Expression analysis of *Pisum sativum* putative defence genes during *Orobanche crenata* infection

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Abstract

The root holoparasitic angiosperm *Orobanche crenata* is a severe constraint to the cultivation of legumes. Breeding for resistance is a difficult task. Understanding the mechanisms underlying host resistance is a fundamental issue for the genetic improvement of legumes. In this work, the temporal expression patterns of 8 defence-genes known to be involved in different metabolic pathways activated during several plant-pathogen interactions were investigated in *Pisum sativum*. Molecular analyses were carried out using quantitative real-time polymerase chain reaction during the initial stages of the parasitization process in susceptible (Messire) and incomplete resistant (Ps624) pea genotypes. Transcriptional changes in response to *O. crenata* revealed induction of genes putatively encoding pathogenesis-related proteins, peroxidase activity and dehydration stress-responsive signalling. This, combined with high constitutive gene expression mediating-phenylpropanoid pathway were observed as part of the defence mechanisms triggered in Ps624 genotype to restrict the growth of the parasite.

Keywords : parasitic plants, plant defence, Real-Time PCR

Introduction

Crenate broomrape (*Orobanche crenata* Forsk.) is a holoparasitic weed that seriously attacks legume crops, such as faba bean, lentils, chickpea and vetch. This parasitic plant is potentially the major constraint for *Pisum sativum* cultivation in the

Mediterranean area and Middle East. The only minor levels of incomplete resistance available in commercial cultivars and the lack of a suitable control method has relegated pea cultivation in infested areas (Pérez-de-Luque *et al.* 2005a).

Genetic resistance remains today as one of the most desirable components in an integrated control strategy. Resistance in strict sense indicates processes which prevent establishment of the parasite. However, resistance to *O. crenata* in legumes is a complex multicomponent event with low heritability making breeding for resistance a difficult task (Rubiales 2003). A detailed knowledge of the mechanisms underlying such resistance during the host-parasite interaction or the incomplete resistance that reduce the negative effects of the parasite on crop yield is necessary to improve breeding programmes. However, despite the enormous economic impact of this disease little is known about the molecular background of this legume-parasite interaction. Initial screening in pea germplasm led to the identification of valuable sources of resistance (Rubiales *et al.* 2005). Histological studies have revealed lignification of host endodermis and occlussion of host vessels as main mechanisms to prevent parasite intrusion at early infection stages during incompatible reactions (Pérez-de-Luque *et al.* 2005b). But so far, studies regarding the dissection of changes in gene expression in parasitized plants and the molecular bases of resistance remain at very preliminary stages. Advances in the knowledge of gene expression in infected roots was initiated demonstrating the specific activation of the PR-1 (pathogenesis-related) and HMGR (3-hydroxy-3-methylglutaryl Coenzyme A reductase) gene promoters during the tobacco defence response to *O. aegyptiaca* [Joel and Portnoy 1998; Westwood *et al.* 1998). Recently *in situ* hybridization techniques have shown the expression of a peroxidase and a β–glucanase involved in resistance [Pérez-de-Luque *et al.* 2006a]. The use of model plants in transcriptional profiling studies is gaining insight into the molecular

regulation of plant-parasitic plant interactions [Vieira Dos Santos *et al.* 2003; Die *et al.* 2007]. Comparative mapping studies have demonstrated a high degree of sinteny between *Medicago truncatula* and pea [Choi *et al.* 2004]. But until now, the transfer of knowledge obtained from model plants to crop legumes has been limited. Target gene approaches based on the knowledge gained from these systems allow the identification of orthologous genes involved in pea defence against *Orobanche* being helpful for crop improvement toward resistance.

Based on data obtained for the model legume plant *M. truncatula* [Die *et al.* 2007] and a previous related publication on pea [Pérez-de-Luque *et al.* 2006a] we focused on the gene expression pattern in roots during the initial stages of the parasitization process from the early contact with *Orobanche* radicles to the well-developed parasite tubercle formation, leading to a detailed temporal expression analysis of eight putative defence genes in pea. Our data is discussed and compared with those previously obtained through hystological and transcriptomic analysis of other plant-parasitic plant systems.

