The Spanish Melon Genomics Initiative

P. Puigdomènech[1], J.A. Martínez-Izquierdo[1], P. Arús[2], J. Garcia-Mas[2], A.J. Monforte[2], F. Nuez[3], B. Picó[3], J. Blanca[3], M. Aranda[4], V. Arnau[5] and A. Robles[6]
[1]Laboratori de Genètica Molecular Vegetal CSIC-IRTA, Departament de Genética Molecular, Barcelona, Spain
[2]Laboratori de Genètica Molecular Vegetal CSIC-IRTA, Departament de Genética Vegetal, Cabrils, Spain
[3]COMAV-UPV, Departamento de Biotecnología, Valencia, Spain
[4]CEBAS-CSIC, Departamento de Biología del Estrés y Patología Vegetal, Murcia, Spain
[5]UV-Departamento de Informática, Valencia, Spain
[6]GAP-UPV, Departamento de Informática de Sistemas y Computadores, Valencia, Spain

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Abstract

Melon is a worldwide extended cucurbit. Spain is the 5th world melon producer and leader in Europe. The melon genome is relatively small and its genetic map has an estimated size of 1,021 cM, which represents approximately 440 kb per cM. These data make it a very attractive species for performing studies at the genomic level. Recently, the "Spanish Genomics and Proteomics Strategic Action Programme" has funded the project "Development of genomic tools in melon for the analysis of resistance to pathogens and fruit quality traits" involving five Spanish laboratories with wide experience in melon research. As a first objective of this project we will sequence 30,000 ESTs from eight cDNA libraries. From these ESTs we will obtain a microarray that will be used for the study of the transcriptome. Another main goal of the project is to increase the current genetic map resolution by mapping 300 new SNPs. We will start a physical map and BAC contigs spanning around 1 Mb will be developed in three regions where clusters of disease resistance genes are located. The establishment of a collection of mutants and a TILLING platform will allow us to undertake studies of reverse genetics. Finally, we will set up a bioinformatics platform. The information obtained will be useful at short or medium term for aspects related to the melon improvement of disease/pest resistance and fruit quality. On the other hand, as synteny levels are expected to be high among Cucurbitaceae species, these results could be applicable to other important cucurbits such as cucumber, watermelon, zucchini or squash. More information about the current status of the project is already available at the web site www.melogen.upv.es

INTRODUCTION

Melon (*Cucumis melo* L.) is a diploid species ($2n=2x=24$) that belongs to the Cucurbitaceae family and is a crop which is widespread throughout temperate, subtropical and tropical climates. It is the most widespread vegetable crop in Spain with regard to surface area and production after tomato, with 38,100 ha and 1,102,400 t in 2004 (FAOSTAT, 2005). Spain is the 5th melon producer worldwide and the leader in Europe.

The melon genome is relatively small. Some studies have estimated its genome size to be 450-500 Mb (Arumuganathan and Earle, 1991), which is similar to that of rice (419 Mb), and three times the size of the model species *Arabidopsis thaliana* (125 Mb). The melon genetic map has an estimated size of 1,021 cM (Oliver et al., 2001; Gonzalo et al., 2005), which represents approximately 440 kb per cM.

Melon's economic importance and the fact that there is little genetic knowledge of it make it a very attractive species for performing studies at a genomic level. Recently, the "National Genomics and Proteomics Strategic Action Programme" is funding the project "Development of genomic tools in melon (*C. melo* L.) for the analysis of resistance to pathogens and fruit quality traits (MELOGEN)" involving five Spanish laboratories with

wide experience in melon research (the Molecular Genetics Department CSIC-IRTA, Barcelona (coordinator); the Plant Genetics Department CSIC-IRTA, Cabrils; the Biotechnology Department COMAV-UPV, Valencia; Stress Biology and Plant Pathology Department CEBAS-CSIC, Murcia; The Informatics Department UV, Valencia; and the Informatics Systems and Computers Department GAP-UPV, Valencia). Some of these research groups have a close relationship with the most important seed companies in Spain, which dedicate important R&D resources to melon breeding.

