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RESEARCH PAPER

DeepSuperSage analysis of the *Vicia faba* transcriptome in response to *Ascochyta fabae* infection

Eva MADRID[1,2], Carmen PALOMINO[1], Anne PLÖTNER[3], Ralf HORRES [3], Björn ROTTER[4], Peter WINTER[4], Nicolas KREZDORN[4] and Ana M. TORRES[1]

[1] Área de Mejora y Biotecnología, IFAPA-Alameda del Obispo, Apdo 3092, 14080 Córdoba, Spain
[2] Institute for Sustainable Agriculture, CSIC, 4084, E-14080 Córdoba, Spain
[3] Molecular BioSciences, Biocenter, Johann Wolfgang Goethe University, Max-von-Laue-Str. 9, D-60438 Frankfurt, Germany
[4] GenXPro GmbH, Altenhöferallee 3, 60438, Frankfurt am Main, Germany

Summary. Understanding the host response to *Ascochyta fabae* in faba bean (Vicia *faba* L.), is crucial to elucidate the biology of host resistance. In an attempt to unravel the faba bean – *A. fabae* interaction, we performed genome-wide transcriptome profiling by deepSuperSAGE that quantified the early transcriptional changes elicited by the fungus in the resistant 29H faba bean genotype. The total number of 26 bp tags obtained was 1,313,009, of which 51,484 were unique sequences (UniTags) and 161 of them corresponded to fungal sequences. Sequences with a full match of the 26 bp revealed 2,222 tags with a significant P-value that were expressed differentialy between inoculated and control leaves. After gene ontology (GO) annotation, 2,143 of these matched to databases sequences (approximately 1/3 into each GO domain). At a 2.7-fold change threshold, 1,197 sequences were significantly differentially expressed in infected as compared to control leaves. Of these, nearly half were up- and the other downregulated. The most enriched GO terms corresponded to tags related with photosynthesis metabolism or structural components. Ten of them can be associated with plant defense, due to their association with responses to the jasmonic acid pathway, pectin esterase activity or gene silencing. Validation of the SuperSAGE data by qPCR of ten differentially expressed UniTags confirmed a rapid increase or decrease in mRNA 8 to 12 hours after inoculation in most of the up-regulated tags and, less consistently, in the downregulated ones. This study represents the most comprehensive analysis of the Ascochyta-response transcriptome of faba bean available to date. The applicability of these tags will increase as more faba bean genomic and cDNA sequences become available.

Key words: SuperSage, Ascochyta, faba bean, transcriptome.

Introduction

Faba bean is a high protein cool season grain legume widely used in human nutrition and livestock feeding. As a legume crop, faba bean supplies an important added value by fixing atmospheric nitrogen in symbiosis with soil bacteria, thus reducing costs and minimizing impact on the environment. Widely distributed in temperate environments around the globe, faba bean is a staple in the diets of many societies in the Middle East, Central and East Asia and North Africa. Traditionally grown in the Mediterranean basin, Latin America and also in northern Europe, faba bean has recently received an increased interest in Northern America and Australia.

According to FAO, all food legume crops have seen increased yields in the last five years. Among them, faba bean has experienced a 14% relative increase, with an average global yield of 1.7 tons ha^{-1}, mostly in environments with irrigation and good agronomic practices. Today, China is the largest producer, followed by North African countries and Australia. Developed countries rank second, after China, in total world production of faba beans. In develop-

Corresponding author: E. Madrid
Fax: +34 957 218503
E-mail: b62mahee@uco.es

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ing countries, however, the growth rate over the last 15 years was only 0.5% per year and, unlike in other legume crops, there is a big yield gap in the range of 3 tons/ha between the top faba bean growing countries and the developing world (Akibode and Maredia, 2011).

Although the agronomic and economic importance of the crop is well demonstrated (faba bean is the fourth biggest traded pulse crop in the developing world), the overall picture for faba bean cultivation has declined in the last years due to different factors such as the high susceptibility to diseases and pests (Akibode and Maredia, 2011). Improvement of yield performance through germplasm enhancement and crop management is a must to contribute substantially to cover nutritional requirement and food security particularly in the developing world.

Faba bean grain production is affected by many pathogens, which vary in incidence and severity from one region to another. Besides crenate broomrape, a very aggressive parasitic angiosperm in the Mediterranean basin, losses in faba bean are mostly caused by aerial and soil fungi. One of the most important diseases is ascochyta blight caused by *Ascochyta fabae* Speg., which is distributed worldwide causing up to 90% yield losses in susceptible cultivars when environmental conditions are favourable for disease development (Sillero *et al*. 2010). Blight symptoms, as dark-brown spots, occur on leaves, stems and pods of infected plants. Developed lesions can penetrate the pod and infect developing seeds causing brown stains, which considerably reduce the market value. The fungus can survive on crop debris, self-sown volunteer plants and infected seeds, and the disease spreads by rain splash and wind-borne spores. Thus, ascochyta blight resistance represents a major constraint to increasing faba bean yield and is a key objective in faba bean breeding programs.

Several sources of resistance to *A. fabae* have been described (Tivoli *et al*. 1988; Hanounik and Robertson, 1989; Sillero *et al*. 2001), and both polygenic and oligogenic inheritance have been suggested (Hanounik and Robertson, 1989; Rashid *et al*., 1991; Kohpina *et al*., 2000). However, the complexity of the resistance and the lack of detailed knowledge about the loci involved in its control have prevented the development of cultivars with durable resistance by classical breeding methods. Because the development of resistant cultivars is challenging, understanding the molecular mechanisms underlying ascochyta resistance is needed for successful knowledge-based faba bean crop improvement.

Faba bean is an understudied crop with one of the largest genomes among legumes (~13,000 Mb), which complicates the development of saturated linkage maps and the identification or location of important genes. Genetic maps are nevertheless available, and progenies from two segregating faba bean crosses have been used to identify genes affecting ascochyta blight resistance. A polygenic control determined by at least two QTLs (Quantitative Trait Loci) with significant proportions of the resistance has been reported and validated in multi-environment trials (Roman *et al*., 2003; Avila *et al*., 2004). Interestingly, one of the QTLs assigned to chromosome III appears to be stable in different genetic backgrounds (Díaz-Ruiz *et al*., 2009).

The prospects for marker assisted selection (MAS) for ascochyta blight resistance in faba bean will depend on the discovery of the genes and regulatory pathways controlling resistance, as well as the development of allele-specific markers that will allow breeders to select the correct phenotype efficiently. DNA microchips yield enormous amounts of data linking cDNA sequences to gene expression patterns but, in the absence of such resources, expressed sequence tags (ESTs) provide an accessible tool for identifying genes expressed in a given pathogen-host interaction. ESTs libraries are limited in faba bean. Currently, no more than 5,510 faba bean ESTs are publicly available at the NCBI (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html, last update July 2012), compared to at least 44,157, 18,576, 9,513 ESTs available for chickpea, pea or lentil, respectively.

