CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

April 1, 2009, to March 31, 2010

GENETIC VARIATION IN LETTUCE

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SUMMARY:

We continue to apply novel methods for detecting, analyzing, and manipulating genetic variation in lettuce. The aim of this project is to ensure that lettuce does not lag behind other crops in benefiting from the application of genomic and biotechnological techniques. We have continued four projects: (i) Introduction of genes into lettuce using *Agrobacterium tumefaciens* and analysis of their expression. (ii) Cloning and characterization of disease resistance genes. (iii) Genetic mapping using a variety of molecular markers with the goal of locating most of the disease resistance genes known in lettuce. (iv) Comparative genomics to identify candidate genes controlling horticultural traits. Our efforts have mainly been focused on projects (ii) through (iv). We have been making extensive use of new high-throughput sequencing and marker technologies. As part of the Compositae Genome Project, over 200,000 EST sequences have been combined with short reads from next-generation sequencing to generate ~50,000 assemblies representing most of the transcribed genes in lettuce. These sequences have been and are being mined for candidate genes for agriculturally important traits. Candidate genes have been mapped relative to over 60 loci for disease resistance, development, and horticulturally important traits using Illumina GoldenGate Single Nucleotide Polymorphism (SNP) assays. Candidate genes which co-segregated with disease resistance phenotypes are being tested for function using RNA interference (RNAi) and several causal genes have been identified. We have been utilizing a custom lettuce Affymetrix genotyping chip with over 6 million overlapping probes representing >35,000 unigenes from lettuce. We are refining an ultra-high density genetic map of over 14,000 loci based on genotyping using hybridizations of genomic DNA to the Affymetrix chip (http://chiplett.ucdavis.edu). We have developed and are curating a database for lettuce as part of the Compositae Genome Project (http://compositdb.ucdavis.edu/) that includes genetic, molecular marker, cultivar, phenotypic and sequence data for lettuce.

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PROJECT TITLE: **GENETIC VARIATION IN LETTUCE**

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OBJECTIVES:

To develop and apply new methods for detecting, analyzing and manipulating variation in lettuce. We continue to pursue these objectives in four projects:

1) Analysis of transgenes in lettuce.

2) Molecular cloning of genes for disease resistance and other horticultural traits.

3) Development of a detailed genetic map and identification of reliable, readily-assayed markers linked to disease resistance genes.

4) Utilization of comparative genomics to identify candidate genes controlling horticultural traits and development of robust molecular markers for them.

In the first three projects we are emphasizing either novel forms of disease resistance or increasing the efficiency of selection for disease resistant genotypes. The fourth project includes a wide range of horticultural traits. Some of these studies are currently funded from Federal grants and gifts from several seed companies. All projects were initiated with CLRP funds and application of the results to lettuce improvement is supported by CLRP funds. All projects impact both crisphead and leafy improvement.

PROCEDURES AND RESULTS:

Transgene Expression in Lettuce

Our transgenic studies remain a lower priority than the other objectives, in part because there is not a major need that can only be addressed by transgenic lettuce and therefore commercial application of transgenes in lettuce is not an immediate priority. Introduction of genes into lettuce using *A. tumefaciens* is routine; in earlier experiments, however, transgenes that expressed well in other plant species were expressed poorly after multiple generations in lettuce. Factors influencing the stability of transgene expression over multiple generations in lettuce are not well understood and require further investigation. We do not currently have projects specifically focused on optimizing the stability of transgene expression in lettuce. However, we continue to generate dozens of transgenics as parts of other projects (see below). These are providing data on transgene expression and stability.

Resistance Candidate Gene Identification and Mapping

In previous years, we identified over 700 candidate resistance genes by mining our database of lettuce ESTs (http://cgpdb.ucdavis.edu) and amplifying conserved domains, using PCR with degenerate oligonucleotide primers, for genes known to function in disease resistance. Of these candidate genes, 291 were mapped. Over half of the 120 NBS-LRR-encoding genes representing ~20 gene families map to resistance phenotypes (McHale *et al*., 2009. *Theor. Appl. Genet*. **118:**565-580).

