Genetic and physical mapping of candidate genes for resistance to Fusarium oxysporum f.sp. tracheiphilum race 3 in cowpea [Vigna unguiculata (L.) Walp].

Permalink
https://escholarship.org/uc/item/9vj2j4k8

Journal
PloS one, 7(7)

ISSN
1932-6203

Authors
Pottorff, Marti
Wanamaker, Steve
Ma, Yaqin Q
et al.

Publication Date
2012

DOI
10.1371/journal.pone.0041600

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

Marti Pottorff¹, Steve Wanamaker¹, Yaqin Q. Ma¹, Jeffrey D. Ehlers¹,², Philip A. Roberts³, Timothy J. Close¹

1 Department of Botany & Plant Sciences, University of California Riverside, Riverside California, United States of America, 2 Bill & Melinda Gates Foundation, Seattle, Washington, United States of America, 3 Department of Nematology, University of California Riverside, Riverside, California, United States of America

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). Biparental 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/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 [1]. 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 [2,3,4]. Fusarium wilt is especially problematic in cowpea production regions within the United States including the southeastern United States and the Central Valley of California [5]. 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 [6]. Several races of Fot have evolved, races 1, 2, 3, and 4, which are identified according to differential interactions on several cowpea genotypes [5,6,7]. Currently, Fot race 3 is the predominant and most widely distributed race [7]. 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 [8,9]. These cultivars were developed using conventional breeding approaches that rely on phenotypic assessments as
Figure 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...
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) [10]. 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 accesses 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 × 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 × 24-125B-1 genetic map (Figure 1, Table 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). 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). 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). 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 biparental mapping study. Fot3-1 spanned 15.4 cM to 18.3 cM on linkage group 6 of the cowpea consensus genetic map (Table 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). 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). 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). 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 biparental mapping study. Fot3-1 spanned 15.4 cM to 18.3 cM on linkage group 6 of the cowpea consensus genetic map (Table 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). 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). 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). Henceforth, the Fot race 3 resistance locus will be referred to as Fot3-1.

Table 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 consensus 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 6 16.88 1_1381          | 771 CH001O04        |
| N/A 6 17.14 1_0895          | N/A 6 17.14 1_1077          | 250 CH046G19        |
| N/A 6 17.14 1_1363          | N/A 6 17.40 1_0897          | 250 CM002B24, CM015O07 |
| 1   50.49 1_0860             | 6   17.82 1_0860            | 250 CH045O10, CM002B24, CM015O07 |
| 1   50.49 1_1484             | 6   17.88 1_1484            | N/A 250 CH076D23, CH093L18 |
| 1   49.42 1_1107             | 6   18.31 1_1107            | 250 CM001C09, CM051M10 |
| N/A 6 19.04 1_0704          | N/A 6 19.54 1_1206          | 250 CM054B04, CH051M10 |

SNP markers are aligned in the order designated by the cowpea consensus genetic map. doi:10.1371/journal.pone.0041600.t001
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 biparental mapping studies, was the only marker with alleles that co-segregated perfectly with a corresponding resistant or susceptible phenotype (Figure 2). The resistant genotype at this locus is associated with the adenine nucleotide which is color-coded green in Figure 2. The susceptible genotype was associated with the guanine nucleotide which is color-coded red in Figure 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 (File S1) (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).

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 3). 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 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 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 3). Soybean loci Glyma15g13290, Glyma15g13300 and Glyma15g13310 were identified between orthologous soybean genes to markers 1_0860 and 1_1212 (Table 3). Glyma15g13290 and Glyma15g13300 were annotated as disease resistance proteins of the NBS-LRR class.

| 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 R²               | Wilting/Stunting   | 10.2   | 10.7   | 10.7   | 8.8    |
|            | IM LOD              | Vascular Discoloration | 4.49  | 4.91   | 4.91   | 3.52   |
|            | IM R²               | 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 R²               | Wilting/Stunting   | 20.2   | 22.7   | 22.7   | 18.4   |
|            | IM LOD              | Vascular Discoloration | 4.44  | 5.16   | 5.16   | 4.67   |
|            | IM R²               | 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 | Vascular Discoloration | 0.005 | 0.0005 | 0.0005 | 0.0005 |
|            | 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 R²               | Wilting/Stunting   | 18.4   | 17.7   | 17.7   | 12.6   |
|            | IM LOD              | Vascular Discoloration | 4.97  | 4.85   | 4.85   | 3.23   |
|            | IM R²               | 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.
doi:10.1371/journal.pone.0041600.t002
while Glyma15g13310 was annotated as an LRR protein (Table 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 [12]. 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 biparental 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 1). The length of the contig is estimated at 885,600 bp (540 nonrepeated 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 (Figure 4, Table 1). SNP marker 1_1107 was identified on two overlapping BAC clones, CH051M10 and CM001C09 (Figure 4, Table 1). Markers 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 4). SNP marker 1_1212 was also identified on BAC clone CM001C09, which it shares with marker 1_0860 (Figure 4). Marker 1_1484, which flanks 1_1107 on the cowpea consensus genetic map, was not identified on the cowpea physical map (Table 1). The significant region of the Fot3-1 locus spanned three overlapping BAC clones, CH093L18, CM001C09 and CH051M10 (Figure 4). 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 4).