Materials and methods

Plant material and inoculation

The susceptible *P. sativum* cv. Messire and incomplete resistant accession Ps624, were selected based on previous experiments (Rubiales *et al.* 2005). A Petri dish assay was carried out according to Pérez-de-Luque *et al.* (2005a): seeds of *P. sativum* were germinated in filter paper and kept in the dark at 20ºC for 5 days. Seedlings with roots between 5-7 cm were placed in squared Petri dishes (12cm x 12cm) containing a sheet of glass-fibre filter paper (GFFP; Whatman International, Kent, UK), and perlite as substrate. When seedlings presented at least one true leaf, they were inoculated with

O. crenata seeds at a density of ~50 seeds $cm^{-2,}$, collected from infested faba bean fields in Córdoba. The synthetic germination stimulant GR24 was applied by adding 3 mL of a 10 ppm solution. *O. crenata* seeds had previously been surface-sterilized (González-Verdejo *et al.* 2005) and stored in the dark at 20 ºC during 8 days to promote conditioning. Dishes were sealed with parafilm, covered with aluminium foil and stored vertically in trays with Hoagland nutrient solution (Hoagland and Arnon, 1950). The plants were maintained in a growing chamber at 20ºC, 14 h photoperiod and irradiance of 200 μmol m^{-2} s^{-1}. Two serial experiments using thirty plants per experiment and genotype were performed. Fifteen plants were infected and the other 15 used as non-infected controls.

Sample collection and nucleic acids isolation

Observations on host-parasite development were taken every week by using a binocular microscope (Nikon SMZ1000; Nikon Europe BV, The Netherlands). Samples from control and infected *P. sativum* whole roots were harvested at: 15 days post-inoculation (dpi), the *O. crenata* radicles contact with the host roots before the attachment; 21dpi, initial stage of tubercle formation once the vascular systems of the two plants are connected; 35dpi, prior to necrosis of most of the developed tubercles in Ps624 genotype. In order to avoid contamination with parasite tissues, host roots were abundantly washed with distilled water and blot dried with filter paper. The most of parasite tubercles from root samples collected at 21 and 35 dpi were carefully removed with a scalpel. Collected samples were frozen in liquid nitrogen. Total RNA samples were isolated from roots (0.1 g) using TRIZOL reagent (Invitrogen, Carlsbad, USA) according to manufacture's protocols from different pools of five plants in order to minimize variation in gene expression among individual plants in both, infected and

non-infected control samples. The integrity of total RNA was checked on 2% (w/v) agarose gels and its quantity as well as purity was determined by an optical density at 260nm and A_{260}/A_{280} absorption ratio using the BioPhotomer (Eppendorf, Germany). Genomic DNA from plants was isolated according to Torres *et al.* (2005) and used for PCR amplification with degenerated primers.

Sequence information and primer design

First, to identify *P. sativum* orthologous of *M. truncatula* defence-related genes, we queried pea ESTs database from the GenBank. Second, specific peroxidase and glucanase primers were derived from *P. sativum* peroxidase (GenBank accesion no. AF396465) and *P. sativum* glucanase (Chang *et al*. 1992). Third, since no pea cellulose synthase sequence was available in databases, a degenerated primer-based strategy was used. The design of the degenerated primers was based on five putative cellulose synthase cDNAs from *M. truncatula*, *Arabidopsis thaliana*, *Eucalypus grandis*, *Vitis vinifera* and *Gossypium hirsutum*. Polymerase chain reaction was performed with primers CellS1 5'-GNTGAYCCNYTNAARGARCC-3' and CellS2 5'-TTRCARAANGGANCCCAYTT- 3' in a reaction volume of 25 µl using a template 1 µl of genomic DNA. The cycling conditions were: 94 ºC for 35 s, 59 ºC for 35 s and 72 ºC for 1 min for 40 cycles. The amplified 179 bp fragment was cloned into the pGEM-T vector system (Promega, USA), sequenced and submitted to the GenBank database under accession no. EU681279.

