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Transcriptional analysis of major chaperone genes in salt-tolerant and salt-sensitive mesorhizobia

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ABSTRACT

Salinity is an important abiotic stress that limits rhizobia-legume symbiosis, affecting plant growth, thus reducing crop productivity. Our aims were to evaluate the tolerance to salinity of native chickpea rhizobia as well as to investigate the expression of chaperone genes *groEL*, *dnaKJ* and *clpB* in both tolerant and sensitive isolates. One hundred and six native chickpea mesorhizobia were screened for salinity tolerance by measuring their growth with 1.5% and 3% NaCl. Most isolates were salt-sensitive, showing a growth below 20% compared to control. An association between salt tolerance and province of origin of the isolates was found. The transcriptional analysis by northern hybridization of chaperone genes was performed using tolerant and sensitive isolates belonging to different *Mesorhizobium* species. Upon salt shock, most isolates revealed a slight increase in the expression of the *dnaK* gene, whereas the *groESL* and *clpB* expression was unchanged or slightly repressed. No clear relationship was found between the chaperone genes induction and the level of salt tolerance of the isolates. This is the first report on transcriptional analysis of the major chaperones genes in chickpea mesorhizobia under salinity, which may contribute to a better understanding of the mechanisms that influence rhizobia salt tolerance.

1. Introduction

Rhizobia are soil bacteria able to establish nitrogen fixing symbioses with legumes. The biological nitrogen fixation contributes to an ecological and sustainable agriculture, as it reduces the need for chemical N-fertilizers and improves crop productivity. One of the major problems that agriculture is currently facing is the effect of abiotic environmental stresses, leading to yield reductions and subsequent economic losses (Ashraf et al. 2008). Among the abiotic stresses, salinity seriously limits the productivity of agricultural crops (Ashraf et al. 2008; Katerji et al. 2009; Grewal 2010) and affects about 80 million ha of arable lands worldwide (Munns and Tester 2008).

Legumes represent a very significant group of crops in agriculture and therefore their tolerance to salt stress is important worldwide. Chickpea (*Cicer arietinum* L.) is one of the most important grain legume crops because it is a relevant protein source in both human and animal diets. Like other legumes, chickpea is very sensitive to salinity, which affects its growth and development (Elsheikh and Wood 1990a).

Salinity may negatively affect the rhizobia-plant symbioses in several processes: growth and survival of rhizobia in soil, root colonization, infection and nodule development and functioning (Kulkarni et al. 2000). The rhizobia isolated from chickpea nodules and cultured *in vitro* are usually much more tolerant to salt than their host (Zahran 1999). Nevertheless, chickpea rhizobia differ in NaCl tolerance; some strains are able to grow at salt concentrations as high as 500 mM NaCl (Kucuk and Kivanc 2008), others cannot grow even when NaCl concentration is lowered to 100 mM NaCl (Elsheikh and Wood 1990a, b; Zurayk et al. 1998; Kucuk and Kivanc 2008).

A major consequence of salt-stress is the loss of intracellular water, which imposes a water deficit because of osmotic effects on a wide variety of metabolic activities (Fatnassi et al. 2011). Proteins are at permanent risk of unfolding, especially when cells are exposed to environmental stress conditions, such as high salt concentration. When protein denaturation occurs, molecular chaperones enable denatured proteins to acquire their native folding faster and more reliably than they otherwise would (Hartl and Hayer-Hartl 2009).

Several classes of chaperones are induced under stress conditions, such as salinity. The DnaK machinery comprises the co-chaperone DnaJ and the nucleotide exchange factor GrpE, whereas the GroEL system includes the co-chaperone GroES (Chaudhuri et al. 2009). ClpB belongs to the Clp family, which

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is ubiquitous among prokaryotes and eukaryotes, acting as both protease and chaperone (Gottesman et al. 1997). Under extreme conditions, ClpB interacts with the DnaK chaperone system catalyzing protein disaggregation and reactivation (Motohashi et al. 1999; Zolkiewski 1999). In *Ensifer meliloti* cells subjected to salt stress, Domínguez-Ferreras et al. (2006) reported the induction of several genes, including *clpB* as well as the repression of some *groESL* operon copies. Additionally, the co-chaperone *dnaJ* was described as being involved in *Rhizobium tropici* salt tolerance (Nogales et al. 2002). However, in chickpea rhizobia, little is known about the expression of chaperone genes under saline conditions.

The present study describes the screening of a collection of Portuguese chickpea rhizobia for salinity tolerance and investigates the gene expression of the well characterized chaperone systems *dnaK–dnaJ*, *groEL–groES* and *clpB*. The transcription of these chaperone genes upon salt shock was analyzed, using sensitive and tolerant mesorhizobia isolates, belonging to several *Mesorhizobium* species.

2. Methods

2.1. Bacterial isolates

A total of 106 chickpea rhizobia isolates were used in the present study, including 94 isolates which were previously characterized (Sup Table 1). Twelve isolates were obtained in this study from root nodules of chickpea trap plants (Table 1), as previously described (Alexandre et al. 2009). Overall, isolates were collected from 26 soil samples (Table 2), covering almost all Portuguese territories with the exception of Azores Islands and Minho province. Four *Mesorhizobium* type strains were also used: *M. ciceri* (UPM-Ca7^T), *M. loti* (LMG 6125^T); *M. amorphae* (ACCC 19665^T) and *M. mediterraneum* (UPM-Ca36^T). All isolates were preserved in 30% (v/v) glycerol at −80 °C and cultured in yeast extract mannitol (YEM) broth (Vincent 1970) for routine use.

