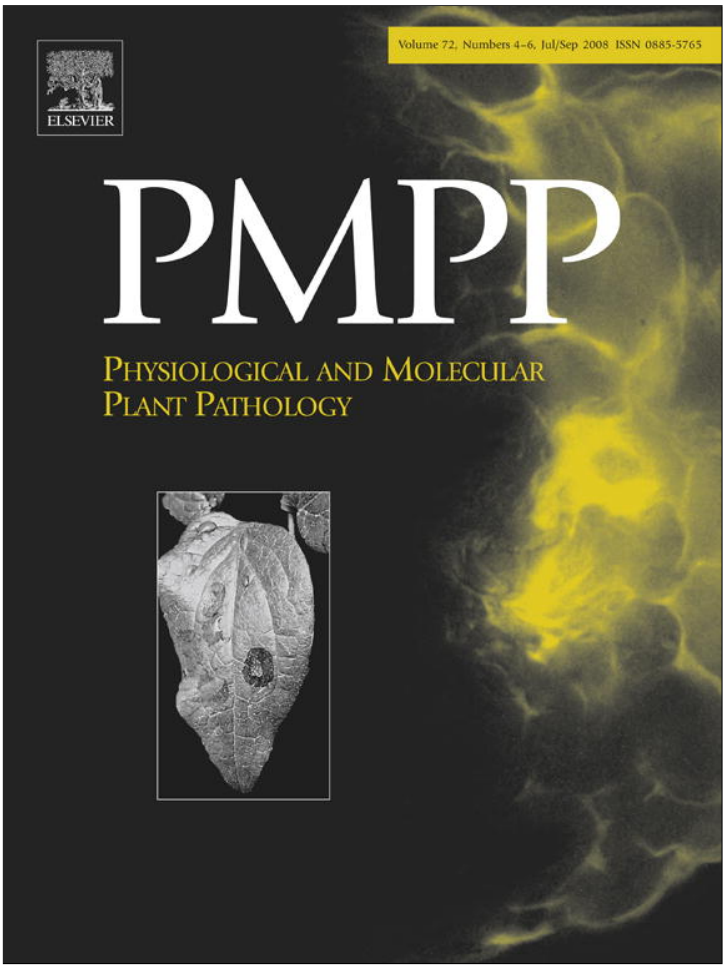

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A *Phaseolus vulgaris* EF-hand calcium-binding domain is induced early in the defense response against *Colletotrichum lindemuthianum* and by abiotic stress: Sequences shared between interacting partners☆

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ABSTRACT

Early in the plant defense response against pathogens, transcription of specific genes is activated. We studied the genes up-regulated in the early resistance reaction of *Phaseolus vulgaris* against *Colletotrichum lindemuthianum*. The effect of the light–dark transition, ultraviolet-(A–B) light, and temperature stress on these genes was also evaluated. A calcium-binding protein and previously identified SUMO genes were over-expressed in the plant reaction to pathogen and under environmental stress. These genes appear to act as integrative signals and convergence points in early plant responses to pathogens and several forms of environmental stress. The calcium-binding protein gene was present in both plant and fungi genomes.

1. Introduction

Following detection of pathogen by plants, considerable physiological adjustment and rapid changes in gene expression take place in host cells to activate defense responses in order to evade microorganism colonization and disease. During this process, key genes coordinate the activation of other defense genes. Current knowledge of plant host signaling pathways, specifically with regard to the genes expressed early in the host defense response is limited. However, the list of genes involved is growing and mutational analysis studies are revealing the genetic complexities of pathogen perception and molecular signaling pathways and subpathways [1–4]. There may be differences between plant species. Some important crops and their relevant pathosystems, such as the *Phaseolus vulgaris* (common beans)/*Colletotrichum lindemuthianum* (a fungus) have been poorly studied at a genetic level. Although the common bean is an important crop legume and source of protein worldwide, little genetic information was available until Ramírez et al. recently reported 7969 different contigs from various common bean plant organs [5].

Plants, like other eukaryotic and many prokaryotic organisms, have evolved a regulation system referred to as "circadian rhythm" that generates a period of rhythmicity of about 24 h. The circadian rhythm modulates the physiology of adaption to the light/dark and temperature periodicity and impacts many aspects of plant metabolism, development, and behavior [6]. Fluctuations in environmental conditions such as photoperiod, relative humidity, and temperature greatly impact the growth rate and development of plants. Based on this, it is expected that the biology of the defense response in plants against pathogen microorganisms can be modulated in a similar way by the light/dark cycle.

Some plant defense response genes are expressed under several abiotic stresses. For example, the *CPN1* gene was proposed as a link between disease resistance and acclimation to low-humidity and low-temperature conditions [7]; the chimeric ATCNGC-encoding gene, designated *ATCNGC11/12*, was identified in the *cpr22* mutant of *Arabidopsis* due to high resistance to

☆ The nucleotide sequences obtained through this work have been submitted to GenBank under the accession numbers DQ118371 for the partial cDNA from plant and EU045571 for the fungal genomic DNA fragment.

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Phytophthora parasitica, but is dependent on low humidity [4,8]; a WRKY transcription factor, a ferredoxin-dependent glutamate-synthase, and several other insect-responsive genes of an unknown function are similarly regulated by UV-B and insect herbivory treatments [9]. These studies show that some defense response genes are transcriptionally activated by pathogens, as well as by different forms of environmental stress, suggesting that signaling components are shared by different stress signaling pathways and suggesting that plant responses to biotic and abiotic stresses often overlap.

The two genes analyzed in this study were isolated from a cDNA library previously obtained by subtractive hybridization from mRNA populations induced in plant tissue in 1–6 h post-inoculation (hpi) in *P. vulgaris/C. lindemuthianum* resistant and susceptible interactions. One of these sequences corresponds to an ubiquitin-like or *SUMO* gene (AF451278) and was previously reported [10]. The SUMO proteins are about 100 amino acids in length and are only 8–15% identical to ubiquitin, but fold into a similar globular structure [11]; these proteins become covalently attached to various intracellular target proteins and modify their localization and/or stability and function [12]. An eight-gene family of SUMO tag proteins was found in *Arabidopsis* [13]. Previous studies have shown that SUMO proteins participate in the response to several forms of biotic or abiotic stress. The SUMO1/2 conjugation is one of the early plant responses to heat stress [12]. In the tomato, a *SUMO* gene is involved in the defense response against pathogen components [14]. Many phytopathogenic bacteria use a type III secretion system (T3SS). The SUMO tag in SUMO protein conjugates is the target for several T3SS effectors. These effectors are cysteine proteases that specifically cleave SUMO, reducing the levels of SUMO–protein conjugates in the host cells [15–17] and interfering with the regulation of host plant proteins during infection [15,18,19]. Biochemical evidence demonstrating that T3SS effectors act as SUMO proteases was found in the study of *Xanthomonas campestris* pv *vesicatoria* effector XopD; this protein encodes a constitutively active cysteine protease with SUMO peptidase activity, suggesting that the primary roll of these effectors is to interfere with plant pathways regulated by SUMO [17]. The second gene analyzed in this study codes for a novel EF-hand calcium-binding protein; this gene is probably involved in the early calcium-induced signaling pathway leading to onset of the plant's defense response, as the conserved Ca^2+ binding motifs observed in this protein have been reported in proteins involved in plant defense reactions [20,21].

