phvul.008G213300	Subtilisin-like serine endopeptidase family protein	1_0661	5	47.18
8	Phvul.008G213400	O-Glycosyl hydrolases family 17 protein	1_0945	5	46.51
8	Phvul.008G214200	Ethylene-forming enzyme	1_0120	5	46.51
8	Phvul.008G214300	Phosphotyrosine protein phosphatases superfamily protein	1_1128	5	45.76
8	Phvul.008G225300	Transmembrane amino acid transporter family protein	1_0205	5	43.29
8	Phvul.008G230100	DHFS-FPGS homolog B	1_1533	5	42.51
8	Phvul.008G230400	Mitochondrial glycoprotein family protein	1_0677	5	42.51
8	Phvul.008G239200	PRP38 family protein	1_1243	5	38.39
8	Phvul.008G242400	Auxin response factor	N/A		
8	Phvul.008G242500	NC domain-containing protein-related	1_0839	5	37.23
8	Phvul.008G242600	Acyl activating enzyme 5	1_1419	5	37.73
8	Phvul.008G243400	TRAM, LAG1 and CLN8 (TLC) lipid-sensing domain	1_0242	5	37.23

Table 4.12 Annotations for the *Mac-11* locus on clone CH038D17 of contig 426 on the cowpea physical map.			
Cowpea BAC node	e- score	*P. vulgaris* locus/cowpea SNP	*P. vulgaris* annotation
CH038D17_VU1.3_NODE_0001	0	Phvul.008G243200/1_0242	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein
CH038D17_VU1.3_NODE_0002	0	Phvul.008G243600	Tetratricopeptide repeat (TPR)-like superfamily protein
CH038D17_VU1.3_NODE_0004	0	Phvul.008G242800	Nijmegen breakage syndrome 1
CH038D17_VU1.3_NODE_0005	2.00E-122	Phvul.008G243800	ABC transporter of the mitochondrion 3
CH038D17_VU1.3_NODE_0007	0	Phvul.008G243000	No functional annotation
CH038D17_VU1.3_NODE_0008	0	Phvul.008G242500/1_0839	NC domain-containing protein-related
CH038D17_VU1.3_NODE_0013	4.00E-116	Phvul.008G243300	Cystathionine beta-synthase (CBS) family protein
CH038D17_VU1.3_NODE_0022	0	Phvul.008G242200	P-loop containing nucleoside triphosphate hydrolases superfamily protein
CH038D17_VU1.3_NODE_0025	3.00E-77	Phvul.008G292600	Photosystem II reaction center protein B
CH038D17_VU1.3_NODE_0029	0	Phvul.008G242400	Auxin response factor
CH038D17_VU1.3_NODE_0040	8.00E-128	Phvul.002G060900	No functional annotation
CH038D17_VU1.3_NODE_0047	0	Phvul.008G242600/1_1419	Acyl activating enzyme 5
CH038D17_VU1.3_NODE_0048	0	Phvul.008G242700	Acyl activating enzyme 5
CH038D17_VU1.3_NODE_0057	0	Phvul.008G242600	Acyl activating enzyme 5

Table 4.13 *Mac-12* on the Sanzi x Vita 7 genetic map, the cowpea consensus genetic map and the cowpea physical map.									
Sanzi x Vita 7 genetic map				Cowpea consensus genetic map vs.4				Cowpea physical map	
LG	cM	Locus	LOD	LG	cM	Locus	Annotation	Contig	BAC(s)
6	0.00	1_1521	3.16	7	4.09	1_1521	Ribosomal protein S5/Elongation factor G/III/V family protein	35	CH096E18, CM047D20
6	0.00	1_0261	3.16	7	4.28	1_0261	TCP family transcription factor	N/A	
6	0.00	1_0763	3.16	7	4.28	1_0763	mRNA splicing factor, thioredoxin-like U5 snRNP	35	CH018P13
6	0.00	1_1418	3.16	7	4.58	1_1418	Microtubule associated protein (MAP65/ASE1) family protein	35	CH075A21, CM061J01
6	0.00	1_1014	3.16	7	5.61	1_1014	No functional annotation	100	CH055K02, CM035H04
6	0.00	1_0006	3.16	7	5.88	1_0006	Ribosomal protein S5 family protein	100	CM050D19, CH006H06, CH088L13
6	0.00	1_1266	3.16	7	5.88	1_1266	UDP-glucosyl transferase 88A1	N/A	
6	0.00	1_1446	3.16	7	5.88	1_1446	UDP-3-O-acyl N-acetylglycosamine deacetylase family protein	84	CM062N22, CM065K24
6	0.37	1_0719	2.99	7	6.28	1_0719	MMS ZWEI homologue 1	84	CH010L23, CM040H24
		N/A		7	9.96	1_0198	Copper amine oxidase family protein	196	CH074C16
		N/A		7	9.96	1_1141	Cytochrome B5 isoform A	196	CH074C16
6	7.61	1_1302	4.38	7	9.96	1_1302	Tetratricopeptide repeat (TPR)-like superfamily protein	N/A	
		N/A		7	13.15	1_0711	HVA22 homologue C	N/A	
		N/A		7	14.35	1_1536	ATP-citrate lyase A-1	N/A	
		N/A		7	14.92	1_0631	Threonyl-tRNA synthetase	N/A	
		N/A		7	15.10	1_0529	Glutathione S-transferase TAU 18	N/A	
		N/A		7	16.97	1_0439	Pyruvate dehydrogenase complex E1 alpha subunit	542	CH007H14
6	17.81	1_0278	3.50	7	17.98	1_0278	Spermidine synthase 1	N/A	
		N/A		7	18.27	1_0723	No functional annotation	N/A	
		N/A		7	18.70	1_0126	Nucleic acid-binding, OB-fold-like protein	542	CH004E16
		N/A		7	18.91	1_0047	Ubiquitin 6	542	CH094E06, CM054C15
		N/A		7	18.91	1_0108	Ubiquitin 6	542	CH094E06
		N/A		7	18.91	1_1150	Signal recognition particle binding	N/A	
		N/A		7	18.91	1_1215	Kelch repeat-containing F-box family protein	N/A	
		N/A		7	19.33	1_1026	Sterol 4-alpha-methyl-oxidase 2-1	N/A	
6	19.00	1_0385	3.38	7	19.91	1_0385	No functional annotation	N/A	
		N/A		7	19.99	1_0659	Uncoupling protein 5	N/A	
		N/A		7	19.99	1_1510	MSCS-like 2	N/A	
6	19.98	1_0279	3.86	7	20.68	1_0279	Zincin-like metalloproteases family protein	1132	CM004O13
		N/A		7	20.82	1_0561	No functional annotation	N/A	
		N/A		7	20.82	1_0644	Phosphoenolpyruvate carboxykinase 1	N/A	
		N/A		7	20.82	1_0564	Mitochondrial substrate carrier family protein	N/A	

		N/A		7	21.57	1_0983	No functional annotation	N/A	
		N/A		7	21.68	1_1482	Shikimate kinase like 2	N/A	
		N/A		7	21.77	1_1414	SPT2 chromatin protein	N/A	
6	21.83	1_0023	4.09	7	22.68	1_0023	Defender against death (DAD family) protein	N/A	
		N/A		7	23.21	1_0168	Ribosomal protein L34e superfamily protein	581	CM014F24
		N/A		7	23.34	1_0384	Translocase of the outer mitochondrial membrane 6	N/A	
6	21.83	1_0906	4.09	7	23.54	1_0906	Phosphoribulokina se	337	CM016L18, CH096J02
		N/A		7	23.54	1_0912	SCAMP family protein	337	CH096J02, CM016L18
		N/A		7	23.70	1_1472	No functional annotation	337	CH096J02
		N/A		7	24.13	1_0755	No functional annotation	337	CM016L18, CM040H16
		N/A		7	24.22	1_0196	Acyl-CoA- binding protein 6	337	CM050J18, CH061D01, CH092O12
		N/A		7	24.22	1_0391	Mob1/phocein family protein	337	CM040H16, CH062O07
6	22.33	1_0708	3.70	7	24.53	1_0708	CLP protease P4	337	CH068C24, CH061D01
		N/A		7	26.62	1_0663	Phosphoserine aminotransferase	N/A	
6	27.37	1_1057	2.92	7	27.33	1_1057	DHHC-type zinc finger family protein	N/A	
6	27.37	1_1035	2.92	7	27.80	1_1035	RNA-binding (RRM/RBD/RNP motifs) family protein	N/A	
		N/A		7	28.21	1_0648	No functional annotation	N/A	
		N/A		7	28.22	1_0696	Aconitase 1	N/A	
6	27.37	1_0477	2.92	7	28.38	1_0477	Rhodanese/Cell cycle control phosphatase superfamily protein	N/A	
		N/A		7	28.84	1_0917	RHO-related protein from plants 1	1276	CM067B17
		N/A		7	29.43	1_0228	Ribosomal protein L2 family	1276	CM067B17
		N/A		7	29.43	1_0641	Regulatory particle non-ATPase 10	1276	CH017F14
6	28.56	1_0270	3.37	7	29.60	1_0270	Ferredoxin- NADP(+)-oxidoreductase 2	N/A	
		N/A		7	30.15	1_0884	NAD(P)-binding Rossmann-fold superfamily protein	N/A	
		N/A		7	30.15	1_1186	Histone H1-3	N/A	
6	30.03	1_0621	2.33	7	31.04	1_0621	Ubiquitin- conjugating enzyme 16	N/A	

Table 4.14 Annotations for cowpea BAC clone CH074C16.			
Cowpea BAC node	*P. vulgaris* locus (cowpea SNP)	Annotation	e-score
CH074C16_VU1.3_NODE_0006	Phvul.003G040400 (1_1141)	Cytochrome B5 isoform A	2.00E-80
CH074C16_VU1.3_NODE_0011	Phvul.003G040700	Fructokinase-like 2	3.00E-85
CH074C16_VU1.3_NODE_0012	Phvul.003G040500	Copper amine oxidase family protein	9.00E-114
CH074C16_VU1.3_NODE_0026	Phvul.011G041500	Vacuolar proton ATPase A3	1.00E-34
CH074C16_VU1.3_NODE_0036	Phvul.003G040700	Fructokinase-like 2	0
CH074C16_VU1.3_NODE_0038	Phvul.003G040600	ATPase E1	2.00E-147
CH074C16_VU1.3_NODE_0040	Phvul.003G041000	Protein kinase superfamily protein	7.00E-51
CH074C16_VU1.3_NODE_0042	Phvul.007G262100	Dihydropterin pyrophosphokinase / Dihydropteroate synthase	0
CH074C16_VU1.3_NODE_0043	Phvul.003G041100	No functional annotation	3.00E-44
CH074C16_VU1.3_NODE_0046	Phvul.004G029000	Structural maintenance of chromosome 3	1.00E-52
CH074C16_VU1.3_NODE_0051	Phvul.003G040500(1_0198)	Copper amine oxidase family protein	2.00E-178
CH074C16_VU1.3_NODE_0055	Phvul.003G040800	6-phosphogluconolactonase 1	5.00E-73
CH074C16_VU1.3_NODE_0077	Phvul.003G041000	Protein kinase superfamily protein	4.00E-88
CH074C16_VU1.3_NODE_0085	Phvul.003G040800	6-phosphogluconolactonase 1	4.00E-118
CH074C16_VU1.3_NODE_0089	Phvul.007G262100	Dihydropterin pyrophosphokinase / Dihydropteroate synthase	4.00E-98
CH074C16_VU1.3_NODE_0090	Phvul.003G040900	Uncharacterized protein family UPF0090	6.00E-69
CH074C16_VU1.3_NODE_0098	Phvul.003G040700	Fructokinase-like 2	0
CH074C16_VU1.3_NODE_0101	Phvul.003G040500	Copper amine oxidase family protein	0
CH074C16_VU1.3_NODE_0125	Phvul.008G105900	Tetratricopeptide repeat (TPR)-like superfamily protein	1.00E-84
CH074C16_VU1.3_NODE_0132	Phvul.003G040900	Uncharacterized protein family UPF0090	1.00E-29
CH074C16_VU1.3_NODE_0136	Phvul.003G040800	6-phosphogluconolactonase 1	1.00E-75
CH074C16_VU1.3_NODE_0137	Phvul.003G041000	Protein kinase superfamily protein	6.00E-20
CH074C16_VU1.3_NODE_0143	Phvul.003G040900	Uncharacterized protein family UPF0090	2.00E-128
CH074C16_VU1.3_NODE_0153	Phvul.003G041000	Protein kinase superfamily protein	1.00E-41
CH074C16_VU1.3_NODE_0169	Phvul.003G040700	fructokinase-like 2	1.00E-76
CH074C16_VU1.3_NODE_0173	Phvul.003G041000	Protein kinase superfamily protein	2.00E-26
CH074C16_VU1.3_NODE_0203	Phvul.003G041000	Protein kinase superfamily protein	4.00E-49
CH074C16_VU1.3_NODE_0212	Phvul.003G041000	Protein kinase superfamily protein	5.00E-104

Table 4.15 Annotations for the *Mac-12* locus on BAC clones CH068C24 and CH061D01 on contig337 of the cowpea physical map.			
BAC clone/node	e- score	*P. vulgaris* locus/cowpea SNP	*P. vulgaris* annotation
CH068C24_VU1.3_NODE_0001	1.00E-179	Phvul.002G131500.1	DZC (Disease resistance/zinc finger/chromosome condensation-like region) domain containing protein
CH068C24_VU1.3_NODE_0002	2.00E-40	Phvul.002G131000.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0003	0	Phvul.002G128800.1/1_0708	Replication factor-A protein 1-related
CH068C24_VU1.3_NODE_0004	3.00E-77	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0005	0	Phvul.002G129900.1	NB-ARC domain-containing disease resistance protein
CH068C24_VU1.3_NODE_0008	4.00E-23	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0009	2.00E-88	Phvul.002G131000.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0011	2.00E-74	Phvul.002G129900.1	NB-ARC domain-containing disease resistance protein
CH068C24_VU1.3_NODE_0013	4.00E-62	Phvul.002G130500.1	NB-ARC domain-containing disease resistance protein
CH068C24_VU1.3_NODE_0015	0	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0017	0	Phvul.002G128400.1	Tetratricopeptide repeat (TPR)-containing protein
CH068C24_VU1.3_NODE_0019	5.00E-126	Phvul.002G131200.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0036	0	Phvul.002G129100.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
CH068C24_VU1.3_NODE_0039	0	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0044	0	Phvul.002G129100.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
CH068C24_VU1.3_NODE_0045	3.00E-21	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0049	8.00E-134	Phvul.002G131000.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0051	0	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0052	4.00E-49	Phvul.002G130100.1	NB-ARC domain-containing disease resistance protein
CH068C24_VU1.3_NODE_0053	8.00E-65	Phvul.002G131200.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0054	0	Phvul.002G131200.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0057	0	Phvul.002G131000.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0063	7.00E-21	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH068C24_VU1.3_NODE_0075	0	Phvul.002G130100.1	NB-ARC domain-containing disease resistance protein
CH061D01_VU1.3_NODE_0001	0	Phvul.002G127500.1	Uncharacterized protein
CH061D01_VU1.3_NODE_0010	0	Phvul.002G128300.1	Ankyrin repeat family protein
CH061D01_VU1.3_NODE_0013	7.00E-42	Phvul.002G128400.1	Tetratricopeptide repeat (TPR)-containing protein
CH061D01_VU1.3_NODE_0021	3.00E-115	Phvul.002G128900.1	Uncharacterized protein
CH061D01_VU1.3_NODE_0055	2.00E-148	Phvul.002G127600.1	Ribonuclease P protein subunit P38-related
CH061D01_VU1.3_NODE_0079	2.00E-46	Phvul.002G127400.1	HAC13 protein (HAC13)
CH061D01_VU1.3_NODE_0098	0	Phvul.002G128000.1	Amino acid permease family protein
CH061D01_VU1.3_NODE_0119	0	Phvul.002G127800.1/1_0196	Leucine-rich repeat protein kinase family protein
CH061D01_VU1.3_NODE_0122	3.00E-43	Phvul.002G128400.1	Tetratricopeptide repeat (TPR)-containing protein
CH061D01_VU1.3_NODE_0124	2.00E-80	Phvul.002G130600.1	NB-ARC domain-containing disease resistance protein
CH061D01_VU1.3_NODE_0135	5.00E-129	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH061D01_VU1.3_NODE_0157	7.00E-28	Phvul.007G021800.1	Radical SAM superfamily protein

CH061D01_VU1.3_NODE_0234	2.00E-120	Phvul.002G130500.1	NB-ARC domain-containing disease resistance protein
CH061D01_VU1.3_NODE_0240	0	Phvul.002G129700.1	LRR and NB-ARC domains-containing disease resistance protein
CH061D01_VU1.3_NODE_0256	1.00E-72	Phvul.002G129900.1	NB-ARC domain-containing disease resistance protein
CH061D01_VU1.3_NODE_0261	0	Phvul.002G128800.1/1_0708	Replication factor-A protein 1-related
CH061D01_VU1.3_NODE_0269	8.00E-86	Phvul.002G131000.1	LRR and NB-ARC domains-containing disease resistance protein
CH061D01_VU1.3_NODE_0271	3.00E-41	Phvul.002G130400.1	NB-ARC domain-containing disease resistance protein
CH061D01_VU1.3_NODE_0282	0	Phvul.002G128200.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
CH061D01_VU1.3_NODE_0346	0	Phvul.002G128400.1	Tetratricopeptide repeat (TPR)-containing protein

Table 4.16 *Mac-13* on the Sanzi x Vita 7 genetic map, the cowpea consensus genetic map and the cowpea physical map.									
Sanzi x Vita 7 genetic map				Cowpea consensus genetic map vs.4				Cowpea physical map	
LG	cM	Locus	LOD	LG	cM	Locus	Annotation	Contig	BAC(s)
		N/A		4	20.20	1_1221	Ribosomal RNA processing 4	445	CH022D17 CH062O11 CH069K06
10	41.89	1_1242	2.15	4	20.72	1_1242	Acyl-CoA N-acyltransferases (NAT) superfamily protein	N/A	
		N/A		4	21.49	1_0027	Photosystem II reaction center W	N/A	
		N/A		4	21.49	1_0153	Alpha/beta- Hydrolases superfamily protein	N/A	
10	48.25	1_0535	2.82	4	22.85	1_0535	Nucleolar RNA-binding Nop10p family protein	N/A	
		N/A		4	22.92	1_1261	Nuclear transport factor 2 (NTF2) family protein	121	CH041G03 CM004E09 CM059K12
10	49.57	1_1092	2.65	4	23.66	1_1092	Ribosomal protein S19	121	CH005N14
10	51.21	1_0646	2.88	4	24.12	1_0646	Regulatory particle AAA-ATPase 2A	116	CM060J02
10	51.21	1_0874	2.88	4	24.40	1_0874	Heat shock cognate protein 70-1	N/A	
		N/A		4	24.43	1_1264	ALWAYS EARLY 4	N/A	
10	51.65	1_0826	3.16	4	24.55	1_0826	Eukaryotic translation initiation factor 3A	N/A	
		N/A		4	25.00	1_0692	Tetratricopeptide repeat (TPR)-like superfamily protein	N/A	
		N/A		4	25.31	1_0403	RNA-binding (RRM/RBD/RNP motifs) family protein	N/A	
10	55.18	1_0106	2.45	4	25.57	1_0106	Ribosomal protein L4/L1 family	383	CM067G06 CM007L11 CM056F01

Table 4.17 Synteny of *Mac-13* with *G. max* chromosomes 3 and 19.					
G. max chromosome	*G. max* locus	Phytozome annotation	Cowpea SNP	LG	cM
3	Glyma03g31080	Terpene synthase family, metal binding domain	N/A		
3	Glyma03g31100	Chalcone-flavanone isomerase	N/A		
3	Glyma03g31110	Terpene synthase, ent-copalyl diphosphate synthase	N/A		
3	Glyma03g31120	N-terminal acetyltransferase	1_1242	4	20.72
3	Glyma03g31520	AUX/IAA family member	N/A		
3	Glyma03g31530	AUX/IAA family member	N/A		
3	Glyma03g31570	Lipase (class 3)	1_0153	4	21.49
3	Glyma03g31580	Photosystem II PsbW protein	1_0027	4	21.49
3	Glyma03g32420	RNA-binding RAS-GAP SH3 binding protein related	1_1261	4	22.92
3	Glyma03g32800	26S protease regulatory subunit	1_0646	4	24.12
3	Glyma03g32850	Hsp70 protein	1_0874	4	24.40
3	Glyma03g32950	Translation initiation factor 3, subunit a (eIF-3a)	1_0826	4	24.55
3	Glyma03g33260	Arginine/serine-rich splicing factor	1_0403	4	25.31
19	Glyma19g34370	AUX/IAA family member	N/A		
19	Glyma19g34380	AUX/IAA family member	N/A		
19	Glyma19g34410	Photosystem II PsbW protein	1_0027	4	21.49
19	Glyma19g34910	Nucleolar RNA-binding protein, Nop10p family	1_0535	4	22.85
19	Glyma19g35150	RNA-binding RAS-GAP SH3 binding protein related	1_1261	4	22.92
19	Glyma19g35510	26S protease regulatory subunit	1_0646	4	24.12
19	Glyma19g35560	Heat shock protein 70 KDA	1_0874	4	24.40
19	Glyma19g35660	AMP dependent ligase/synthetase	1_0826	4	24.55
19	Glyma19g35820	O-linked N-acetylglucosamine transferase	1_0692	4	25.00
19	Glyma19g36180	60S ribosomal protein L4	1_0106	4	25.57

Table 4.18 Synteny of *Mac-13* with *P. vulgaris* chromosome 1.					
P. vulgaris chromosome	*P. vulgaris* locus	Phytozome annotation	Cowpea SNP	LG	cM
1	Phvul.001G147300	Auxin-responsive GH3 family protein	N/A		
1	Phvul.001G147700	BSD domain-containing protein	1_1221	4	20.20
1	Phvul.001G151900	Terpenoid cyclases/protein prenyltransferases superfamily protein	N/A		
1	Phvul.001G152000	Chalcone-flavanone isomerase family protein	N/A		
1	Phvul.001G152100	Terpenoid cyclases/protein prenyltransferases superfamily protein	N/A		
1	Phvul.001G156500	AUX/IAA transcriptional regulator family protein	N/A		
1	Phvul.001G157600	Ethylene responsive element binding factor 3	N/A		
1	Phvul.001G160200	Ethylene response factor 1	N/A		
1	Phvul.001G160300	Ethylene response factor 1	N/A		
1	Phvul.001G160500	Ethylene response factor 1	N/A		
1	Phvul.001G164600	Nuclear transport factor 2 (NTF2) family protein	1_1261	4	22.92
1	Phvul.001G164900	Auxin-induced protein 13	N/A		
1	Phvul.001G167500	Ribosomal protein S19	1_1092	4	23.66
1	Phvul.001G172600	RNA-binding (RRM/RBD/RNP motifs) family protein	1_0403	4	25.31
1	Phvul.001G174300	Ribosomal protein L4/L1 family	1_0106	4	25.57

Table 4.19 Annotations for the *Mac-13* locus on clones CH062O11 and CH069K06 of contig445 on the cowpea physical map.			
BAC clone/node	e- score	*P. vulgaris* locus/cowpea SNP	*P. vulgaris* annotation
CH062O11_VU1.3_NODE_0001	0	Phvul.001G147700	BSD domain-containing protein
CH062O11_VU1.3_NODE_0003	2.00E-135	Phvul.001G148000	No functional annotation
CH062O11_VU1.3_NODE_0004	0	Phvul.001G147600/1_1221	Ribosomal RNA processing 4
CH062O11_VU1.3_NODE_0012	5.00E-65	Phvul.001G148100	No functional annotation
CH062O11_VU1.3_NODE_0013	2.00E-162	Phvul.001G147800	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
CH062O11_VU1.3_NODE_0014	7.00E-71	Phvul.001G147900	No functional annotation
CH062O11_VU1.3_NODE_0019	3.00E-93	Phvul.001G148200	GTP binding Elongation factor Tu family protein
CH062O11_VU1.3_NODE_0020	0	Phvul.003G048700	Mitochondrial transcription termination factor family protein
CH062O11_VU1.3_NODE_0022	2.00E-35	Phvul.010G156400	Phosphatidic acid phosphatase-related / PAP2-related
CH062O11_VU1.3_NODE_0024	4.00E-50	Phvul.001G147200	Major facilitator superfamily protein
CH062O11_VU1.3_NODE_0025	0	Phvul.001G147300	Auxin-responsive GH3 family protein
CH062O11_VU1.3_NODE_0034	2.00E-59	Phvul.001G147200	Major facilitator superfamily protein
CH062O11_VU1.3_NODE_0036	7.00E-85	Phvul.003G048700	Mitochondrial transcription termination factor family protein
CH062O11_VU1.3_NODE_0037	6.00E-127	Phvul.001G147400	DNAse I-like superfamily protein
CH062O11_VU1.3_NODE_0038	8.00E-44	Phvul.001G148100	No functional annotation
CH062O11_VU1.3_NODE_0040	2.00E-45	Phvul.001G148100	No functional annotation
CH069K06_VU1.3_NODE_0005	0	Phvul.001G147700	BSD domain-containing protein
CH069K06_VU1.3_NODE_0011	0	Phvul.001G147600/1_1221	Ribosomal RNA processing 4
CH069K06_VU1.3_NODE_0014	1.00E-85	Phvul.001G147900	No functional annotation
CH069K06_VU1.3_NODE_0015	3.00E-163	Phvul.001G147800	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
CH069K06_VU1.3_NODE_0019	1.00E-50	Phvul.001G147200	Major facilitator superfamily protein
CH069K06_VU1.3_NODE_0026	0	Phvul.001G147400	DNAse I-like superfamily protein
CH069K06_VU1.3_NODE_0028	0	Phvul.001G147300	Auxin-responsive GH3 family protein
CH069K06_VU1.3_NODE_0041	3.00E-49	Phvul.001G147200	Major facilitator superfamily protein
CH069K06_VU1.3_NODE_0051	2.00E-59	Phvul.001G147200	Major facilitator superfamily protein
CH069K06_VU1.3_NODE_0051	6.00E-35	Phvul.001G147200	Major facilitator superfamily protein
CH069K06_VU1.3_NODE_0059	0	Phvul.001G146700	Homolog of yeast autophagy 18 (ATG18) G
CH069K06_VU1.3_NODE_0062	2.00E-46	Phvul.001G147100	Major facilitator superfamily protein
CH069K06_VU1.3_NODE_0065	3.00E-134	Phvul.001G146800	Protein kinase family protein
CH069K06_VU1.3_NODE_0067	2.00E-118	Phvul.007G037800	CwfJ-like family protein
CH069K06_VU1.3_NODE_0079	6.00E-50	Phvul.001G147200	Major facilitator superfamily protein
CH069K06_VU1.3_NODE_0080	1.00E-84	Phvul.001G146900	Receptor-like kinase in flowers 3
CH069K06_VU1.3_NODE_0082	0	Phvul.001G147000	Receptor-like kinase in flowers 3
CH069K06_VU1.3_NODE_0083	0	Phvul.001G146900	Receptor-like kinase in flowers 3

Table 4.20 Candidate genes identified in *Mac-10*, *Mac-11*, *Mac-12* and *Mac-13* loci.				
QTL	Trait	Resource	Locus	Candidate gene
Mac-10	Macrophomina	Synteny with soy bean	Glyma05g01280	WRKY72 transcription factor
	Macrophomina	Synteny with soy bean	Glyma17g10630	WRKY72 transcription factor
Mac-11	SD photoperiod	Cowpea genetic map	SNP 1_0926	Cytochrome b-c1 complex, subunit 8 protein
	SD photoperiod	Cowpea genetic map	SNP 1_1253	Phototropic-responsive NPH3 family protein)
	SD photoperiod	Synteny with common bean	Phvul.008G292600	Photosystem II reaction center protein B
	SD photoperiod	Cowpea physical map	Phvul.008G292600	Photosystem II reaction center protein B
	Macrophomina	Synteny with soy	Glyma02g40650	Auxin response factor
	Macrophomina	Synteny with Medicago	Medtr5g084140	ARF with a B3 DNA binding domain
	Macrophomina	Synteny with common bean	Phvul.008G242400	Auxin response factor
	Macrophomina	Cowpea physical map	Phvul.008G242400	Auxin response factor
Mac-12	SD photoperiod	Cowpea physical map	Phvul.003G040400 SNP 1_1141	Cytochrome B5 isoform A
	Macrophomina	Cowpea physical map	Phvul.003G040700	Fructokinase-like 2
	Macrophomina	Cowpea physical map	Phvul.003G041000	Protein kinase superfamily proteins
	Macrophomina	Cowpea physical map	Phvul.003G040500 (SNP 1_0198)	Copper amine oxidase family protein
	Macrophomina	Cowpea physical map	Phvul.003G040800	6-phosphogluconolactonase 1
	Macrophomina	Cowpea physical map	Phvul.003G041000	Protein kinase superfamily protein
	Macrophomina	Cowpea physical map	Phvul.002G131500	DZC (Disease resistance/zinc finger/chromosome condensation-like region)
	Macrophomina	Cowpea physical map	Phvul.002G130100 Phvul.002G130500 Phvul.002G130600 Phvul.002G129900	NB-ARC domain-containing disease resistance protein
	Macrophomina	Cowpea physical map	Phvul.002G129700 Phvul.002G131000 Phvul.002G131200	LRR and NB-ARC domains-containing disease resistance protein
	Macrophomina	Cowpea physical map	Phvul.002G127800 SNP 1_0196	Leucine-rich repeat protein kinase family proteins
Mac-13	SD photoperiod	Cowpea genetic map	SNP 1_0027	Photosystem II reaction center W protein
	Macrophomina	Synteny with soy bean	Glyma03g31520 Glyma03g31530 Glyma19g34370 Glyma19g34380	AUX/IAA family genes
	Macrophomina	Synteny with common bean	Phvul.001G147300	Auxin-responsive *GH3* family protein

	Macrophomina	Synteny with common bean	Phvul.001G156500	AUX/IAA transcriptional regulator family protein
	Macrophomina	Synteny with common bean	Phvul.001G164900	Auxin-induced protein 13
	Macrophomina	Synteny with common bean	Phvul.001G152100 Phvul.001G151900	Terpenoid cyclases/protein prenyltransferases superfamily protein
	Macrophomina	Synteny with common bean	Phvul.001G152000	Chalcone-flavanone isomerase family protein
	Macrophomina	Synteny with common bean	Phvul.001G157600	Ethylene responsive element binding factor 3
	Macrophomina	Synteny with common bean	Phvul.001G160200 Phvul.001G160300 Phvul.001G160500	Ethylene response factor 1
	Macrophomina	Cowpea physical map	Phvul.001G147300	Auxin-responsive *GH3* family protein

Figure 4.1 Examples of *M. phaseolina* disease symptoms. 1A. Seedling damping-off. 1B. Reddish- brown lesions which occur at the soil line. 1C. Mature plant with reddish-brown lesions and curled leaves. 1D. Mature plant which has died due to Macrophomina. 1E. Charcoal rot (or ashy stem blight) on mature plant stem. 1F. Reddish-brown discoloration and rot of vascular system.

