Involvement of the glycine-rich RNA-binding proteins (GRP) in osmotic stress response during seed germination: a comparison between GRP 2 and GRP 7

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ABSTRACT

The plant glycine-rich RNA-binding proteins (GRP) are a major class of proteins known to be implicated in plant response to abiotic and biotic stress. Recent studies regarding this type of proteins focused mostly on two of them, namely GRP2 and GRP7, which are mainly involved in osmotic and oxidative plant adaptation mechanisms. The main purpose of this research was to assess the involvement of these two glycine-rich RNA-binding proteins (GRP2 and GRP7) in seed germination capacity under drought stress conditions using the model plant *Arabidopsis thaliana*. The plant material was composed out of seed batches obtained from the T-DNA insertion *Arabidopsis thaliana* mutant lines labelled grp2 (knock-out mutant for the glycine-rich RNA-binding protein 2), grp7-1 (knock-out mutant for the glycine-rich RNA-binding protein 7), and the ecotype Columbia 0 (Col0), which was used as control. For the germination assay, the control medium ½ MS (Murashige&Skoog) was supplemented with three different concentrations (100mM, 200mM and 300mM) of mannitol. The germination was assessed each day during one week. After this period, the germination rate was estimated. Three independent replicates were used and the statistical analysis, namely the ANOVA test, was made using the Graph Pad Prism software (trial version). We observed a difference between the two mutants: grp2 seeds germinated similarly on the control medium as well as on the medium supplemented with various mannitol concentrations, while grp7-1 seeds showed a poor germination rate on all three mannitol culture mediums. The germination rate of the grp2 seeds was also increased, on all the culture mediums used, in comparison to the control Col0 seeds. In the present research work, we demonstrated that even though the two proteins GRP2 and GRP7 belong to the same family, their implication in the seed germination capacity under drought stress is opposite, one enhancing the germination, while the other is reducing it.

Keywords: *abiotic stress, Arabidopsis thaliana, drought, glycine-rich proteins (GRP).*

INTRODUCTION

The global population is increasing day by day and it is estimated that it will reach 9 billion till the year 2050. This situation will lead to some problems like world hunger due to the increasing of the areas for building residences and lowering the ones for agriculture. There are also the environmental factors which affect more and

more the agricultural production. It is therefore of crucial importance to decrease all causes leading to lower yields of cereals and crop plants.

The main causes that lead to almost 70% loss of the crop plants are produced by abiotic stressors such as salinity, drought, and very high or very low temperatures. These have also increased in the last decade due to the global climate change

problems. Salinity along with drought is the main condition that threatens production (Hossain *et al.*, 2003). In order to economically exploit affected areas, it is important to search for crop plants that will thrive in such soils and increase the yield production (Ndakidemi and Mako, 2009). Temperature stress has also become a major concern for plant scientists around the world due to the already mentioned climate change problems. Temperature stress causes devastating effects on plant development and metabolism, especially because these processes have different optimum temperature ranges in each plant species (Shah *et al.*, 2011).

Living organisms are incapable to control the environmental factors. This aspect leads them to the development of survival strategies. The most important strategies refer to: stress avoidance (when the organisms are capable of moving) and stress tolerance (in case of plants which are organisms lacking the mobility response mechanism). During the large period of evolution plants developed various biochemical, physiological and molecular mechanisms to respond to stress factors. The biochemical and molecular stress respond mechanisms undergo the gene regulation processes (Miranda, 2011).

Gene activation to environmental stimuli plays an important role in plant adaptation to adverse conditions and favours the appearance of specific proteins (Sachs *et al.*, 1986). Plants respond to these tensions through various biochemical and physiological processes like: decreased or increased number of stomata, carbon fixation efficiency and effectiveness of the mechanism of storing light process which intensifies respiration and increases the number of proteins involved in stress tolerance (Shinozaki K and Yamaguchi-Shinozaki K, 2007).

Arabidopsis thaliana is established since the 1940s as a model organism in genetics. The use of this specific plant species is characterized by several advantages: it has a relatively small genome of 125 megabases; whose sequencing was completed in 2000. The genome consists predominantly of coding DNA sequences, has only five pairs of chromosomes ($2n = 10$), of which detailed maps have already been created and it has a short generation cycle of only eight weeks (from the germination of the seed to production of seeds). Due to the similarities of the model plant

with other higher plant species a series of loss of function and gain of function mutants for several genes of interest were created from the three most important *Arabidopsis* ecotypes found in the spontaneous flora: Columbia, Landsberg and Wassilewskija (Boyce *et al.*, 2001). These mutants were produced using the transfer DNA (T-DNA) mutation technique which introduces a known fragment of DNA into the genome of the plant with the help of a bacterium called *Agrobacterium tumefaciens*.

