Heat-resistant protein expression during germination of maize seeds under water stress

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ABSTRACT. Low water availability is one of the factors that limit agricultural crop development, and hence the development of genotypes with increased water stress tolerance is a challenge in plant breeding programs. Heat-resistant proteins have been widely studied, and are reported to participate in various developmental processes and to accumulate in response to stress. This study aimed to evaluate heat-resistant protein expression under water stress conditions during the germination of maize seed inbreed lines differing in their water stress tolerance. Maize seed lines 91 and 64 were soaked in 0, -0.3, -0.6, and -0.9 MPa water potential for 0, 6, 12, 18, and 24 h. Line 91 is considered more water stress-tolerant than line 64. The analysis of heat-resistant protein expression was made by gel electrophoresis and spectrophotometry. In general, higher expression of heat-resistant proteins was observed in seeds from line 64 subjected to shorter soaking periods and lower water potentials. However, in the water stress-tolerant line 91, a higher expression was observed in seeds that were subjected

to -0.3 and -0.6 MPa water potentials. In the absence of water stress, heat-resistant protein expression was reduced with increasing soaking period. Thus, there was a difference in heat-resistant protein expression among the seed lines differing in water stress tolerance. Increased heat-resistant protein expression was observed in seeds from line 91 when subjected to water stress conditions for longer soaking periods.

Key words: *Zea mays*; Late embryogenesis; Protein abundance; Heat shock

INTRODUCTION

Among the various types of abiotic stressors to which plants are subjected, low water availability is one of the stressors that most limits development of agricultural crops (Shao et al., 2008). Development of genotypes with high tolerance to water stress is a challenge in plant breeding programs, since the stress tolerance mechanisms are controlled by a large variety of genes that are expressed at different stages of plant development (Roy et al., 2011).

Water stress tolerance varies according to the crop growth stage. However, it is important to identify genotypes that present mechanisms of water stress tolerance early, which justifies studies of water stress during germination (Thakur and Sharma, 2005). The use of molecular techniques to identify metabolic changes and gene expression, combined with conventional breeding should be employed to develop new cultivars with higher water stress tolerance (Shanker et al., 2014). Although gene expression is an excellent method for studying plant responses under abiotic stress conditions, protein levels are usually not related to the transcript number inside the cell, since several post-transcriptional changes may occur (Timperio et al., 2008). Therefore, proteomic analysis has been a valuable tool in plant response studies.

Under stress conditions, various responses are generated, including changes in expression of certain genes that induce metabolic changes. In this context, heat-resistant proteins such as late embryogenesis abundant (LEA) proteins (Battaglia et al., 2008) and heat-shock proteins (HSP) (Wang et al., 2004) play important roles in these responses, and also protect plants under stress conditions.

Although there is great stability in the electrophoretic heat-resistant protein patterns in some species such as maize (José et al., 2005), gene expression that codes for heat-resistant proteins may vary among cultivars with different levels of water stress tolerance. In a study of two wheat cultivars contrasting in their water stress tolerance, Kaur et al. (2014) found that six of the ten analyzed LEA protein genes were more expressed under water stress conditions in the tolerant cultivar. Ristic et al. (1991) found a HSP band of approximately 45 kDa in leaves from a heat- and water stress-tolerant maize line that was not expressed in the susceptible lines.

Heat-resistant protein expression during seed development has been studied previously. For example, Andrade et al., (2013) observed that there are differences in heat-resistant protein expression patterns in different maize seed lines as well as at different development stages. In addition, protein expression in maize lines that are tolerant to high drying temperatures was detected early by electrophoresis (Andrade et al., 2013).

However, more studies are needed to fully understand the protein levels and the activity of these proteins during germination under stress conditions. This study aimed to evaluate heat-resistant protein expression under water stress conditions during the germination of maize seed lines differing in water stress tolerance.

MATERIAL AND METHODS

The study was conducted at the Central Seed Laboratory, Agriculture Department in Universidade Federal de Lavras (UFLA), in Lavras, MG, Brazil. We used two maize lines, 64 and 91, provided by the maize breeding program of Geneseeds Genetic Resources Ltda. Seeds were produced during the 2011/2012 harvest, in the experimental area of UFLA Agriculture Department. The seeds had previously been classified according to water stress tolerance (Abreu et al., 2014). Line 64 was classified as low tolerant and line 91 classified as tolerant to water stress.

To simulate water stress, polyethylene glycol (PEG 6000) solutions were used at water potentials of 0.0, -0.3, -0.6, and -0.9 MPa, in which 0.0 MPa corresponds to the control treatment. Determination of the amount of PEG 6000 was performed according to the equation proposed by Michel and Kaufmann (1973). Soaking periods were 0 (dry seeds), 6, 12, 18, and 24 h. The initial water content of the seeds (12.5%) was determined following the rules for seed analysis (Brasil Ministério da Agricultura, Pecuária e Abastecimento, 2009).

