Characterization of cDNAs encoding two glycine-rich proteins in chickpea (Cicer arietinum L.): accumulation in response to fungal infection and other stress factors

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Abstract

In chickpea plants infected with the pathogenic fungus Ascochyta rabiei [Pass.] Labr. several mRNAs for two glycine-rich proteins (GRPs) were identified by differential cDNA screening. The main part of the deduced amino acid sequences of the 14.6 kD GRP1 and the larger GRP2 consists of glycine-rich repetitive elements essentially as found for GRPs in other plants. Tyrosine residues in conserved positions inside these repetitive motifs suggest an involvement of the GRPs in a polymerization process by oxidative cross-linking, i.e. cell wall fortification. Both GRP transcripts are induced by infection with A. rabiei, showing a maximum of expression 5 days post infection. Wounding of leaves and the stress of water treatment (performed as a control) also seem to induce the accumulation of GRP transcripts. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Glycine-rich proteins (GRPs) are a class of simple-structured proteins, often consisting of repetitive amino acid patterns with a high glycine content. They resemble animal keratins or silk fibronins, i.e. proteins that are characterized by both strength and flexibility. GRPs were first described by Condit and Meagher for petunia in 1986 [1]. Until now, GRPs and/or their corresponding genes have been identified in a variety of plants including both monocotyles and dicotyles [2]. Their specific function is still a topic of discussion but most of them are thought to be structural cell wall components like HRGPs (hydroxyproline-rich glycoproteins) or PRPs (proline-rich proteins). Glycine-rich proteins were found mainly in the vascular tissue [3,4]. For some GRPs the localization in the cell walls of vascular elements has been shown [5]. In addition to developmental regulation GRPs seem to be expressed in response to a variety of abiotic stress factors, including cold [6–8], water stress [7,9] and wounding [10]. GRPs also seem to play a role in plant–microbe-interactions: Their induction after bacterial [11], viral [12–14] and fungal [8] infection has been reported. Here we describe the isolation and characterization of cDNAs encoding two GRPs which accumulate in chickpea (Cicer arietinum L.) plants in response to fungal infection and other stress factors.

2. Materials and methods

2.1. Library construction and selection of cDNA-clones

In order to identify genes involved in the de-
defense mechanisms of chickpea against the infection with the fungus *Ascochyta rabiei* [Pass.] Labr. (cause of the ‘Ascochyta blight’ disease), two cDNA libraries were constructed from poly(A)RNA extracted from 18-day-old chickpea plants (resistant cultivar ILC 3279) 4 days after infection with spore-suspensions of *Ascochyta rabiei* cultivar XXI or after treatment of plants with sterile water, respectively [15]. These libraries were compared by differential dot-blot hybridization with radioactively-labelled probes constructed from chickpea cDNA. Clones that showed significant differences in signal intensity when hybridized to the two cDNA libraries were transfected into *E. coli* for further investigation and sequencing.

### 2.2. Growth, infection and wounding of chickpea plants

To induce germination, chickpea seeds (resistant cultivar ILC 3279) were watered for 12 h and kept in darkness for 2 days at high humidity. Seedlings then were planted in a standard soil–sand mixture (2:1) and grown for two weeks (12 h light/darkness, 20°C). On the 14th day of growth the plants were either sprayed with a suspension of *Ascochyta rabiei* cultivar XXI spores (10^6 spores/ml, 500 µl (5 × 10^5 spores)/plant) or treated with sterile water (control). After the treatment the plants were kept at high humidity to allow the fungal spores to germinate.

Plants were wounded at the same developmental stage when infection and water-treatment were performed (14 day old). All fully developed leaves of the experimental plants were cut two times, avoiding to wound the rachis. Those plants were kept separated from the infected and water-treated plants to avoid unwanted interference of effects such as ethylene signalling.

