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RESEARCH ARTICLE

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Transcriptome profiling of lentil in response to *Ascochyta lentis* infection

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Abstract

Aim of study: The purpose was to identify some general and genotypic-specific defense responses, in order to obtain a set of candidate genes presumably involved in the resistance.

Area of study: The experiment was carried out in León, Spain.

Material and methods: We have analyzed the response of three lentil genotypes to *Ascochyta lentis* (isolate AL 84) at transcriptomic level using the Massive Analysis of cDNA Ends (MACE) technique: the susceptible cultivar 'Lupa', the moderately resistant 'ILL5588' and the resistant wild accession 'BG 16880' (*L. culinaris* subsp. *orientalis*).

Main results: MACE results yielded a total of 50,935 contigs. The average number of detected contigs in each of the six samples was approximately of 40,000. In response to *Ascochyta* infection, the jasmonic acid pathway and the lignin biosynthesis were up-regulated in resistant genotypes, while they were down-regulated in the susceptible one. The response to chitin, the salicylic pathway and the auxin response were activated only in the resistant *L. c. culinaris* genotype, while the giberellin synthesis was only induced in the susceptible *L. c. culinaris* cv. 'Lupa'. A set of 18 lentil gene sequences putatively involved in the response to the pathogen were validated by RT-qPCR.

Research highlights: It can be concluded that in response to the infection by *Ascochyta*, the lignin biosynthesis and the JA pathway were critical for the resistance, while the giberellin synthesis seems to be related with susceptibility to the pathogen.

Additional keywords: *Lens culinaris*; *Lens orientalis*; transcriptomic; MACE.

Abbreviations used: ABA (abscisic acid); ET (ethylene); FDR (false discovery rate); GA (gibberellic acid); GO (gene ontology); JA (jasmonic acid); MACE (massive analysis of cDNA ends); NLR (nucleotide-binding domain and leucine rich repeat containing protein); RT-qPCR (quantitative reverse transcription PCR); SA (salicylic acid).

Authors' contributions: PGG, FV, FJV, LESM, CP, AIG and MPV designed the study and participated in the data interpretation. PGG, FV, FJV and AIG carried out the *Ascochyta* inoculations and RNA extraction. RH, NK, BR and PW carried out the MACE experiment and the general bioinformatics analyses. LESM and CP performed specific bioinformatics analyses and made substantial contributions to the manuscript. PGG and MPV drafted the manuscript. All authors read and approved the final manuscript.

Supplementary material (files S1 to S10) accompanies the paper on SJAR's website.

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Introduction

Ascochyta blight is one of the main lentil diseases in most of the countries producing this crop. This disease, caused by the fungus *Ascochyta lentis* (syn *A. fabae* f. sp. *lentis*, teleomorph *Didymella lentis*), affects all above ground parts of the plant and is characterized by necrotic lesions. On susceptible cultivars under favorable conditions, it can lead to breakage of the stems and severe yield reduction. Seed quality may also

be reduced through seed discoloration or retardation of seed development. Chemicals can control Ascochyta blight, but besides the environmental problems and the inputs increase related with the use of fungicides, the development of resistant cultivars is considered a more efficient and sustainable approach (Davidson & Kimber, 2007).

Ascochyta lentis is a host-specific pathogen (Peever, 2007) and it is considered as a necrotrophic fungus, although a short biotrophic period cannot be completely excluded (Tivoli & Banniza, 2007; Sari *et al.*, 2017). The pathogenicity of this type of fungus and the resistance of the plant could be related to the production of specific fungal toxins and plant receptors or detoxifying molecules. Whatever the mechanism is, it should include the recognition of the pathogen in order to trigger the defense response. The recognition can be based on interactions between pathogen-associated molecular patterns (PAMPs) and the corresponding plant pattern recognition receptors (PRRs) in the pattern-triggered immunity (PTI), or alternatively it could be based on pathogen effectors and plant receptors, in special nucleotide-binding domain and leucine rich repeat containing (NLR) proteins, in the effector-triggered immunity (ETI). Both ways result in the activation of the plant immune response, mainly by the jasmonic acid (JA) and ethylene (ET) dependent pathways, which act in a synergistic way (JA/ET pathway). The salicylic acid (SA) signaling pathway, and probably the abscisic acid (ABA) and gibberellic acid (GA) signaling pathways, also have a role in the final output (Mengiste, 2012; Abdullah & Akhtar, 2016).

Gene expression analyses in lentil revealed that the JA and SA signaling pathways play a central role in determining the resistance/susceptibility to *A. lentis*, although the ET, SA, GA and ABA pathways might also be involved (Mustafa *et al.*, 2009; Ford *et al.*, 2017). Nevertheless, at least for some genotypes, only the JA pathway genes could explain the different reaction against *Ascochyta* in resistant *vs* susceptible lentil cultivars (Sari *et al.*, 2017). These observations generally agree with the results obtained in other legumes after their host-specific *Ascochyta* infection, as in *Pisum* (Fondevilla *et al.*, 2014, 2018), *Lathyrus* (Almeida *et al.*, 2015), *Vicia* (Ocaña *et al.*, 2015) or *Cicer* (Madrid *et al.*, 2012).

