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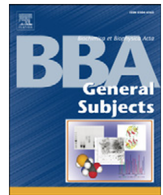

# An antifungal peptide from *Coffea canephora* seeds with sequence homology to glycine-rich proteins exerts membrane permeabilization and nuclear localization in fungi

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ABSTRACT

*Background:* The superfamily of glycine-rich proteins (GRPs) corresponds to a large and complex group of plant proteins that may be involved in many developmental and physiological processes such as RNA biogenesis, stress tolerance, pollen hydration and plant-pathogen interactions, showing defensive activity against fungi, bacteria and viruses.

*Methods:* In this study, the peptides from *Coffea canephora* seeds were extracted according to the methods of Egorov et al. (2005). The purified peptide was submitted for amino acid sequencing and antimicrobial activity measurement.

*Results:* The purified peptide with a molecular weight of 7 kDa, named *Cc*-GRP, was observed to display homology to GRPs. The *Cc*-GRP–fungi interaction led to morphological changes and membrane permeability, including the formation of pseudohyphae, which were visualized with the aid of SYTOX green dye. Additionally, *Cc*-GRP also prevented colony formation by yeasts. Antifungal assays of *Fusarium oxysporum* and *Colletotrichum lindemuthianum*, observed by light microscopy, showed that the two molds exhibited morphological changes after the growth assay. *Cc*-GRP coupled to FITC and its subsequent treatment with DAPI revealed the presence of the peptide in the cell wall, cell surface and nucleus of *F. oxysporum*.

*Conclusions and general significance:* In this work we purified, characterized and evaluated the in vitro effect on fungi of a new peptide from coffee, named *Cc*-GRP, which is involved in the plant defense system against pathogens by acting through a membrane permeabilization mechanism and localized in the nuclei of fungal cells. We also showed, for the first time, the intracellular localization of *Cc*-GRP during antimicrobial assay.

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## 1. Introduction

Recently, a novel group of proteins in plants characterized by a high content of repetitive sequences containing glycine residues, called glycine-rich proteins (GRPs), has been studied and a wealth of new GRPs have been identified [1]. The expression of these proteins is modulated by both abiotic and biotic factors. Interaction with or infection by pathogenic organisms, such as viral [2] or fungal infections [3], also modulates GRP expression. GRPs are classified based on their general structure, accounting for the arrangement of glycine repeats and conserved regions [1,4,5].

In most of these proteins, a signal peptide has been identified as well as other specific structures, such as cold shock response domains or RNA-binding regions [6].

The different patterns of expression of GRPs and their subcellular localization suggest an involvement in different physiological processes. Among the numerous roles for GRPs are their proposed activities against microorganisms. This activity has been seldom reported, but it has been demonstrated in filamentous fungi [7,8] and gram-negative bacteria [9]. Attacking pathogens, such as viruses [2] and fungal infections, increase the expression of GRPs. Root knot nematode Meloidogyne incognita infection in alfalfa results in the induction of a GRP possessing an RNA-binding motif [10]. The GRPs with RNA-binding regions may be involved in either RNA processing or the control of gene expression [11]. While searching for peptides with antimicrobial activity, two glycine-rich peptides containing GGH repeats were isolated from the roots of *Capsella bursa-pastoris*. The two peptides, named Sheperin I and II, exhibited activity against several bacteria and fungi. Both peptides are encoded by a

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single gene, herein referred to as CbGRS1. This gene is expressed exclusively in the roots [12].

The diversity in structure, expression pattern and subcellular localization of plant GRPs provides a very strong indication that these proteins may play very different roles in plants, and only in recent years, when the first functional data were published, was this statement confirmed. However, the question of how the glycine-rich domains of these proteins contribute to their functional roles in plants still remains unknown [5].

In this work, we report the characterization and purification of a new Glycine-rich peptide from *Coffea canephora* seeds, *Cc*-GRP, which has in vitro fungicidal activity. We also show the membrane permeabilization and intracellular localization properties of *Cc*-GRP resulting from this antifungal activity.

## 2. Materials and methods

### 2.1. Plant materials

Seeds from the *C. canephora* (p. ex Fr.) cultivar Robusta Tropical (EMCAPER – 8151) were collected at the pinhead stage from coffee trees on the experimental farm of INCAPER, Linhares, ES, Brazil.