Loci for resistance to as many diseases as possible, particularly downy mildew, LMV, corky root, *Fusarium* and *Verticillium* wilts, and big vein, are being placed onto the consensus map relative to candidate genes. So far, a total of 52 phenotypic resistance loci have been mapped relative to these 291 candidate resistance genes (Fig. 1). The majority of resistance phenotypes are linked to NBS-LRR-encoding genes.

Mapping of Resistance to Big Vein

In collaboration with Dr. Ryan Hayes (USDA Salinas), we have identified three chromosomal regions contributing to resistance against big vein. Dr. Hayes developed a population of 89 RILs from crossing cvs. Parade and Pavane. Cultivar Pavane is resistant to big vein. Plants were evaluated in three field experiments for disease severity as the percentage of plants with symptoms and calculating the area under the disease progression curve (AUDPC). A genetic map was constructed for this population using 255 polymorphic SNPs analyzed with the Illumina GoldenGate assay. Three chromosomal regions associated with resistance to big vein were identified on linkage groups 3a and 4a (Fig. 2). These three quantitative trait loci (QTLs) together explained 56% of the phenotypic variation observed.

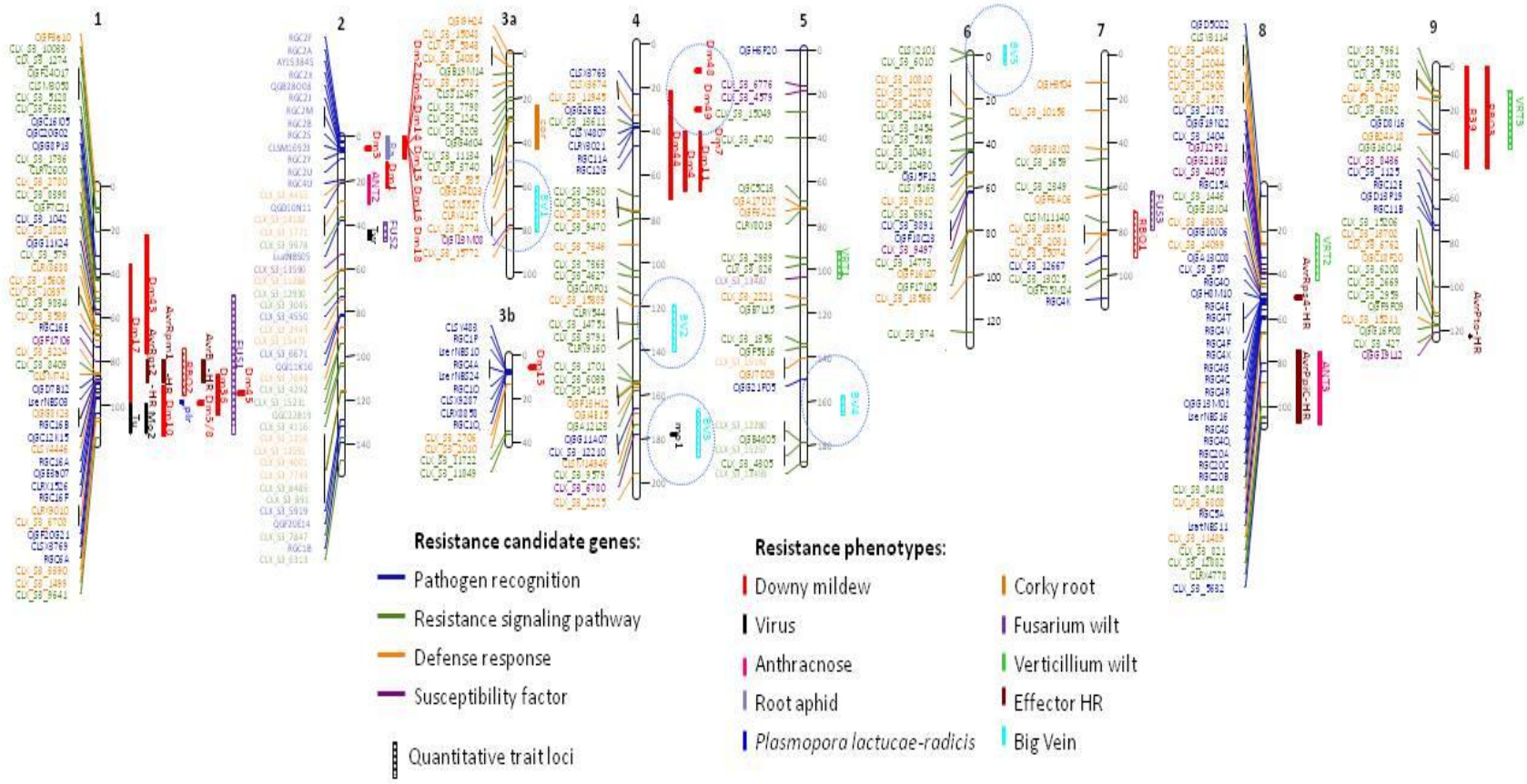
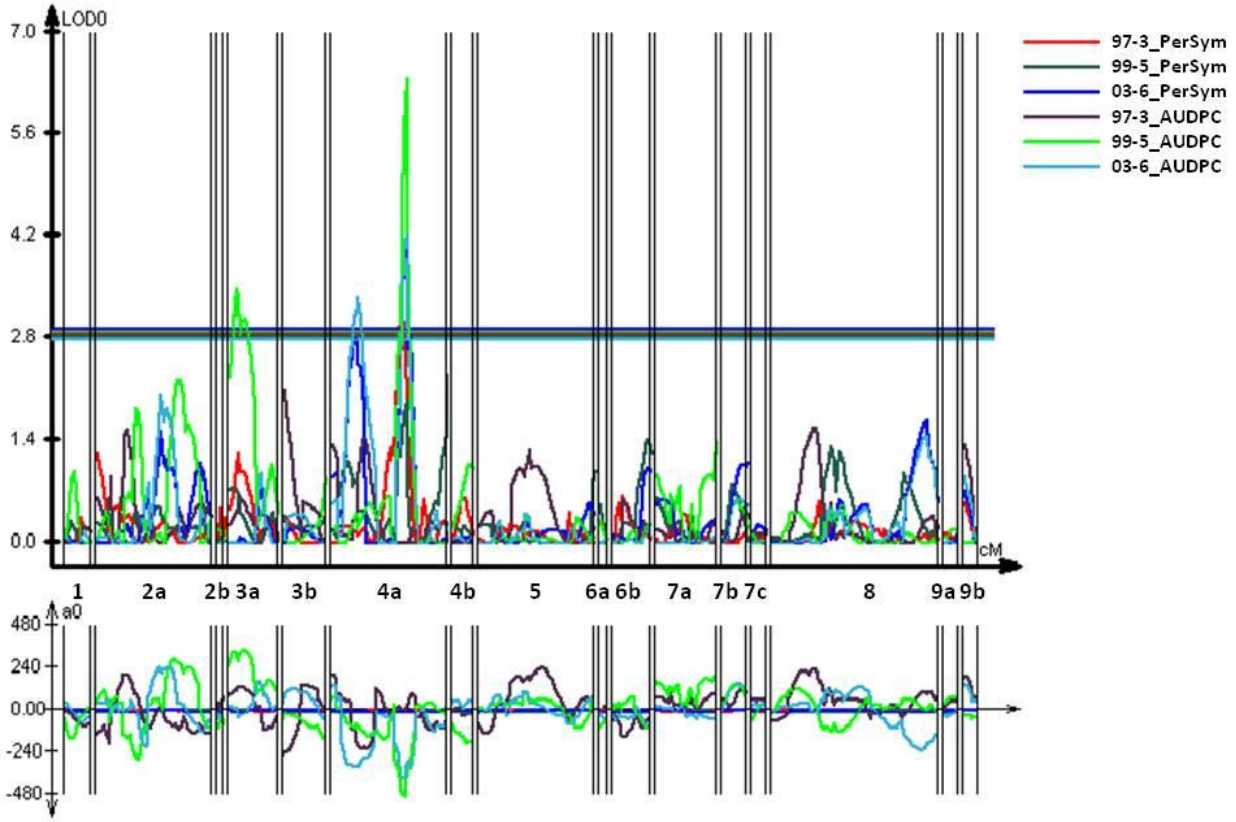


Figure 1. Lettuce genetic map displaying 291 mapped candidate resistance genes and 52 resistance phenotypes. Dotted blue circles indicate resistance phenotypes added over the past year to those reported previously (CLGRB report 2008-2009).

