

# Characterization of Transcription Factors Following Expression Profiling of *Medicago truncatula*–*Botrytis* spp. Interactions

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**Abstract** *Medicago truncatula* may be used as a model plant to study the pathosystem *Vicia faba*/*Botrytis* spp. This study aims to investigate the profiling of the transcription factors (TFs) of *M. truncatula* involved in the interaction with *Botrytis cinerea* and *Botrytis fabae*. The response of two genotypes of *M. truncatula* to the pathogens was first established, defining genotype A17 as partially resistant and genotype Esp162 as susceptible. We found that *B. fabae* is more aggressive as a pathogen of *M. truncatula* than *B. cinerea*. The profiling of the TFs involved in the interaction was subsequently carried out using an available genome guide qPCR-based platform for the quantitative measurement of 1,084 *M. truncatula* TFs. Microscopic studies showed that the infection process had started 24 h after inoculation. A total of 126 TFs showed significant differences in their expression after inoculation irrespective of the genotype, while 37 TFs were differentially expressed between the resistant and the susceptible genotypes and 70 TFs showed different levels of expression between the genotypes unaltered by infection. This has allowed characterization at the transcriptional regulation level of the differential response of two genotypes of *M. truncatula* to two very similar pathogens differing in their aggressiveness. These results will be valuable to increase the functional knowledge of the *M. truncatula* genome and to help in breeding programmes of *V. faba* for resistance to *Botrytis* spp.

**Keywords** Legumes · Chocolate spot · Disease resistance · Transcriptomics · Gene regulation

## Introduction

Faba bean (*Vicia faba* L.) is one of the most important legume crops providing protein for both human and animal consumption in many regions of the world (Duc et al. 2010; Rubiales 2010). However, the production of this crop is limited by various factors including diseases, which plays an essential role (Stoddard et al. 2010). Chocolate spot is one of the most important foliar diseases affecting the crop; under propitious conditions (i.e. mild temperatures around 15–22 °C and relative humidity higher than 80 %), this disease may cut down yields drastically (Villegas-Fernández et al. 2009). The foliar disease is caused by *Botrytis fabae* Sard., which is related to the necrotrophic fungus *Botrytis cinerea* Pers.: Fr. *B. cinerea* is a polyphagous pathogen with a wide range of host (more than 200 species), including strawberries, grapes and tomatoes (Staats et al. 2005). This pathogen may also infect faba bean plants producing chocolate spot symptoms, but without serious consequences (Harrison 1988).

Spraying crops with fungicides is the only available measure to effectively control chocolate spot. However, fungicides are often too expensive and not environmentally adequate (Stoddard et al. 2010). A number of agronomic practices such as intercropping can reduce this infection (Fernandez-Aparicio et al. 2011) but needs to be further adjusted. Growing resistant varieties becomes a key strategy when dealing with this disease since they could replace chemical measures. Although various researches have been carried out to find the sources of resistance and incomplete-resistant varieties have been introduced in some countries, further work is needed (Sillero et al. 2010). It is important to detect plants with a higher degree of resistance and look into the mechanisms

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accounting for such resistance. This will help to understand plant protection against infection. Characterising mechanisms of resistance are comprehensive; it covers from the macroscopic level to the molecular level. Unfortunately, there has been no important development of infrastructure, both in terms of knowledge and physical capacity, to carry out molecular studies of *V. faba* (Varshney et al. 2009).

*Medicago truncatula* Gaertn is a well-established model plant for legumes, and a considerable amount of molecular and physiological information is available (Rose 2008; Young and Udvardi 2009). *M. truncatula* has been described as a promising system for studying plant–pathogen interactions and some studies show interesting results (Rispaill et al. 2010; Tivoli et al. 2006b). With regard to *Botrytis* spp., to our knowledge, these pathogens have not been reported to damage *M. truncatula* in nature, so it presents as a non-host resistance to *Botrytis* spp. Non-host resistance has been widely researched in different pathosystems (some examples: Hadwiger (2008); Die et al. (2009); Lipka et al. (2008)) and this type of resistance is largely based on the response by the plant at the molecular level (Holub and Cooper 2004; Nurnberger and Lipka 2005). Nevertheless, it is possible to bring about infection on *M. truncatula* by *Botrytis* spp. under controlled conditions in the laboratory (Ellwood et al. 2007); so, breaking the natural non-host resistance of *M. truncatula* to *Botrytis* spp. The study of *M. truncatula*/*Botrytis* spp. interactions might provide useful information for the better understanding of the *V. faba*/*B. fabae* pathosystem.

Transcription factors (TFs) are crucial in plant development and differentiation, they regulate the expression of complex traits in plants (Udvardi et al. 2007), such as resistant responses to biotic stresses (Singh et al. 2007). Identifying the TFs involved in these responses would be an important step to unravel the intricate mechanisms responsible for resistance, and could open the door to manipulation for the future of the metabolic pathways implicated (Iwase et al. 2009). TFs present low expression levels compared to those of other genes; hence, a very sensitive technique such as quantitative real-time PCR (qPCR), which is estimated to be at least 100-fold more sensitive than DNA arrays in transcripts detection (Czechowski et al. 2004), is required for the study of TFs. In the case of *M. truncatula*, a genome guide qPCR-based platform for a quantitative measurement of 1,084 TF genes is available (Kakar et al. 2008) and has been employed in various studies (Gao et al. 2010; Madrid et al. 2010; Verdier et al. 2008).

The objective of this work was to carry out a gene expression profile of *M. truncatula* transcription factors and identify those involved in the interaction with pathogens *B. fabae* and *B. cinerea*. The evaluation of the differential responses of two *M. truncatula* genotypes to the pathogens was first established under laboratory-controlled conditions.