Finally, the gene-specific primer sets used for real-time reverse transcription (RT)-PCR were designed with a calculated Tm of 60 ± 0.5 ºC, GC% between 20% and 80% and amplification products not larger than 100 bp (Table 1**)**.An orthologous of the *M. truncatula* elongation factor-1α (*ef-1α*, TC106845, The Institute for Genomic

Research; TIGR) was used as constitutively expressed gene for transcript normalization with primers *efa1* 5'-AAGCTAGGAGGTATTGACAAG-3' and *efa2* 5'-ACTGTGCAGTAGTACTTGGTG-3'.

Two step real-time RT-PCR

Total RNA (1µg) was reverse-transcribed using the QuantiTec Reverse Transcription Kit (Quiagen, Germany), according to the manufacturer's instructions. Genomic DNA was eliminated during this procedure by RNase-free DNase I treatment. In order to ensure equal starting cDNA amounts, real-time PCR amplification of *ef-1α* was run for all different templates and depending on the C_T (threshold cycle), three-fold to ten-fold serial dilutions of cDNA were prepared to obtain similar C_T values for products due to equal starting amounts of cDNA, before initiating real-time PCR experiments. Polymerase chain reactions were performed in a 96-well plate with a Mx3000P Real-Time PCR System (Stratagene, 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. The following standard thermal profile was used for all PCR reactions: polymerase activation (95 °C for 10 min), amplification and quantification cycles repeated 40 times (95°C for 1min, 60°C for 1min). Each measurement was performed in triplicate and the C_T was determined.

Verification of amplified products

Specificity of the primer amplicons was checked by melting-curve analysis performed by the PCR machine after 40 amplification cycles (60 to 95 ºC with one

fluorescence read every 0.6 ºC). All investigated RT-PCR products that showed only single peaks and no primer-dimer peaks or artifacts were considered for further analysis. In order to confirm the plant origin of the transcripts, amplification products were checked on 2% (w/v) agarose gel using cDNA from Messire infected roots (21dpi) and cDNA from *Orobanche* nodules (21dpi) developed in Messire plants. A primer pair was used as *Orobanche* expressed control gene *ocr1* 5'-GTCTGCAGTAGTATGTTGCAT-3' and *ocr2* 5'-GACAAATTCCTCAAAATCTTC-3'.

Data analysis

Data were analysed using the Mx3000P analysis software version 3.00 (Stratagene, USA). All amplification plots were analysed with an R_n threshold of 0.035 to obtain C_T values for each gene-cDNA combination. The PCR efficiency (E) 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 $(1+E)=10^{slope}$ (Ramakers *et al.* 2003). Primer efficiency values with an R^2 value less than 0.997 were ignored. The expression levels of the gene of interest (GOI) relative to the *ef-1α* were calculated for each cDNA sample using the equation: relative ratio $_{GOI/ef\text{-}1\alpha}$ = (E_{GOI} $^{-CT_{GOI}}$)/($E_{ef\text{-}1\alpha}$ $^{-CTef\text{-}1\alpha}$). The values of six infected and six control samples (from the two independent experiments) were used in a Student's *t* test to calculate probabilities of distinct induction or repression and the average ratio of these values was used to determine the fold change in transcript level in infected samples compared with non-infected control plants as described by McGrath *et al.* (2005).

Results

The susceptible *P. sativum* cv. Messire and the incomplete resistant accession Ps624 were selected and used to monitor the transcript accumulation of genes encoding several defence-related proteins assayed by real-time reverse transcription (RT)-PCR strategy. Real-time PCR reactions resulted in a single product with the specific temperature shown in Table 1. Amplification products were obtained using cDNA from pea root tissues but no products were detected using cDNA from *O. crenata* nodules, proving that the gene expression observed was transcribed in roots of *P. sativum* (Fig. 1). The different mechanisms of resistance to *O. crenata* in Ps624 accession were reflected by a low number of established tubercles per plant that presented a delay in tubercle development in accordance with previously characterized differences in resistance to broomrape (Castillejo *et al*. 2004; Pérez-de-Luque *et al.* 2005a). Moreover, most of the tubercles formed became necrotic (Table 2) and died 35 days after inoculation (Fig. 2).