Recently, Khorramdelazad *et al.* (2018) and Sari *et al.* (2018) have utilized the RNA-seq approach for the study of transcriptome changes in lentil after the infection by *A. lentis*. In this way, a set of candidate genes involved in the different stages of the defense response has been identified, from the pathogen recognition to the systemic acquired resistance. Although the response was genotype-specific, showing up the existence of several resistance mechanisms in lentil (Sari *et al.*, 2018), some common characteristics are found in the resistant/tolerant genotypes, such as an earlier and faster detection and signalling response to the infection, and a higher expression level of structural defense-related genes, in special those coding for proteins involved in the reinforcement of cell walls (Khorramdelazad *et al.*, 2018).

An alternative technology to analyze gene expression is the Massive Analysis of cDNA Ends (MACE) because it is considered one the most cost-effective methodologies for the studies on changes in gene expression (Zajac *et al.*, 2015). This technique has proved its usefulness in other plant species and it yielded data on candidate genes further validated by qPCR (Yakovlev *et al.*, 2014; Zajac *et al.*, 2015; Weiβ *et al.*, 2017; Weiβ & Winkelmann, 2017). Our main objective was to obtain a general overview of the changes in gene expression that occur in other interesting lentil genotypes showing different levels of resistance when they are infected by *Ascochyta* using the MACE technique. The purpose was to identify some general and genotypic-specific defense responses, in special the common gene expression changes showed by the resistant genotypes, in order to obtain a set of candidate genes presumably involved in the resistance. This group of genes will provide the basis for the development of genic markers useful in breeding programs, and for further studies on mapping and expression changes of the candidate genes in other lentil genotypes.

Material and methods

Three samples of *Lens culinaris* have been used, two of them belong to the cultivated species *L. c.* subsp. *culinaris* (accession 'ILL5588' and cv. 'Lupa') and one to the wild ancestor *L. c.* subsp. *orientalis* (accession 'BG 16880'). 'Lupa' is a Spanish cultivar, 'ILL5588' is an accession from the International Center for Agricultural Research in the Dry Areas (ICARDA) lentil collection and 'BG 16880' is an accession from the Spanish Centro Nacional de Recursos Fitogenéticos (CRF). The response of the three genotypes to the *A. lentis* infection was different: *L. c. orientalis* showed resistance to the fungus, 'ILL5588' could be considered as moderately resistant, and 'Lupa' was highly susceptible to the pathogen.

Thirty plants of each accession were grown in sterile conditions until inoculation (15 days after germination) with a conidial suspension of the isolate AL 84 of *A. lentis* (ATCC® 46981™) following the method described in Ford *et al.* (1999). Other 30 plants

per accession were grown in the same conditions and similarly treated, but without the presence of the pathogen, to use them as controls. Twenty four hours after inoculation, the aerial parts of the corresponding plants were pooled, frozen in liquid nitrogen, grinded and stored at -80ºC until total RNA extraction was carried out. According to Roundhill *et al.* (1995), it is around 24 hours after infection when the first signs of disturbance in the plant cell cytoplasm in reaction to the hyphae penetration are detected. Also at this time the magnitude of oxidative burst seems to be related with the level of the plant resistance (Sambasivam *et al.*, 2017) and the differences in expression changes among lentil genotypes are more apparent (Khorramdelazad *et al.*, 2018; Sari *et al.*, 2018). Total RNA was extracted using the method described in Chang *et al.* (1993).

The RNA of each corresponding genotype and treatment were pooled separately to produce six different MACE cDNA libraries. The libraries were constructed using the "MACE-Kit" (GenXPro GmbH, Frankfurt am Main, Germany) as described in Zawada *et al.* (2014). In short, cDNA was obtained from ≈5 µg of total RNA, then randomly fragmented and biotinylated 3′ ends were captured after binding to a streptavidin matrix. A library ready for high-throughput sequencing was prepared using TrueQuant adapters included in the kit. The library consisted of 50–1000 bp-long fragments derived from the 3′-end of the cDNAs. The 3′ ends of the libraries were sequenced on a HiSeq 2000 (Illumina) with 100 cycles to generate the MACE tags, each tag representing one single transcript molecule. The Illumina-derived sequence reads were processed with GenXPro's in-house MACE analysis pipeline. Briefly, the elimination of duplicate PCR-derived tags, identified by TrueQuant technology, was carried out. Then, low-quality sequences were deleted, and finally the removal of sequencing adapter primers and poly(A)-tails was carried out using an in-house Python script.

Reads from all samples were assembled using the Trinity RNA-Seq *de novo* Assembly (Grabherr *et al.*, 2011). The resulting contigs of the assemblies longer than 100 bp were annotated by BLAST (BLAST 2.2.25+) to different sources, using in all cases the default parameters implemented in the NCBI BLAST tool and a threshold e-value of 10^{-5}. The contigs were compared by BLASTX, first to all *Fabaceae* Swissprot and afterwards to all *Fabaceae* Trembl protein sequences from Uniprot (http://www.uniprot.org/). Contigs that did not have a match to one of these databases were annotated by BLASTN to all *Lens* mRNA sequences from NCBI. Contigs were hereafter again used as reference for annotation and quantification of the MACE tags. Only uniquely mapped reads were taken into account. In order to compare the different samples, a normalization of the raw counts was performed according to Anders & Huber (2010).