Our aim in this study was to gain insight into the early molecular mechanisms activated in the plants in response to pathogens; we analyzed the *P. vulgaris*/*C. linemuthianum* pathosystem and identified transcripts that accumulated in the resistant interaction. Accumulation of transcripts from the two genes described above, as well as other genes previously reported to be induced in response to several abiotic stresses [7,22], such as UV, cold, and high temperature conditions, was assessed. Transcripts from the novel EF-hand calcium-binding protein encoding gene and from the previously reported *SUMO* gene were differentially regulated by light/dark transition and by extreme temperatures and UV abiotic stresses. Both plant and fungus express the novel calcium-binding protein encoding gene. The significance of these results is discussed.

2. Material and methods

2.1. Pathogen culture and plant growth conditions

Cultures of *C. lindemuthianum* were maintained on PDA agar at 25 °C. Spore suspensions in water supplemented with 0.01% Tween 20 were prepared from two-to-three week-old cultures grown on Petri plates. Spore concentration was determined with a hemacytometer. Bean seeds were germinated in humid chambers, transferred to sterile soil mixture in separate pots and kept under greenhouse conditions.

2.2. Plant infection with the C. lindemuthianum pathogen

Primary leaves from 25-cm high, 15-day-old seedlings were inoculated on adaxial and abaxial sides with water or conidial suspension (1.5×10^6 conidia ml^{-1} in 0.01% Tween 20) and kept in a bioclimatic chamber equipped with illumination (six 20-W tubes) at 26 °C, 70% relative humidity (RH), and 12 h light/12 h dark photoperiod. Disease symptoms were evaluated on a five-point scale where 0–1 was scored as resistant and 2–4 was susceptible. For molecular studies of early expressed genes, primary leaves were harvested 0.5, 1, 2, 4, and 6 hpi, were ground in liquid nitrogen, and stored at −70 °C until use. For microscopy studies, plant tissue was sampled at 14, 24, and 54 hpi and processed for histological observations. The pathotypes 2 and 1472 of *C. lindemuthianum*, virulent and avirulent, respectively, grown on Michigan Dark Red Kidney bean, were a kind gift from Dr. June Simpson from CINVESTAV-IPN Irapuato Unit.

2.3. Histological processing

Leaf fractions from inoculated and control seedlings were sampled 4 h post-inoculation and fixed in FAA (3.7% formalin, 50% ethanol, 5% acetic acid in water). Fungal structures were stained for 5 min with 0.1% cotton blue in lactophenol. Plant tissues were faded in methyl salicylate–ethanol at methyl salicylate concentrations of 25, 50, and 75% for 1 h. Tissue sections of approx. 1 cm^2 were mounted in pure methyl salicylate and analyzed by Nomarski interference contrast microscopy.

2.4. Plant growth conditions for light/dark normal transition studies

Plants used for the light/dark normal transition studies were grown in a bioclimatic chamber under the illumination, temperature, and photoperiod conditions described above. The 15-day-old seedlings were inoculated at the beginning of the day (light, 6:00 a.m.) or the night (dark, 18:00 p.m.) with the virulent or avirulent pathotype.

2.5. Abiotic stress growth conditions

For UV experiments, 15-day-old seedlings were exposed for 4 h to UV supplied by a UV lamp (15 W, 15B; Toshiba, Japan) that radiates at wavelengths > 290 nm. The UV-B and UV-A light dose in seedlings exposed at 35 cm from the light source was 2.19 W/m^{-2} (7.88 kJ m^{-2}) in a range in the spectrum of energy between 290 and 400 nm with a major concentration in 320–370 nm (measured using a spectroradiometer, Bentham Instruments, Ltd., UK). This experiment was carried out at 26 °C, ~70% RH. The primary leaves were sampled 4 h afterwards.

For cold or hot condition experiments, 15-day-old seedlings were maintained at 8 °C or 38 °C, respectively, in the bioclimatic chamber with the illumination and photoperiod described. Temperature exposure began at 10:00 a.m., with sampling of the primary leaves 4 h afterwards. Leaf tissues were harvested and ground in liquid nitrogen to a fine powder and stored at −70 °C until used.

2.6. RNA extraction and analysis of gene expression

Total RNA was extracted from primary bean leaves using the method described by Chomczinsky and Sacchi [23]. For early gene

expression studies, total RNA from primary bean leaves sampled at 0.5, 2, 4, and 6 hpi were pooled. For the calcium-binding protein light/dark kinetic expression analysis, the plant tissue was sampled with 2 h intervals throughout 6–12 hpi of light period and 2–12 hpi of dark period. Total RNA from early times (0.5–6 hpi) of day (6:30, 8:00 10:00, and 12:00 a.m.) was pooled. For the *SUMO* gene analysis, plant tissue was sampled at 2 h intervals throughout the 24 h experiment. Plant tissues were harvested and ground in liquid nitrogen to a fine powder and stored at −70 °C until used. These experiments were performed three times. For abiotic stress studies, total RNAs were obtained from primary leaves subjected to 4 h of extreme temperature or UV treatments.

In the Northern blot assays, 8 μg of total RNA was fractionated by agarose–formaldehyde denaturing gel electrophoresis, transferred to nylon membranes (Immobilon, Millipore, Bedford, MA) by capillary transfer, and hybridized with random primed P^{32}-dCTP amplified cDNA fragment (REDIPRIME II, Random Primer DNA labeling kit, Amersham Biosciences, Piscataway, NJ). For light/dark 24 h experiments, 18 μg of total RNA was used per lane. The same membranes were used for the second cDNA probe hybridization after stripping the first probe in boiling 0.5% SDS. For the early time points (2 and 4 hpi) for the *SUMO* gene, a separate membrane was processed. For the abiotic stress assays, the same membrane was used for an additional cDNA probe hybridization, after stripping the first probe. The autoradiograms were analyzed using the software ImageJ 1.36b [24]. The probe hybridization signal was normalized with respect to the 28S rRNA bands in the ethidium bromide image. The Northern blot assays for the light/dark studies were performed three times with total RNAs from independent experiments; the presented images correspond to the third experiment, in these cases, the 28S rRNA as loading control in the ethidium bromide image is useful for both genes. Plots were constructed with data collected from the image analysis of each of the three independent experiments. Results were analyzed using ANOVA, with a cutoff P of 0.05 to indicate significance. The t test was used to assess the differences in independent comparisons between treatments and the paired t test was used for light and night and in comparisons of resistant and susceptible interactions at each time point of the plant tissue sampling.

2.7. DNA genomic hybridization

Plant genomic DNA used for blot analysis was isolated from 15-day-old seedlings using the cetyl-trimethylammonium bromide method (CTAB) [25]. Fungal genomic DNA was extracted from mycelium using a CTAB-based method [26]. Southern analysis was performed in nylon membranes (Immobilon, Millipore) using 8 μg of plant genomic DNA and 8 μg of fungal DNA. The plant DNA was digested with *Eco*RI, *Eco*RV, or *Hind*III, whereas the fungal DNA was digested with *Eco*RV, *Sal*I, or *Hind*III. DNA was transferred from agarose gels to nylon membrane by capillary transfer. The membrane was hybridized at 65 °C with the random primed P^{32}-dCTP amplified cDNA fragment (REDIPRIME II, Random Primer DNA labeling kit, Amersham Biosciences, Piscataway, NJ). Biomax MS imaging film (Eastman Kodak Company, Rochester, NY) was exposed to the blot for 72 h and developed.

2.8. Cloning and sequencing

The cDNA fragments obtained from subtractive hybridization were inserted in the pGEM T-easy vector (Promega, Madison, WI) and used to transform *Escherichia coli* (DH5α) by the $CaCl_2$ procedure [27]. Miniprep DNA corresponding to the clone 106 was purified and the insert was sequenced using an automatic sequencer (ABI Prism 377 DNA Sequencer, Applied Biosystems, Foster City, CA) at the sequencing facility of the CINVESTAV-IPN Irapuato Unit.

