学位論文

Study of molecular mechanisms regulating anhydrobiosis in a tardigrade, *Hypsibius dujardini*.

（ ヤマクマムシの乾眠誘導機構の研究　）

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Abstract

Upon desiccation, some tardigrades enter an ametabolic dehydrated state called anhydrobiosis and can survive a desiccated environment in this state. For successful transition to anhydrobiosis, some anhydrobiotic tardigrades require pre-incubation under high humidity conditions, a process called preconditioning, prior to exposure to severe desiccation. Although these tardigrades are thought to sense mild dehydration and prepare for transition to anhydrobiosis during preconditioning, the molecular mechanisms governing such processes remain unknown. In this doctoral study, I investigated molecular mechanisms regulating anhydrobiosis, using an anhydrobiotic tardigrade, *Hypsibius dujardini*, which requires preconditioning to enter anhydrobiosis. In Chapter 1, I used a chemical genetic approach to investigate the signaling pathways required for successful transition to anhydrobiosis in *H. dujardini*. I first demonstrated that inhibition of transcription or translation drastically impaired anhydrobiotic survival, suggesting that *de novo* gene expression is required for successful transition to anhydrobiosis in this tardigrade. I then screened 81 chemicals and identified 5 chemicals that significantly impaired anhydrobiotic survival after severe desiccation, in contrast to little or no effect on survival after high humidity exposure only. In particular, cantharidic acid, a selective inhibitor against protein phosphatase (PP) 1 and PP2A, exhibited the most profound inhibitory effects. Two other PP1/PP2A inhibitors also significantly and specifically impaired anhydrobiotic survival, suggesting that PP1/PP2A activity plays an important role for anhydrobiosis in this species. The identified inhibitory chemicals could provide novel clues to elucidate the regulatory mechanisms underlying anhydrobiosis in tardigrades requiring preconditioning. In Chapter 2, I investigated transcriptional response during preconditioning in *H. dujardini* using RNA-sequencing. I identified 146 upregulated genes during preconditioning and classified these genes as primary and secondary response genes based on the sensitivity to the treatment with a translation inhibitor. Using qRT-PCR, I verified consistent upregulations of

four primary response genes even when *de novo* translation was inhibited by inhibitor treatment. Pre-treatment with cantharidic acid inhibited the upregulation of these primary response genes. This result indicates that PP1/PP2A is involved in the signaling of desiccation stimuli to the initial transcriptional response. This is, to my knowledge, the first report suggesting the involvement of PP1/PP2A activity in transcriptional response to desiccation stress in a tardigrade. This study opens avenues leading to the molecular understanding of the mechanisms regulating transition to anhydrobiosis in the tardigrade.

Contents

Abbreviations

2-APB ……...2-aminoethyl diphenylborinate

MNS ……3,4-Methylenedioxy-β-nitrostyrene

CA …….cantharidic acid

CAHS ……....Cytoplasmic Abundant Heat Soluble

FPKM ……Fragments Per Kilobase of exon model per million Mapped reads

MAHS ……...Mitochondrial Abundant Heat Soluble

PP1/PP2A ……..protein phosphatase 1 and protein phosphatase 2A

RH …….relative humidity

SAHS ……Secretory Abundant Heat Soluble

DMSO ……dimethyl sulfoxide

stMQ…….....sterilized Milli-Q

General Introduction

Through evolutions, organisms have acquired suitable traits to adapt to new environments. A large-scale example in evolutionary history is the transition to land from sea, where life is believed to have originated. To colonize the land, several animal lineages are thought to have independently acquired protective mechanisms against desiccation [Stabelli et al. 2013]. To avoid deleterious water loss, most animals utilize their mobility to escape from a desiccated environment, and retain their body water by the proper intake of water and the prevention of surface water evaporation [Dingle 1996; Machin 1967]. In contrast, some small animals such as tardigrades and nematodes, whose mobility is limited and whose large surface/volume ratio enhances evaporation, have adapted to tolerate a loss of body water to withstand a desiccated environment [Watanabe 2006]. When encountering desiccation, these animals lose water and enter a metabolically inactive dehydrated state referred to as anhydrobiosis, and resume their metabolic activity upon rehydration.

Tardigrades are tiny animals comprising the phylum Tardigrada, which comprises more than 1000 species [Degma et al. 2015]. All tardigrades are principally aquatic and require surrounding water to grow and reproduce, though some species have anhydrobiotic abilities. When desiccated, anhydrobiotic tardigrades contract their bodies longitudinally with the loss of body water, to form a compact shape called a tun, and are able to tolerate almost complete dehydration (Fig. 1A) [Møbjerg et al. 2011]. Upon rehydration, dehydrated tardigrades in tun formation appear to absorb water gradually and start to move a head and legs within approximately 1 hour.

