Gene expression analysis of molecular mechanisms of defense induced in *Medicago truncatula* parasitized by *Orobanche crenata*

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Abstract

The infection of *Medicago truncatula* Gaertn. roots with the obligate parasite *Orobanche crenata* Forsk. is a useful model for studying the molecular events involved in the legumes-parasite interaction. In order to gain insight into the identification of gene-regulatory elements involved in the resistance mechanism, the temporal expression pattern of ten defense-related genes was carried out using real-time quantitative reverse-transcription polymerase chain reaction assays. The induction of all of the analyzed transcripts significantly increased over a range from 2- to 321-fold higher than the control depending on the gene and time point. The transcriptional changes observed in response to *O. crenata* infection suggest that resistance could rely on both, the induction of general defense-related genes and more specific responses.

Abbreviations: CCR, cinnamoyl CoA reductase; CHS, chalcone synthase; dpi, days post-inoculation; EST, expressed sequence tag; HSP, heat shock protein; MtEf-1a, elongation factor 1-alpha; PPR, pentatricopeptide repeat; RT-PCR, real-time reverse transcription PCR; SSH, suppression subtractive hybridization; TC, tentative consensus; UBQ, ubiquitin

Keywords : Gene expression; *Medicago truncatula*; *Orobanche crenata*; Real-time RT-PCR

1. Introduction

The holoparasitic angiosperm *Orobanche crenata* Forsk. is considered an important constraint to legume crops in the Mediterranean area [28]. Resistance to *O. crenata* in legumes is a multigenic character with very low heritability, which makes breeding for *Orobanche* resistance a difficult task [27]. Some genomic studies have aimed to identify and locate *Orobanche* resistance genes/QTLs in molecular maps in legumes [25, 30]. Nevertheless, the host response to parasitic plants seems to require a deeper understanding of the complex defense multifactorial process. There is a strong consensus that detailed knowledge of the molecular mechanisms underlying the host-parasite interaction is necessary to improve legume breeding programs. However plant-parasitic plant studies are limited compared to other plant–pathogen systems, and little is known so far about the changes in gene expression in parasitized plants. The

identification and subsequent characterization of legume genes, which are either specifically and/or differentially expressed within the plant upon parasitization, are the first steps to understanding this highly complex interaction. Several defense responses to the parasitization process by *Orobanche* spp. include increased levels of Pathogenesis-Related proteins (PR-proteins) [12], *hmg2*, a defense-related isogene of 3-hydroxy-3methylglutaryl CoA reductase (HGMR) [33] and a set of several defense genes involved in the jasmonate and ethylene signalling pathways [32]. Recent in situ hybridization studies have shown that a peroxidase and a β-glucanase are differentially expressed in cells of pea roots resistant to *O. crenata* [20]. The advent of high-throughput technologies is providing large-scale surveys leading to a more integrated view of gene expression responses. To improve our understanding of the mechanisms underlying resistance during pathogenic interactions, cDNA libraries have been generated from plants inoculated with various pathogens with the aim to increase the availability of the potential number of defense-related genes. However, one problem linked to random EST collections is the underrepresentation of genes that are weakly expressed, but might be key regulators of the defense response. Techniques, such as suppression subtractive hybridization (SSH), overcome this limitation by equalizing the abundance of cDNAs within the target population and enable the generation of cDNA libraries that are enriched for sequences overrepresented in certain RNA samples [3].

Non-host resistance is presumed to be a complex, multicomponent form of resistance, including both constitutive and inducible defenses. Non-host resistance is highly effective and durable, making this type of resistance of great interest for agriculture [8]. The combination of different resistance mechanisms into a single cultivar appears to be a powerful strategy. Plants often respond in similar ways in host and non-host interactions, and several components of the signalling pathways can be shared between host resistance and non-host resistance [29]. We have chosen the model legume *Medicago truncatula* Gaertn. as a suitable species for a transcriptomic approach because of its ideal characteristics, such as its small, diploid genome, rapid generation time, self-fertility and ease of seed production [2]. *M. truncatula* is being used to increase understanding of plant interactions with other organisms [26]. Furthermore, its close phylogenetic relationship to other legumes such as pea, lentil and faba bean is attractive so the non-host interaction of *M. truncatula-O. crenata* can be used to improve our understanding of the nature of resistance in legume-parasite interactions [16, 21].