Recent advances in next-generation sequencing (NGS) combined with techniques for the quantification of gene expression are opening new avenues for genome-wide transcriptome studies and target gene identification in organisms from which massive nucleotide sequence information is not yet available. Based on the Serial Analysis of Gene Expression (SAGE) first described by Velculescu *et al*., (1995), SuperSAGE is an improved method of digital gene expression profiling, characterized by the generation of 26 bp tags through the type III restriction enzyme *Eco*P15I (Matsumura *et al*., 2005). SuperSAGE provides a powerful tag-based method for obtaining a quantitative profile of genes expressed in the biological material of interest. It allows absolute

quantification of mRNA abundance by counting the relative frequencies of individual tag fragments from expressed transcripts. This method is especially useful for expression profiling in organisms in which little genome information is available, like faba bean, because the 26 bp tag sequences enables tag-to-gene annotation with higher specificity than in the predecessor techniques SAGE or LongSAGE.

Combination of SuperSAGE with NGS allows detection and analysis of very low abundant transcripts that could play fundamental roles in a specific biological process. SuperSAGE was successfully applied in whole-genome transcript profiling of plant species such as rice (Matsumura *et al*., 2003), banana (Coemans *et al*., 2005), chili pepper (Hamada *et al*., 2008), chickpea (Molina *et al*., 2008, 2011), tobacco (Gilardoni *et al*., 2010) and tropical crops as cowpea, soybean or sugarcane (Kido *et al*., 2010).

SuperSAGE was recently used in within the framework of the ERA-NET Plant Genomics project: LEGRESIST (http://www.genxpro.info/science_and_technologies/Legresist/), with the aim of resolving the transcriptome of different legumes crops such as faba bean, chickpea, lentil and grass pea (*Lathyrus sativus* L.) during infection with their corresponding *Ascochyta* pathogens (Kahl *et al*., 2009). Here we report the results of the SuperSAGE approach, combined with massively parallel pyrosequencing on the Illumina GAII sequencing platform, for quantifying early transcriptional changes elicited by *A. fabae* in the resistant 29H faba bean genotype as compared to the non-infected tissue. The main objective was to identify genes differentially regulated in the response to pathogen perception. To confirm the reliability of SuperSAGE and identify candidate genes involved in ascochyta blight resistance, qPCR experiments for selected differentially expressed tag sequences were performed. The results provide a foundation for the identification of novel regulators associated with the ascochyta-faba bean interaction and of potential targets for molecular breeding in this crop.

Materials and methods

Plant material and *A. fabae* inoculation

The SuperSAGE analysis was carried out using the faba bean line 29H described as resistant to *A. fabae* in several studies (Tivoli *et al*., 1988; Maurin and Tivoli, 1992; Bond *et al*., 1994; Sillero *et al*., 2001). Seeds of the genotype 29H were pre-germinated and sown with three replicates, three plants each, in 14 cm-diameter pots, using a 1:1 mixture of sand and peat. All plants were grown in a controlled condition chamber at 20–22ºC. Half of the seedlings were inoculated when the third leaf was fully expanded, by spraying a suspension of *A. fabae* spores prepared with tap water (5×10^5 conidia mL^{-1}) with 0.03% (v:v) of Tween-20. A monoconidial isolate of *A. fabae*, CO99-01, originating from Córdoba, Spain, was used. Plants were incubated for 48 h in the dark at 100% relative humidity, and then kept at 20ºC, under a photoperiod of 14 h light and 10 h dark, with light intensity of 148 mmol m^{2-1} s^{-1} at the leaf canopy. Uninoculated replicated plants in the same conditions were included in the assay as controls. To confirm that *A. fabae* infection had been effective, inoculated plants were checked for expected disease symptoms at 15 days and compared to controls.

RNA isolation and construction of SuperSAGE libraries

Leaves were harvested at 4, 8 and 12 hours after inoculation. These harvesting time points were chosen because penetration and successful establishment of the disease in faba bean occurs rapidly after spore germination, with the minimum period of leaf wetness for infection being 4 h at 20–25ºC (Pritchard *et al*., 1989). Therefore, early defence responses occur immediately or shortly after contact with a pathogenic organism. Entire leaf tissue was taken from plants at these time points, and immediately frozen in liquid nitrogen. An identical collection of leaf tissue was accomplished in the non-inoculated lines.

Total RNA was extracted from separate tissue samples for each time point from both inoculated and non-inoculated plants using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The RNA was stored at -80ºC until use. RNA was quantified by the absorbance at OD 260 using Evolution 500 UV-visible spectrophotometer (Thermo Electron Corporation, San Josè, CA, USA). The absorbance ratio at OD260/280 and OD260/230 was used to assess the purity of all RNA samples. Only RNA samples with OD260/280 ratio (protein contamination ranging between 1.8 and 2.0) and OD260/230 (organic pollutant) higher than 2.0 were used for the further analysis. RNA integrity was verified on 2% agarose gel electrophoresis and ethidium bromide staining.

SuperSAGE libraries were produced at GeXPro GmbH, essentially as described by Matsumura *et al*. (2010), but with the implementation of GenXPros PCR-bias-proof technology "TrueQuant" to distinguish PCR copies from original tags. The TrueQuant method involves individual barcoding of each template-molecule prior to PCR, copies are identified by sequence-similarity of the barcode-tag combination. Sequencing was performed on an Illumina GAII machine.

Tags quantification and data analysis

For each library, 26 bp long tags were extracted from the sequences using the GXP- Tag sorter software provided by GenXPro GmbH, Frankfurt am Main, Germany. Tags were counted using a pearl script ("GenXProgram"). Differential expression of the tags and transcripts was calculated using a Bio_Sage script (pearl) (http://search.cpan.org/~scottzed/Bio-SAGE-Comparison 1.00/lib/Bio/SAGE/Comparison.pm), based on Audic and Claverie (1997). For fold-change (FC) calculations the libraries were normalized to 1,000,000 tags and the FC for each tag was calculated by dividing the number of tags in the normalized inoculated library (I) by the number of tags in the normalized control library (C) (I vs. C). Tags absent in one of the libraries (tag count = 0) were set to 0.05 for calculation of the fold-change.