### 2.3. Northern-analysis

Total RNA was isolated from 0.5 g of chickpea leaves (fresh weight) using the ‘RNAqueous-MIDI™-Kit’ (Ambion, USA). Concentration of RNA was determined by UV absorbance. The RNA was seperated by gelelectrophoresis (20 µg RNA per lane, 1% agarose in MOPS-buffer, 12.5% Formalin (37% formaldehyde)) and blotted to an uncharged nylon-membrane (Qiagen, Germany) by alkaline transfer [16]. The RNA was heat-fixed at 80°C for 30 min.

Visualization of the seperated rRNA by ethidiumbromide- (gel) and methyleneblue-staining (membrane) were performed as a loading control.

T7-RNA polymerase (‘RNA Transcription Kit’, Stratagene, USA) was used to construct digoxigenin-labelled antisense RNA probes (DIG RNA Labelling Mix, Boehringer, Germany) by in vitro transcription of the GRP-encoding cDNA clones.

Hybridization was performed under highly stringent conditions (68°C, 12–18 h, buffer: 5 × SSC, 0.02% SDS, 0.1% laurylsarcosine, 2% Blocking-Reagent (Boehringer, Germany), 50% formamide). Unspecifically bound probe was removed by two washes at room temperature (2 × SSC, 0.1% SDS, 15 min) followed by two washes at 68°C (0.1 × SSC, 0.1% SDS, 20 min).

Digoxigenin was immuno-localized with ‘Anti-DIG-AP’ and the substrate ‘CDPstar™’ (Boehringer, Germany) according to the manufacturers instructions. Chemiluminescence resulting from the processing of the substrate by the alkaline phosphatase was visualized by exposure of the membranes to X-ray films.

### 3. Results

By differential hybridization we found over 100 cDNA clones that appeared to be increased or decreased in the level of expression in response to infection. These clones were named ‘INR’ (infection responding) [15]. By sequencing and comparison to databases 14 clones with significant homologies to glycine-rich proteins were identified, mostly among those earlier classified as ‘increased expression’ after infection. Eleven of these cDNAs encode for a GRP of 148 amino acids with a glycine content of 36%, among them three full-length clones (INR5, INR20, INR41). The deduced 14.6 kD protein was named GRP1. The three other clones contain an open reading frame for a larger GRP of more than 208 amino acids (GRP2, > 20 kD) with an incomplete N-terminal region. The amino acid sequences of GRP1 (deduced from cDNA clone INR20, see EMBL # AJ00756) and GRP2 (deduced from cDNA clone INR23, see EMBL...
Theoretical pI of 5.78 was computed for GRP1. Computer-aided prediction of the secondary structure of both proteins lead to no significant results, although the repetitive amino acid pattern (see below) suggests a regular structure.

Both proteins show the same basic pattern: a N-terminal region with structural similarities to putative cell wall signal peptides of GRPs from other plants [11,12] (37 amino acids in GRP1) is followed by a region of glycine-rich repeats. The similar structure of the two proteins and their repetitive elements is shown in Fig. 1. The predominant motif of the repetitive elements in both proteins is GGGNYGX, where in most cases, X is asparagine or histidine. GRP1 contains ten repeats of the GGGNYGX element whereas GRP2 has 16 repetitions of this motif resulting in the larger size of GRP2. Besides the main repetitive element both GRP sequences also include some smaller glycine-containing motifs (e.g. GGH, GGN, GGY). Similar repetitive elements are common among GRPs in other plants [4,17,18].

Database comparison of the chickpea GRP sequences revealed high homologies to a large number of GRPs from other plants (i.e. bean, tomato, tobacco, rice), especially several *Medicago* species. The N-terminal region of GRP1 shows a similarity of over 80% to the N-terminus of the protein MFSTRESS, a GRP from *Medicago falcata* L. induced by environmental stress [18] (Fig. 2). Overall similarity of the GRPs from the two members of the Fabaceae family is 62%.