The *p*-values for differential gene expression between the samples were calculated using the DEGSeq R/Bioconductor package (Wang *et al.*, 2010). The values for controlling the False Discovery Rate (FDR) were estimated according to Benjamini & Hochberg (1995). A change in a contig expression was considered statistically significant when the FDR value was ≤ 0.05.

The functional annotation of the sequences was carried out using the MACE2GO software, available at GenXPro's server at http:\\tools.genxpro.net. The enrichment of the gene ontology (GO) categories was based on the genes that showed differential expression at $p \leq 0.05$, and the statistical significance was calculated using the Fisher's exact test. The results of the GO enrichment analysis were summarized and plotted using the REVIGO (Reduce plus Visualize Gene Ontology) software that clusters the terms by their relationship and avoids the redundancy, making easier the results interpretation (Supek *et al.*, 2011).

In order to test the reliability of the MACE results, RT-qPCR was carried out for 18 genes. Three biological and three technical replicates for each genotype and treatment (control and infected) were obtained in the same conditions as in the MACE experiment and used for this analysis. Total RNA was extracted using the same method as in the MACE experiment, and after a DNase digestion, RNA quality and quantity was evaluated by electrophoresis and NanoDrop spectrophotometry. To be sure of the complete absence of DNA contamination, we followed the method described in Vaghefi *et al.* (2013). For each sample, 1 µg of RNA was reverse transcribed using the High-Capacity RNA-o-cDNA™ kit (Applied Biosystems™) following the manufacturer's instructions, and the cDNA obtained was diluted 1:10 to be used in the RT-qPCR reactions. Three technical replicates per sample were prepared and each one contained 7.5 µL of Power SYBR™ Green PCR Master Mix (Applied Biosystems™), 2 µL of cDNA and 0.4 µM of each primer in a total volume of 15 µL. The RT-qPCR was performed in a StepOnePlus Real-Time PCR instrument (Applied Biosystems™) using the following program: 95 ºC for 10 min, 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min and finally a melting stage from 60 ºC to 95 ºC with increasing steps of 0.3 ºC. The amplification efficiency for each primer pair was estimated by the software LingRegPCR v2017.1 (Ruijter *et al.*, 2009). The analysis of gene expression changes was carried out with the Comparative C_T ($\Delta\Delta C_T$) method implemented in StepOne™ software v2.3 (Life Technologies).

Most of the primers for RT-qPCR were designed from specific MACE contig sequences using the software Primer3Plus (Untergasser *et al.*, 2007). For the reference gene, we used the primer pair LcActin-257 described in Sari *et al.* (2017) because it has been validated in RT-qPCR experiments dealing with *Ascochyta* infection in lentil. The information about the primer sequences, PCR efficiencies and foldchanges can be found in the File S1 [suppl.].

Results

General characteristics of the MACE experiment

The sequencing data generated by MACE were deposited in the NCBI BioProject repository under accession ID PRJNA356810. A summary of the general numbers of reads and contigs obtained is shown in Table 1. The experiment yielded 11,416,403 reads that were assembled *de novo* in 50,935 contigs. The contig sequences are presented in the File S2 [suppl.] and the data for each sample in the File S3 [suppl.]. For each of the six samples, the number of detected contigs was similar (~ 40,000), only the sample "Lupa control" yielded a slightly lower number of contigs (36,023). The final transcriptome was made of 43,970 different contigs when considering as different only those contigs that matched different genes or sequences in the BLAST analysis. Contigs that matched the same sequence in the BLAST analysis were considered as coming from the same transcript, and the reads were summed up for the differential gene expression analysis.

Most of the contigs (70 %) were shared between the three genotypes (Fig. 1A), and only a small proportion (~ 7 %, ranging from 784 to 1,303 contigs) was exclusive of a single genotype. The obtained data for control and infected plants from the three genotypes are shown in the Fig. 1B and 1C, respectively. When comparing the contigs detected in a specific genotype, a high proportion was common between the control and infected sample (Fig. 1D), and only a small percentage (between 2.5 % to 4.5 %) presented a significant change in expression, always with a greater number for the up-regulated contigs.

Table 1. Summary of the results obtained in the MACE experiments for the six *Lens* samples.

Sample[1]	Nº of reads	Nº of contigs	Nº of different contigs
'ILL5588' control	2,433,891	40,283	33,381
'ILL5588' infected	2,096,925	41,678	34,770
'Lupa' control	1,240,587	36,023	29,197
'Lupa' infected	1,755,745	40,651	33,767
L. c. orientalis control	2,045,990	39,946	33,047
L. c. orientalis infected	1,843,265	40,615	33,725
Total	11,416,403	50,935	43,970

[1] 'ILL5588' and 'Lupa' are *L. c. culinaris*.

Functional annotation of the contigs

The results of the functional annotation are shown in the File S4 [suppl.]. Approximately 16 % of the contigs matched a well-characterized sequence, 13 % matched genes coding for putative proteins with GO term, while the remaining 71 % were uncharacterized RNAs or related to genes coding for uncharacterized proteins.

The BLAST hits mainly matched well-characterized gene sequence from legume species, reflecting the phylogenetic proximity with lentil, although it was noticeable that 193 contigs without a BLAST hit in the plant databases, matched sequences from *Ascochyta*.

