Characterization of a Class I Chitinase from Saffron (*Crocus sativus* L.)

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Abstract

Bacterial expression of a saffron (*Crocus sativus* L.) class I chitinase catalytic domain resulted in an active enzyme that showed in vitro antifungal properties when assayed against saffron fungal pathogens isolated from field-grown plants.

INTRODUCTION

Plants react to pathogen attack by producing a number of proteins believed to be important in protecting them from the deleterious effects of the stress. One family of these proteins are chitinases. Supporting evidence for the defensive role of chitinases includes inhibition of fungal growth in vitro and in vivo (Roberts et al., 1986; Shlumbaum et al., 1986) and that over expression of chitinase in transgenic plants increased resistance to fungal attack (Broglie et al., 1991; Xu et al., 1996). In saffron plant the corm is a subterranean organ with characteristics that make it particularly prone to pathogen attack, it is a very rich sugar contained organ (Chrungoo and Farooq, 1985) and it is in direct contact with soil, an environment where pathogens thrive in great numbers. Furthermore, the corm is fundamental for the immediate survival and for the successful vegetative reproduction of the whole plant due to the sterile nature of this triploid plant. Therefore an adequate protection of this organ against pathogen attack is critical and the production of an antifungal molecule could be part of a local defence strategy.

MATERIALS AND METHODS

Plant and Fungal Material

In vitro corms and callus were obtained from central merystemic stem segments (1cm x 1cm) (Piqueras et al., 1999).

Saffron plants were collected from Tarazona outfields (Albacete, Spain). Infected corms were ground to homogeneity and powdered tissue was inoculated in LB liquid media during 72 h and then plated in potato dextrose agar and incubated at 24º C during 72 h. After this time fungus were morphologically determined by microscopy.

Determination of *Fusarium oxysporum* fsp. *tuberosi*

We performed PCR using genomic DNA extracted as Doyle and Doyle (1990) from *F. oxysporum* isolated from infected corms. A pair of primers corresponds to a region of a fungal β-tubulin gene (TUB1: 5´-AAC ATG CGT GAG ATT GTA AGT-3´ and TUB2: 5´-TCT GGA TGT GTT GGG AAT CC-3´) were used to amplified this region. After PCR amplification products were separated on a 1.5 % agarose gel, purified using Wizard SV™ Gel and PCR Clean-Up System (Promega), ligated into pGEMT-Easy™ vector (Promega) and sequenced in an ABI PRISM™ 310 genetic analyser using T7 and SK primers of the vector. Blast of the obtained sequence in the database of NCBI showed that the clone has 98 % of homology with the tuberosi pathotype of *F. oxysporum* tubulin gene (emb/AF008546).

Expression of ChitCD in *E. coli*

A clone identified as a class I chitinase by nucleotide sequence homology with other chitinases from the NCBI database was obtained from a cDNA library of saffron

corm. Based on sequence data the pair of primers BCDF: 5´-GATGAATTCTTGCTTAAATATGTTCCA-3´ containing a *BamHI* site and ECDR: 5´-GTTGGATCTTCCTTTCCCGGATTCGC-3´ containing an *EcoRI* site were designed to amplify the sequence by PCR from saffron genomic DNA. The *EcoRI* and *BamHI* sites in pGex™-2TK vector (Amersham Pharmacia Biotech) were used for insertion of the PCR product. The resulting construct pGex-2TK-*ChitCD* contains the chitinase catalytic domain *ChitCD* translationally fused to the glutation-S-transferase (*GST*) *gene*. *E.coli* BL21 (Promega) harbouring the recombinant expression plasmid was grown to A_{600}=0.4 before induction with 1 mM isopropyl ß-D-thiogalactopyranoside (IPTG). After induction cells were allowed to grow for 3 h at room temperature. Cells were collected at 6000 g for 5 min at 4º C, frozen at –80º C, and lysed. Most of the protein expressed under these conditions was accumulated in insoluble inclusion bodies. A soluble protein fraction was extracted from the cells with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM EDTA and an insoluble protein fraction was obtained from the cell debris with 1mM EDTA, 1.5 % sarcosyl, 0.1 mM PMSF. Soluble protein extract was filtered through 0.45 µm (Millipore) and 1 % Triton X-100 was added. This sample was applied directly onto a Glutation Sepharosa 4B™ column (Amersham Pharmacia Biotech), and the bound proteins were eluted with 10 mM reduced glutation in 50 mM Tris-HCl pH 8.