The water content in desiccated tardigrades depends on the surrounding humidity. Exposure to low humidity conditions causes a severe decrease in the water content of tardigrades. Some anhydrobiotic tardigrades, typically known as xerophilic species (species inhabiting an environment with low water availability), can tolerate immediate exposure to low

humidity conditions from a fully hydrated state. On the other hand, most other anhydrobiotic tardigrades inhabiting moist environments cannot tolerate such drastic dehydration and require pre-exposure to high-humidity conditions where only mild dehydration occurs for several hours or days prior to severe dehydration in a low humidity environment. This process is referred to as preconditioning. The requirement of preconditioning to withstand severe dehydration depends on tardigrade species and is well correlated with the water availability of their habitats [Wright 1991]. Therefore I hypothesized that the habitable environments of anhydrobiotic tardigrades may depend on their anhydrobiotic styles i.e., whether they require preconditioning to enter anhydrobiosis or not. Accordingly, comparison of the molecular mechanisms regulating anhydrobiosis between preconditioning-requiring tardigrades and non-requiring tardigrades will give us the insight into how organisms extend their habitats.

The tardigrade *Ramazzottius varieornatus* which inhabits easy to dry moss such as *Bryum argenteum* can withstand severe desiccation without preconditioning (Fig. 1B) [Horikawa et al. 2008]. The mean life span of this tardigrade under rearing conditions is about 35 days and an adult produces totally 7 to 8 eggs on average [Horikawa et al. 2008]. *R. varieornatus* has an anhydrobiotic ability at all developmental stages [Horikawa et al. 2008]. Our previous transcriptomic analysis revealed that *R. varieornatus* exhibits few changes in the gene expression profile during dehydration, suggesting that this species constitutively synthesizes the protective molecules required for anhydrobiosis, at least at the transcriptional level (unpublished data). In contrast, the tardigrade *Hypsibius dujardini* which inhabits humid environment such as benthic sediments in a pond requires preconditioning to tolerate severe dehydration (Fig. 1B) [Wright 1989; Gabriel et al. 2007]. Gabriel et al. (2007) reported that the generation time of *H. dujardini* is 13 to 14 days at room temperature and each adult produces approximately 3 eggs per laying. The oviposition synchronizes with molting and embryos develop in the shed exoskeleton until hatching. This species is thought to sense environmental desiccation and prepare for upcoming severe dehydration during preconditioning. Therefore, *H.*

dujardini could have signal transduction systems to induce its successful transition to anhydrobiosis. Almost nothing is known, however, about the molecular mechanisms regulating anhydrobiosis in tardigrades. *H. dujardini* is easy to maintain in the laboratory, and the strain is established [Gabriel et al. 2007] and used for expressed sequence tag and genomic projects, providing a wealth of sequence information (http://www.ncbi.nlm.nih.gov/nucest/?term=hypsibius+dujardini) [Boothby et al. 2015; Koutsovoulos et al. 2015, a preprint uploaded in bioRxiv server]. Therefore, this species is suitable for dissection of the molecular mechanisms regulating anhydrobiosis in tardigrades.

In my doctoral studies, I first used a transcription and a translation inhibitor and found that *de novo* gene expression is required for successful transition to anhydrobiosis in *H. dujardini*. I performed further chemical screening and identified five chemicals that specifically impaired anhydrobiotic survival. Among them, cantharidic acid, a selective inhibitor against two serine/threonine phosphatases, protein phosphatase (PP) 1 and PP2A (PP1/PP2A), showed much stronger inhibitory effect. Two other selective inhibitors of PP1/PP2A also specifically inhibited anhydrobiotic survival, suggesting the requirement of PP1/PP2A activity for anhydrobiosis in *H. dujardini*. This is the first report to suggest that *de novo* gene expression and PP1/PP2A activity are required for successful transition to anhydrobiosis in the tardigrade. The identified chemicals could be powerful tools for investigating the detailed molecular mechanisms regulating anhydrobiosis in *H. dujardini* and possibly other tardigrades as well.

I next performed comparative transcriptome analyses during preconditioning, and identified 146 upregulated genes. These upregulated genes were classified as primary response genes and secondary response genes based on their sensitivity to translation inhibitor treatment. Consistent upregulation even after treatment with a translation inhibitor was confirmed for at least four putative primary response genes based on quantitative reverse transcription polymerase chain reaction (qRT-PCR). The upregulation of these primary response genes was inhibited by pre-treatment with the PP1/PP2A inhibitor cantharidic acid, suggesting that

PP1/PP2A is involved in the regulation of the initial transcriptional response to desiccation, as primary response genes are thought to be first transcribed upon desiccation stress. The identified primary response genes could be useful for investigating initial transcriptional regulation of anhydrobiosis through the identification of the cis-regulatory elements responsive to desiccation and the corresponding transcription factors.