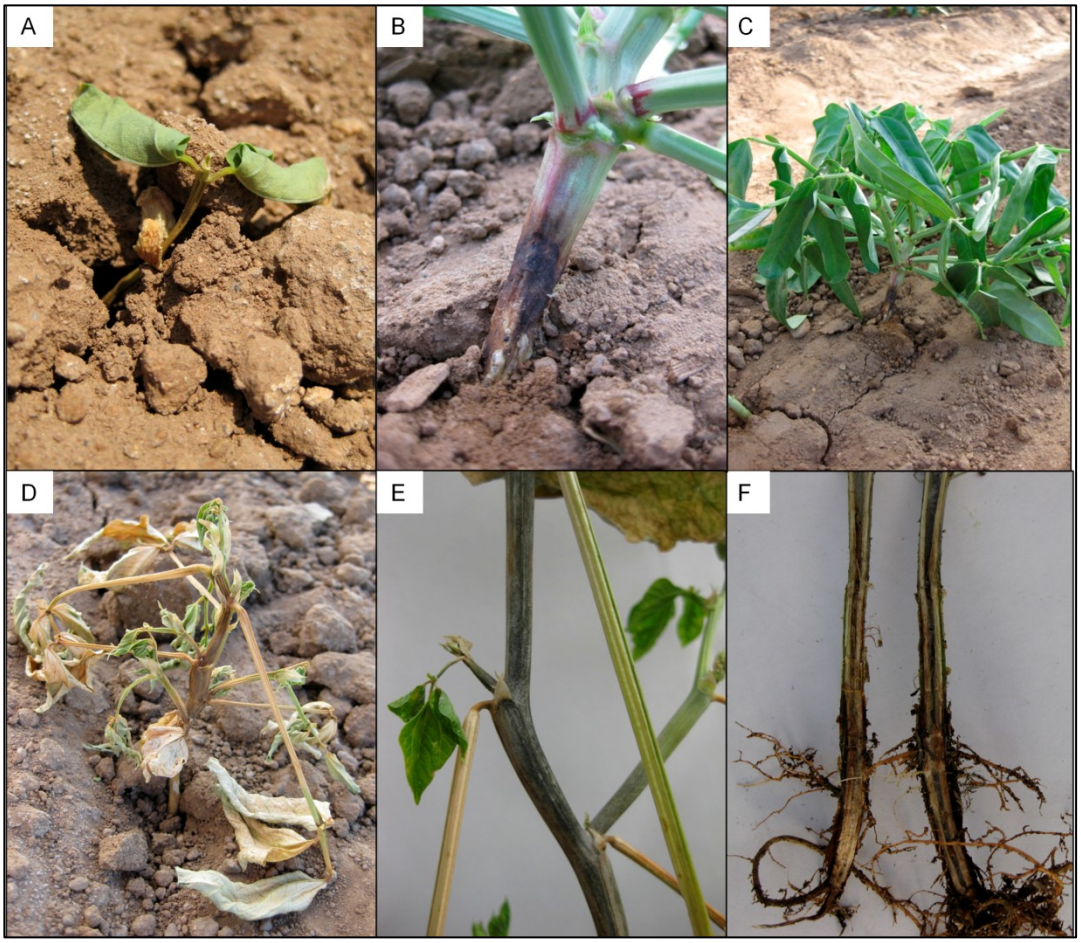


Figure 4.2 *Mac-10* locus in the Sanzi x Vita7 genetic map. *Mac-10* QTL (only Interval Mapping analysis is shown) was observed in the 2009 field experiment for the percent surviving phenotype, 1 week post germination. *Mac-10 s*panned approximately 4.64 cM, from 35.44 cM to 40.08 cM on linkage group 2. SNP marker 1_ 0381 (position 36.5 cM) was the most significant (red) on the linkage group. The significance threshold of 2.0 is indicated by the horizontal broken line.

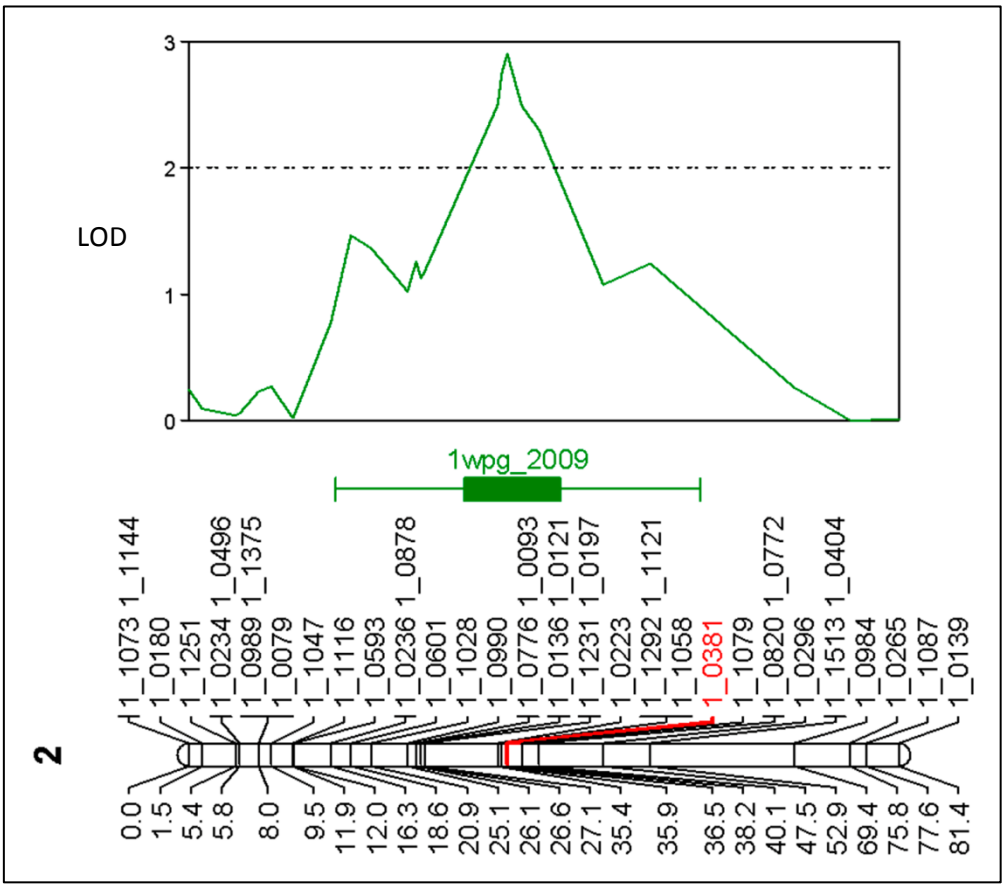


Figure 4.3 Macrophomina resistance, drought tolerance and maturity-related QTLs on the cowpea consensus genetic map. QTLs were positioned on the cowpea consensus genetic map using SNP markers identified in the QTL analyses. *Mac-10*, *Mac-11*, *Mac-12* and *Mac-13* (labeled light green) were identified in the Sanzi x Vita 7 population in *M. phaseolina* infested fields in Riverside, California. *Mac-1*, *Mac-2*, *Mac-3*, *Mac-4*, *Mac-5*, *Mac-6*, *Mac-7*, *Mac-8* and *Mac-9* (labeled magenta) were previously identified in the IT93K-503-1 x California Blackeye 46 population in *M. phaseolina* infested fields in Riverside, California and using greenhouse inoculation experiments (Muchero et al. 2011). Maturity-related leaf senescence QTLs, *Mat-1* and *Mat-2* (labeled dark green) and the seedling-stage drought tolerance QTLs, *Dro-1*, *Dro-3*, *Dro-3*, *Dro-7*, *Dro-8* and *Dro-10* (labeled blue) were previously identified in drought field experiments in Coachella, California using IT93K-503-1 x CB46 population (Muchero et al. 2009 and 2011). The most significant marker for each QTL is highlighted in the corresponding color on the linkage group. SNP marker 1_0464 labeled red was the most significant marker for both the *Mac-4* and the *Dro-10* QTL. SNP marker 1_0678 labeled red was the most significant marker for both the *Mac-7* and *Mat-1* loci. SNP marker 1_0804 labeled red was the most significant marker for both *Mac-6* and *Mat-2* loci.

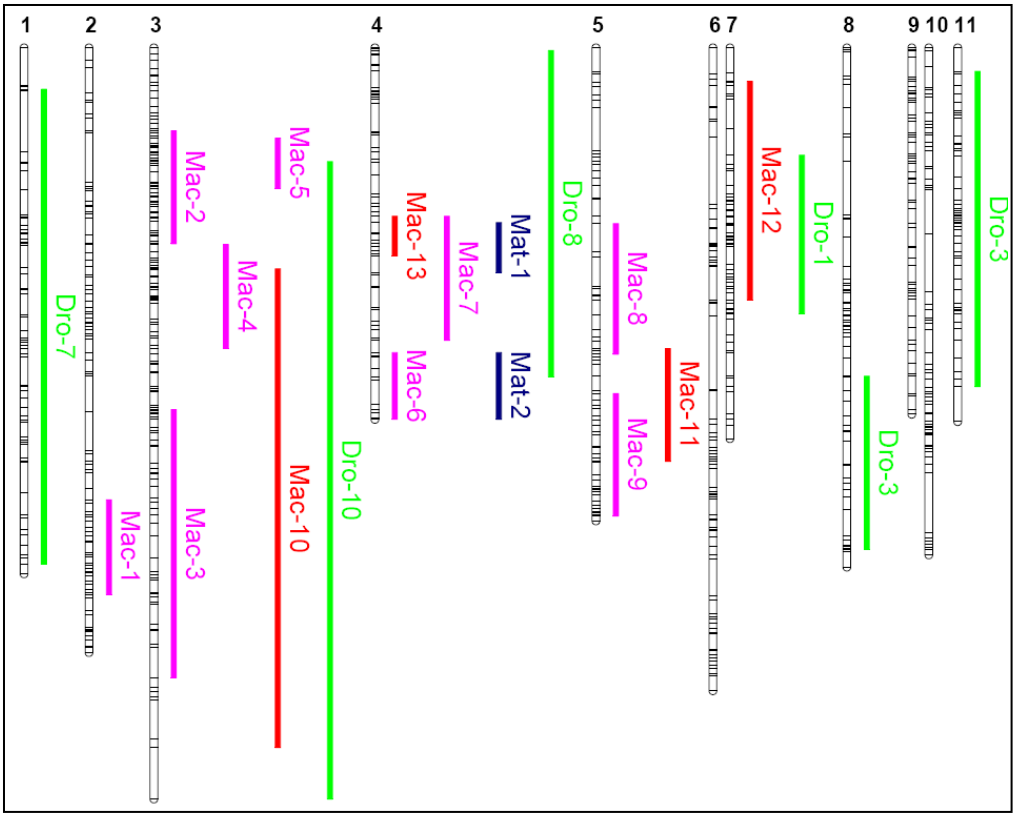


Figure 4.4 Synteny of *Mac-10* with *G. max*. Synteny was examined for the *Mac-10* locus between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the soybean genome. The *Mac-10* locus which spanned 74.80 cM to 78.61cM on the cowpea consensus genetic map vs. 6 linkage group 3 was determined to be syntenic with soybean chromosomes 5 and 7. The syntenic locus in soybean chromosome 5 extended from soybean locus Glyma05g00400 to Glyma05g02190, in which a WRKY72 transcription factor was observed in the region and considered as a candidate gene. The syntenic locus in soybean chromosome 7 extended from soybean locus Glyma17g08630 to Glyma17g11060 where a WRKY72 transcription factor was observed. The syntenic map was drawn using HarvEST: Cowpea database using a cut off e-score value of -10 and a minimum number of 10 lines drawn per linkage group.

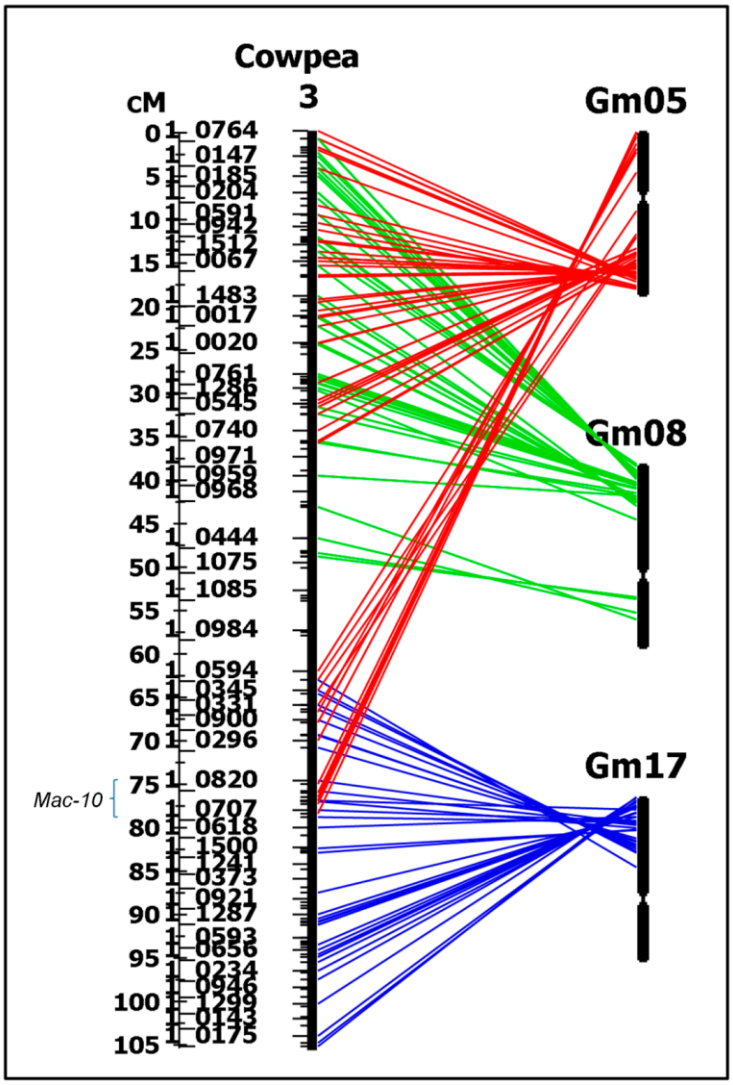


Figure 4.5 *Mac-11* locus in the Sanzi x Vita7 genetic map. The *Mac-11* QTL (Interval Mapping analysis shown) was observed in both the 2009 and 2010 field experiments and mapped to linkage group 5 on the Sanzi x Vita7 genetic map. The 2009 percent survivor data is plotted in green, the 2009 percent seedling damping off data is plotted in red, and the 2010 percent survivor data is plotted in fuchsia. The most significant markers for the 2009 and 2010 experiments are labeled in red. The significance threshold of 2.0 is indicated by the horizontal broken line.

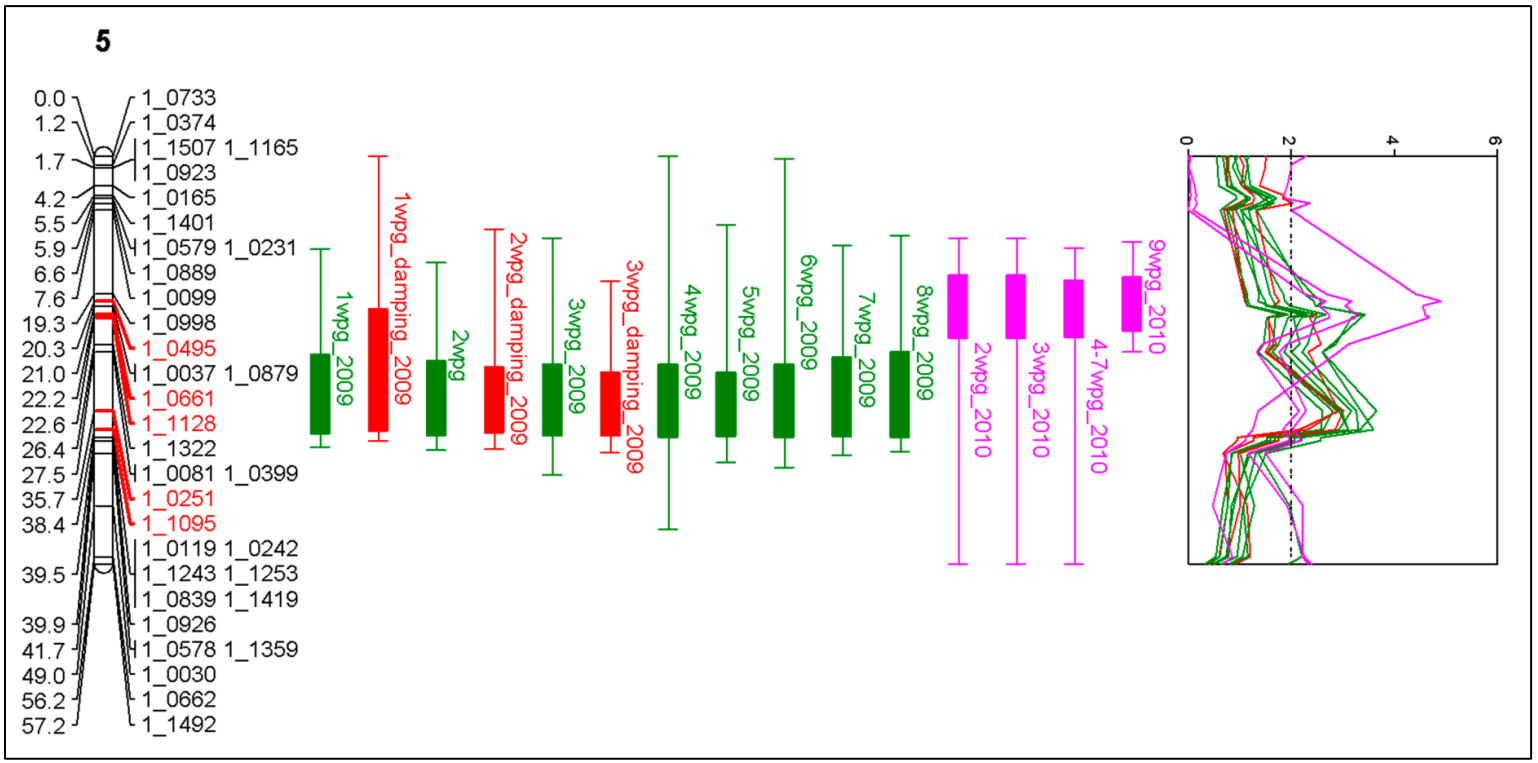


Figure 4.6 Marker-trait association of the *Mac-11* locus. A marker-trait association of the *Mac-11* locus was analyzed using fourteen cowpea genotypes which differ in their photoperiod sensitivity, days to maturity and tolerance to Macrophomina. Vita 7, IT93K-503-1, Apagbaala, IT98K-499-39, Suvita-2, Moussa Local and Iron Clay are obligate short-day photoperiod sensitive, late-maturing and Macrophomina-resistant. Sanzi, IT82E-18(Big Buff), CB46, CB27, UCR 24, 524-B and Bambey 21 are day-neutral, early-maturing and Macrophomina-susceptible. The *Mac-11* locus spanned from 37.23 cM to 49.10 cM on the cowpea consensus genetic map LG5 (depicted horizontally). SNP marker 1_1419 (position 37.73 cM) alleles co-segregated with the Macrophomina-resistant and Macrophomina-susceptible genotypes. The Macrophomina-resistant genotypes were associated with the cytosine nucleotide (orange) and the Macrophomina-susceptible genotypes were associated with the guanine nucleotide (red). The guanine/cytosine SNP for marker 1_1419 is at position 560 in the cowpea unigene 3720 which is annotated as an AMP dependent ligase/synthetase.

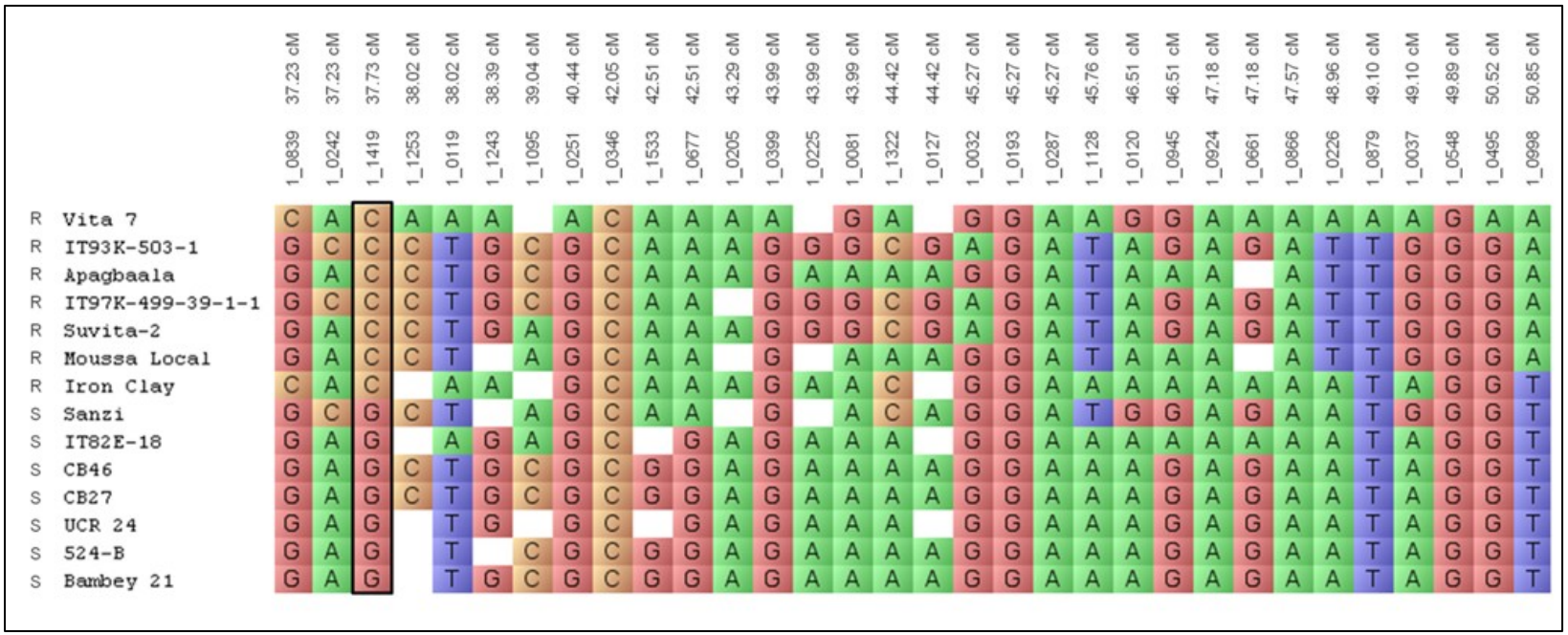


Figure 4.7 Synteny of *Mac-11* locus with *G. max* chromosomes 2 and 14. Synteny was examined for the *Mac-11* locus between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Mac-11* locus which spanned 37.04 cM to 50.85 cM on the cowpea consensus genetic map linkage group 5 was determined to be syntenic with soybean chromosomes 2 and 14. The syntenic locus in soybean chromosome 2 extended from soybean locus Glyma02g40530 to Glyma02g43640 in which an auxin response factor was observed in the region and considered as a candidate gene. The syntenic locus in soybean 14 extended from soybean locus Glyma14g04890 to Glyma14g39280 in which another auxin response factor was observed. The syntenic map was drawn using HarvEST: Cowpea database using a cut off e-score value of -10 and a minimum number of 10 lines drawn per linkage group.