In a preliminary study, a suppression subtractive hybridization was carried out in order to obtain a first comprehensive description of the gene expression changes induced in *M. truncatula* during the interaction with the parasitic plant *O. crenata* [4]. This transcriptomic approach led to the identification of a significant number of *M. truncatula* genes in response to the parasite taking host samples pooled at three time points. Nevertheless, a large amount of information on the temporal aspects of gene expression can be lost when just an overall view of the parasitization process is considered. For this reason, genes whose expressions were shown to be strengthened after SSH experiments have been retained for a kinetic analysis of their regulation by real-time reverse transcription (RT)-PCR strategy. We focused on the gene expression pattern in roots from the earliest contact with *Orobanche* radicles to the well-developed parasite tubercle formation, leading to a detailed temporal expression analysis of ten putative defense genes.

2. Results

The transcript accumulation for several genes in *M. truncatula* during specific stages of infection by *O. crenata* was assessed by real-time RT-PCR. Three time points (15, 21 and 35 days) were assessed to develop a kinetic study of the gene expression pattern in *Medicago* roots, from the initial contact with *Orobanche* radicles to the well-developed parasite tubercle (*Figure 1*). The choice of the genes studied was dictated by the fact that all of them have been obtained from a cDNA library enriched for genes up-regulated in *M. truncatula* roots infected by *O. crenata* [4]. Each primer set, designed to amplify a particular defense-related gene, had successfully amplified a unique putative product when DNA (data not shown) and cDNA (*Supplemental Figure 1*) were used as templates in conventional RT-PCR runs resulting in single products with the specific temperature shown in *Table I.*

2.1 Stability of expression for the candidate reference genes

The general expression levels of the candidate reference genes were calculated based on mean real-time PCR threshold cycle values. *MtEf-1a* was the most highly expressed gene in the root tissues studied. *UBQ* and *PPR* had lower levels, but had similar expression levels between them, which were close to that of the genes being analyzed. Single-factor ANOVA and linear regression analyses of C_T values were used to evaluate the stability of the gene expression [1]. The mean expression level for each gene in each sample was regressed against the overall means for the different tissue samples (*Supplemental Figure 2*). Three out of ten genes assessed in this study were included in this estimation. The ANOVA F-test of differences among templates indicated that none of the reference genes showed significant variation in expression over the time points and treatments. *MtEf-1a* had a coefficient of variation (CV) above 5%, and the other

two genes had CV below 3.5%. A summary of statistics is shown in *Table II.* The combination of both a constant expression level (low slope) and high predictability (low CV) led us to consider *UBQ* as the most adequate internal control in our experiment. Thus, the relative expression ratio of defense-related genes in the infected plants versus the controls was estimated by normalizing against *UBQ*.

2.2 Gene expression pattern in M. truncatula infected roots and sequence analysis

With no exception, all the RT-PCR reactions resulted in a single product at the predicted size confirmed by melting point analysis performed at the end of the real-time RT-PCR and the corresponding agarose gel. The gene expression patterns of infected plants relative to non-infected plants determined by quantitative real time RT-PCR are shown in *Figure 2*. The induction of a chalcone synthase (*CHS*, TC106550) reached a relative expression level of 120.41 in infected roots at 35 days post-inoculation, the highest comparative expression level measured in this study. Comparing infected roots with the corresponding non-infected controls, all studied transcripts showed a marked accumulation at least in one of the time points analyzed. Grouping the genes into functional categories according to Journet et al. [13], an up-regulation was maintained throughout all of the experiments for the 'cell wall modification' category with a cinnamoyl CoA reductase gene (*CCR*, TC106830). The most strongly *Orobanche*-induced gene, a protein with unknown function (TC110611), had more than a 320-fold difference in expression levels in infected roots compared with non-infected controls at 35dpi again. To identify the genomic sequence of the genes with the highest relative and comparative expression levels and to obtain the first information about the genomic origin of the two novel ESTs that did not reveal homologies to any other sequence after blastx search, we compared the cDNA sequences against available sequences of the

ongoing *M. truncatula* genome-sequencing project (http://medicago.org/genome/cvit_bacs.php). Three contigs on chromosomes 4, 6 and 7 were found (*Table III*). The genomic sequence containing the complete cDNA sequences presented introns for TC110611 and EH058490, but not for TC106550.

