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The HSP70 chaperone machines of *Chlamydomonas* are induced by cold stress

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ABSTRACT

The responses of *Chlamydomonas reinhardtii* cells to low temperatures have not been extensively studied compared with other stresses. Like other organisms, this green alga has heat shock protein 70s (HSP70s) that are located in chloroplast, mitochondrion and cytosol. To test whether temperature downshifts affected HSP70s synthesis, we used real-time PCR and protein gel blot analysis. *C. reinhardtii* cells exposed to cold stress show increased HSP70s mRNA levels. Genes encoding other components of HSP70 chaperone machines (e.g. CGE1, CDJ1, HSP90C and HSP90A) are also up-regulated in response to decreased temperature. We demonstrated that the accumulation of all analyzed mRNA occur more slowly and with reduced amplitude in cells exposed to cold than in cells treated with heat. Furthermore, *C. reinhardtii* cells display the splicing of the CGE1 transcript that was dependent on low temperature. Finally, the transcription regulator of *C. reinhardtii* HSF1 is also cold-responsive, suggesting its role in the transcriptional regulation of HSP genes at low temperature.

1. Introduction

Variations in the surrounding temperature are the most common stress for plants. Low temperature constitutes a key factor affecting plant growth and development. Plants have evolved a mechanism to enhance tolerance to freezing during exposure to periods of low temperatures. This process is known as cold acclimation (Thomashow, 1999). The plants can orchestrate production of osmolytes and antifreezing proteins, synthesis of enzymes to reduce ROS concentrations, and mechanisms that protect cellular membranes (Kaplan et al., 2004; Lissarre et al., 2010; Miura and Furumoto, 2013; Tarkowski and van den Ende, 2015). Moreover, heat shock proteins (HSPs) have also been shown to accumulate in response to low temperature (Timperio et al., 2008; Renaut et al., 2006). To reveal more fundamental components of the cold acclimation in plants it is valuable to study the process in plant models that are evolutionarily apart. As analysis of the regulation of cold acclimation process in the green lineage has yet mostly been performed on higher plants (Chinnusamy et al., 2007; Zhu et al., 2007; Lissarre et al., 2010), we have carried out a study on the unicellular green alga *Chlamydomonas reinhardtii*.

The response of C. *reinhardtii* to sudden temperature downshifts has not been extensively studied compared with heat stress conditions (Schroda et al., 2015). Physiologically, the cold shock response of *C. reinhardtii* manifests itself as an acclimation phase during which cell division completely stops immediately after the temperature downshift (Lapina et al., 2013). In cold-treated *C. reinhardtii* cells, gluconeogenesis and starch biosynthesis pathways are activated leading to a pronounced starch and sugar accumulation (Valledor et al., 2013). During cold acclimation phase of many prokaryotes and eukaryotes, several cold shock proteins, which help the cells to adopt to low temperature, are produced (Nakaminami et al., 2006, 2009; Sasaki et al., 2007; Phadtare and Severinov, 2010). In contrast to bacteria and higher plants, *Chlamydomonas* contains a single cold shock domain protein termed NAB1 that appeared to be not involved in low temperature adaptation (Mussgnug et al., 2005). This implies that the cold-specific early response is mediated by a different and as yet uncharacterized regulatory mechanism.

In all organisms the molecular basis of the response to many different stresses includes rapid accumulation of heat shock proteins (HSPs). Moreover, HSPs have also been shown to accumulate in response to low temperature (Timperio et al., 2008; Renaut et al., 2006). HSP70s family comprises one subset of the best-studied chaperones in *Chlamydomonas*. These proteins function in all major subcellular compartments of the cell, and the HSP70B, HSP70C and HSP70A are targeted to the chloroplast, mitochondrion and cytosol, respectively (Drzymalla et al., 1996; Harris, 2008). Numerous

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studies have elucidated HSP70 chaperone functions under heat stress conditions and in protein metabolism of the alga (Schroda et al., 2015). Our intention was to characterize the expression of *Chlamydomonas* HSP70 chaperone machines in response to low temperature treatment. Furthermore, we found indications that the heat shock factor 1 (HSF1), is also cold-inducible. Our data further support the idea that HSPs may have important roles during cold acclimation of plants.

2. Material and methods

2.1. Culture conditions

The wall-less strain cw15-325 (cw15*arg7-8*) used in all experiments was kind gift from Prof. M. Schroda (University of Kaiserslautern, Germany). Algae were grown photoheterotrophically in Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) supplemented with 50 mg L^{-1} of arginine at a light intensity of 45 μE m^{-2} s^{-1} at 22 °C

2.2. Cold and heat treatments

Based on a preliminary growth inhibition test with a range of temperatures, 7 °C was selected as exposure cold temperature. For cold stress experiments, algae grown at constant illumination at 22 °C to exponential phase were harvested by centrifugation, resuspended in TAP medium precooled to 7 °C, and incubated at the same temperature for different periods. The cells were analyzed during a 10 points time course from 0 h to 96 h aiming to monitor both short (1–8 h) and long term (16–96 h) responses. Control cells were cultivated at constant illumination at 22 °C.