Glycine-rich proteins (GRPs) can be localized in the cell walls of many higher plants and are thought to be part of a group of structural proteins which form the cellular wall along with extensins and proline-rich proteins (Ringli *et al.*, 2001). The glycine-rich proteins are associated with multiple independent physiological processes because of their diverse sub-cellular localization. The expression patterns of GRP genes are both diverse and highly tissue specific (Magioli *et al.*, 2001). A subset of the GRP genes encodes proteins that bind nucleic acids. The *Arabidopsis thaliana* genome encodes eight glycine-rich RNA-binding proteins (GR-RBP1 to GR-RBP8). These proteins are characterized by containing an RNA recognition motif (RRM) or an RNA/DNA-binding cold shock domain (CSD) at the N-terminus and a glycine-rich domain at the C-terminus (Lorković and Barta, 2002). They are thought to play a prominent role in germination and seedling development of *Arabidopsis thaliana* under freezing conditions (temperature lower than 0 degrees) (Kim *et al.*, 2008). However, their normal developmental roles have not been studied in detail.

MATERIALS AND METHODS

In order to accomplish our project aims we examined wild-type *Arabidopsis thaliana* and two mutant knock-out lines.

Plant material: The Col0 (Columbia 0) strain was selected as wild type, as it is the most widely used. The knock-out mutant lines used in this study are labeled grp2 (SALK_048476C – bought from the Nottingham Arabidopsis Stock Center NASC) and grp7-1 (obtained from Prof. Dr. Dorothee Staiger, University of Bielefeld, Germany). These mutants were obtained through the T-DNA insertion technique and are verified knock-out lines for AtGRP2 and AtGRP7 protein.

In vitro germination assays: In order to assess the involvement of two most researched glycine-rich proteins (AtGRP2 and AtGRP7) in plant response to drought (one of the most aggressive abiotic stress factor) we evaluated the germination capacity of the seeds obtained from the wild-type Col0 and the two mutant lines taken into consideration in this study.

The $\frac{1}{2}$ Murashige&Skoog (1962) culture medium containing growth vitamins but not sugar (used as control) was supplemented with three different concentrations of mannitol (substance known to induce osmotic stress to plants) ranging from 100mM to 300mM.

To reduce the infection rate of the biological material (the collected seeds) had to be surface sterilized. The sterilization took place under the following steps: 1 ml of 70% ethanol was added into the Eppendorf tube where the seeds were collected. After a 2 min shake they were centrifuged at a 14000rpm speed for another 2 min. The supernatant was removed and 900 μ l Bleach combined 200 μ l 10% (w/v) Triton X was added. After another centrifugation a three time wash with sterilized water followed. The seeds were left covered with aluminum foil for 2 days in the refrigerator for stratification

In this experiment, we used batches of fifty seeds per line. Even though the UPOV legislation regarding seed germination tests implies the use of batches containing 100 seeds per replicate the motivation of using batches of only 50 seeds per replica was that our study interest was focused more on the evaluation of the impact of the glycine-rich proteins on the germination than the evaluation of the seed germination regarding the culture process. We observed the germination during a 7 days period, counting each day the seeds and calculating the germination rate.

Statistical analysis: We used three independent replicates and we analyzed statistically our results using the Graph Pad Prism v6 software (trial version), namely the ANOVA test.

RESULTS AND DISCUSSION

In order to assess the germination capacity of the seeds collected from the wild-type *Arabidopsis thaliana* (Col0) and the two selected knock-out lines (grp2 and grp7-1) we first investigated the seeds under the microscope to check for

Fig.1. Seeds collected from *Arabidopsis thaliana* plants. A: Seeds originated from the wild type Col0; B: Seeds originated from the grp2 knock-out mutant; C: Seeds originated from the grp7-1 knock-out mutant.

differences in their development and then we set them to germinate on simple $\frac{1}{2}$ MS culture media.

There were no differences observed in regards to the developmental status of the seeds collected from the wild-type Col0 and the two mutant knock out lines (grp2 and grp7-1) (Figure 1).

Even though we could observe from the first days that the grp2 seed had a slightly rapid germination, there were no significant differences observed regarding the germination rate of the mutant lines compared to the wild type Col0. By the end of the experiment all seeds germinated to full percentage (Figure 2 A).

The response of the *Arabidopsis thaliana* mutants and wild type Col0 to drought stress was tested by observing the germination capacity of the seeds on $\frac{1}{2}$ MS culture media supplemented with three different concentrations of mannitol varying among 100mM and 300mM.

None of the seeds started to germinate by day one, still by day two we noticed a slightly difference in the germination capacity of the grp2 line and wild type Col0 compared to the grp7-1 knock-out line. By day four, in the middle of the observation period only 10 grp7-1 seeds had germinated in contrast to almost 25 (50%) Col0 and 30 (60%) grp2 seeds. By the end of the observation time, all of the grp2 seeds were fully germinated contrasting with the results obtained in case of the grp-1 mutant line where only 30 (60%) seeds were capable to fully germinate (Figure 2 B).

By using the second concentration of mannitol, we were able to conclude the fact that the grp2 seeds are more stable in the germination capacity under drought stress compared to the grp7-1 and even the wild-type Col0.