Gene expression patterns in susceptible pea genotype

The penetration of *O. crenata* radicles, parasite attachment and further development of tubercles into host roots led to a transient induced of selected genes during the three time-points addressed shown in Table 3. The induction of a gene encoding a dehydrin-like protein (*dhl*) exhibited a 1302.92-fold difference in expression level in infected Messire roots at 35dpi compared with the corresponding controls, which was the highest difference expression level measured in this study. A remarkable higher level of glutathione S-tranferase gene (*gst*) was detected during the initial

contacts with the *Orobanche* radicles (15dpi) and the developed tubercles stage (35dpi) in infected Messire plants when compared to infected Ps624 plants (Fig. 3).

Gene expression patterns in incomplete resistant pea genotype

Significant induction in Ps624 accession ranged from at least two-fold and up to 22-fold difference in expression level between infected and control plants. Genes identified more than 2-fold change relative expression in resistant compared with susceptible genotype were tentatively classified as associated with the molecular resistant response. A distribution of the ratios [(Ps624 infected/*ef-1α*)/(Messire infected/*ef-1α)*] using the 2-fold cutoff is shown in Fig. 3. Expression levels were higher in Ps624 for all transcripts analysed at least in one of the time points studied except for glutathione S-tranferase gene (*gst*) which showed remarkable higher level in Messire plants 15 and 35dpi and the hypersensitive reaction 203J gene (*hsr203J*) which was 4.73-fold difference 35dpi in infected Messire compared to infected Ps624 plants.

Interestingly the highest comparative expression level detected in Messire genotype for the *dhl* gene (1302.92-fold difference between infected and non-infected plants) did not reach the relative level observed in Ps624 that showed a 22.39-fold change up-regulation in infected plants. Thus, in spite of this high up-regulation in both genotypes, the *dhl* gene was induced finally to similar levels in both infected genotypes (Fig. 3).

Discussion

In the present work, a molecular approach to compare the expression patterns of some defence-related genes known to be expressed in response to parasitic plants infection was addressed by RT-PCR strategy. Two pea genotypes differing in their

sensitivity to *O. crenata* were selected and used to monitor the gene expression patterns from the earliest contact with *Orobanche* radicles to the well-developed parasite tubercle.

The role of hypersensitive-like reaction (HR) in resistance of legumes to *O. crenata* has been debated in the past on the basis of the appearance of necrotic lesions during the interaction with vetch (Goldwasser *et al.* 1997) or chickpea (Rubiales *et al.* 2003). Pérez-de-Luque et al. (2005b), have shown that unsuccessful penetration of *O. crenata* seedlings during the initial steps in the interaction and the necrosis of the established tubercles cannot be attributed to cell death in the host. In this study, we evaluated transcript accumulation of *hsr203J* gene, usually employed as molecular marker of the hypersensitive response (Gopalan *et al*. 1996; Pontier *et al*. 2001). Induction of *hsr203J* was detected in both infected genotypes at 15dpi during the first contacts between host and parasite. However, the up-regulation was maintained throughout all the experiment only in Messire infected plants when no significant death or darkened tubercles were observed. This, casts doubt on the active role of *hsr203J* as a resistance mechanism. In this sense, some authors have suggested that *hsr203J* would be a negative regulator of the HR (Tronchet *et al*. 2001; Nasir *et al.* 2005). This protein might function as a scavenger for ROS-derived compounds (Tronchet *et al*. 2001) produced by an oxidative burst following parasite penetration into host roots. Oxidative stress, in the absence of HR, has already been shown during the interaction *A. thaliana*-*O. ramosa* (Vieira Dos Santos *et al*. 2003). It may be hypothesized that oxidative stress is induced by the penetration of *Orobanche*, generated upon the cell-wall degradation of the host cells during the compatible reaction. According to this model, Messire responds probably to the infection by both, a detoxification mechanism involving *gst* and induction of *hsr203J* implicated in cell protection.