2.2. Rhizobia characterization: 16S rRNA gene-based phylogeny, plasmid number and symbiotic effectiveness

Amplification of the 16S rRNA gene and subsequent sequence alignments, as well as phylogenetic analyses were performed as described before (Alexandre et al. 2009). The plasmid profiles were analyzed by horizontal agarose gel electrophoresis using a two-comb system, as described by Laranjo et al. (2001). Symbiotic effectiveness (SE) was determined as the ratio of shoot dry weight (SDW) of inoculated plants minus SDW of non-inoculated control plants and SDW of non-inoculated nitrogen-supplemented control plants minus SDW of non-inoculated control plants (Gibson 1987). Symbiotic effectiveness is presented as percentages.

2.3. Salt stress tolerance

The salt tolerance of bacterial isolates was screened by evaluation of their growth based on optical density (OD) readings at 540 nm. The YEM medium was supplemented with 1.5% and 3% NaCl for stress conditions. For control conditions, standard YEM was used. After overnight growth in YEM, bacterial cultures were standardized to an initial OD of 0.03 and grown for 48 h at 28 °C. Three replicas per treatment were done.

2.4. Statistical analysis

In order to compare differences in isolate tolerance, optical density values were converted into percentage values, considering growth at control conditions as 100%. Average value and standard deviation of the three replicas were calculated. Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, USA). The Kruskal–Wallis test was used in order to explore the relationship between stress tolerance (continuous dependent variable) and categorical independent variable, as for instance species group or province of origin. To identify categories that differ significantly from others, three different *post hoc tests* (Tamhane, Dunnett T3 and Games-Howell) were used. To detect structure in the relationships between categorical variables, the correspondence analysis (CA) was conducted as an exploratory data analysis technique (Benzécri 1973). Isolates were divided into three classes: sensitive (growth < 10%), tolerant (growth between 10 and 20%) and highly tolerant (growth > 20%), in order to investigate the relationships between these classes and isolates province of origin. Spearman's correlations were performed in order to determine if any of the soil characteristics were related with salt-tolerance.

2.5. RNA extraction and northern hybridization

RNA extraction was performed using cell cultures in exponential growth phase, submitted to a salinity shock in YEM supplemented with 5% NaCl, for one hour. Control RNA was extracted from cells grown in YEM. Total RNA extraction was performed according to the protocol for Rapid Isolation of RNA from Gram-negative Bacteria (Ausubel et al. 1997).

The nonradioactive DIG system (Roche Applied Science) was used for northern experiments. RNA samples were denatured in a loading buffer (50% deionized formamide; 6.1% formaldehyde; 1× MOPS) and separated by electrophoresis on a 1.5% agarose gel containing 2% formaldehyde in 1× MOPS (20 mM MOPS buffer, 5 mM sodium acetate, 2 mM EDTA, pH 7.0). After electrophoresis, capillary transfer into a positively charged nylon membrane (Roche Applied Science) was carried out in 20× SSC (3 M NaCl; 300 mM sodium citrate, pH 7.0). RNA was fixed by baking the membrane at 120 °C for 30 min. The *groEL* and *dnaKJ* RNA probes were obtained as previously described (Alexandre and Oliveira 2011). The *clpB* RNA probe was obtained using a gene fragment of 1388 bp that was amplified using the primers clpB-F (5′-CGCCGAACCAAGAACAATCC-3′) and clpB-R (5′-ACCCTCCTCATAGCCGACAT-3′) (Stabvida). The PCR reaction was prepared with 2 U Taq DNA polymerase (Fermentas), 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Invitrogen), 20 pmol of each primer and DNA of *Mesorhizobium* sp. MAFF 303099. The amplification program used was: 3 min at 95 °C for initial denaturation; 30 cycles of 1 min at 94 °C, 50 s at 62 °C and 2 min at 72 °C and a final extension step at 72 °C during 5 min. The PCR product was purified using the GFX^{TM} PCR DNA or Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. The *clpB* gene fragment was cloned using pGEM-T Easy Vector System (Promega) following the manufacturer's instructions. All RNA probes were obtained by *in vitro* transcription labeling, using DIG Northern Starter Kit (Roche Applied Science). The DNA probe for 16S rRNA was labeled using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science). The 16S rRNA gene PCR amplification was performed using DNA of *M. mediterraneum* Ca36^T, as previously described (Alexandre and Oliveira 2011).

Hybridizations were carried out overnight at 68 °C, after a pre-hybridization period of 30 min at the same temperature. For the 16S rRNA detection, the membranes were re-hybridized overnight at 50 °C with a DNA probe. After hybridization, stringency washes and immunological detection were performed according to the manufacturer's instructions.

Hybridization signals were analyzed using ImageQuantTL^{TM} v7.01 (GE Healthcare). The 16S rRNA signal was used as internal control for the amount of total RNA loaded. To determine the expression levels, the ratio between transcript signals and the corresponding 16S rRNA signals was calculated and the fold difference