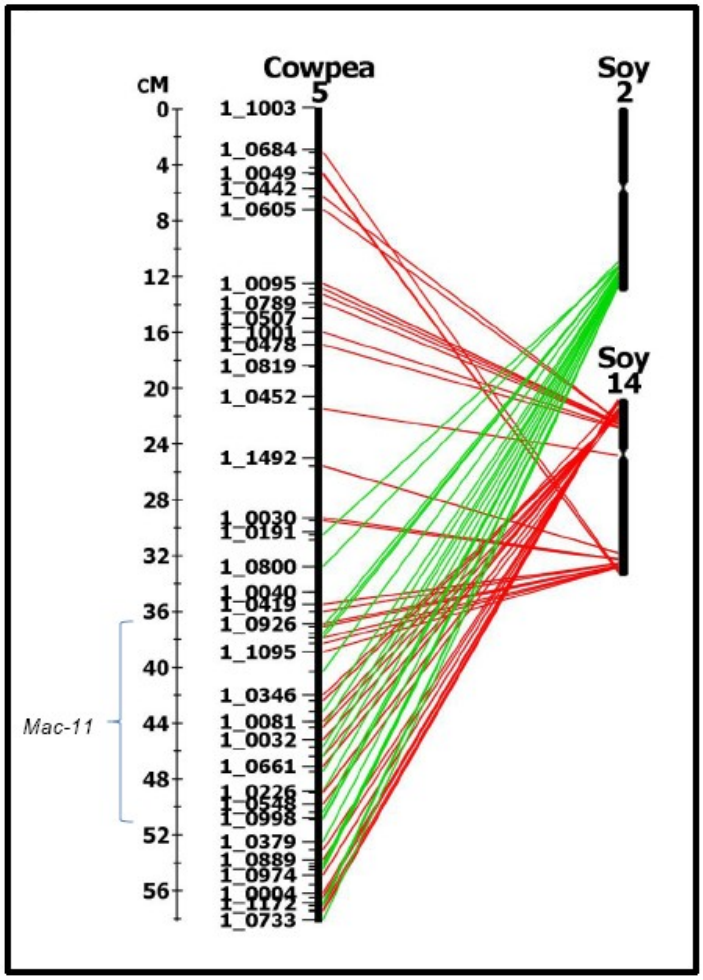


Figure 4.8 Synteny of *Mac-11* locus with *M. truncatula* chromosome 5. Synteny was examined for the *Mac-11* locus between cowpea and *M. truncatula* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Mac-11* locus was determined to be syntenic with Medicago chromosome 5, spanning from Medtr5g083440 to Medtr5g085190 in which an auxin response factor was identified in the region and as a candidate gene. The syntenic map was drawn using HarvEST: Cowpea database using a cut off e-score value of -10 and a minimum number of 10 lines drawn per linkage group.

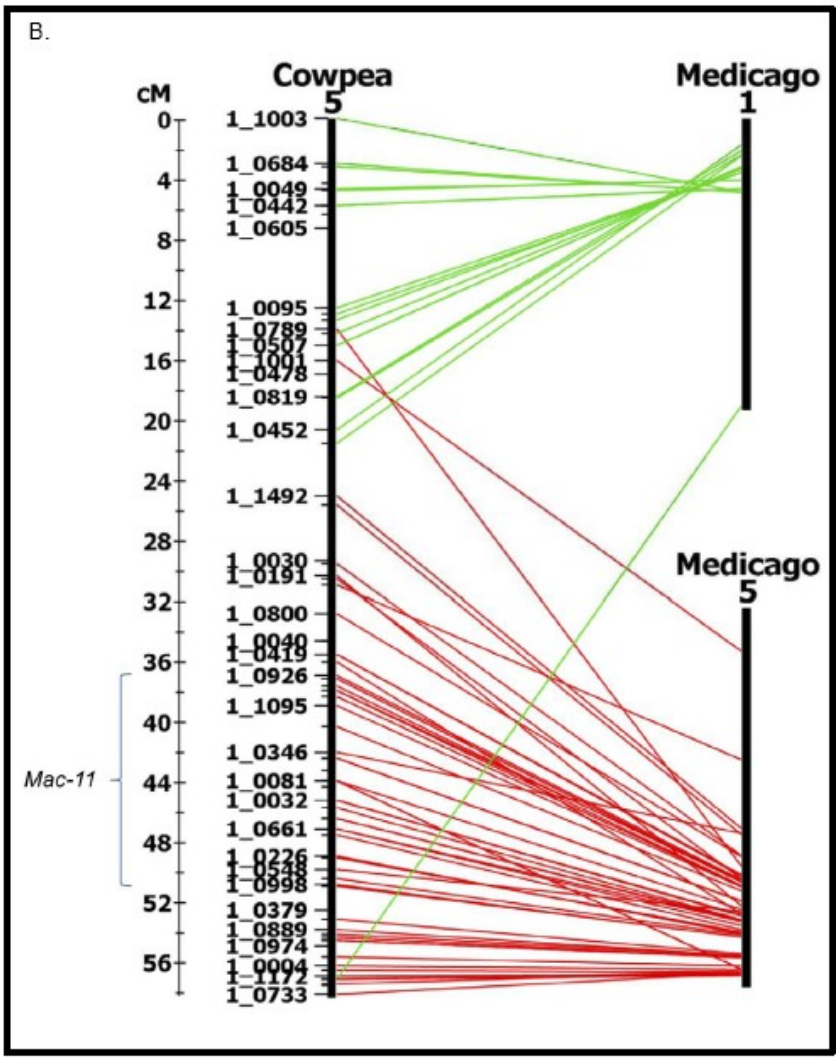


Figure 4.9 Synteny of *Mac-11* locus with *P. vulgaris* chromosome 8. Synteny was examined for the *Mac-11* locus between cowpea and *P. vulgaris* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Mac-11* locus was highly syntenic with common bean chromosome 8, extending from locus Phvul.008G203600 to Phvul.008G243400 in which an auxin response factor was observed and considered as a candidate gene for the *Mac-11* locus. The syntenic map was drawn using HarvEST: Cowpea database using a cut off e-score value of -10 and a minimum number of 10 lines drawn per linkage group.

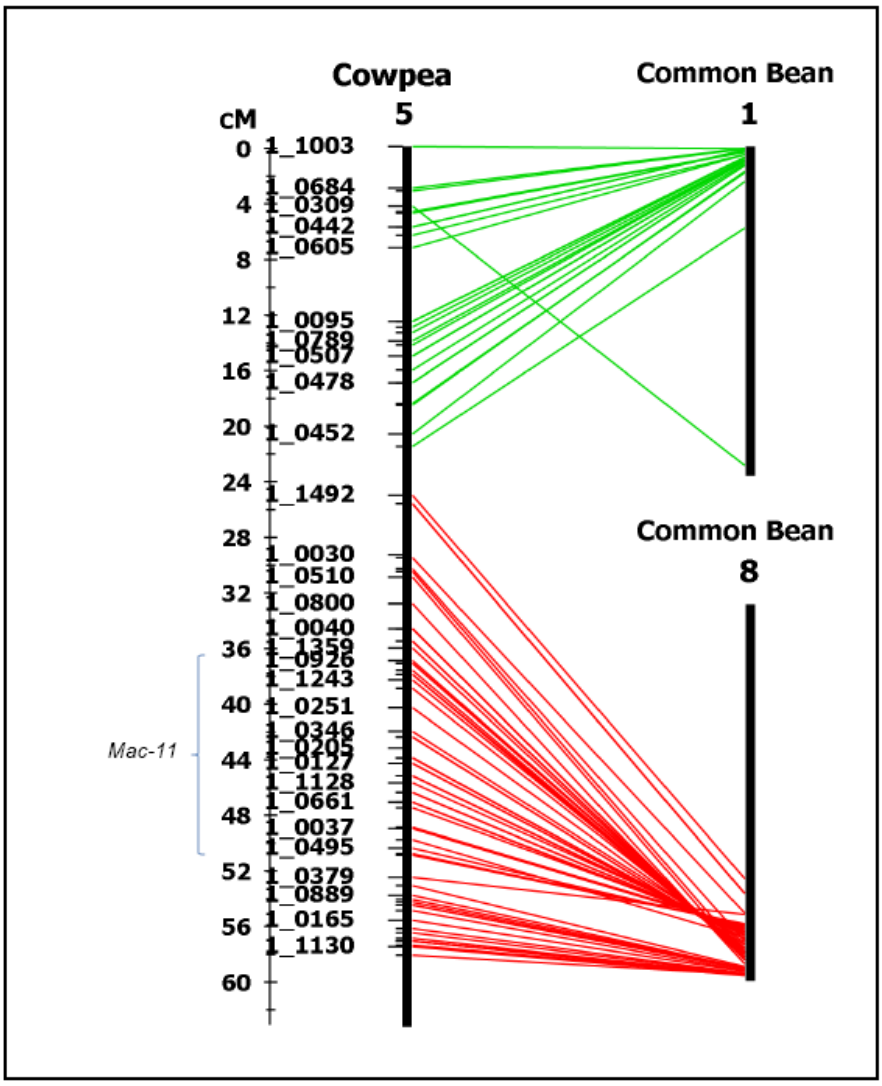


Figure 4.10 *Mac-12* locus in the Sanzi x Vita7 genetic map. The *Mac-12* QTL (Interval Mapping analysis shown) was observed in both the 2009 and 2010 field experiments and mapped to linkage group 6 of the Sanzi x Vita7 genetic map. For the 2009 experiment, *Mac-12* was observed for the entire experiment and SNP marker 1_1302 was the most significant marker and is highlighted in red. In the 2010 field experiment, *Mac-12* was observed inconsistently. SNP markers 1_0559, 1_0708 and 1_0278 were the most significant markers (red) on the linkage group. The 2009 percent survival data are plotted in green, the 2009 percent seedling damping off data are plotted in red, and the 2010 percent survivor data are plotted in fuchsia. The significance threshold of 2.0 is indicated by the horizontal broken line.

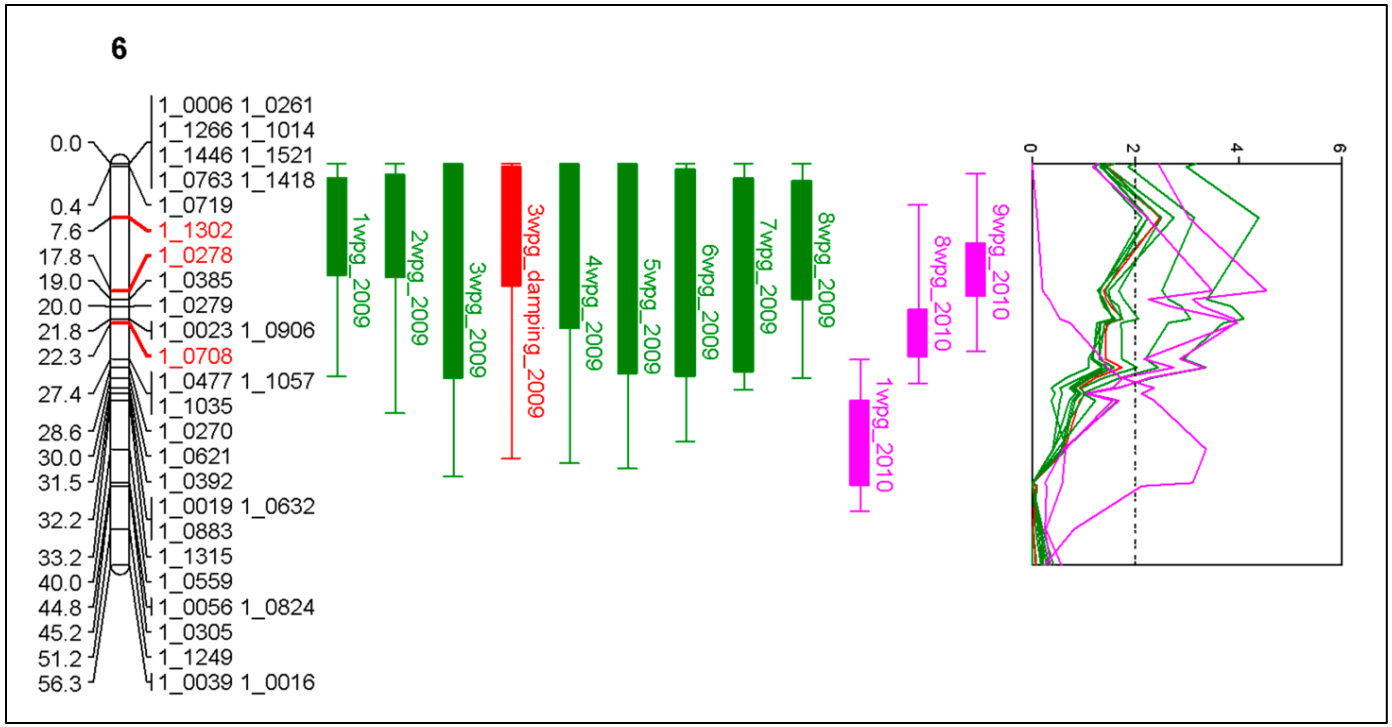


Figure 4.11 *Mac-13* locus in the Sanzi x Vita7 genetic map. The *Mac-13* QTL (Interval Mapping analysis shown) was observed in the 2010 field experiment from 2 to 9 weeks post germination. *Mac-13* spanned from 41.89 cM to 55.18 cM on linkage group 10. SNP marker 1_0826 (red) was the most significant marker. The significance threshold of 2.0 is indicated by the horizontal broken line.

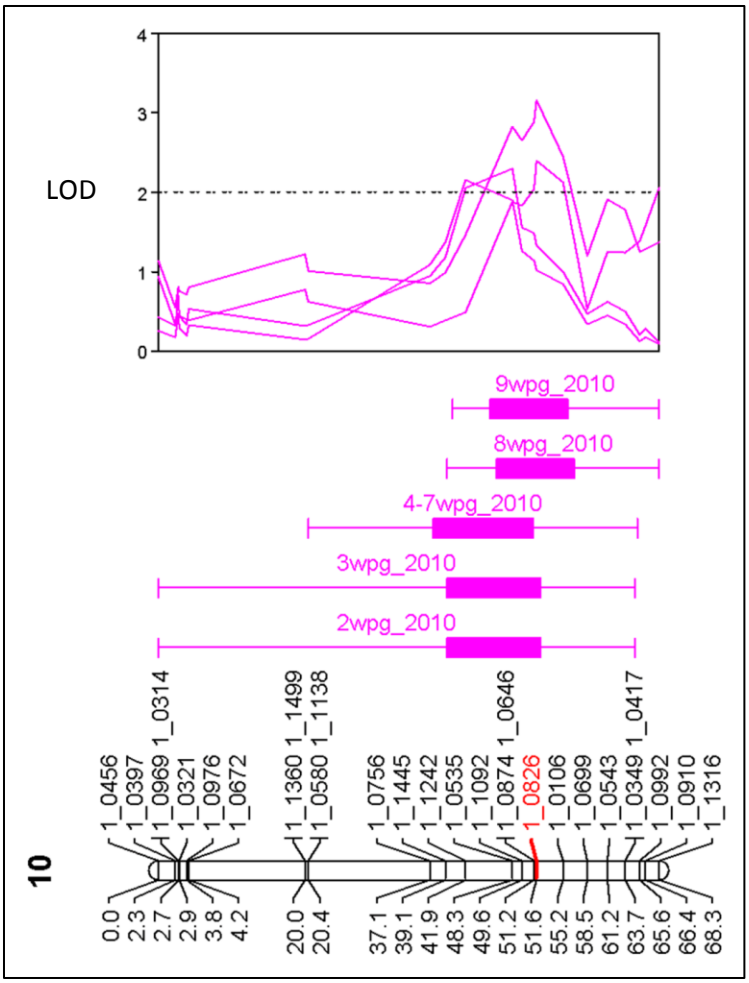


Figure 4.12 Marker-trait association in the *Mac-13* locus. A marker-trait association of the *Mac-13* locus was analyzed using fourteen cowpea genotypes which differ in their photoperiod sensitivity, days to maturity and tolerance to Macrophomina. Vita 7, IT93K-503-1, Apagbaala, IT98K-499-39, Suvita-2, Moussa Local and Iron Clay are obligate short-day photoperiod sensitive, late-maturing and Macrophomina-resistant. Sanzi, IT82E-18(Big Buff), CB46, CB27, UCR 24, and 524-B are day neutral, early-maturing and Macrophomina-susceptible. The *Mac-13* locus which spanned from 20.72 cM to 25.57 cM on the cowpea consensus genetic map linkage group 4 which is depicted horizontally. SNP marker 1_1242 (position 20.72 cM) alleles co-segregated with the Macrophomina-resistant and Macrophomina-susceptible genotypes. The Macrophomina resistant genotypes were associated with the cytosine nucleotide which is color-coded red and the Macrophomina-susceptible genotypes were associated with the thymine nucleotide which is color-coded green. The thymine/cytosine SNP is at position 685 in the cowpea unigene 4217 which was annotated as an N-terminal acetyltransferase and can be viewed in HarvEST: Cowpea.

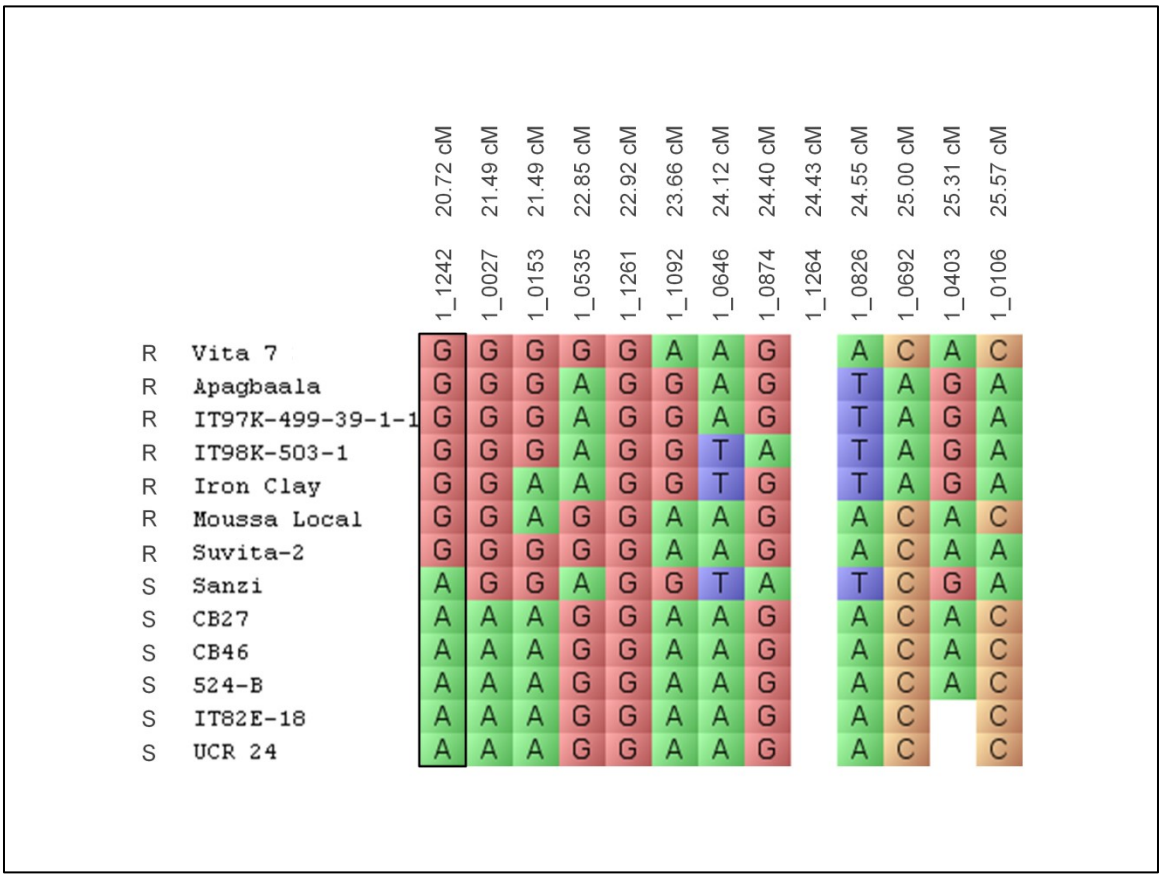


Figure 4.13 Synteny of *Mac-13* locus with *G. max* chromosomes 3 and 19. Synteny was examined for the *Mac-13* locus between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Mac-13* locus on the cowpea consensus genetic map linkage group 4 (20.72 cM to 25.57 cM was determined to be syntenic with soybean chromosomes 3 and 19. The syntenic locus in soybean chromosome 3 spanned from soybean loci Glyma03g31080 to Glyma03g33270. Candidate genes observed in the region included terpene synthase genes, chalcone-flavanone isomerase and AUX/IAA family genes. The syntenic locus in soybean 19 spanned from soybean locus Glyma19g34380 to Glyma19g36180. Two AUX/IAA family genes were observed in the region and were considered as candidate genes. The syntenic map was drawn using HarvEST: Cowpea database using a cut off e-score value of -10 and a minimum number of 10 lines drawn per linkage group.

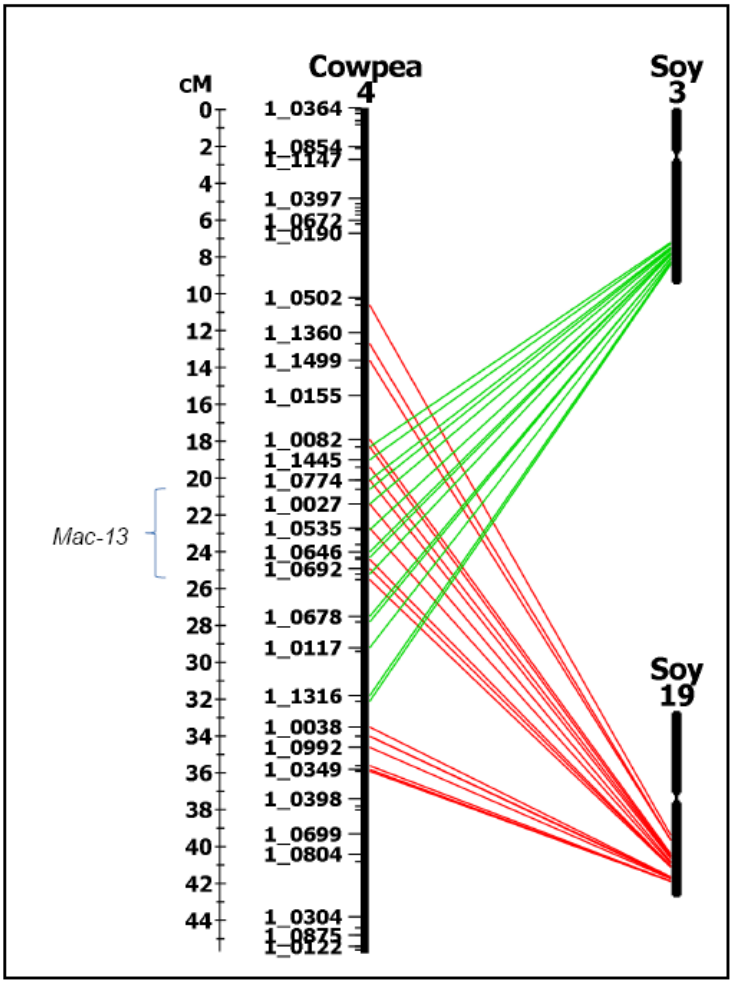
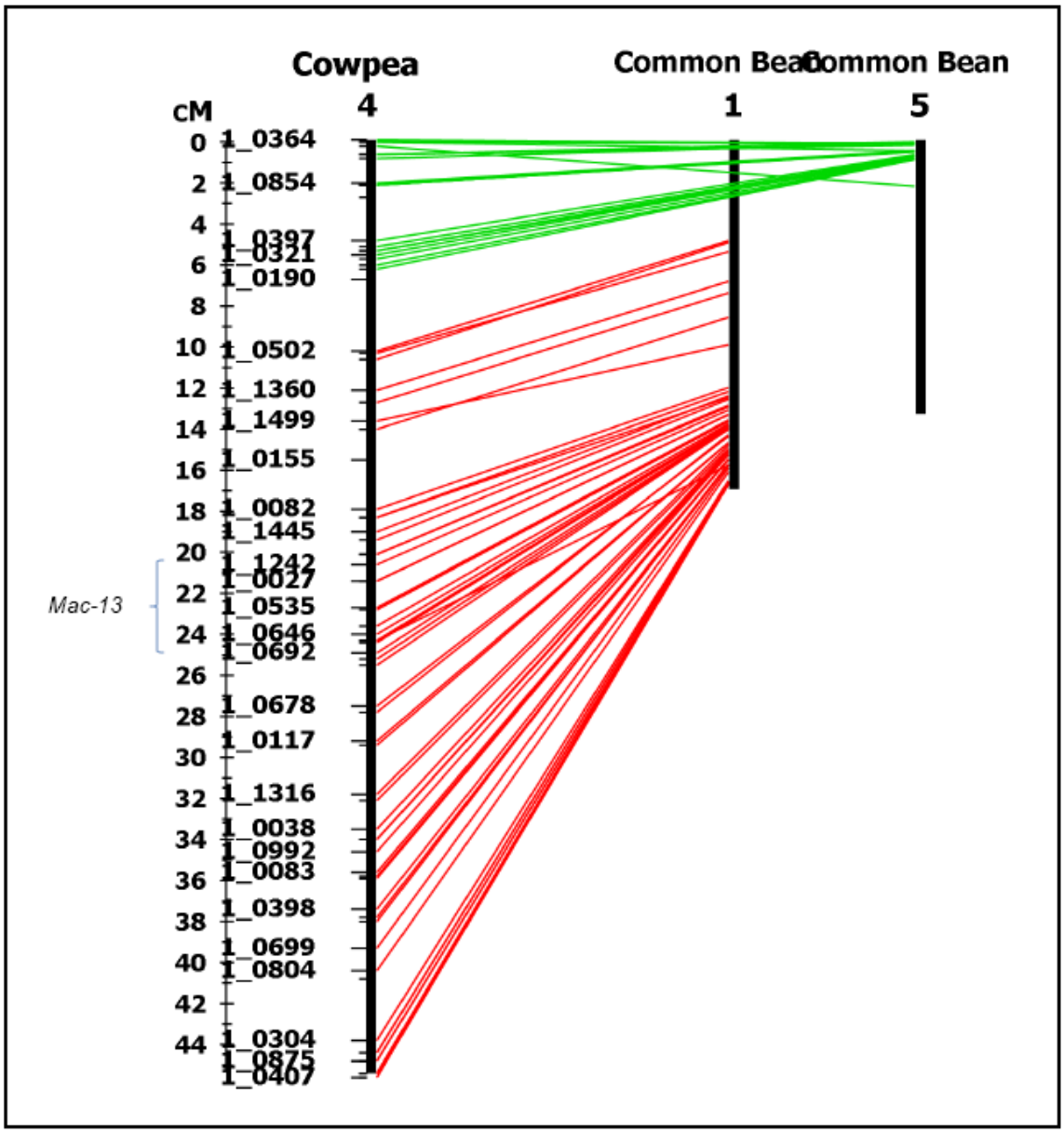


Table 4.14 Synteny of *Mac-13* locus with *P. vulgaris* chromosome 1. Synteny was examined for the *Mac-13* locus between cowpea and *P. vulgaris* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. A syntenic relationship was observed for the *Mac-13* locus with *P. vulgaris* chromosome 1, extending from Phvul.001G147300 to Phvul.001G174300. Several auxin-induced genes, terpenoid cyclases, chalcone-flavanone isomerase and ethylene responsive genes were observed in the syntenic locus and were considered candidate genes. The syntenic map was drawn using HarvEST: Cowpea database using a cut off e-score value of -10 and a minimum number of 10 lines drawn per linkage group.



Chapter 5

Leaf Morphology in Cowpea [*Vigna unguiculata* (L.) Walp]: QTL Analysis, Physical Mapping and Identifying a Candidate Gene using Synteny with Model Legume Species

Abstract

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. 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. 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.

Introduction

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 majority of the 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 (Inaizumi et al. 1999).

