Clinorotation Affects Induction of the Heat Shock Response in *Arabidopsis thaliana* Seedlings

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ABSTRACT

Clinorotation used to simulate microgravity effects in ground-based experiments is considered as a mild stress factor for plants. We have assumed that it might influence the plant tolerance to other stressful factors. To test this, *Arabidopsis thaliana* seedlings were grown on a horizontal clinostat (2 rpm) or under stationary conditions (control), and then were subjected to heat treatment. The kinetics of gene expression of cytosolic HSP70s and HSP90s during exposure to 37°C for 0.5-2 h was examined by RT-qPCR to estimate level of the heat shock reaction. It was shown that clinorotation caused the minor increase in transcript abundance of five AtHSP70s and AtHSP90-1 under normal temperature, as well as a faster onset and enhancement of their induction during heat shock. The heat tolerance was evaluated as a function of seedling survival after exposure to 45°C for 45 min. Seedlings grown under clinorotation were determined to withstand heat treatment better than seedlings grown under stationary conditions. The obtained data support the assumption that clinorotation may provide

cross-protection of plants against fluctuations in environmental conditions.

INTRODUCTION

Steady growing of plants as an autotrophic link of Life Support Systems is one of the key issues for long-term manned space missions (Fert et al., 2002; Wheeler, 2010). Their ability to cope with spaceflight environments is thus of critical importance. It has been shown that microgravity, an unusual factor for plants, does not prevent their growth and development but causes changes in structural and functional organization of cells, and is considered as a mild stressor (Medina et al., 2011; Kordyum, 2014). However, its influence on the development of adaptive reaction to other unfavorable factors is not well understood. To study effects of reduced gravity in plants in ground-based experiments, we used clinorotation. This study is focused on how clinorotation might influence a conserved reaction of cells to elevated temperatures, known as the heat shock response. A key component of this response is upregulation of heat shock proteins (HSP) (Vierling, 1991; Sørensen et al., 2003; Wang et al., 2004). Functioning as molecular chaperones, they protect and reactivate protein structures affected by stress events.

It has been found that spaceflight factors, including microgravity, activate HSP expression in plant cells (Paul et al., 2005, 2012; Zupanska et al., 2013; Hausmann et al., 2014). In our previous work, some temporary increase in the

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HSP70 and HSP90 levels in pea seedlings for the first hours of clinorotation was determined (Kozeko and Kordyum, 2007). In addition, seedlings grown under permanent rotation showed a perceptible increase in HSP induction in response to high temperatures, when compared to seedlings grown motionlessly (Kozeko, 2008).

Numerous investigations have confirmed the importance of HSPs in plant tolerance to heat, as well as to a range of other stressful factors (Vierling, 1991; Sørensen *et al.*, 2003; Wang *et al.*, 2004). Moreover, their involvement in cross-protection between different types of stress has been examined (Banti *et al.*, 2008; Montero-Barrientos *et al.*, 2010).

In the present work, we have studied the influence of clinorotation on gene expression of HSP70 and HSP90 during the following heat shock treatment, as well as thermotolerance in *Arabidopsis thaliana* seedlings. HSP70s are considered as the principal stress-induced HSP family in many species (Sørensen *et al.*, 2003). HSP90s also respond to damaging factors (Miloni and Hatzopoulos, 1997; Wang *et al.*, 2004). In *A. thaliana*, there are five homologues of HSP70s (DnaK subfamily) and four homologues of HSP90s that are localized to the cytosol. Among these AtHSP70-4, AtHSP70-5, and AtHSP90-1 are highly induced by heat (Miloni and Hatzopoulos, 1997; Lin *et al.*, 2001; Sung *et al.*, 2001). Refer to Table 1 for gene names and AGI codes.

Table 1. Primers of target genes used for RT-qPCR.