The 20 most abundant GO terms for the three categories (biological process, molecular function and cellular component) are represented in the Fig. 2. For the category "biological process", the most represented GO term was the very general one "metabolic process". Some more specific terms, possibly related with the response of the plants to the pathogen infection can be found within the "biological processes" (oxidation-reduction process, phosphorylation, defense response, etc.). In the "molecular function" category, the terms related with "binding" of ATP, nucleotides and metal ions were the most abundant, although other terms related with oxidoreductase and kinase activities were also represented. The "cellular component" category showed a high abundance of terms related with the cellular membranes.

Contigs showing differential expression after the *A. lentis* infection

Contigs were considered differentially expressed when a statistically significant change in the number of normalized reads after the *A. lentis* infection was observed (FDR ≤ 0.05), and were clustered in groups showing similar changes of expression. In all groups, only contigs with a full description were considered and when several contigs matched the same sequence, only one is shown (File S5 [suppl.]).

Among the groups, those constituted by contigs which are exclusively up-regulated in the resistant genotypes are especially interesting because they would include the genes directly involved in an effective defense response (File S5 [suppl.], sheets

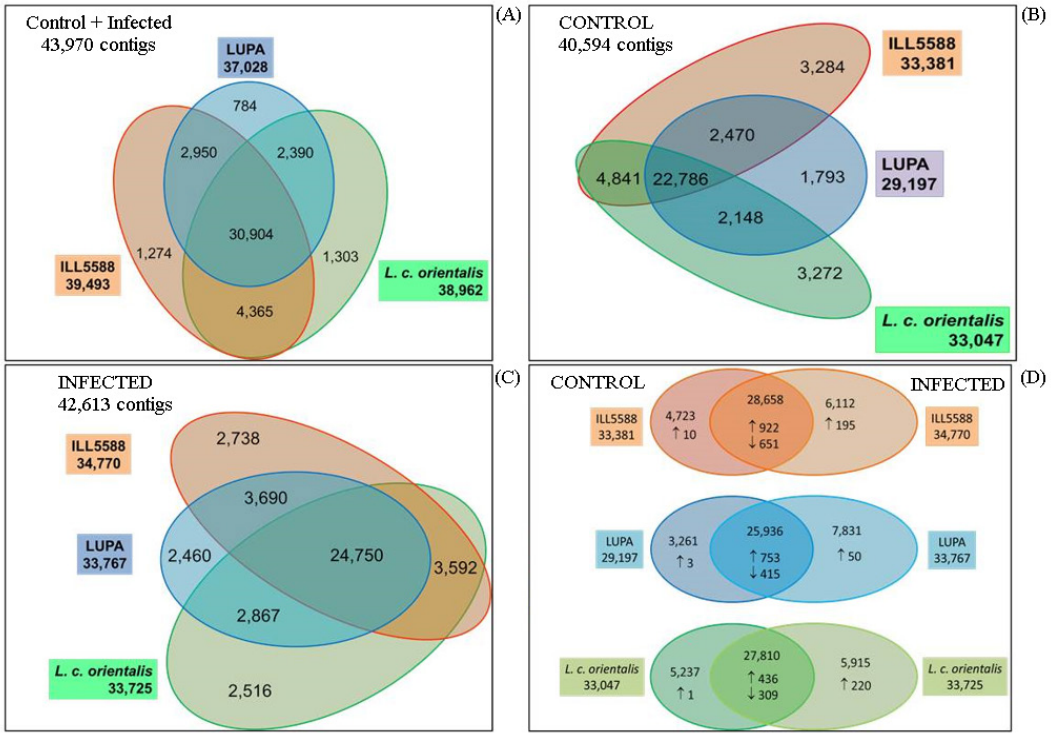

Figure 1. Number of different contigs detected in total (control + infected) (A), control (B) and infected (C) samples, and comparison control *vs.* infected within samples (D). The total number of different contigs in each sample is presented in the rectangles. The number of contigs with a significant change in the expression (↑, up-regulated, ↓, down-regulated, FDR ≤ 0.05) when comparing controls *vs*. infected with *A. lentis* is shown (D).

"up in R, down in S", "up in R, no change in S"). Several contigs found in this group seem to be directly involved in the different levels of plant defense. Thus, some sequences seem to be related with the protection against the oxidative stress caused by the ROS species (peroxidase, aldose reductase or superoxide dismutase). Contigs for WRKY transcription factors, which in *Arabidopsis* are directly involved in the resistance response to necrotrophic fungi (Buscaill & Rivas, 2014), were also found. In addition, there were sequences presumably coding for enzymes involved in the synthesis of monoterpernes, which protect the plant against pathogens when accumulated in leaves or roots (geranyl-diphosphate synthase), for enzymes that degrade the fungal cell walls (glucan 1,3-beta-glucosidase, glucosylceramidase), or for enzymes involved in the lignification and defense against fungal pathogens (laccases).

It is also worth mentioning the contigs related with hormone pathways involved in defense. The cysteine-rich receptors-like protein kinases might trigger the ABA and SA signaling pathways (Burdiak *et al.*, 2015; Lu *et al.*, 2016). The activation of the ET pathway was reflected in the up-regulation of the contig c7470_g1_s1, which would code for an ethylene response factor. The up-regulation of the contig c9517_g1_s1, probably involved in the synthesis of a patatin-related phospholipase A, suggests a defense through the JA pathway (Canonne *et al.*, 2011).