2.3 In silico expression profiling analyses

The composition of the ESTs-corresponding TC in the *M. truncatula* Gene Index Project (MtGI), shows that the three ESTs aligned into TC106550 derived from the root system, (two were obtained in *Rhizobium meliloti* association and one derived from a developing root library). However, the composition of the EST sequences supports that the TC110611 gene is not up-regulated in non-infected samples, because none of the three ESTs was derived from non-inoculated or non-treated libraries. Furthermore, the TC-composition strengthens the specificity of TC110611 in the defense response; one EST derives from aphid-infected shoots, one from leaves infected with *Colletrotrichum trifolii* and one from methyl jasmonate (MeJA)-elicited roots. Hence, although the expression of TC110611 is not root-specific, it is likely to be strongly induced during the defense response.

3. Discussion

We performed real-time RT-PCR assays to analyze changes in gene expression patterns in *M. truncatula* during its interaction with *O. crenata.* Several factors that may affect the reliability of real-time RT-PCR were carefully controlled in conducting the experiments: i) absence of genomic DNA contamination on all RNA reverse-transcribed samples confirmed by PCR using primers designed on the intron sequence; ii) efficiency of cDNA synthesis assessed by real-time PCR amplification 5'/3' ratio; iii) specific primer sets designed that amplified unique single products confirmed by melting curve and gel analyses; iv) and PCR efficiency of each primer pair in each individual reaction with correlation coefficients higher than 0.997. This robust strategy and analysis allowed for a reliable calculation of the relative level of gene transcripts. In order to confirm that the internal control gene expression levels remained constant during the infection, we investigated a suite of three reference control genes to normalize real-time PCR data. These genes have been used as internal controls in many experimental systems. However, it is unusual in the studies to provide any validation of these genes prior their application. The most appropriate internal gene for use as a reference control should be tested and validated first for the specific experiment in order to analyze more reliable patterns of expression.

With the aim of characterizing the defense responses to *O. crenata*, we conducted a time-course study in the root tissue during the interaction with the model legume *M. truncatula* for several defense-related genes. Identification of key stages in the process is a major element for detailed temporal expression analysis. In this sense, once the parasitic plant becomes attached to the host, responses acting after parasite penetration and during tubercle development have been addressed as potentially key to neutralizing the parasite [11]. Several resistant phenotypes were identified in legumes including pre-

235 penetration, post-penetration and post-establishment mechanisms (see for review [24]).
236 During the *Orobanche* attachment to the roots (15dpi), the patterns of gene expression
237 reported here reflect the host perception of the parasite and the activation of several
238 mechanisms to protect itself. The induction of *CHS*, an enzyme of the phenylpropanoid
239 pathway leading to phytoalexins production [7], the defense-related protein GATase
240 [36], a ripening-related protein with a domain for Bet v I allergen that belongs to a
241 protein family including the pathogenesis-related protein of the PR-10 group [19], a
242 *CCR* involved in cell wall reinforcement through the biosynthesis of lignin [15], and the
243 induced expression of a gene with no previous link to defense responses, a magnesium
244 transporter, demonstrate that at least from this earliest point, the parasite is detected and
245 defensive mechanisms are activated. But this raises questions about the early response
246 being too slow or ineffective to prevent the *Orobanche* development at this stage.
247
248 Previous studies have pointed to the prevalence of physical barriers as defense
249 mechanisms to prevent the penetration of the parasite into the root vascular cylinder [5,
250 20]. Changes in gene expression patterns involved in cell wall reinforcement during
251 *Orobanche* attack has been previously described in *A. thaliana* [31] and the legume
252 *Lotus japonicus* [10] using a subtractive hybridization approach. Recently, a proteomic
253 work in *M. truncatula* also identified some proteins belonging to the 'cell wall' group
254 (M. Castillejo, personal communication). The authors speculate that the active role in
255 the defense response that could be making the host cell more resistant to infection and
256 penetration. In this sense, the permanently induced expression of the *CCR* gene during
257 our experiment, especially during the 21 and 35dpi (more than 28-fold and 62-fold
258 difference of expression level in infected roots compared with the corresponding
259 controls, respectively), indicates the reinforcement of physical barriers associated with