2.9. Gene amplification by PCR from fungal and plant genomic DNA and sequencing

Oligonucleotide primers were designed based on the sequence for the novel calcium-binding protein encoding gene from plant for the amplification of a 376-bp fragment. The primers used were forward, 5′-AGGCAAACATGGAAGTGACA-3′, and reverse, 5′-TGGGAAATACAAGAATGATGAAG-3′. Amplification by PCR was performed using three different temperature gradients. Each 50-μL PCR amplification reaction contained a 1× concentration of TaqPCR reaction buffer (Sigma), 2.5 U of Red*Taq* DNA polymerase, 40 ng of DNA template, 200 μM each deoxynucleoside triphosphate, and 0.5 mM each primer. The cycling conditions were 95 °C for 5 min, followed by 35 amplification cycles: 95 °C for 1 min; 55 °C, 57 °C, or 59 °C in gradient, independent reactions for 1 min; 72 °C for 45 s, and a final extension step at 72 °C for 5 min in a gradient thermal cycler (Gradient Palm-Cycler, Corbett Research, Corbett Life Science). PCR products were visualized after electrophoresis in a 1.2% agarose gel containing 0.2 μg/mL of ethidium bromide by transillumination with UV light. The fragments were purified by a gel extraction procedure (Wizard SV Gel and PCR Clean-Up System, Promega, Madison, WI), cloned in pGEM T-easy vector (Promega, Madison, WI), and sequenced in automatic sequencer (ABI Prism 377 DNA Sequencer, Applied Biosystems).

2.10. Database search and sequence alignments

The basic local alignment search tools (TblastN and BlastP), available online at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov), were used with the clone 106 fragment as the query sequence. Sequences with major homologies were aligned with the high-accuracy program, MUSCLE v. 3.6 [28]. Phylogenetic evolutionary analysis was conducted using MEGA version 3.1 [29] by the neighbor-joining inference method and bootstrap test. The sequence was also analyzed for the presence of possible motifs on the ExPASy (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics (http://www.isb-sib.ch/) using the ExPASy ScanProsite and ScanMotif programs.

3. Results

3.1. Cytology of plant–pathogen interactions

In order to evaluate the behavior of the two *C. lindemuthianum* pathotypes in the interaction with the Michigan Dark Red Kidney, primary leaf tissues of inoculated seedlings were sampled at 12, 24, and 54 hpi and examined by Nomarski interference contrast microscopy. Susceptible and resistant interactions characteristic of both fungal pathotypes on the Michigan Dark Red Kidney remained stable as previously reported [10]. Both pathotypes showed germinative capacity at 12 hpi; but at 24 hpi, cellular structures of pathotype 1472 appeared seriously damaged with cell debris around collapsed spores. At 54 hpi and later, the tissue from the seedlings inoculated with the pathotype 1472 remained healthy. In contrast, extensive necrosis was observed in vascular tissue from leaves inoculated with pathotype 2 (Fig. 1). Samples from leaves infected with pathotype 1472 are referred to here as resistant; samples from leaves infected with pathotype 2 are referred to as susceptible.

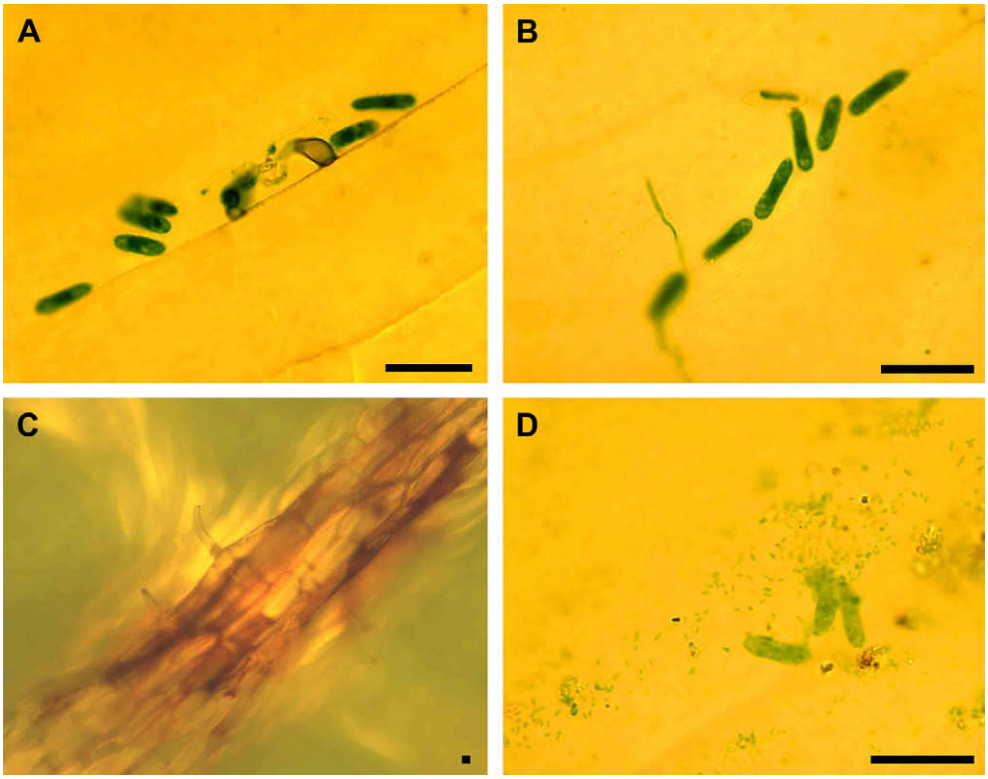

Fig. 1. Behavior of *Colletotrichum lindemuthianum* races 2 and 1472 on Michigan Dark Red Kidney leaves. Race 2 in plant-susceptible interactions at (A) 12 and (C) 54 hpi; race 1472 in plant-resistant interactions at (B) 12 and (D) 24 hpi. In C, the necrotrophic phase in the virulent fungus on veins of bean leaves can be seen. In D, cellular debris from spores of the avirulent fungus is apparent. Bars = 10 μm.

3.2. Differential gene expression observed by northern blotting with cDNA fragment from clone 106

From a subtracted cDNA library, clones showing early (0.5–6 hpi) differential expression between resistant and susceptible interactions and control plants were assessed by northern blot assay. The hybridization signal was analyzed by ImageJ 1.36 software and the data were plotted to assess signal differences. The clone 106 carrying a 505-base pair (bp) insert corresponds to a gene that shows a two-fold increase in expression in resistant plant reaction compared to the susceptible interaction and more than a two-fold increase in expression compared to the basal expression in the control plant (Fig. 2). The sequencing of the insert and the analysis of sequences revealed that the fragment contained part of the coding region, the 3′ untranslated region (UTR), and the poly-A tail of a message (data not shown).