The resistance to *A. lentis* could also be related to the capacity of the plant of maintaining the expression of some key genes. Thus, the contigs showing down-regulation in the susceptible 'Lupa' but without expression changes in the more resistant genotypes could provide valuable information. In this group (File S5 [suppl.], sheet "no change in R, down in S"), some may be related with the lack of an effective defense response against *Ascochyta*. Examples of these functions are CC-NBS-LRR resistance protein, serine/threonine protein kinase, calcium and calcium/calmodulin-dependent serine/threonine-protein kinase, calmodulin protein kinase or auxin-induced protein AUX28. The role of calmodulin in the defense may be related to the *MLO* gene family response which has an important role in the resistant defense against fungus in several plant species (Kim *et al.*, 2002). Likewise, the down-regulation of some genes of the auxin pathway in the susceptible genotype has shown that a correct auxin signaling is needed against necrotrophic pathogens (Fu & Wang, 2011).

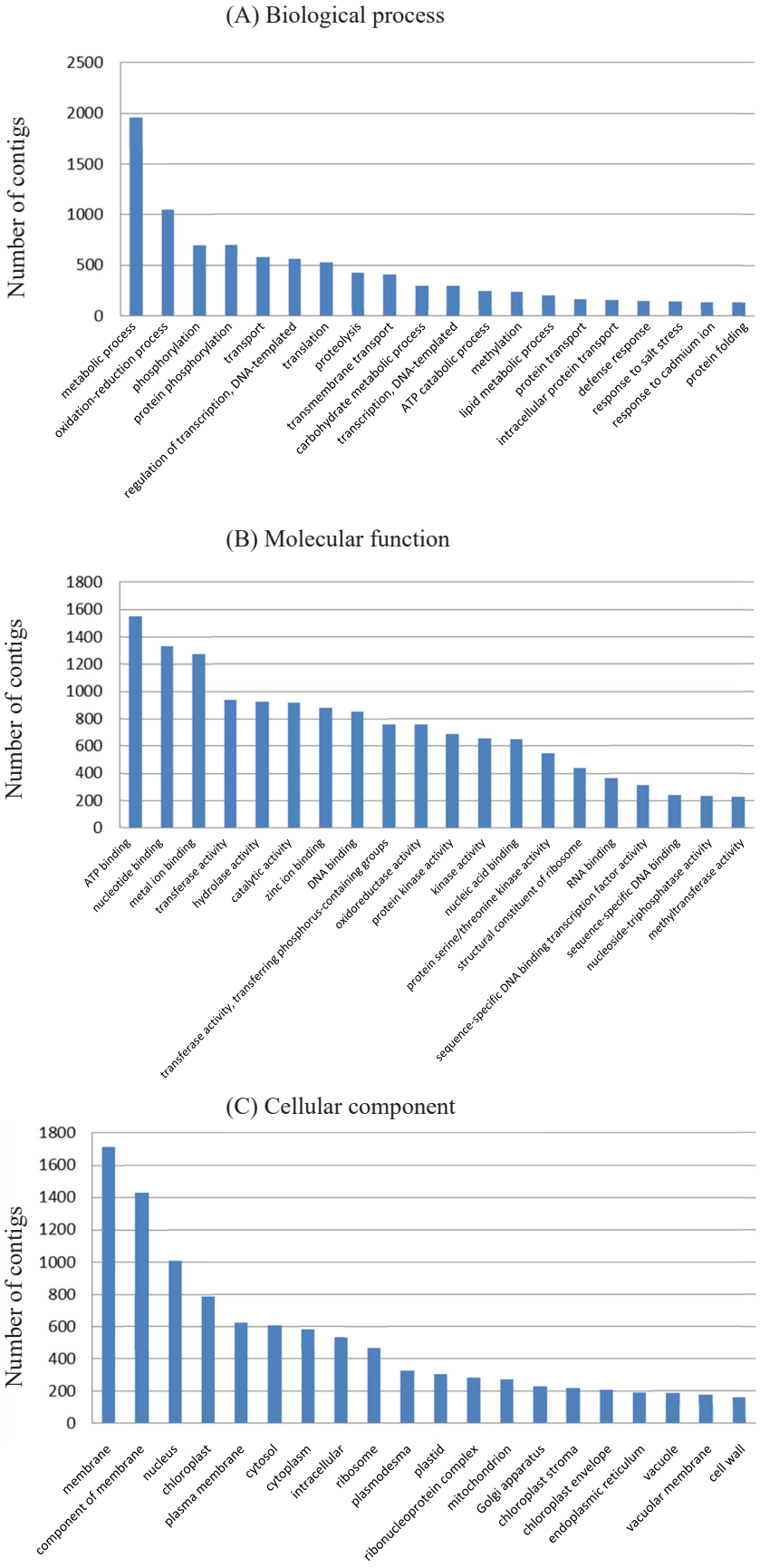

Figure 2. Number of contigs included in the 20 most frequent GO terms for the three main categories: (A) biological process, (B) molecular function and (C) cellular component.

