

Gene coding for an elongation factor is involved in resistance against powdery mildew in common bean

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Abstract

Key message Genetic control of the resistance response against powdery mildew in common bean was studied combining genetic, genomic and transcriptomic analyses. A candidate resistance gene in cultivar Porrillo Sintetico was proposed.

Abstract The species causing the fungal disease powdery mildew (PM) in the local common bean crop was identified as *Erysiphe polygoni* through the molecular analysis of the internal transcribed spacer region. A genetic analysis of the resistance in cultivar Porrillo Sintetico was conducted using different F_{2,3} populations, and a dominant gene conferring total resistance against a local PM isolate was physically located between 84,188 and 218,664 bp of chromosome Pv04. An in silico analysis of this region, based on the common bean reference sequence, revealed four genes candidate to be involved in the resistance reaction. Relative expression levels of these genes after PM infection showed a significant over-expression of the candidate gene *Phvul.004G001500* in the resistant genotype Porrillo Sintetico. This gene was re-sequenced in the parental genotypes X2776 and Porrillo Sintetico to explain their different

phenotypic responses against PM. Several substitutions where identified in exon regions, all of them synonymous, so differences in the produced amino acid sequence were not expected. However, a total of 37 mutations were identified in non-coding regions of the gene sequence, suggesting that intron variation could be responsible for the different gene expression levels after PM infection. No evidence of other regulatory mechanisms, such as alternative splicing or methylation, was identified. Candidate resistance gene *Phvul.004G001500* codes for an elongation factor that is not a typical gene related to recognition of specific pathogens in plants, suggesting its involvement in the resistance through plant immune system.

Introduction

Common bean (*Phaseolus vulgaris* L.) is an important legume for direct human consumption worldwide (FAO 1999; Broughton et al. 2003). It is an autogamous diploid ($2n=2x=22$) species with a relatively small genome (~587 Mb) that has recently been assembled and automatically annotated, with 27,197 protein coding loci identified (Schmutz et al. 2014; Vlasova et al. 2016).

Powdery mildew (PM) is a widespread plant disease causing significant yield losses in both agricultural and horticultural crops. Geographical distribution of this disease is increasing rapidly in different parts of the world, affecting large areas. This expansion has been related to global climate change because PM pathogen populations evolve quickly, showing a high rate of variation due to the coexistence of sexual and asexual stages and their high dispersal capability. In fact, PMs are a useful model for studying effects of climate change on plant diseases (Glawe 2008). PM fungi are obligate biotrophic parasites that invade susceptible

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plant cells without triggering cell death, which suggests a highly adapted mechanism of parasitism, avoiding detection and/or evading host defenses. Symptoms are easily recognizable as greyish powdery superficial growth, generally occurring on younger plant material and the upper sides of leaves (Schwartz et al. 2005). PM disease can be caused by a broad range of genera and species of Ascomycete fungi of the order *Erysiphales* (Glawe 2008). Some species are highly specific, whereas others infect a broad range of host species. There is controversy concerning the common bean PM's causal agent, as it has frequently been ascribed to *Erysiphe poligona* DC (Rezende et al. 1999; Schwartz et al. 2005), but recent studies suggest that it is closer to *Erysiphe diffusa* (Cooke & Peck) U. Braun & S. Takam, formerly *Microsphaera diffusa* Cke. & Pk. (Almeida et al. 2008). The identification of the species is difficult by classical methods based on the phenotypes of hyphae, conidia and conidiophores, and the host range, due to the complexity of the genus. A powerful method for fungal species identification is the analysis of sequence variation in the nuclear ribosomal internal transcribed spacer (ITS), which is used as a universal DNA barcode marker for fungi (Schoch et al. 2012).

The most effective control tool for this disease is the use of resistant cultivars. Differences between common bean varieties in responses against PM were identified for the first time in naturally infected fields (Dundas 1936, 1941; Schwartz et al. 1981). Recently, an evaluation test conducted under controlled greenhouse conditions was proposed (Trabanco et al. 2012). To date, not many resistance sources have been identified in common bean. Porrillo Sintetico is one of the most resistant cultivars described, showing a total resistance response in the field and in controlled evaluations (Schwartz et al. 1981; Trabanco et al. 2012). Before starting a breeding program for the development of resistant varieties is important to know about pathogen diversity, variations in the reactions among cultivars, and the type of resistance inheritance. Genetic analyses showed a qualitative mode of PM resistance inheritance (Trabanco et al. 2012). Two dominant resistance genes were identified and located in the genetic linkage map of common bean, the *Pm1* gene that confers total resistance at the end of linkage group (LG) Pv11, and the *Pm2* gene that confers intermediate resistance at LG Pv04 (Pérez-Vega et al. 2013). These genes show an epistatic relationship, *Pm1* masking the response of *Pm2*. Previous genetic analyses suggested that the resistance reaction in Porrillo Sintetico was conferred by two dominant and independent genes, one conferring total resistance, and another gene conferring intermediate resistance, with moderate mycelial development on leaves, although the genetic location of these genes remained unknown (Trabanco et al. 2012). Relative genetic positions inferred for the PM resistance genes *Pm1* and *Pm2* overlap with resistance gene analog regions (RGAs), where resistant genes against different diseases have been located