## Materials and methods

### Evaluation of *Medicago truncatula* genotypes

The responses of two accessions of *M. truncatula* to separate inoculation with two isolates of *B. fabae* and *B. cinerea* were assessed at the seedling stage under laboratory-controlled conditions. Two consecutive series of experiment were performed, with four replications per genotype in the first experiment and ten replications in the second experiment (each replication consisting of a single plant). A third experiment was carried out to perform microscopic observations of the first stages of the infection at two different times (with three replications per time). Management of plant and pathogen material and inoculation procedures were the same for the three experiments.

### Plant material

Two genotypes of *M. truncatula* supplied by Dr. J. M. Prospero (INRA, Montpellier, France), were employed for this study, namely, line A17 (cultivar ‘Jemalong’) and line Esp162. These accessions had presented the most extreme reactions to the disease in a previous evaluation of a germplasm collection of *M. truncatula* (data not shown). Plants were grown in a growth chamber at 20 °C with a photoperiod of 14 h of visible light (150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density) and 10 h of darkness.

### Pathogen

A monospore isolate of *B. cinerea* (Bc-CA-06) and another one of *B. fabae* (Bf-CO-05) were used in these tests. The *B. cinerea* isolate was multiplied on 9-cm Petri dishes with potato dextrose agar medium (Oxoid) and kept at 19 °C with a cycle of 12 h of visible light + near-ultraviolet radiation and 12 h of darkness for 10 days, after which time, a carpet of sporulating mycelium covered the surface of the plate. The *B. fabae* isolate was grown in modified V8 medium as described by Villegas-Fernández et al. (2012) until sporulating mycelium was attained.

### Inoculation

A spore suspension of each isolate was obtained by adding glucose solution (1.2 % w/v) to the plates where the fungi had sporulated (10–15 ml per dish) and then scraping the surface of the medium with a needle to dislodge the spores. The suspension was filtered through a sieve and the spore concentration was adjusted to 1,150,000 spores/ml with a hemacytometer slide. Tween-20 was finally added (0.03 % v/v).

Plants were sprayed with approximately 2.5 ml of the spore suspension, taken to an incubator and kept in the

darkness for 48 h at 20 °C with approximately 100 % continuous relative humidity. The plants were transferred to a growth chamber under the same conditions; the pots in the trays were covered with plastics whose inner sides were sprinkled daily with water in order to maintain a relative humidity greater than 95 %.

#### *Disease-response evaluation*

Evaluation was made at two time points: 2 and 8 days after inoculation (2 and 8 dai, respectively). These points were chosen on the basis of previous studies, in which the appearance timing of symptoms had been established. Sporulation would start 8 days after inoculation, giving rise to a second cycle of infection that would interfere with the evaluation, so no more evaluations were made beyond that time point. The recorded parameter was disease severity (DS), expressed as the percentage of foliar surface of each plant covered with lesions, and a correction to increase by 50 % the weight of the sporulating lesions, when present:

$$\text{Disease severity} = (\% \text{ non-sporulating lesions}) + 1.5 \times (\% \text{ sporulating lesions})$$

#### *Microscopy observations*

Pathogens spore germination was determined at the microscopic level. Plant samples were taken twice (24 and 48 h) after inoculation. One complete leaf was detached from each plant, its three leaflets separated and processed according to Rubiales and Moral (2004): they were laid with the adaxial surface up on filter paper dipped in fixative (1:1, absolute ethanol/glacial acetic acid, v/v). After several changes of the fixative, the leaflets were transferred to filter paper, moistened with tap water for at least 2 h, then to lactoglycerol (1:1:1, lactic acid/glycerol/water, v/v/v) for at least, another 2 h. A drop of trypan blue in lactoglycerol (0.1 %, w/v) was placed on a cover glass; the sample was carefully laid with the adaxial surface toward the cover glass and then mounted in lactoglycerol on a microscope slide. The percentage of germinated spores was determined under  $\times 200$  magnification (a minimum of 100 spores per leaflet was observed; those with a germ tube and those without were counted, and the proportion was subsequently calculated).

#### *Data analysis*

The statistical software employed was Statistix 8 (Analytical Software, Tallahassee, FL, USA). The factorial analyses of variance (factors being genotype and pathogen) were carried out for the studied parameters in the evaluation experiments (i.e., severity and percentage of germination) at different

times. Data were arcsine transformed as required. The means were separated using least significant difference (LSD) test ( $p=0.05$ ).

#### *Gene expression profiling of transcription factors*

A large-scale profiling of transcription factors involved in the interaction of *M. truncatula*–*Botrytis* spp. was carried out. The tested *M. truncatula* genotypes were A17 and Esp162. Three different treatments were applied to six plants of each genotype: (1) inoculation with a *B. fabae* isolate, (2) inoculation with a *B. cinerea* isolate and (3) no inoculation. Three biological replications were performed.

#### *Plant material, inoculation and sampling*

Plant and pathogen management and inoculation were carried out in the same way as described in the evaluation experiment. Non-inoculated plants were sprayed with the glucose and Tween-20 solution. Sampling was made 48 h after the inoculation (after taking plants out of the incubator). All the leaves of the six plants subjected to the same treatment were detached and pooled, thus making up an individual sample, immediately frozen in liquid nitrogen for storage at  $-80$  °C.

#### *Total RNA extraction and cDNA synthesis*

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Its integrity was checked via denaturing agarose gel electrophoresis (2 % w/v) and the RNA concentration and purity were spectrophotometrically determined. To remove genomic DNA contamination, 200  $\mu\text{g}$  of total RNA were treated with RNase-free DNase RQ I<sup>TM</sup> (Promega, Madison, USA); the RNA integrity, concentration and purity were again determined. PCR with primers specific for *M. truncatula* ubiquitin gene was employed to confirm that no genomic DNA remained (Kakar et al. 2008). First-strand cDNA was synthesised with the aid of SuperScript<sup>TM</sup> III reverse transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol.

#### *Real-time PCR*

A collection of primer pairs corresponding to 1,084 putative transcription factors (TFs) in *M. truncatula* was generated using available genomic databases (Kakar et al. 2008). Polymerase chain reactions were carried out as described by Kakar et al. (2008) in optical 384-well plates with an ABI PRISM<sup>®</sup> 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR<sup>®</sup> Green to monitor double-stranded DNA (dsDNA) synthesis. Four housekeeping genes (*MtUbi*, *MtGapdh*, *MtPdj2*, *MtEfl1*) were replicated twice in each plate.