Figure 2. QTL associated with resistance to big vein. PerSy = Percent symptoms. AUDP = Area under the disease progression curve. Measurements were made in three field experiments carried out during 1997, 1999 and 2003 (97, 99 and 03). The upper graph shows the probability (LOD) score (y axis) for each of the nine chromosomes aligned along the x axis. The horizontal bar indicates the significance threshold derived by permutating the data. The lower graph shows the allelic contribution from each parent (Parade above and Pavane below).



In collaboration with Yoichi Kawazu (National Institute of Vegetable and Tea Science, Mie, Japan) we have studied the resistance to big vein in a second population, cv. Thompson x cv. Cisco. We identified QTLs for resistance in this population but their chromosomal locations, on chromosomes 5 and 6 (Fig. 1), were different from those identified in the Parade x Pavane cross. This indicates that Pavane and Thompson may represent different sources of resistance for big vein. The QTLs in both populations require validation by repeated the phenotyping in additional generations. If the different QTLs are confirmed from each cross, there is the possibility of combining these QTLs through marker assisted selection (MAS) to provide higher levels of resistance.

Mapping of Resistance to Downy Mildew

We are continuing to map resistance phenotypes using bulk segregant analysis (CLRB variation report 2008-2009). Additional bulks of resistant and susceptible individuals from two F_2 populations from crosses between novel *L. serriola* donors and recurrent cv. Salinas were genotyped with the Illumina GoldenGate SNP assay. Both resistances mapped to LG4 in the same cluster of resistance phenotypes as *Dm4, Dm7, Dm11* and *Dm44* (Figure 1).

Additional bulks of resistant and susceptible individuals from three F_2 populations resulting from crosses between cv. Salinas as the recurrent parent and several novel *L. serriola* donors have been genotyped using the Illumina GoldenGate SNP assay and markers have been identified that distinguish these bulks. These markers will be analyzed using single-stranded conformational polymorphism (SSCP) analysis in segregating populations to determine their precise genetic positions. Markers tightly linked to the resistance loci will be used as markers for selection in our breeding program.

Functional Analysis of Candidate Resistance Genes

Post-transcriptional gene silencing (PTGS, RNAi) involves expression of double-stranded RNA fragments of target genes leading to the degradation of the mRNA transcribed from the endogenous (target) genes and consequently to the loss of the target gene's activity. RNAi targeted to the LRR-encoding region of *RGC2B* (that encodes the *Dm3* resistance specificity) had previously shown that *Dm14*, *Dm16*, *Dm18* and *Ra* are also members of the *RGC2* family (Wroblewski *et al.*, 2007. *Plant J.* **51**:803-18). We have been generating transgenic plants silenced for other resistance gene candidates (*RGC*s) that co-segregate with resistance phenotypes in order to test the resistance function of each *RGC* (Table 1). These RNAi lines are being used as tester stocks in crosses to resistant lines to identify genes involved in resistance to several diseases.

These experiments have shown that different subsets of resistance specificities may be silenced by different domains of the same RGC2 gene. *Dm16* but not *Dm6* was silenced by a fragment of the LLR domain of *Dm3*; however, the converse was true for RNAi using the NBS domain of *Dm3*. RNAi using a fragment of the NBS domain of *Dm7* also silenced *Dm4* and *Dm11* indicating that all three resistance phenotypes are conferred by the same *RGC* family.

Table 1. Summary of experiments using RNAi to test the resistance conferred by several *RGC*s. Sequences from domains of 25 *RGC* genes that co-segregate with a resistance phenotype have been transformed into Cobham Green. T_1 lines were crossed to multiple resistant cultivars. Silencing is monitored by analyzing activity of the *GUS* reporter gene in *Agrobacterium*-mediated transient assays (a fragment of the *GUS* gene is included in each RNAi gene construct, seeWroblewski *et al.*, 2007) and progeny are tested for loss of phenotype. The protein domain encoded by the gene segment used to generate the RNAi construct is indicated.