(Ferrier-Cana et al. 2003; David et al. 2009; Ferreira et al. 2013; Meziadi et al. 2016). At a genomic level, these regions are rich in genes encoding proteins containing nucleotide binding site (NBS)-leucine-rich repeat (LRR) domains, which specifically recognize an avirulence gene product of the pathogen (Young 2000; Dangl and Jones 2001; Marone et al. 2013). Plants can recognize potential pathogens through two perception systems. One, named pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), detects conserved microbial molecules through pattern-recognition receptors, leading to PAMP-triggered immunity (PTI). The other evolved to recognize microbial virulence effectors, usually through intracellular resistance proteins (R proteins), causing effector-triggered immunity (ETI), which is associated with gene-for-gene interactions, and vertical or race-specific resistance (Heath 2000; Jones and Dangl 2006; Dodds and Rathjen 2010). The most studied resistant model in plants includes R genes coding for NBS-LRR domains that are involved in specific plant-pathogen interactions (Marone et al. 2013). Currently, widespread plant genome sequencing is helping in the identification of new mechanisms and resistance genes involved in PTI. It has even been suggested that PTI may be more important than ETI in inhibiting pathogen growth and can be a more stable form of resistance (Heath 2000). However, how expression of genes involved in the resistance response is regulated against stress is still poorly understood. Genes that code for multiple transcript variants from a single gene through different splice sites (alternative splicing) have been identified as a part of a regulatory mechanism present in many R genes (Dinesh-Kumar and Baker 2000; Jordan et al. 2002). DNA methylation, in higher eukaryotes, characterized by the presence of 5-methylcytosine (m⁵C) nucleotides, plays an important role in gene regulation in plants in response to biotic stresses (Chinnusamy and Zhu 2009; Downen et al. 2012). DNA methylation has also been identified as an important mechanism for host DNA defenses against the incorporation of “parasitic” or foreign DNA (Yoder et al. 1997; Colot and Rossignol 1999).

The aim of this study was to investigate the genetic control of the resistance response against PM in the bean cultivar Porrillo Sintetico, combining genetic, genomic and transcriptomic analyses.

Materials and methods

Plant material

Two populations derived from different crosses were used, X2776×G122 and X2776×Porrillo Sintetico. Both populations were obtained by self-pollinating the corresponding F₁ seeds, and then individually self-pollinating the resulting

F₂ seeds. Each F₂ plant was used for DNA analyses and the corresponding F₃ seeds were used to characterize the PM reactions of the corresponding F₂ plants (F_{2,3} family). Population X2776×G122 contained 90 F_{2,3} families. X2776 is a breeding line developed in SERIDA (Asturias, Spain), belonging to the fabada market class. G122 is a landrace cultivar collected from India, having cranberry bean-type seeds. Population X2776×Porrillo Sintetico was formed by 160 F_{2,3} families. Porrillo Sintetico is a small-seeded black breeding line, included in the black turtle market class that shows resistance to PM (Trabanco et al. 2012).

Inoculation procedure and disease scoring

A local isolate of PM maintained in the SERIDA station was used. It was grown and maintained on plants of the susceptible bean cultivar Xana in spore-proof chambers. Resistance tests were carried out according to Trabanco et al. (2012). Plant responses were recorded as infection type (IT) following a 0–4 scale (Fig. 1). Plants showing the IT0 response were considered resistant, plants showing IT1, IT2, or IT3 were considered to have intermediate resistance, and plants showing IT4 were considered susceptible. The reaction was evaluated in 10–20 F₃ seedlings per F₂ plant 10–12 days after inoculation. Families having an unclear classification after the first tests were re-evaluated to confirm their genotypes. In each test, families were individually randomized throughout the whole climate room, and the lines Cornell 49242 (IT0), A195 (IT1), TO (IT2), X2776 (IT3), and Xana (IT4), were included as references for different reaction types.

DNA extraction and molecular marker analyses

Plant and fungal DNA were extracted using the FastDNA kit (MP Biomedicals, Illkirch, France) following the supplier's instructions. DNA concentrations were quantified

photometrically using a Biomate 3 UV–visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Previously described insertion/deletions (InDel) and microsatellites (SSR, simple sequence repeat) markers were used (Moghaddam et al. 2014; <http://bic.css.msu.edu/Genetics.cfm>). Other markers were designed in this work from the bean sequenced genotype G19833 (*Phaseolus vulgaris* v1) available at <http://www.phytozome.net> (Goodstein et al. 2012; Schmutz et al. 2014) using the web application BatchPrimer3 v1.0 with default parameters (You et al. 2008; <http://probes.pw.usda.gov/batchprimer3/>). All markers designed were specific. Polymerase chain reaction (PCR) amplifications were performed in 20 µl solution containing 25 ng of genomic DNA, 100 mM Tris–HCl, 100 mM KCl (pH 8.3), 4 mM MgCl₂, 0.2 mM each dNTP (Bioline, London, UK), 0.2 µM each primer, and 1.25 U of Biotaq DNA polymerase (Bioline). Amplifications were performed in a Veriti Thermal Cycler (Applied Biosystems, Life Technologies, Foster City, CA, USA). For newly designed primers, the conditions recommended by the OligoAnalyzer software were used (<http://www.uku.fi/kuulasma/OligoSoftware/>). PCR products were resolved on 2% agarose or 6% polyacrylamide gels, stained with SYBR Safe (Invitrogen, Life Technologies, CA, USA) and visualized under UV light.

Linkage analyses

The goodness-of-fit of observed to expected ratios was tested using chi-squared. MAPMAKER Macintosh version 2.0 software (Lander et al. 1987) was used for linkage analyses and map construction considering a log of the likelihood ratio (LOD) threshold of 3.0 and a recombination fraction of 0.25. The 'order' and 'compare' commands were used to identify the most probable loci order within a linkage group. The 'ripple' command was used to verify the order and establish a log-likelihood value. Distances between ordered loci (in centimorgans, cM) were calculated using the Kosambi mapping function.

