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Chromosome 10 in the tomato plant carries clusters of genes responsible for field resistance/defence to *Phytophthora infestans*

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ABSTRACT

The main objective of the present study was to reanalyse tomato expression data that was previously submitted to the Tomato Expression Database to dissect the resistance/defence genomic and metabolic responses of tomato to *Phytophthora infestans* under field conditions. Overrepresented gene sets belonging to chromosome 10 were identified using the Gene Set Enrichment Analysis, and we found that these genes tend to be located towards the end of the chromosome 10. An analysis of syntenic regions between *Arabidopsis thaliana* chromosomes and the tomato chromosome 10 allowed us to identify conserved regions in the two genomes. In addition to allowing for the identification of tomato candidate genes participating in resistance/defence in the field, this approach allowed us to investigate the relationships of the candidate genes with chromosomal position and participation in metabolic functions, thus offering more insight into the phenomena occurring during the infection process.

1. Introduction

Tomato late blight (LB) caused by *Phytophthora infestans* is one of the most damaging diseases of tomatoes and constitutes a significant amount of research area worldwide. Very early, it became evident that there were differences between tomato and potato isolates of *P. infestans* and that host specialisation was occurring within the pathogen population [1–5]. The mechanisms necessary to achieve this specialisation remain unknown [6–8]. Currently, three dominant clonal lineages of *P. infestans* in North America (US-7, US-11 and US-17) are tomato-specialised [7,9,10]. The tomato-specialised isolates are more damaging pathogens on tomatoes because they reproduce more rapidly and out-compete non-specialised isolates in field epidemics [11].

Tomato-specialisation evolved in the field, and the increased pathogenicity and extended biotrophy characteristic of these isolates can be observed in the field as well as in the laboratory [12]. Unfortunately, there is sometimes a discrepancy between results reported from growth chamber studies and the phenotype observed in the field. For example, transgenic plants expressing osmotin or other pathogenesis-related (PR) genes were reportedly resistant to *P. infestans* in growth chamber tests [13]. However, when these plants were evaluated in field tests, no such resistance was detectable. The inability to detect some host responses in the field is not due to high variance in the field because typically, differences in resistance among potato clones are more reliably detected in carefully controlled field tests than in growth chamber tests [14]. Instead, it seems likely that some of the responses that are observed in the growth chamber and not in the field might be artifactual compared to the field situation. Research on tomato resistance to LB has lead to the identification of *ph-1*, *ph-2* and *ph-3* as important race-specific resistance genes; they are located in chromosomes 7, 10 and 9, respectively. Among them *ph-3* is the strongest and has already been introduced into breeding lines of commercial tomatoes [15–17]. However, their genomic and transcribed sequences remain unknown. In addition to those genes, some QTLs have been reported to confer race-nonspecific resistance to LB in tomato [16]. Despite of that, there is still a lack of knowledge on the genetics and genomics of LB resistance in tomato.

Therefore, our main objective was to combine recent and traditional statistical methods to better describe the transcriptional activities of tomato defence genes, which are important during the response of the plant to *P. infestans* under field conditions. We also aimed to discern trends among the expression data. Different statistical methods for the analysis of microarray data have been proposed and carried out to reveal differentially expressed genes between treatments (e.g., between inoculated and not inoculated plants). Most of these approaches are based on *t*-tests and their associated p-values. However, determining significant expression changes above random signals remains difficult. Currently, many statistics are based on the concept of false discovery rate (FDR) which avoids a specified percentage of false discoveries and offers a criterion to support significant differences [18]. Gene expression differences between inoculated and not inoculated plants were determined using the most widely used statistical method to detect differential gene expression: Significance Analysis of Microarray

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(SAM) [19]. Furthermore, overrepresented gene sets were searched among differentially expressed genes. Chromosomal regions that are associated with the overexpressed gene sets were identified using Gene Set Enrichment Analysis (GSEA) [20]. GSEA focuses on gene sets that share a common feature (e.g., biological function, chromosome location, or regulation) [20]. Here, only chromosomes were identified as overrepresented gene sets. In addition to allowing for the identification of important genes participating in resistance using a robust statistical method, this approach allowed us to investigate their relationships with their chromosomal location to gain a global insight into the genomic phenomena occurring during the infection process and the relationship of gene sets implicated in resistance to other plant species, which had not been reported until now.