The contigs showing an identical change of expression in the three genotypes (File S5 [suppl.], sheets "up in R and S" and "down in R and S") are supposed to be involved in general mechanisms of response against the pathogen. Among the common up-regulated sequences, at least 17 were related with genes described in other plant species as part of the defense mechanisms. Thus, four contigs have similarity with genes coding for pathogenesis related proteins (PR) or disease resistance response proteins. Among them, it would be worth remarking the contigs c112_g1_s1 and c24313_g1_s1, which matched with the genes coding for the *L. culinaris* pathogenesis-related protein 4 and pathogenesis-related protein 10.

GO enrichment analysis

In order to get a wider picture of the affected processes by the fungus infection, a GO enrichment analysis was carried out. For this study, we have used the *p*-value ≤ 0.05 as indication of a change in the gene expression instead the FDR value to get a higher number of data for each GO term.

The significant enriched GO terms in each genotype are shown in File S6 [suppl.]. A long list of GO terms was obtained, but many terms were closely related. Therefore the enriched GO terms were plotted using REVIGO software (Supek *et al.*, 2011), which clusters similar GO terms based in their semantic similarity. The enriched GO terms for the "biological process" category in the three genotypes are presented in the Fig. 3, the other two categories are shown in the File S7 [suppl.].

Ascochyta lentis sequences

Some contigs did not match any sequence in the plant databases and were virtually undetected in control plants, while they showed a significant increase in infected plants. In order to explore if they had a fungal origin, these contigs were BLAST compared against the *A. lentis* genome (INSDC RZGK00000000.1, assembly Alentis_Al4) and the *Ascochyta rabiei* genome database (AscRab1.0, INSDC Assembly GCA_001630375.1, Verma *et al.*, 2016) and the sequences from this species published by Fondevilla *et al.* (2015). A total of 193 contigs matched to fungal sequences (File S8 [suppl.]).

Besides some sequences related with genes involved in the general functions of the fungus (ribosomal, membrane or mitochondrial proteins), some other could be implicated in the pathogenicity since several matched sequences in the PHI database (Pathogen-Host Interaction database, http://www.phi-base.org/). According to this database the corresponding genes are involved in the virulence phenotype. For example, Contig292 was related with the major protein of the Woronin body, a structure derived from the peroxisomes that is initially required for a proper development and functioning of appressoria, and subsequently necessary for the survival of hyphae during invasive growth and host colonization (Soundarajan *et al.*, 2004), and contig c34174_g1_s1 was related with an alcohol oxidase that has been described as a pathogenicity factor in *Cladosporium fulvum* (Segers *et al.*, 2001).

RT-qPCR validation

In order to test the reliability of the expression changes observed in the MACE experiment, 18 sequences were analyzed with RT-qPCR in three biological and three technical replicates of each genotype and condition (control and infected). The genes were chosen because of their putative role in defense and/or their abundance in the MACE results. In all cases, the amplification efficiency was at least a 90 % (File S1 [suppl.]). The reference gene (*LcActin-257*), validated by Sari *et al.* (2017), also showed a stable expression in the MACE contigs that matched it. For the analyzed genes, the correlation between the observed foldchanges after the infection in MACE (File S9 [suppl.]) and RT-qPCR was positive and statistically significant in the three genotypes (0.69 for 'Lupa', 0.80 for 'ILL5588' and 0.62 for *L. c. orientalis*) (File S10 [suppl.]).

Discussion

Because one of the causes of the limited production of lentil worldwide is the fungal disease caused by *A. lentis*, we were interested in the analysis of the differences in response to the infection in susceptible and resistant cultivars.

In this study, the changes in the lentil transcriptome after the *A. lentis* infection were estimated in new lentil materials by using the MACE technique for the first time in this species. This approach has allowed us to obtain high-quality information, not only on the expressed genes in the plant genotypes, but also on their expression levels.

The significant and positive correlation between the expression changes observed in the MACE and RT-qPCR experiments in the three genotypes supports the reliability of the obtained results in the transcriptome profiling using the MACE technique. Moreover, the resolution of the MACE technique is highlighted by the fact that also sequences from the pathogen were identified, although they were in a small proportion in the total RNA sampled from infected plants.