In Vitro Antifungal Assay

Assays for antifungal activity toward *Phoma* sp., *Beauveria* sp. and *Fusarium oxysporum* were carried out in petri plates containing potato dextrose agar (39 mg/ml). Fungi were plated (4 mg/ml of mycelia in sterile water) by extension. Wells of 0.9 cm ∅ were made in the medium plates and antifungal proteins were inoculated at a concentration of 30 µM. After 72 h at 24º C degradation zones were visualized.

For $IC_{(50)}$ (inhibitory concentration 50) determination, different doses of the purified antifungal protein were added separately to aliquots containing 6 ml potato dextrose agar at 45º C, mixed rapidly, and poured into separate petri dishes. After the agar was cooled down, a small amount of *Phoma* mycelia, the same amount to each plate, was inoculated. Buffer without protein served as a negative control. After incubation at 24º C for 72 h, the area of the mycelia colony was measured and the inhibition of fungal growth determined. $IC_{(50)}$ was defined as the concentration of protein that provokes an inhibition of mycelia growth of about 50%.

Biological Stress Treatment

Infections of plant material were performed using fungal zoospores at a final concentration of 10^6 spores/ml during 48 h.

Expression Analysis

Expression analyses were performed by RT-PCR and southern blot. Total RNA was isolated from different saffron tissues using Trizol™ (GibcoBRL) reagent. Five micrograms were reverse transcribed with oligo(dT) using Ready-To-Go™ You-Prime First-Strand Beads kit (Amersham Pharmacia Biotech) 20 µl of the reverse transcriptase reaction products were subjected to PCR amplification using the primers described above. As a control of loading and RNA integrity, a pair of primers that amplified a region of a 18S RNA in plants was used. Blots for southern analyses were conducted as described by Sambroock and Russell (2001). Amplification products of PCR were resolved on agarose gel and blotted onto Boehringer-Mannheim™ positively charged nylon membranes. Blots were probed with DIG-labelled DNA and results were visualized by Roche DIG System™ using CSPD™ as substrate. Luminescence signal in the reacting bands was counted with a Photo-CaptMW System™ (Vilbert Lourmat), and the background counts of the membrane were subtracted. The amount of cDNA loaded in each lane was calibrated by hybridisation with the 18S probe. The values obtained with the control probe were used to calculate the relative intensity of the signals for chitinase in each lane.

RESULTS

We have isolated fungal pathogens from infected saffron corms collected from Tarazona (Albacete, Spain) outfields and identified them as the fungus *Cladosporium cladosporoides*, *Rhizomucor* sp., *Paecilomyces* sp., *Phoma* sp., *Beauveria* sp., *Aspergillus terreus*, *Aspergillus niger*, *Penicillium chrysigenum* and *Fusarium oxysporum,* the latter is known as one of the most important plant pathogenic species causing diseases in many agricultural and horticultural crops. Some races of *F. oxysporum* have high pathogenity against saffron as *F. oxysporum* fsp. *gladioli* that was identified as one of the most nocive pathogens in Italian saffron crops (Di Primo and Capelli, 2000). We determined the pathotype of *F. oxysporum* isolated from Spanish corms as *F. oxysporum* fsp. *tuberosi* by a method based in PCR.

From a cDNA library of saffron corm we have identified a class I chitinase. Bacterial expression of the catalytic domain of this chitinase resulted in an enzyme that showed in vitro antifungal activity against pathogens as *Phoma* and *Beauveria* (Figure 1) with a calculated $IC_{(50)}$ value against *Phoma* of 0.66 μM (Figure 2). Induction of the chitinase was examined in infected corms by RT-PCR and southern blot. Saffron chitinase is expressed in corm, also in aseptic generated in vitro corms, callus as in other organs of the plant like roots and leaves although a faint or none expression was detected in petals, stigmas and stamens (Figure 3). Induction of the chitinase occurred when corms were infected with fungus like *Phoma* and *Beauveria* (Figure 4 A) and no induction occurred against *F. oxysporum* fsp. *tuberosi* although it was observed when infection was performed in callus (Figure 4 B).