## Data analysis

SDS 2.0 software (Applied Biosystems) was used to analyse the data. C<sub>q</sub> values were those corresponding to a threshold of 0.3. Amplification reactions with more than one melting curve peak were not further considered. Posterior analysis included the determination of PCR efficiency (E) with the help of LinReg software (Ramakers et al. 2003). Only reactions whose efficiencies were greater than 1.6 were chosen for this analysis. Software application *BestKeeper* (Pfaffl et al. 2004) was employed to identify the most stable housekeeping genes for use as reference genes.

Gene expression value was calculated as E<sup>C<sub>q</sub></sup>. The normalisation index in each plate was defined as the geometric mean of the expression values of the reference genes in the plate; expression level for every gene was subsequently normalised by dividing the values by that index. Finally, factorial analyses of variance were carried out for these normalised expression values; factors were genotype and pathogen (with three levels: inoculation with *B. fabae*, inoculation with *B. cinerea* and no inoculation). Means were separated using LSD test ( $p=0.05$ ). These statistical analyses were performed using Statistix 8 (Analytical Software, Tallahassee, FL, USA).

The TFs showing significant differences only for pathogen were clustered using a k-means clustering analysis. A data matrix with the log<sub>2</sub> of the ratios of the inoculated/non-inoculated expression values was employed to generate a Euclidean distance matrix. Cluster v3.0 (Eisen et al. 1998) was used for clustering and TreeView v1.05 software was used for visualising.

## Results

### Evaluation of *Medicago truncatula* genotypes

Before carrying out the expression profiling of TFs of *M. truncatula*, it was necessary to identify genotypes of *M. truncatula* showing differential reaction to infection by *Botrytis* spp. It was equally important to characterise the differential aggressiveness of both pathogenic species (*B. cinerea* and *B. fabae*) against *M. truncatula*. Those were the objectives of the first experiment under controlled conditions, confronting two genotypes of *M. truncatula* against two isolates of *B. cinerea* and *B. fabae*. The genotypes were chosen on the basis of previous tests (data not shown).

First symptoms of infection appeared as soon as 2 dai. These were small necrotic spots covering leaf tissue. They were not abundant, so severity was still low, being the highest values just around 7%. Nevertheless, at this early stage, it was already possible to discriminate between genotypes, though still not between isolates: genotype A17 presented very low severity compared to that of genotype Esp162, while both

*B. fabae* and *B. cinerea* showed similar aggressiveness (Fig. 1a shows the results for each genotype/pathogen combination). The factorial analysis of the variance for severity confirmed this, showing significant genotypic effects, but not detecting differences between isolates. No interaction between genotype and pathogen was detected either (Supplementary Table 1).

Symptoms had increased at 8 dai. Not only did they consist of necrotic spots, but also whole necrotic areas had developed in some leaves, resembling aggressive lesions typical of infection by *B. fabae* on faba bean (Harrison 1988). Some of these lesions had even started sporulating. Again, these symptoms were more frequent in genotype Esp162 (Fig. 2), reaching very high levels in some cases (~60%); on the contrary, severity values for genotype A17 ranged from 6 to 12% (Fig. 1b). On this occasion, however, it was possible to discriminate between the pathogens: the isolate of *B. fabae* was more aggressive than the isolate of *B. cinerea*. All this was confirmed by the factorial analysis of variance (Supplementary Table 2). Actually, interaction between genotype and pathogen was detected. This interaction is explained because the response of A17 to both pathogens was quite similar; whereas the differences in aggressiveness between *B. cinerea* and *B. fabae* were found on genotype Esp162.

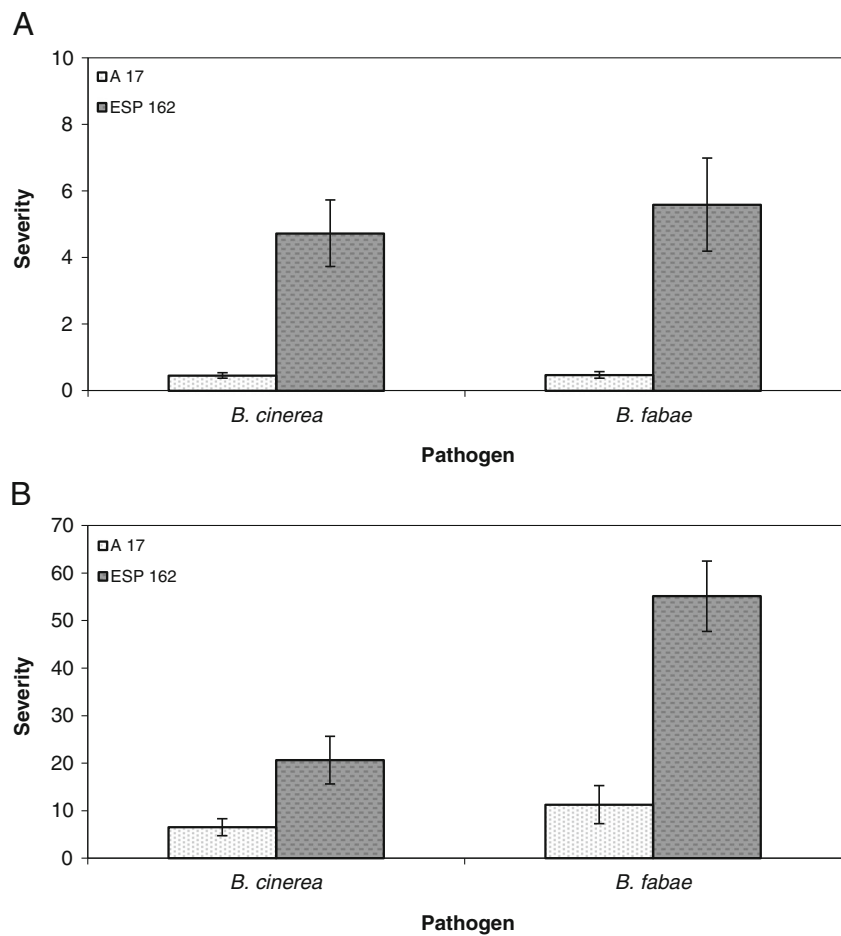
This first experiment, hence, permitted the identification of two genotypes of *M. truncatula* showing differential responses to *Botrytis* spp.: genotype A17, presented a reaction that can be defined as resistant, while genotype Esp162 was susceptible to the pathogens. Additionally, the superior aggressiveness of *B. fabae* over *B. cinerea* was established.