This project started with a significant knowledge base obtained by the project's various partners: a) a wide collection of Cucurbits germplasm (exotic germplasm, landraces and cultivars) kept in the Genebank of COMAV-UPV and breeding lines obtained by Semillas Fitó S.A. The COMAV is the leader of the Cucurbits Working Group of the European Network of Genetic Resources of the IPGRI. This group coordinates the activities related to genetic resources of cucurbits in Europe (Picó et al., 2002); b) wide experience in the molecular breeding of melons against viruses and fungi by CEBAS-CSIC and COMAV-UPV (Nuez et al., 1999; Díaz et al., 2002; Dias et al., 2002, 2004; Marco et al., 2003; Pico et al., 2005); c) a saturated genetic map constructed by PGD CSIC-IRTA, with transferable markers based on known DNA sequences (RFLPs, SSRs and SNPs) (Oliver et al., 2001; Gonzalo et al., 2005). This map was obtained using a group *Inodorus* 'Piel de Sapo' market class T111 breeding line and the Korean accession PI 161375 as parental lines, and includes some genes involved in important fruit traits (Monforte et al., 2004) and disease resistance (Garcia-Mas et al., 2001); d) several mapping populations obtained by PGD CSIC-IRTA (a population of doubled-haploid lines, DHL, and a collection of near-isogenic lines); e) a BAC library from the DHL population with 23,000 clones with an average size of 141 kb, representing the melon genome six times, and with contigs around disease resistance genes (Morales et al., 2005; van Leeuwen et al., 2003, 2005) (MGD CSIC-IRTA); f) a preliminary small collection of ESTs; g) experience with computer applications for protein analysis and algorithm development for DNA analysis by GAP-UPV and UV (Arnau and Marín, 2003; Arnau et al., 2005).

Additional basic tools and information obtained by other research groups were also available when this project began: several melon genetic maps using parental lines of the cantaloupe type (Wang et al., 1997; Perin et al., 2002b; Silberstein et al., 2003), the development of microsatellite markers (Danin-Poleg et al., 2001; Ritschel et al., 2004), the position of fruit quality traits (Perin et al., 2002a) and disease resistance clusters in the melon genome (Brotman et al., 2002), as well as the map-based cloning of disease resistance genes (Joobeur et al., 2004).

The central objective of the Spanish Melon Genomics Initiative is to develop several basic genomic tools that will facilitate and accelerate genetic analysis in melon. We will also employ these tools to obtain applicable information for aspects related to the improvement of disease/pest resistance and fruit quality.

The analysis of gene function requires the existence of mutant alleles for the genes analysed. Plant breeding also requires genetic variability which is not always naturally available. This fact can be significant for the cucurbit family, a group of species relatively isolated among cultivated crops. For this reason, one of the main goals of this project is to develop and use a mutant collection using the TILLING approach (McCallum et al., 2000). This collection will be used to undertake studies of reverse genetics for several candidate genes.

EST collections represent an important tool in structural and functional genomic projects. These collections allow the construction of microarrays, which are very powerful tools for high-throughput genetic expression studies (Seki et al., 2001). We will sequence 30,000 ESTs from eight cDNA libraries (obtained from fruits in different ripening stages, and from leaves and roots infected with different pathogens). From these ESTs we will construct a microarray with thousands of unigenes that will be used for the study of the transcriptome in different physiological processes.

Saturated genetic maps based on high-quality markers are very useful for map-

based cloning. SNPs are the best marker type for detecting polymorphism and they can be developed from genes with a known function, which gives them an added value. SNPs can also be implemented in DNA chips (Cho et al., 1999), thereby allowing high-throughput genotyping. These properties make SNPs the most promising marker type, and they will be the reference marker type in the near future. In this project we will generate a high-resolution genetic map based primarily on SNPs developed from the set of ESTs previously obtained. With this functional genetic map we will start a physical map by anchoring BAC clones with markers present in the genetic map. BAC contigs will be developed in regions where clusters of disease resistance genes are located.

Finally, we will develop bioinformatics analysis tools to process the large amount of data that will be generated in the project. All this data will be included in a database. More information about the current status of the project is available on the project's web site: www.melogen.upv.es.

The tools developed as a result of the initiative may also be employed by other research groups working with melon. In addition, as synteny levels are expected to be high among Cucurbitaceae species (van Leeuwen et al., 2003; Park et al., 2005), these results might also be applied to other important cucurbits such as cucumber, watermelon, zucchini or squash.

CURRENT STATUS OF THE GENOMIC TOOLS DEVELOPED BY THE SPANISH MELON GENOMICS INITIATIVE

Collection of Phenotypic Variants

The establishment of the mutant collection and the TILLING platform has been initiated. Twenty thousand melon seeds of the 'Piel de Sapo' M62-113 line have been mutagenized. Different doses of Ethylmethanesulfonate (EMS) (from 0.5% to 1.5%) have been assayed to find the optimal concentration of mutagen in order to saturate the genome with mutations. M1 plants are now being selfed to obtain a collection of 5,000 M2 mutant families. DNA bulks from these families will be prepared and used to look for allelic variation. The COMAV genebank will host this mutant collection which may become a very important tool not only in this project, but also for the entire scientific community working on melon.

cDNA Libraries, ESTs Collection, and Microarrays

Eight normalized cDNA libraries were obtained using polyadenilated RNAs extracted from leaves, roots and fruits in different conditions:

- Fruits of 'Piel de Sapo', both immature (15 days after pollination) and mature (45 days after pollination).
- Leaves of 'Piel de Sapo', both healthy and infected with *Cucumber mosaic virus* (CMV).
- Healthy roots as well as roots infected with the fungus causing melon collapse, *Monosporascus cannonballus*, from a susceptible genotype ('Piel de Sapo'), and a resistant genotype (subsp. *agrestis*).