Sequence homology alignment

The 26-bp tags were annotated to the following databases in the respective order: Vicia_29_136_uniprot_viridiplantae_annotation (Assembled data of normalized cDNA), MTGI.032511_3'CATG (TIGR Medicago), MTGI.032511, all_TIGR_DFCI_PLANT_3'CATG and NCBI_ENTREZ_FUNGI_REFSEQ. For each database, a trimmed version termed "3'GATC" was first established, limiting the entries to the last 3' CATG-motif until the very 3'end, which is the most likely site at which the SuperSAGE tag is produced. When no annotation to the 3'-CATG- database was possible, the untrimmed database was used for annotation. All_TIGR DFCI_PLANT_3'CATG corresponds to all TIGR plant-entries (status: February 2012). In order to optimize the annotation, four rounds of annotation using BLAST were performed with decreasing stringency. The first round required 26 bp sequence similarity, the following rounds required 25, 24 and 23 bp sequence similarity, respectively. Counts from tags with the same hit were summed up to calculate the transcript-frequency and normalized to 1,000,000.

Confirmation of SuperSAGE expression profiles via qPCR

Parallel RNA extractions of the same tissue, from which the SuperSAGE libraries were derived, were carried out as described in the previous section. Leaves from line 29H collected at two additional time points (24 and 48 h) after inoculation were included in the analysis. Approximately 4 μg of total RNA were reverse transcribed using the M-MLV reverse transcription enzyme (Invitrogen), in combination with oligodT (dT12–18) according to manufacturer's instructions. To confirm the total absence of DNA, cDNA was used as template for PCR amplification using a primer pair from chalcone synthase gene spanning an intron. The primers were: CHS Fw1-Rev1 (5'-TGGTTAGTGTGTCTGAAATCCGC-3' and 5'-CCAACAAGACTGTCCAAGTGAGT-3') (Gutiérrez, 2008). Resulting cDNA was quantified with Nanodrop spectrometer measurement (NANODROP, Willmington DE, USA). SYBR green oligonucleotide deduction was carried out with the software package Primer 3 (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000) using selected 26 bp tags as starting points.

In order to normalize the data the three reference genes actin1 (*ACT1*), cyclophilin (*CYP2*) and eukaryotic elongation factor 1-alpha (*ELF1A*), were used (Gutiérrez *et al*., 2011). The real-time quantitative PCR (qPCR) reactions used the FastStar Universal SYBR Green PCR master mix (Roche Diagnostic) according to manufacturer's instructions. qPCR amplifications were carried out in a 7500 HT sequence detection System (Applied Biosystems, Foster City, CA, USA) with the following temperature profile: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec. and 60°C for 1 min. (annealing and elongation). No-template controls were included. Amplicon quality was checked by an additional melting curve gradient with fluorescence measures after each temperature step. The amplification of the target genes at each cycle was monitored by SYBR green fluorescence. The Ct, defined as the PCR cycle at which a statistically significant increase of report-

er fluorescence is first detected, was used as a measure for the starting copy numbers of the target gene. Relative quantification of the targets was performed by the comparative ΔΔCt method. The efficiency of each primer pair was checked for all templates using LinReg software. The RT-PCR data were normalized with the relative efficiency of each primer pair.

Data analysis

The gene expression stability of ACT1, CYP2 and ELF1A reference genes was tested using geNorm (Vandesompele *et al*., 2002) as described in its manual. Briefly, the geNorm software provides a measure of gene expression stability (M), using the principle that the expression ratio of two ideal internal control genes is identical in all the tested samples. Genes with M value lower than 1.5 were considered stable. The expressions of these reference genes were used for calculating a normalization factor of each sample according to Vandesompele *et al*., (2002). Differences in transcript level between genotypes and treatments were assessed for each time using analysis of variance (ANOVA).

Results

Generation of SuperSAGE libraries

The total number of SuperSAGE 26 bp tags, obtained after removal of PCR-copies by TrueQuant and singleton-tags, was 1,313,009 reads, comprising 970,568 from the *A. fabae* inoculated (I) library and 342,441 tags from the control (C) (Figure 1). Less than 4% of the tags represented 51,484 unique transcripts, so-called UniTags (44,799 in the I and 28,481 in the C library). One hundred and sixty one out of the 51,484 Unitags (representing 0.3% of the total), were annotated to the fungal sequence entries available at NCBI. These Tags were also extracted from the analysis.

Only 8.8% of the tags could be annotated to the existing TIGR database entries, 2,815 of them to *Medicago* and 1,702 to the remaining TIGR plant databases. This was not unexpected, because faba bean ESTs are limited and public databases currently contain no faba bean stress-related sequences associated with the expression in leaf tissue. To overcome this limitation, two normalized cDNA libraries were developed after inoculating leaves from the faba bean genotypes 29H and Vf136, considered to be resistant and susceptible to *A. fabae*, respectively (Torres AM, personal communication). The cDNA libraries proved valuable for allowing the correct annotation of a great percentage of UniTag sequences (21,032 or 40.9%), while the remaining 25,774 tags, revealed no hits to any of the target databases (Figure 1). The development of the normalized cDNA libraries will be reported elsewhere and will be further used for functional analysis of differentially expressed genes, development of markers for map-based cloning and annotation of future genomic sequences. As mentioned above, this genomic resource has also facilitated the production of qPCR assays for validation of differentially expressed transcripts. Among the annotated UniTags, 68.4% could be matched perfectly with 26/26 bps by basic local alignments (BLAST), 19.4% with one mismatch (25/26), 6.5% with two mismatches (24/26) and 5.7% with three mismatches (23/26).

Significantly differentially expressed tags ($P \leq 0.05$) that matched with 26 bp to a database entry were extracted from the previous library and considered for further analysis, yielding a total of 166,115 tags (124,229 in the I library and 41,886 in the C library). These tags represent 2,222 unique sequences, each detected at least twice in the combined libraries (1,846 and 1,864 in inoculated and control samples, respectively). Almost 90% of the sequences (1,980) could be annotated by using the new faba bean cDNA libraries with longer ESTs faba bean sequences. The remaining 242 sequences (11%) matched with GenBank DNA sequence databases (Table 1).