lignification. However, the transcript accumulation of other genes analyzed suggests the co-induction of several defense mechanisms. Thus, an increase in the abundance of transcripts for a small heat shock protein was observed at 21dpi. Heat stress proteins (HSPs) act as molecular chaperones by preventing aggregation and enhancing the refolding of denatured proteins [18]. The fact that small HSPs may protect cells from the dehydration effects [22] would be in accordance with the already developed connection with the host vascular tissues at this time point and the decrease of water and nutrient supply. The induction of water stress genes could be very important as a defensive strategy and point to an overlap at the molecular level of the pathogen-responsive signal transduction pathways with those connected with abiotic stress already reported for other pathogens [34, 35]. In spite of the strong evidence supporting the host senses the parasite and considering that the parasite penetrates rapidly through the host tissues, it is tempting to question whether *Medicago* spends too much time recognizing the intrusion of *Orobanche* cells in their tissue. Although the host has the appropriate genes to trigger the defense, a delayed defensive response seems to be unsuccessful in preventing the penetration of the parasite and development of several tubercles.

Interestingly, the most strongly *Orobanche*-induced gene in this study was one EST (TC110611) without a known function (more than 320-fold expression level in infected roots at 35dpi). Database analysis of the MtGI revealed that all different ESTs aligned to TC110611 were derived from *M. truncatula* tissues challenged upon pathogen or elicitor treatments. The broad range of pathogenic interactions in which this gene was observed suggests its role is involved in common defense responses. Further

284 experimental evidence is needed to characterize its function during the interaction
285 legumes-*Orobanche*.
286
287 One significant outcome is the detailed temporal expression of two novel genes without
288 homologies to any previously identified EST sequence. In our previous transcriptomic
289 study, we identified several cDNA clones with no homology on an amino acid level in
290 the EST databases to date by the subtractive hybridization method. A blastn search for
291 both genes within the presently available *M. truncatula* BAC sequence collection
292 confirmed the host plant origin for one of the two ESTs analyzed here, and significant
293 induction ($P \leq 0.05$) was observed in the *M. truncatula* roots after the vascular systems of
294 the two plants are connected at 21dpi. Therefore, they are extremely interesting for
295 further plant-parasitic plant interactions and could reveal novel mechanisms.
296
297 **4. Conclusion and Remarks**
298 The strategy of coupling EST sequencing with in silico and experimental expression
299 analyses is a powerful tool for identifying *M. truncatula* genes specifically regulated in
300 the defense response and represents a first step towards the characterization of genes
301 specifically involved in this interaction that have been previously isolated through
302 suppression subtractive hybridization.
303 Following invasion of *Medicago* tissues by the parasitic plant *O. crenata*, a range of
304 defense mechanisms is triggered to restrict their growth along the main initial stages of
305 the infection process. These include physical barriers to prevent parasite intrusion based
306 on the reinforcement of cell walls by lignification and the induction of gene expression-
307 mediating pathways leading to phytoalexin production, activation of pathogenesis-
308 related proteins and a cross-talk of more general plant defense-related responses, such

as abiotic stress-responsive signalling pathways. The different temporal accumulation of novel gene transcripts with no putative homologues identified so far in other plant genomes represents a resource for further research. Besides their importance for functional studies, specific genes represent the starting point for unravelling the molecular mechanism of the transcriptional induction during the parasitic plant infection. Moreover, considering *M. truncatula* sinteny with economically important grain legume crops, these results could be helpful in the study of other legume-*O. crenata* interactions which are to some extent resistant to broomrape, providing insights into the molecular mechanisms of host plant responses to parasitization.