EST sequencing from these eight cDNA libraries has been initiated. As of this communication 14,271 ESTs have been sequenced and analysed using the bioinformatics tools developed for this project (9,519 unigenes have been obtained from these ESTs).

The construction of a melon microarray will be initiated after finishing the bioinformatic analysis of the 30,000 ESTs sequenced during this project. The unigenes included in the microarray will represent the three tissues, in different biological conditions, which were used for the construction of the cDNA libraries. We will PCR amplify the 4,000 unigenes with universal primers. These cDNAs will be delivered to the Transcriptome Service of "Serveis Científico-Tècnics" of Barcelona University, in order to be printed on a Genetix Qarray microarray. This microarray will also be available for the scientific community working on melon to undertake transcriptomic studies in other physiological processes.

Genetic and Physical Maps

The last version of the melon genetic map included 327 transferable markers (226 RFLPs, 97 SSRs and three SNPs) which provided a map density of 3.11 cM/marker (Gonzalo et al., 2005). This density is in the process of being increased by means of mapping 300 new SNPs. The preliminary search for SNPs in melon ESTs, performed by PGD CSIC-IRTA, indicates that the average frequency of SNPs between the parents of the map ('Piel de Sapo' and PI 161375) was one every 441 bp. Seventy-five percent of the polymorphisms were located in introns and the 3' untranslated regions (Morales et al., 2004). To date, 50 new SNPs have been discovered from the preliminary collection of ESTs available at the beginning of the project. These are being mapped using CAPS or 'Single primer extension' (SNaPshot) technologies with the DHL population of the melon genetic map. The new ESTs sequenced during the project will be selected according to their homology to genes involved in biotic stress, abiotic stress, fruit development, nutritional content (sugar, vitamin, carotenoids) and degradation of the fruit cell wall, and will be used to generate 250 new SNPs.

A functional genetic map with 500 markers will be established by mapping the 300 new SNPs and 200 RFLPs obtained from cDNAs that have been sequenced. We will begin the physical mapping with the search for positive BACs for the 500 markers of the functional map.

Bioinformatics Platform

We are constructing a bioinformatics platform that will group and order both the information generated in the project as well as existing data on melon and cucurbits. The database will be accessible through the web site melogen.upv.es. By the end of the project, it will include the EST analysis, the expression profiles obtained with the microarrays and the genetic and physical map data.

We have developed an EST pipe perl script that automates the preprocessing, the clustering and the annotation of the EST data. This script stores all the relevant results in a relational database that also holds information about the genetic maps and markers. All this data is accessible through a web site that is also linked to this database.

We are using a cluster server in which different mapping process strategies are being tested to improve the application performance by efficiently using the available server hardware resources. We also are creating our own software when needed and we are working on the parallelization on the software that is being used in the project.

GENOMIC ACTIVITIES PERFORMED BY THE SPANISH MELON GENOMICS INITIATIVE

The tools to be developed will be used in this project for aspects related to the improvement of melon for disease/pest resistance and fruit quality.

Identification of Mutants for Selected Genes by SNP Analysis with the TILLING Platform

We will confirm the functionality of the TILLING platform by searching for mutants in four selected genes: a) elF(iso)4E, the loss of function of which may yield plants resistant to potyvirus; b) Phytoene Desaturase (PDS), where a knock-out will cause the suppression of carotene biosynthesis, producing plants susceptible to photobleaching; c) two genes involved in fruit size, but without a priori detailed knowledge about the effect of the mutations, fw2.2 and the 3-hydroxy-3-methylglutaryl-coenzime A reductase (HMG-CoA). Shortly, to search for mutants in these genes we will design specific primers to amplify regions where it is most likely that mutations will occur involving a change in the amino acid and a consequent effect in the protein. Heteroduplex originating in the bulks which include mutant alleles will be identified by digestion with endonuclease Cel I which cuts single base pair mismatching. Several individuals of each selected M2 family will be screened with Cel I to identify plants carrying the mutation. The mutation will be validated by sequencing, and mutant plants will be selfed to perform phenotypic studies.