Heat shock treatments were conducted by rapidly shifting cultures from growth conditions to a water bath at 40 °C.

At each harvesting time the cells were observed under a differential interference contrast (DIC) (microscope Leica DRMXA, 100× objective, camera Leica DC500) without fixation. The number of cells was recorded with use of a counting chamber and the viable cells were estimated microscopically with use of 0.05% (v/v) Evans blue (DIA-M, Russia) as described earlier (Zalutskaya et al., 2015). The fresh weight was estimated gravimetrically.

2.3. Protein and chlorophyll content

Cell culture samples (2–4 10^6 cells mL^{-1} in 10 mL) were harvested by centrifugation, the supernatant was discarded, and the pellet was resuspended in 0.1 M DTT, 0.1 M Na_2CO_3. Protein content was determined using the method described by Popov et al. (1975). Chlorophyll was extracted from whole cells with acetone. 0.6 mL of culture was centrifuged and the pellet resuspended in 83% acetone to extract pigments. Cellular debris was pelleted by centrifugation and chlorophyll a and b levels were determined spectrophotometrically (SmartSpec Plus, BioRad) in the supernatant, by measuring optical absorbance at 645 nm and 663 nm. Calculations of total chlorophyll (μg mL^{-1}) were performed as previously described (Harris, 2008).

2.4. Real-time PCR

Total RNA was isolated from algal cells as described by Ermilova et al. (2010). Protocols for complementary DNA synthesis and for real-time PCR were described previously (Zalutskaya et al., 2015). Gene-specific primers are listed in Table 1. All PCR reactions were performed using the CFX96 Real-Time PCR Detection System (Bio-Rad) and SYBR Green I. Abundance of transcript of each analyzed gene was normalized to the endogenous reference gene *RACK1* (GenBank accession number: XM_001698013.1). Fold difference was calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Values were obtained from at least two biological replicates; each replicate was analyzed three times.

2.5. Protein gel blot analysis

The protein concentration was determined by staining with amido black, using BSA as a standard (Popov et al., 1975). SDS-PAGE was performed using a protocol of Laemmli (Laemmli, 1970). Gel blot analysis was performed as described previously by Ermilova and co-workers (Ermilova et al., 2013). The dilutions of the primary antibodies used were as follows: 1:2000 anti-HSP70B, 1:5000 anti-CrPII, 1:6000 anti-CGE1, 1:5000 anti HSP90C and 1:3000 anti-HSF1. A 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit serum (Sigma) was used as a secondary antibody. The peroxidase activity was detected by an enhanced chemiluminescence assay (Roche).

3. Results

3.1. Expression of HSP70B is inducible by cold-shock

The minimal temperature for continued growth of *C. reinhardtii* cw15-325 averaged out 15–14 °C, depending on media conditions. Shift from the normal growth range to temperatures below 14 °C results to arrest of cell division (Fig. 1A). However, lethal low temperatures for *C. reinhardtii* averaged out below 0 °C. At 22 °C, cells had a mean diameter of 8.3 μm, which increased to 10.8 μm during the 96 h cold treatment (Fig. 1C). The average chlorophyll content per cell was 3.1 pg. It increased by 22% to 3.8 pg during 96 h of incubation. Additionally, after 72 h of cold treatment the fresh weight and protein content slightly increased (Fig. 1B, insert). Hence, while cell division was arrested during cold stress, cells continued to grow at a slow rate. Therefore, to some extent, *C. reinhardtii* uses molecular mechanisms to cope with temperature from 14 to 0 °C resulting in viable but not dividing cells. We chose 7 °C because this temperature was sufficient to block the cell division, yet this temperature was not to inhibit cell viability of cw15-325 strain.

To test if HSP70B is controlled by cold-shock at the transcriptional level, *Chlamydomonas* cells were grown on TAP medium in the light for 72 h at 22 °C and then exposed to 7 °C. Levels of *HSP70B* mRNA were induced after only 3 h of cold treatment and increased to maximal levels within 16–24 h of exposure to cold (Fig. 2A). The highest level, 4.9 times that of the control, was achieved at 16 h. After 24 h of cold treatment, *HSP70B* transcript level decreased again approximately 1.4–1.8 times higher than the control level. For a comparison, the kinetics of *HSP70B* mRNA accumulation at 40 °C is shown (Fig. 2A, insert). The transcript level rose rapidly to approximately 13 times that of the control after 30 min and decreased markedly after 2 h. The transient nature of *HSP70B* mRNA accumulation in response to cold in *C. reinhardtii* is similar to that seen with some members of HSPs in *S. cerevisiae* and higher plants, maximum expression is achieved after about 4–24 h of treatment and decreased back to control (Aguilera et al., 2007; Sung et al., 2001; Cao et al., 2012).

A temperature downshift resulted in an increased abundance of HSP70B protein as detected with use of protein gel blot analysis (Fig. 2B, upper row). As a control, the levels of CrPII (Fig. 2B, lower row) were unaltered. The data suggest that the *HSP70B* gene is induced by cold treatment. Moreover, the response of *HSP70B* to low temperature was much slower than its response to heat-shock temperature.