2. Results

2.1. Field response of tomato plants to P. infestans leads to the upregulation of pathogenesis-related (PR) and other defence-related genes

The microarray data were downloaded from the Tomato Expression Database (http://ted.bti.cornell.edu/), experiment E022. Applying SAM to tomato microarray data, comparing *P. infestans* inoculated and not-inoculated IL 6–2 tomato plants showed that differences between the mean expression in both conditions remained constant over time (Additional file 1). We were able to detect a subset of known infection markers, with an FDR of 0.03, after 36 h of inoculation. Thus, this FDR value was chosen as a threshold for all subsequent statistical tests (for the complete list of infection markers see Additional file 2).

We only identified upregulated genes across the sampled time points that passed with the selected FDR threshold. We also observed a greater number of differentially expressed genes at 36 h compared to 60 h. Among the induced genes, we found WRKY transcription factors, the Pti genes *Pti4* and *Pti5* and PR genes, *PR1*, *PR5* and *PR10* and *1-aminocyclopropane-1-carboxylate synthase*. We detected a late induction (60 h) of divinyl ether synthase genes, which was previously reported for a partially compatible interaction of tomato [12]. This finding, based on our experiments under controlled conditions, suggests that in the field, divinyl ethers are most likely involved in tomato defence against *P. infestans*, but at late stages of the interaction.

2.2. Tomato chromosome 10 constitutes a response beacon to P. infestans infection in the field

GSEA was conducted for all time points comparing inoculated and non-inoculated plants. Two types of gene sets were used: metabolic pathways (Additional file 3) and chromosome assignment (Additional file 1); out of this we found overrepresented genes only for chromosomes. We also found that among upregulated genes, those belonging to chromosome 10 were overrepresented (FDR 0.013).

2.3. Upregulated genes are not randomly distributed along chromosome 10

We performed a Wilcoxon signed rank test to evaluate whether upregulated genes, at each time point, were randomly distributed along chromosome 10. At time points 0, 12 and 36 the test indicated that the upregulated ones, belonging to this chromosome were randomly located on the chromosome. However, at time point 60 h, the distribution of these genes showed enough evidence to reject the null hypothesis of random distribution in chromosome 10 (p-value = 0.0023) indicating that a cluster of genes activated during infection could exist on chromosome 10. There was no evidence to reject the hypothesis of random distribution of upregulated genes in other chromosomes (data not shown).

2.4. Pathogenesis-related genes are co-located at the chromosome level between tomato an Arabidopsis thaliana

Given that induced genes in chromosome 10 were arranged in a non-random manner, we were interested in evaluating whether this was a conserved characteristic among some angiosperms, i.e., if pathogen-response genes are clustered in their genomes and their location. To test this idea we performed a synteny analysis between tomato chromosome 10 and all *A. thaliana* chromosomes. Comparative genomics studies between *Solanum lycopersicum* and *A. thaliana* have shown that a high number of orthologous genes (44.5%) and reorganisation events are shared by these two species [21]. We identified that most of the shared regions between these two species, Multiple Unique Matches (MUMs), are located towards the chromosome ends; the number of MUMs along the chromosomes of either tomato or Arabidopsis closely mirrors the gene density in each species. A relative large amount of MUMs was present in all of the *A. thaliana* chromosomes (Fig. 1), particularly on chromosomes 2 and 3, this holds true even when taking into account gene content in each chromosome. These chromosomes showed more and longer MUMs than the other comparisons. These alignments also showed that the shared regions were located at the end of the chromosome 10 above the region of 60 Mbp. Across the shared regions described by MUM's were found again genes involved in pathogen responses (e.g., protein lectine kinases and D-phospolypases). Following a gene ontology over-representation analysis, we found that terms such as "abiotic stress response", "multicellular organismal development", "response to endogenous stimulus" and "kinase and transcription factor activity", among others, were overrepresented (Figs. 2A and B). A microsynteny analysis revealed that the number of micro-syntenic regions is reduced when the spacer length increases (Additional file 4). To be conservative, a distance of less than 100 nt between two MUM's was defined as a micro-syntenic region, thus a total of 1192 regions were detected (Additional file 4). The majority of these regions were located at the end of the chromosome, in agreement with our previous analysis. The list of genes identified in regions longer than 1000 nt is available in Additional file 5.