Abundance of UniTags and annotation to public databases

UniTags were classified in abundance groups according to their copy number (Molina *et al*., 2008). UniTags present at <2, >2 - ≤50, >50 ≤500, >500 - ≤5,000 and >5,000 copies per million (copies.million^{-1}) were considered as very low-, low-, mid-, high- and very high-abundant tags, respectively. The frequency distribution of the 2,222 tags showed that only 0.14% occurred at very high copy numbers (>5,000 counts/million); high abundant tags (between 500 and 5,000 copies/million$^{-1)}$ represented 1.1%, whereas 17% and 62.5% of the transcripts were present at mid- and low- abundant frequencies (>50 and <500 and less than 50 copies/million, respectively). Finally, 19.3%

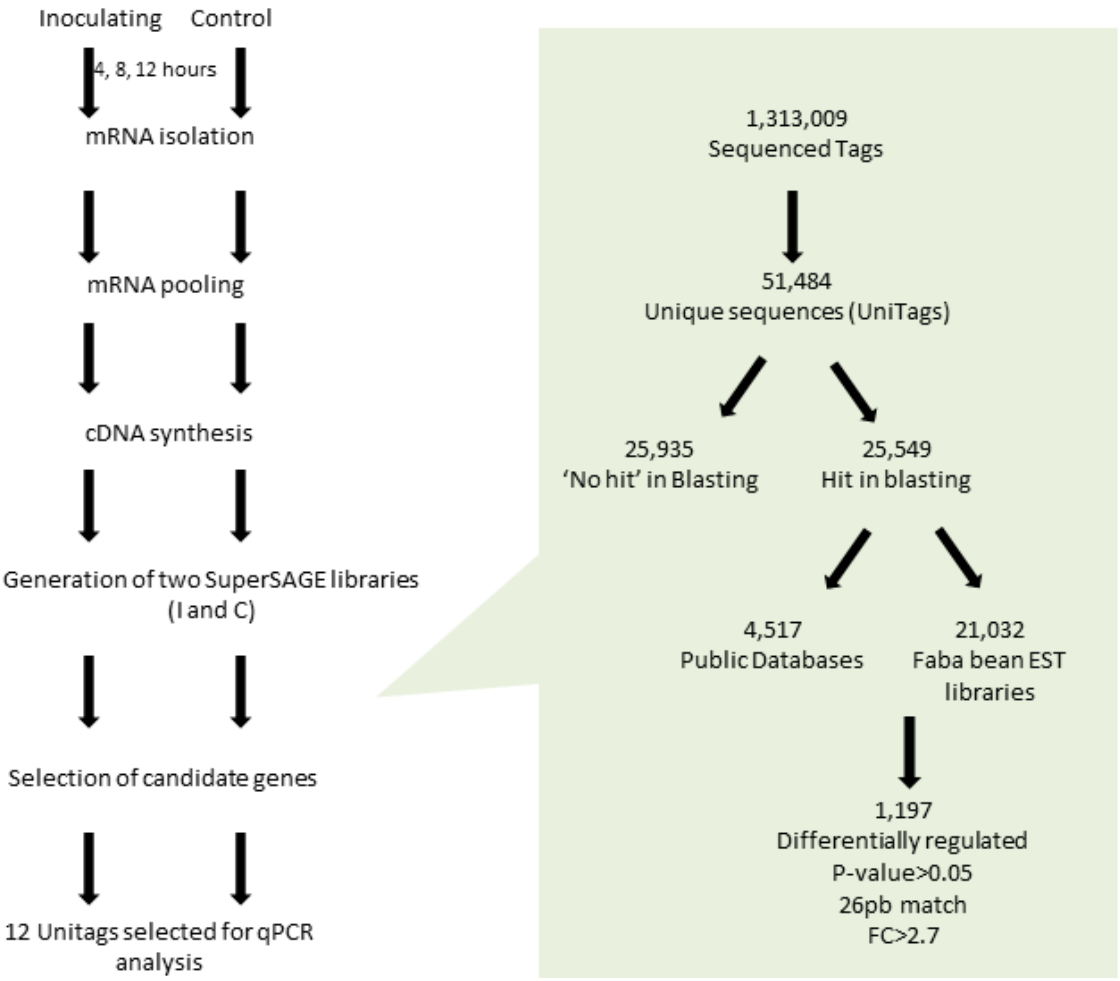

Figure 1. Schematic representation of the approach used for identification of regulatory genes by SuperSAGE. Two SuperSAGE libraries were generated from faba bean leaves of the resistant genotype Vf29H. Plants were harvested 24 hours after *Ascochyta fabae* inoculation. Replicated plants in the same conditions were included in the assay, to be used as control. From these libraries, 1,197 unique mRNAsequences (UniTags) were defined as differentially expressed (FC, fold-change).

of the transcript-tags were found at very low expression levels (0 to 2 copies.million-1) (Table 1). Thus, the strongest changes in expression levels occurred primarily in genes expressed at low to intermediate levels.

Differential gene expression in response to ascochyta blight inoculation of faba bean and assignment to Gene Ontology (GO:) functional categories

We calculated the natural logarithm of expression ratios of the 26 bp tags from inoculated versus control plants, as well as the significance levels (*P*-values) according to Audic and Claverie (1997) of up- and down-regulation for each selected transcript (26 bp matching, $P<0.05$). Differences in abundance of tags in control and stressed leaves were considered relevant at a 2.7-fold change (Molina *et al*., 2008). At this threshold 1,197 (53.9%) tags were significantly differentially expressed in inoculated versus control leaves. Of these, 376 and 358 of the tags were exclusively detected in the control or the inoculated library, respectively. Among the 1,197 tags differentially expressed, 608 (27.36%) were up-, and 589 (26.5%) were down-regulated. A total of 352 tags (15.8%) showed a more than 8-fold difference in

Table 1. Features of SuperSAGE libraries from control and inoculated leaves.

Library	Control	Inoculated	Total	(%)
Sequenced Tags	41886	124229	166115	100
Nº of unique transcript (Unitags)	1864	1846	2222	100
Public databases			242	10.9
Faba bean libraries			1980	89.1
Abundance classes of UniTags[a]				
Very high-abundant: >5000 cpm	3	3	6	0.14
High-abundant: >500≤5,000	25	23	48	1.1
Mid- abundant: >50≤500	246	508	754	17
Low-abundant: ≤50	1590	1187	2777	62.5
Very low-abundant: >0≤2	358	501	859	19.3

[a] Values normalized to 1 million tags (cpm).

expression (P<0.05, 26 bp matching), 307 being up-, and 45 down-regulated by infection. As depicted in the Venn diagram (Figure 2), a considerable number of tags occurred exclusively in either the control tissue or under infection conditions.

The 40 most significantly up- and down-regulated transcripts are listed in Table 2 and Table 3, respectively. Many transcripts could not be annotated, because they either matched to anonymous ESTs, or did not hit any entry in the database.