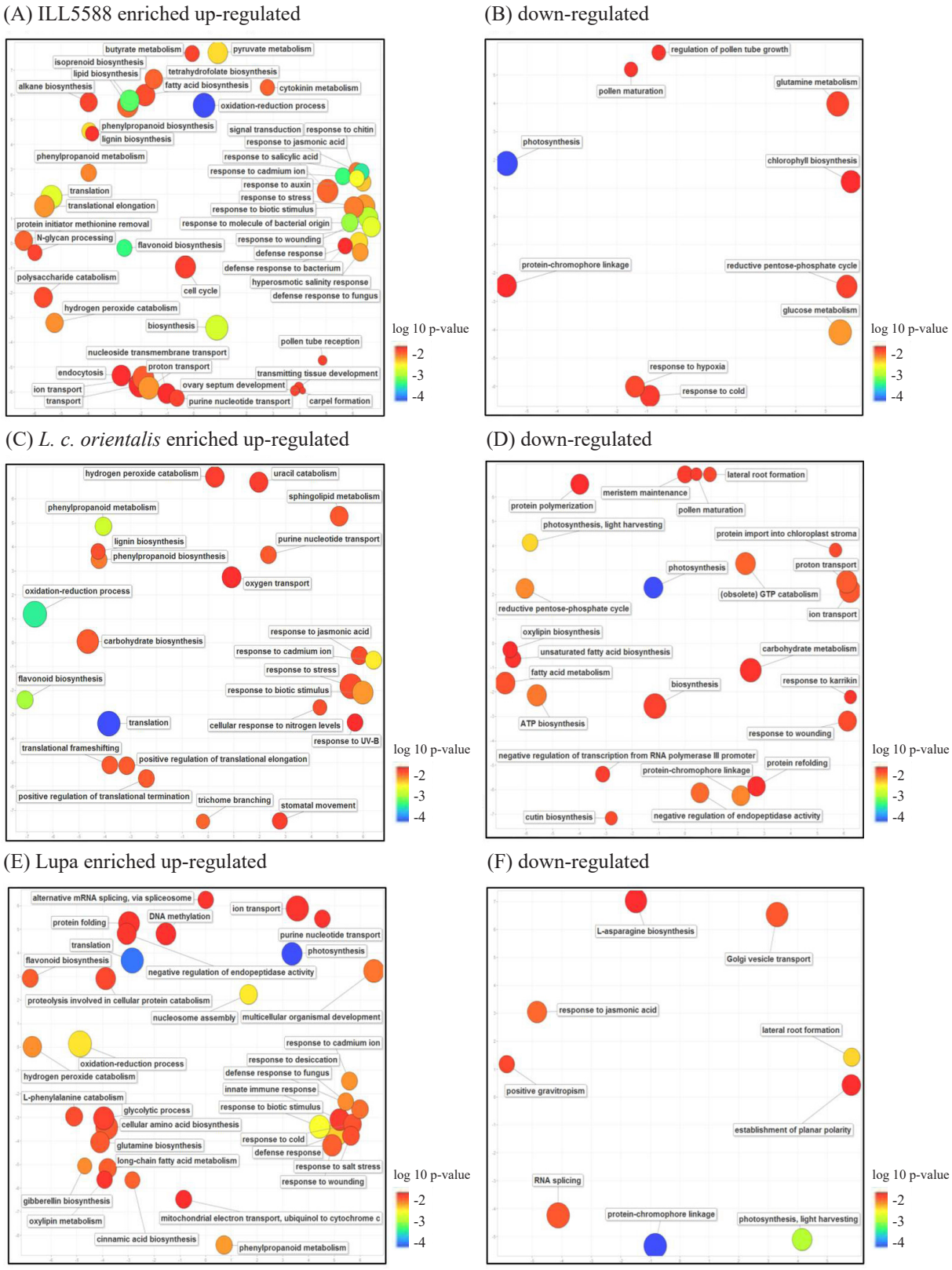

Figure 3. REVIGO plots of the enriched GO terms associated to contigs showing a significant expression change after *Ascochyta lentis* infection the category "*biological process*". (A) Enriched up-regulated GO terms in the genotype 'ILL5588', (B) enriched down-regulated terms in 'ILL5588', (C) enriched up-regulated terms in the genotype *Lens culinaris orientalis*, (D) enriched down-regulated terms in the genotype *L. c. orientalis*, (E) enriched up-regulated terms in the genotype 'Lupa', (F) enriched down-regulated terms in 'Lupa'.

The length of the MACE contigs, mainly between 100 and 1000 bp, was enough to allow the identification of homologous genes in the databases. It is remarkable that a 48 % of the sequences did not match any characterized sequence, maybe due to the fact that MACE mainly covers the 3' end of a transcript that often only contains the 3' UTR, which makes difficult to get a protein annotation.

Even with a limited functional annotation, these data yielded a number of candidate genes involved in the resistance response based on the type of identified genes and the expression changes in the three genotypes analyzed. The key resistance genes are probably distinct in different genotypes, as illustrated by the different genes used in the lentil breeding programs (Pérez de la Vega *et al.*, 2011; Sudheesh *et al.*, 2016), although some common mechanisms seemed to have a role, such as the activation of the JA pathway (Sari *et al.*, 2018).

The way in which *A. lentis* is recognized by the plant and how this fact triggers the defense response is not yet resolved. Although a change in a gene expression after the infection does not necessarily imply a direct role of that gene in the resistance response, when the changes are similar in several resistant genotypes and different from the changes in susceptible ones, it seems logical to consider the gene as involved in the resistance. Based on this rationale this study has pointed out several genes probably involved in the response between the fungus recognition by the plant until the accumulation of antifungal compounds in plant cells.

Similarly, a change in gene expression in the same direction for all genotypes (resistant or susceptible) can be the signature of those genes involved in general mechanisms of response against pathogens, but not the ultimate responsible for the resistance, or alternatively by-products due to the stress. This set of genes included candidate genes previously described as responsible for the resistance, such as some genes coding for PR proteins (*PR4* and *PR10a*). The RT-qPCR data did not show a significant change of expression for these genes with the exception of the up-regulation of *PR4* in the susceptible genotype (File S9 [suppl.]).

Although these results suggest that these genes are not main candidates for the differences in resistance response among the three genotypes analyzed in this work, it would be possible that the resistance depended not only in the magnitude of the expression change, but also in the absolute quantity of a gene product inside the cell. Besides, we cannot rule out that the resistance response would be determined by the different alleles borne in the three genotypes or by post-transcriptional modifications no detected in the sequences obtained with the MACE technique.