3.3. Hybridization with fungal and plant digested genomic DNA

In order to determine whether this gene originated from the plant or fungal genome, a Southern blot assay was performed with 8 μg of genomic DNA from plant tissue digested with *Eco*RI, *Eco*RV, or *Hind*III and the same amount of DNA from the pathogen digested with *Eco*RV, *Sal*I or *Hind*III. One or two bands were observed in DNA

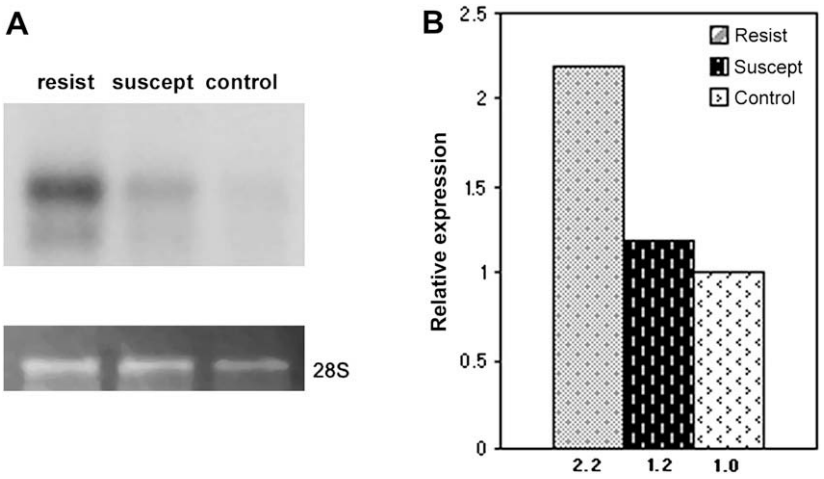

Fig. 2. Northern blot probed with the cDNA fragment from clone 106 and relative transcript expression levels in each treatment. (A) Hybridization signal with P^{32}-dCTP labeled cDNA fragment on total RNA from resistant, susceptible, and control plants sprinkled with water. (B) Plot representing the relative levels of expression.

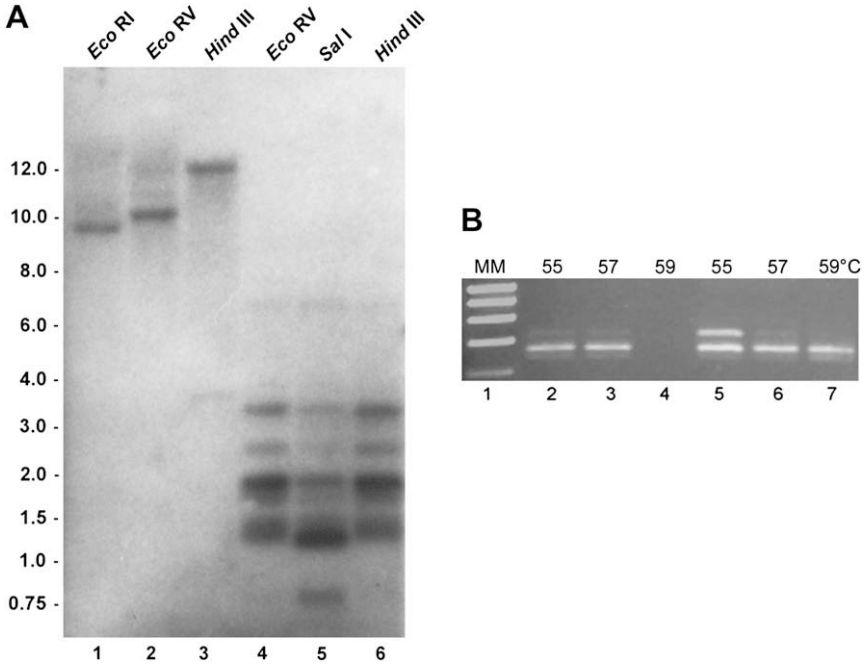

Fig. 3. Southern blot analysis using a cDNA fragment from clone 106 as probe and fragment amplified by PCR from plant and fungal genomic DNA. (A) Bean genomic DNA (8 μg) was digested with *Eco*RI (lane 1), *Eco*RV (lane 2), or *Hind*III (lane 3). Fungus genomic DNA (8 μg) was digested with *Eco*RV (lane 4), *Sal*I (lane 5), or *Hind*III (lane 6). The digested DNA was separated in a 0.8% agarose gel. The sizes in kbp of a marker DNA are specified on the left. (B) The amplified fragment of the novel calcium-binding protein gene from fungal and plant genomic DNA obtained by PCR at several gradient annealing temperatures (55, 57, 59 °C); lanes 2–4 in fungal genomic DNA, lanes 5–7 in plant genomic DNA; lane 1, 1 kbp ladder molecular marker.

from plant and four or five bands from the fungal DNA (Fig. 3A). In the northern blot assay, one intense band and a second less intense one of lower molecular weight were observed (Fig. 2A). Large differences were observed in the sizes of fragments among plant and fungi. In the DNA from plant, fragment sizes ranged between 9.8 and 12 kb, whereas in the DNA from fungus the sizes ranged from 0.75 to 7 kb. The smaller sizes of fragments from DNA from fungi indicate that restriction endonuclease sites occur with higher frequency in fungi than in plants.

3.4. Amplification of a region of the fungal genomic DNA with similarity to the plant gene

Oligonucleotide primers were designed based on the sequence for the novel protein encoding gene from plant. The PCR reaction performed with fungal genomic DNA template gave a product of 377 bp using the two temperature gradients (55 and 57 °C) (Fig. 3B). The sequence of the fungal fragment (acc. EU045571) showed 99% of similarity with the sequenced region of the plant gene. The fungal fragment exhibits a change from cytosine to guanine at one location in the 319 bp portion of the ORF and there was an insertion of a thymine in the 57 bp portion of the UTR relative to the plant sequence. The sequence of the plant DNA product was identical to that obtained before. No intronic regions were amplified in genomic DNA from either species.

3.5. The cDNA fragment similar to EF-hand calcium-binding protein genes of plant origin

A search for homology in the NCBI GenBank using BlastN and BlastP algorithms showed that the novel gene aligns with calcium-binding proteins, calmodulin-like, or caleosins from plants and has less similarity to hypothetical proteins of fungal origin. Using the conserved domain architecture retrieval tool (CDART), the new gene from *P. vulgaris* was aligned with 41 plant and fungal genes, which were calcium-binding proteins, caleosin, or hypothetical proteins. The alignment of the predicted amino acid sequence of this new gene, obtained using the MUSCLE (v. 3.6) program, exhibited 46–65% amino acid identities to other plant calcium-binding proteins and 38–44% identities to hypothetical proteins from fungi (Fig. 4A).

The cladogram of the phylogenetic tree based on amino acid sequences of accessions from plants and fungus with the highest identities to the novel protein has three clades: *Oryza sativa* (BAD46173.1), *Arabidopsis thaliana* (NP_564996.1), *Cynodon dactylon* (AAS48644.1), *P. vulgaris* (new accession AAZ23153.1), *C. lindemuthianum* (new accession EU045571), and *Lilium longiflorum* (ABK40508.1) in a multifurcating clade; *Sesamum indicum* (AAF13743.1), *Hordeum vulgare* (AAQ74237.1), and *Glycine max* (AAB71227.1) in another clade; and the fungus *Phaeosphaeria nodorum* (EAT82449.1) and *Magnaporthe grisea* (XP_365408.1) in a more distant clade (Fig. 4B).

Using the MotifScan tool, we searched the PROSITE database on the ExPASy Proteomics Server with the derived amino acid sequence. A number of motifs were recognized in the novel protein: an EF-hand calcium-binding domain profile in the region of 37–53 amino acids, a caleosin profile in the region of 2–99 amino acids, a tyrosine kinase phosphorylation site in the positions of 4–12 amino acids, and two tyrosine sulfation sites between 5–19 and 33–47 amino acids (data not shown).