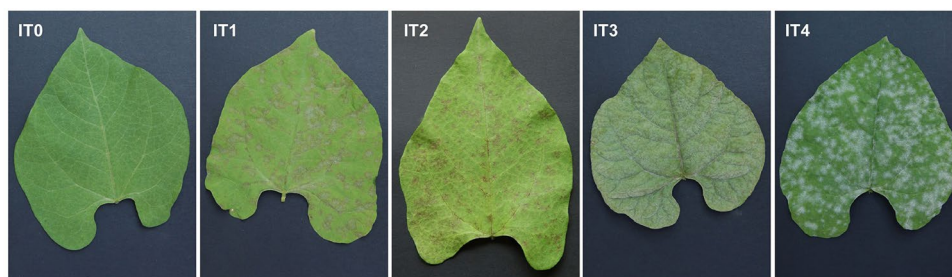


Fig. 1 Scoring scale for PM symptom evaluation. Five infection types (IT) were considered in the classification of responses to PM in common bean: IT0, primary leaves with no visible symptoms; IT1, primary leaves with necrotic reactions on leaves and no mycelial

development; IT2, primary leaves with necrotic reactions and moderate mycelial development; IT3, primary leaves with moderate mycelial development and no sporulation; IT4, primary leaves with abundant mycelial development and profuse sporulation

RNA extraction and reverse transcriptase (RT)-PCR analysis

Total RNA was isolated using the GeneJet Plant RNA Purification Kit (Thermo Fisher Scientific). RNA qualities and concentrations were determined by gel electrophoresis and using a spectrophotometer. Purified RNA was further treated with RNase free DNase I (Thermo Fisher Scientific) to remove any contaminating genomic DNA according to the manufacturer's instructions. Approximately 2 µg of DNA-free RNA was used for cDNA synthesis with the First Strand cDNA Synthesis Kit for RT-PCR AMV (Roche, Madrid, Spain) according to the manufacturer's instructions. The oligo (dT) primer supplied with the kit was used.

RT-qPCR

Seeds of the parental lines X2776 and Porrillo Sintetico were surface sterilized using 10% sodium hypochlorite solution for 2 min, rinsed with distilled water and planted in 15-cm plastic pots (1.5 L volume) containing peat:perlite (80:20). For each genotype, half of the seedlings were used as a control, without inoculate, and the other half were inoculated with PM as described above. Each sample was represented by leaves from four plants. Inoculated plants were maintained after collecting leaf samples to verify the presence of PM symptoms. The experiment was repeated three independent times. RT-qPCR reactions were conducted in a Stratagene Mx3000P machine (Agilent Technologies, Santa Clara, CA, USA) using the Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies). Data were analyzed with MxPro qPCR software (Agilent Technologies). Each sample was amplified in four independent replicates. The *insulin degrading enzyme* (*IDE*, *Phvul.001G133200*) and *actin* (*Act11*) genes were used as *housekeeping* genes in expression experiments. These genes were proposed by Borges et al. (2012) as reference internal control genes for RT-qPCR normalization in common bean during biotic stresses. Significant differences in the obtained results were investigated using Student's *t* test and ANOVA with the SPSS V12 software. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method, where $\Delta C_t = C_t$ gene of interest $- C_t$ internal control, $\Delta\Delta C_t = \Delta C_t$ inoculum $- \Delta C_t$ control, and 2 is the maximum PCR efficiency (Livak et al. 2001).

DNA sequencing and sequence analyses

The internal transcribed spacers (ITS1 and ITS2) and the 5.8 S rRNA were re-sequenced in the local PM isolate using the primers ITS1 and ITS4 (White et al. 1990). The obtained sequences were aligned with the sequences

deposited in GenBank (<http://blast.ncbi.nlm.nih.gov/>) and UNITE databases (Kõljalg et al. 2013; <http://unite.zbi.ee>) using the BLAST (Basic Local Alignment Search Tool) algorithm. *Phvul.004G001500* was re-sequenced in genotypes X2776 and Porrillo Sintetico. In all cases, PCR products were purified with Illustra Exoprostar 1-Step (GE Healthcare Life Sciences, Barcelona, Spain) following the supplier's instructions, and sequenced with a 3130XL Genetic Analyzer (Scientific and Technical Services, University of Oviedo, Asturias, Spain). Fragments were sequenced in both forward and reverse directions. Sequencing results were analyzed using the Sequence Scanner V2 (Applied Biosystems) software, and the consensus sequence was generated using the BioEdit Sequence Alignment Editor (Hall 1999).

Alternative splicing

Alternative splicing experiments were conducted using cDNA samples obtained from leaf tissues before PM infection. To test the possibility of alternative spliced transcripts in the gene *Phvul.004G001500*, two primers were designed with Batchprimer3 (You et al. 2008) using the sequences of the fifth and last exons: ExCr4_1500-F (5' CGTGTGGT GCTTTGGTACG 3') and SpliCr4_1500-R (5' TTAGGC AACTTGAGCGACAG 3'), respectively. The sizes of the amplified fragments were estimated on 2% agarose gels using a 100-bp marker (Bioline).