Once this had been done, the next step was the identification of the best time point for the collection of samples for the profiling of TFs. The microscopic observation of the early stages of the interaction plant/pathogen was deemed useful for this purpose.

The spores of *Botrytis* spp. germinate producing hyphae that grow on the leaf surface until they penetrate the plant epidermis. Then, they grow in the mesophyll, branching in different directions and killing cells to feed on them. We wanted to focus on the initial stages of infection, so the aim of the microscopy experiment was to evaluate spore germination after inoculation. We tested both genotypes of *M. truncatula*, in case any difference in the behaviour of the pathogens on them could be detected.

The rate of spore germination of *B. fabae* was below 30% in both A17 and Esp162 at 24 h after inoculation, lower than that of *B. cinerea* (~80% for both genotypes of *M. truncatula*). At 48 h after inoculation, the rate of spore germination for *B. fabae* had risen up to 60%, still lower than the rate for *B. cinerea*, which had only increased slightly (Fig. 3). The analysis of variance confirmed these differences for factor pathogen at both time points, whereas it did not detect any difference for genotype or the interaction genotype–pathogen

**Fig. 1** Severity values for two genotypes of *M. truncatula* (A17 and Esp162) 2 days (a) and 8 days (b) after inoculation with either *B. cinerea* or *B. fabae*. Vertical bars represent means of standard deviations (LSD test for arcsin  $\sqrt{x}$  transformed data;  $p < 0.05$ )



(Supplementary Tables 3 and 4). Therefore, the differential reaction to infection by *Botrytis* spp. of these genotypes of *M. truncatula* is not related to the process of germination of the spores of the pathogens.

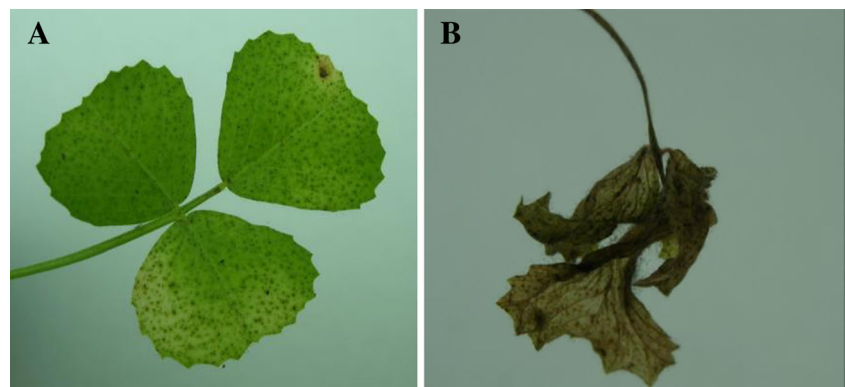
Considering the results of these two experiments, we chose the time point for the collection of samples for the profiling of transcription factors at 48 h after inoculation. At this time, a considerable number of pathogen spores are germinated and some symptoms have become visible,

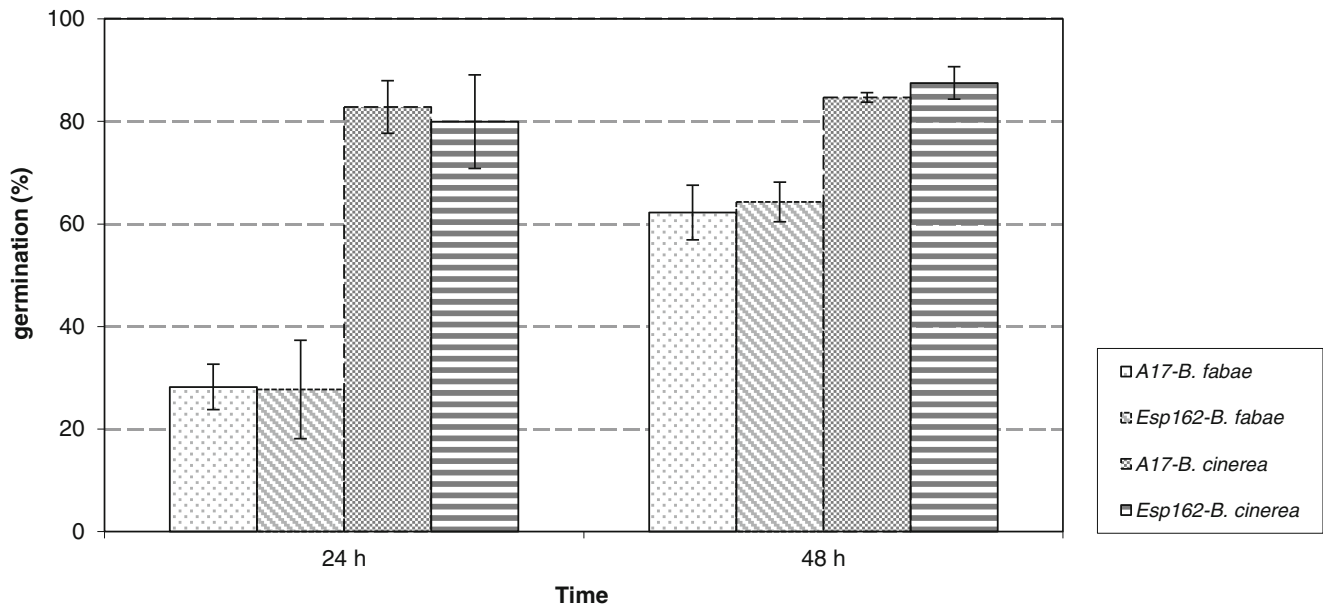
showing that the infection is already under way, but still in its initial stages.