From the protein–protein analysis, some proteins could be annotated in the shared regions in chromosomes 2 and 3 in *A. thaliana*. The analysis showed an interesting group of proteins related with host defence: i) syntaxin SYP122 a salicylate (SA)-associated defence protein [35] ii) CZF1, a transcriptional factor involved in defence to chitin and stress; iii) S6K2 a serine/threonine protein kinase and RAPTOR1 a target of rifampicin (TOR) regulation protein, both linked to the control of many cellular functions related with stress defence [37]; iv) LOH2, a protein mainly related with the sphingolipid metabolism, target of the AAL fungal-toxin that triggers programmed cell death [38]. Glycosyl transferases, protein kinases (calcium dependent), transporters (ammonium and membrane associated) and ATPase family protein were also included among the proteins identified in the shared regions.

3. Discussion

Molecular interactions between *P.* infestans and its hosts are complex, and the holistic study of them has traditionally been impaired by technology and statistical analysis methods. Thus, we combined different sets of data (gene expression and additional genomic data) and a robust and versatile array of statistical methods to give a comprehensive understanding at the whole genome level of the tomato — *P. infestans* interaction under field conditions. We discovered that there is an important level of synteny of pathogenesis-related genes between tomato and the distant model species *A. thaliana,* and that a set of genes that are upregulated in the tomato transcriptome after the infection with the oomycete are clustered in chromosome 10. Moreover, the results of the Wilcoxon test allowed us to determine that genes upregulated during infection, after 60 h of inoculation are not randomly distributed on the tomato chromosomes but aggregated in this chromosome. This