However, comparative analysis of regulated genes revealed quantitative and qualitative differences in the gene expression profiles between the two infected genotypes (Fig. 3). Genes identified more than 2-fold change expression in resistant compared with susceptible genotype were tentatively classified as associated with the molecular resistant response. An early induction 15dpi was detected for a ripening-related protein with a domain for Bet v I allergen belonging to a group of protein family including pathogenesis-related protein of the PR-10 group (Moiseyev *et al.* 1997). Inducible expression, RNase activity and ligand-binding activities have linked Bet v I allergen to plant defence as well as to abiotic stress (Samac and Graham 2007). A recent work, using a proteomic approach to investigate the *M. truncatula–O. crenata* interaction, led to the identification of Bet v I allergen associated to resistance (MA Castillejo, unpublished data). Another observation from 15dpi was the accumulation of peroxidase transcripts which persisted at 21dpi. There is strong evidence supporting the implication of peroxidases in plant resistance to parasitic plant (Goldwasser *et al.* 1999; Vieira Dos Santos *et al.* 2003; Castillejo *et al.* 2004). The formation of protein cross-links of the cortical cell walls have been suggested to be involved in resistance (Echevarría-Zomeño *et al.* 2006; Pérez-de-Luque *et al.* 2006a). Thus, the peroxidase activivity induction observed is likely to be implicated in cell wall reinforcement through oxidative cross-linking of structural proteins conferring mechanical barriers to the invading parasite.

Combined with the physical barriers, the induction of several genes mediating other mechanisms of resistance takes place after the vascular connections have been established. Since *Orobanche* must overcome such activated barriers, this could explain first, the delayed development of the few established individuals and finally, the death of the tubercles. In this sense, there was a notably up-regulation of *dhl* gene in Ps624

(11.34-fold induced, the most strongly *Orobanche*-induced gene in this genotype at 21dpi). Dehydrins are members of a protein family expressed during dehydration-stress and have been identified in a range of species including pea (Roberton and Chadler 1992). Although their specific role remains challenging areas for further study, this protein might comprise part of the alterations in host metabolism necessary to overcome the water deficiency caused by the parasite.

However, the accumulation of *dhl* transcripts cannot explain the necrosis of *O. crenata* tubercles. Two main factors have been suggested to be involved in resistance : vessel occlusion (Pérez-de-Luque *et al.* 2005b; Pérez-de-Luque *et al.* 2006b) and/or accumulation of toxic compounds such as phenolics (Serghini *et al.* 2001; Echevarría-Zomeño *et al.* 2006). Peroxidases are known to be involved in the cell wall modification discussed above but also appear to be implicated in this late resistance form. The peroxidases polymerize polysaccharides and polyphenols to produce stable vascular occluding gels (Crews *et al.* 2003). Recent reports have related vessel oclussion in pea resistance to high peroxidase activity (Pérez-de-Luque *et al.* 2005a; Mabrouk *et al.* 2007). The increase in abundance of transcript for peroxidase 21dpi (11.88-fold difference, the most strong difference between infected genotypes at this time-point) seems to highlight the important role that this enzyme plays in defence against *Orobanche*. Curiously three genes not up-regulated in the resistant genotype showed higher relative expression values in Ps624 when the two genotypes were compared. Chalcone synthase is located in the phenylpropanoid pathway leading to synthesis of phenolic compounds or phytoalexins production. The derived products may confer mechanical and chemical barriers to *Orobanche* suggesting the important role of the phenylpropanoid pathway in the elicited defence (Griffitts *et al.* 2004, Pérez-de-Luque *et al.* 2006a; Echevarría Zomeño *et al.* 2006; Lozano *et al.* 2007). Cellulose synthases

are responsible for the biosynthesis of one of the principal polysaccharides of the cell wall and the role in defence of β-glucanases has been pointed out by releasing oligosaccharides elicitors (Esquerré-Tugayé *et al.* 2000). Increased levels of cellulose synthase and β-glucanase have been detected in *Medicago* and pea resistant to *O. crenata*, respectively (MA Dita, pers. comm.; Castillejo *et al.* 2004). Although no up-regulation was observed in infected plants, high expression levels in Ps624 might suggest that a higher constitutive level for some transcripts expression in the incomplete resistant genotype could help the plant in priming defence reactions against pathogens more rapidly.