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 [13]. 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 (File S2). 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 (File S3). The BES of CH051M10 was also identified after

**Figure 2. Marker-trait association of 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. doi:10.1371/journal.pone.0041600.g002
BLASTing to the assembled sequence (File S3). 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 (File S4).

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 (File S5). 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 (File S6). 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 4). 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 4). 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 (File S7). 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

**Figure 3. 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 Glyma09g0210 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. doi:10.1371/journal.pone.0041600.g003
Table 3. Synteny of Fot3-1 with Glycine max chromosomes 9 and 15.

| G. max chromosome | G. max locus | Phytome 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 |

doi:10.1371/journal.pone.0041600.t003

Discussion

In this study, we report the identification of the Fot3-1 locus which confers resistance to F. oxysporum 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 [16,17,18,19,20] which fits the gene-for-gene hypothesis whereby pathogen and host express complementary dominant genes [21]. The alteration or loss to either the host’s resistance gene or pathogen’s avirulence gene leads to disease [21]. 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) [22,23] or a coiled-coil motif (CC-NBS-LRR or non TIR-NBS-LRR) [22,23]. Currently, two genes have been cloned which confer resistance to F. oxysporum f.sp. lycopersici (Fol) race 1 in tomato which was determined to be a CC-NBS-LRR disease resistance gene [24]. 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 [25].

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 [26]. The majority of RLK have serine/threonine kinases and LRR motifs [27]. The
receptor-like cytoplasmic kinase (RLCK) disease resistance genes include PBS1, Pti and Pto [28]. PBS1 confers resistance against Pseudomonas syringae pv phaseolicola in Arabidopsis [29]. Pti and Pto both confer resistance to the bacterium Pseudomonas syringae pv tomato [30,31]. Xa21 is a LRR RLK and confers resistance against Xanthomonas campestris pv oryzae in rice [32]. Lrk10 which confers resistance to the fungus, Puccinia recondite in wheat was also determined to be a serine/threonine protein kinase [33]. The I-3 locus which confers resistance to F. oxysporum f.sp. lycopersci race 3 in tomato, was determined to be positioned within a large cluster of S-locus receptor-like kinases (SRLK) [34]. Interestingly, we recently identified TIR-NBS-LRR proteins and leucine-rich repeat serine/threonine protein kinases in the Fot4-1 and Fot4-2 syntetic 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 intraspecific 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 F10 generation. CB27 is a cultivar which was bred for resistance to F. oxysporum f.sp. tracheiphilum races 3 and 4 [8]. C93W-24-125B is a breeding line from Cameroon and is highly susceptible to Fot race 3 [35,36]. 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-cm² plugs were cut from frozen Fusarium-containing PDA plates and transferred aseptically to flasks containing 500 ml 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 1.0×10⁷ microconidia per ml using a hemocytometer. Greenwood

Figure 4. 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. doi:10.1371/journal.pone.0041600.g004
experiments were conducted using a modified root-tip inoculation method as previously described [37]. Ten greenhouse grown seeds per line were planted in seedling 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 5). The severity of the disease was evaluated on a zero to five rating scale (Table 5). The extent of the disease symptoms were evaluated individually then averaged to determine the disease severity for each RIL.

The California Blackeye 27 × 24-125B-1 population and genotypes CB27, CB46, Iron Clay, SH49-10-1-1, SH50-17-9-1-1 (also known as California Blackeye No. 50), SH50-7-9-2, IT93K-503-1, 24-125B-1, CB5, Bambev 21, IT82E-18/Big Buff and IT84S-2049 were genotyped at the F8 generation or above using biallelic 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 × 24-125B-1 population was created previously and is included in both cowpea consensus genetic map vs.2 [10] and vs. 3 [15]. The map was generated using 339 SNP markers and 90 individuals and consisted of sixteen linkage groups and spans approximately 600 cM total distance [15]. The cowpea consensus genetic map vs. 5 [15] 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 [15]. 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 [15]. 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 × 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

Table 4. 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 S-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 |

doi:10.1371/journal.pone.0041600.t004
bi-parental mapping [38]. 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 [39].