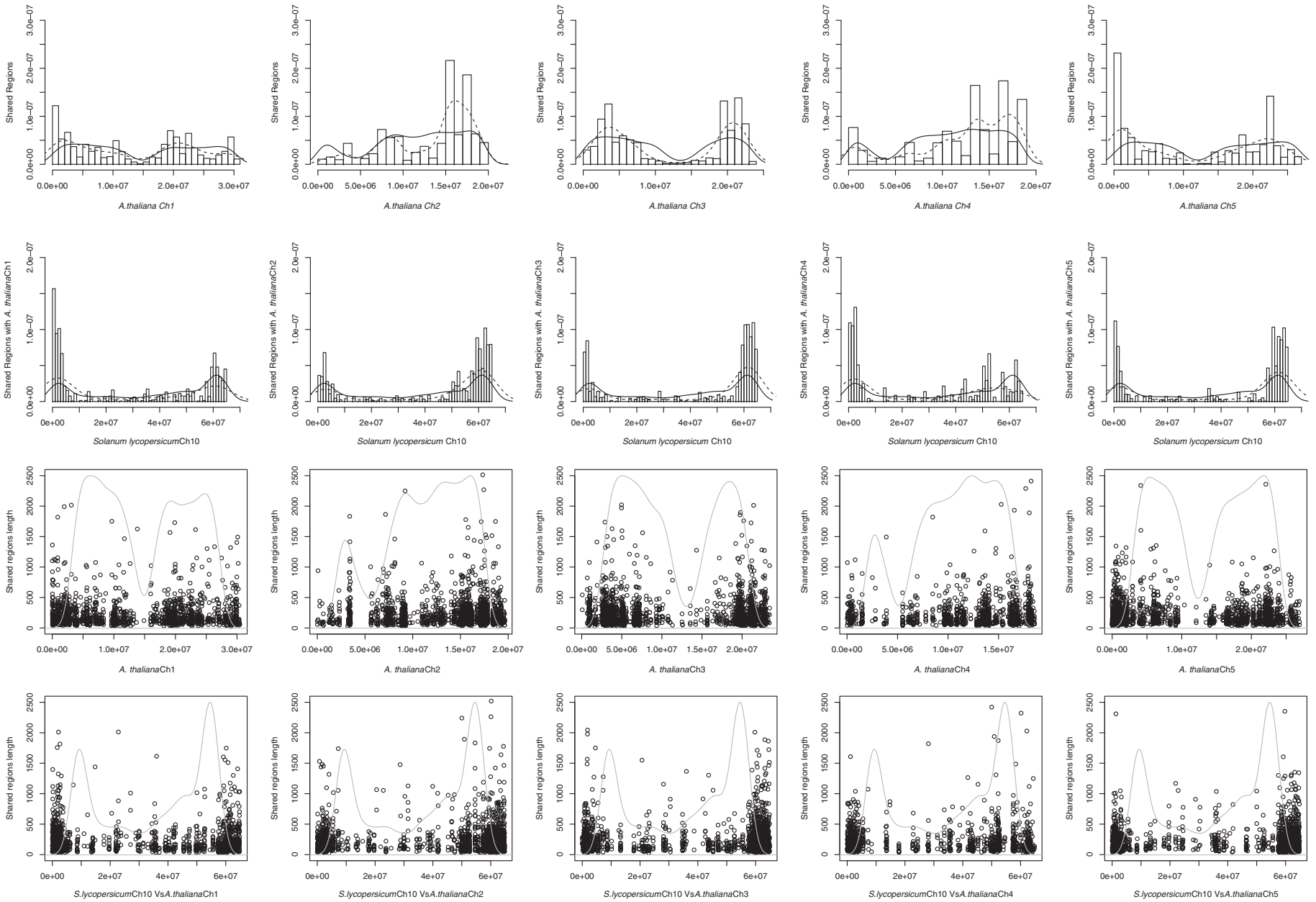

Fig. 1. Synteny paired analysis across *A. thaliana* chromosomes (columns) and *S. lycopersicum* Chr10. Histograms (first and second rows) and dot plots (third and fourth rows) show the number of collinear region and the length (pb), respectively according to the chromosome length. On the histograms, we added an accessory density graph to present the distribution of the shared regions, dashed line. The continuous line in the graphics represents the gene density in the same windows. It is clear that the density of MUMs along the chromosomes closely mirrors gene density.