Bisulfite sequencing: gene methylation

The presence of CpG islands in the sequence of the *Phvul.004G001500* gene was tested using the software Methprimer and with the default parameters (Li and Dahiya 2002; <http://www.urogene.org/methprimer/index1.html>). This software was also used to design specific primers to amplify the regions of interest (BSF-F:5' TAAGGA TATAGGTTTGAATAATGAT 3', and BSF-R:5' TTACAC TAAAAAATAAACCTCATAAATAAT 3'). Methylation experiments were conducted in DNA samples obtained from leaf tissues before PM infection. The bisulfite treatment of approximately 1 µg of genomic DNA was performed using the Cells-to-CpG™ Bisulfite Conversion Kit (Applied Biosystems) following the manufacturer's instructions. The bisulfite treatment of single-stranded DNA converts unmethylated cytosines into uracil, while methylated cytosines remains unconverted. After treatment, the regions of interest were amplified and sequenced with a 3130XL Genetic Analyzer (Scientific and Technical Services, University of Oviedo). By comparing the sequence of the bisulfite-treated DNA with that of untreated DNA, the methylation profile is determined: conversion of a cytosine to thymine indicates non-methylated cytosines; in contrast,

the absence of cytosine to thymine conversions indicates methylation.

Results

Characterization of the local PM isolate

To identify the PM species used in this work, a partial sequence of the ribosomal DNA, including ITS1, 5.8 S ribosomal and ITS2, was analyzed using the standard set of primers ITS1+ITS4. The genomic sequence obtained was added to GenBank (<http://www.ncbi.nlm.nih.gov>) as accession KU320678. This sequence was aligned with that of *E. polygona* (GenBank accession GU570595.1), and *E. diffusa* (GenBank accession FJ378880.1), the two main PM causal agents reported in common bean. The sequence alignment revealed that the local PM isolate was most similar to *E. polygona* (Fig. S1). Two nucleotide differences (one deletion and one substitution) were found between the local PM isolate and *E. polygona* (score = 1182, *e*-value = 0.0), and five nucleotide differences (one deletion and four substitutions) were found between the local PM isolate and *E. diffusa* (score = 1166, *e*-value = 0.0).

Observed segregation of PM resistance

The PM resistance response was analyzed in the $F_{2:3}$ populations from X2776 × G122. X2776 showed an IT3 response, or intermediate resistance, against PM, whereas G122 showed an IT4 response, or susceptibility. The observed segregation was: 26 families with all of the seedlings having IT3 responses (homozygous for the X2776 allele), 37 families with seedlings having IT3 and IT4 responses (heterozygous), and 27 families with all of the seedlings having IT4 responses (homozygous for the G122 allele). This ratio fit the expectations for one gene ($\chi^2_{1:2:1} = 2.87$, $p = 0.24$).

The segregation of the PM response was also analyzed in the $F_{2:3}$ population of X2776 × Porrillo Sintetico. Porrillo Sintetico showed an IT0 response, or total resistance, against PM. The observed segregation of PM resistance in the $F_{2:3}$ population of X2776 × Porrillo Sintetico showed 37 families with all of the seedlings having IT0 responses, 69 families with all of the seedlings having IT3 and IT0 responses, and 44 families with all of the seedlings having IT3 responses. This ratio fit the expectation for one gene ($\chi^2_{1:2:1} = 1.61$, $p = 0.45$). The segregation ratio observed and the absence of susceptible (IT4) families, can be explained if X2776 and Porrillo Sintetico share the gene conferring the IT3 response, and Porrillo Sintetico carries a second epistatic gene that confers total resistance, thereby

masking the effects of the gene conferring intermediate resistance.

Linkage genetic analyses

Molecular markers located in the positions of LGs Pv04 and Pv11, in which PM resistance genes *Pm1* and *Pm2* had been mapped (Pérez-Vega et al. 2013), were analyzed in both populations. In population X2276 × G122 the gene conferring intermediate resistance was significantly linked to markers located on chromosome Pv11 and showed an independent segregation of molecular markers located on chromosome 4 (Table S1). In the population X2276 × Porrillo Sintetico the gene conferring total resistance was significantly linked to markers located on chromosome Pv04 and showed an independent segregation of molecular markers located on chromosome Pv11 (Table S2). A high-density linkage map was created for LG Pv04 in the X2776 × Porrillo Sintetico population (Fig. 2a). The PM resistance gene conferring the IT0 response was flanked by markers IND4_00798 (0.4 cM) and IND4_02324 (1.8 cM), which were physically located on chromosome Pv04 at 79,827 and 232,391 bp, respectively. The genomic sequence of chromosome Pv04 between these two markers was analyzed to design new molecular markers and approach the resistance gene. A total of 13 microsatellite markers were developed, of which 5 were polymorphic (Table S3) and included in the genetic linkage map (Fig. 2b). A co-segregation was observed between PM resistance gene and SSR markers Chr4_SSR4, Chr4_SSR8, Chr4_SSR10, Chr4_SSR15, and Chr4_SSR25.

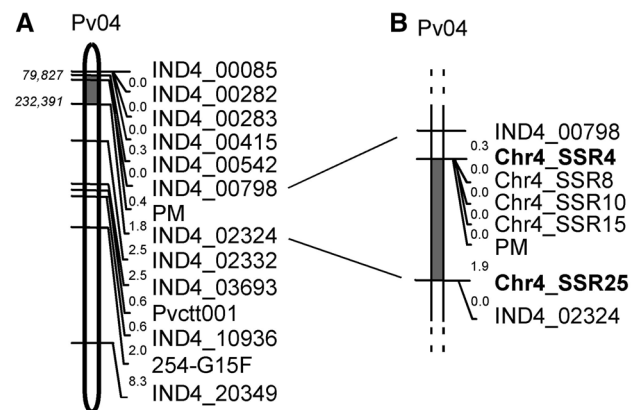


Fig. 2 Genetic linkage map obtained in the X2776 × Porrillo Sintetico $F_{2:3}$ population. Map distances are expressed in centiMorgans using the Kosambi mapping function. **a** Genetic linkage map obtained for LG Pv04, including the gene conferring the IT0 response against PM (PM, resistance gene). **b** Detail of the genetic linkage map corresponding to markers located between 79,827 and 232,391 bp of chromosome 4