DISCUSSION

Stimulation or induction of chitinase expression by pathogen attack is often observed (Bishop et al., 2000; Collinge et al., 1993). The character of this expression can be systemic or local which depends on the infecting pathogen, its virulence and also on the particular chitinase class (Gerhardt et al., 1997; Meier et al., 1993).

The saffron chitinase subject of this study is induced in corm and callus by fungal infection. However, the different levels of accumulation of the chitinase transcript in the infected corms by *Phoma* and *Beauveria* and its absence in the corms infected by *Fusarium oysporum* f.sp *tuberosi* suggest an incompatible interaction between *C. sativus* and some fungi like *Phoma* and *Beauveria*, while *F. oxysporum* fsp. *tuberosi* could acts as virulent pathogens that in general induce chitinases to a lesser extent and slower response (Meier et al.,1993). Differential induction of chitinase mRNA has been reported before, in *Phaseolus vulgaris* hypocotyls different induction of chitinase was observed after infection with spores of an avirulent and virulent race of *Colletotrichum lindemuthianum* (Hedrick et al., 1998), in a similar way Rasmussen et al. (1992) showed the differential induction pattern of two (resistant and susceptible) cultivars of *Brassica napus* chitinase mRNA against *Phoma lingam* infection. Antifungal in vitro properties of the saffron chitinase supported the importance of this protein in the plant defence mechanisms, furthermore data show that an increase in chitinase activity occurred in saffron against pathogen attack and that the differential gene activation depends on pathogen-plant interaction.

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Figures

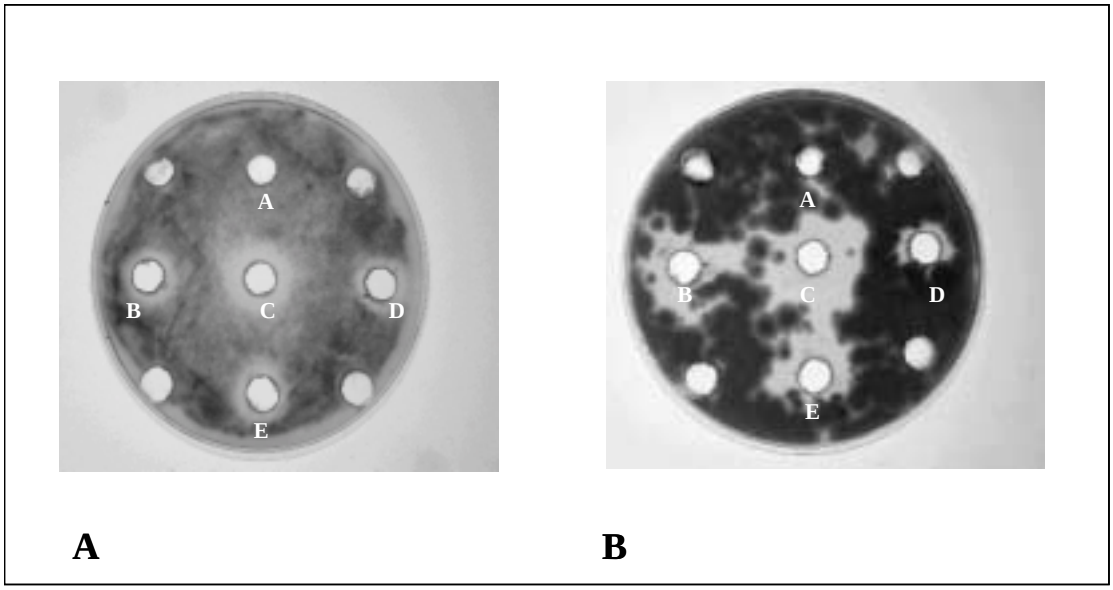

Fig. 1. Inhibitory activity of different proteins toward *Beauveria* (A) and *Phoma* (B). A: control (NaAc 0.2 M pH 5.5), B: ChitCD, C, E: Corm protein extracts, D: *Nicotiana tabacum* class I chitinase.