Gene name	AGI code	Primer sequences (5' to 3')
<i>AtHSP70-1</i>	AT5G02500	F: AAACCCTAGCCGCCTTATTC R: GATAGCTGGTCCTTCTCCTTTAC
<i>AtHSP70-2</i>	AT5G02490	F: AGCTTGTGAGAGAGCAAAGAG R: ACGGGTGATTGGAGAATAGA
<i>AtHSP70-3</i>	AT3G09440	F: GACATTAGTGGAACCCGAGAG R: GTCTGAGCCGTAGATGACAAAG
<i>AtHSP70-4</i>	AT3G12580	F: AGGGCACGAACAAAGGACAACAAC R: TCAGCCGACACATTCAGGATACCA
<i>AtHSP70-5</i>	AT1G16030	F: GGAGCTATCTCTGGGCTTAATG R: GGCCTTCGTACCCTTCTTATC
<i>AtHSP90-1</i>	AT5G52640	F: GTTACCCTATCTACCTTTGGACCG R: CTGCTTGTTGATGAGTTCCAC
<i>AtHSP90-2</i> ,	AT5G56030	To detect mRNA of three genes in total: F: GCTACCCAATCTCTCTCTGGATT R: GTACTCCTCCTTGTTGATCTCCTC
<i>AtHSP90-3</i> ,	AT5G56010	
<i>AtHSP90-4</i>	AT5G56000	
<i>AtUBQ5</i>	AT3G62250	F: AACCTTGAGGTTGAATCATCC R: GTCCTTCTTTCTGGTAAACGT

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Heat Treatments

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0) seeds were sterilized with 70% ethanol and hypochlorite solution (3% Cl), washed five times with sterile water, incubated in the dark at 4°C for 2 d, and plated with equal spacing on germination medium (0.5 Murashige

and Skoog mineral salt complex, 1% sucrose, 0.8% agar) in Petri plates. Seeds were allowed to germinate and grow on a horizontal clinostat (2 rpm) or under stationary conditions (control) at $22 \pm 1^\circ\text{C}$, photoperiod of 16 h light/8 h dark, and light intensity $\sim 110 \mu\text{mol m}^{-2}\text{s}^{-1}$. After 12 d of growth, rotated and control seedlings were subjected to heat treatments.

For seedling survival assay, plates with 12-d-old seedlings, grown as described above, were

exposed to 45°C for 45 min. Following the heat treatment, seedlings were returned to the stationary conditions and their survival was recorded for the next 6 d of incubation. The number of viable seedlings that were still green and generated new leaves was quantified to determine the survival rate. This assay was also performed for 5-d-old seedlings.

For analysis of gene expression, 12-d-old seedlings grown, as described above, were exposed at 37°C for 0.5, 1, and 2 h and then frozen at -70°C. Three independent experiments were performed for each experimental condition.

RT-qPCR

Total RNA was extracted from seedlings using innuPREP Plant RNA Kit (Analytik Jena). 1 µg of each sample was reverse transcribed into cDNA using RevertAid First Strand cDNA synthesis Kit (ThermoSci) with oligo(dT)18 primer. Real-time amplification was performed using the Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific) and the iQ5 amplifier (Bio-Rad). The following PCR program was employed: 95°C for 2 min, then 35 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 40 s. *UBQ5* was used as the reference gene for all assays. Primer pairs, as outlined in Table 1, were designed using IDT PrimerQuest Tool (<https://eu.idtdna.com/Primerquest/Home/Index>). The conserved sequences of highly homologous *AtHSP90-2*, *AtHSP90-3*, and *AtHSP90-4* were used to design the common primers for them. Specificity of each primer pair and quality of RT-qPCR products was checked by melting curve analysis and agarose gel electrophoresis. Quantity of each transcript was measured in three independent biological samples with three analytical replicates. *UBQ5* expression was used to normalize the transcript levels in each sample. Relative expression of each gene was calculated by the comparative $\Delta\Delta C_t$ method relatively to the transcript level of the control at 22°C.

Data Analysis and Statistics

For data analysis, the mean and standard deviation were calculated for each group. A two-way ANOVA was used to determine significant differences for the effects of clinostat rotation and high temperature on transcript levels ($p < 0.05$). For the seedling survival and effect of clinostat rotation on *HSP* expression at normal temperature, the means for the control

and experimental groups were compared using Student's t-test ($p < 0.05$).