Synteny was examined between cowpea and *G. max* using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes as described previously [10]. Syntenic relationships between the cowpea, soybean, *Medicago truncatula* and *Arabidopsis 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 *HindIII* and *MboI* (Amplicon Express, Pullman, WA). Contigs were assembled using the snapshot method of DNA fingerprinting [40] 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 1640 bp (personal communication, Ming Cheng Luo).

BAC clones CH051M10 and CH093L18 were sequenced using an Illumina GAII or HiSeq 2000 sequencer, respectively, at the Institute of Integrative Genomics 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 Diagenode 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 [13] 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.
located at position 693, parenthesized and in bold.

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).

Supporting Information

File S1 SNP Marker 1_1107 sequence. cDNA sequence of P12 assembly unigene 12265 which is housed in Harvest: Cowpea database (http://harvest.ucr.edu). The adenine/guanine SNP is P12 assembly unigene 12265 which is housed in Harvest: Cowpea database (http://harvest.ucr.edu). The adenine/guanine SNP is located at position 693, parenthesized and in bold.

File S2 FASTA file for cowpea BAC clone CH051M10. (TXT)

File S3 BLAST of cowpea SNP markers and BES to cowpea BAC clone CH051M10. (DOCX)

File S4 Soybean candidate genes BLASTed to cowpea BAC clone CH051M10. (DOCX)

References

1. Armstrong GM, Armstrong JK (1981) Formae specialis and races of Fusarium oxysporum causing wilt disease in: Nelisen P, Toussaint T, Cook R, editors. Fusarium Diseases, Biology and Taxonomy University Press: USA: Pennsylvania State University Press. 391–399.
2. Summerell BA, Leslie JP, Liew ECY, Laurence MH, Bullock S, et al. (2011) Fusarium species associated with plants in Australia. Fungal Diversity: 1: 27.
3. Assunção IP, Michereff SJ, Mizubuti ESG, Brommelschenkel SH (2003) Influence of Fusarium wilt intensity on cowpea yield. Fitopatologia Brasileira 28: 615–619.
4. Armstrong GM, Armstrong JK (1980) Cowpea wilt Fusarium oxysporum f. sp. tachypodii race 1 from Nigeria. Plant Disease 64: 954–955.
5. Hare WW (1953) A new race of Fusarium causing wilt of cowpea. Phytopathology 43: 291.
6. Patel BV (1995) Fungal, bacterial and viral diseases of cowpeas in the USA. In: Singh SR, Rache KO, editors. Cowpea research, production and utilization Chichester, UK: John Wiley and Sons. 205–213.
7. Smith SN, Helms DM, Temple SR, Frate C (1999) The distribution of Fusarium wilt of blackeye cowpeas within California caused by Fusarium oxysporum f. sp. tachypodii race 1. Plant Disease 83: 694–694.
8. Ehlers JD, Hall AE, Patel PN, Roberts PA, Matthews WC (2000) Registration of ‘California Blackeye 27’ cowpea. Crop Science 40: 854–855.
9. Ehlers JD, Sanden BI, Frate CA, Hall AE, Roberts PA, Registration of ‘California Blackeye 50’ cowpea. Journal of Plant Registrations 9: 236.
10. Muchero W, Diop NN, Bhat PR, Fenton RD, Wamamaker S, et al. (2009) A consensus genetic map of cowpea [Vigna unguiculata (L.) Walp.] and synteny based on EST-derived SNPs. Proceedings of the National Academy of Sciences USA 106: 18153–18164.
11. Miller I, Shaw P, Stephen G, Bayer M, Cardle L, et al. (2010) Flapjack-graphical genotype visualization. Bioinformatics 26: 3133–3134.
12. Choi HK, Mun JH, Kim DJ, Zhu H, Baek JM, et al. (2004) Estimating genome conservation between crop and model legume species. Proceedings of the National Academy of Sciences USA 101: 15209–15294.
13. Zerboino D, Birney E (2006) Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Research 18: 821–829.
14. Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter 9: 208–218.
15. Diop N, Ehlers J, Wamamaker S, Muchero W, Fatokum C, et al. (2012) An improved consensus genetic linkage map of cowpea [Vigna unguiculata (L.) Walp.] In: Boukar O, Ouhily O, Fatokum C, Lopez K, Tamo M, editors. Enhancing cowpea value chains through research advances. Ibadan, Nigeria: International Institute of Tropical Agriculture (IITA). pp. In press.
16. Zink FW, Thomas CE (1996) Genetics of resistance to Fusarium oxysporum f. sp. melonis races 0, 1, and 2 in muskmelon line MR-1. Phytopathology 80: 1230–1232.
17. Rubio J, Hajj-Meussa E, Kharat M, Moreno MT, Millan T, et al. (2003) Two genes and linked RAPD markers involved in resistance to Fusarium oxysporum f. sp. ciceri race 6 in chickpea. Plant Breeding 122: 180–191.
18. McGrath DJ, Gillespie D, Wardley L (1987) Inheritance of resistance to Fusarium oxysporum f. sp. lycopersici races 2 and 5 in Lycopersicon pennellii [Fusarium wilt of tomato]. Australian Journal of Agricultural Research 38: 729–733.
19. Scott JW, Jones JP (1969) Monogenic resistance in tomato to Fusarium oxysporum f. sp. lycopersici race 3. Phytopathology 49: 49–53.
20. Saffari M, Abou-Alsed M, Katan J, Zamil D (1991) RFLP mapping of II, a new locus in tomato conferring resistance against Fusarium oxysporum f. sp. lycopersici race 1. Theoretical and Applied Genetics 82: 22–26.
21. Flor HHI (1971) Current status of the gene-for-gene concept. Annual Review of Phytopathology 9: 275–296.
22. Meyers BC, Dickerman AW, Michelson RW, Siwamakrishnan S, Sobral BW, et al. (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. The Plant Journal 20: 317–332.
23. Pan Q, Wendel J, Fuhr R (2000) Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cerial genomes. Journal of Molecular Evolution 50: 203–213.
24. Simons G, Groenendijk J, Wijnbrandy, Reijmans M, Groen J, et al. (1998) Dissection of the Fusarium I2 gene cluster in tomato reveals six homologs and one active gene copy. The Plant Cell Online 10: 1055–1068.
25. Joobeur T, King JJ, Nolin SJ, Thomas CE, Dean RA (2004) The fusarium wilt resistance locus Fw-2 of melon contains a single resistance gene with complex features. The Plant Journal 39: 293–297.
26. Geer P, Hanjet T, Lautberg RA (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. Annual Review of Cell Biology 10: 251–337.
27. Becraft PW, King JJ, Nolin SJ, Thomas CE, Dean RA (1994) The fusarium wilt resistance locus Fw-2 of melon contains a single resistance gene with complex features. The Plant Journal 39: 293–297.
28. Meyers BC, Dickerman AW, Michelson RW, Siwamakrishnan S, Sobral BW, et al. (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. The Plant Journal 20: 317–332.
29. Pan Q, Wendel J, Fuhr R (2000) Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. Journal of Molecular Evolution 50: 203–213.
30. Simons G, Groenendijk J, Wijnbrandy, Reijmans M, Groen J, et al. (1998) Dissection of the Fusarium I2 gene cluster in tomato reveals six homologs and one active gene copy. The Plant Cell Online 10: 1055–1068.
Resistance to Fusarium Race 3 in Cowpea