3.6. Kinetics of mRNA accumulation of EF-hand calcium-binding protein and SUMO genes throughout light and dark normal periods

We evaluated mRNA expression kinetics of the novel protein by analysis of the hybridization signal obtained in three independent northern blot assays, corresponding to three independent experiments. The *EF-hand calcium-binding protein* gene showed different expression kinetics in resistant compared to susceptible plants and controls (Fig. 5). The ANOVA test showed significant differences ($P = 0.05$) between treatments (data not shown). Differences were first observed in the dark period: the level of transcript increased in

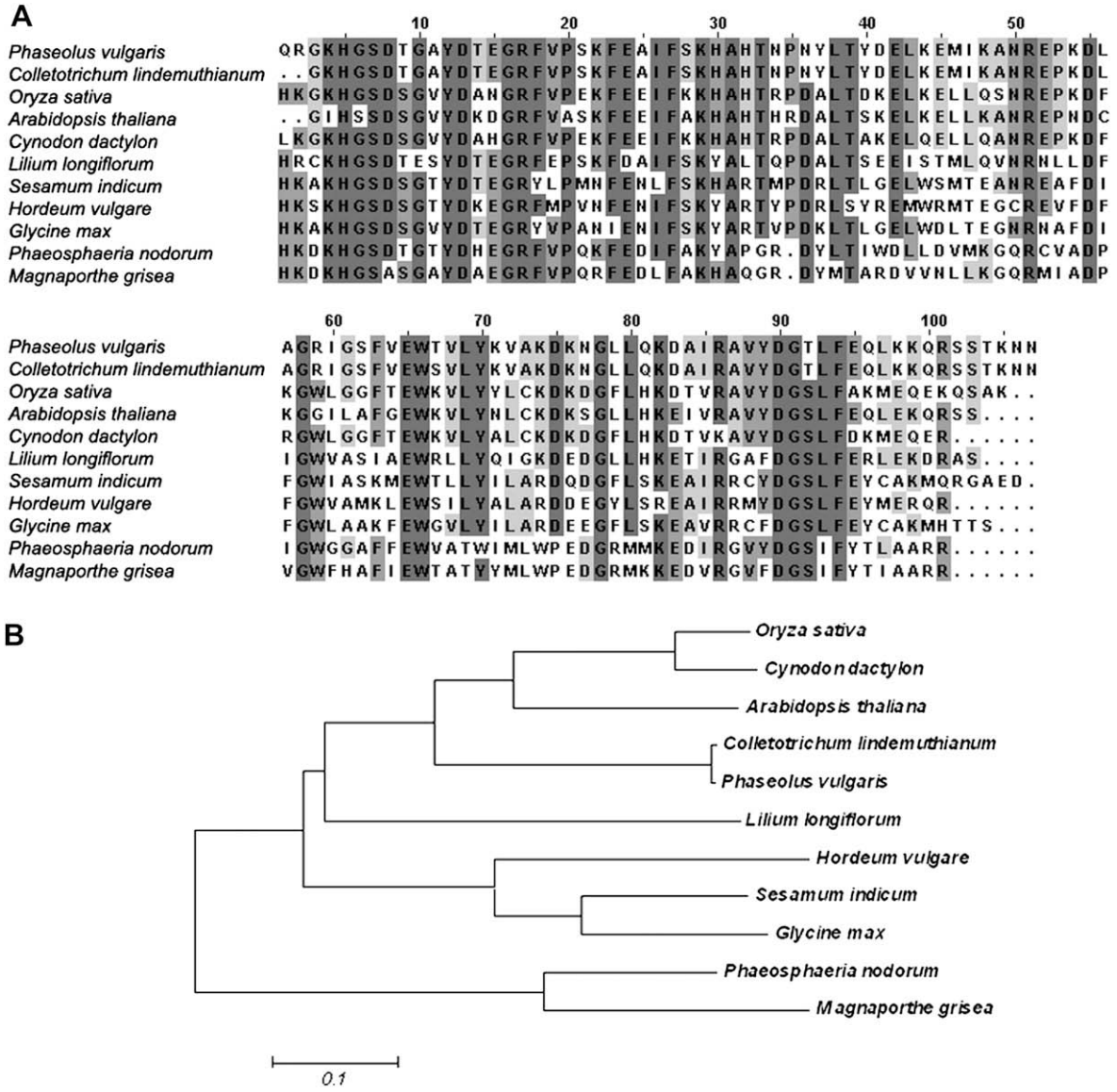

Fig. 4. Amino acid sequence analysis of *Phaseolus vulgaris* EF-hand calcium-binding protein (acc. DQ118371). (A) The derived amino acid sequence of the *P. vulgaris* calcium-binding protein was compared to those of other plant calcium-binding, calmodulin-like, or caleosin proteins from NCBI GenBank that showed high identities; two sequences from fungus were also included. Conserved amino acid residues are shaded and residue numbering refers to the *P. vulgaris* EF-hand calcium-binding protein sequence. The accession numbers of the sequences used are as follows: *P. vulgaris* (AAZ23153.1), *Colletotrichum lindemuthianum* (EU045571), *Arabidopsis thaliana* (NP_564996.1), *Hordeum vulgare* (AAQ74237.1), *Glycine max* (AAB71227.1), *Oryza sativa* (BAD45232.1), *Cynodon dactylon* (AAS48644.1), *Lilium longiflorum* (ABK40508.1), *Sesamum indicum* (AAF13743.1), *Magnaporthe grisea* (XP_365408.1), and *Phaeosphaeria nodorum* (EAT82449.1). (B) The phylogeny reconstruction analysis by neighbor-joining inference method and bootstrap test. The tree has three clades: *O. sativa*, *C. dactylon*, *A. thaliana*, *P. vulgaris*, *C. lindemuthianum* and *L. longiflorum* in a clade; *H. vulgare*, *G. max*, and *S. indicum* in a monophyletic group; and *M. grisea* and *P. nodorum* in a distant third clade.

resistant interactions, whereas in the susceptible interactions and control plants there were no significant changes. Transcript accumulated to higher levels at early times (0.5–6 hpi) in light conditions in the resistant interaction than in the susceptible interactions and control plants (Fig. 2). In susceptible plants, the EF-hand calcium-binding protein transcript was increased at the end of the light condition (8–12 hpi) relative to the beginning, but did not surpass the levels observed in the resistant interaction at early times of light or in the dark periods (Fig. 5). Stable and relatively low transcript levels were maintained in the susceptible interaction and control treatment samples throughout the light and dark period. In the control plants, lower transcript levels were observed throughout the light period than in the dark. A peak at the beginning of the light cycle was observed in the resistant interaction and control treatments. The resistant interaction showed statistical differences with respect to the susceptible interaction and control plants in the dark period ($P = 0.05$). Furthermore, in the resistant interaction statistically significant differences were observed between the light and dark periods (Fig. 5).

Higher SUMO transcript levels were observed in the resistant interaction than in susceptible and control plants at the 12 times analyzed (Fig. 6). High levels of transcript were observed at early times of the light period (0.5–6 hpi) in the resistant interaction in this pathosystem, as have been previously reported [10]. Interestingly, although constant intermediate transcript levels were observed in susceptible samples, transcript levels reached those found in resistant samples at 10 hpi in light and dark conditions (Fig. 6B). SUMO transcripts showed a noticeable peak at the beginning of the light cycle (6:00 a.m.), although this was less obvious in controls than in pathogen-treated samples (Fig. 6B). Statistic differences were obtained in independent comparisons between resistant and susceptible interactions and control plants (data not