For each treatment, 50 seeds were separated, weighed, distributed on sheets of germitest paper moistened with PEG 6000 solutions, and rolled. To calculate the PEG 6000 solution volume, a ratio equivalent to 2.5 times the mass of the dry germitest paper was used. The rolls were then kept in plastic bags throughout the soaking period to avoid water evaporation and to maintain the desired water potential. Subsequently, the seeds were placed to germinate in a BOD-type germination chamber, set at a 25°C constant temperature.

After soaking, seeds were again weighed and placed in gerbox boxes containing approximately 50 g silica gel in each until 13% water content was reached. The final water content was determined using the formula suggested by Hampton and TeKrony (1995). Finally, embryos from dry seeds were extracted with a scalpel. Thus, two replicates of 12 embryos for each treatment were extracted and stored in a deep freezer at -86°C.

Heat-resistant protein extraction

For heat-resistant protein extraction, 100 mg embryos were macerated in a porcelain mortar kept on ice in the presence of liquid nitrogen. Samples were placed in 1500-µL microtubes. After adding an extraction buffer solution (50 mM Tris-HCl, pH 7.5; 500 mM NaCl; 5 mM $MgCl_2$, and 1 mM PMSF) in the ratio of 1:10 (material weight: volume of extraction buffer), the sample was homogenized using a vortex. The homogenates were then centrifuged at 13,000 *g* for 30 min, at 4°C. The supernatant, which was transferred to a new microtube, was incubated in a water bath at 85°C for 15 min and centrifuged again. The supernatant was transferred into new microtubes and the pellet was discarded.

Expression of heat-resistant proteins by electrophoresis

Seventy microliters of the extract and 40 µL sample buffer (composed by 2.5 mL glycerol, 0.46 g SDS, 20 mg bromophenol blue, adding up to a volume of 20 mL extraction buffer solution) were placed in a boiling water bath for 5 min (Andrade et al.

2013). Subsequently, 50 µL of each sample (extract and sample buffer) were added per well on an SDS-PAGE polyacrylamide gel (12.5% separating gel and 6% concentrating gel). The electrophoresis run was performed at 150 V for 6 h. Gels were stained for 12 h in Coomassie brilliant blue 0.05% (Alfenas, 2006), and destained in 10% acetic acid solution. As a reference standard, we used BenchMark™ Protein ladder, Novex, by Life Technologies, Carlsbad, CA, USA, with 220, 260, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kDa calibration standards. The polyacrylamide gel bands were analyzed using a standard protein as a reference, based to band intensity variation, and band presence or absence, following the methods of José et al. (2005).

Quantification of heat-resistant proteins by spectrophotometry

The determination of heat-resistant proteins was made using a BioTek™ Eon™ Microplate Spectrophotometer, Winooski, VT, USA following the method proposed by Bradford (1976). Readings were made at 595 nm wavelength, using the Gen5 v.2 Data Analysis Software by BioTek. To do so, a calibration curve as made using bovine serum albumin at a concentration of 1 mg/mL. Based on this standard curve, the total quantifications of heat-resistant proteins from the other samples were made. Triplicate for each of the two biological replicates were used. For the heat-resistant protein extraction, the methodology described by Andrade et al. (2013) was used, with the exception of the addition of sample buffer. For the readings, 200 µL Coomassie Blue G-250 reagent and 10 µL extracted sample were used. Measurement results are expressed in mg/g seed embryo. The results were analyzed using a completely randomized design in a 4 x 5 factorial arrangement (four water potentials and five soaking times). The data analysis of variance was performed using the statistical program Sisvar (Ferreira, 2011), at 5% probability by the F-test. The comparison of means was performed using the Scott-Knott test, at 5%.

RESULTS

In line 64, which was classified as having a low water stress-resistance, the highest expression of heat-resistant proteins was found in the non-soaked seeds (0 h; Figure 1A). Under this condition, 107 mg/g heat-resistant proteins were observed. The protein expression in the control seeds (0 MPa), varied based on soaking time. In particular, a decreased expression was observed in seeds soaked for 12 h compared to both shorter and longer soaking times.

In the -0.3 MPa water potential treatment, there was an observed reduction in protein expression following 12 and 24 h of soaking. In contrast, in seeds subjected to -0.6 MPa water potential, a reduction was observed following 12 h of soaking. However, for seeds subjected to -0.9 MPa water potential, no differences were observed in protein expression regardless of soaking time and, in general, smaller amounts of protein were detected than those in the other water potential treatments.