In silico analysis

The genetic map indicated that the PM resistance gene is flanked by markers Chr4_SSR4 and Chr4_SSR25, corresponding to the physical positions of 84,202 and 218,664 bp, respectively, on chromosome 4. A total of seven functional genes were predicted in this chromosomal region (Fig. 3a), from gene *Phvul.004G001100* to gene *Phvul.004G001700*. The four genes having functional annotations, *Phvul.004G001200*, *Phvul.004G001300*, *Phvul.004G001400*, and *Phvul.004G001500*, were considered as candidates involved in the resistance response. The remaining three genes having no functional annotations were not considered in the analysis. A gap of 68,873 bp missing values was present in the G19833 genome between genes *Phvul.004G001600* and *Phvul.004G001700*. This gap is also present in the genome of the genotype BAT93 recently published (Vlasova et al. 2016).

Relative expression analysis

The possible involvement of each one of the four candidate genes was tested through expression analyses (RT-qPCR) in both parental genotypes, X2776 and Porrillo Sintetico, using two treatments: control without inoculation, and inoculated samples (analyzed 72 h after inoculation). For each one of the four genes, primers located in a coding region were developed (Table S4). Two *housekeeping* genes, *Act11* and *IDE*, were tested as internal control (Table S5). In the first of three independent experiments conducted, the *Act11* gene was discarded as it showed significant differences between treatments in cultivar Porrillo Sintetico. The *IDE* gene showed not significant changes on its expression and was used as internal control in all of

the experiments. Table 1 shows the results of the analyses of variance for the expression levels of the four candidate genes. Only the *Phvul.004G001500* gene was differentially expressed after inoculation (treatment effect) and for the interaction genotype $\times$ treatment, suggesting that this gene could be related to the different responses against PM infection between genotypes X2776 and Porrillo Sintetico. Significant differences were observed in the interaction genotype $\times$ run. This can be explained due to the natural variation of the physiological state of the tested plants and of the infectivity of the inoculum, which are difficult to standardize among repetitions. Figure 4 shows the results of the gene expression using the $2^{-\Delta\Delta C_t}$ method. In agreement with the ANOVA, no significant differences were found for genes *Phvul.004G001200*, *Phvul.004G001300*, and *Phvul.004G001400*, while gene *Phvul.004G001500* was significantly up-regulated in the resistant genotype Porrillo Sintetico 72 h after the PM infection.

Re-sequencing of the *Phvul.004G001500* gene

Gene *Phvul.004G001500*, annotated in the genotype G19833 (Chr04:95644.0.100091) contains 7 exons and 6 introns (Fig. 3b). To investigate any variation between X2776 and Porrillo Sintetico that could justify their different responses against PM, the *Phvul.004G001500* gene was re-sequenced in both genotypes using a set of 8 primer pairs (Table S6). The genomic sequences obtained for genotypes X2776 and Porrillo Sintetico were added to GenBank (<http://www.ncbi.nlm.nih.gov>) and correspond to accessions KU320679 and KU320680, respectively. A sequence alignment with reference genotype G19833 showed 7 synonymous substitutions involving exon regions in X2776 (Fig. S2), and 3 synonymous

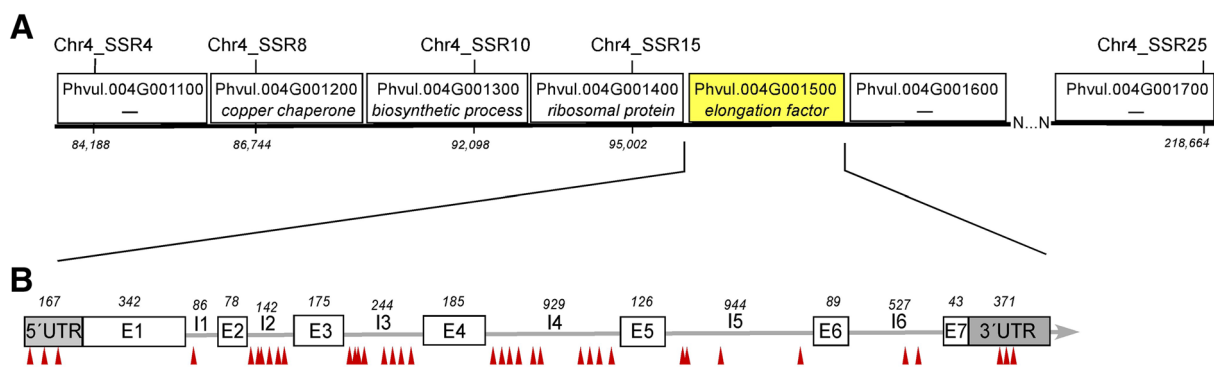


Fig. 3 **a** Genes predicted between 84,188 and 218,664 bp of chromosome 4 in the bean sequenced genotype G19833 (*Phaseolus vulgaris* v1; <http://www.phytozome.net>), and estimated physical positions (in bp) of markers Chr4_SSR4, Chr4_SSR8, Chr4_SSR10, Chr4_SSR15, and Chr4_SSR25. The annotated function for each locus is indicated in *italics*. N missing base pair values. **b** Schematic representation

of gene *Phvul.004G001500*. UTR, untranslated regions. Exons (E) are drawn as *boxes* and named from 1 to 7. Introns (I) are drawn as *diagonal lines* and named from 1 to 6. The size in bp of each region is indicated. *Triangles* indicate the localization of mutations in the sequence between X2776 and Porrillo Sintetico