RESULTS

In order to test whether clinorotation influences the heat shock response, seedlings grown under stationary conditions (control) or clinostat rotation for 12 d were exposed to 37°C for 0.5-2 h. Levels of mRNAs of cytosolic HSP70s and HSP90s in seedlings were quantified using RT-qPCR. We used one set of primers for *AtHSP90-2*, *AtHSP90-3*, and *AtHSP90-4*, since their sequences are highly homologous and we could not synthesize specific primers for each gene. In addition, these HSP90s are known to show similar pattern of gene expression at both normal and high temperatures (Miloni and Hatzopoulos, 1997). Therefore, we measured the total mRNA level of these three genes to characterize gene expression of constitutive cytosolic HSP90s.

Figure 1 shows that exposure to 37°C resulted in rapid induction of HSP genes in control and rotated seedlings, but with differences in magnitudes of their expression. The mRNA level of constitutive *AtHSP70-1* steadily increased at a low rate over the duration of heat exposure, and only a tendency to its enhancement after clinorotation was observed. Induction of constitutive *AtHSP70-2*, *AtHSP70-3*, as well as *AtHSP90-2*, *AtHSP90-3*, and *AtHSP90-4* (in total) reached the maximum levels at 1 h of heat treatment, while thereafter the mRNA amounts declined. At the same time, a higher expression rate for *AtHSP70-2* and *AtHSP70-3* and, conversely, a tendency to decline for expression of *HSP90s* were determined in rotated seedlings, when compared to control ones. Inducible *AtHSP70-4*, *AtHSP70-5*, and *AtHSP90-1* showed strong induction by 30 min of heat treatment, its significant increase within the next 30 min and a steady high level for the second hour. After clinorotation, the induction of these genes occurred faster and reached a higher level, especially for *AtHSP70-4* and *AtHSP70-5*.

As illustrated in Figure 2, comparison of the transcript abundance in rotated and control seedlings at $22 \pm 1^\circ\text{C}$ (0 h of heat exposure) showed an increase of 2- to 7-fold under clinorotation, with the exception of constitutive HSP90s.

The heat tolerance of seedlings was evaluated as a function of their survival after an

Figure 1. An effect of clinostat rotation on induction of *HSP70* and *HSP90* genes in *Arabidopsis thaliana* (Col) seedlings during heat shock. 12-d-old seedlings grown under stationary conditions (control) or clinorotation were exposed at 37°C for the indicated times. Expression levels were assessed using RT-qPCR, and normalized with respect to *UBQ5* mRNA. Relative mRNA amounts were calculated as a fold change to the control (= 0 h of the heat treatment). Data are means of three independent biological samples with three analytical replicates $\pm$ s.d. The effect of high temperature was significant for all the HSPs; the effect of clinorotation was significant for *AtHSP70-2*, *AtHSP70-3*, *AtHSP70-4*, *AtHSP70-5*, and *AtHSP90-1* (Two-way ANOVA, $p < 0.05$).

Figure 2. An effect of clinostat rotation on expression of *HSP70* and *HSP90* genes in *Arabidopsis thaliana* (Col) seedlings. The bars present the fold change of transcript abundance in 12-d-old seedlings grown under clinorotation relative to the control (seedlings grown under stationary conditions, = 1; dotted line). *HSP90 c* represents *AtHSP90-2*, *AtHSP90-3*, and *AtHSP90-4* (in total). Bars denoted with an asterisk (*) indicate differences between the rotated and control seedlings that are statistically significant, compared with the control (Student's t-test, $p < 0.05$). Expression levels were assessed using RT-qPCR and normalized with respect to *UBQ5* mRNA. Data are means of three independent biological samples with three analytical replicates $\pm$ s.d.