Resistance Locus	Targeted domain	# RGCs with RNAi transgenics	# RGCs tested for change in phenotype	Resistance genes silenced
Dm3 cluster	*Dm3* NBS	1	1	*Dm3*, *Dm14*, *Dm16*, *Dm18*, *Ra*
Dm3 cluster	*Dm3* LRR	1	1	*Dm3*, *Dm6*, *Dm18*
Dm5/8 cluster	NBS	7	7	0
Dm5/8 cluster	LRR	7	7	*Dm5/8*, *Dm45*
Dm5/8 cluster	TIR	1	1	0
Dm7 cluster	NBS	1	1	*Dm7*, *Dm4*, *Dm11*, *Dm44*
Dm7 cluster	LRR	1	1	0
Dm7 cluster	TIR	1	0	0
Locus conferring AvrRps4-HR	LRR	3	3	0
ANT3, locus conferring AvrPpiC-HR	NBS	1	1	0
Tvr1/*Fus1*	NBS	1	0	0

Mapping of Candidate Genes Relative to Horticultural Traits

In past years, horticultural traits have been mapped relative to candidate genes in our core $F_{7:8}$ RIL mapping population derived from a cross between *L. sativa* cv. Salinas and *L. serriola* acc. UC96US23. QTL analysis was performed on data from this core mapping population gathered in three separate growing seasons, 2002 to 2004. QTL analysis identified chromosomal regions involved with horticultural traits such as flowering time and leaf shape. One hundred and fourteen candidate genes in lettuce with similarity to 93 genes of known function in Arabidopsis were mapped. To date, 35 of these mapped candidate genes co-locate with QTL at $P<0.05$ and 29 of these genes co-locate to QTL at the more stringent $P<0.01$ (See 2008-2009 report for details).

We have expanded the original core $F_{7:8}$ RIL mapping population to include more than 134 additional RILs for further mapping and higher resolution QTL analysis. In 2009, we trialed the 134 new RIL families in Davis, CA (Fig. 3). Twenty RIL families that had been included in previous trials were included as controls. A broad range of horticultural traits including heading, bolting, branching, flowering, leaf shape, shattering and spines were measured. We are combining the data collected in 2009 with previous data sets from more than 100 additional RIL families. The increased size of the mapping population (over 200 RIL families) will allow the refinement of the QTL positions.

Figure 3. Field trial for 134 additional Salinas x *L. serriola* RIL families, Davis 2009.

We are continuing to combine the chromosomal positions of resistance and horticultural phenotypes with markers and candidate genes into a single integrated map for lettuce. This involves the analysis of multiple populations in collaboration with several researchers (Table 2). We have constructed genetic maps for the Parade x Pavane, Thompson x Cisco, (Salinas 88 x La Brillante) x Salinas 88, Pacific x La Brillante, PI251246 x Salinas, Valmaine x Salinas, Salad Bowl x CGN14263, Iceberg x Saladin, Iceberg x Grand Rapids, Cobham Green x LS238, Cobham Green x LS241 and PI502595 x Bibb populations. QTL analyses for traits segregating in these populations are underway.

We welcome researchers who wish to collaborate in the analysis of existing populations, particularly the core $F_{7:8}$ RIL mapping population derived from *L. sativa* cv. Salinas x *L. serriola* acc. UC96US23 as well as analysis of additional populations, for traits that have not been studied yet.

Table 2. Populations being analyzed for disease resistance and horticultural traits.