### 2.2. Fungi

*Candida albicans* (CE022) and *Candida tropicalis* (CE017) were obtained from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. Both yeast and fungi were maintained on Agar Sabouraud (1% peptone, 2% glucose, and 1.7% agar-agar). The fungal isolates *Fusarium oxysporum* and *Colletotrichum lindemuthianum* were kindly supplied by CNPAF/EMBRAPA, Goiania, Goias, Brazil. The fungi were also maintained on Agar Sabouraud (1% peptone, 2% glucose, and 1.7% agar-agar).

### 2.3. Purification of Cc-GRP

Extraction of the *Cc*-GRP peptide from coffee seeds was performed as described by Egorov et al. [8]. The resulting suspension was centrifuged at 15,000 ×g (at 4 °C), and the supernatant was extensively dialyzed, recovered by freeze drying and resuspended in 50 mM Tris–HCl (pH 8.0). The purification steps were done as described by Zottich et al. [13]. Protein content was determined as described by Bradford [14].

### 2.4. Tricine gel electrophoresis

Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (tricine–SDS–PAGE) was performed according to the method of Schägger and von Jagow [15].

### 2.5. Amino acid sequence analysis

For the amino acid sequence analysis, peptides from the H1 fraction were separated by tricine–SDS–PAGE, transferred to a polyvinylidene difluoride (PVDF, Millipore) membrane and stained with Ponceau-S (0.1%). The 7 kDa band was excised from the membrane and briefly washed as follows: in 100 μl of water, then in 400 μl of methanol with vortexing and finally in 100 μl of chloroform with vortexing. After the last wash was removed, the membrane was air dried. The N-terminal amino acid sequences of the peptides blotted onto PVDF were

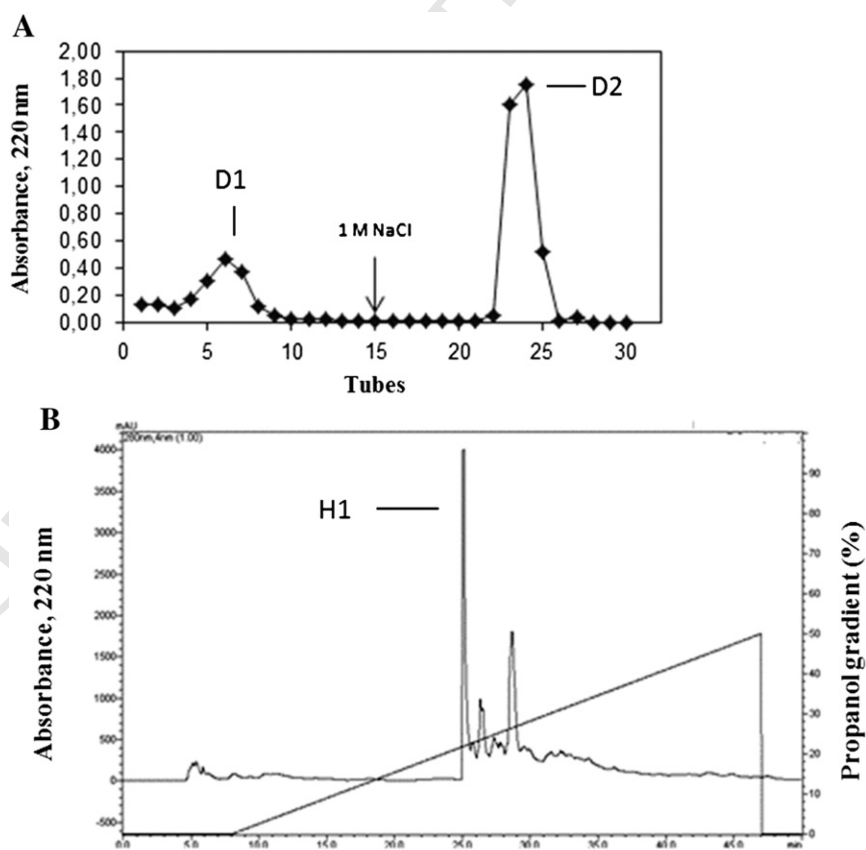


**Fig. 1.** Purification of *Cc*-GRP from *Coffea canephora* seeds. (A) Partial purification of the antifungal peptide from *C. canephora* seeds by anion-exchange DEAE-sepharose chromatography. The column was equilibrated, and the sample was initially eluted with 50 mM Tris–HCl, pH 8.0, followed by the same buffer containing 1 M NaCl at 60 ml/h. (B) RP-HPLC chromatography. The DEAE-sepharose fraction (D1) was applied to a C18 reverse-phase column and run in a Shimadzu apparatus. Elution was performed as described in Section 2.3. The oblique line represents the propanol gradient, and the other line represents the protein elution profile at 220 nm.

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