To obtain gene function categories of the differentially expressed UniTags, gene ontology (GO) annotation was performed by BLASTX (using the corresponding annotated nucleotide sequences as queries) against the non-redundant GenBank and UniProtKB/TrEMBL protein databases. For this analysis we used UniTags that showed a maximum of 2 mismatches (24/ 26) with entries in the GenBank nucleotide database. As a result, 2,143 Tags matched to an amino acid sequence entry (e-value <1e $^{-10}$) in the GenBank and UniProtKB/TrEMBL databases: ~32% matched into molecular function category, ~37% into cellular component and ~31% into biological process. Among the most prevalent GO biological processes, ~21% of the UniTags grouped into the cellular process category (including regulation, response to stimulus and cell communication), ~19% classified into metabolism (primary metabolic and biosynthetic process), ~17% into binding, ~14% into catalytic activity (e.g. hydrolase, transferase or lyase activity), ~13% into response to stimulus (for example response to endogenous and chemical stimulus, biotic and abiotic stresses and detection of stimulus), ~9% into regulation of biological processes and ~9% into developmental processes. A survey of the most enriched GO terms pointed towards tags exhibiting a strong differential expression (Table 4).

Validation of SuperSAGE data by qPCR

A subset of 10 differentially expressed UniTags (Table 5) was selected for further analysis based on three criteria: 1) strong and significant changes in the FC values (either up- or downregulated in I versus C); 2) match to known regulatory components in the databases; 3) homology with a sequence present in the normalized cDNA library, allowing a reliable primer design. The selected sequences were used to design gene-specific primers to 1) validate the SuperSAGE data and 2) study the kinetics of mRNA induction by real time qPCR. Total RNA was extracted from inoculated or control leaves of the resistant genotype 29H at 4, 8, 12, 24 and 48 hours after inoculation.

qPCR analysis revealed that mRNA levels of the 10 selected UniTags were consistent with a rapid increase or decrease between 8-12 hours after inoculation (Figure 3). Interestingly, some transcripts showed either no or minimal induction by inoculation at the

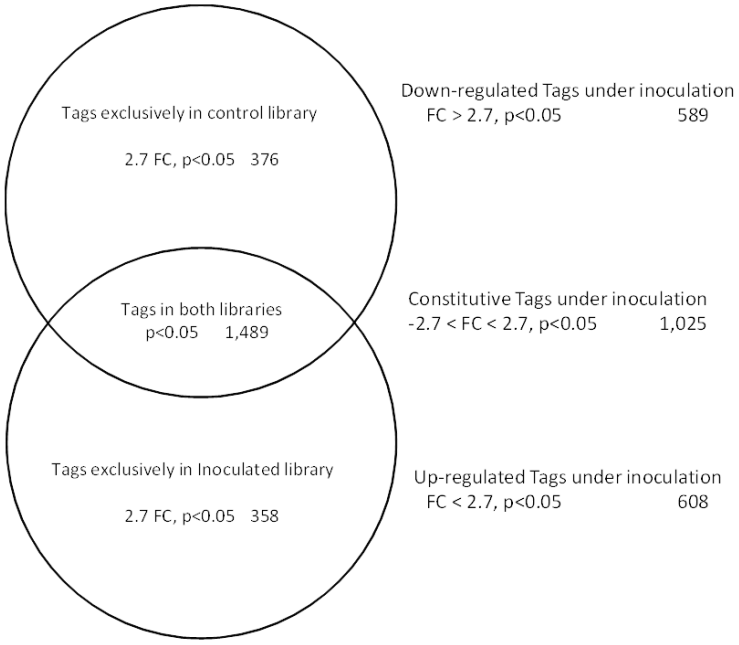

Figure 2. Venn diagram of the quantitative tags classification into down-regulated, constitutively expressed, and up-regulated transcripts in control and inoculated faba bean leaves. Only tags matching 26 bp and with a $P<0.05$ were taking into account to build the diagram

beginning of the infection (4–8 hours after inoculation) when the fungal spores are still penetrating.

The differential regulation could be confirmed in most cases for up-regulated Tags, except for Tag 283953, coding for an ethylene receptor transcription factor (ERTF), which showed the same expression in inoculated and control leaves (Figure 3). For down-regulated UniTags, differential expression could be confirmed only in two of the five sequences tested, corresponding to tags 27480 and 332014 (Figure 3). In the remaining three sequences, the expression was higher in the inoculated leaves.

Discussion

In this study, SuperSAGE was used to characterize the faba bean transcriptome in response to infection by the fungal pathogen *A. fabae*. We analyzed expression of approximately 50,000 transcripts from control and inoculated faba bean leaves, with the main objective of identifying factors with potential regulatory functions after infection with *A. fabae*. To our knowledge, this is the first global analysis of gene expression in this plant-pathogen interaction.

With >342,441 and >970,568 sequenced tags generated in the control and infected library, respectively, a deep analysis of the polyadenylated transcriptome of faba bean is now available. As shown in Table 1, nearly 80% of transcripts were present in less than 50 copies per million and only a small portion of transcripts (<2%), were represented by more than 500 copies per million Since each tag represents a single mRNA-molecule, this depth also gives insights into the expression levels of rare transcripts. Because the amount of transcript molecules per cell varies depending on the cell type, it is not possible to estimate the level of missed transcripts. However, the fact that many singletons were found even in the infected library clearly indicates that very rare transcripts may have escaped the analysis. These might include transcripts of highly interesting pleiotropic genes such as those encoding transcription factors, that may be present at only 0.001 copies per cell (Czechowski *et al*., 2004). Therefore, the detection of rare transcript should be improved by increasing the number of sequenced transcripts.

The different amounts of tags in the two libraries result in different resolutions of the transcription

Table 2. Top 40 up-regulated tags.