All these mechanisms are based on the assumption that the host recognizes the pathogen and reacts against it. The induction of *dhl* or *gst* genes as early as 15dpi in the susceptible genotype demonstrates that the parasite is apparently detected and defensive mechanisms are activated. But this raises the question as such a response is too slow or ineffective to prevent the *Orobanche* development. It has been related a delayed response to reduced input into the plant signal recognition system (Tao *et al.* 2003) or an active process of defence genes supression (Caldo *et al*. 2004). So far, there is no convincing evidence that parasitic plants suppress the response of the host. The perceived signal input is greater in incompatible reactions (unsuccessful attachment-penetration and darkening of established tubercles) and therefore the output signal is greater. The observation that gene activation in the host does not mount an effective defence against *Orobanche* might indicate that the invasion is recognized only partially.

Conclusion and remarks

This work describes a first transcriptomic approach with the aim to study gene expression patterns in *P. sativum* after infection with the parasitic plant *O. crenata.* The

complexity of resistance to parasitic plants in legumes is a consequence of the coordinated induction of several mechanisms. Following invasion of *P. sativum* tissues by *O. crenata*, a range of defence mechanisms are triggered to restrict their growth. Induction of defence genes in host plants is underlying the perception of the parasite by the host, even in the case of compatible reaction. But gene activation in this case is not sufficient to result in host resistance. If the transcript inductions observed are expressed as functional proteins, the defence response comprises reinforcement of cell walls, activation of pathogenesis-related proteins and phenylpropanoid pathway. Upregulation of genes involved in this mechanisms combined with high constitutive expression values determines a more effective defence against the parasite. Further experiments have to be done to understand the biological function of genes involved in the basic mechanisms governing resistance to parasitic plants. Understanding the function of genes plays an essential role in the characterization of disease processes and this will be of great importance in directing pea breeding programmes and developing resistant crops.

Acknowledgments

The authors acknowledge the financial support provided by the Spanish project INIA2007-0009.

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FIGURE LEGENDS

Fig. 1. Transcript accumulation of (1) cellulose synthase, (2) hsr203J, (3) peroxidase, (4) glutathione S-transferase, (5) chalcone synthase, (6) dehydrin-like protein, (7) β-glucanase and (8) ripening-related protein genes in cDNA from *P. sativum* infected roots and *O. crenata* nodules. A control *O. crenata* expressed gene (9) was used. No amplification products appeared using cDNA from *O. crenata* demonstrating that transcripts detected are of *P. sativum* origin.

Fig. 2. Parasitization process in the dish system. **(A)** Radicles (r_d) of germinated *O. crenata* seeds (s) contacting with susceptible Messire roots (h) 15dpi. **(B)** Initial stages of tubercle formation (t_i) in Messire roots 21dpi. **(C)** Developed *O. crenata* tubercles (t) in Messire roots showing (f) initial floral spike formation 35dpi. **(D)** Necrotic *Orobanche* tubercle in incomplete resistant Ps624 roots (h) 35dpi.

Fig. 3. Transcriptional changes in parasitized *P. sativum* roots. A distribution of the normalized expression (Ps624 inoculated/*ef-1α*) *vs.* (Messire inoculated/*ef-1α*) is shown. Ratios between the two infected genotypes statistically significant ($P \leq 0.05$) are presented.

TABLE LEGENDS

Table 1.
Primer sequences used in real-time PCR for amplifying defence-related genes in *Pisum sativum*.

[a] unique sequence for primer design

Table 2.
Tubercle number and % necrotic tubercles of *O. crenata* on pea roots at 35 days post inoculation in Petri dish assays. Data shown as mean ± SE. Values are mean of 10 replicates in two independent experiments.

[a] Included the S2 (crown-roots start to develop), S3 (bud <1 cm) and S4 (first development of floral spike) developmental stages according to ter Borg *et al.* (1994).

Table 3.
Gene expression patterns in *P. sativum* roots on the basis of real-time RT-PCR experiments. Values shown indicate average relative expression ratio to control (average data from two independent experiments with three technical replicates). Bold text indicates statistically significant induction ($P \leq 0.05$).

n.d. = no products detected using cDNA of non-infected or infected roots.