#### Gene expression profiling of transcription factors

The objective of this experiment was to identify those transcription factors of *M. truncatula* involved in the interaction with *Botrytis* spp. The two chosen genotypes of *M. truncatula* present differential responses to *Botrytis* spp., and the

**Fig. 2** Leaves of genotype Esp162 of *Medicago truncatula* infected by *Botrytis fabae* (8 days after inoculation), showing typical necrotic symptoms (a) and complete necrosis with sporulation (b)





**Fig. 3** Germination values for spores of *B. fabae* and *B. cinerea* isolates on two genotypes of *M. truncatula* (A17 and Esp162) 24 and 48 h after inoculation. Vertical bars represent means of standard deviations

pathogens (*B. fabae* and *B. cinerea*) present differential aggressiveness; hence, our study had to consider both the effect of plant genotype and of pathogen.

A total of 874 out of the 1,084 TF-encoding transcripts (i.e. 80.6 %) could be amplified. The analysis with Bestkeeper allowed choosing *MtGapdh*, *MtPdj2* and *MtEfl* as the best reference genes, since they were the most stable across the different biological situations tested. These three genes were used to calculate the index for normalisation. The ensuing analyses of variance showed that 170 amplified transcripts showed significant differences only for genotype (Supplementary Table 5), 126 amplified transcripts showed significant differences just for pathogen (Supplementary Table 6) and, finally, 37 amplified transcripts showed significant differences for factors genotype and pathogen (Table 1). In Table 2, information about the proportions of the families of TFs of *M. truncatula* present in these three groups is provided.

A k-means clustering was performed on the 126 genes showing significant differences for pathogen in order to facilitate their analysis. This resulted in their classification into seven different clusters (Fig. 4). These clusters correspond with the different patterns of reaction to inoculation by the pathogens. Clusters 1 and 5 comprise TFs which are similarly down-regulated on infection by either *B. fabae* or *B. cinerea* (down-regulation were higher in cluster 1 than in cluster 5); in cluster 2, on the contrary, up-regulated TFs after infection by both pathogens are found; induced TFs only after inoculation with *B. fabae* are present in cluster 3; cluster 4 includes those TFs which undergo repression after infection, being this repression higher for *B. fabae* than for *B. cinerea*; finally, cluster 6 presents the repressed TFs after infection with *B. cinerea* and cluster 7 shows the repressed TFs after infection with *B. fabae*.

These various behaviours are shown in Fig. 4. As for the family distribution, cluster 2 includes three genes belonging to the Zn-Finger (C<sub>2</sub>H<sub>2</sub> type) family, two genes belonging to the WRKY family, one gene belonging to the AP2/EREBP family, one gene to the BHLH family and one gene to the HD-like family. In cluster 3, five AP2/EREBP, four MYB, two Zn-finger (one C<sub>2</sub>H<sub>2</sub> type and one CO-like type), one GRAS and one NAC are found, among others. In cluster 4, five AP2/EREBP, two Myb, two BHLH, one Zn-Finger (YABBY type), one bZIP, one PHD and one TCP are found. Clusters 1, 5, 6 and 7 are made up of TFs belonging to different families, no family standing out in particular.

As for the 37 genes showing significant differences for genotype and pathogen, most of them presented down-regulation, its degree depending on the genotype. Only three genes showed up-regulation for resistant genotype A17 after inoculation.

These results show that nearly a fifth of the 874 amplified TFs (126 for pathogen and 37 for both genotype and pathogen) are involved in the interaction *M. truncatula/Botrytis* spp.

## Discussion

In this work, we have studied the interaction between *M. truncatula* and two important causal agents of diseases in legumes at the level of the regulation of transcription. Given that *M. truncatula* is not a natural host of these pathogens, the first step was to set up a system that reproduces an effective infection process. Although the procedures have been described before (Ellwood et al. 2007), no differential genotypic responses to *Botrytis* spp. had been previously reported. We

**Table 1** Transcription factors that have presented significant differences in expression for both genotype and pathogen after inoculation with either *B. fabae* or *B. cinerea*. Their ascription to the different families of transcription factors of *Medicago truncatula* is provided. Different ratios of expression levels are shown