Tag_id	FC[a]	Seq, Description	Seq, Length	#Hits	Min. e-value	Mean similarity (%)
CATGAAACATTAGGAACAAATTTTCC	-12.3	polyamine oxidase	200	2	1.15E-13	97
GATCCTTCTACAGCTTCTTGCTCAAC	-12.1	---NA---	165	0		
CATGGCGCATCCATAACAAAACCTCT	-10.8	ethylene-responsive transcription factor	380	2	4.65E-26	89
CATGAATAATTCACATAAATGGAACG	-10.8	---NA---	153	0		
CATGAGTTGAAATTTTGTTGTAACTT	-10.6	---NA---	192	0		
CATGTTATTCTGAAACAACATTTTGT	-10.5	---NA---	296	0		
CATGATCAGATAAATAAATTCTTTGT	-10.4	---NA---	191	0		
GATCATCTGCTAGCAATAACAATATC	-10.3	---NA---	203	0		
CATGGAATGAGCTTCTCAATGCAGCA	-10.2	---NA---	209	0		
CATGTAATAGGCCTCTCTGATGTTTT	-10.2	gdsl esterase lipase at5g14450-like	316	20	2.82E-20	85
CATGTATATATTTTTAGTAATATTTA	-10.2	---NA---	157	0		
GATCATTTTCCTAAAGTATTCATTTC	-10.1	---NA---	278	0		
GATCATTCTCCCTCTACTACCAATTG	-9.9	uncharacterized protein LOC100796062	151	12	1.59E-15	80
CATGTATACTGTAATTTTACTATGAA	-9.9	---NA---	188	0		
CATGTGCTATGCAAATAATATTTGAA	-9.9	---NA---	213	0		
CATGCTTAATTTTAAAAAAAAAAAAA	-9.9	---NA---	192	0		
CATGTTACTAGAGAAATGTAATTGAA	-9.9	---NA---	156	0		
GATCTATTCTGTTACTATTACTAGAT	-9.8	hypothetical protein MTR_3g028260	361	11	4.60E-16	81
CATGCCCTTGTAATATAACTTGCTAA	-9.8	---NA---	221	0		
CATGTATTTTACACGACTATTTATTT	-9.7	---NA---	170	0		
GATCCTCTCACTCAACTTCTTCTAGC	-9.7	---NA---	236	0		
GATCAGGGTATAGTTTATAGGAATTT	-9.7	---NA---	247	0		
CATGTAATTACTAAGCAAGTGAGGGA	-9.6	---NA---	173	0		
CATGTCTTACTCAAATTAGCCATTTA	-9.6	---NA---	174	0		
GATCAGATGCTATATGCAATTGTTTT	-9.6	---NA---	345	0		
GATCAAAACCCCTCGGAGACTAGACT	-9.6	e3 ubiquitin-protein ligase ring1-like	651	20	1.67E-58	80
CATGCCTCCAGATGCTCAACACGTAA	-9.5	glutamate decarboxylase	1423	20	0	96
CATGTCATTTATTTTTGTCATTAAAA	-9.5	---NA---	244	0		
CATGAGCCAAATCTGTTTTGTCAGAA	-9.5	---NA---	348	0		
CATGTAATTTGCTTTTTTGAACGAAA	-9.5	---NA---	279	0		

(*Continued*)

Table 2. *Continues.*

Tag_id	FC[a]	Seq, Description	Seq, Length	#Hits	Min. e-value	Mean similarity (%)
CATGTTCAAACACTGAGAACAAGGTG	-9.5	hypothetical protein MTR_3g028470	333	13	8.72E-28	83
CATGGATGTTATTTTTAATGAGAAAA	-9.4	---NA---	182	0		
GATCAGCTGGCTTACAAAATCTGCTG	-9.4	hypothetical protein MTR_8g094730	182	1	9.65E-11	92
GATCCTATCAACCCTATATAAAACTT	-9.4	---NA---	231	0		
CATGAGTATATTTACTAAGGTTTTAA	-9.4	---NA---	232	0		
CATGTATATATTGAATTTGTCAATGA	-9.4	1-orf2 protein	915	20	8.12E-91	56
CATGTATGCTGCTCTATATTGGAATT	-9.4	---NA---	450	0		
GATCAAGGGTCAATCTTAAATTCACC	-9.4	---NA---	244	0		
CATGGACTTCCTCTCATAGTTCAATC	-9.4	cytochrome p450	347	20	3.60E-27	85
CATGTAAACAAGTTTACTGAAAAAAA	-9.4	leucine-rich repeat protein kinase	406	5	1.32E-17	74

[a] FC, Fold change calculated by dividing the number of tags in the normalized inoculated library (I) by the number of tags in the normalized control library.

profiles. In the deeper-sequenced infected library, 23,003 tags were identified which are solely found

Table 3. Top 40 down-regulated tags.

Tag_id	FC[a]	Seq, Description	Seq, Length	#Hits	Min. e-value	Mean similarity (%)
GATCTTCGGGGAGGAATATTCCCCTA	9.2	---NA---	177	0		
CATGGTCCAGAGTATGCCACTTTGGG	9.2	---NA---	174	0		
CATGACAGACAAAAAAAAAAAAAAAA	9.0	---NA---	154	0		
GATCTGTTCTTCCCACCTTCAATTCT	8.9	---NA---	266	0		
CATGTTGTATATATAGTTATTTGAGG	8.7	dentin sialophospho protein	893	20	2E-51	67
GATCAGGTTGGTACTGATGATACTGC	8.5	protein	879	20	1E-147	77
GATCCTTCTATCCTAGGAATGTTTTT	8.5	f-box family protein	306	7	1E-22	73
GATCGTATCGAAATCGATTAGATAGA	8.5	pentatricopeptide repeat-containing protein	814	12	2E-19	65
GATCAATGTTGACAAACCAAAATCCA	8.2	---NA---	787	0		
GATCCTGTTGAAAAAAAAAAAAAAAA	8.2	---NA---	297	0		
CATGAACTGAAATTGGGAAGCTTCCC	8.2	prh26 protein	1270	20	9E-159	89
CATGAAGATTGGCCTGCTAATAGTTA	8.2	small heat shock protein	296	20	5E-38	73
CATGACTAAATCAAGATTTGAATTGC	8.2	---NA---	192	0		

(*Continued*)

Table 3. *Continues.*

Tag_id	FC[a]	Seq, Description	Seq, Length	#Hits	Min. e-value	Mean similarity (%)
CATGCAAAATTGACGATGTATATGTG	8.2	hypothetical protein MTR_4g130400	948	7	8E-91	58
CATGGAATCGAGAGCTCCAAGTGGGC	8.2	hypothetical protein MTR_5g051030	171	8	8E-19	95
CATGGTGGATGGGGGTATTCCCAATT	8.2	uncharacterized protein LOC100804791	372	2	7E-18	78
CATGTACAGAAAGCAGCACAAGAAGG	8.2	---NA---				
CATGTCAAGTGAGAGAGGGAGGTTTA	8.2	udp glucose:flavonoid 3-o-glucosyltransferase	356	20	1E-22	73
CATGTGTGATAAAATTGTGCAACGAA	8.2	---NA---	258	0		
CATGTTAGTTCAACAAAAAAAAAAAA	8.2	---NA---	172	0		
GATCAGCAGCCGCACCATTTCCATTA	7.9	homeobox-leucine zipper protein hat7-like	559	20	2E-62	72
GATCATTGCTAATTGGTGGTTGTTTG	7.9	---NA---	275	0		
GATCGAGCGCTCTTGCGCCGAAGATG	7.9	---NA---				29
GATCTAAGAAGAAGTTTCGTAAATAT	7.9	---NA---	337	0		
GATCTGTTCAGGTTTTAATTCATCAC	7.9	---NA---	390	0		
GATCTTATTATCTTGAGAAATTAGTT	7.9	---NA---	255	0		
GATCTTTCTGGACTGTCTTTATAAAT	7.9	hypothetical protein MTR_7g017370	151	1	3E-07	64
CATGAAGAGTTATTATCTCTCAGCTT	7.9	---NA---	390	0		
CATGAAGCAAAAAATAGTTATAGTGT	7.9	metal ion binding	413	20	1E-29	79
CATGACAAATCAGGAAGCTGCCGAGA	7.9	protein phosphatase 2c 68	821	20	2E-115	86
CATGAGAAAGAAACAGAGCGAGAGAT	7.9	---NA---	626	0		
CATGCATTGGTGAAAGGGCAAGATTT	7.9	uncharacterized protein LOC100807468	430	20	2E-21	79
CATGCTCCATCCGCTTCCATTACTCT	7.9	secologanin synthase-like	167	20	4E-16	88
CATGTAAAATTGAGATGGTGAGATTT	7.9	---NA---	258	0		
CATGTGAAGGAACTTTATATTTATCG	7.9	---NA---	223	0		
CATGTTTGCATCTGTTGTTATACCAC	7.9	31 kda ribonucleoprotein	364	4	3E-29	78
CATGTTTGTTTCAAAAAAAAAAAAAA	7.9	---NA---	368	0		
GATCAACAAAAGTTAGCATCCGAGTT	7.5	ankyrin repeat domain-containing protein 13c-b-like	969	20	6E-163	75
GATCAACCCAAAATCAAAACAACAAC	7.5	---NA---	152	0		
GATCAACTGTTTAAATGATATCCATT	7.5	---NA---	168	0		