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 (Barrett 1987). Cowpea shoots and leaves are rich sources of calcium, phosphorous and Vitamin B (Maynard 2008). 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 (Dugje et al. 2009).

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 (Krishnaswamy et al. 1945; Jindla and Singh 1970; Ojomo 1977; Kohle 1970; Fery 1985b; Oluwatosin 2002). 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) (Muchero et al. 2009a; Lucas et al. 2011). 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 (AM). The identified and confirmed QTLs will facilitate cultivar improvement using marker-assisted selection (MAS) 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

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 5.1).

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 (Oluwatosin 2002; Ojomo 1977; Kohle 1970; Saunders 1960). Although the F_1 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 (Krishnaswamy et al. 1945; Jindla and Singh 1970; Ojomo 1977; Kohle 1970; Fery 1985a; Oluwatosin 2002). 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

(Table 5.2, Table 5.3, Figure 5.1). 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 5.3). 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 (Harland 1919). Ojomo 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, Ogundiwin 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 5.3). The *Hls* locus spans from 25.57 cM to 35.96 cM on the cowpea consensus genetic map linkage group 4 (Table 5.3). 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 5.3). 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 5.3). In addition, there was a slight difference in the order of the SNP markers in the Sanzi x Vita7 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 (Table 5.4). PI 632882, CB27, Bambey 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 (Table 5.4). 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 5.2). The allele for the hastate genotype at this locus was the thymine nucleotide (color coded blue in Figure 5.2). The allele for the sub-globose genotype was the cytosine nucleotide (color coded red in Figure 5.2). 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) (Figure 5.3). 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 5.2).

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 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 has been used in fidelity. In order to view each individual 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.

A high co-linearity was observed for the *Hls* locus with Medicago chromosome 7 (Table 5.5, Figure 5.4). Eight Medicago genes orthologous to cowpea SNP markers were identified in the syntenic region of Medicago chromosome 7 (Table 5.5). The syntenic region spanned from Medtr7g084010 locus to Medtr7g134530 locus which corresponded to 29.30 cM to 35.96 cM of the *Hls* locus on the cowpea consensus genetic map (Table 5.3, Table 5.5). The region which spanned from Medicago genes orthologous to cowpea SNP markers 1_1013 to 1_0349 were in the same linear order as on the cowpea consensus genetic map (Table 5.3, Table 5.5). The region spanning between Medicago 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 (Barkoulas et al. 2007) 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 5.5). 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 (Table 5.6, Figure 5.4). 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 5.6). 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 5.6). 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_0417 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 5.6). 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 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 5.6). 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) (Guitton and Berger 2005). 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 (Wang et al. 2006), flower organ development (Goodrich et al. 1997; Chanvivattana et al. 2004; Schubert et al. 2006) and leaf development (Goodrich et al. 1997; Katz et al. 2004).

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 (Goodrich et al. 1997) and EZA1/SWINGER is expressed in regions of dividing cells and meristems during vegetative and reproductive development (Chanvivattana et al. 2004). CLF has been shown to directly target and repress the floral

homeotic gene, AGAMOUS (AG), and a homeobox gene, SHOOTMERISTEMLESS (STM) (Schubert et al. 2006; Katz et al. 2004). SWN has been shown to have partially redundant functions with CLF and therefore may also be involved in regulating leaf development (Chanvivattana et al. 2004). 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 (Chanvivattana et al. 2004). 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 (Kearsey and Farquhar 1998). 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 phenotyping 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 5.5, 5.6 and 5.7). 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 (Table 5.7). 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 5.8). The genomic sequences for Glyma03g38320 and Glyma19g40430 returned a high alignment with contig C27664167 and scaffold28398 (Table 5.8). 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 5.8). Interestingly, unigene 21752 was obtained from leaf and shoot meristems used for a mature pre-flowering developmental stage cDNA library from cowpea varieties DanIla, 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 genetic map identified several BAC contigs which incompletely span the *Hls* region (Table 5.3). The most significant SNP marker from the QTL analysis, 1_0910, was identified in BAC clone CH050F07 of contig821 (Table 5.3). 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 5.3). 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 5.3). 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.

Materials and 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 F_{10} 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 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 5.5) 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 cm^3 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 F_8 generation using bi-allelic SNP markers from the 1536 Illumina GoldenGate Assay as previously described (Muchero et al. 2009). All genotypes used for the marker-trait association study were SNP genotyped at the F_8 generation or above as previously described (Muchero et al. 2009).

Genetic maps

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 (Lucas et al. 2011). 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 (Lucas et al. 2011). The consensus vs. 4 map consists of ten RIL populations and two F_4 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 (Van Ooijen 2004). 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 (Voorrips 2002).

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 *Hls* locus were visualized using GGT 2.0 software (Van Berloo 2008). The cowpea consensus genetic map vs.4 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://phymap.ucdavis.edu/cowpea). It consists of two BAC clone libraries developed using restriction enzymes *Hind*III and *Mbo*I (Amplicon Express, Pullman, WA). Contigs were assembled using the snapshot method of DNA fingerprinting (Luo et al. 2003a) 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 1640bp (personal communication, Ming Cheng Luo).

List of abbreviations

BAC: bacterial artificial chromosome; BES: BAC end sequence; bp: base pairs; cM: centimorgans; EST: expressed sequence tags; EZA1: ENHANCER OF ZESTE; LG: linkage group; LOD: logarithm of (base 10) of odds; MAS; marker-assisted selection; Mb: megabases; MTP: minimum tiling path; Pc-G: Polycomb-group protein; QTL: quantitative trait locus; RIL: recombinant inbred line; SNPs: single nucleotide polymorphism; SWN: SWINGER.

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Author contributions:

MP conducted the greenhouse and field experiments. MP analyzed the genetic inheritance, QTL analysis, marker-trait association, candidate gene analysis using synteny and comparison of the cowpea consensus genetic map and physical map. CF provided the RIL population. MP, JDE, PAR and TJC participated in the design, interpretation of data and writing of the manuscript.

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Table 5.1 Inheritance of leaf shape in Sanzi x Vita 7 population.					
Experiment	Hastate	Sub-globose	Ratio	x^2	p-value
Greenhouse	58	60	1:1	0.03	0.85
Field	59	57	1:1	0.03	0.85

Table 5.2 QTL analysis of the *Hls* locus in the Sanzi x Vita 7 population.							
Experiment	Analysis	1_0106	1_1316	1_0417	1_0349	1_0992	1_0910
Greenhouse	IM LOD	27.32	28.8	24.18	24.18	31.21	33.82
	IM R^2	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	28.77	22.44	22.44	28.57	30.89
	IM R^2	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

Table 5.3 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		4	27.60	1_0678	1014	CH021P21
		N/A		4	27.90	1_1209	N/A	
		N/A		4	29.30	1_0117	N/A	
		N/A		4	29.51	1_0128	N/A	
15	63.65	1_1316	28.80	4	31.88	1_1316	N/A	
		N/A		4	32.21	1_0157	N/A	
		N/A		4	33.57	1_0038	926	CM002I07, CM052G13
		N/A		4	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		4	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.

Table 5.4 Cowpea accessions with a hastate or sub-globose leaf phenotype.			
Hastate or "strip" leaf shape	Source/Origin	Sub-globose leaf shape	Source/Origin
Vita7/ PI 580806/ TVu 8461	IITA/Nigeria	Sanzi	Ghana
PI 632869/ TVNu 435	Malawi	California Blackeye 27 (CB27)	United States
PI 632875/ TVNu 523	Zambia	Bambey21	Senegal
PI 632876/ TVNu 531	Tanzania	PI 418979/ HAN CHUI YEN	Shaanxi, China
PI 632878/ TVNu 554	Zambia	PI 448337/ TVu 5018	Niger
PI 632899/ TVNu 113	Tanzania	PI 448682/ TVu 5473	Niger
PI 632910/ TVNu 109	Tanzania	PI 580445/ TVu 7382/ UCR 4734	Nigeria
PI 632913/ TVNu 353	Zambia	PI 580510/ TVu 7684/ UCR 4785	Nigeria

Table 5.5 The *Hls* syntenic region in *Medicago truncatula* chromosome 7.

Medicago locus	Position (bp)	Phytozome annotation	Cowpea SNP	LG	cM
Medtr7g084010	MtChr7: 18093097 - 18096342	Glycosyltransferase	1_1316	4	31.88
Medtr7g127710	MtChr7: 30002559 - 30004421	Small nuclear ribonucleoprotein G	1_0117	4	29.30
Medtr7g130340	MtChr7: 30448639 - 30451565	Tetrahydrofolate dehydrogenase/cyclohydrolase	1_1013	4	34.09
Medtr7g132610	MtChr7: 30739419 - 30778183	Histidine kinase	1_0910	4	34.09
Medtr7g132800	MtChr7: 30863955 - 30868447	Glycosyl hydrolase family 3 C terminal domain	1_0992	4	34.69
Medtr7g133020	MtChr7: 30974729 - 30981121	SWN (SWINGER); transcription factor	N/A	N/A	N/A
Medtr7g134340	MtChr7: 31708007 - 31710614	Peptidyl-prolyl cis-trans isomerase	1_0083	4	35.66
Medtr7g134420	MtChr7: 31747440 - 31752793	Papain family cysteine protease	1_0417	4	35.96
Medtr7g134530	MtChr7: 31793943 - 31799643	ATP-dependent RNA helicase	1_0349	4	35.87

Table 5.6 The *Hls* syntenic region in *Glycine max* chromosomes 3 and 19.					
G. max chromosome	*G. max* locus	Phytozome annotation	Cowpea SNP	LG	cM
3	Glyma03g34240	Protein phosphatase type 2A	1_1209	4	27.90
3	Glyma03g34420	UDP glycosyl transferase	1_0678	4	27.60
3	Glyma03g35490	Small nuclear ribonucleoprotein G	1_0117	4	29.30
3	Glyma03g36050	Glycosyl transferase	1_1316	4	31.88
3	Glyma03g36560	60S ribosomal protein	1_0157	4	32.21
3	Glyma03g37080	Tetrahydrofolate dehydrogenase	1_1013	4	34.09
3	Glyma03g38320	EZA1 (SWINGER); transcription factor	N/A	N/A	N/A
3	Glyma03g38520	Cysteine proteinase	1_0417	4	35.96
3	Glyma03g38550	ATP-dependent RNA helicase	1_0349	4	35.87
19	Glyma19g36180	60S ribosomal protein	1_0106	4	25.57
19	Glyma19g36250	40S ribosomal protein S23	1_0061	2	24.10
19	Glyma19g38130	Small nuclear ribonucleoprotein G	1_0117	4	29.30
19	Glyma19g38170	Ubiquitin extension protein 2 (UBQ2)	1_0128	4	29.51
19	Glyma19g38720	Glycosyl transferase	1_1316	4	31.88
19	Glyma19g39170	Protein phosphatase	1_1349	3	39.80
19	Glyma19g39240	60S ribosomal protein L21	1_0157	4	32.21
19	Glyma19g39570	60S ribosomal protein L19	1_0038	4	33.57
19	Glyma19g39710	Tetrahydrofolate dehydrogenase	1_1013	4	34.09
19	Glyma19g40080	60S ribosomal protein L19	1_0038	4	33.57
19	Glyma19g40090	Histidine kinase	1_0910	4	34.09
19	Glyma19g40300	Glycosyl hydrolase family	1_0992	4	34.69
19	Glyma19g40430	EZA1 (SWINGER); transcription factor	N/A	N/A	N/A
19	Glyma19g41120	Cysteine proteinase	1_0417	4	35.96
19	Glyma19g41150	ATP-dependent RNA helicase	1_0349	4	35.87

Table 5.7 Summary of significant markers in the *Hls* locus.							
Analysis	Description	Sanzi x Vita 7			Cowpea genetic map		
		SNP	LG	cM	SNP	LG	cM
Synteny	Flanking marker to candidate gene	N/A			1_1013	4	34.09
QTL analysis	Most significant marker in QTL analysis	1_0910	15	67.54	1_0910	4	34.09
Synteny	Flanking marker to candidate gene	1_0992	15	67.20	1_0992	4	34.69
Synteny	Flanking marker to candidate gene	N/A			1_0083	4	35.66
Marker-trait association	Co-segregated with genotype/phenotype	1_0349	15	66.46	1_0349	4	35.87
Synteny	Flanking marker to candidate gene	1_0417	15	66.46	1_0417	4	35.96

Table 5.8 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

Figure 5.1 *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.

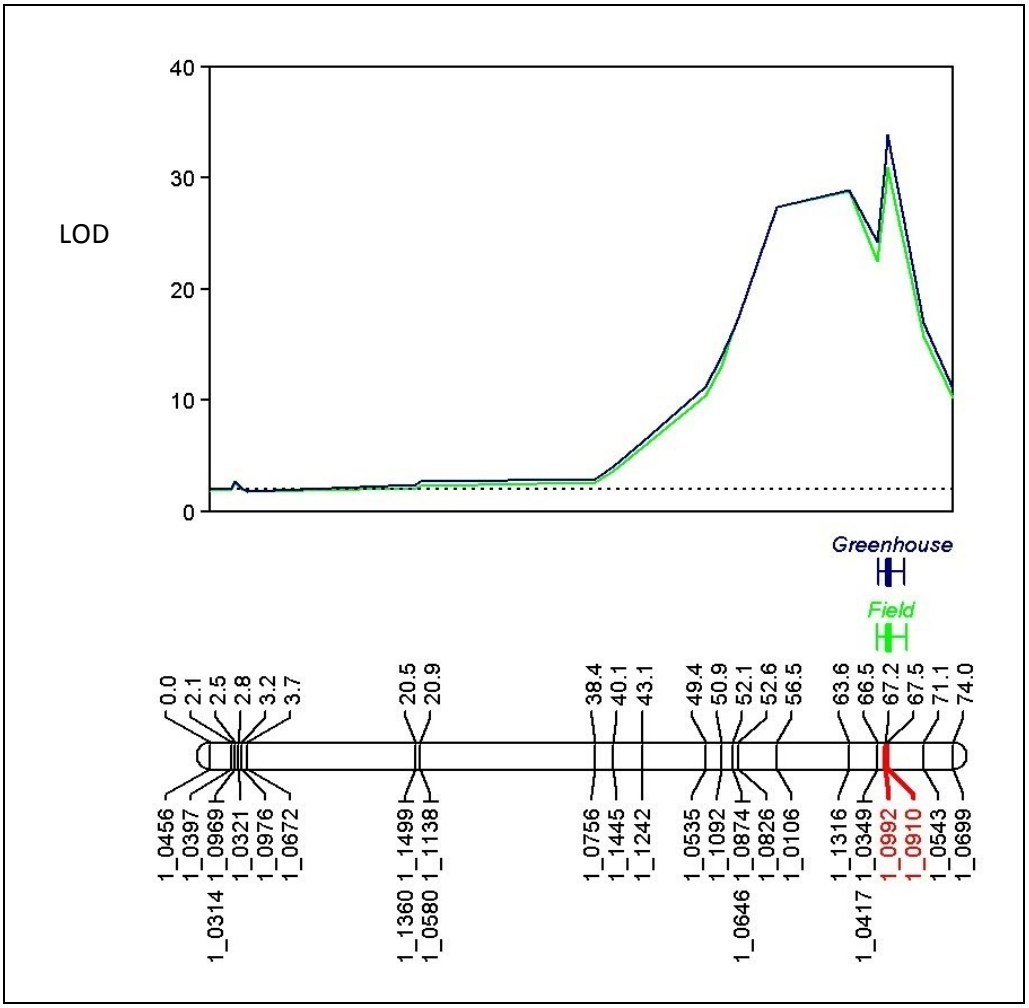


Figure 5.2 Marker-trait association in the *Hls* locus. The *Hls* locus on the cowpea consensus genetic map linkage group 4 is depicted vertically along with cowpea genotypes which differ in hastate or sub-globose leaf shape. Red colored blocks indicate the "AA" allele, blue colored blocks indicate the "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)

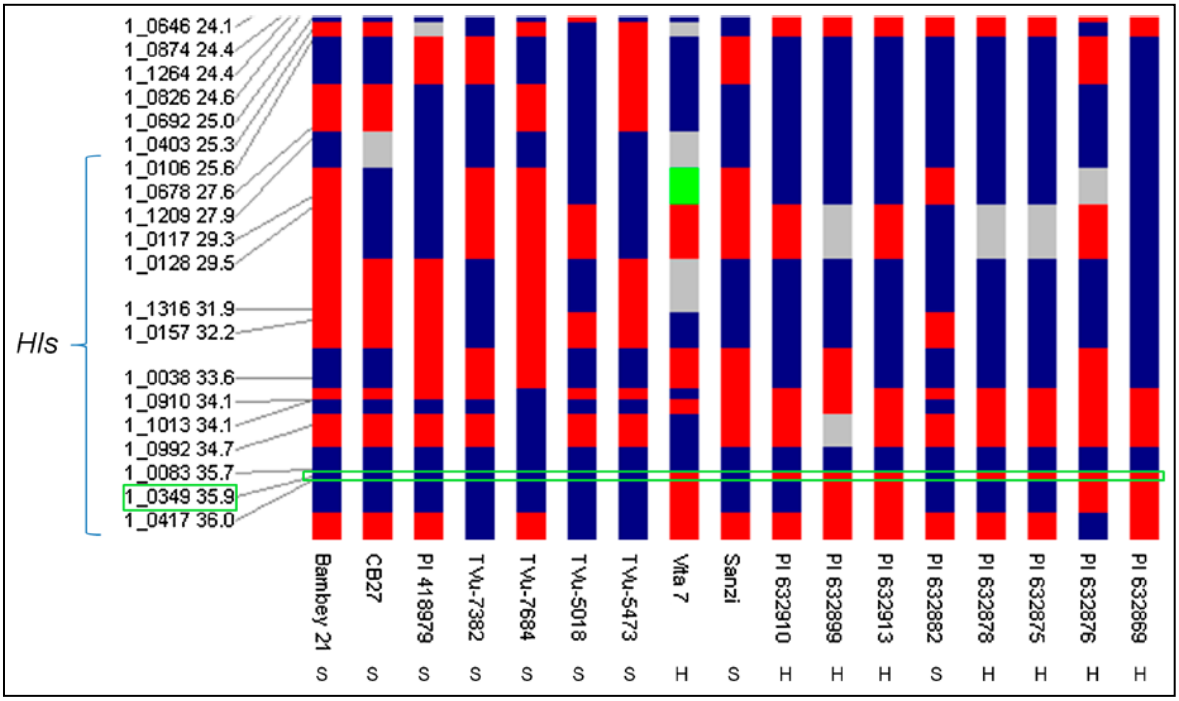


Figure 5.3 SNP marker 1_0349 sequence

CATTGCTCGCGCGAAGACCGGTACTGGAAAGACGCTAGCGTTCGGAATTCCAGTTATTA
AAGGCCTCACTGAAGTTGAAGATGAGCCTTCTCTCAGGAGGTCTGGTAGGCTTCCCAGA
GTTTTGGTGCTGGCCCCTACGAGGGAGTTGGCGAAGCAAGTGGAGAAGGAGATAAAGGA
ATCTGCTCCTTATCTCAGCACTGTTTGTGTTTATGGCGGTGTTTCTTATGTTACTCAGC
AGAGTGCTCTTTCACGAGGTGTAGATGTGGTGGTCGGGACCCCAGGGAGAATAATTGAC
TTGATTAATGGGAAGAGCCTTAAGCTGAATGAGGTTCAGTATTTGGTGCTTGATGAAGC
AGATCAGATGCTTGCTGTTGGGTTTGAGGAGGATGTGGAAGTGATTTTAGAGAACCTCC
CTTCTCAGAGGCAGAGCATGCTTTTCTCTGCCACCATGCCTGCTTGGGTGAAGAAGTTG
GCGAGAAAATATTTGAACAACCCACTCACAATTGATTTGGTTGGTGATGAAGAAGAAAA
GCTCGCTGAAGGGATAAAACTTTTTGCTATATCAGCCACTGCCACTTCAAAGCGGACAA
TTCTCTCTGATCTCGTAACTGTTTATGCAAAGGGTGGGAAGACTATTGTATTTACACAG
ACAAAAAAAGATGCTGATGAAGTATCACTGTCATTAACAAATAGTATAACGTCTGAAGC
ACTGCATGGTGATATATCTCAGCATCAGAGAGAAAGAACATTGAATGGTTTTCGGCAAG
GAAAATTCACAGTGCTTGTTGCTACTGATGTTGCAGCTCGTGGACTTGATATTCCCAAT
GTTGATTTGATTATCCATTATGAGCTTCCCAATGATCCTGAGACTTTCGTACACCGCTC
TGGTCGTACTGGTCGTGCTGGAAAACAAGGTACTGCCATTCTGTTGTACACCAGTAGCC
AGAGGAGAACAGTTAGATCCCTTGAACGTGATGTAGGCTGCAAGTTTGAATTTGTTAGT
CCGCCAGCTATGGAAGAGGTCTTGGAGTCATCTGCGGAGCAGGTTGTTGCCACACTTGG
TGGAGTTCATCCCGAATCTATCCAGTTTTTCACCCCAACTGCACAAAAACTGATCGAAG
AACAAGGAACAACTGCCCTTGCCGCTGCCCTTGCACAACTTAGTGGATTTTCCCGACCT
CCATCATCCCGGTCTCTTATCACCCACGAACAGGGATGGACTACGTTGCAACTAATTCG
GGATTCGGAGAATAGTAGATATTTTTCAGCAAGATCAGTCACTGGGTTTCTTTCTGATG
TTTTTTCATCAGCTGCCGATGAAGTTGGAAAAATCCATATAATTGCAGATGAAAGGGTT
CAAGGAGCCGTTTTTGATCTTCCCGAGGAGATTGCTAAAGAGTTGCTTACTAAGGACAT
ACCACCTGGTAACACCATTTCCAAGATCACCAAGCTACCTCCTTTGCAAGACGATGGGC
CTCCAAGTGATTTCTATGGAAGGTTCTCTGACAGAGAACGTGGTAACCGAAGAGGATCT
ACTTCTAGGGGAGGTTTTAGTTCTAGGGGAGGTGGTTTTGCTTCTAGGGACCGGAGAGG
TTTTAAATCCTCACGGGGATGGGATGGGGAAGACTCTGATGATGACGACTTCAGTGATC
GATCTAGTAGGAGAGGTGGTAGAAATTTTAAATCTGGCGGCAATAGCTGGTCTCGAGCA
GGAGGTAAAAGTGGTGGAGATGATTGGCTAATTGGGGGTAGACGATCAAGCCGGCCTTC
ATCATCAGACAGATTCGGAGGGGCCTGTTTCAATTGTGGGGAATCTGGTCATCGTGCAT
CAGATTGTCCAAACTCTTCAAACCGGCGAAGCTTTTTTTAAGTTCCCACATTTTTTTGG
GGCGCCGCTTTGACCATGACGGACATGAACTTGTGCCACTGTTATTGGCCTGATGGGTT
CCGGAAAATTGAAGCATGCTTACCGAAAAGAGTTACAGAAGCAATATTAGTTTGCATCT
CACGTGTTGGCGTGATCTCCGTGGGGACCTCCTTTGTCGTCCTCTTTTTTGTGTCTCAA
TGAAATTTAGTATTTGTTTGGGCTTAAGAATAGTGCTGTATCTTCTTTTTCGGGTT**(C/T)**GGTTTAAGAGGTTAGTTGTATGGTCCTGTATTCTTCTCAACTTATTATTTAACATCT
TTTTGAACCTTCCCGGTTTAGGAACAGACTGGAAAAATGAATGAAAGATGAAATCCTAA
AGGTTTATGCAAAAAAAAAAAAAAAAA

Figure 5.4 Synteny of the *Hls* locus with *Medicago truncatula* and *Glycine max*. Synteny was examined for the *Hls* locus between cowpea and *M. truncatula* and cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes. The *Hls* locus which spans 25.57 cM to 35.96 cM on linkage group 4 of the cowpea consensus genetic map was syntenic with Medicago chromosome 7. The syntenic locus spanned from Medicago locus Medtr7g084010 to Medtr7g134530. A candidate gene was identified in the highly significant syntenic region of *Hls*, Medtr7g133020, which was annotated as an ortholog of the Arabidopsis EZA1/SWINGER (SWN) gene. Two syntenic loci were identified for the *Hls* locus in soybean chromosomes 3 and 19. The syntenic region in soybean chromosome 3 spanned from the soybean locus Glyma03g34240 to Glyma03g38550. An orthologous candidate gene was observed in the most significant region of the syntenic *Hls* locus, Glyma03g38320, which was annotated as an ortholog of the Arabidopsis EZA1/SWINGER (SWN) gene. The syntenic *Hls* locus on soybean chromosome 19 spanned from Glyma19g36180 to Glyma19g41150 where another soybean ortholog of the EZA1/SWINGER (SWN) gene, Glyma19g40430, was observed. The syntenic map was drawn using HarvEST: Cowpea database (http://harvest.ucr.edu) using a cut off e-score value of -10 and a minimum number of 10 lines drawn per linkage group. Colored lines indicate cowpea genes orthologous to genes on *M. truncatula* and *G. max* chromosomes.

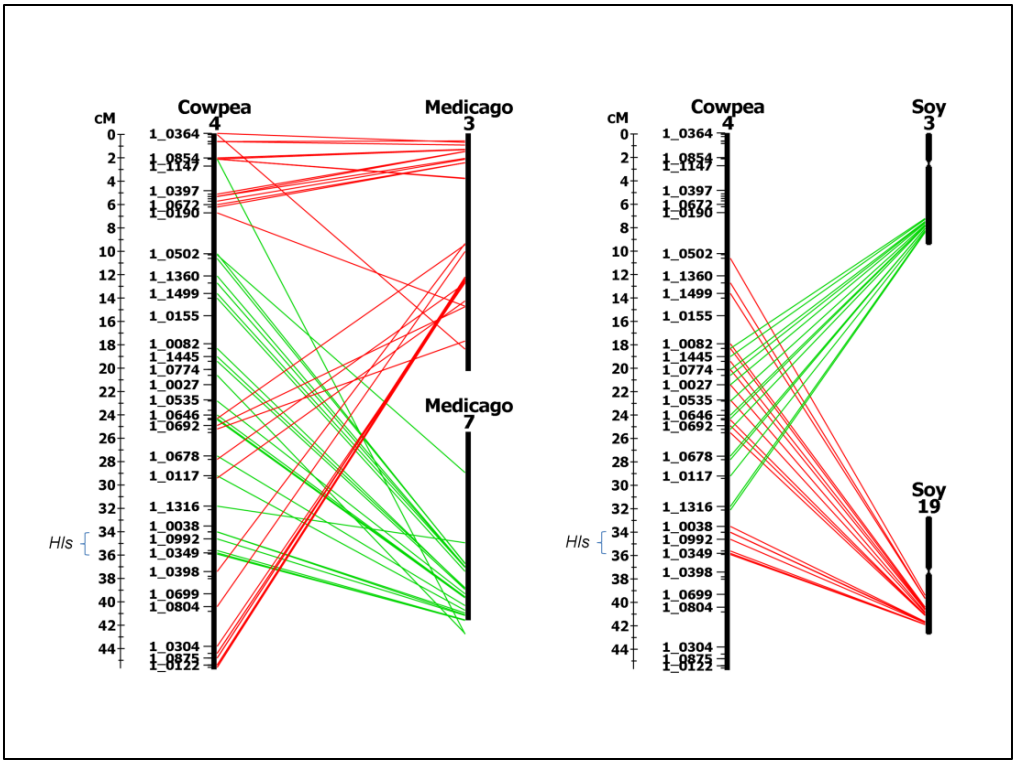
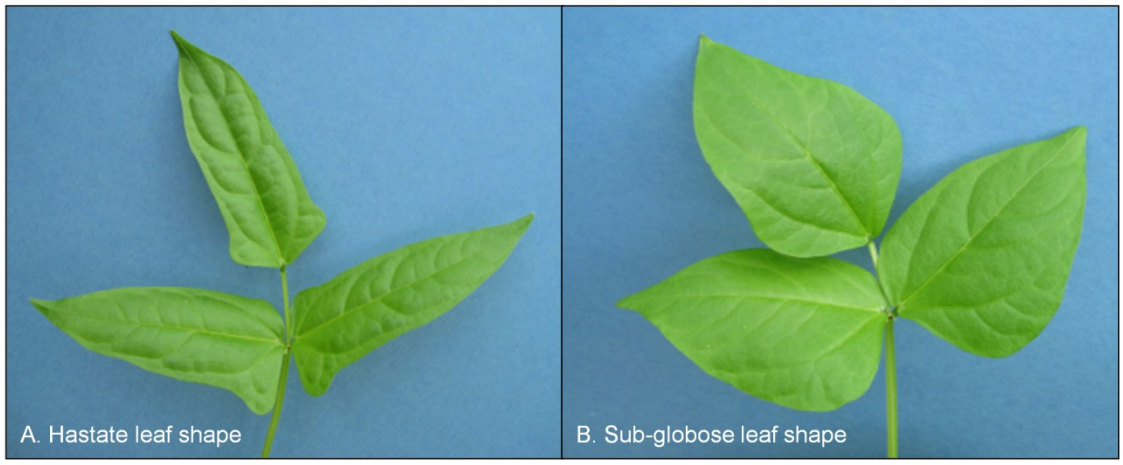


Figure 5.5 Hastate and sub-globose leaf shapes segregating in the Sanzi x Vita7 population.