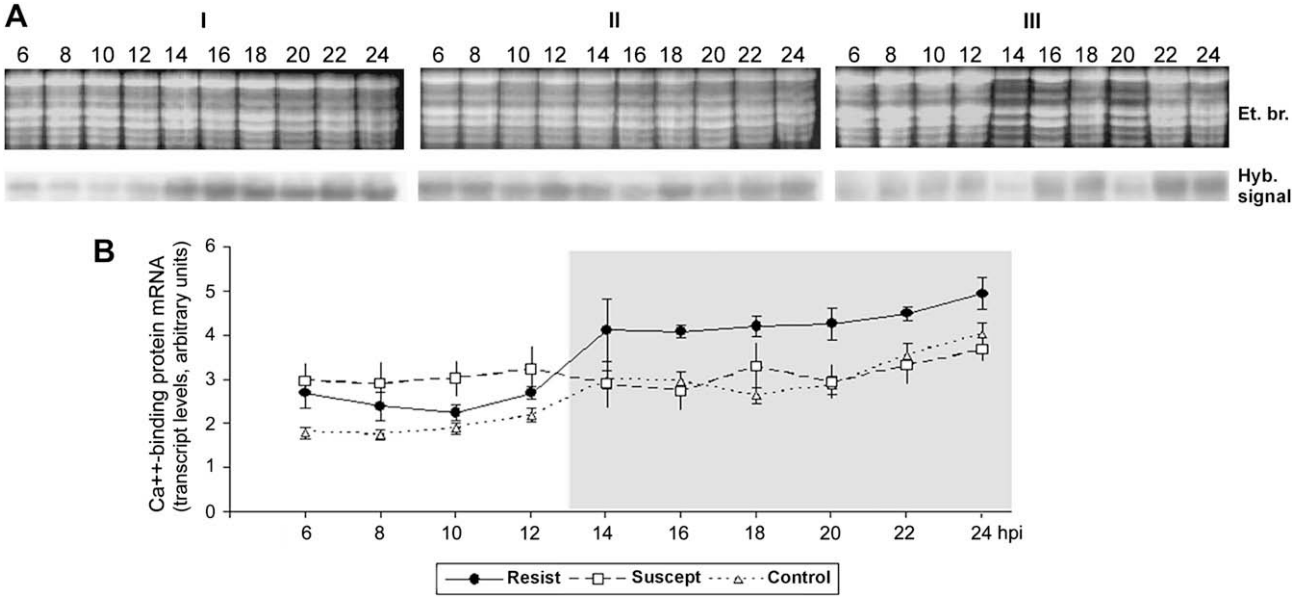

Fig. 5. Northern blot assay with total RNA for the EF-hand calcium-binding protein mRNA and the mRNA expression kinetics. (A) In the upper panel, total RNA was stained with ethidium bromide; in the bottom panel, the hybridization signal with the radiolabeled cDNA probe for the EF-hand calcium-binding protein gene is shown. From left to right: I, resistant interaction; II, susceptible interaction; and III, control plants. (B) mRNA expression kinetics for the EF-hand calcium-binding protein throughout the 24 h day. Values are expressed as means of three independent experiments (±SE), in white background for day (light) period and in gray background for night (dark) period. The level of expression in the plot indicates transcript abundance relative to the 28S rRNA.

shown). At each time point, statistically significant differences were obtained throughout the experiment, except at 10 hpi in light and dark periods, when the level expressions were almost equivalent (Fig. 6B).

3.7. Common bean SUMO and EF-hand calcium-binding protein gene transcripts accumulate after UV exposure

Northern blots were performed on total RNA sampled at 2, 4, and 6 hpi in the common bean susceptible and resistant interactions with *C. lindemuthianum* or after 4 h UV exposure. Both the SUMO and the calcium-binding protein gene transcripts accumulate, the calcium-binding protein at lower levels than SUMO, in the abiotic- and biotic-stressed plants. The highest transcript levels were observed in the resistant interaction, but transcript levels were notably higher after UV exposure. Lower levels were consistently observed in the susceptible plant–pathogen interaction (Fig. 7).

3.8. SUMO and EF-hand calcium-binding protein gene transcripts accumulate after cold and heat treatments

Northern blot analysis was also performed with total RNA isolated from leaves after 4 h cold (8 °C) or heat (38 °C) treatment.

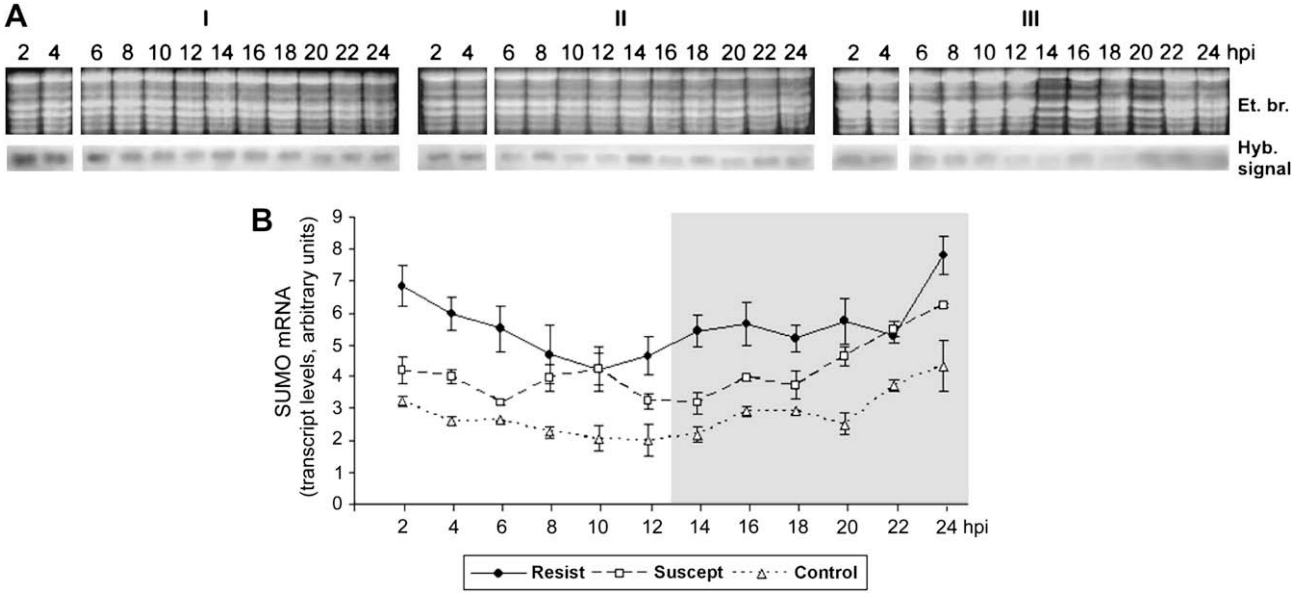

Fig. 6. Northern blot assay with total RNA evaluating SUMO mRNA expression levels and kinetics. (A) In the upper panel, total RNA was stained with ethidium bromide; in the bottom panel, the hybridization signal with the radiolabeled cDNA probe is shown. From left to right: I, resistant interaction; II, susceptible interaction; and III, control plants. (B) mRNA expression kinetics for SUMO mRNA throughout the 24 h day; the values are expressed as means of three independent experiments (±SE), in white background for day (light) period and in gray background for night (dark) period. The level expression was plotted as transcript abundance relative to the 28S rRNA.

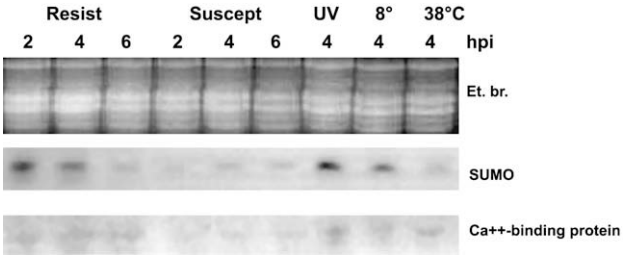

Fig. 7. Northern blot assay for the SUMO and EF-hand calcium-binding protein mRNAs obtained from plants challenged with pathogen and in abiotic stress. Hybridization signals in the northern blot assays in resistant and susceptible plant–pathogen interactions from 2, 4, and 6 hpi samples, in comparison with the abiotic stresses UV, cold (8 °C), and hot (38 °C) temperature conditions after 4 h treatment. Upper panel, total RNA stained with ethidium bromide. Bottom panel, hybridization signals with the SUMO (upper line) and with the EF-hand calcium-binding radiolabeled cDNAs (lower line) used as probes.