Transcriptome Analysis

The melon microarray will be used to study the transcriptome in three particular cases, namely: nearly-ripe fruit (45 DAP) of 'Piel de Sapo' versus unripe fruit (15 DAP); *Monosporascus cannonballus* inoculated roots using susceptible ('Piel de Sapo') and resistant (*agrestis* subspecies) melon genotypes; and CMV, *Melon necrotic spot virus* (MNSV), *Watermelon mosaic virus* (WMV), and *Cucurbit yellow stunting disorder virus* (CYSDV) inoculated leaves versus non-inoculated ones, or viral inoculated leaves versus systemic leaves from the same infected plant. In the latter case, several viral strains and susceptible and resistant melon genotypes will also be used. Diverse poly(A) RNA preparations will be obtained from the different tissues and will be used by "Serveis Científico-Tècnics" of Barcelona University to prepare the probes for the microarray experiments. Data from the microarray experiments will be obtained by reading the hybridization reactions by means of an Array Scanner and will be analysed using the bioinformatics tools developed in the project.

Fine Mapping and Cloning of Disease Resistance Genes

Current melon breeding is mainly focused on the incorporation of disease resistance into commercial germplasm and on the study of fruit quality traits. Some of the partners on this project as well as other research groups are involved in mapping melon genes and QTLs as the first step in isolating them (Dias et al., 2004; Joobeur et al., 2004; Monforte et al., 2004; Brotman et al., 2005; Morales et al., 2005; Perchepied et al., 2005).

In melon, several regions that contain known disease resistance genes and homologous sequences have been detected (Garcia-Mas et al., 2001; Brotman et al., 2002, 2005; van Leeuwen et al., 2003): a) region of the genes *Vat* (aphid resistance) and *Pm-w* (powdery mildew resistance) and where the resistance gene homologue MRGH63 is located, in linkage group 4; b) region of linkage group 7, where the genes *Fom-1* (resistance to *Fusarium*), *Prv* (resistance to *Papaya ringspot virus*, PRPV), and the resistance gene homologue MRGH21 are located; c) region of the *nsv* gene that confers resistance to MNSV in linkage group 11.

The genetic and physical maps to be developed will be used during this project to characterize these regions of the genome. The BAC library will be screened with the 500 markers (RFLPs and SNPs) from the functional map. We will construct contigs starting with the positive BACs for the resistance gene homologues in the three regions described previously. BAC contigs will be constructed using chromosome walking technology using the BAC ends that will be sequenced. Our estimations are that 15 BAC clones of average insert size (140 kb) will be enough to cover a region of 1 Mb for each of the three contigs. We estimate that the physical map will cover around 25% of the melon genome.

At the moment, the group has accomplished the physical mapping of the gene that confers resistance to MNSV (Morales et al., 2005), laying the foundations for its characterization.

Fine mapping of interesting regions and/or the availability of a physical map will also be of interest for performing synteny studies in cucurbits.

STARTING A WORLDWIDE GENOMICS INITIATIVE ON CUCURBITS

Last June, the Spanish Melon Genomics Project (MELOGEN) organized a Workshop in Barcelona (PGD IRTA-CSIC) in order to discuss the possibility of setting up a worldwide genomics initiative on cucurbits. Following other successful examples, such as those undertaken by the Solanaceae or Legume communities, it would be of great interest to organize an international network based on melon genomics and extended to other important cucurbits such as cucumber, watermelon and squash, Spain also being the leader in production in Europe.

Public researchers from different countries and private seed companies were invited with the idea of putting together all of the different research interests in this group of species, covering areas such as genetics, genomics, disease resistance, biochemistry, germplasm, breeding, etc. The National Genome Programs in Cucurbits were presented

(Spain, Israel, USA, France and Japan), and many groups from these and other countries (The Netherlands, Poland) presented their genomic activities in cucurbits and other research topics with the species of this family. Private Companies (Clause Tézier, De Ruiter Seeds, Enza Zaden, Keygene, Nunhems, Rijkzwaan, Sakata, Semillas Fitó, Seminis, Syngenta, Takii, Western Seed, Zeta Seeds) participated in a round table showing their elevated interest in cucurbit genomic research. The high number of attendants and the diversity of the topics presented reflect the interest in the creation of an International Cucurbit Genomic Initiative.

This workshop was an excellent opportunity for advancement in cucurbit genomics. A steering committee was established. It was preliminarily composed of the representatives of each of the main countries involved in genome programs in cucurbits (Pere Puigdomenech, CSIC-IRTA, Spain; Jordi Garcia-Mas, CSIC-IRTA, Spain; Nurit Katzir, ARO, Israel; Jack Staub, University of Wisconsin, USA; Michel Pitrat, INRA Avignon, France; and Hiroshi Ezura, University of Tsukuba, Japan). This steering committee will be extended to include representatives from other interested countries, and both public and private interests will be considered. One of the first actions to be implemented by the steering committee will be the creation of an international Cucurbitaceae database which will include the main information produced by the different national genome projects.

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