Chapter 6

Identification of candidate genes and molecular markers for heat-induced brown discoloration of seed coats in cowpea [*Vigna unguiculata* (L.) Walp].

Abstract

Heat-induced browning (*Hbs*) of seed coats is caused by high temperatures which discolor the seed coats of many legumes, affecting the visual appearance and quality of seeds. The genetic determinants underlying *Hbs* in cowpea are unknown. We identified three QTL associated with the heat-induced browning of seed coats trait, *Hbs-1*, *Hbs-2* and *Hbs-3*, using cowpea RIL populations IT93K-503-1 (*Hbs* positive) x CB46 (*hbs* negative) and IT84S-2246 (*Hbs* positive) x TVu14676 (*hbs* negative). *Hbs-1* was identified in both populations, accounting for 28.3% -77.3% of the phenotypic variation. SNP markers 1_0032 and 1_1128 co-segregated with the trait. Within the syntenic regions of *Hbs-1* in soybean, Medicago and common bean, several ethylene forming enzymes, ethylene responsive element binding factors and an ACC oxidase 2 were observed. *Hbs-1* was identified in a BAC clone in contig 217 of the cowpea physical map, where ethylene forming enzymes were present. *Hbs-2* was identified in the IT93K-503-1 x CB46 population and accounted for 9.5 to 12.3 % of the phenotypic variance. *Hbs-3* was identified in the IT84S-2246 x TVu14676 population and accounted for 6.2 to 6.8 % of the phenotypic variance. SNP marker 1_0640 co-segregated with the heat-induced browning phenotype. *Hbs-3* was positioned on BAC clones in contig512 of the cowpea physical map, where several ACC synthase 1 genes were present. The identification of loci determining heat-induced browning of seed coats and co-segregating molecular markers will enable transfer of *hbs* alleles into cowpea varieties, contributing to higher quality seeds.

Introduction

Heat-induced browning is a phenomenon caused by high temperatures which discolor the seed coats of many legumes. The brown discoloration affects the visual appearance and quality of seeds which reduces its commercial value. The heat-induced brown discoloration affects the seed coats of soybean (Wang et al. 2002), common bean (Park and Maga 1999; Junk 2005) (Elsadr et al. 2011), cowpea (Hall and Patel 1988), faba bean (Nasar-Abbas et al. 2009) and lentil (Matus et al. 1993). The genetic determinants underlying heat-induced brown discoloration of seed coats in cowpea as well as other legumes is currently unknown.

In cowpea, the brown discoloration only appears on the seed coat and does not affect the underlying cotyledons (Hall and Patel 1988). In general, heat-induced browning can appear as patches or blotches, at the ends of the seed or over the entire surface of the cowpea seed (Hall and Patel 1988). Hall and Patel (1988) using microscopic and visual inspection noted that the tissue in the center of the brown discoloration appeared to be sunken and the seeds that were affected had rough textured seed coats. The brown discoloration of the seed coat has been observed in green seeds of fully matured cowpea pods, however, the brown discoloration is more distinct on dry matured seeds (Hall and Patel 1988).

Hall and Patel (1985) studied the genetic inheritance of heat-induced browning in three cowpea populations derived from crosses with the heat-induced browning genotype, Tvu 4552; Tvu 4552 x CB5, Tvu 4552 x Bambey 21 and Tvu 4552 x PI 204647. It was

confirmed with backcross populations that the *Hbs* trait is controlled by a single nuclear gene dominant to normal non-browning seed coat phenotype (*hbs*) (Hall and Patel 1988). The rate of the brown discoloration can be temperature controlled (Nielsen and Hall 1985; Hall and Patel 1988). An increase in night temperatures especially during pod-filling produced more browning of seeds with rough textured seed coats (Nielsen and Hall 1985).

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 genomic resources have been integrated by using a 1536-SNP genotyping platform and include an EST-derived SNP consensus genetic map (Muchero et al. 2009a; Diop et al. 2012; Lucas et al. 2011), known syntenic relationships between cowpea, *Medicago truncatula*, *Glycine max, Phaseolus vulgaris* and *Arabidopsis thaliana*, and a cowpea EST sequence collection housed in HarvEST:Cowpea database (Close and Wanamaker 2001). The cowpea physical map has been anchored to the consensus genetic map using the same SNP genotyping platform and sequenced BAC clones (Luo et al. 2007). In addition, more than 500 diverse cowpea accessions have been SNP genotyped and a first draft of the cowpea genome sequence has been assembled (Close and Wanamaker 2005). These resources will enable dissection of underlying genetic components of target agronomic traits using genetic and physical mapping.

In this study, we identified three QTL, *Hbs-1*, *Hbs-2* and *Hbs-3*, associated with heat-induced browning of seed coats using the cowpea RIL populations IT93K-503-1 x CB46 and IT84S-2246 x TVu14676. SNP markers were identified which co-segregated with the heat-induced browning of seed coats phenotype in the *Hbs-1* and *Hbs-3* loci, and could be used for indirect selection in breeding a higher quality cowpea grain. Additionally, ethylene forming enzymes were identified as a cowpea candidate gene for the *Hbs-1* locus and an ACC synthase 1 gene was identified as a cowpea candidate gene for the *Hbs-3* locus.

Results

QTL analysis

QTL analysis of two phenotypic datasets for the IT93K-503-1 (*Hbs*) x CB46 (*hbs*) population identified two loci for the heat-induced browning phenotype. We designated the major locus as *Hbs-1* and the minor locus as *Hbs-2*. *Hbs-1* spanned 35.21cM to 76.13 cM on linkage group 8 of the IT93K-503-1 x CB46 individual genetic map (Table 6.1, Figure 6.1). The most significant region (2-LOD) spanned 60.09 cM to 60.53 cM (0.44 cM total length) (Table 6.1, Figure 6.1). SNP markers 1_0032 and 1_1128 were the most significant markers for both experiments (Table 6.1). Marker 1_1128 had the highest association with the heat-induced browning phenotype and accounted for 62.8% (LOD score 20.01) and 77.3% (LOD score 30.19) of the phenotypic variance in the two experiments, respectively (Table 6.1). The corresponding *Hbs-1* locus was positioned on the cowpea consensus genetic map where it spanned 25.14 cM to 57.58 cM on linkage group 5; the most significant region (2-LOD) spanned from 45.27 cM to 45.76 cM (Table 6.2, Figure 6.2). The minor locus, *Hbs-2*, spanned from 36.82 cM to 51.79 cM on linkage group 3 of the individual map (Table 6.3, Figure 6.3). SNP marker 1_1342 accounted for the highest percent phenotypic variance of 9.5 % (LOD 2.11) and 12.3 % (LOD 2.77) (Table 6.3). *Hbs-2* was positioned on the cowpea consensus genetic map where it spanned from 31.28 cM to 58.09 cM on LG6 (Table 6.4, Figure 6.2). *Hbs-2* overlapped SNP markers 1_1346 (55.50 cM) and 1_0437 (57.41 cM) which were previously identified within the stage II heat-tolerance QTL *Cht-3* (Lucas et al. 2013a).

Heat shock proteins (HSP), DNA J heat shock family protein (DNA J HSP) and hydroxyproline-rich glycoprotein family (HPR) were identified as potential candidate genes within the syntenic loci of *Cht-3* in soybean (Lucas et al. 2013a). The overlap of the *Hbs-2* locus with the *Cht-3* locus does not concur with the findings of Hall and Patel (1988) in which the heat-induced browning of seed coats trait was not linked with heat tolerance in early floral bud development. However, this QTL mapping study and Lucas et al. (2013) used different cowpea populations than the study by Hall and Patel (1988).

The heat-induced browning of seed coats trait was mapped in the IT84S-2246 (*Hbs*) x TVu14676 (*hbs*) population using two phenotypic datasets, wherein one major and one minor QTL were identified. The major locus was found to overlap directly with the IT93K-503-1 x CB46 *Hbs-1* locus on the cowpea consensus genetic map, spanning from 34.66 cM to 56.58 cM on linkage group 5 (Table 6.2, Figure 6.2). The most significant region (2-LOD) spanned from 45.27 cM to 46.51 cM (Table 6.2, Figure 6.2). Therefore, the major heat-induced browning locus identified in the IT84S-2246 x TVu14676 population will also be referred as *Hbs-1*. *Hbs-1* spanned 24.98 cM to 59.60 cM on linkage group 9 of the IT84S-2246 x TVu14676 individual map (Table 6.5, Figure 6.4). SNP 1_0032 was the most significant marker for both experiments, accounting for 28.3% and 34.1% of the phenotypic variance and LOD scores of 9.54 and 12.05, respectively (Table 6.5). The minor locus which spanned 17.79 cM to 20.97 cM on linkage group 3 was designated as *Hbs-3* (Table 6.6, Figure 6.5). SNP markers 1_0280, 1_1534 and 1_1404 shared the same marker bin and accounted for 6.2 % and 6.8 % of the phenotypic

variance with LOD scores of 1.85 and 2.02, respectively (Table 6.6). The *Hbs-3* locus was positioned on the cowpea consensus genetic map where it spanned 36.0 cM to 37.96 cM on linkage group 1 (Table 6.7, Figure 6.2).

Marker-trait association within the *Hbs-1* and *Hbs-3* loci

Cowpea genotypes which differ in their phenotype to heat-induced browning of seed coats were chosen for a marker-trait association to narrow the *Hbs-1* and *Hbs-3* loci. IT93K-503-1, IT84S-2246, IT93K-2046, TVu-4552, TVx-3236, TVu-53 and TVu-15315 were positive for the heat-induced browning phenotype and are referred to as *Hbs* (Table 6.8). TVu-14676, CB5, CB27, CB46, 524B and Bambey 21 were negative for the heat-induced browning phenotype and are referred to as *hbs* (Table 6.8). Within the most significant region of the *Hbs-1* locus, which extended from 45.27cM to 46.51 cM on LG5, 2 out of 6 SNP markers co-segregated with *Hbs* (positive) and *hbs* (negative) genotypes (Figure 6.6). The *Hbs* positive genotypes had the adenine nucleotide at the 1_0032 locus, while the *hbs* negative genotypes were associated with the guanine nucleotide (Figure 6.6). The adenine/guanine SNP in marker 1_0032 is at position 469 of cowpea unigene 5294 and can be viewed in HarvEST:Cowpea (Close and Wanamaker 2001). For SNP marker 1_1128, the *Hbs* positive genotypes had the thymine nucleotide which is color-coded blue while the *hbs* negative genotypes had the adenine nucleotide and were color-coded green (Figure 6.6). The thymine/adenine SNP in marker 1_1128 is position 950 of cowpea unigene 4874 and can be viewed in HarvEST:Cowpea (Close and

Wanamaker 2001). SNP markers 1_0032 and 1_1128 could both be used as molecular markers to screen against the heat-induced browning of seed coats trait in cowpea.

The same *Hbs* positive and *hbs* negative genotypes were used to narrow the *Hbs-3* locus, which spanned 36.00 cM to 37.96 cM on LG1. The alleles for SNP marker 1_0640 co-segregated with *Hbs* positive and *hbs* negative phenotypes (Figure 6.7). The *Hbs* positive genotypes were associated with the adenine nucleotide while *hbs* negative genotypes were associated with the guanine nucleotide (Figure 6.7). The SNP for marker 1_0640 is position 348 and can be viewed in HarvEST: Cowpea (Close and Wanamaker 2001). SNP marker 1_0640 could also be used for screening germplasm and breeding material against the minor heat-induced seed coat browning locus.

Theoretically, these three "tagged SNPs" could be used to genotype the *Hbs-1* and *Hbs-3* haplotype blocks to determine the phenotype, rather than the sixty-six SNP markers within the *Hbs-1* QTL and eleven SNP markers within the *Hbs-3* QTL. However, a larger and more diverse set of cowpea germplasm would be needed to test and validate this approach.

Synteny of *Hbs-1* with *G. max*, *M. truncatula* and *P. vulgaris*

The *Hbs-1* locus was examined for synteny with the soybean genome and a high co-linearity was observed for soybean chromosomes 2 and 14 (Figure 6.8). Soybean orthologs for eleven out of twenty-three SNP markers were identified in the co-linear region of soybean chromosome 2, spanning from Glyma02g42560 to Glyma02g43640, which corresponded to 44.42 cM to 46.51 cM of the *Hbs-1* locus (Table 6.9). The region

surrounding the soybean orthologs to SNP markers 1_1128 and 1_0032 was examined on the soybean genome browser on the Phytozome website (Goodstein et al. 2012) for genes associated with heat stress. Several soybean loci were closely linked with the soybean ortholog for cowpea SNP 1_1128 and were considered candidate genes for the heat-induced browning of seed coats phenotype: Glyma02g43500 was annotated as an ATERF3/ERF3 (ethylene responsive element binding factor); Glyma02g43560, Glyma02g43580 and Glyma02g43600 were annotated as ethylene-forming enzymes (EFE) (Table 6.9). The syntenic locus on soybean chromosome 14 spanned from Glyma14g05250 to Glyma14g06330 which corresponded to 44.42 cM to 47.18 cM of the *Hbs-1* locus on the cowpea consensus genetic map (Table 6.9). Soybean locus Glyma14g05470 was annotated as an ATERF3/ERF3 (ethylene responsive element binding factor 3) and was considered a putative candidate gene for the *Hbs-1* locus (Table 6.9). Other putative soybean candidate genes for the *Hbs-1* locus included Glyma14g05350, Glyma14g05360 and Glyma14g05390, which were annotated as EFEs (Table 6.9).

The *Hbs-1* locus was syntenic with *M. truncatula* chromosome 5 where it spanned from Medicago locus Medtr5g018870 to Medtr5g093060, which corresponded to 44.42 cM to 47.18 cM of the *Hbs-1* locus on the cowpea consensus genetic map (Table 6.10, Figure 6.9). Several Medicago genes were observed in the region of Medicago orthologs to SNPs 1_0032 and 1_1128 and were considered as putative candidate genes for the *Hbs-1* locus; Medtr5g092410, Medtr5g092450 and Medtr5g092470 were annotated as ethylene

response factor 3 (ERF3), Medtr5g092480 was annotated as ERF11 and Medtr5g092760 was annotated as an EFE (File 6.10).

SNP markers for the *Hbs-1* locus were examined in the common bean genome to determine if a syntenic relationship existed; the *Hbs-1* locus was highly co-linear with common bean chromosome 8, extending from locus Phvul.008G213300.1 to Phvul.008G214300.1 (Table 6.11, Figure 6.10). The marker-order between cowpea and common bean was conserved, although the gene order was inverted (Table 6.11). Phvul.008G213800.1 and Phvul.008G213900.1 were slightly upstream from co-segregating SNP marker 1_1128 and were annotated as ethylene-forming enzyme and ACC oxidase 2 (Table 6.11).

***Hbs-1*, *Hbs-2* and *Hbs-3* on the cowpea physical map**

The cowpea physical map (Luo et al. 2007) which has been anchored to the cowpea consensus genetic map via SNP markers and sequenced BAC clones was used to identify a contig which overlapped the *Hbs-1, Hbs-2* and *Hbs-3* loci. Significant markers from the QTL analysis and closely linked markers from the cowpea consensus genetic map identified cowpea BAC contigs and clones which overlapped the heat-induced browning QTLs. SNP markers 1_1128 and 1_0120 which were identified in BAC clone CM018C23 of contig217 positioned the *Hbs-1* locus on the physical map (Table 6.2). BAC clone CM018C23 has 84 fingerprint bands which estimated its size as 137,760 bps (Luo et al. 2007). Annotations for BAC clone CM018C23 revealed the presence of three ethylene-forming enzymes (EFE) (Table 6.12). The fact that EFEs and other genes

involved in the biosynthesis of ethylene were identified in the syntenic loci of *Hbs-1* in cowpea, soybean, Medicago and common bean reinforces the utility of syntenic relationships in identifying candidate genes between the legume species.

The length of the *Hbs-2* QTL was quite extensive and many contigs overlapped the region. The most significant marker from the QTL analysis, SNP 1_1343 was imbedded in BAC clone CH082B14 of contig606 (Table 6.4). However, no genes of interest were identified in the clone.

The *Hbs-3* locus was also positioned on the cowpea physical map where BAC contigs 674, 512 and 661 incompletely spanned the *Hbs-3* locus (Table 6.7). SNP marker 1_0640 which co-segregated with the heat-induced browning phenotype in the *Hbs-3* locus was not identified in the cowpea physical map. However, SNP 1_0640 (37.96) shared the same marker bin as SNP 1_0396 (37.96 cM) on the cowpea consensus genetic map, so it was considered the closest marker to the *Hbs-3* locus (Table 6.7). SNP 1_0396 was identified in BAC clone CM042F21 of contig 512, but no genes associated with heat stress were found in the annotations. SNP marker 1_0383 (37.64 cM) which is 0.32 cM away from SNP 1_0640 (37.96 cM) was identified in BAC clones CH047M01 and CM014K16 which are also contained within contig512 (Table 6.7). Annotations for CH047M01and CM014K16 revealed the presence of two 1-aminocyclopropane-1-carboxylate (ACC) synthase 1 genes as possible candidate genes for the *Hbs-3* locus (Table 6.13).

Discussion

Candidate genes for the *Hbs-1* and *Hbs-3* loci

Synteny between cowpea, soybean, Medicago and common bean as well as the integrated genomic resources for cowpea were used to identify ethylene forming enzymes (EFE) as the cowpea candidate gene for the *Hbs-1* locus and an ACC synthase 1 gene for the *Hbs-3* locus. The plant hormone ethylene has long been associated with plants' ability to systematically relay information regarding abiotic and biotic stress. The biosynthesis of ethylene is dependent on the rate-limiting step; conversion of S-adenosylmethionine (AdoMet)(SAM) to ACC by the enzyme (ACC) synthase (ACS) (Kende 1993). Thus, ACS is considered to be the most important enzyme in this pathway. EFEs are involved in the secondary reaction forming ethylene; oxidation of ACC to ethylene (Yang and Hoffman 1984; Kende 1989).

The importance of ethylene closely associated with heat stress has been indicated by several studies. Researchers examining heat-induced oxidative damage in *Arabidopsis* showed that ethylene, abscisic acid (ABA) and salicylic acid (SA) were key to protecting against heat-induced stress; an ethylene-insensitive mutant *ethylene resistant 1* (*etr-1*) showed an increase of susceptibility to heat (Larkindale and Knight 2002). In another study, *Arabidopsis* insensitive mutants to ethylene signaling, *etr-1* and *ethylene insensitive 2* (*ein2*), showed a significant reduction in tolerance to basal-thermotolerance compared to wild-type (Larkindale and Huang 2005). Munne-Bosch et al. (2004) sought to determine if airborne ethylene such as found in highly polluted areas affected plant

stress tolerance. They observed that ethylene-fumigated holm oak trees showed much less tolerance to heat stress and heat stress combined with drought stress than controls (Munné-Bosch et al. 2004). Ethylene treated oak trees showed oxidative stress at 35 °C whereas the controls showed a heat tolerance up to 50 °C (Munné-Bosch et al. 2004). Additionally, ethylene treated trees showed more visual leaf area damage than controls (Munné-Bosch et al. 2004). Investigators of a heat-susceptible hard red winter wheat found that there was a 6-fold increase of ethylene in wheat kernels vs. no change in a heat-tolerant wheat cultivar 'Halberd' (Hays et al. 2007). Similarly, a 7-fold increase of ethylene was produced in embryos and a 12-fold increase of ethylene was found in the flag leaf of the heat-susceptible wheat genotype (Hays et al. 2007). The fact that ethylene is involved in heat stress regulation in many plant species makes ACC synthase and EFEs plausible candidate genes for regulating heat-induced browning of seed coats in cowpea. It is interesting to note that the two candidate genes for heat-induced brown discoloration in cowpea are two enzymes intricately involved in the ethylene biosynthesis pathway.

Conclusion

In this study, we report the identity of three loci, *Hbs-1, Hbs-2 and Hbs-3*, associated with heat-induced browning of seed coats in cowpea. The major heat-induced browning locus, *Hbs-1*, was observed in both RIL populations, IT93K-503-1 x CB46 and IT84S-2246 x TVu14676. *Hbs-2* was a minor locus identified in the IT93K-503-1 x CB46 population, while *Hbs-3* was a minor locus observed in the IT84S-2246 x TVu14676 population. Parental lines IT93K-503-1 and IT84S-2246 both exhibited the heat-induced browning of seed coats trait. The genetic and physical mapping and identity of candidate genes for the *Hbs-1* and *Hbs-3* loci were conducted utilizing integrated cowpea consensus genetic and physical maps as well as syntenic relationships with soybean, Medicago and common bean. The major locus, *Hbs-1*, was narrowed to cowpea BAC clone CM018C23 of BAC contig 217 on the cowpea physical map, where ethylene-forming enzymes (EFE) were present and considered as putative cowpea candidate genes. The minor locus, *Hbs-3* was positioned on BAC clones CM042F21, CH047M01 and CM014K16 of contig512 of the cowpea physical map where ACC synthase 1 genes were present and considered as potential candidate genes.

The practical outcome of this study was the identification of molecular markers 1_0032 and 1_1128 co-segregating with the *Hbs-1* phenotype and SNP marker 1_0640 which co-segregated with the *Hbs-3* phenotype. The heat-induced browning phenotype is present in about 20% of the elite IITA breeding lines. Since the *Hbs* phenotype is not manifested unless the breeding material is exposed to the appropriate heat conditions, having

markers to screen against the trait at the seedling stage would limit the number of plants needed to be grown to maturity. This would enable an efficient selection process to ensure that cowpea cultivars being bred do not carry the heat-induced browning trait. These approaches should expedite variety development by at least halving the current traditional breeding selection process which relies on time-consuming and costly phenotyping under heat stress conditions. Future goals include functional analysis of cowpea *Hbs-1* and *Hbs-3* candidate genes, which enhance our understanding of the heat-induced browning phenomenon as well as provide a 'perfect marker' which would further improve marker-assisted breeding efficiency.

Materials and methods

Plant populations

The IT93K-503-1 (*Hbs-1*) x California Blackeye '46' (*hbs-1*) population consisted of 113 lines which were advanced to the F_{10} generation using single seed decent. IT93K-503-1 is an advanced breeding line developed by International Institute for Tropical Agriculture (IITA) which features several important traits, including drought tolerance (Muchero et al. 2009b), resistance to *Fusarium oxysporum* f.sp. *tracheiphilum* (Fot) race 3 (Pottorff et al. 2012b) and 4 (Pottorff et al. 2013), and resistance to *Macrophomina phaseolina* (Muchero et al. 2011). California Blackeye 46 is a California bred variety from University of California, Davis and has important qualities such as resistance to Fot race 3 (Davis and Frate 2007). The F_9 and F_{10} generation were phenotyped for the heat-induced browning trait using a set of 99 RILs.

The IT84S-2246 (*Hbs*) x TVu14676 (*hbs*) consisted of 136 RILs which were advanced to the F_8 generation using single seed descent. IT84S-2246 is an IITA breeding line which has strong resistance to several root-knot nematodes including *Rk*-virulent *M.incognita* and *Rk*-aggressive *M. javanica* (Ehlers et al. 2002), aphids, bruchids and thrips and several other diseases (Singh et al. 1997). TVu14676 is a cowpea cultivar developed by IITA and is resistant to the parasitic plant *Striga gesnerioides* races SGl, SG2, SG3 and SG5 (Timko et al. 2007). The F_9 and F_{10} generation were phenotyped for the heat-induced browning of seed coats trait using 131 and 134 out of 146 RILs. All cowpea

materials were available from the University of California Riverside cowpea germplasm collection.

Experiments and phenotyping

Greenhouse experiments were conducted at the University of California Riverside, Citrus Research Station. Two independent experiments for each population were conducted to phenotype for the heat-induced browning of seed coats trait. Seeds of parents and RILs were planted into separate 18.93 L pots filled with University of California Soil Mix II (Matkin and Chandler 1957) and watered daily. Greenhouse day temperatures varied with the mean daily maximum of 35°C and a mean nightly minimum of 24°C. The seeds were harvested when mature after the pods had dried.

Heat-induced browning was phenotyped by a visual inspection of dried seeds obtained from mature plants exposed to high temperatures under greenhouse conditions. Brown discoloration covering the entire surface of the seed or in smaller patches was considered positive for the heat-induced browning trait and were recorded as a "1". Seeds which did not display brown discoloration at all were considered negative for the trait and were recorded as a "0".

SNP genotyping

The IT93K 503-1 x CB46 and IT84S-2246 x TVu14676 populations were genotyped at the F_8 generation using bi-allelic SNP markers using the 1536 Illumina GoldenGate Assay as previously described in Muchero et al. (2009). The cowpea cultivars used for the marker-trait association study were SNP genotyped at the F_8 or higher generation.

Genetic maps

The genetic map for the IT93K 503-1 x CB46 RIL population was previously created and is included in the cowpea consensus genetic map vs.2 (Muchero et al. 2009a), vs.3 (Diop et al. 2012), and vs.4 (Lucas et al. 2011). The individual map was generated using 114 RILs and 374 SNP markers and consisted of seventeen linkage groups which spanned approximately 639.6 cM (Lucas et al. 2011). The genetic map for IT84S-2246 x TVu14676 was also previously included in the vs.2, vs.3, and vs.4 maps. The individual map was generated using 136 RILs and 345 SNP markers and consists of fourteen linkage groups which span approximately 666.9 cM (Lucas et al. 2011). The cowpea consensus genetic map vs. 4 (Lucas et al. 2011) was used for this study and is an updated version of the vs.2 and vs.3 maps. The vs. 4 consensus map consisted of ten RIL populations and two breeding populations which increased the marker density and improved the marker order (Lucas et al. 2011). The map is 680 cM in length and contains 1107 markers with an average of 0.65 cM between markers (Lucas et al. 2011). The current SNP-based cowpea linkage map is included in a publicly available browser called HarvEST:Cowpea (Close and Wanamaker 2001).