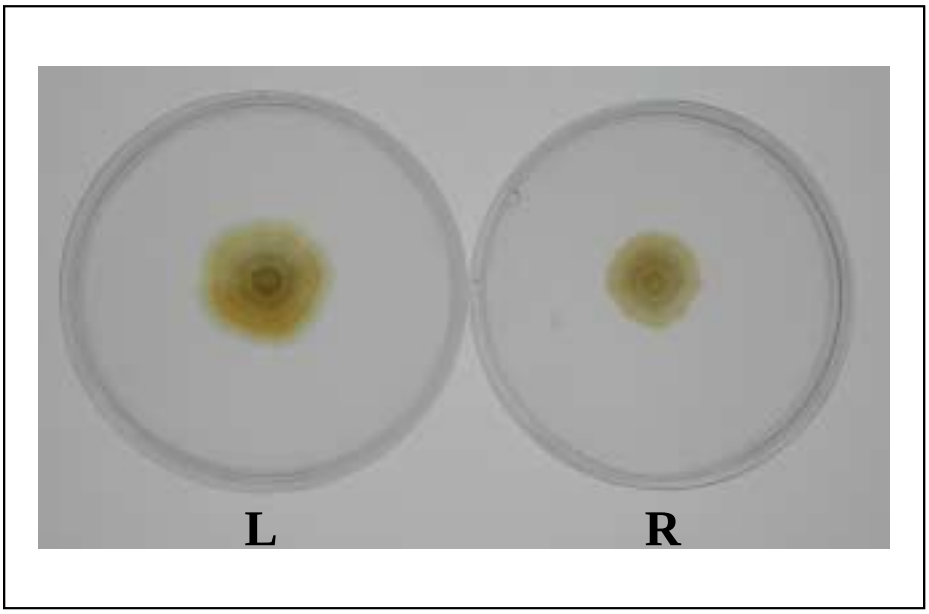

Fig. 2. Determination of $IC_{(50)}$ value of antifungal activity of ChitCD (*C. sativus* class I chitinase catalytic domain) toward *Phoma*, L (left): control, R (right): antifungal protein at 0.66 μM.

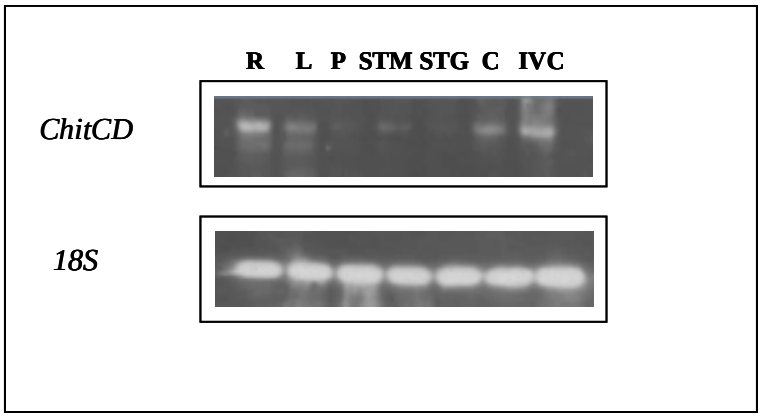

Fig. 3. Expression analysis of saffron class I chitinase in different plant tissues, **R**: roots, **L**: leaves, **P**: petals, **STM**: stamens, **STG**: stigmes, **C**: callus, **IVC**: in vitro generated corm. Analysis was done by RT-PCR and southern blot, hybridisation was done with specific PCR derived probe *ChitCD* and 18S ribosomal plant probe serves as a control.