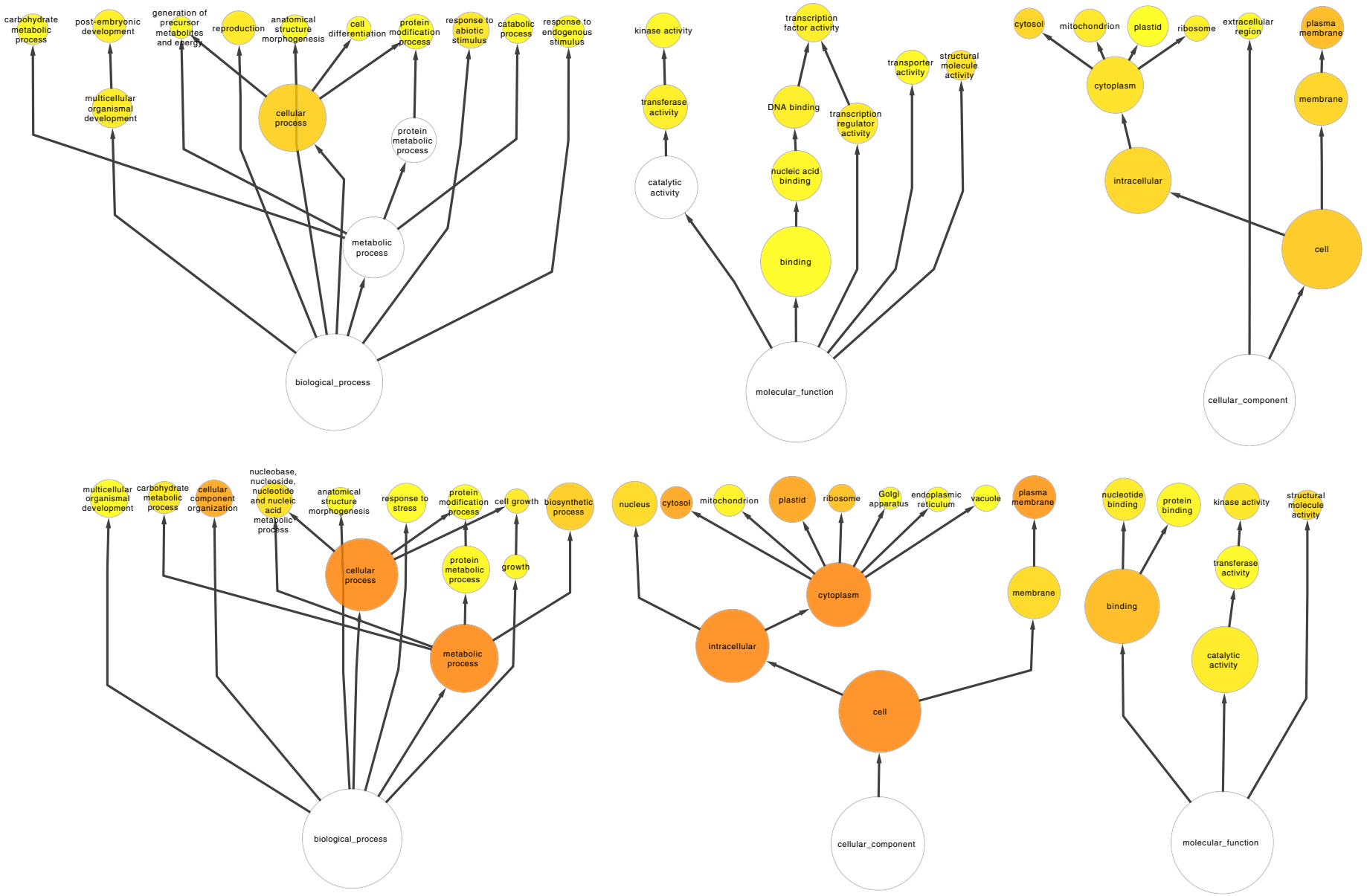

Fig. 2. Gene ontology hierarchy category assignment over the shared regions between *S. lycopersicum* Chr10 and *A. thaliana* chromosomes 2 and 3. Full alignment between tomato chromosome 10 and *A. thaliana chromosomes 2* (A) and 3 (B) using BiNGO. Dark orange categories are the most significantly overrepresented. White nodes are not significantly overrepresented, and the area of the node is proportional to the number of genes annotated to the corresponding GO category.

finding suggests the existence of resistance islands in this genomic region. It was interesting to find by the GSEA analysis that chromosome 10 genes were co-upregulated, whereas some genes, such as those from chromosome 6 were not involved as a group in the response to *P. infestans*. This finding is relevant given that the introgression line used for microarray analyses, IL6-2 belongs to a series of lines that have a segment of *Lycopersicon pennellii* chromosome 6 introgressed into an *Lycopersicon esculentum* (M82) *P. infestans* susceptible background [22]. The differentially expressed genes observed in both chromosomes suggest that there is a correlation of expression between key defence genes in chromosome 6 (DCP-1 like decapping protein, EDS1, monoxygenase and transcription factors ethylene-responsive, WRKY and b-zip like) and defence-conserved clusters of genes in chromosome 10. A thorough examination of the genes exhibiting differential expression in chromosomes 6 also indicates a correlation between the genes from both chromosomes to activate defence. The chromosome 6 genes that exhibit altered expression upon pathogen infection were shown previously to encode key regulators/components of basal- and race-specific disease resistances [23–25]. Additionally, these genes can regulate each other, as it has been shown with monoxygenases that positively regulate the EDS-1 pathway [25].

Previous studies report a correlation between the genomic regions on chromosomes 6 and 10 and pathogen resistance in tomatoes [26,27]. Danesh et al. published findings that were based on studies conducted in plants that were inoculated through the roots with *Pseudomonas solanacearum* [26]. Analyses on F2 progenies confirmed that partial resistance loci could be mapped on these two chromosomes [26]. Several other studies confirmed the relevance of genes in these two chromosomes to defence processes. Grube and collaborators reported on the Ph-2 gene in chromosome 10, a gene conferring race resistance to *P. infestans* in tomato [27]. Smart et al. detected a quantitative trait locus (QTL) on chromosome 6 of the desert tomato (*L. pennellii*) [28]. This QTL accounted for 25% of the phenotypic variance in the population related to resistance to *P. infestans*. QTLs have also been detected for early resistance to *P. infestans* in tomato genome in chromosomes 3, 4, 5, 8, 10 and 11 (reviewed in Foolad [29]), which is in agreement with our results.