[a] FC, Fold change calculated by dividing the number of tags in the normalized inoculated library (I) by the number of tags in the normalized control library.

Table 4. List of the most enriched Go terms showing the number of differentially up and down-regulated Tags found on each one.

GO-Term	Description	Transcripts	Differential expressed	Up regulated	Down regulated	Enrichment P-value	GO-Level(s)
GO:0009753	Response to jasmonic acid stimulus	17	5	4	1	0.008	4
GO:0046910	Pectinesterase inhibitor activity	3	2	1	1	0.018	4
GO:0030076	Light-harvesting complex	14	4	4	0	0.020	4.5
GO:0016458	Gene silencing	8	3	1	2	0.021	3.6
GO:0034357	Photosynthetic membrane	64	10	6	4	0.025	4.5
GO:0010287	Plastoglobule	22	5	5	0	0.026	6.8
GO:0031984	Organelle subcompartment	56	9	5	4	0.028	4
GO:0055035	Plastid thylakoid membrane	56	9	5	4	0.028	6
GO:0031976	Plastid thylakoid	56	9	5	4	0.028	5.6
GO:0009534	Chloroplast thylakoid	56	9	5	4	0.028	6.7
GO:0009535	Chloroplast thylakoid membrane	56	9	5	4	0.028	7
GO:0042651	Thylakoid membrane	56	9	5	4	0.028	5.6
GO:0009769	Photosynthesis, light harvesting in photosystem ii	9	3	3	0	0.029	6.7
GO:0044436	Thylakoid part	67	10	6	4	0.034	4.5
GO:0009579	Thylakoid	67	10	6	4	0.034	4.5
GO:0040029	Regulation of gene expression, epigenetic	10	3	1	2	0.039	6.7
GO:0044435	Plastid part	70	10	6	4	0.044	4.6

in this library, while only 6,685 tags are found in the control library. For rare transcripts in the infection-specific library, it is thus not possible to state that they are attributable to the treatment, whereas the unique tags found in the control library are likely to be linked to the treatment and growth conditions.

SuperSAGE identified early resistance response genes with a reasonable success rate according to the tag annotation, thus advancing our understanding of the global transcriptional changes occurring after *A. fabae* infection. However, changes at the gene expression level are not necessarily a direct indication of the involvement of a gene in a biological process. For this reason, functional analysis of each of the differentially expressed genes is a key step to understand both the mechanism underlying the plant defence response and the biological significance of the global changes in gene expression.

Annotation of nearly 41% of the UniTags was possible because of the availability of a normalized faba bean cDNA library. Because only 5,415 faba bean EST sequences are publicly available at present, a large number of significant hits represented fully uncharacterized database accessions (50.37%), limiting functional interpretation of the faba bean transcription profiles. The annotation frequency was even lower for low abundant tags, since cDNA/EST libraries mainly include highly abundant transcripts.

In agreement with the leaf tissue used for the analysis, a high proportion of the most abundant

Table 5. List of the 10 UniTags selected for qPCR. It is indicated the holologe *Medicago truncatula* (Mt) chromosome.

Tag_ID	Seq. Description	GI number	Mt Chromosome	SS fold change
Tag 283953	Ethylene-responsive transcription factor	XP_003605655.1	4	-10.8
Tag 40395	NA	NA	-	-10.06
Tag 74487	ATP-dependent metalloprotease	XP_003628399.1	8	-9.15
Tag 81915	Alpha-1 4-glucan-protein synthase	XP_003614188.1	5	-4.09
Tag 18581	NA	NA	-	-2.83
Tag 6557	Hypothetical protein MTR_7g061260	XP_003623107	7	3.3
Tag 1513	Transmembrane protein	XP_003600055.1	3	6.3
Tag 221927	NA	NA	-	7.86
Tag 332014	Pentatricopeptide repeat-containing protein	XM_003614062	5	8.45
Tag 27480	bZIP transcription factor bZIP122	NP_001235033.1	4	8.45

transcripts corresponded to genes required for energy production and photosynthesis. Interestingly, some UniTags were associated with response to jasmonic acid, one of the molecules involved in plant immunity signalling (Pieterse *et al*., 2009; Birkenbihl *et al*., 2011; Ibrahim *et al*., 2011), as well as with pectin esterase activity and gene silencing. Pectin esterase was shown to play a role in the plant response to pathogen attack (Vorwerk *et al*., 2004). Up-regulation of genes encoding pectin esterase was reported in *Glycine max* (Ibrahim *et al*., 2011) and *Arabidopsis thaliana* during the early stages of infection by the root-knot nematode *Meloidogyne incognita* (Jammes *et al*., 2005). Other examples for the role of pectins in pathogen defence include the induction of pectin methylesterase inhibitors in *Arabidopsis* resulting in increased resistance to the necrotrophic fungus *Botrytis cinerea* (Lionetti *et al*., 2007). Further investigations are needed to corroborate the implication of these tags in the faba bean resistance response.

Examination of the differently expressed group of genes suggests that some SuperSAGE-tagged genes were transiently up- or down-regulated early after the onset of the resistance response, and therefore changes in their expression levels could not be detected during a normal infection process. Because the RNA samples derived from a mixture of cells at different stages of the resistance response, the magnitude of the change in gene expression could be masked, thereby overlooking a transient up-regulation of gene expression in the normal infection samples (Hamada *et al*., 2008). This could explain some of the differences between the SuperSAGE and the qPCR expression data found in this study.