Statistical analyses

Kruskal-Wallis and Interval Mapping analysis packages of MapQTL 5.0 software were used to conduct the QTL analysis (Van Ooijen 2004). 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 at 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 determine the span of QTLs using 1-LOD and 2-LOD to determine left and right margins. QTLs were visualized using MapChart 2.2 software (Voorrips 2002).

Synteny

Synteny was examined between cowpea and *G. max*, cowpea and *M. truncatula* and cowpea and *P. vulgaris* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes. Syntenic relationships between the different genomes can be examined in HarvEST:Cowpea database (Close and Wanamaker 2001). The soybean, Medicago and common bean annotations were taken from the Phytozome webpage (Goodstein et al. 2012). 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 (Close and Wanamaker 2001). Due to limited resolution in the software images, not all markers are presented in the screenshot images output from Harvest: Cowpea. The linkage group must be magnified using the HarvEST: Cowpea software in order to view each individual marker.

Marker-trait association

Genotypic data comprised of cowpea varieties and their SNP call for each locus of the cowpea consensus genetic map were visualized using Flapjack software (Milne et al. 2010).

Cowpea physical map

The physical map was developed using an advanced African breeding line IT97K-499-35 (Luo et al. 2007). It consists of two BAC clone libraries developed using restriction enzymes *Hind*III and *Mbo*I (Amplicon Express, Pullman, WA). Contigs were assembled using the snapshot method of DNA fingerprinting (Luo et al. 2003b) and assembly was 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 (Luo et al. 2007). The cowpea physical map manuscript is currently in preparation. The size of the BAC clones was estimated by multiplying the number of unique bands generated from the fingerprinting assay by 1640bp (personal communication, MC Luo).

Raw sequence data were generated for cowpea BACs using an Illumina HiSeq 2000 sequencer by John Weger at the Institute of Integrative Genome Biology, University of California, Riverside from DNA samples prepared by Yaqin Ma (UCR). Sequence assemblies of each BAC were generated by Stefano Lonardi from paired-end 100 base reads using the combinatorial pooling method described previously (Lonardi et al. 2013). A NODE is defined as a sequence or contig which can be consistently reconstructed using the sequencing reads (Zerbino 2010a; Zerbino and Birney 2008). All sequence data

are publicly available via the Harvest:Cowpea database (Close and Wanamaker 2001) and version 0.03 of the assembled cowpea genome (Close and Wanamaker 2005). Cowpea genome version 0.03 which contained approximately 200 Mb of assembled scaffolds and contigs covered about 97% of previously identified cowpea genes is available for BLAST searches and sequence retrieval (Close and Wanamaker 2005).

List of abbreviations

ACC: 1-aminocyclopropane-1 carboxylic acid; ACS: 1-aminocyclopropane-1 carboxylic acid synthase; BAC: bacterial artificial chromosome; bp: base pairs; cM: centiMorgan; DNA J HSP: DNA J heat shock family protein; EFE: ethylene forming enzyme; ERF: ethylene responsive element binding factor; EST: expressed sequence tags; HPR: hydroxyproline-rich glycoprotein family; HSP: heat shock proteins; LG: linkage group; LOD: logarithm (base 10) of odds; MAS; marker-assisted selection; Mb: megabases; QTL: quantitative trait locus; RIL: recombinant inbred line; SNP: single nucleotide polymorphic sequence.

The authors declare that they have no competing interests.

The work in this paper did not require approval from an ethics committee.

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Authors' contributions

MP, JDE, TJC and PAR conceived and designed the experiments. MP and JDE conducted the experiments. MP drafted the manuscript and performed the statistical analyses. MP, JDE, TJC, and PAR analyzed the data. All authors read and approved the final manuscript.

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Table 6.1 QTL analysis of *Hbs-1* in the IT93K-503-1 x CB46 population.							
Experiment	LG	cM	Locus	IM analysis		Kruskal-Wallis analysis	
				LOD	R^2	F-test	p-value
F_9	8	40.67	1_0030	3.64	15.6	16.245	0.0001
F_9	8	44.29	1_0419	4.56	19.2	19.774	0.0001
F_9	8	46.87	1_0242	6.15	25	25.472	0.0001
F_9	8	47.38	1_1419	6.15	25.1	25.472	0.0001
F_9	8	56.01	1_0677	11.97	43.5	43.212	0.0001
F_9	8	56.01	1_1533	11.97	43.5	43.212	0.0001
F_9	8	58.56	1_1322	16.48	55.6	53.946	0.0001
F_9	8	58.67	1_0127	16.49	55.7	53.946	0.0001
F_9	8	58.67	1_0225	16.49	55.7	53.946	0.0001
F_9	8	58.71	1_0081	16.5	55.7	53.946	0.0001
F_9	8	60.09	1_0032	19.99	62.8	60.21	0.0001
F_9	8	60.53	1_1128	20.01	62.7	60.21	0.0001
F_9	8	64.33	1_0226	11.63	42.1	41.119	0.0001
F_9	8	64.56	1_0037	11.62	42	42.613	0.0001
F_9	8	65.84	1_0998	9.9	36.9	37.943	0.0001
F_9	8	66.04	1_0588	9.9	36.9	36.157	0.0001
F_9	8	68.86	1_0379	9.89	37	36.904	0.0001
F_9	8	71.44	1_0579	7.18	28.7	27.787	0.0001
F_9	8	71.81	1_0387	6.33	25.6	24.675	0.0001
F_9	8	72.48	1_1401	5.67	23.3	23.063	0.0001
F_9	8	74.43	1_0923	5.01	21.1	20.774	0.0001
F_9	8	75.86	1_0078	4.26	18.2	17.438	0.0001
F_9	8	76.13	1_1130	4.22	18	17.358	0.0001
F_{10}	8	35.21	1_1492	2.27	10.1	9.808	0.005
F_{10}	8	40.67	1_0030	4.54	19.1	19.774	0.0001

F_{10}	8	44.29	1_0419	5.61	23.1	23.649	0.0001
F_{10}	8	46.87	1_0242	7.44	29.5	29.864	0.0001
F_{10}	8	47.38	1_1419	7.44	29.5	29.864	0.0001
F_{10}	8	56.01	1_0677	14.25	49.5	48.822	0.0001
F_{10}	8	56.01	1_1533	14.25	49.5	48.822	0.0001
F_{10}	8	58.56	1_1322	24.19	70	66.818	0.0001
F_{10}	8	58.67	1_0127	24.2	70	66.818	0.0001
F_{10}	8	58.67	1_0225	24.2	70	66.818	0.0001
F_{10}	8	58.71	1_0081	24.21	70	66.818	0.0001
F_{10}	8	60.09	1_0032	30.16	77.3	73.769	0.0001
F_{10}	8	60.53	1_1128	30.19	77.3	73.769	0.0001
F_{10}	8	64.33	1_0226	16.44	54.2	52.537	0.0001
F_{10}	8	64.56	1_0037	16.42	54.1	54.174	0.0001
F_{10}	8	65.84	1_0998	13.94	47.8	48.822	0.0001
F_{10}	8	66.04	1_0588	13.92	47.7	46.722	0.0001
F_{10}	8	68.86	1_0379	13.93	47.9	47.75	0.0001
F_{10}	8	71.44	1_0579	10.28	38.5	37.219	0.0001
F_{10}	8	71.81	1_0387	9.13	34.7	33.738	0.0001
F_{10}	8	72.48	1_1401	8.26	32	31.77	0.0001
F_{10}	8	74.43	1_0923	7.4	29.5	29.066	0.0001
F_{10}	8	75.86	1_0078	6.47	26.5	27.391	0.0001
F_{10}	8	76.13	1_1130	6.41	26.2	27.177	0.0001

Table 6.2 *Hbs-1* in IT93K-503-1 x CB46 and IT84S-2246 x TVu14676 individual maps, cowpea consensus genetic map, and the cowpea physical map.

IT93K-503-1 x CB46				IT84S-2246 x TVu14676				Cowpea consensus genetic map				Cowpea physical map	
LG	cM	SNP	LOD	LG	cM	SNP	LOD	LG	cM	SNP	Annotation	contig	BAC(s)
8	60.09	1_0032	30.16	9	49.51	1_0032	12.05	5	45.27	1_0032	SecE/sec61- gamma protein transport protein	N/A	N/A
		N/A			N/A			5	45.27	1_0193	Ribosomal protein S4 (RPS4A) family protein	N/A	N/A
		N/A			N/A			5	45.27	1_0287	Glycine-rich protein 3	N/A	N/A
8	60.53	1_1128	30.19		N/A			5	45.76	1_1128	Phosphotyrosine protein phosphatase	217	CM018C23
		N/A		9	50.69	1_0120	10.41	5	46.51	1_0120	Ethylene-forming enzyme	217	CM018C23
		N/A				N/A		5	46.51	1_0945	O-Glycosyl hydrolases family 17 protein	N/A	N/A
		N/A		9	50.69	1_0661	10.41	5	47.18	1_0661	Subtilisin-like serine endopeptidase family protein	N/A	N/A

Table 6.3 QTL analysis of *Hbs-2* in the IT93K-503-1 x CB46 population.							
Experiment	LG	cM	Locus	IM analysis		Kruskal-Wallis analysis	
				LOD	R^2	F-test	p-value
F9	3	50.837	1_1343	2.77	12.3	12.3	0.0005
F9	3	51.792	1_0871	2.61	11.7	11.7	0.001
F10	3	36.824	1_0794	2.15	9.6	9.6	0.005
F10	3	50.837	1_1343	2.11	9.5	9.5	0.005
F10	3	51.792	1_0871	1.98	8.9	9.15	0.005

Table 6.4. *Hbs-2* in the IT93K-503-1 x CB46 genetic map, cowpea consensus genetic map and cowpea physical map.

IT93K-503-1 x CB46				Cowpea consensus genetic map vs.4				Cowpea physical map	
LG	cM	SNP	LOD	LG	cM	SNP	Annotation	contig	BAC(s)
3	51.79	1_0871	1.98	6	31.28	1_0871	Transketolase family protein	N/A	
		N/A		6	31.42	1_0300	Bifunctional inhibitor/lipid-transfer protein	N/A	
		N/A		6	31.42	1_0635	Light harvesting complex photosystem II	N/A	
		N/A		6	31.42	1_0927	Tetratricopeptide repeat (TPR)-like protein	855	CM018P09
		N/A		6	31.42	1_1331	Aldolase-type TIM barrel family protein	N/A	
		N/A		6	31.42	1_1368	Methyl-CPG- binding domain 8	855	CM018P09
		N/A		6	31.42	1_1530	CD2-binding protein-related	855	CM018P09
		N/A		6	33.04	1_0307	T-complex protein 11	265	CM005F11, CH084P15
		N/A		6	41.95	1_0911	Mitogen-activated protein kinase 16	1117	CM024D19, CM012O18
		N/A		6	42.06	1_1367	RNA-binding KH domain-containing protein	240	CM013I01
		N/A		6	45.75	1_0691	NIFU-like protein 2	N/A	
		N/A		6	46.27	1_0830	Fibrillin	N/A	
		N/A		6	47.25	1_0897	Na+/H+ antiporter 6	250	CM015O07, CM002B24
		N/A		6	47.41	1_1363	Eukaryotic aspartyl protease family protein	250	CM015O07
3	39.59	1_0860	1.79	6	47.86	1_0860	Vesicle- associated membrane protein 726	250	CH076D23, CH093L18
3	39.66	1_1484	1.79	6	47.93	1_1484	Kinase-related protein of unknown function	250	CH076D23, CH093L18
3	39.74	1_1107	1.58	6	48.31	1_1107	Chloroplastic NIFS-like cysteine desulfurase	250	CH051M10, CM001C09, M019E01
		N/A		6	49.14	1_0704	Glycolipid transfer protein	250	CH051M10
		N/A		6	49.60	1_0544	Peroxidase superfamily protein	N/A	
3	39.82	1_1443	1.56	6	49.85	1_1443	Peroxidase superfamily protein	N/A	
3	40.15	1_0290	1.55	6	50.34	1_0290	Ribosomal protein L13 family protein	551	CM030A23, CM054O22
3	40.15	1_1020	1.55	6	50.34	1_1020	Root FNR 1	551	CM030A23, CM054O22
3	39.23	1_0148	1.57	6	50.57	1_0148	GTP binding	551	CM054022, CM030A23
3	40.15	1_0701	1.55	6	50.92	1_0701	Putative type 1 membrane protein	N/A	
3	40.42	1_0026	1.55	6	51.21	1_0026	Tubulin beta 8	691	CH024L08
		N/A		6	51.21	1_1090	6- phosphogluconate dehydrogenase	551	CM030A23
		N/A		6	51.81	1_0538	fatty acid hydroxylase 1	691	CH071M11
3	36.82	1_0794	2.15	6	54.05	1_0794	Rab5- interacting family protein	N/A	
		N/A		6	54.61	1_0124	ATP-citrate lyase B-1	N/A	
		N/A		6	54.61	1_1244	DNA-binding enhancer protein-related	38	CH074G16
		N/A		6	54.78	1_1080	Phytochrome B	N/A	
		N/A		6	54.99	1_1194	No functional annotation	N/A	
		N/A		6	55.25	1_0760	ATPase, F1 complex	N/A	
		N/A		6	55.50	1_1346	Ubiquitin-like superfamily protein	N/A	
		N/A		6	57.41	1_0437	Bacterial sec-independent translocation protein	240	CH077I19, CM023J02
		N/A		6	57.97	1_0015	Acyl carrier protein 4	707	CM054P06
3	50.84	1_1343	2.11	6	58.09	1_1343	Ribosomal protein L18ae/LX family protein	606	CH082B14

Table 6.5 QTL mapping of *Hbs-1* in the IT84S-2246 x TVu14676 population.							
Experiment	LG	cM	Locus	IM analysis		Kruskal-Wallis analysis	
				LOD	R^2	F-test	p-value
F9	9	24.98	1_1492	2.39	7.9	10.171	0.005
F9	9	30.84	1_0800	1.83	6.2	8.121	0.005
F9	9	33.77	1_0877	2.09	7	9.199	0.005
F9	9	33.77	1_0362	2.09	7	9.199	0.005
F9	9	33.77	1_0040	2.09	7	9.199	0.005
F9	9	34.63	1_1359	2.24	7.4	9.832	0.005
F9	9	40.41	1_0119	3.83	12.4	16.381	0.0001
F9	9	40.41	1_1243	3.83	12.4	16.381	0.0001
F9	9	41.23	1_0251	3.83	12.4	16.381	0.0001
F9	9	45.29	1_1533	6.34	19.7	26.002	0.0001
F9	9	45.29	1_0677	6.34	19.7	26.002	0.0001
F9	9	47.48	1_0225	8.95	26.6	35.178	0.0001
F9	9	47.48	1_0081	8.95	26.6	35.178	0.0001
F9	9	49.51	1_0032	12.05	34.1	45.032	0.0001
F9	9	50.69	1_0661	10.41	30.3	39.953	0.0001
F9	9	50.69	1_0120	10.41	30.3	39.953	0.0001
F9	9	51.67	1_0226	9.36	27.7	36.523	0.0001
F9	9	51.67	1_0037	9.36	27.7	36.523	0.0001
F9	9	51.94	1_0495	9.36	27.7	36.523	0.0001
F9	9	52.16	1_0998	9.78	28.7	37.915	0.0001
F9	9	52.28	1_0588	9.78	28.7	37.915	0.0001
F9	9	53.78	1_0379	6.8	21	27.7	0.0001
F9	9	57.07	1_0974	5.34	16.9	22.268	0.0001
F9	9	57.07	1_1401	5.34	16.9	22.268	0.0001
F9	9	57.82	1_0387	5.34	16.9	22.268	0.0001
F9	9	57.82	1_0579	5.34	16.9	22.268	0.0001
F9	9	59.60	1_0923	4.52	14.5	19.116	0.0001
F10	9	24.98	1_1492	3.81	12.4	15.896	0.0001
F10	9	30.84	1_0800	2.45	8.2	10.741	0.005
F10	9	33.77	1_0877	2.71	9	11.829	0.001
F10	9	33.77	1_0362	2.71	9	11.829	0.001
F10	9	33.77	1_0040	2.71	9	11.829	0.001
F10	9	34.63	1_1359	2.9	9.6	12.591	0.0005
F10	9	40.41	1_0119	3.94	12.9	16.841	0.0001
F10	9	40.41	1_1243	3.94	12.9	16.841	0.0001
F10	9	41.23	1_0251	3.94	12.9	16.841	0.0001
F10	9	45.29	1_1533	5.56	17.6	23.08	0.0001
F10	9	45.29	1_0677	5.56	17.6	23.08	0.0001
F10	9	47.48	1_0225	7.81	23.9	31.257	0.0001

F10	9	47.48	1_0081	7.81	23.9	31.257	0.0001
F10	9	49.51	1_0032	9.54	28.3	37.093	0.0001
F10	9	50.69	1_0661	8.2	24.9	32.595	0.0001
F10	9	50.69	1_0120	8.2	24.9	32.595	0.0001
F10	9	51.67	1_0226	7.36	22.7	29.676	0.0001
F10	9	51.67	1_0037	7.36	22.7	29.676	0.0001
F10	9	51.94	1_0495	7.36	22.7	29.676	0.0001
F10	9	52.16	1_0998	7.74	23.7	31.01	0.0001
F10	9	52.28	1_0588	7.74	23.7	31.01	0.0001
F10	9	53.78	1_0379	5.25	16.7	21.942	0.0001
F10	9	57.07	1_0974	5.42	17.2	22.574	0.0001
F10	9	57.07	1_1401	5.42	17.2	22.574	0.0001
F10	9	57.82	1_0387	5.42	17.2	22.574	0.0001
F10	9	57.82	1_0579	5.42	17.2	22.574	0.0001
F10	9	59.60	1_0923	4.69	15.1	19.788	0.0001

Table 6.6 QTL analysis of *Hbs-3* in the IT84S-2246 x TVu14676 population.							
Experiment	LG	cM	SNP marker	IM analysis		Kruskal-Wallis analysis	
				LOD	R^2	F-test	p-value
F_9	3	17.79	1_0280	1.85	6.2	8.193	0.005
F_9	3	17.79	1_1534	1.85	6.2	8.193	0.005
F_9	3	17.79	1_1404	1.85	6.2	8.193	0.005
F_9	3	20.97	1_0640	0.71	2.4	3.066	0.1
F_{10}	3	17.79	1_0280	2.02	6.8	8.925	0.005
F_{10}	3	17.79	1_1534	2.02	6.8	8.925	0.005
F_{10}	3	17.79	1_1404	2.02	6.8	8.925	0.005
F_{10}	3	20.97	1_0640	0.84	2.9	3.625	0.1

Table 6.7 *Hbs-3* in IT84S-2246 x TVu14676 individual map, cowpea consensus genetic map, and the cowpea physical map.

IT84S-2246 x TVu14676				Cowpea consensus genetic map vs.4				Cowpea physical map	
LG	cM	SNP	LOD	LG	cM	SNP	Annotation	contig	BAC(s)
3	17.79	1_0280	2.02	1	36.00	1_0280	Mitochondrial acyl carrier protein 2	674	CM059B13
		N/A		1	36.02	1_0630	Thioredoxin F2	N/A	N/A
3	17.79	1_1534	2.02	1	36.02	1_1534	High chlorophyll fluorescent 109	674	CM059B13 CH093M18
		N/A		1	36.25	1_0059	No functional annotation	674	CM013N21
		N/A		1	36.25	1_0312	2-phosphoglycolate phosphatase 1	674	CM013N21
		N/A		1	36.56	1_0532	Chloroplastic acetylcoenzyme A carboxylase 1	674	CM059B13 CH093M18
3	17.79	1_1404	2.02	1	36.56	1_1404	No functional annotation	N/A	N/A
		N/A		1	36.99	1_0726	Uncharacterized protein family	N/A	N/A
		N/A		1	37.64	1_0383	Ribosomal L5P family protein	512	CM042F21 CM014K16
		N/A		1	37.96	1_0396	No functional annotation	512	CM042F21
3	20.97	1_0640	0.84	1	37.96	1_0640	Ubiquitin- conjugating enzyme 5	N/A	N/A

Table 6.8 Origins of genotypes used for the marker-trait association in the *Hbs-1* and *Hbs-3* loci.

Cultivar	Reference	Country of origin	Type	*Hbs* phenotype	*Hbs-1* 1_0032 (45.27 cM/LG5)	*Hbs-1* 1_1128 (45.76 cM/LG5)	*Hbs-2* 1_0640 (37.96 cM/LG1)
IT84S-2246 (PI 582519)	Not published	Nigeria	IITA breeding line	Positive	AA	TT	AA
IT93K-503-1	Not published	Nigeria	IITA breeding line	Positive	AA	TT	AA
IT93K-2046	Not published	N/A	IITA breeding line	Positive	AA	TT	AA
TVu-4552	Hall and Patel (1985)	Nigeria		Positive	AA	TT	AA
TVx-3236 (PI 632845)	Hall and Patel (1985)	N/A	IITA breeding line	Positive	AA	TT	AA
TVu-53	Not published	Nigeria		Positive	AA	TT	AA
TVu-15315	Not published	Chad		Positive	AA	TT	AA
TVu-14676	Not published	Botswana	Traditional cultivar/landrace	Negative	GG	AA	GG
CB5	Hall and Patel (1985)	U.S.A.	Improved variety	Negative	GG	AA	GG
CB27	Not published	U.S.A.	Improved variety	Negative	GG	AA	GG
CB46	Not published	U.S.A.	Improved variety	Negative	GG	AA	GG
524B	Not published	U.S.A.	Improved cultivar (CB5 x CB3)	Negative	GG	AA	GG
Bambey 21	Hall and Patel (1985)	Senegal	Improved variety	Negative	GG	AA	GG

Table 6.9 Synteny of *Hbs-1* in *Glycine max* chromosomes 2 and 14.					
G. max chromosome	*G. max* locus	Phytozome annotation	Cowpea locus	cM	LG
Gm02	Glyma02g42560	Vesicle coat protein clathrin, heavy chain	1_0127	44.42	5
Gm02	Glyma02g43500	ATERF3/ERF3 (Ethylene responsive element binding factor 3)	N/A	N/A	N/A
Gm02	Glyma02g43550	Tyrosine phosphatase family	1_1128	45.76	5
Gm02	Glyma02g43560	EFE (Ethylene forming enzyme)	1_0120	46.51	5
Gm02	Glyma02g43580	EFE (Ethylene forming enzyme)	N/A	N/A	N/A
Gm02	Glyma02g43600	EFE (Ethylene forming enzyme)	N/A	N/A	N/A
Gm02	Glyma02g43640	Glycosyl hydrolases family	1_0945	46.51	5
Gm14	Glyma14g05250	Subtilase family	1_0661	47.18	5
Gm14	Glyma14g05270	Subtilase family	1_0661	47.18	5
Gm14	Glyma14g05300	Glycosyl hydrolases family	1_0945	46.51	5
Gm14	Glyma14g05350	EFE (Ethylene forming enzyme)	N/A	N/A	N/A
Gm14	Glyma14g05360	EFE (Ethylene forming enzyme)	N/A	N/A	N/A
Gm14	Glyma14g05390	EFE (Ethylene forming enzyme)	1_0120	46.51	5
Gm14	Glyma14g05400	Tyrosine phosphatase family	1_1128	45.76	5
Gm14	Glyma14g05470	ATERF3/ERF3 (Ethylene responsive element binding factor 3)	N/A	N/A	N/A
Gm14	Glyma14g05800	SecE/Sec61-gamma subunits	1_0032	45.27	5
Gm14	Glyma14g06330	CIRCADIAN PROTEIN CLOCK/ARNT/BMAL/PAS	1_1322	44.42	5

Table 6.10 Synteny of *Hbs-1* in *Medicago truncatula* chromosome 5.					
M. truncatula chromosome	*M. truncatula* locus	Phytozome annotation	Cowpea locus	cM	LG
5	Medtr5g018870	40S ribosomal protein S4 (RPS4B)	45.27	1_0193	5
5	Medtr5g090360	Clathrin, heavy-chain linker	44.42	1_0127	5
5	Medtr5g091490	Protein transport protein SEC61 gamma subunit	45.27	1_0032	5
5	Medtr5g091750	Glycine-rich protein	45.27	1_0287	5
5	Medtr5g091880	Glycine-rich protein	45.27	1_0287	5
5	Medtr5g092410	ERF3 (Ethylene responsive element binding factor 3)	N/A		
5	Medtr5g092450	ERF3 (Ethylene responsive element binding factor 3)	N/A		
5	Medtr5g092470	ERF3 (Ethylene responsive element binding factor 3)	N/A		
5	Medtr5g092480	ERF11 (ERF DOMAIN PROTEIN 11)	N/A		
5	Medtr5g092680	Tyrosine specific protein phosphatase family protein	45.76	1_1128	5
5	Medtr5g092760	EFE (Ethylene forming enzyme)	46.51	1_0120	5
5	Medtr5g093030	Glycosyl hydrolases family	46.51	1_0945	5
5	Medtr5g093060	Subtilisin/kexin-related serine protease	47.18	1_0661	5

Table 6.11 Synteny of *Hbs-1* in *P. vulgaris* chromosome 8.					
P. vulgaris chromosome	*P. vulgaris* locus	*P. vulgaris* annotation	Cowpea SNP	LG	cM
8	Phvul.008G213300.1	Subtilisin-like serine endopeptidase family protein	1_0661	5	47.18
8	Phvul.008G213400.1	O-Glycosyl hydrolases family 17 protein	1_0945	5	46.51
8	Phvul.008G213800.1	Ethylene-forming enzyme	N/A		
8	Phvul.008G213900.1	ACC oxidase 2	N/A		
8	Phvul.008G214200.1	Ethylene-forming enzyme	1_0120	5	46.51
8	Phvul.008G214300.1	Phosphotyrosine protein phosphatases superfamily protein	1_1128	5	45.76

Table 6.12 Annotations for the *Hbs-1* locus on cowpea BAC clone CM018C23 of contig217 of the cowpea physical map.			
BAC Clone/NODE	e-score	*P. vulgaris* locus/cowpea SNP	Annotation
CM018C23_VU2.3_NODE_0001	2.00E-69	Phvul.008G214100.1	Ankyrin repeat family protein
CM018C23_VU2.3_NODE_0002	0	Phvul.008G214000.1	Ankyrin repeat family protein
CM018C23_VU2.3_NODE_0003	1.00E-142	Phvul.008G213700.1	Calcineurin-like metallo-phosphoesterase superfamily protein
CM018C23_VU2.3_NODE_0004	3.00E-79	Phvul.008G214300.1/1_1128	Phosphotyrosine protein phosphatases superfamily protein
CM018C23_VU2.3_NODE_0011	3.00E-143	Phvul.008G214000.1	Ankyrin repeat family protein
CM018C23_VU2.3_NODE_0013	8.00E-124	Phvul.007G135600.1	Ethylene-forming enzyme
CM018C23_VU2.3_NODE_0021	8.00E-92	Phvul.008G214000.1	Ankyrin repeat family protein
CM018C23_VU2.3_NODE_0022	1.00E-47	Phvul.008G214000.1	Ankyrin repeat family protein
CM018C23_VU2.3_NODE_0024	2.00E-151	Phvul.008G214200.1/1_0120	Ethylene-forming enzyme
CM018C23_VU2.3_NODE_0030	6.00E-147	Phvul.008G213800.1	Ethylene-forming enzyme

Table 6.13 Annotations for the *Hbs-3* locus on cowpea BAC clone CM042F21, CH047M01 and CM014K16 of contig512 of the cowpea physical map.