Evidence has been reported that chromosome 10 in the tomato has arisen from chromosomal rearrangements, or more precisely that it has suffered a parametric inversion after divergence from the potato [30,31]. Moreover, some studies showed that divergence of genes on chromosome 10 is greater between the two species, than that observed in genes on collinear chromosomes [30]. These findings and the fact that we found an enrichment of differentially expressed (upregulated) genes on chromosome 10 due to *P. infestans* inoculation could indicate that resistance mechanisms specific to tomato have been developed on this chromosome after speciation, although previous research has shown that chromosomal location of resistance genes could be broadly conserved during speciation [27].

Divinyl ether synthase genes have been shown to accumulate on potato leaves infected with *P. infestans,* where they inhibit the mycelial growth of the pathogen [32]. It was found that the ability of a potato cultivar to accumulate divinyl ethers during infection correlates with higher plant resistance to infection. Based on these early findings our results suggest that in the tomato at early stages of infection, suppression of divinyl ether genes could facilitate disease establishment and that their late expression could be related to cell death as suggested by Weber et al. [32].

4. Conclusions

Several genes were found to be upregulated during infection with *P. infestans*. Most of the genes appeared at 36 h after inoculation in the field. Additionally, a set of genes in chromosome 10 acts in concert at late stages of the infection (60 h).

Regarding specific resistance genes, our results suggest that in the tomato at early stages of infection, a suppression of divinyl ether genes occurs and could facilitate disease establishment. In late stages of infection, the expression of divinyl ether genes could be related to cell death.

5. Material and methods

5.1. Microarray data

The data sets were obtained from the Tomato Expression Database website (http://ted.bti.cornell.edu/), experiment E022. In this study we only used experiments that were carried out using the TOM1 DNA chip (available at http://ted.bti.cornell.edu/cgi-bin/TFGD/miame/home.cgi). The accession numbers and description of the experiments used are available in the aforementioned database. For the differential expression analysis, we focused on the experiments where gene expression profiling of tomato infection by *P. infestans* in the field was studied. The tomato line used in the experiment is IL6-2, that belongs to a series of introgression lines that have a segment of *L. pennellii* chromosome 6 introgressed into an *L. esculentum* (M82) *P. infestans* susceptible background [22]. The goal of that experiment was to gain insight into the molecular basis of the compatible interaction between *P. infestans* and its hosts (with a major emphasis on the role of gene suppression). For this comparison, four time points were available at 0, 12, 36 and 60 h with 8 replicates of each condition (32 experiments).

5.2. Calibration of microarray data

As intensities are not directly comparable between different chips, data were calibrated by the method proposed by Huber et al. [33] by means of the vsn R Bioconductor package [34] that is available from their website, http://www.bioconductor.org/. The Huber's method performs more than simply calibrating the microarray data based on the mean, but it allows for the stabilisation of variance. The variances of the transformed intensities become nearly independent of their expected values [33].

The transformation is similar to the logarithm in the high intensity range, but does not affect differences between conditions at low intensities values, as the logarithm transformation does [33]. The transformation and calibration are performed as follows: $h_x = arsinh\left(\frac{x}{s}\right)$, where x is the measured intensity and s is the standard deviation of intensities in the replicates of each experiment.

5.3. Differential expression between inoculated and non inoculated plants

For the detection of differential expression, a modified *t*-test proposed by Tusher and coworkers [19] was used. The Significance Analysis of Microarrays (SAM) is used to detect differentially expressed genes by assigning a score (analogue to the t statistic, called d_i) to each gene based on the change in the mean expression relative to the standard deviation of repeated measures of each condition. To determine if this score is greater than a given threshold, the probability (p-value) is calculated based on permutations of the repeated measures to generate a probability distribution. Furthermore, the percentage of genes that could have passed this threshold simply by chance is also calculated and reported as the false discovery rate (FDR) [19], providing a double filter for false positives. Moreover, we tuned the FDR threshold in this study based on biological knowledge as explained in the following section.