In the line 64 zymograph (Figure 2A), no band of approximately 60 kDa molecular weight was observed in dry seeds (2A). In general, a higher expression of heat-resistant proteins could be observed in seeds subjected to 0 and -0.3 MPa water potentials. In the control treatment, higher intensity bands of 60 and 40 kDa molecular weight were observed after 6 h of soaking.

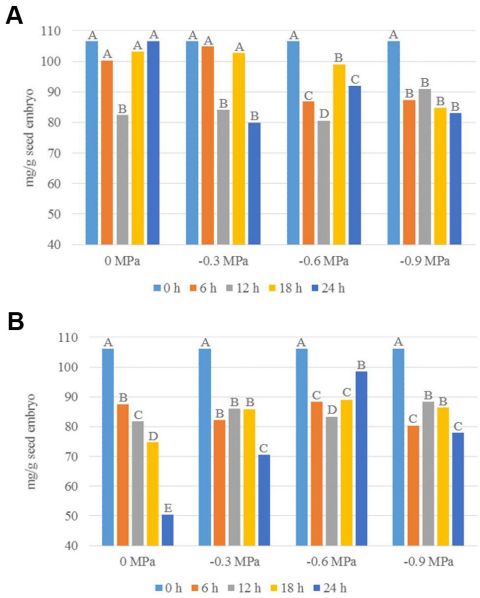

Figure 1. Quantification (mg/g seed embryo) of lines 64 (**A**) and 91 (**B**) heat-resistant proteins in maize seed embryos submitted to 0, -0.3, -0.6, and -0.9 MPa water potentials during 0, 6, 12, 18, and 24 soaking hours. Means followed by the same letter were grouped by the Scott-Knott test ($P < 0.05$).

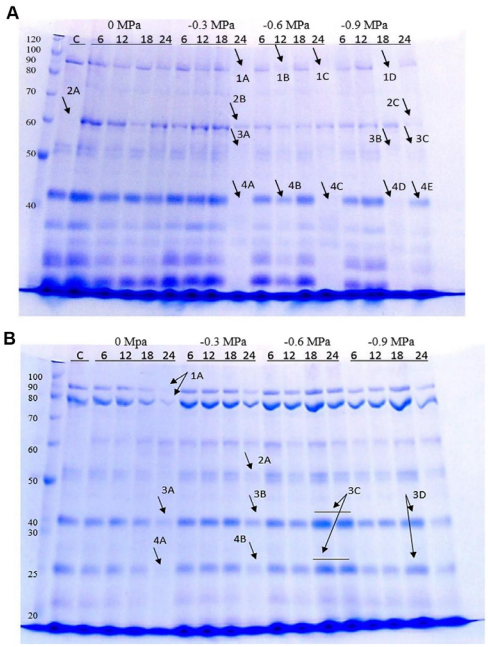

Figure 2. Patterns of lines 64 (**A**) and 91 (**B**) heat-resistant proteins, extracted from maize seeds embryos submitted to 0, -0.3, -0.6, and -0.9 MPa water potentials during 0, 6, 12, 18 and 24 soaking hours. Arrows correspond to specific isoforms.

In seeds subjected to -0.3 MPa water potential, a similar pattern of protein expression was observed after 6, 12, and 18 h of soaking. However, following 24 h of soaking, a suppression in bands of 90, 50, and 40 kDa (1A, 3A, and 4A) was observed, in addition to a reduction in intensity of the 60 kDa band (2B). In seeds subjected to soaking under more negative potentials, a general reduction in heat-resistant protein expression was observed. In seeds subjected to -0.6 MPa water potential, this reduction can be seen in bands with 90 kDa molecular weight following 12 and 24 h of soaking (1B and 1C), and 40 kDa after 12 h of soaking (4B). The band of approximately 40-kDa molecular weight is also absent in seeds soaked for 24 hours (4C). Following the -0.9-MPa water potential treatment, a reduction in protein expression for bands of 90- and 50-kDa molecular weights was observed following 18 h of soaking (1D and 3B), and for bands of 60, 50, and 40 kDa, following 24 h of soaking (2C, 3C, and 4E). The 40-kDa molecular weight band was also absent in seeds subjected to 18 h of soaking (4D). This corresponds well to the smaller amounts of heat-resistant proteins observed for this water potential (Figure 2A).

Line 91, which is considered water stress-tolerant, higher levels of heat-resistant proteins in dry seeds were observed (Figure 1B). In the control treatment (0 MPa), a gradual reduction in the protein expression was observed as a function of soaking time. In the -0.3 MPa water potential treatment, no significant differences among the 6, 12, and 18 h soaking times were observed. However, following 24 h of soaking, a reduction of protein expression was found. In the analysis of seeds subjected to the -0.6 MPa water potential, the lowest protein expression was observed following 12 h of soaking. In the -0.9 MPa water potential treatment, lower expression was observed in seeds soaked for 6 and 24 h. For the remaining soaking periods, no significant differences were observed.