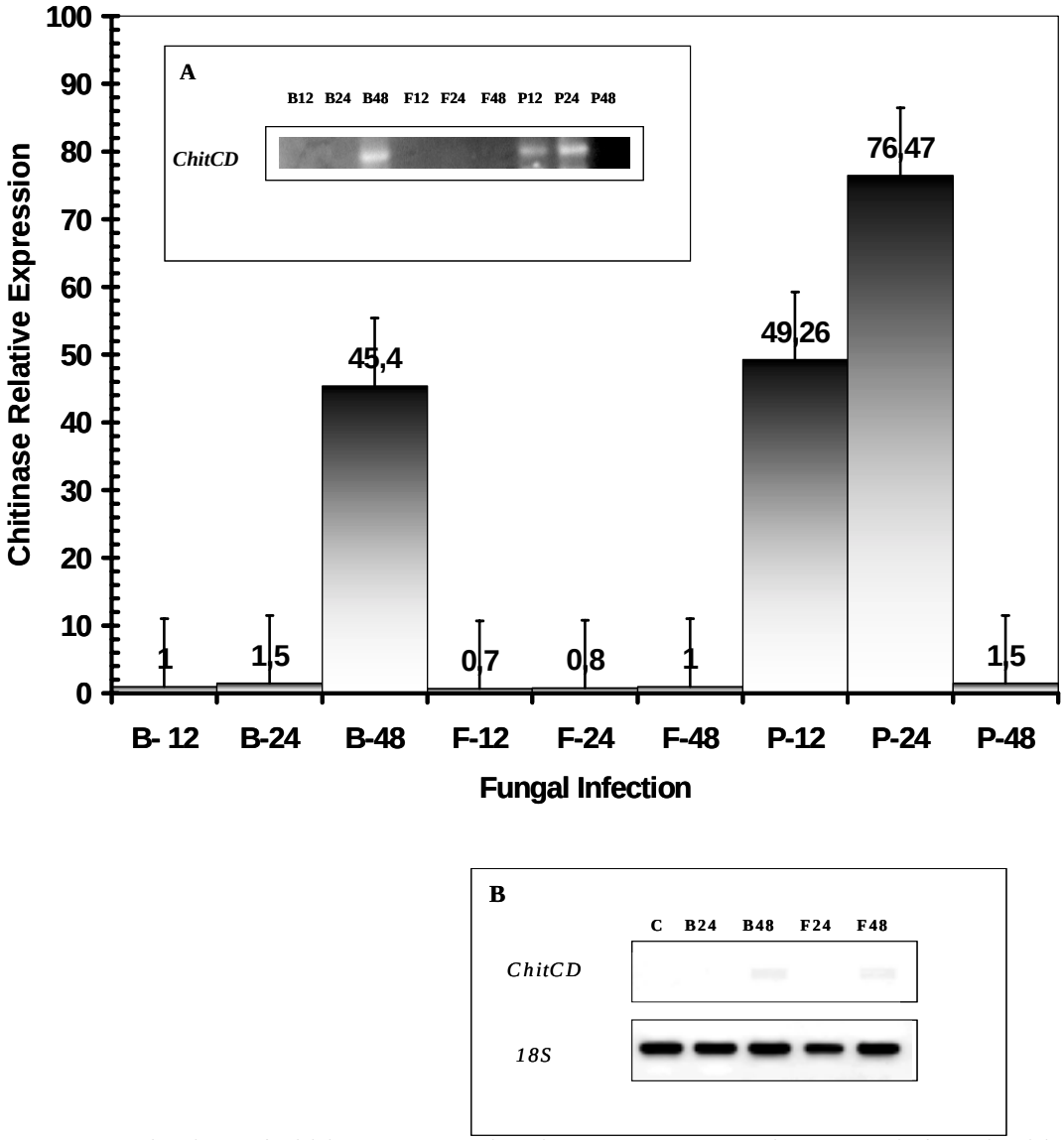

Fig. 4. **A**. Induction of chitinase expression in corms generated *in vitro* infected with *Beauveria* (B), *F.oxysporum fsp. tuberosi* (F) and *Phoma* (P), during different time sets (12, 24, 48 h). Expression analyses were performed by RT-PCR and southern blot using *ChitCD* as a probe, results were visualized by luminescence method, signal in the reacting bands was counted with an image analyser and the background counts of the membrane were subtracted. The amount of cDNA loaded in each lane was calibrated by hybridisation with 18S riboprobe. The values obtained with the riboprobe were used to calculate the relative intensity of the signals for chitinase in each lane. **B**. Induction analysis by RT-PCR, cDNA was obtained from saffron callus infected with *Beauveria* (B), *F.oxysporum fsp. tuberosi* (F) and without treatment (C) after 24 and 48 hours.