Table 1 Analyses of variance for expression levels

	Phvul.004G001200			Phvul.004G001300			Phvul.004G001400			Phvul.004G001500		
	df	F	p	df	F	p	df	F	p	df	F	p
Genotype	1	3.41	ns	1	27.64	*	1	564.99	**	1	3.00	ns
Treatment	1	0.00	ns	1	1.64	ns	1	23.69	*	1	21.32	*
Run	2	0.10	ns	2	8.16	*	2	7.30	ns	2	1.79	ns
G*T	1	6.56	ns	1	2.86	ns	1	13.08	ns	1	196.43	**
G*R	2	7407.30	**	2	10.57	ns	2	3.08	ns	2	68.92	*
T*R	2	900.34	**	2	16.63	ns	2	0.12	ns	2	10.41	ns
G*T*R	2	0.01	ns	2	1.70	ns	2	4.49	*	2	0.31	ns

Analyses of variance for the expression levels of *Phvul.004G001200*, *Phvul.004G001300*, *Phvul.004G001400*, and *Phvul.004G001500*. Genotypes, X2776 and Porrillo Sintetico; Treatments, control (without inoculation) and stressed (72 h after inoculation with PM); Run, three independent experiments

ns not significant, df degrees of freedom

* $p < 0.05$; ** $p < 0.01$

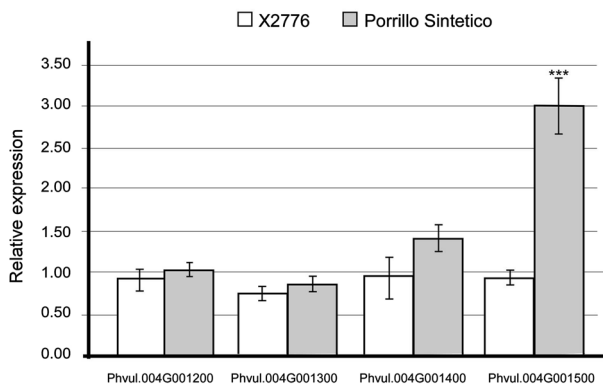


Fig. 4 Relative expression levels of genes *Phvul.004G001200*, *Phvul.004G001300*, *Phvul.004G001400* and *Phvul.004G001500* in the genotypes X2776 and Porrillo Sintetico using the $2^{-\Delta\Delta C_t}$ method. Data points are average values of three biologically independent experiments, with four replicates per sample in each experiment. The standard error is shown. Asterisks indicate the statistical significance calculated with Student's *t* test (*** $p < 0.001$)

substitutions involving exon regions in Porrillo Sintetico (Fig. S3). Then, changes in the produced amino acid sequence between X2776 and Porrillo Sintetico were not expected. Concerning non-coding regions, Porrillo Sintetico showed, relative to X2776, a total of 37 mutations: 4 insertions, 26 substitutions and 7 deletions (Fig. 3b, Fig. S4). The major difference is a deletion of 28 bp in intron 5 of the gene, which includes an alternative splice donor site (TGTGGTGGTTGTCAGTGTAAC TATTGGT).

Phvul.004G001500 gene regulation

To investigate the regulatory mechanism of the *Phvul.004G001500* gene involved in the PM response, its

expression was explored considering alternative splicing and methylation.

Alternative splicing Based on the alternative splice donor site found in intron 5 of the cultivar Porrillo Sintetico, the possibility of an alternatively spliced transcript was tested. Primers ExCr4_1500-F and SpliCr4_1500-R that anneal, respectively, to exons 1 and 7 of the *Phvul.004G001500* gene were developed. In the case of intron retention, the most frequent way of splicing, different sized transcripts should be expected. A unique fragment of 1032 bp was amplified from X2776 and Porrillo Sintetico (Fig. S5). This result showed no evidence of alternative spliced transcripts, constitutively expressed, for gene *Phvul.004G001500*.

Methylation A CpG island, or a region susceptible to being methylated, was identified in the *Phvul.004G001500* gene sequence of the reference genotype G19833 using the software Methprimer with the default parameters (Fig. S6A). This CpG island spanned from 111 to 221 bp and included part of the 5'-UTR (1–167 bp) and part of the exon 1 (168–342 bp). After bisulfite conversion, this region was amplified with primers BSF-F and BSF-R, and sequenced in the genotypes X2776 and Porrillo Sintetico. In both genotypes, all of the cytosines included in the CpG island were converted to thymines after the bisulfite DNA treatment (Fig. S6B, S6C). This result showed no evidence of the involvement of methylation in the *Phvul.004G001500* regulation.

Discussion

PM fungi include a complex and diverse group of genera and species of obligate pathogens, making PM difficult to research because they cannot be routinely cultivated on artificial media and should be maintained “in vivo” using

susceptible cultivars. The current taxonomic system for PM fungi uses not only morphological information, which should be determined by a specialist, but also molecular data. A sequence analysis of the ITS-rDNA region revealed that the local PM isolate used in this work was more closely related to the species *E. polygoni* than to *E. diffusa*, the two main species identified as pathogenic in common bean (Rezende et al. 1999; Almeida et al. 2008). The ITS region is the formal fungal barcode for the exploration of fungal diversity in environmental samples (Schoch et al. 2012), but the more than 300,000 public fungal ITS sequences make it difficult to establish a taxonomic classification. Only about half of these sequences were annotated at the species level, and more than 10% of the publicly identified fungal ITS sequences have been shown to be incorrectly annotated (Nilsson et al. 2006). To optimize the molecular identification of fungi the UNITE database (Kõljalg et al. 2013) was used in this work, in which sequences from the international nucleotide database were unified and their taxonomic and ecological annotations were corrected as much as possible.