The recognition of the pathogen is usually mediated by the interaction of a pathogen molecule (*i.e.*, chitin) by a plant receptor, usually a wall associated receptor-like kinase (Mengiste, 2012). At least 10 contigs in our work belonged to this kind of receptors, but they were in low copy number and their expression changes were not significant. On the other hand, the recognition can also be mediated by interactions between fungal effectors and R-proteins, many of which belong to the NBS-LRR gene family. We found 42 contigs related to NBS-LRR genes, and most of them did not show significant changes after the infection.

After the pathogen recognition, several hormones pathways, mainly SA, ABA and the synergistic JA and ET, would mediate the defense response. Our data point to a high number of contigs matching genes related with those signalling pathways. For instance, 33 contigs in 'ILL5588' contain the GO term 0009751 (response to salicylic acid) and four out of the 33 were down-regulated and 11 up-regulated after infection, or 142 contigs contain the GO term 0006952 (defense response) among which seven were down-regulated and 34 up-regulated (File S6 [suppl.]).

A general view of the processes that change significantly after the infection was obtained by summarizing the enriched GO terms using the software REVIGO (Fig. 3 and File S7 [suppl.]). In 'ILL5588' (moderately resistant), many up-regulated enriched GO terms were related with the plant defense, some were general terms such as "defense response", "response to biotic stimulus" or "response to stress", but others were more specifically related in the response to *Ascochyta* (defense response to fungus, response to chitin, response to SA, response to JA, response to auxin). Other up-regulated terms seemed to suggest the way in which the plant is controlling the fungus invasion, such as reinforcing the cell wall (lignin biosynthesis) or storing toxic antifungal compounds, most of them derived from flavonoids, isoprenoids, phenylpropanoids or fatty acids (Amil-Ruiz *et al.*, 2011). One of the highest up-regulated enriched terms was "oxidation-reduction process", probably related with the accumulation of reactive oxygen species (ROS) in the early stages of the fungus infection. The role the oxidative burst and the associated cell death is not clear in the necrotrophic pathogenicity, although the general idea is that the early activation of ROS production induces the defense mechanisms, but a sustained production and the associated cell death promotes susceptibility to necrotrophs and resistance to biotrophs (Mengiste, 2012). In lentil, the resistance has been associated to both responses: the inhibition, such as in the cultivar 'CDC Robin' (Sari *et al.*, 2017) and the promotion, such as in 'ILL7537' (Khorramdelazad *et al.*, 2018), of the programmed cell death.

In 'ILL5588', the down-regulated enriched terms are mainly related with photosynthesis functions, a usual response after a pathogen invasion (Huot *et al.*, 2014).

L. c. orientalis shared several up-regulated enriched GO terms with 'ILL5588', and although they were in a lower number and belonged to a more general GO level, some coincidences suggest a common mechanism of defense with 'ILL5588' (lignin biosynthesis, response to JA, flavonoid biosynthesis, or phenylpropanoid biosynthesis). Many down-regulated terms were related with photosynthesis as found in 'ILL5588'. One of the down-regulated terms (cutin biosynthesis) seems to be directly related with the defense, since cutin is supposed to protect all the aerial parts of the plants. However, although the relationships of cutin with the response mechanisms is proved, its role its controversial: while in tomato the level of cutin in the cuticle is correlated with the resistance to *Botrytis cinerea*, in *Arabidopsis* mutants with lower levels of cutin in the leaves showed an increase in the resistance to *B. cinerea* (Fich *et al.*, 2016).

A remarkable difference between the susceptible 'Lupa' and the resistant genotypes was the down-regulation of the response to JA and the up-regulation of the genes for gibberellin biosynthesis. Gibberellin promotes the degradation of DELLA proteins, which strengths the SA signaling pathway and weakens the JA pathway, thus increasing the susceptibility to necrotrophs (Navarro *et al.*, 2008). The role of DELLA proteins in the resistance to *A. lentis* is supported by Khorramdelazad *et al.* (2018), who found an over-expression of the coding genes in the resistant genotype ILL7537, suggesting a main role in the systemic acquired resistance signaling.

Summarizing the results for these three *Lens* genotypes, it can be concluded that in response to *Ascochyta*, the reaction by the host plant was multiple and although it presents genotype-associated peculiarities, some common points can be observed. Thus, the JA pathway was activated in resistant genotypes, while it was down-regulated in the susceptible one, and the processes related with the lignin biosynthesis are also induced exclusively in the two resistant genotypes. The response to chitin, the salicylic pathway and the auxin response were activated only in the resistant *L. c. culinaris* genotype 'ILL5588', while the giberellin synthesis was only induced in the susceptible *L. c. culinaris* cv. 'Lupa'. Finally, the general defense responses and the metabolism of ROS products were activated in all the three genotypes. Because the hormone pathways are interconnected, either synergism (JA and auxin pathways) or antagonism (JA and SA pathways, SA and auxin pathways) among them, the balance of all signals would be the determinant of the level of resistance.

Since similar results have been previously published in other lentil genotypes (Khorramdelazad *et al.*, 2018; Sari *et al.* 2018), we can conclude that our results agree with the up-regulation of JA signaling as a general response of the resistance to Ascochyta blight infection in lentil.

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