ID	Family	R(ni)/S(ni)		R(Bf)/S(ni)		R(Bc)/S(ni)	
		R(ni)	S(ni)	R(Bf)	S(ni)	R(Bc)	S(ni)
1040.m00021	MADS	0.82	0.36	0.29	0.39	0.58	
1061.m00001	MADS	0.29	0.51	0.33	0.48	0.55	
1084.m00001	ARID	0.46	0.39	0.56	0.34	0.50	
1103.m00014	CCHC (Zn)	12.71	3.47	1.13	3.22	0.79	
1161.m00029	Tc/PD	15.71	0.62	0.88	0.61	0.96	
1221.m00017	BHLH	1.77	0.13	0.05	0.23	0.27	
1241.m00015	C <sub>2</sub> C <sub>2</sub> (Zn)	2.07	0.11	0.14	0.28	0.27	
1323.m00024	S1Fa-like	0.95	6.06	3.92	3.12	1.74	
1392.m00018	HMG	0.67	0.40	0.41	0.58	0.52	
1405.m00027	BTB/POZ	1.43	0.46	0.44	0.59	0.68	
1564.m00034	CCAAT-HAP3	0.72	0.47	0.67	0.25	0.27	
1733.m00014	AS2	0.80	0.28	0.49	0.36	0.52	
221.m00129	MADS	0.25	0.44	0.53	0.53	0.68	
891.m00010	C <sub>2</sub> C <sub>2</sub> (Zn)	0.19	0.26	0.41	0.39	0.93	
924.m00009	MADS	0.32	0.38	0.52	0.44	0.63	
925.m00001	HLH	1.02	0.42	0.64	0.47	0.72	
939.m00019	HB	0.98	0.49	1.25	0.51	1.13	
956.m00012	TrpR	0.85	0.48	0.78	0.56	0.62	
980.m00009	C <sub>2</sub> H <sub>2</sub> (Zn)	0.24	0.56	0.46	0.63	0.38	
986.m00012	MADS	0.77	0.19	0.72	0.23	0.87	
989.m00004	AS2	0.43	0.50	0.43	0.58	0.45	
1011.m00010	NAC	2.99	0.61	0.70			
1365.m00012	Myb	0.64	0.68	0.65			
1443.m00022	NAC	1.41	0.61	1.31			
1524.m00006	Myb	3.12	1.12	2.48			
1684.m00031	Myb	1.17	0.32	0.59			
1699.m00013	C <sub>2</sub> H <sub>2</sub> (Zn)	1.59	0.53	1.23			
201.m00219	C <sub>2</sub> C <sub>2</sub> (Zn)	0.62	0.75	0.52			
1325.m00003	PHD	0.82			0.41	0.69	
1381.m00017	Myb	0.69			0.40	0.65	
1429.m00034	C <sub>2</sub> C <sub>2</sub> (Zn)	0.55			0.57	0.66	
1588.m00008	HB	0.50			0.54	0.71	
1701.m00020	AS2	0.40			0.67	0.52	
225.m00189	Myb	0.71			0.49	0.64	
944.m00008	BHLH	0.81			0.52	0.68	

ID identification number for each transcription factor, R resistant genotype A17, S susceptible genotype Esp162; ni not inoculated, BF inoculated with *B. fabae*, BC inoculated with *B. cinerea*

have identified two genotypes of *M. truncatula* that may be defined as partially resistant (A17) and susceptible (Esp162) to *Botrytis* spp. under test conditions. The severity values of their differential responses are similar to those obtained by genotypes of *V. faba* in experiments under controlled conditions (Villegas et al. 2012). It is remarkable that *B. fabae* has

**Table 2** Percentages of the genes that have shown significant differences for genotype, pathogen or both genotype and pathogen after inoculation with *Botrytis* spp., belonging to the different families of transcription factors of *Medicago truncatula*

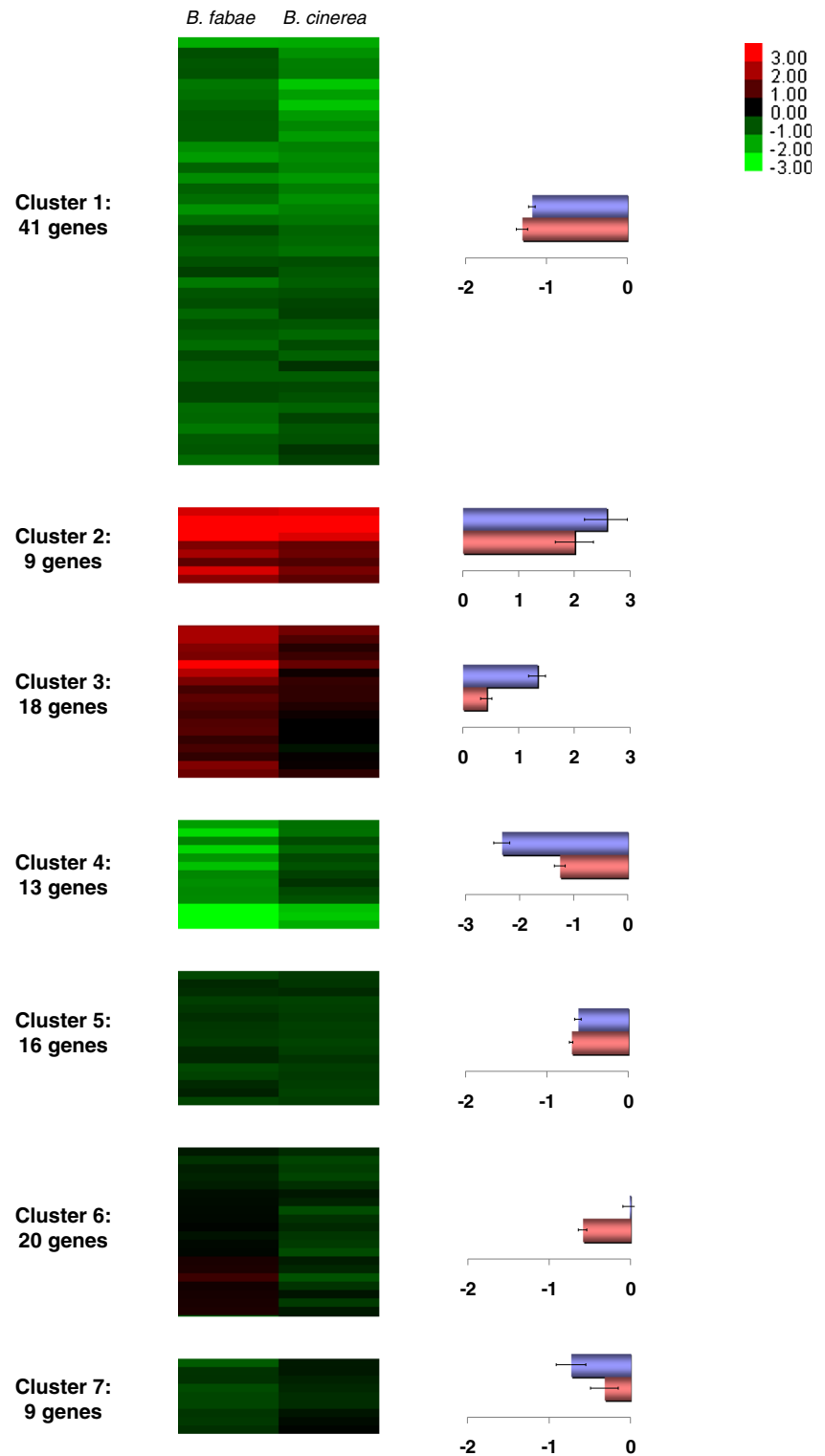
Genotype		Pathogen		Genotype and pathogen	
Family	%	Family	%	Family	%
Myb	12.94	Myb	11.90	C <sub>2</sub> H <sub>2</sub> (Zn)	17.14
CCHC (Zn)	11.76	AP2/EREBP	9.52	MADS	14.29
HD-like	7.65	C <sub>2</sub> H <sub>2</sub> (Zn)	7.94	Myb	14.29
PHD	5.29	HB	6.35	AS2	8.57
AP2/EREBP	4.71	C <sub>2</sub> C <sub>2</sub> (Zn)	5.56	BHLH	5.71
C <sub>2</sub> H <sub>2</sub> (Zn)	4.71	BHLH	3.97	HB	5.71
WRKY	4.71	BZIP	3.97	NAC	5.71
HB	3.53	GRAS	3.97	CCHC	2.86
Jumonji	3.53	ABI3-VP1	3.17	PHD	2.86
BZIP	2.94	DHHC (Zn)	3.17		
NAC	2.94	WRKY	3.17		
BHLH	2.35	NAC	2.38		
ARF	2.35	HMG	2.38		
Others	30.59	Others	32.55	Others	22.86