BAC Clone/NODE	e-score	*P. vulgaris* locus/cowpea SNP	*P. vulgaris* gene model
CM042F21_VU2.3_NODE_0001	0	Phvul.008G060300.1	Brassinosteroid-responsive RING-H2
CM042F21_VU2.3_NODE_0002	0	Phvul.008G059900.1	Protein of unknown function
CM042F21_VU2.3_NODE_0004	0	Phvul.008G060100.1	Ferric reduction oxidase 2
CM042F21_VU2.3_NODE_0005	0	Phvul.008G059800.1	Plant U-Box 15
CM042F21_VU2.3_NODE_0007	5.00E-84	Phvul.008G060500.1	Transmembrane protein-related
CM042F21_VU2.3_NODE_0008	9.00E-146	Phvul.008G059200.1/1_0383	Ribosomal L5P family protein
CM042F21_VU2.3_NODE_0009	3.00E-67	Phvul.008G059300.1	Peptidase family M48 family protein
CM042F21_VU2.3_NODE_0010	0	Phvul.008G059400.1	Protein kinase superfamily protein
CM042F21_VU2.3_NODE_0016	0	Phvul.008G060000.1	Fringe-related protein
CM042F21_VU2.3_NODE_0023	0	Phvul.008G059500.1/1_0396	Protein of unknown function
CH047M01_VU1.3_NODE_0002	7.00E-140	Phvul.008G058200.1	Alpha/beta-Hydrolases superfamily protein
CH047M01_VU1.3_NODE_0003	0	Phvul.008G058300.1	WRKY family transcription factor
CH047M01_VU1.3_NODE_0007	0	Phvul.002G179300.1	Polynucleotidyl transferase
CH047M01_VU1.3_NODE_0015	0	Phvul.008G058400.1	ACC synthase 1
CH047M01_VU1.3_NODE_0019	5.00E-131	Phvul.008G058700.1	Cysteine-rich RLK (RECEPTOR-like protein kinase) 29
CM014K16_VU2.3_NODE_0003	0	Phvul.008G058500.1	Heavy metal transport/detoxification superfamily protein
CM014K16_VU2.3_NODE_0012	4.00E-140	Phvul.008G058200.1	Alpha/beta-Hydrolases superfamily protein
CM014K16_VU2.3_NODE_0014	0	Phvul.008G058400.1	ACC synthase 1
CM014K16_VU2.3_NODE_0016	0	Phvul.008G059400.1	Protein kinase superfamily protein
CM014K16_VU2.3_NODE_0019	0	Phvul.008G058600.1	Cysteine-rich RLK (RECEPTOR-like protein kinase) 29
CM014K16_VU2.3_NODE_0019	7.00E-24	Phvul.007G052500.1	Cysteine-rich RLK (RECEPTOR-like protein kinase) 29
CM014K16_VU2.3_NODE_0022	1.00E-82	Phvul.008G058000.1	WRKY DNA-binding protein 24
CM014K16_VU2.3_NODE_0023	0	Phvul.008G058300.1	WRKY family transcription factor
CM014K16_VU2.3_NODE_0025	6.00E-33	Phvul.008G059400.1	Protein kinase superfamily protein
CM014K16_VU2.3_NODE_0027	0	Phvul.008G059100.1/1_0383	Protein of unknown function
CM014K16_VU2.3_NODE_0058	3.00E-67	Phvul.008G059300.1	Peptidase family M48 family protein
CM014K16_VU2.3_NODE_0059	0	Phvul.008G058900.1	NAD(P)-binding Rossmann-fold superfamily protein
CM014K16_VU2.3_NODE_0003	0	Phvul.008G058500.1	Heavy metal transport/detoxification superfamily protein
CM014K16_VU2.3_NODE_0012	4.00E-140	Phvul.008G058200.1	Alpha/beta-Hydrolases superfamily protein
CM014K16_VU2.3_NODE_0014	0	Phvul.008G058400.1	ACC synthase 1
CM014K16_VU2.3_NODE_0016	0	Phvul.008G059400.1	Protein kinase superfamily protein
CM014K16_VU2.3_NODE_0019	0	Phvul.008G058600.1	Cysteine-rich RLK (RECEPTOR-like protein kinase) 29
CM014K16_VU2.3_NODE_0019	7.00E-24	Phvul.007G052500.1	Cysteine-rich RLK (RECEPTOR-like protein kinase) 29
CM014K16_VU2.3_NODE_0022	1.00E-82	Phvul.008G058000.1	WRKY DNA-binding protein 24

Figure 6.1 *Hbs-1* in the IT93K-503-1 x CB46 population. The heat-induced browning of seed coats locus, *Hbs-1*, was identified using datasets from two experiments. *Hbs-1* spanned 60.09 cM to 60.53 cM on linkage group 8. SNP markers 1_0032 and 1_1128 were the most significant markers in the locus and are highlighted in red on the linkage group. However, 1_1128 had the highest association with the heat-induced browning phenotype, accounting for 62.8% and 77.3% of the phenotypic variance. The significance threshold is indicated by the horizontal broken line.

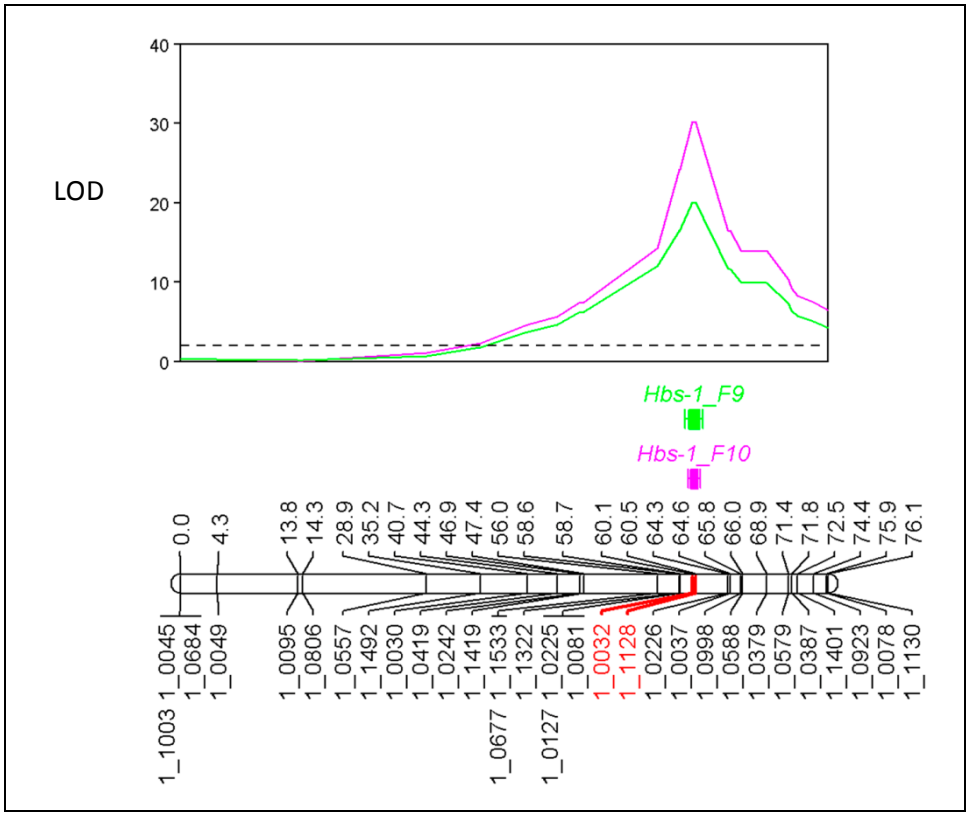

Figure 6.2 *Hbs-1*, *Hbs-2* and *Hbs-3* on the cowpea consensus genetic map. Heat-induced browning of seed coats QTLs were positioned on the cowpea consensus genetic map using SNP markers identified in the QTL analyses. *Hbs-1* and *Hbs-2* (labeled light blue) were identified in the IT93K-503-1 x CB46 population. *Hbs-1* and *Hbs-3* (labeled magenta) were identified in the IT84S-2246 x TVu14676 population. The most significant SNP marker for each QTL is highlighted in the corresponding color on the linkage group. SNP marker 1_0032 is labeled red since it was the most significant marker for both the *Hbs-1* locus identified in IT93K-503-1 x CB46 and the IT84S-2246 x TVu14676 population.

Figure 6.2 *Hbs-1*, *Hbs-2* and *Hbs-3* on the cowpea consensus genetic map.

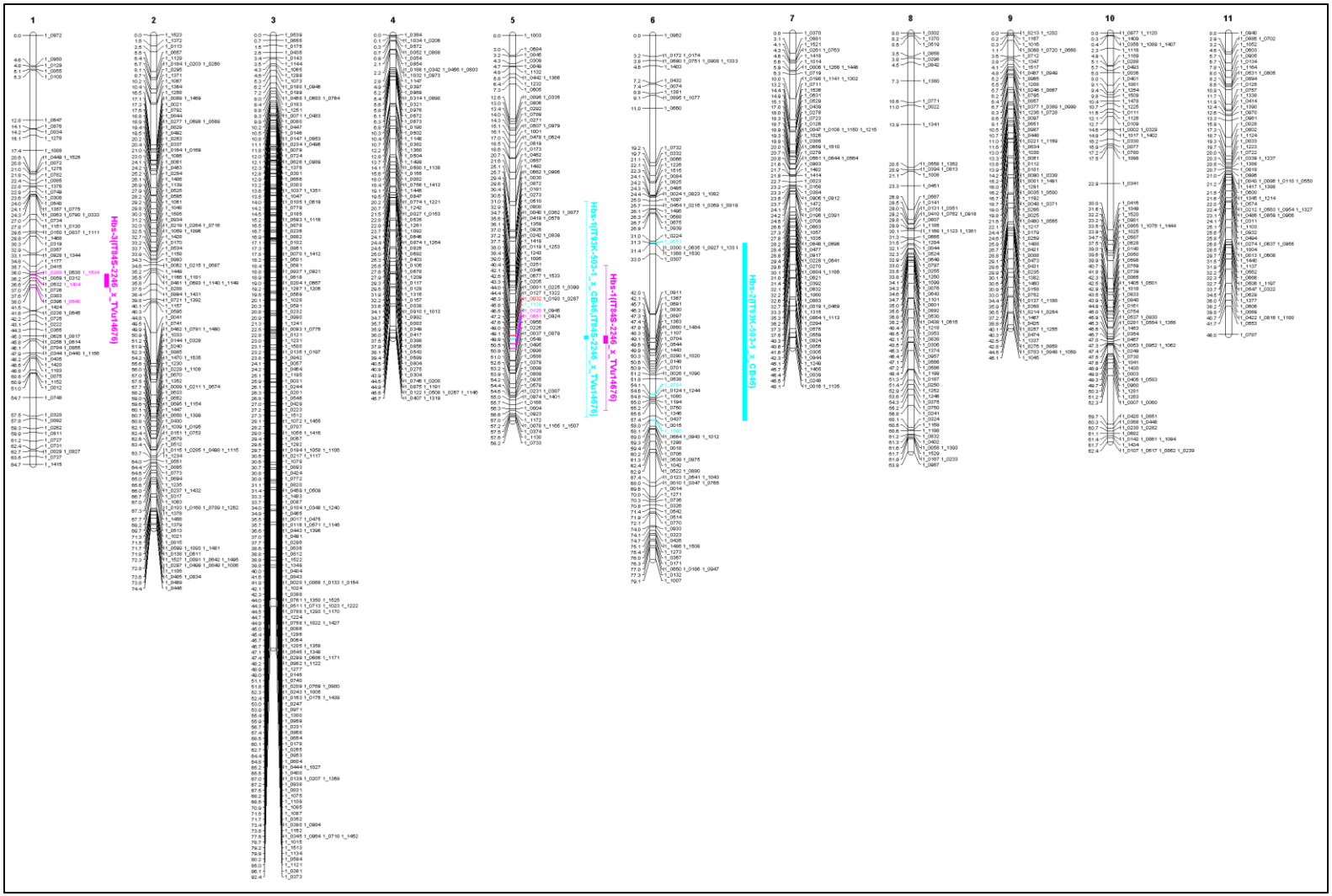

Figure 6.3 *Hbs-2* in the IT93K-503-1 x CB46 population. The heat-induced browning of seed coats locus, *Hbs-2*, was identified using datasets from two experiments. *Hbs-2* spanned 36.82 cM to 51.79 cM on linkage group 3. SNP marker 1_1342 accounted for the highest percent phenotypic variance of 9.5 % (LOD 2.11) and 12.3 % (LOD 2.77) and is highlighted in red on the linkage group. The significance threshold is indicated by the horizontal broken line.

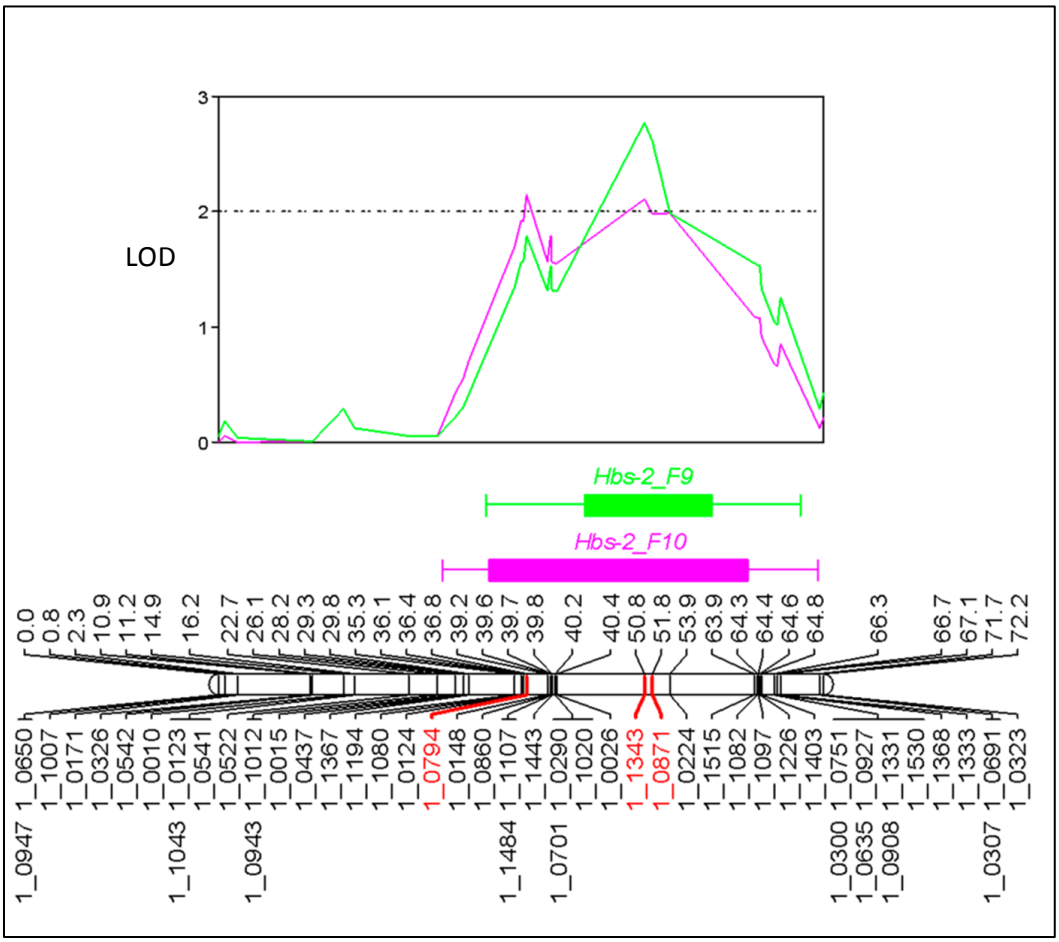

Figure 6.4 *Hbs-1* in the IT84S-2246 x TVu14676 population. The major locus for the heat-induced browning of seed coats phenotype was mapped using datasets from two experiments. *Hbs-1* spanned 49.51 cM to 50.69 cM on linkage group 9. SNP marker 1_0032 was the most significant marker for both experiments accounting for 28.3% and 34.1% of the phenotypic variance and is highlighted in red on the linkage group. The significance threshold is indicated by the horizontal broken line.

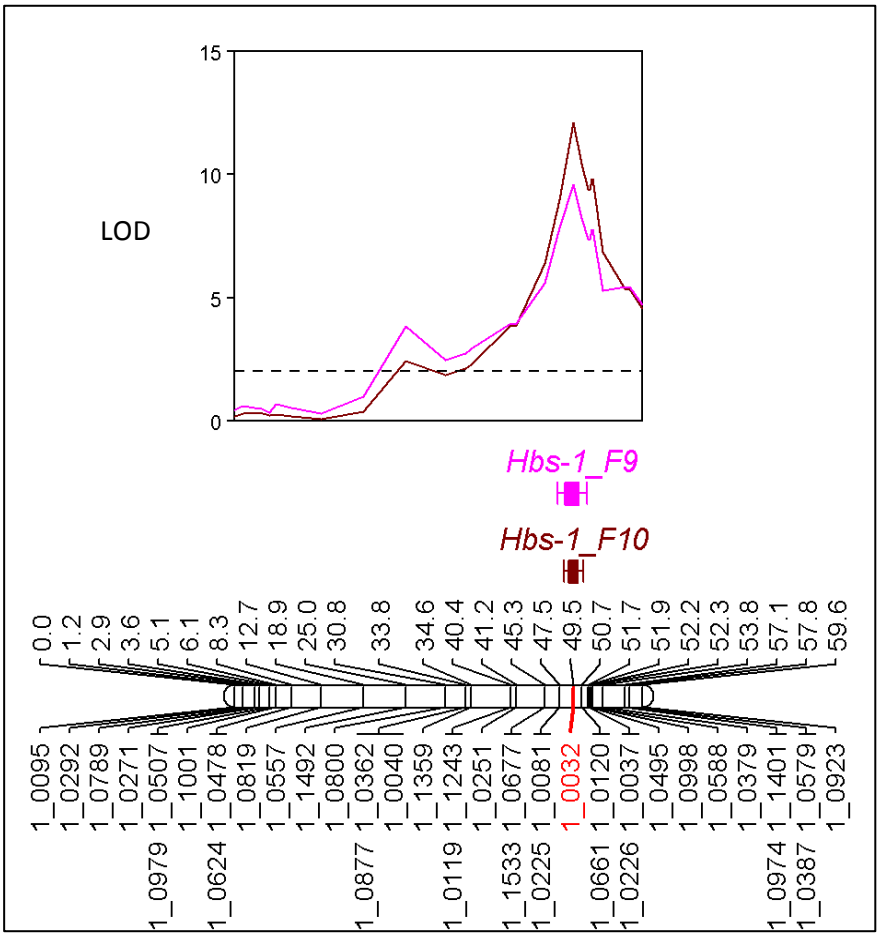

Figure 6.5 *Hbs-3* in the IT84S-2246 x TVu14676 population. The minor locus for the heat-induced browning phenotype was mapped using two experimental datasets (Interval Mapping analysis shown). *Hbs-3* spanned 8.67 cM to 20.97 cM on linkage group 3. SNP markers 1_0280, 1_1534 and 1_1404 shared the same marker bin, accounting for 6.2 % and 6.8 % of the phenotypic variance and are highlighted in red on the linkage group. The significance threshold is indicated by the horizontal broken line on the graph.

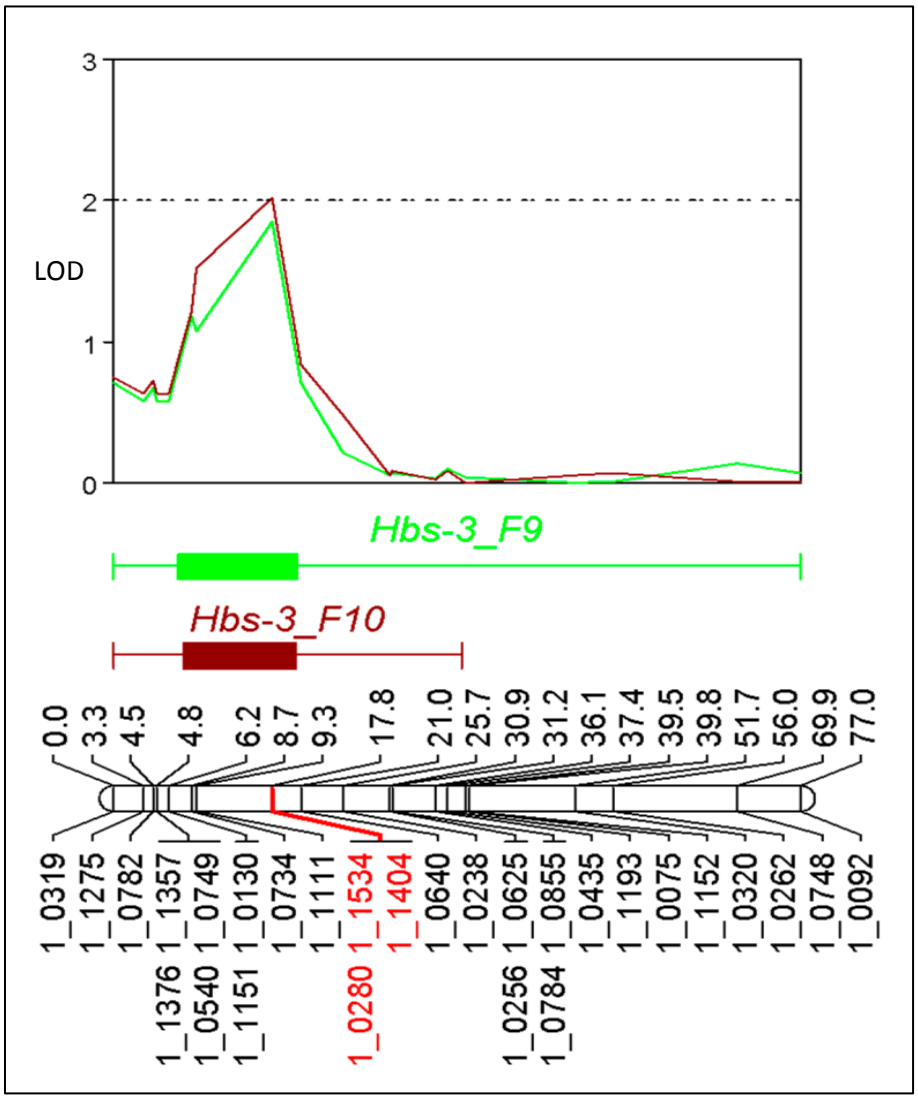

Figure 6.6 Marker-trait association of the *Hbs-1* locus. A marker-trait association of the *Hbs-1* locus was analyzed using thirteen cowpea genotypes which differ in their response to heat-induced browning of seed coats phenotype. IT93K-503-1, IT84S-2246, IT93K-2046, TVu-4552 and TVx-3236, TVu-53 and TVu-15315 were positive for the heat-induced browning phenotype and are referred to as *Hbs*. TVu-14676, CB5, CB27, CB46, 524B and Bambey 21 are negative for the heat-induced browning phenotype and are referred to as *hbs*. The most significant region of the *Hbs-1* locus spanned from 45.27 cM to 47.18 cM on the cowpea consensus genetic map linkage group 5. SNP markers 1_0032 and 1_1128 alleles co-segregated with the positive (*Hbs*) and negative (*hbs*) genotypes. The *Hbs-1* positive genotypes were associated with the adenine nucleotide (color-coded green) at the 1_0032 locus, while *hbs-1* negative genotypes were associated with the guanine nucleotide (color-coded red). The adenine/guanine SNP in marker 1_0032 is position 469 of cowpea unigene 5294 which was annotated as an AMP dependant ligase/synthetase and can be viewed in HarvEST: Cowpea. At the 1_1128 locus, the *Hbs-1* positive genotypes were associated with the thymine nucleotide (color-coded blue) while the *hbs-1*-negative genotypes were associated with the adenine nucleotide (color-coded green). The thymine/adenine SNP in marker 1_1128 is position 950 of cowpea unigene 4874, which was annotated as an ubiquitin-protein ligase and can be viewed in HarvEST: Cowpea.

Figure 6.6 Marker-trait association of the *Hbs-1* locus.

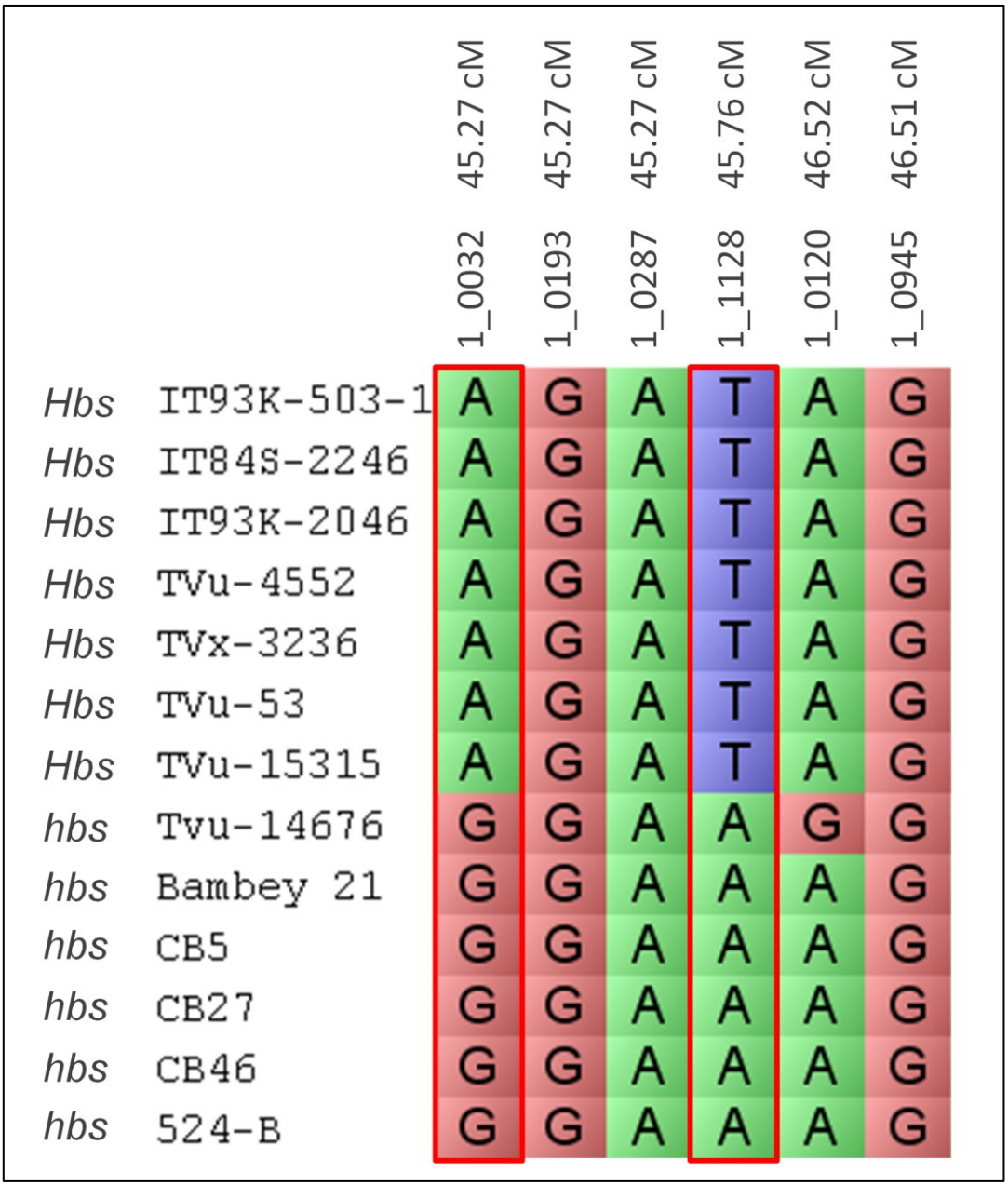

Figure 6.7 Marker-trait association of the *Hbs-3* locus. A marker-trait association of the *Hbs-3* locus was analyzed using thirteen cowpea genotypes which differ in their response to heat-induced browning of seed coats phenotype. IT93K-503-1, IT84S-2246, IT93K-2046, TVu-4552 and TVx-3236, TVu-53 and TVu-15315 were positive for the heat-induced browning phenotype and are referred to as *Hbs*. TVu-14676, CB5, CB27, CB46, 524B and Bambey 21 are negative for the heat-induced browning phenotype and are referred to as *hbs*. The *Hbs-3* locus spanned from 36.00 cM to 37.96 cM on the cowpea consensus genetic map linkage group 1. SNP marker 1_0640 alleles co-segregated with the positive (*Hbs*) and negative (*hbs*) genotypes. The *Hbs-3* positive genotypes were associated with the adenine nucleotide (color-coded green) while the *hbs-3* negative genotypes were associated with the guanine nucleotide (color-coded red). The adenine/guanine SNP in marker 1_0640 is position 348 of cowpea unigene 2077, which was annotated as a 60S ribosomal protein and can be viewed in HarvEST: Cowpea.