The heat-resistant protein expression in line 91 showed expression patterns that differed from those observed in line 64 (Figure 2B). In seeds soaked in water (0 MPa), there was a reduction in band intensity with increased soaking period, which was also observed in the spectrophotometry quantification (Figure 1B). In seeds soaked for 24 h, this reduction was evident in bands of 90-, 80-, 40-, and 25-kDa molecular weight (1A, 3A, and 4A). In seeds subjected to -0.3 MPa water potential, the highest heat-resistant protein expression was found in seeds soaked for 6, 12, and 18 h. However, when seeds were soaked for 24 h, a reduced intensity was seen in the 50-, 40-, and 25-kDa molecular weight bands (2A, 3B, and 4B), indicating lower expression in seeds subjected to this treatment. In the -0.6 MPa water potential treatment, a higher expression was observed following the longer soaking periods (18 and 24 h). This agrees with the results obtained using spectrophotometry. Under this condition, there was an increase of band intensity at 40- and 25-kDa molecular weights (3C). Greater expression can also be observed in seeds subjected to -0.9 MPa water potential for 18 h (3D).

DISCUSSION

Heat-resistant proteins have been extensively studied and are considered to participate in various developmental processes and to be accumulated during stress responses (Battaglia and Covarrubias, 2013). Although these proteins are stable in maize seeds (José et al., 2005), they have been suggested to participate in various plant development processes related to water stress.

In this study, using spectrophotometry techniques, higher quantities of heat-resistant proteins were observed in dry seeds in both lines 64 and 91. In general, a lower protein expression was observed in line 64, which was classified as low water stress-tolerant, after

soaking at various water stress potentials, especially in the most negative potentials. The same trend was observed for protein expression assessed using electrophoresis techniques.

Small polymorphisms for heat-resistant proteins were found by Abreu et al. (2014), when studying heat-resistant protein extraction in protruded seeds under water stress conditions. Albuquerque et al. (2009) also found a reduction in band intensity of heat-resistant protein in seeds soaked for a longer time, near the root protrusion.

In line 91, which is considered water stress-tolerant, a gradual reduction of heat-resistant proteins with soaking time was observed both using the electrophoretic and spectrophotometric techniques. This was expected, since the maximal expression of LEA proteins in seeds occurs at drying after maturation and is reduced progressively at the beginning of soaking and subsequent germination. In contrast to line 64, a higher protein expression was observed in line 91 when the seeds were subjected to different levels of water stress, as shown in the electrophoresis results. This same trend was observed by Kaur et al. (2014), who observed that six of ten LEA protein genes studied were more expressed in tolerant wheat cultivars when subjected to water stress. In line 91, it was observed the presence of highlighted lower molecular weight bands after 18 and 24 h of soaking in -0.6 MPa water potential and 18 h in -0.9 MPa water potential.

According to Sun et al. (2012), water stress induces heat-resistant protein expression, such as HSP. Ristic et al. (1991) found a HSP band of approximately 45 kDa in maize leaves from a heat and water stress-tolerant line. This same band was not expressed in susceptible lines (Ristic et al., 1991). In line 91, an increase of low molecular weight bands was observed under water stress conditions. This was also observed by Abreu et al. (2014), who used seed root protrusions as a material for protein extraction. In this study, they also found that lines 91 and 64 responded to water stress conditions with an increase and decrease in catalase enzyme activity, respectively (Abreu et al., 2014). Under prolonged hydration conditions, LEA proteins can act as antioxidant molecules (Caramelo and Iusem, 2009). According to Rosa et al. (2005), high drying temperature tolerance in maize seeds is associated with catalase enzyme activity and increased heat-resistant protein expression.

According to Hong-Bo et al. (2005), water stress induces ABA hormone production, which is considered an important positive regulator for both the induction and dormancy maintenance in soaked seeds (Finch-Savage and Leubner-Metzger, 2006). Through an assessment of maize lines subjected to water stress induced by PEG 6000, Abreu et al. (2014) observed that line 91 had a high physiological quality under favorable germination conditions. However, when subjected to water potentials lower than -0.6 MPa, a reduced germination was observed, which might be due to ABA accumulation (Abreu et al., 2014). Under favorable soaking conditions, LEA protein expression is reduced over the germination progress. However, under water stress conditions, an increase in gene expression of several LEA proteins may occur. Thus, heat-resistant protein expression is dependent on the genotype and the condition under which the germination process occurs.

CONCLUSIONS

We observed a difference in heat-resistant protein expression among different maize lines differing in water stress tolerance. In the absence of water stress, heat-resistant protein expression is reduced with increasing soaking duration. The highest heat-resistant protein expression was observed in line 91, when subjected to water stress for longer soaking periods.

Conflicts of interest

The authors declare no conflict of interest.

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