A previous genetic analysis using F_2 populations suggested that the cultivar Porrillo Sintetico carried two independent dominant genes against PM, one gene conferring complete resistance, the IT0 response, and another gene conferring intermediate resistance, or the IT3 response (Trabanco et al. 2012). Results obtained in this work corroborate this hypothesis, and allowed for the localization of these genes in the genetic map. In the $F_{2:3}$ population X2776×G122 (IT3×IT4) the gene conferring intermediate resistance to PM was located in LG Pv11, closely linked to marker SH13b (recombination fraction=0.11, LOD=17.72). The *Pm1* gene conferring total resistance against PM was mapped at this position in the Cornell 49242 genotype, also linked to the SH13b marker (Pérez-Vega et al. 2013). This marker is linked to genes conferring resistance to anthracnose (Campa et al. 2014), a fungal disease caused by *Colletotrichum lindemuthianum* (Schwartz et al. 2005), and it tags a chromosome region that corresponds to a RGA including a complex cluster of genes having NBS-LRR domains (Schmutz et al. 2014). The localization of a gene involved in the resistance response against PM in this chromosomal region, in which genes against different diseases have been mapped, is not unexpected (Ferreira et al. 2013; Meziadi et al. 2016). In the $F_{2:3}$ population X2776×Porrillo Sintetico (IT3×IT0) the segregation ratio observed for the gene conferring total resistance fitted the expectation for one gene, indicating that both parental lines differed in the gene involved in the genetic control of total resistance and shared the gene conferring intermediate resistance. This result is expected if both genes show an epistatic relationship, where the gene conferring total resistance masks the effect of the gene conferring intermediate

resistance. A dominant epistatic relationship between genes involved in the PM resistance response in common bean was also reported in the cultivar Cornell 49242 (Trabanco et al. 2012; Pérez-Vega et al. 2014). The presence of a gene conferring total PM resistance in Porrillo Sintetico was supported by the results of a breeding program conducted to introgress this gene into line X2776 using a backcrossing method (Trabanco et al. 2012). Over the course of this breeding program, in each backcrossed generation, the segregation ratio observed fit that expected for one dominant gene, and after 6 backcrossed generations, total resistance against PM was successfully introgressed into X2776. In the current work, the gene conferring total PM resistance in Porrillo Sintetico was located in LG Pv04, delimited by molecular markers Chr4_SSR4 and Chr4_SSR25 (physical positions 84,202 and 218,664 bp, respectively).

In silico analysis of this chromosomal region revealed seven genes that were automatically annotated (Schmutz et al. 2014). Based on the functional annotations of these genes, the possible implication of four of them in the resistance response was tested through expression analyses. *Phvul.004G001500* was significantly upregulated in the resistance genotype Porrillo Sintetico 72 h after PM infection, suggesting its involvement in the resistance response. A gene expression analysis, either in response to a pathogen infection or between pathogen resistant and susceptible plants, is an important genetic approach to discover, and to functionally characterize genes that play roles in host pathogen interactions (Somssich et al. 1986; Fritze-meier et al. 1987). The in silico analysis of this chromosomal region also included a gap of 68,873 missing values (Schmutz et al. 2014; Vlasova et al. 2016). It is not possible to rule out the possibility that another linked gene coded by the chromosomal region including the gap of missing values could also be involved in the resistance response against PM. If this chromosomal region is inherited as a block, without recombination events, in the $F_{2:3}$ population X2776×Porrillo Sintetico, two different but closely linked genes involved in the PM resistance trait, would segregate as one single gene. With the available information on the common bean sequence, this possibility cannot be discarded. The implication of gene *Phvul.004G001500* in the PM response should be validated through virus-induced gene silencing. Notwithstanding, small-seeded black bean cultivars have been described showing high resistance levels against viruses and, in particular, the Porrillo Sintetico cultivar has been used as a resistance source against Bean Golden Mosaic Virus (Morales and Singh 1991), thus Porrillo Sintetico probably shows a resistant response against the plant viral vectors used for the application of virus induced gene silencing associated technology. Another way to validate candidate genes in plants is using transformation technology, although the common bean is a recalcitrant

species to in vitro regeneration, and to date, there is no routine genetic transformation system (Hnatuszko-Konka et al. 2014).

Differential transcript levels ultimately result from DNA variation, directly, in the observed gene or, indirectly, in other genes that act in regulating its expression and through epigenetic DNA modifications. Candidate gene *Phvul.004G001500* was re-sequenced from genotypes X2776 and Porrillo Sintetico to identify genomic variation that could be responsible for the phenotypic variation observed between these genotypes in their PM response. At the sequence level, several synonymous substitutions involving coding regions were identified between both genotypes and the reference genome G19833, so the produced amino acid sequence is not modified. Other mutations (substitutions or insertion/deletions) were identified in the UTR or intron regions that could be responsible for the different *Phvul.004G001500* expression levels between genotypes X2776 and Porrillo Sintetico after PM infection. It is well documented that introns can have positive effect on gene expression in plants (Simpson and Filipowicz 1996; Fiume et al. 2004) through a process named intron-mediated enhancement (IME; Mascarenhas et al. 1990). For instance, in the plant–pathogen interaction *Solanum tuberosum* L.-*Phytophthora infestans* (causal agent of late blight) SNPs were associated with resistant genes differentially expressed after the oomycete fungal infection (Muktar et al. 2015), or in common bean one single-nucleotide polymorphism (SNP) in the promoter region has been identified as responsible for different expression levels of β -phaseolin protein (Panduranga et al. 2016). Alternative splicing (Jordan et al. 2002; Dinesh-Kumar et al. 2000) and gene methylation (Yoder et al. 1997; Chinnusamy and Zhu 2009; Downen et al. 2012) play a key role in plant gene expression levels under stress. In this work, no evidences for these two different regulatory mechanisms against PM were found. However, the involvement of other regulatory elements in the control of *Phvul.004G001500* expression cannot be discarded. Many *cis*-sequences have been identified in plants as regulatory elements which control gene expression levels through interactions with DNA (Bilas et al. 2016).