5. Material and Methods

5.1 Plant material, growth conditions and inoculation

Seeds of *Medicago truncatula* Gaertn. genotype SA4087 (incomplete late-acting resistance mediated by necrosis of the parasite tubercles) were sterilized in commercial bleach (20% in sterile water) for 10 minutes and scarified with a sterile scalpel. Then the seeds were placed in sterile water at 4ºC for 48 hours to synchronize germination. The water was replaced three times to help germination according to the Seed Germination Protocol at *Medicago truncatula* resource (www.medicago.org). Seedlings with roots between 2-5 cm were placed in square Petri dishes (12cm x 12cm) containing a sheet of glass fiber filter paper (GFFP; Whatman International, Kent, UK) and perlite as the substrate. When seedlings presented at least one true leaf, they were inoculated with *Orobanche crenata* Forsk. seeds (Córdoba population) previously surface-sterilized with formaldehyde according to González-Verdejo et al. [6]. They were stored in the dark at 20ºC for eight days to promote conditioning of the seeds necessary for germination. Three mL of a 0.034 mM solution of the synthetic germination stimulant GR24 was applied [9]. Dishes were sealed with parafilm, covered with aluminium foil to prevent roots and broomrape seeds from the light, placed vertically with the germinating host plant upwards in trays with Hoagland nutrient solution and kept in the growing chamber at 20ºC with 14 h light. Two independent experiments using thirty plants per experiment were performed. Fifteen of theses plants were inoculated and the other fifteen were used as non-inoculated controls.

5.2 Sample collection and RNA isolation

Whole root samples from control and infected *M. truncatula* plants were harvested at different time points according to Die et al. [4]. Briefly, 15 days post-inoculation (dpi),

365 the *O. crenata* radicles contacted the host root and adhered to it through the formation
366 of an attachment organ; 21dpi, the outer part of the seedling developed into a tubercle
367 once a physiological bridge between the vascular system of the two plants was formed;
368 35dpi, prior to necrosis, most of developed parasite tubercles (*Figure 1A*). Collected
369 samples were immediately frozen in liquid nitrogen. Total RNA samples isolated from
370 roots were prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according
371 to manufacture's protocols from different pools of five roots (both infected and control
372 roots) to minimize variation in gene expression among the individual plants. The
373 resulting RNA preparations were stored at -80ºC until use. The integrity of total RNA
374 was checked on 2% (w/v) agarose gels, and its quantity and purity were determined by
375 an optical density reading at 260nm and the OD_{260}/OD_{280} absorption ratio using a
376 Nicolet Evolution 500 spectrophotometer (Thermo Electron Corporation, Cambridge,
377 UK).
378
379 *5.3 cDNA synthesis and Quality Control*
380 Total RNA (1µg) was reverse-transcribed using the QuantiTec Reverse Transcription
381 Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. The
382 absence of genomic DNA contamination was subsequently confirmed by PCR using
383 primers (5'-GTCCTCTAAGGTTTAATGAACCGG-3' and 5'-
384 GAAAGACACAGCCAAGTTGCAC-3') designed on the intron sequence of the
385 reference gene *ubiquitin* (*UBQ* TC102473; *M. truncatula* Gene Index Project, MtGI,
386 http://compbio.dfci.harvard.edu/tgi/) [14]. The efficiency of cDNA synthesis was
387 assessed by real-time PCR amplification of the *UBQ* control gene (5'-
388 GCAGATAGACACGCTGGGA-3'; and 5'-AACTCTTGGGCAGGCAATAA-3';
389 primer efficiency 95.26% ± 3.26%, amplicon length 120bp). The 5'/3' ratio of *UBQ*

cDNA was determined by real-time RT-PCR with two primer pairs that amplify in the 5' region or 3' region, according to $(1+E_{UBQ5'})^{C_T UBQ5'}/(1+E_{UBQ3'})^{C_T UBQ3'}$, where E represents the PCR efficiency for the 5' and 3'primers, and C_T is the threshold cycle values for both primers, approximately $2^{\Delta C_T(UBQ5'-UBQ3')}$. Ratios in the range of 1-2 are considered satisfactory, and the cDNA is suitable for RT-PCR analysis [14]. This is an important control step prior to downstream quantification assays to overcome erroneous expression ratios when different 3'/5' ratios are compared, when the amplicons are not located immediately upstream to the RT initiation site or when the amplification site is unknown. Two other internal control genes were used for comparing transcript levels, *elongation factor 1-alpha* and *pentatricopeptide repeat* (*MtEf-1a* TC106485; *PPR* TC96273; MtGI).