Our outcomes show that SuperSAGE is a valuable filter to select candidate genes in response to a biotic stress. However, further studies are required to validate some of the expression patterns observed. Moreover, to solve annotation problems and increase data resolution, new strategies for transcriptome sequencing should be explored in order to improve sequence assembly, gene prediction and subsequent functional analysis. This issue is of special relevance in faba bean, where limited sequence information is available.

The qPCR results corroborated several transcripts related with the plant response to *A. fabae*. These include genes potentially related to stress response such as a bZIP (basic leucine zipper) transcription factor that was repressed at 12 h after infection, as well as an ethylene response transcription factor (ERTF) that showed no difference between inoculated and control plants. bZIP proteins constitute a large family of transcription factors in plants that regulates stress response and hormone signalling (Kim, 2006) while ERTFs have been shown to modulate multiple responses to abiotic and biotic stress, taking part in regulation of metabolism, developmental processes, and/or defense responses (Fukao *et al*., 2006; Andriankaja *et al*., 2007; Oñate-Sánchez *et al*., 2007; Hattori *et al*., 2009).

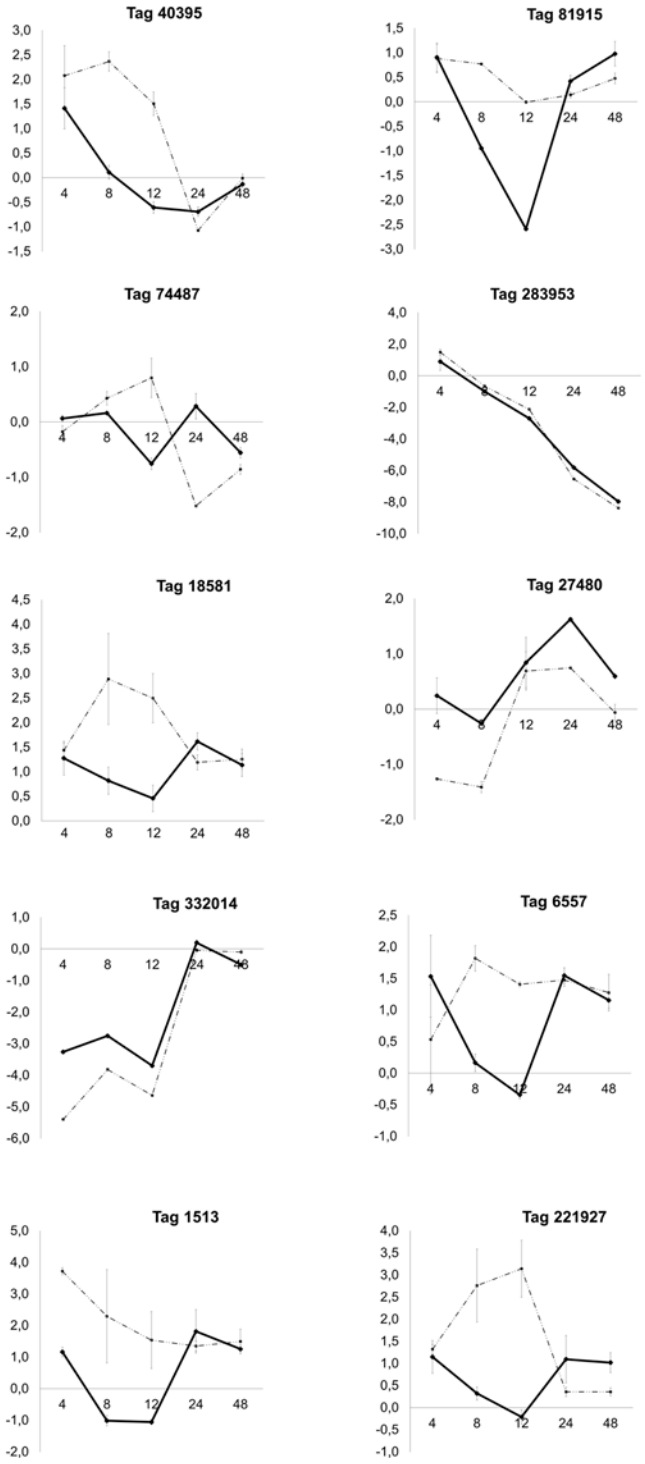

Figure 3. Analysis of mRNA accumulation corresponding to selected UniTags by qPCR. Examples of the kinetics of mRNAs for 10 UniTags analyzed by qPCR 4, 8, 12, 24 and 48 hours after inoculation with *A. fabae*. Relative mRNA quantification was performed using ACT1, CYP2 and ELF1A, as a reference genes for normalization. The data values point was set arbitrary to 1. Transcripts levels were analyzed in three biological replicates (n = 3).

Other annotated differentially regulated genes are involved in general responses. Thus, the alpha 1,4-glucan protein synthase has a possible role in the synthesis of plant cell wall polysaccharides. It was found to be associated with the cell wall with high concentrations in plasmodesmata (Wald *et al*., 2003) and is involved in the cellulose biosynthetic process. ATP-dependent metalloproteases play a crucial role in regulating the heat-shock transcription factor σ^{32} (Herman *et al*., 1995; Tomoyasu *et al*., 1995). Pentatricopeptide repeat (PPR) proteins function in mitochondria or plastids gene expresssion (Nakamura *et al*., 2004). Some of these proteins play a role in post-transcriptional processes within organelles and are thought to be sequence-specific RNA-binding proteins (Meierhoff *et al*., 2003; Mili *et al*., 2003; Delannoy *et al*., 2007). Studying the transcriptional response of these genes in a susceptible faba bean genotype is the next step to further investigate their implication in the plant resistance response.

Although there were numerous un-annotated and unknown tags, our results highlight the usefulness of next-generation sequence data to investigate genome wide expression profiles in orphan legume species such as faba bean. The applicability of these tags will increase as more faba bean genomic and cDNA sequences become available. Functional analysis of the differentially expressed genes and comparison of our data with results from genome-wide expression profiling in related crops will provide a deeper insight into the molecular mechanisms involved in the legume-*Ascochyta* pathosystems. New sequencing-based techniques like Massive Analysis of cDNA Ends (MACE) and RNAseq at a sufficiently deep scale will further improve the understanding of the faba bean transcriptome.

The quantitative nature of *A. fabae* resistance resistance highlighted by the number of QTLs identified so far (Roman *et al*., 2003; Avila *et al*., 2004; Díaz-Ruiz *et al*., 2009) makes breeding for this trait difficult. Co-location of some of these candidate genes in the available faba bean mapping populations may offer breeders a new avenue to incorporate *Ascochyta* resistance QTLs efficiently into a single marker-based breeding scheme.

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