Phvul.004G001500 codes for an elongation factor (EF) thermostable according to the protein analysis through evolutionary relationships (PANTHER) classification system (Schmutz et al. 2014). EFs are a set of proteins that play important roles in the cell cycle process. A total of 72 genes coding elongation factors have been annotated in common bean. There is considerable evidence of their multifunctionality, including their involvement in peptide chain elongation, the quality control of newly produced proteins, ubiquitin-dependent protein degradation, the organization of the actin cytoskeleton, and in modulating the expression

of genes against both abiotic and biotic stresses (Ejiri 2002; Sasikumar et al. 2012; Fu et al. 2012; Li et al. 2014). EFs are a multigene family that is highly conserved among different species, between mammals and plants, and even between eukaryotes and prokaryotes. In mammals, serious diseases, such as cancer, diphtheria and Alzheimers, have been associated with changes in EFs (Ejiri 2002). In plants, EF-Tu (thermostable) was proposed as a factor capable of triggering the innate immune response through oxidative bursts, callose deposits, and ethylene biosynthesis, thereby inducing resistance against several pathogens (Kunze et al. 2004; Zipfel et al. 2006; Furukawa et al. 2014).

The multifunctionality of EFs clouds the interpretation of mechanisms that may be involved in the PM resistance response. As biotrophic fungi, PMs invade epidermal cells, penetrating the host cell wall and then developing a highly specialized infection structure called a haustorium. This organ invaginates the host plasma membrane, forming the extrahaustorial membrane, which is the focal point of host-pathogen interactions, the trafficking of nutrients from the host to the pathogen, and is essential for the growth and reproduction of biotrophic pathogens (Hückelhoven 2005; Eichmann and Hückelhoven 2008; Hückelhoven and Pastruga 2011). Plant defenses against PM can occur at different steps. Early defense prevents penetration of the conidial spore, such as the *mildew resistance locus* (*Mlo*) first described in barley (Büschges et al. 1997). *Mlo* genes act during the invasion of the first epidemial cell, prior to haustoria formation, and code for a set of integral membrane proteins that are crucial for effective host penetration. *mlo* loss-of-function is related to a broad-spectrum PM resistance that is effective in the pre-invasive stage, and is recessively inherited. In a different step of the infection, an unusual PM resistance protein, RPW8, has been described in *Arabidopsis* (Xiao et al. 2001; Wang et al. 2009; Hückelhoven and Pastruga 2011). RPW8 is a host protein specifically targeted to the extrahaustorial membrane, and is related to H₂O₂ accumulation, reducing oxidative damage to the host cell, and to the callose encasement that constrains haustoria development, resulting in a resistance response. Oxidative burst and callose deposition functions have been related to EFs, and could act in a way similar to RPW8 to confer PM resistance. However, the movement of the RPW8 protein to the extrahaustorial membrane is dependent on actin cytoskeletal functions. The organization of the actin cytoskeleton is a function described for EFs (Condeelis 1995; Gross and Kinzy 2005), and their involvement in this step could also be related to the PM resistance response. In fact, the cytoskeleton has been proposed as a regulator and target of biotic interactions in plants that are related to plant defense signaling (Takemoto and Hardham 2004; Janda et al. 2014). The different expression levels in cultivar Porrillo Sintetico of the *housekeeping* gene *Act11*,

which invalidated its use as reference gene in this work, could be related to cytoskeletal organization. The fact that the gene conferring the total resistance response against PM would act through the plant immune system could explain its epistatic relationship with the gene conferring intermediate resistance, located in an RGA that includes a complex cluster of genes having NBS-LRR domains, thus it could act through a specific R gene.

This is the first report that, through direct genetic analysis, maps a resistance gene in common bean which codes for an EF. The multifunctionality of EFs opens up the possibility of investigating more durable and multiple-stress-resistance breeding in common bean to improve the responses against different diseases caused by other fungi or bacteria. This strategy has been successfully used in tomato, wheat, and rice, in which transferring an *Arabidopsis thaliana* EF-Tu receptor provided good resistance against various bacterial pathogens (Lacombe et al. 2010; Schoonbeek et al. 2015; Lu et al. 2015). Even the possibility of improving responses against biotic stresses, such as heat tolerance, through EFs can be explored, as it has been demonstrated that different types of EFs are differentially expressed under temperature-related stress conditions in wheat, pea, and soybean leading to a better tolerance to temperature stress in cultivars that accumulate more EF (Ristic et al. 2008; Singh et al. 2004; Chung et al. 2009).

Author contribution statement AC and JJF conceived and designed the experiments; AC performed experiments; AC analyzed the data; AC and JJF wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interests.

Ethical standards Authors declare that the experiments conducted in this work comply with the current laws of the country in which they were performed.

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