turned out to be more aggressive than *B. cinerea*. The former is a specialist pathogen that infects a few species of the Fabaceae, mainly *V. faba* (Staats et al. 2005), while the latter is a generalist pathogen responsible for diseases in legumes such as *Lens culinaris* and *Medicago sativa* (Tivoli et al. 2006a; Williamson et al. 2007), not posing a serious challenge for *V. faba*. The behaviour of *M. truncatula* resembles more of *V. faba* than that of other legumes in the case of these pathogens, thus making it a good model plant for the study of the *V. faba/Botrytis* spp. pathosystem.

The microscopy studies have shown that *B. cinerea* spores reached the maximum level of germination at 24 h after inoculation; the spores of *B. fabae* need longer time to germinate, always maintaining a lower rate of germination than *B. cinerea*. This might account for similar severities caused by both pathogens only 48 h after inoculation; later, however, after the number of germinated spores of *B. fabae* has increased, this pathogen proves to be more aggressive.

The availability of this biological material has allowed us to investigate in two different directions: firstly, through the identification of genes of *M. truncatula* involved in the process of infection by *Botrytis* spp., focusing on specific reactions to each pathogen, different in aggressiveness as they are; and secondly, through the identification of genes that characterise the differential response of each genotype when confronted with a given pathogen. Timing for sampling was chosen on the basis of this macroscopical and microscopical information, considering that 48 h after the inoculation, infection had just started to take place, and the plant would be reacting to it.

**Fig. 4** Heat maps of the expression profiles ( $\log_2$  of the ratio inoculated/non-inoculated) of the TFs presenting significant differences for pathogen, separated in the clusters obtained by K-means clustering (colour scale is shown: *green* for down-regulation and *red* for up-regulation and *black* for values close to 0); the number of genes included in each cluster is indicated on its *left*. The average of the ratios for each cluster is represented to its *right*, *blue bar* being for *B. fabae* and *red bar* for *B. cinerea*



Nearly 20 % of the 874 amplified genes changed their expression levels 48 h after inoculation with either *B. fabae* or *B. cinerea* (37 genes showed significant differences for genotype and pathogen, while 136 genes showed for only pathogen, regardless of the genotype). This proportion is

higher than that (~13 %) found for *Uromyces striatus* (Madrid et al. 2010) in the only other study reported so far to have used the *M. truncatula* TF qPCR platform to assess the response to fungal diseases. We can hypothesise that this difference is because both *B. fabae* and *B. cinerea* are



necrotrophic pathogens that quickly penetrate plant tissue, grow inside it and feed on plant cells after killing them, while *U. striatus* is a biotrophic fungus that takes a longer period to expand inside and damage the plant, therefore causing higher physiological alteration at the beginning of infection by *Botrytis* spp.

Cluster analysis allowed the grouping of the TFs that differentially responded to infection. Most of these TFs were down-regulated (99 out of 126, 57 independently of the pathogen); they are of various types, belonging to different families, and though their repression could be due to the hampering of biological processes by the pathogen, it is also true that down-regulation may be an integral part of a defensive response by plants (Bustos et al. 2010). Further work would be necessary to elucidate this issue, and establish the role of each of these TFs under these circumstances. As for the nine genes up-regulated after infection with both pathogens, six of them belong to families related to stress responses: AP2/EREBP, which is a unique family to plants, characterised by the presence of the AP2 DNA-binding domain, and is known to be linked to the response to abiotic and biotic stresses (Dietz et al. 2010); C<sub>2</sub>H<sub>2</sub> or Cys<sub>2</sub>/His<sub>2</sub>-type zinc finger proteins, which have been implicated in the response to different stresses, both biotic and abiotic (Kielbowicz-Matuk 2012); and WRKY, also an exclusive family to plants, whose members constitute what has been called a ‘web of plant defence regulators’ against pathogens (Eulgem 2006). These genes might constitute the core of the defensive reaction of *M. truncatula* to *Botrytis* infection.