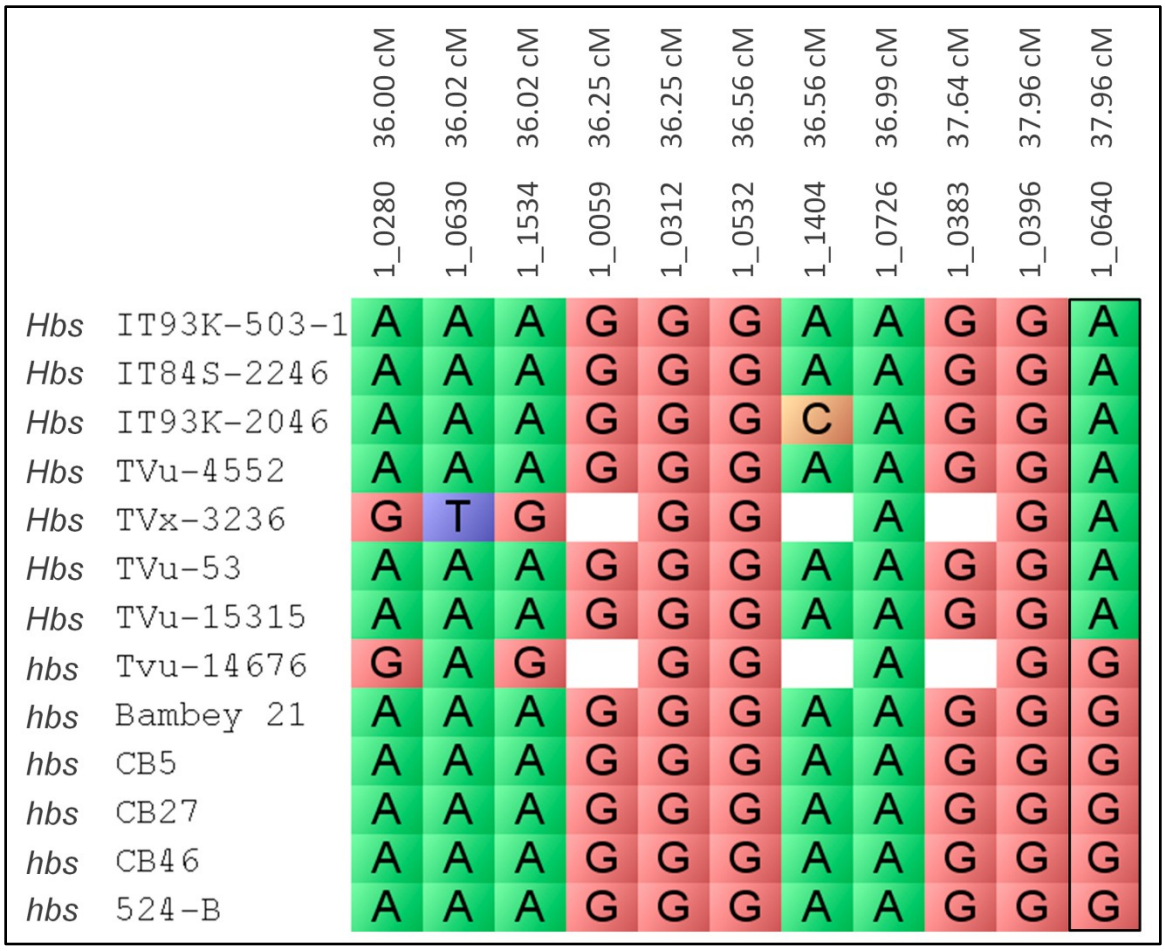

Figure 6.8 Synteny figure of *Hbs-1* locus with *G. max*. Synteny was examined for the *Hbs-1* locus between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Hbs-1* locus which spanned 45.27 cM to 47.18 cM on the cowpea consensus genetic map linkage group 5 was determined to be syntenic with soybean chromosomes 2 and 14. The syntenic locus in soybean chromosome 2 extended from soybean locus Glyma02g42560 to Glyma02g43640 which corresponded to 44.42 cM to 46.51 cM of the *Hbs-1* locus. The syntenic locus on soybean chromosome 14 spanned from Glyma14g05250 to Glyma14g06330 which corresponded to 44.42 cM to 47.18 cM of the *Hbs-1* locus on the cowpea consensus genetic map. Ethylene responsive element binding factor 3 and 11 and ethylene forming enzymes were observed in the syntenic regions of soybean and were considered candidate genes for the *Hbs-1* locus.

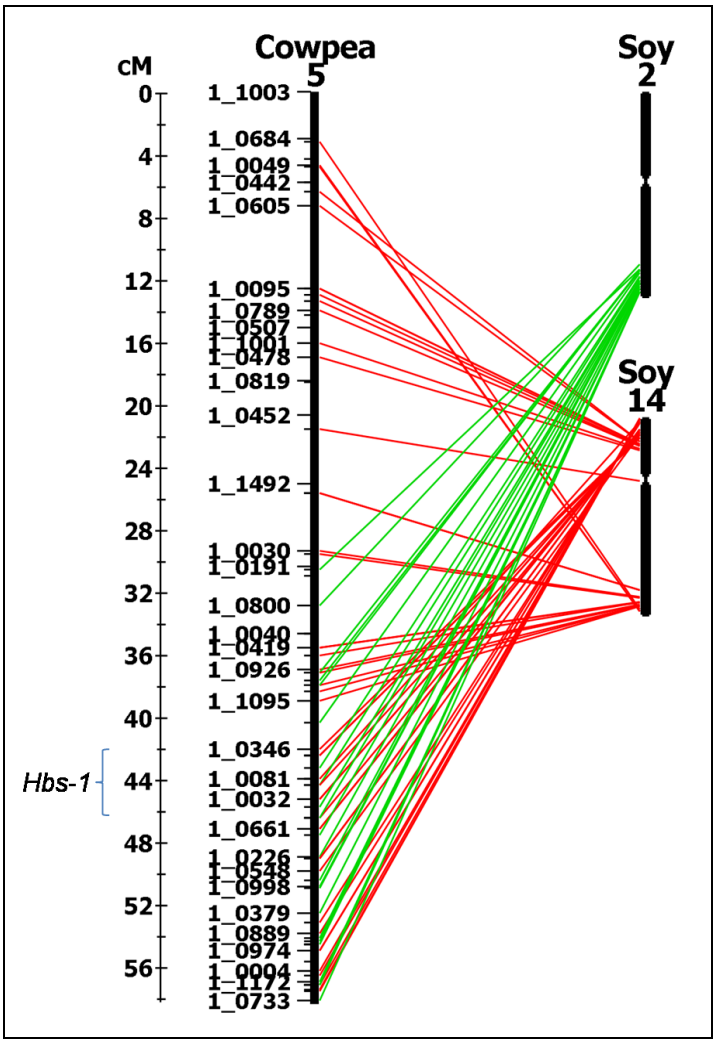

Figure 6.9 Synteny of *Hbs-1* locus with *M. truncatula*. Synteny was examined for the *Hbs-1* locus between cowpea and *M. truncatula* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Hbs-1* locus which spanned 45.27 cM to 47.18 cM on the cowpea consensus genetic map linkage group 5 was determined to be syntenic with *M. truncatula* chromosome 5 where it spanned from Medicago locus Medtr5g018870 to Medtr5g093060. Ethylene response factor 3 (ERF3) and an ethylene forming enzyme were present in the locus and were considered candidate genes.

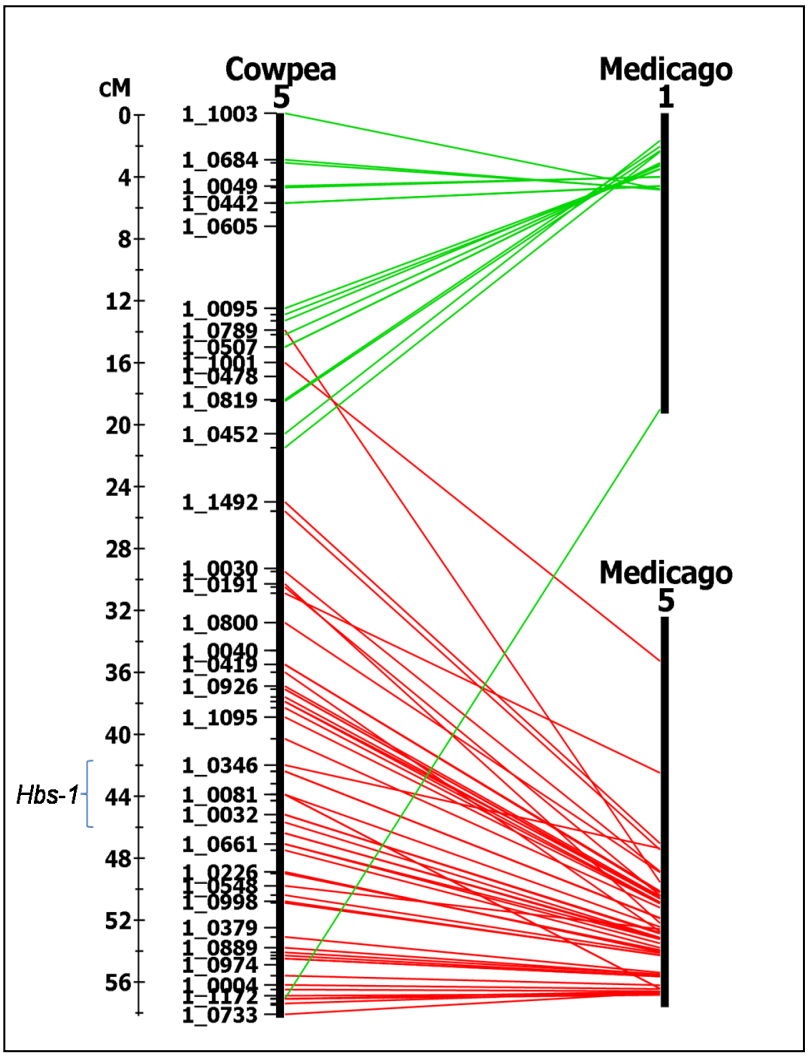

Figure 6.10 Synteny figure of *Hbs-1* locus with *M. truncatula*. Synteny was examined for the *Hbs-1* locus between cowpea and *M. truncatula* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genome. The *Hbs-1* locus which spanned 45.27 cM to 47.18 cM on the cowpea consensus genetic map linkage group 5 was determined to be syntenic with *M. truncatula* chromosome 5 where it spanned from Medicago locus Medtr5g018870 to Medtr5g093060. Ethylene response factor 3 (ERF3) and an ethylene forming enzyme were present in the locus and were considered candidate genes.

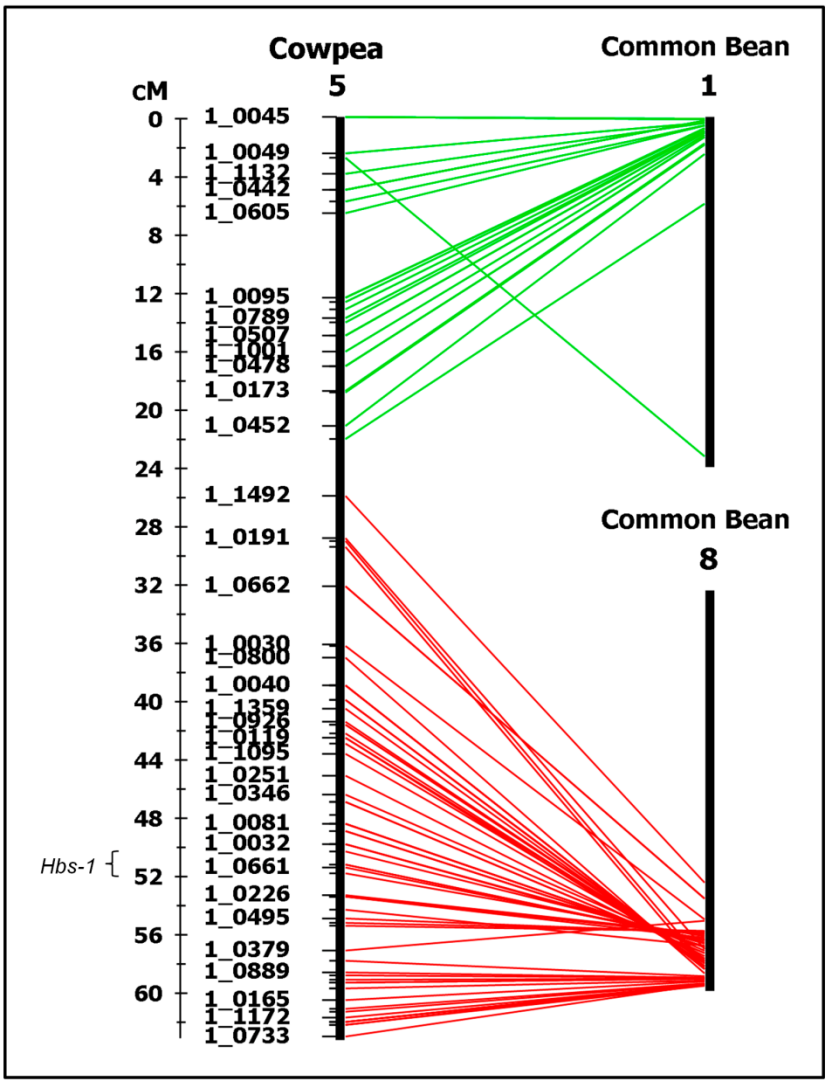

Figure 6.11 Heat-induced browning of seed coats phenotype. Cowpea genotypes which are positive for the *Hbs* trait manifest a brown discoloration either partially or over the entire surface of the seed coat when exposed to high temperature heat during flowering. A. RIL number 9 from the IT93K-503-1 x CB46 population which is positive for the heat-induced browning of seed coats (*Hbs)* trait is shown. B. RIL number 8 from the IT93K-503-1 x CB46 population which is negative for the heat-induced browning of seed coats (*hbs*) trait is shown.

Chapter 7

Conclusion

Cowpeas are an important drought-tolerant legume crop in many areas of the world. However, like most crop plants, cowpea is afflicted with numerous diseases including fungal diseases, which can cause considerable damage to productivity and yield. Identification of loci for disease resistance and other agronomic traits and underlying candidate genes can contribute to improved cowpea varieties.

The main objectives of my studies were to map and identify QTL associated with resistance to two fungal pathogens, *F. oxysporum* f.sp. *tracheiphilum* race 3 and race 4 and *M. phaseolina* in cowpea. Other sub-projects involved studying the leaf morphology trait and the heat-induced brown discoloration of seeds trait in cowpea. All QTLs were positioned on the cowpea consensus genetic map, cowpea physical map and syntenic relationships with *G. max*, *M. truncatula* and *P. vulgaris* were analyzed. Molecular markers which co-segregated with the trait as well as candidate genes were identified.

Fusarium race 3 summary

Fot3-1 was identified in the RIL population CB27 x 24-125B-1 and confers resistance against *F. oxysporum* f.sp. *tracheiphilum* race 3. *Fot3-1* spanned 15.4 cM to 18.3 cM on linkage group 6 of the cowpea consensus genetic map and accounted for 25.2% – 27.8 % of the phenotypic variance. A marker-trait association panel of Fot race 3 resistant and susceptible genotypes identified SNP marker 1_1107 as co-segregating with Fot race 3

resistance and narrowed the locus to a 1.2 cM region. Macro and microsynteny was observed for the *Fot3-1* locus region with *G. max* where several (NBS-LRR) disease resistance proteins, leucine-rich repeat serine/threonine protein kinases and a leucine-rich repeat protein were observed in the syntenic regions of soybean chromosomes 9 and 15. *Fot3-1* was identified on the cowpea physical map on BAC clone CH093L18, spanning approximately 208,868 bp on BAC contig250. The *Fot3-1* locus was narrowed to 0.5 cM distance on the cowpea genetic map linkage group 6, flanked by SNP markers 1_0860 and 1_1107. Cowpea BAC clone CH093L18 was sequenced and four sequences with similarity to leucine-rich repeat serine/threonine protein kinases were present and considered as candidate genes for the *Fot3-1* locus.

Fusarium race 4 summary

Two independent loci which confer resistance to Fot race 4 were identified, *Fot4-1* and *Fot4-2*. *Fot4-1* was identified in the IT93K-503-1 x CB46 population and accounted for 32.6% - 46.5% of the phenotypic variance. *Fot4-1* was positioned on the cowpea consensus genetic map, spanning 21.57 cM to 29.40 cM on linkage group 5. The *Fot4-2* locus was identified in both the CB27 x 24-125B-1 and CB27 x IT82E-18(Big Buff) populations which validated the QTL. In the CB27 x 24-125B-1 population, *Fot4-2* accounted for 37.6% - 40.2% of the phenotypic variance, whereas in the CB27 x IT82E-18 (Big Buff) population, *Fot4-2* accounted for 18.9% - 27.1% of the phenotypic variance. *Fot4-2* was positioned on the cowpea consensus genetic map on LG3, spanning from 64.44 to 80.23 cM. The positioning of *Fot3-1*, *Fot4-1* and *Fot4-2* on the cowpea

consensus genetic map established that the loci were independent of each other. Synteny was examined for the *Fot4-1* and *Fot4-2* loci with *G. max*. Two TIR-NBS-LRR disease resistance proteins and a leucine-rich repeat serine/threonine protein kinase were observed in the syntenic region of *Fot4-1* locus and six copies of leucine-rich repeat serine/threonine protein kinases and a TIR-NBS-LRR disease resistance protein was observed in the *Fot4-2* syntenic locus. *Fot4-1* and *Fot4-2* were coarsely positioned on the cowpea physical map.

Macrophomina summary

The Macrophomina study identified four major QTLs, *Mac-10*, *Mac-11*, *Mac-12* and *Mac-13*, in the Sanzi x Vita 7 population. The *Mac-10* locus accounted for 9.9% of the phenotypic variance and spanned 27.24 cM to 86.07 cM on cowpea LG3. Synteny was observed for *Mac-10* with soybean chromosomes 5 and 17 in which WRKY72 transcription factors were observed. The *Mac-11* locus accounted for 10% - 16.3% of the phenotypic variance and spanned 37.04 cM to 50.85 cM on cowpea LG5. Within the *Mac-11* locus, SNP marker 1_1419 was observed co-segregating with late-maturity Macrophomina-resistant genotypes. Synteny of the *Mac-11* locus was observed with *G. max*, *M. truncatula* and *P. vulgaris* where auxin response factors were present. *Mac-11* was identified within BAC clone CH038D17 of contig426 on the cowpea physical map; CH038D17 annotations revealed that an auxin response factor was present. The *Mac-12* locus accounted for 8.5% to 15.1% of the phenotypic variance and spanned 4.09 cM to 31.04 cM on cowpea LG7. The *Mac-13* locus accounted for 10.8% of the

phenotypic variance and spanned 20.72 cM to 25.57 cM on cowpea LG4. Within the *Mac-13* locus, SNP marker 1_1242 was identified as co-segregating with late-maturity Macrophomina-resistant genotypes. Syntenic relationships for the *Mac-13* locus with *G. max* and *P. vulgaris* revealed the presence of AUX/IAA family member genes and auxin-responsive *GH3* family proteins. *Mac-13* was positioned within BAC clones CH062O11 and CH069K06 of contig445 on the cowpea physical map, where auxin-responsive *GH3* family proteins were identified.

Mac-10, *Mac-11* and *Mac-13* co-located in the general vicinity of previously mapped Macrophomina QTLs on the cowpea consensus genetic map. *Mac-10* was the only QTL which was observed during the seedling stage and hints to seedling-stage Macrophomina resistance. *Mac-12* was the only newly discovered Macrophomina locus and was observed during the entire cowpea season, which may confer a broad spectrum resistance during the entire plants growth and development.

Leaf morphology summary

Leaf morphology was studied in the Sanzi x Vita 7 population. The *Hls* (hastate leaf shape) locus accounted for 71.5% - 74.7% of the phenotypic variance and spanned 25.57 to 35.96 cM on cowpea LG4. A marker-trait association within the *Hls* locus identified SNP marker 1_0349 alleles co-segregating with the hastate or sub-globose leaf phenotype. High co-linearity was observed for the syntenic *Hls* region with *M. truncatula* chromosome 7 and *G. max* chromosomes 3 and 19, in which orthologs for the EZA1/SWINGER (AT4G02020.1) gene were present. The *Hls* locus was positioned 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.

Heat-induced brown discoloration of seed coats summary

The heat-induced browning trait was studied in the IT93K-503-1 x CB46 and IT84S-2246 x TVu14676 populations in which three loci were identified, *Hbs-1*, *Hbs-2* and *Hbs-3*. The major locus, *Hbs-1*, was identified in both populations and accounted for 62.8% -77.3% of the phenotypic variance and spanned 45.27 cM to 47.18 cM on LG5 on the cowpea consensus genetic map. A marker-trait association identified two SNPs, 1_0032 and 1_1128, which co-segregated with the heat-induced browning of seed coats phenotype and could be used as molecular markers to screen against *Hbs-1*. The syntenic regions of *Hbs-1* with *G. max*, *M. truncatula* and *P. vulgaris*, revealed the presence of several ethylene forming enzymes (EFE), ethylene responsive element binding factors and an ACC oxidase 2 gene. *Hbs-1* was identified on the cowpea physical map within BAC clone CM018C23 of contig 217 where ten copies of EFE and an ACC oxidase 2 gene were present. *Hbs-2* was the second locus associated with the heat-induced browning trait in the IT93K-503-1 x CB46 population, which accounted for 9.5 to 12.3 % of the phenotypic variance. *Hbs-2* was positioned on the cowpea consensus genetic map where it spanned from 31.28 cM to 58.09 cM on LG6. *Hbs-3* was a minor locus identified in the IT84S-2246 x TVu14676 population and accounted for 6.2 to 6.8 % of the phenotypic variance. The *Hbs-3* locus was positioned on the cowpea consensus genetic map where it spanned 36.0 cM to 37.96 cM on linkage group 1. SNP marker

1_0640 was identified within the *Hbs-3* locus co-segregating with the heat-induced browning phenotype. *Hbs-3* was positioned on BAC clones in contig512 of the cowpea physical map, where several ACC synthase 1 genes were present.

Future prospective research

The results from the QTL mapping studies for the Fot race 3 and race 4 resistance, Macrophomina resistance, leaf morphology and heat-induced browning of seed coats traits have opened up several areas of future research in cowpea as well as application to breeding programs. The identification of highly significant molecular markers within trait loci can be utilized in MAS breeding schemes to optimize the genetic improvement of cowpea via different strategies which include pedigree backcrossing and marker-assisted recurrent selection (MARS). These approaches could expedite variety development by halving the current traditional breeding selection process. However, before the markers can be utilized in MAS breeding schemes, the markers must be tested and validated in select cowpea genotypes to make certain there is a tight linkage of the marker with the trait, ensuring that the marker will select for the correct phenotype. This is especially important in the loci in which we couldn't find a marker which co-segregated 100% with the phenotypes.

Cowpea-specific candidate genes were identified for the *Fot3-1*, *Mac-11*, *Mac-13, Hbs-1* and *Hbs-3* loci which could offer a more precise method of introgressing the positive traits into elite cowpea cultivars. Identification of the gene or genes responsible for the trait of interest would enable the use of "perfect markers", whereby the marker is

designed specifically from the gene(s) underlying the trait which could decrease "linkage drag" or introgression of possible closely linked negative traits within the region. Additional strategies could include developing and validating haplotype profiles within the QTLs identified. This would ensure that only positive alleles for the trait are targeted as well as positive alleles of closely flanking markers.

Macrophomina resistance in cowpea would be a good candidate for implementing MAS within breeding programs since the trait is quantitative and the phenotypic selection requires laborious screening which can be further complicated by G x E effects. Quantitative disease resistance can offer a broad spectrum resistance to other pathogens as well as a "durable resistance" which may last longer than qualitative disease resistance which relies on single genes and can be overcome by evolving pathogen populations. The *Mac-10* and *Mac-12* loci would be good targets to further investigate since together they could provide Macrophomina tolerance at the seedling-stage as well as throughout the entire developmental process of cowpea. Additionally, these two loci were not associated with late-maturing Macrophomina resistance, which would be a positive trait to introgress into future Macrophomina-resistant cowpeas. However, more work will need to be undertaken to understand Macrophomina resistance in cowpea. Continual efforts in genetic mapping studies using other cowpea populations as well as the use of association mapping studies would ensure a collection of Macrophomina resistance loci as well as provide a global view of quantitative disease resistance in cowpea. In addition, an awareness of the photoperiod sensitivity of cowpea germplasm must be factored into any future greenhouse screening or field experiments.

Functional analysis of candidate genes

The identification of cowpea candidate gene sequences for the *Fot3-1* (leucine-rich repeat serine/threonine protein kinase), *Mac-11* (auxin response factor) and *Mac-13* (auxin-responsive *GH3* family protein) and *Hbs-1* (ethylene forming enzymes or an ACC oxidase 2 gene) loci, has opened up several new areas in cowpea research. A more fundamental research approach can be undertaken for the *Fot3-1*, *Mac-11*, *Mac-13* and *Hbs-1* loci since we were able to identify the cowpea gene sequences on BAC clones. This will enable functional analysis of the candidate genes which will enable a more fundamental understanding of the gene and the phenotype.

Gene expression profiles of the candidate genes in different genetic backgrounds and under different environmental conditions could give us more insight to how or if the gene is affecting the phenotype. Once the candidate genes are cloned from the resistant and susceptible parent, gene expression could be linked to polymorphisms observed in the sequences between the resistant and susceptible parent, which could then be statistically linked with the phenotypic variation of the trait being studied.

Currently, there are a several molecular protocols for the transformation of cowpea (Ivo et al. 2008; Popelka et al. 2006; Muthukumar et al. 1996; Garcia et al. 1986) which would enable complementation experiments to test candidate genes. Another technique which could be used to functionally analyze candidate genes would be virus induced gene silencing (VIGS), which has been used to validate a resistance gene against the plant parasite *S. gesnerioides* race 3 (Li and Timko 2009). In addition, due to the close

syntenic relationships between cowpea, soybean, common bean and Medicago, characterization and validation of candidate genes underlying QTL in cowpea can be transferred to the other legumes.

Future improved cowpea varieties

In the next few years, the 45k SNP genotyping array for the cowpea consortium will enable cowpea breeders and researchers to mine data, map traits and implement highly accurate MAS in breeding programs which should make vast improvements in cowpea cultivar development for the Sub-Saharan Africa production regions as well as the United States and other countries.

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