SUMO gene transcripts accumulated to higher levels after cold stress than after heat stress. In contrast, the calcium-binding protein gene transcripts accumulated to higher levels after heat than cold stress, although changes were less pronounced than for SUMO (Fig. 7).

4. Discussion

4.1. Cytology of plant–pathogen interactions

In this study, interactions between common bean (cv. Michigan Dark Red Kidney) and virulent and avirulent pathotypes of *C. lindemuthianum* (pathotypes 2 and 1472, respectively) were evaluated. Microscopy results show that the integrity of conidia and appressoria of the pathogen was structurally affected in the resistant interaction (Fig. 1) as previously reported [10]. This data indicates that this system is a reliable and stable model for susceptible and resistant interactions with common bean and allowed us to perform molecular studies.

4.2. Transcripts of a novel gene encoding an EF-hand calcium-binding domain accumulate in the resistant common bean-pathogen interaction

Using a subtracted cDNA library, previously obtained from plant tissue 0.5–6 hpi in a bean/*C. lindemuthainum* pathosystem, and by means of RNA blot hybridization experiments with total RNA sampled from leaf tissue, we searched for genes differentially expressed in resistant vs. susceptible interactions. We isolated a clone of 505 bp with homology to an *EF-hand calcium-binding protein* gene (acc. DQ118371) that was expressed at two-fold higher level in the resistant interaction compared to the susceptible interaction; transcript levels were also higher than those in control plants (Fig. 2). By comparison to available sequences, we determined that the gene fragment had significant homology to known calcium-binding proteins and caleosin encoding genes found in numerous plants. Sequence comparisons with accessions of fungal origin gave low identity scores of, at best, 44% (Fig. 4).

4.3. A sequence of plant origin found in infecting fungi

To establish whether the novel *EF-hand calcium-binding protein* gene was present in the plant or the infecting fungi, we performed a Southern analysis on digested genomic DNA from plant tissue and an equivalent amount of digested DNA from the pathogen. Our probe hybridized to DNA from plant and, surprisingly, also to DNA from fungus. The size of genome of common bean is estimated at between 450 and 600 Mbp (http://lft-45.esalq.usp.br/BEST/descript.html), whereas the *C. lindemuthianum* genome, according to published data [30], is less than 100 Mbp. As a consequence, in an equivalent amount of DNA from bean plant and *C. lindemuthianum*, there are 4–6 times the numbers of genomic copies in the fungal DNA sample than in plant DNA sample. The data from the Southern analysis clearly suggests that a novel calcium-binding protein encoding gene is present in both organisms, and the differences in genome sizes explain the intense hybridization signal obtained with fungal DNA. The differences in the fragment sizes between plant and fungi detected in the Southern assay are due mainly to the frequency of occurrence of restriction enzyme sites in the two genomes and is also indirect evidence of differences in genome sizes.

We analyzed the GC nucleotide content in a collection of mRNAs in the NCBI GenBank for *C. lindemuthianum* fungus and in a collection of mRNAs for common bean genes. The GC content in the open reading frame (ORF) for the fungus genes averaged 57%, whereas the average for the plant genes was 44%. The content of GC nucleotides in the partial ORF for clone 106 was 38%. The phylogenetic tree, constructed with sequences from accessions with the major identities shows three clades and places the species *O. sativa* (BAD45232.1), *A. thaliana* (NP_564996.1) and *C. dactylon* (AAS48644.1) as the nearest neighbors to *P. vulgaris* (AAZ23153.1) and at some distance from the second clade containing the other three plant species; the most distant species *M. grisea* and *P. nodorum* were in a third clade (Fig. 4B). Due to the higher homology of this new calcium-binding protein gene with sequences of plant origin than with those of fungi, the similarity of the GC nucleotide content of this gene to genes from plants, and the PCR amplification at high annealing temperatures, we assume that this calcium-binding protein gene is originally from the bean genome. We did observe a strong hybridization signal in DNA from fungi, but how the sequence was transferred to the fungus from the plant is an important unanswered question. It has been shown that specialized structures referred to as conidial anastomoses tubes (CATs) are potential agents of gene exchange between individuals of the same and different species of filamentous fungi, including *C. lindemuthianum* [31,32], but this is the first evidence of genetic material exchange with the host.

4.4. A novel gene possibly involved in early calcium-induced signaling in the plant defense response

Due to the homology of our novel gene with calcium-binding protein encoding genes, we hypothesize that this gene is involved in early calcium-induced signaling leading to the onset of the plant defense response. It is well known that shortly after microorganism recognition, an inward flux of Ca^{2+} occurs in plant cells [33–35]. There is much evidence that calcium-binding proteins, including calmodulin (CaM), CaM-like proteins (CMLs), and calcium-dependent protein kinases (CDPKs), are involved in the transduction of calcium signals that originate after avirulence gene products or pathogen-associated molecular patterns (PAMPs) are recognized [35,36]. The barley *Mlo* gene, which acts as plant defense and cell death modulator, and the rice *OsMlo* gene are calmodulin binding proteins [37,38]. In the MLO protein, a domain mediates a Ca^{2+}-dependent interaction with calmodulin *in vitro* and loss of calmodulin binding halves the ability of MLO to regulate defense against powdery mildew *in vivo* [37].

The CDPKs are calcium sensor responders [33] that contain a C-terminal, calmodulin-like domain that in most cases contains a conserved Ca^{2+} binding motif; proteins containing these domains constitute a family of predominantly calcium-dependent Ser/Thr protein kinases that are widespread in the plant kingdom. A number of CDPKs are known to be involved in plant defense

responses [20,21]. Plants also possess a unique set of CaM, CMLs, and several unique CaM target proteins. Plants have more proteins related to Ca^{2+} signaling than any other known organism [36]. The novel *EF-hand calcium-binding protein* gene over-expressed in *P. vulgaris* when infected with avirulent *C. lindemuthianum* may be acting as a calcium sensor relay or calcium sensor responder, transducing the early signal of the intracellular Ca^{2+} increase to downstream components in the signaling network for the assembly of the defense reaction.

4.5. Light/dark regulation of the EF-hand calcium-binding protein and SUMO gene expression

Previous studies have shown a light/dark regulation in the level of expression of some genes involved in stress [39] or pathogen defense responses [40–42]. In this study, higher levels of the novel EF-hand calcium-binding protein transcript were expressed in the resistant plant–pathogen interaction and transcript levels were also influenced by light/dark (day/night) periods. In resistant plants, higher transcript levels were observed under light at 2–4 hpi (Fig. 2), followed by a decrease under light at 6–12 hpi; throughout the dark period, increased transcript levels were maintained compared to susceptible and control plants (Fig. 5). Similar rapid increases and temporal fluctuation in transcript accumulation were observed for several defense genes involved in the phenylpropanoid pathway in soybean after inoculation with *Pseudomonas syringae* [43]. The kinetics of transcript accumulation exhibited in the plant resistance interaction in this study suggest that, firstly, transcripts for the new calcium-binding protein rapidly accumulate after the pathogen is sensed by the plant. Secondly, the transient, early rise in transcript accumulation (at 2–4 hpi) probably contributes to the activation of an important signaling cascade in the plant defense response that is followed by the second peak after 14 hpi or in the dark period. The sustained over-expression in the dark period in the resistant interaction suggests that this signaling pathway must be resumed again late in the day or at night (14–24 hpi) to guarantee an effective resistant plant–pathogen interaction. It may be that in the dark condition, this calcium-binding protein has other biological functions, possibly also related to defense.