5.4. Definition of a threshold for differential gene expression detection based on infection markers

As the $FDR = FP/_{(FP+FN)}$, where FP are false positives and FN are false negatives, indicates the percentage of false positives (i.e., genes

that are declared as differentially expressed but are, in fact, not differentially expressed); this value should be as low as possible. Values of FDR below 10% (0.1) are desirable. It is important to note that a more stringent FDR value will yield fewer differentially expressed genes to evaluate. To determine a good trade off between reliability and the number of differentially expressed genes, we identified the highest FDR (but below 10%) for all genes known to be infection markers on the tomato, based on a literature search and direct string comparison of these markers against the list of identified genes in previous steps. This approach for selecting the FDR threshold is based both on a statistical criteria (p-value<0,05 and FDR<0,1) and a biological criteria (differential expression of infection markers) and assures therefore a very low rate of false positives (less than 3%) in tradeoff with the detection of shifts in gene expression of known infection markers.

5.5. GSEA

Gene Set Enrichment Analysis (GSEA) is based on the detection of groups of genes that show differential enrichment between two or more conditions [20]. The method orders genes based on their differential expression and searches clustering of genes on top (upregulated) or bottom (downregulated) of the list. If genes belonging to one set are randomly distributed, no enrichment of the set among differentially expressed genes is declared. The detection of gene sets that are enriched or depleted is performed by calculating an enrichment score for each set that is related to the grouping of genes at one of the ends of the list [20]. The additional information obtained by this method is the differential expression of groups of genes, which is more informative than the differential expression of one gene alone.

GSEA was conducted for both conditions (inoculated vs. not-inoculated) by pooling all time points for two types of gene sets: chromosomes and metabolic pathways. These gene sets were generated as explained in the following section.

5.6. Construction of gene sets

5.6.1. Genomic information about genes in TOM1

Microarray target protein sequences were downloaded from NCBI protein sequence database. Protein sequences were mapped using BLAST [35] and BLAT [36], to the current tomato genome sequence available at http://solgenomics.net/genomes/Solanum_lycopersicum/genome_data.pl, build 2.30. The best hit either via BLAST or BLAT was taken to assign each protein to a single genome locus.

5.6.2. Functional annotation of genes in TOM1

GO categories and metabolic pathway assignments were identified to target proteins in TOM1. For GO category designation, we used an indirect approach that involved the identification of protein domains using Pfam [37] and the pfam2GO mapping file available from Gene Ontology (The Gene Ontology Consortium, 2000). Protein domains were identified using HMMER3.0 [38] and Pfam v24.0. Only hits with a score higher or equal to the gathering threshold available for each Pfam HMM were considered significant.

In-house scripts were used to assign each gene in the data set to a given metabolic pathway. Genes acting as enzymes were identified from original data set and then mapped against KEGG functional categories and pathways, release 58.0.

5.7. Wilcoxon Signed Rank Test for random organisation of differentially expressed genes on chromosomes

For chromosomes that were determined to be enriched in the *P. infestans* inoculated phenotype by GSEA, the question as to whether the upregulated genes on these chromosomes were randomly distributed on the chromosome was addressed by a Wilcoxon Signed Rank Test. The test was conducted by i) assigning a rank according to their position on the chromosome to all *n* upregulated genes, ii) creating *n* random ranks and iii) comparing both rank sets under the null hypothesis that random ranks are equal to observed ranks.

5.7.1. Synteny analysis

To identify the physical localisation of homologous regions between *S. lycopersicum* and *A. thaliana* a synteny analysis was performed. For this we used the chromosome 10 from the former, downloaded from Solgenomics network (http://solgenomics.net/), and compared it against each chromosome of *A. thaliana* using MAUVE with default parameters [39]. The identification of shared regions in *A. thaliana* was performed using the protein table file downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/). The Gene Ontology statistical overrepresentation was performed using BiNGO. Protein–protein interactions were loaded from STRING database [40]. In order to identify micro-syntenic regions between homologous sites in chromosome 10 of *S. lycopersicum* and chromosome 3 in *A. thaliana* based on MUM's, a saturation curve was constructed with different spacer lengths between MUM's. Genes in regions longer that 1000 nt were identified using the gene feature file (GFF) made available by the genome sequencing projects.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2013.02.001.

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