Of special interest is the differential response observed with the two pathogens. Apart from the nine genes previously mentioned, 18 TFs are also up-regulated when infection is caused by *B. fabae*. Again, among them, we find genes belonging to the families related to reaction to stresses: AP2/EREBP and C<sub>2</sub>H<sub>2</sub>, as previously seen; GRAS, a family of proteins that, though not known to bind to a specific DNA sequence, are classified as transcriptional regulators, and some of its members are described as essential for the activation of stress-inducible promoters (Fode et al. 2008); NAC, which has been reported as key in regulating stress perception (Jensen et al. 2010); and Myb (one homeodomain-like), a family widely represented in plants whose members have been assigned many roles, in some cases linked to defence regulation (Dubos et al. 2010). All of these points to an increase in the defence responses in *M. truncatula* when confronted with *B. fabae* higher than with *B. cinerea*. Our results show that *B. fabae* is more aggressive than *B. cinerea*; therefore, we may assume that the plant unchains additional defensive mechanisms to tackle a more serious challenger. It is more remarkable considering that 24 h after inoculation, there are more germinated spores of *B. cinerea* than of *B. fabae*, therefore, the number of infection sites is, in principle, higher for the former than for the latter; however, it seems that the

plant recognises *B. fabae* as a more dangerous foe even at this early stage and reacts accordingly. In the case of the *V. faba*/*Botrytis* spp. pathosystem, it has been suggested that *B. fabae* is more aggressive than *B. cinerea* because it is capable of metabolising the phytoalexin (wyeron acid) produced by the plant (Harrison 1988). It has also been reported that *B. fabae* produces a phytotoxin (regiolone), which is more toxic against *V. faba* than the equivalent toxin in *B. cinerea* (isosclerone) (Evidente et al. 2011). Our hypothesis suggest that something similar could happen in the case of *M. truncatula*/*Botrytis* spp., therefore leading the plant to resort to extra defence resources in an attempt to stop infection. Finally, it is also noticeable that there are different genes down-regulated depending on the type of pathogen (20 genes for *B. cinerea* and 9 genes for *B. fabae*). As in the previous case, it is difficult to know if this is part of a defence response or if it is a reflection of the hindrance of the different physiological processes by each pathogen. Nevertheless, it would not be likely to expect that *B. cinerea* would hamper more physiological processes than *B. fabae*, given that the former has turned out to be less aggressive a pathogen than the latter. So, it is possible to assume that most of those 20 genes, down-regulated after infection by *B. cinerea*, are somehow related to defence mechanisms. All these results reveal that 48 h after inoculation, although no difference is detected at the macroscopic level; however, at the molecular level, the plant–pathogen interaction is specific for each *Botrytis* species, anticipating the diverse reaction clearly obvious after some days with the difference in aggressiveness of each pathogen.

As for the differential response of the tested genotypes to infection, it is noteworthy that most of the genes presenting different behaviours for both the genotype and the pathogen are down-regulated. Although it is difficult to determine whether this repression is part of the defence reaction, there are some cases pointing to this direction: TFs 1443.m00022, 1699.m00013 and 939.m00019 are all down-regulated in resistant genotype A17 and up-regulated in susceptible Esp162. The same could be said of other genes as 980.m00009 or 989.m00004, which show lower levels of expression in non-inoculated A17 than in non-inoculated Esp162, and experience further repression in A17 after inoculation with both pathogens; the low expression may be related to a more effective defensive response. On the contrary, there are some genes that, in spite of undergoing down-regulation, present higher expression levels in infected A17 than in infected Esp162. We could assume that the down-regulation is, on this occasion, a consequence of malfunction due to the presence of the pathogen, and that the resistant genotype has managed to alleviate this effect. On the other hand, only three TFs undergo up-regulation after inoculation, two of these are common to the infection by *B. fabae* and *B. cinerea* (1103.m00014 and 1323.m00024) and the third TF is exclusively up-regulated after *B. fabae* inoculation

(1524.m00006). All of them reach higher levels of expression in A17 than in Esp162 after inoculation, therefore, they may be suggested as taking part in the defensive reaction against *Botrytis* spp.

Apart from these induced responses to infection, constitutive defences should not be disregarded. *M. truncatula* presents, in principle, non-host resistance to *Botrytis* spp.; although we have managed to overcome this resistance and achieve infection, it is likely that the resistant reaction shown by A17 is largely based on non-host resistance. It is generally accepted that non-host resistance consists of two components: constitutive and induced resistance. The constitutive resistance is based on a series of different elements, ranging from structural barriers to inhibitory metabolites or proteins (Ferreira et al. 2006). We found a great number of TFs (170) showing constitutive differences between the two genotypes regardless of inoculation with the pathogens. This result is in contrast with that of Madrid et al. (2010), who only reported nine TFs presenting constitutive differences between their tested genotypes; this suggests that the genotypes selected for our work differs greatly one from the other. It is probable that some of these 170 TFs are involved in constitutive resistance to infection. Among these TFs, there are members of families related to plant resistance, such as C<sub>2</sub>H<sub>2</sub>, AP2/EREBP, WRKY or NAC; NAC has been reported to be involved in non-host resistance (Uma et al. 2011).

Finally, it is of interest to notice that only 2 of the 13 defence-related genes pointed out by Madrid et al. (2010) appeared as influenced by infection in our results. TF 1082.m00015 is up-regulated in the resistant response to *U. striatus*, while 1063.m00010 is repressed. In our case, the behaviour is quite similar, since 1082.m00015 is up-regulated and 1063.m00010 is down-regulated after inoculation with both *B. fabae* and *B. cinerea* (with no difference between genotypes). In any case, it is not surprising that most of the TFs involved in the reactions to each of the diseases are not the same: the studied systems are very different, both for the type of pathogen and for the genotypes of *M. truncatula* chosen (it is remarkable that A17 is susceptible to *U. striatus* and presents resistance to *Botrytis* spp.).

In conclusion, this work has established a system to study *M. truncatula/Botrytis* spp. interactions that may be a useful model for the *V. faba/Botrytis* spp. pathosystem. The profiling of transcription factors has permitted the identification of, on the one hand, those involved on the differential responses to *B. fabae* and *B. cinerea* and, on the other hand, those participating in the resistance to these pathogens. Future work should focus on assigning a clearer role to these TFs, describing and integrating the pathways in which they may take part, and complementing these results with additional molecular and pathological studies. This would increase the functional knowledge of the *M. truncatula* genome and help in breeding programmes of *V. faba* for resistance to chocolate spot.

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