Figure 3. An effect of clinostat rotation on heat shock survival of *Arabidopsis thaliana* (Col) seedlings. 12-d-old seedlings grown under stationary conditions (control) (A, B) or clinorotation (C, D) at $22 \pm 1^\circ\text{C}$ were exposed at 45°C for 45 min and recovered under the stationary conditions. (A, C) seedlings before the heat treatment and (B, D) seedlings after a 6-d recovery period (B, D). (E) Survival rate of the seedlings after the heat treatment. Data are means $\pm$ s.d. ($n = 3$ plates with ~ 25 seedlings each; * Student's t-test, $p < 0.05$).

exposure to 45°C for 45 min. This temperature is specified as a standard to detect alterations in *A. thaliana* plant thermotolerance (Silva-Correia et al., 2014). Figure 3 depicts that different

survival rates were observed in heat-treated seedlings grown under clinorotation compared with stationary conditions. A portion of rotated seedlings were able to generate new leaves over

the 6-d post-exposure period, whereas control seedlings showed almost complete death.

Similar results were obtained with 5-d-old seedlings as illustrated in Figure 4.

Figure 4. An effect of clinostat rotation on heat shock survival of *Arabidopsis thaliana* (Col) seedlings. (A) 5-d-old seedlings grown in the stationary conditions or under clinostat rotation at $22 \pm 1^\circ\text{C}$ were exposed at 45°C for 45 min, and recovered under the stationary conditions for 3 d. (B) Survival rate of the seedlings after the heat treatment. Data are means $\pm$ s.d. ($n = 3$; * Student's t-test, $p < 0.05$).

DISCUSSION

HSPs/chaperones play a role in protecting cells against multiple stressful conditions (Sørensen *et al.*, 2003; Wang *et al.*, 2004). Their upregulation during the heat shock response provides resumption of protein homeostasis and leads to a higher level of thermotolerance (Vierling, 1991). Moreover, an increasing number of studies demonstrate that HSPs contribute to cross-protection between heat stress and other types of stress in plants (Banti *et al.*, 2008; Montero-Barrientos *et al.*, 2010). In this study, we applied heat treatment following clinorotation of seedlings. As previously mentioned, both factors can activate HSP synthesis. The obtained results showed that clinorotation caused the minor increase in transcript abundance of five cytosolic AtHSP70s and AtHSP90-1 under normal temperature, as well as a faster onset and enhancement of their induction during heat shock. Notably, the strongest effect was revealed for inducible *AtHSP70-4*, *AtHSP70-5*, and *AtHSP90-1*, which are especially important for stress tolerance (Sørensen *et al.*, 2003). In contrast, the total expression of constitutive *AtHSP90-2*, *AtHSP90-3*, and *AtHSP90-4* was not altered under clinorotation and showed less induction by heat treatment in rotated seedlings, when compared to seedlings grown under stationary conditions.

A weak increase in the expression levels of several HSPs in seedlings grown under clinorotation at normal temperature likely reflects adaptation of cells to rotation. It is noteworthy that clinorotation may have multiple impacts on organisms, including not only simulation of the microgravity effects, but also mechanical and electromagnetic influences, etc. However, in our previous work with pea seedlings, comparison of the effects of horizontal and vertical rotation showed small but statistically significant differences in HSP70 and HSP90 levels that could be attributed to simulated microgravity (Kozeko and Kordyum, 2006).

As a whole, the time course of HSP expression during heat exposure was in good agreement with the previous findings by other researchers (Miloni and Hatzopoulos, 1997; Sung *et al.*, 2001). At that, the magnitudes of *AtHSP70s* and *AtHSP90-1* induction were higher in rotated seedlings relative to control ones. These findings correlate with our previous

Western-blot data indicating enhanced induction of HSP70 and HSP90 in pea seedlings in response to high temperatures (38–45°C) after long-term clinorotation (Kozeko, 2008). Thus, increasing the heat shock response under the influence of clinorotation was shown at both transcriptional and translational levels. However, the obtained results could indicate two opposite situations. On the one hand, the higher inducibility of several HSPs may reflect higher sensitivity of rotated seedlings to heat shock; on the other hand, it may result from pre-adaptation of seedlings under clinorotation. To test that, the seedling survival assay was carried out. Its results showed that seedlings grown under clinorotation withstood exposure to the near lethal temperature better than seedlings grown under stationary conditions. These data support the assumption that clinorotation may provide cross-protection of plants against fluctuations in environmental conditions, which correlates with the data on HSP expression.

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