Even though the seeds collected from the mutant line, grp2 showed a slightly influenced germination capacity all the seeds were able to fully germinate by the end of the observation period. In contrast, the seeds obtained from the grp7-1 knock out mutant struggled to germinate under this mannitol concentration, only 20 (40%) seeds being able to fully germinate by the end of the experiment.

The higher mannitol concentration had a negative influence also on the wild-type Col0 seeds. Only half of the seed batch used in each of the replicates tested was able to fully germinate by the end of our observation period (Figure 2 C).

The highest concentration of mannitol used allowed us to draw some important conclusions regarding our study. The results obtained using the 300mM substance concentration, sustained the fact that mannitol had no influence on the germination capacity of the seeds collected from the grp2 knock-out mutant. In contradiction, the germination capacity of the seeds obtained from the grp7-1 knock-out mutant was very much influenced by this high concentration of substance. We could observe that less than 15 (30%) seeds were able to fully germinate by the end of the experiment. In addition, the germination capacity of seeds gathered from the wild-type Col0 was considerably affected by the high concentration of mannitol. The germination rate was of less than 50% represented by the 23 seeds which were able to germinate on every replicate by the end of the observation period (Figure 2 D).

Some studies regarding the involvement of the glycine-rich RNA-binding proteins in abiotic stress conditions were focused before on AtGRP2 and AtGRP7 but mainly on their response to cold temperatures and lesser on their reaction to osmotic stress conditions such as drought and salinity. Our research focused on the germination capacity of some *Arabidopsis thaliana* mutants for these two types of GRPs, namely the knock-out mutant for AtGRP2 and AtGRP7 concluded the fact that these types of proteins are involved in the plant response to abiotic stress conditions.

In 2006 Cao and his group shown that the GRP7 protein is involved in the regulation of abscisic acid and osmotic stress responses. The *grp7-1* knock-out plants for this protein expressed a high sensitivity when exposed to ABA and different concentration of NaCl and mannitol. The seed germination rate was significantly lower on these types of media compared to the control and also the root length was highly affected (Cao *et al.*, 2006).

Two years later in 2008 a research conducted by Kim and coworkers using another *grp7* knock-out line complemented the results obtained by the previously mentioned research group. The results obtained by them suggest the fact that the knock-

out mutants are able to germinate and survive on media with high concentrations of NaCl and mannitol in comparison to the wild type. They also concluded that the overexpressing line had a lower fresh weight comparing to the wild type and knock-out line and that their cotyledons turned brown when maintained for a longer period of time on the stress media (Kim *et al.*, 2008).

The results obtained during this project confirm the affirmation done by Cao and co-workers concerning the germination capacity of the AtGRP7 knock-out mutant.

Still there are contradictions with Kim's provided outcomes regarding the knock-out mutant but this may be due to the fact that they worked with a different mutant line than that used

Fig.2. Graphical representation of the germination capacity of the seeds originated from the wild type *Col0* and the two mutants *grp2* and *grp7-1* *Arabidopsis thaliana* plants. Mean germination is represented by the mean of the seed germination capacity of three independent replicates scored for seven days. The statistical significance is indicated for the $p < 0.05$.

during our research and also due to the different concentrations of mannitol tested.

Kim and his group researched the involvement of the GRP2 protein in response to osmotic stress like salt and drought and in response to ABA. Their results showed that the *grp2* knock-out mutant has a weaker germination capacity than the wild-type and the overexpressing line concluding the fact that the GRP2 protein is involved in seed germination under salt and drought stress conditions but not in the seedling development (Kim *et al.*, 2007).

However, these results were in contrast to ours, which showed an increase germination rate of the *grp2* knock-out mutant in regard to the wild type Col0 on all culture media supplemented with different concentrations of mannitol. These differences may have occurred due to the distinct lines of transgenic seeds used during the research as well as the diverse concentration of inhibitors.

Other authors focused on assessing the role of glycine-rich proteins in abiotic stress response in other plant species than the model plant *Arabidopsis thaliana*. Long and coworkers (2013) demonstrated that the overexpression of a glycine-rich protein found in alfalfa namely MsGRP confers sensitivity to transgenic *Arabidopsis* plants in regard to salt, abscisic acid (ABA) and drought stress. NtRGP-3 a glycine-rich protein from *Nicotiana tabacum* is very homologous to two *Arabidopsis* GRP proteins AtGRP2 and AtGRP4 which affect seed germination under salt and drought stress; NtRGP-3 also is therefore likely to be involved in seed germination and seedling growth due to its expression induced by cold and drought (Chen *et al.*, 2010).

CONCLUSION

The results obtained during this project support the affirmation that even though the two glycine-rich proteins investigated GRP2 and GRP7 belong to the same family of proteins they have different functions in the seed germination process. We could conclude the fact that under drought stress conditions the seed germination process is enhanced through the knock-out of the GRP2 protein in contradiction to the knock-out of protein GRP7 which suppresses the germination process. Take into consideration that our results are in accordance with some of the already published results but also in contradiction with others we can say that it is better to keep investigating these

results further and maybe also using other abiotic stressors like salinity before we state anything for sure. Still these results are an opening pathway in the direction of our present project and in further projects.

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