33. Feuillet C, Schachermayr G, Keller B (1997) Molecular cloning of a new receptor-like kinase gene encoded at the Lr10 disease resistance locus of wheat. The Plant Journal 11: 45–52.

34. Hemming MN, Basuki S, McGrath DJ, Carroll BJ, Jones DA (2004) Fine mapping of the tomato I-3 gene for fusarium wilt resistance and elimination of a co-segregating resistance gene analogue as a candidate for I-3. Theoretical and Applied Genetics 109: 409–418.

35. Hall AE, Cisse N, Thiaw S, Elawad HOA, Ehlers JD, et al. (2003) Development of cowpea cultivars and germplasm by the Bean/Cowpea CRSP. Field Crops Research 82: 103–134.

36. Kitch LW, Boukar O, Ehlers JD, Shade R, Murdock LL (2001) Registration of “C93W-24–125B” cowpea germplasm. Crop Science 40: 854–855.

37. Rigert KS, Foster KW (1987) Inheritance of resistance to two races of Fusarium wilt in three cowpea cultivars. Crop Science 27: 220–224.

38. Van Ooijen JW (2004) MapQTL® 5, Software for the mapping of quantitative trait loci in experimental populations. Kyazma BV, Wageningen, Netherlands.

39. Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. Journal of Heredity 93: 77–78.

40. Luo MC, Thomas C, You FM, Hsiao J, Ouyang S, et al. (2003) High-throughput fingerprinting of bacterial artificial chromosomes using the snapshot labeling kit and sizing of restriction fragments by capillary electrophoresis. Genomics 82: 378–389.

41. Zerbino DR (2010) Using the Velvet de novo assembler for short-read sequencing technologies. Current protocols in bioinformatics/editorial board, Andreas D Basevanis [et al] Chapter 11: Unit 11.15.