The influence of light conditions on the over-expression of defense genes is not unusual: a pathogenesis-related protein from *Nicotiana tabacum* (*prb*-1b) exhibits high expression in the dark [40]; the pathogen-inducible *SsPto* gene from *Solanum surattense* is down-regulated by dark treatment [44]; and a rice *NPR1* homologue (*NH1*) was shown to exhibit light sensitivity [45]. These data and our results suggest that this novel *EF-hand calcium-binding protein* gene could be involved in the molecular mechanisms that are responsible for the onset of the defense reaction and that the kinetic expression of this gene in the resistant interaction is co-regulated by the light/dark (day/night) transition. Furthermore, our data suggests a potential interrelationship between the light/dark cycle and the successful onset of the pathogen-induced signaling defense pathways.

Previously, we identified several bean genes that are over-expressed during a resistant plant's reaction to anthracnose; among these, a *SUMO* (*S*mall *u*biquitin-like *mo*difier) gene (acc. AF451278) that was evaluated in this study. Our results reinforce the previous data regarding the participation of this gene in the defense response of bean plants to anthracnose. In relation to light/dark progress throughout the day, the *SUMO* gene was maintained at higher transcript levels throughout 12 h day/night periods in the resistant interaction than in susceptible and control plants (Fig. 6B). In contrast to the *EF-hand calcium-binding protein* gene, the *SUMO* gene mRNA kinetics, in this pathosystem, were not subject to co-regulation by the light/dark elapse, with the exception of a peak at the beginning of light cycle in the plant–pathogen interactions and at early times post-inoculation in the resistant interaction. Comparing the plant–pathogen interactions, statistical differences are clear in the over-expression of *SUMO* in the resistant plant reaction throughout the experiment. Surprisingly, at 10 hpi in day or night periods, the statistic difference vanished (Fig. 6B). These data could be due to punctual phases of optimization in the plant response to the changing environmental conditions of light/dark and/or temperature.

It is well known that circadian regulation is important for optimizing an organism's response to its environment and enhancing its fitness [46]. For example, a collection of 23 genes involved in phenyl-propanoyds biosynthesis are co-regulated, displaying a peak before dawn under conditions that do not represent stress for the plant [47]. In our study, the peak at the beginning of light cycle in mRNA levels for the *SUMO* gene (Fig. 6) suggests that the SUMO protein may alter the plant physiology before the sun rises. In contrast, the calcium-binding protein mRNA was highly expressed at night and diminished in the day (Fig. 5), a pattern very different from that of the *SUMO* gene expression. Although this study covered only 24 h, the mRNA kinetics of the *EF-hand calcium-binding protein* gene clearly differed from those of the SUMO transcript, suggesting that a different circadian clock mechanism controls the mRNA accumulation of these two genes. This is not surprising because by physiological analyses, it has been found that the plant circadian timing system comprises many copies of the circadian clock with at least one clock in each major plant organ [48,49] and these distributed circadian clocks are thought to function autonomously in regulating related or unrelated mechanisms [49].

4.6. Differential induction of the SUMO and EF-hand calcium-binding protein genes in UV and extreme temperature conditions

Northern blotting results suggest that *SUMO* and the *EF-hand calcium-binding protein* genes are regulated by several abiotic stresses. The SUMO transcript was significantly over-expressed in UV and cold conditions; to a lesser extent, the novel EF-hand calcium-binding protein gene transcript was over-expressed in UV and at high temperatures (Fig. 7). The SUMO conjugation system operates in plants in response to several abiotic stresses, including heat shock. In *Arabidopsis*, the SUMO1/2 conjugate expression increases substantially at early times (2–30 min) after exposure of seedlings to the temperature of 37 °C, suggesting that heat shock-induced SUMO conjugation is reversible [12]. In our study, the SUMO transcript level expression was analyzed in common bean seedlings 4 h after exposure to a temperature of 38 °C and a low level of transcript was observed. The level of mRNA and the respective protein for a given gene do not necessarily correlate, especially in a system where the protein function (e.g., SUMO conjugation) is reversible. In the *P. vulgaris/C. lindemuthianum* pathosystem, it will be necessary to study mRNA expression kinetics over a broader time window and to determine protein levels.

Ca^{2+} and CaM are important components in mediating specific signals in plant response to high temperatures. Heat shock stress in wheat leads to up-regulation of the *CaM1-2* gene and the *hsp26* and *hsp70* genes 10 min after heat shock at 37 °C [50]. Calcium-binding proteins, like calcineurin B-like (CBL) proteins, are important relays in calcium signaling. Analyses of loss-of-function and CBL1-over-expression in *Arabidopsis* indicate that this calcium sensor protein has a crucial function in abiotic stress responses, especially with respect to expression of cold-regulated genes [51]. It has also been reported that calcium efflux from an intracellular pool is involved in UV-A and UV-B signaling pathways that regulate *CHS* expression [52] and that calmodulin and elevations of cytosolic-free calcium are involved in the UV-B induction of *CHS* in parsley cells [53,54].

The previously reported data and the data obtained in this study suggest that the two gene transcripts evaluated here may represent another link between plant disease resistance reaction and plant acclimation response to UV and to extreme environmental conditions. These genes may serve as activators of a shared common signaling network that allows the plant to protect itself against pathogen attacks and against these forms of abiotic stress, in fashions similar to those reported for the chimeric *ATCNGC11/12* gene [4], the *CPN1* defense response gene [7], and the CASAR8.2A gene [22].

There is not an exact parallelism in the expression of the novel *EF-hand calcium-binding* gene and the *SUMO* gene, as the highest expression for the *SUMO* upon UV treatment does not correspond with that for the *EF-hand calcium-binding protein* gene. After heat treatment, the EF-hand calcium-binding protein mRNA levels surpass the SUMO mRNA levels (Fig. 7). Clearly, at extreme temperature conditions, there is a differential induction of these two genes. These results suggest that in the plant response to UV and cold temperatures, the SUMO signaling pathway responds positively and is of greater importance under the hot temperature condition, whereas the calcium-binding protein signaling pathway is of more importance at higher temperature and UV and less critical at cold temperatures. These data suggest that these two genes are involved in different signaling cascades, which converge with a degree of overlap in the response programs for pathogen defense and UV protection. A similar convergence of signaling pathways had been reported for systemin and oligosaccharide elicitors in response to biotic or abiotic stress, and for mitogen-activated protein kinases (MAPKs) in *Lycopersicon peruvianum* suspension-cultured cells in response to UV-B irradiation [55]. There is a balanced interplay between parallel signaling branches with fine-tuning by different sets of partially overlapping reactions in the MAPK- and CDPK-dependent cascades [56] and in the hormone signaling and reactive oxygen species pathways there is a crosstalk between biotic and abiotic stress signaling [57].

In summary, based on our results and on existing literature for these bean SUMO and EF-hand calcium-binding proteins, we hypothesize that the genes we analyzed are involved in the defense response against pathogens, and in the early molecular events that lead to the assembly of the overall defense reaction. These genes, especially the EF-hand calcium-binding protein, are subject to co-regulation by the light/dark transition. We also conclude that both genes function in plant acclimation to extreme temperature and UV conditions and act as integrative signals and convergence points in early plant responses to pathogens and several forms of environmental stress. Interestingly, the novel calcium-binding protein encoding gene is present both in the plant and in the fungi that infects it.

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