In order to verify the inducibility of the GRP mRNAs northern-hybridizations were performed using digoxigenin-labelled RNA-probes constructed from the GRP cDNA-clones INR20 (representing the GRP1 open reading frame flanked by 19 bp on the 5’-end and 200 bp of non-coding region on the 3’-end) and INR51 (a GRP2 cDNA clone that is 29 bp shorter on the

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**Fig. 1.** Repetitive amino acid structure of the two chickpea GRPs. (A) Deduced amino acid sequence of GRP1; (B) deduced amino acid sequence of GRP2. **Bold** = main repetitive element (GGGNYGX).

**Fig. 2.** Comparison of the N-terminal regions of GRP1 (*Cicer arietinum*) and MFSTRESS (*Medicago falcata*). ‘.’, identical amino acid; ‘.’, chemically similar amino acid; **bold** = region with putative signal peptide.
5′-end but otherwise identical to INR23). To test the specificity of the probes a variety of sequenced and identified chickpea cDNA clones were separated by gelelectrophoresis along with the GRP cDNAs and blotted to a nylon membrane. Upon hybridization to the probes used only the GRP cDNAs showed signals, whereas no hybridization to any other cDNA could be observed (data not shown). The northern results are shown in Fig. 3. A cross-hybridization to both GRP transcripts occurs, especially when using the larger probe constructed from INR51 for hybridization. Nevertheless, a strong and transient induction with a maximum 5 days post infection (dpi) was found for both GRPs. The smaller GRP1 mRNA (transcript size about 0.8 kb) appears to respond faster and accumulates to higher levels than the larger GRP2 transcript (about 1 kb). Such results are qualitatively reproducible, although in different kinetics slight quantitative differences especially in the controls could be observed (as for probe INR51 in Figs. 3 and 4). Such differences in the level of expression in the controls may result from developmental regulation or other stress factors (see Section 4).

In an effort to determine whether or not the obvious, although slight raise in the northern signals obtained with the water treated control plants is due to developmental regulation of the GRPs or the stress effected by the water-treatment (including a change of the incubator vessel), total RNA from completely untreated plants of the same age was also isolated. In addition, we isolated RNA from wounded plants. The results of the comparative northern-analyses are shown in Fig. 4. Spraying of plants with water as well as wounding of leaves seem to induce accumulation of both GRP mRNAs (the GRP2 transcript again showing a weaker inducibility), whereas in untreated plants up to 2 days after onset of the experiment only the larger transcript of GRP2 is visible. These significantly weaker
signals in the untreated plants may be due to developmental regulation of the GRPs.

4. Discussion

Regarding the amino acid sequences of the two GRPs, it is noteworthy that each main repetitive motif in both proteins contains the amino acid tyrosine in a conserved position. The phenolic rings of this acid are the putative essential sites for oxidative cross-linking of structural cell wall proteins like HRGPs (hydroxyproline-rich glycoproteins) or GRPs during incorporation into the complex network of phenolic substances of the cell wall [3], maybe as part of a cell wall fortification process. In chickpea cell cultures, the insolubilization of two cell-wall proteins (a putative HRGP and a proline-rich protein (PRP)) after elicitor-induced oxidative burst has been demonstrated [19].

Because of the comparatively late maximum of expression (5 dpi) it is not very likely that the GRPs in chickpea are involved in the direct defense against infection with *A. rabiei*. This and the inducibility by wounding suggest that they may rather play a role in a more general stress-response system, for example as part of a cell wall repair mechanism (as suggested for bean and soybean in reference [5]). It also appears possible that they are responsible for the more effective sealing of vascular elements during the late phases of *A. rabiei* growth in the apoplastic space [20].

Gene activation as shown for GRP1 and GRP2 in the control plants sprayed with water (Figs. 3 and 4), which especially in case of GRP2 (probe INR51) led to an induction nearly as high as triggered by infection or wounding, most likely results from multiple stress factors which are difficult to demonstrate in detail. However, the effects of such ‘manipulation stress’ must be taken in consideration in related studies.

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