5.4 Real-Time RT-PCR Conditions and Data Analysis

Polymerase chain reactions were performed in a 96-well plate with an Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) using SYBR Green to monitor dsDNA synthesis. Reactions contained 0.5 µl 50x SYBR Green Solution, 12.5 µl 2x SensiMix (dT) (Quantace, London), 2.5 µl of cDNA and 200 nM of each gene-specific primer in a final volume of 25 µl. A master mix of sufficient cDNA and SYBR Green Solution was prepared prior to dispensing into individual wells to reduce pipetting errors and to ensure that each reaction contained an equal amount of cDNA. An electronic Eppendorf multipipette was used to dispense the cDNA-containing master mix. The following standard thermal profile was used for all PCR reactions: polymerase activation (95°C for 10 min) was followed by amplification and quantification cycles repeated 40 times (95°C for 30 sec, 55°C for 1 min, 72°C for 30 sec). All RT-PCR runs contained negative controls with no cDNA template in order to exclude or detect possible contamination. The specificity of the primer pairs was checked by melting-

curve analysis performed by the PCR machine after 40 amplification cycles (55 to 95 ºC with one fluorescence read every 0.5 ºC).

Data were analyzed using the Mx3000P analysis software version 3.00 (Stratagene). All amplification plots were analyzed with an R_n threshold of 0.035 to obtain C_T values for each gene-cDNA combination. In order to compare data from different PCR runs or cDNA samples, gene expression levels for each cDNA sample were normalized to the internal control gene *UBQ*, which was the most constant of the three reference genes included in each PCR run. The average C_T value for *UBQ* was 23.22 (±0.74) for all plates/templates measured in these experiments. The gene-specific primers used for RT-PCR (*Table I*) were designed with a calculated Tm of 53 ± 0.5ºC, amplification products not larger than 380bp, and the primer sequences were unique in the MtGI. The PCR efficiency of each primer pair in each individual reaction was estimated from the data obtained from the exponential phase of each individual amplification plot and the equation $(1+E)=10^{slope}$ [23]. Primer efficiency values with an R^2 value less than 0.997 were ignored. The expression levels of each gene of interest (GOI) relative to the reference gene *UBQ* were calculated for each cDNA sample using the equation: relative ratio $_{GOI/UBQ} = (E_{GOI}^{-CT_{GOI}})/(E_{UBQ}^{-CT_{UBQ}})$ as described by McGrath et al. [17]. The values of six control and six infected samples (from the two independent experiments) were used in a Student's *t*-test to calculate the probabilities of distinct induction or repression, and the average ratio of these values was used to determine the change in transcript level in inoculated samples compared with control samples.

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Figure Legends

Figure 1. Morphological changes during parasitization process. *Orobanche crenata* parasitizing roots of *M. truncatula* **(A)** 15, **(B)** 21 and **(C)** 35 days post-inoculation. (s) *Orobanche* seed; (rd) *Orobanche* radicle; (h*) Medicago* roots; (ti) initial stage of tubercle formation; (t) well-developed parasite tubercle.

Figure 2. Transcriptional changes during *Orobanche* parasitization. Gene expression levels in infected roots on the basis of real-time RT-PCR experiments relative to the level of *UBQ* gene. The values above the graph show the ratios in transcript abundance in inoculated vs. control roots. Bold text indicates statistically significant induction ($P \leq 0.05$). Roman numerals represent functional categories according to Journet et al. (2002): (I) Cell wall; (III) Membrane transport; (VI) Secondary metabolism; (X) Signal transduction; (XII.A) Defense and cell rescue; (XII.B) Abiotic stimuli; (XII.C) Unknown function; (XIII) No homology. Symbols in panels represent the mean and SD of two independent experiments.