Disease & Trait	Population	Collaborators
Downy mildew *Bremia lactucae*	Cobham Green x LS238 Cobham Green x LS241	B. Maisonneuve, INRA , France
	Iceberg x Saladin	P. Hand, HRI, UK
	Iceberg x Grand Rapids	I. Simko, USDA, Salinas
Corky root , *Sphingomonas suberifaciens*	Green Lakes x Diana	
Lettuce Mosaic Virus	Balady Aswan Green x Salinas PI226514 x Salinas 88 PI226514 x Clemente	R. Hayes, USDA, Salinas
X. campestris pv. vitians	Reine des Glaces x Delsay	B. Maisonneuve, INRA.
	Little Gen x Salinas 88 Little Gem x Clemente	R. Hayes, USDA, Salinas
Big Vein	Thompson x Cisco	Y. Kawazu, NIV&TS, Japan
	Parade x Pavane	R. Hayes, USDA, Salinas
Verticillium wilt *Verticillium dahliae*	Pavane x Parade Salinas 88 x La Brillante Pacific x La Brillante	R. Hayes, USDA, Salinas
Lettuce drop *Sclerotinia minor* *Sclerotinia sclerotiorum*	PI261245 x Salinas Little Gen x Salinas 88 Little Gem x Clemente	R. Hayes, USDA, Salinas
Fusarium wilt *Fusarium oxysporum*	Valmaine x Salinas	T. Gordon, UC Davis
Basal rot *Botrytis cinerea*	Salad Bowl x CGN14263	
Lettuce dieback Lettuce necrotic stunt virus	Valmaine x Salinas	I. Simko, USDA, Salinas
Shelf life	Iceberg x Saladin	D. Pink, HRI, UK
	Salinas x UC96US23	G. Taylor, U. Southampton
Rib discoloration	Emperor x El Dorado	S. Jenni, AAFC, Canada
Leaf miner	PI502595 x Bibb	B. Mou, USDA, Salinas
Nutritional content, Vit A, C & E, antioxidant & folate	Diplomat x Margarita Green Towers x Western Red Leaf	D. Still, CSU Pomona

Massively Parallel Genetic Analysis using an Affymetrix Gene Chip

In collaboration with Dr. Allen van Deynze (UC Davis), with support from the UC BioStar program, Enza Zaden, Rijk Zwaan and Vilmorin, we developed a 6.6 million oligonucleotide Affymetrix array for high-throughput, massively parallel genotyping of lettuce. The lettuce chip contains sequences for detecting polymorphisms in approximately 35,000 unigenes in lettuce and facilitates rapid SNP discovery, genotyping, mapping, and gene expression analysis. We completed hybridizations in duplicate to genomic DNA of 213 RILs from our core Salinas x *L. serriola* UC96US23 mapping population. We have developed custom algorithms and scripts in order to process these data. We are now in the process of refining an ultra-dense genetic map based on at least 14,000 transcribed sequences.

Sequencing

We have used 'next-generation' sequencing for several projects. We have sequenced and assembled the transcriptome from both *L. sativa* cv. Salinas and *L. serriola* UC96US23. We now have over 52,000 assemblies representing the majority of the transcribed genes of lettuce as well as SNPs differentiating the parents of the core mapping population. We are also sequencing the gene space (genomic sequence reduced for highly repeated sequences) of cv. Salinas. In addition, we are sequencing several isolates of *Bremia lactucae* and using the sequence information to search for genes that determine the virulence of isolates of downy mildew to different cultivars of lettuce.

The Compositae Database (Compositdb)

We have continued to curate publicly-accessible databases for lettuce. The Compositdb contains several searchable databases for lettuce accessible through http://compositdb.ucdavis.edu/ and is the main portal for distributing information generated by the Compositae Genome Project (CGP). Lettdb provides genetic and DNA sequence information as well as gel images for markers and descriptions and sequences of RFLP probes and oligonucleotide primer sequences for PCR-based markers. Lettcv archives extensive genetic, passport and performance data on lettuce cultivars. The CGP database (http://compgenomics.ucdavis.edu/compositae_index.php) contains extensive sequence and related information as well as links to lettuce linkage maps (lettuce genetic map viewer) and marker information. Morphodb (http://compgenomics.ucdavis.edu/morphodb_index.php) is an archive of and provides access to phenotypic information on *Lactuca* species; this database utilizes standard ontologies to facilitate searches across databases. The database is being revised to facilitate access to marker information for breeding purposes from a disease-centric perspective (Fig. 4).

Figure 4. Entry page being developed to allow data access from a disease perspective.