The findings in this study provide the strategy to compare the molecular regulatory mechanisms between two distinctive anhydrobiotic tardigrades, *H. dujardini* and *R. varieornatus*. Such comparative analyses will give us the insight into how organisms extend their habitats.

Chapter 1

Identification of chemicals inhibiting anhydrobiotic survival and suggested involvement of *de novo* gene expression and PP1/PP2A activity in anhydrobiosis in *Hypsibius dujardini*.

Introduction

For successful transition to anhydrobiosis, many anhydrobiotic animals require preconditioning prior to severe dehydration [Crowe 1975; Wright 1989; Lapinski & Tunnacliffe 2003; Shannon et al. 2005]. During preconditioning, animals are thought to sense environmental desiccation and prepare for upcoming severe dehydration. Some anhydrobiotic animals, such as the sleeping chironomid, *Polypedilum vanderplanki,* and the anhydrobiotic nematode, *Aphelenchus avenae,* accumulate huge amounts of a non-reducing disaccharide, trehalose, reaching almost 10% to 18% of the dry weight during the preconditioning period [Higa & Womersley 1993; Watanabe et al. 2002]. The accumulated trehalose is thought to contribute to protecting biomolecules from dehydration stress [Crowe 2002]. In the anhydrobiotic tardigrade *Richtersius coronifer,* trehalose is accumulated up to 2.3% of the dry weight upon desiccation, though the accumulated amounts in tardigrades are generally much lower than those in anhydrobiotic arthropods or nematodes [Westh & Ramløv 1991]. Additionally, energy deprivation by treatment with a mitochondrial uncoupler severely impairs the anhydrobiotic survival of *R. coronifer*, suggesting the presence of energetically controlled processes during the transition to anhydrobiosis [Halberg et al. 2013]. However, the regulatory mechanisms governing the production of protective molecules and inducing the orchestrated transformation to an adaptive dehydrated state remain largely unknown.

H. dujardini is an anhydrobiotic tardigrade which requires longer preconditioning in a high humidity condition to acquire tolerance against severe desiccation [Wright 1989]. This implies the presence of regulatory mechanisms to induce anhydrobiosis in this species in response to preconditioning. Therefore, I investigated the molecular mechanisms regulating anhydrobiosis, using *H. dujardini*.

In this chapter, I used a chemical genetic approach and suggested that *de novo* gene expression is required for entering anhydrobiosis in *H. dujardini*. In addition, I identified 7

chemicals that significantly and specifically impaired the anhydrobiotic survival of this tardigrade. Among them, three different kinds of inhibitors against PP1/PP2A inhibited anhydrobiotic survival, suggesting an important role of PP1/PP2A activity in anhydrobiosis in this species. The inhibitory chemicals identified in this study could be powerful tools for further elucidation of the molecular regulatory mechanisms of tardigrade anhydrobiosis.

Materials and Methods

Animals

The Z151 strain of *H. dujardini* was purchased from Sciento (UK) and maintained at 18°C. Previously established YOKOZUNA-1 strain of *R. varieornatus* was maintained at 22°C [Horikawa et al. 2008]. Both strains were reared on 1.2% agar plates overlaid with volvic water containing *Chlorococcum* sp. (Sciento, UK) or *Chlorella vulgaris* (Recenttec K. K., Japan) as food. Water and food were replaced once or twice a week.

Chemicals

α-amanitin, cycloheximide, J-8, and cantharidic acid were purchased from Enzo Life Sciences (USA). Triptolide was purchased from MedChem Express (USA). 3,4-Methylenedioxy-β-nitrostyrene (MNS), 2-aminoethyl diphenylborinate (2-APB), and okadaic acid were purchased from Santa Cruz Biotechnologies (USA). Tautomycetin was purchased from Tocris Bioscience (USA). The 81 chemicals used for the screening were provided by the Drug Discovery Initiative, The University of Tokyo (Japan) and are listed in Table 1. All chemicals were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical, Japan; special grade) as a stock solution and stored at -20°C. Chemical solutions at the appropriate concentrations were prepared by diluting stock solutions in sterilized Milli-Q (stMQ) water just prior to chemical treatment. The final concentration of DMSO in all chemical solutions was adjusted to 1%.

Desiccation tolerance assay

All procedures were essentially performed at the rearing temperature (18°C for *H. dujardini*, 22°C for *R. varieornatus*). For desiccation, a nylon net filter (Millipore, USA; pore size 11 μm, 25 mm in diameter) was placed on Whatman 3MM filter paper (GE Healthcare, UK; 25 mm

diameter) in a plastic dish (35 mm diameter), and tardigrades were dropped onto the net filter with 125 µl of stMQ water. Approximately 20 *H. dujardini* or 15 *R. varieornatus* were placed on each filter in the experiments other than chemical screening. For preconditioning, the dishes were immediately transferred in a sealed plastic box moistened at a defined relative humidity (RH) and incubated for 0 to 4 days. RH was controlled by equilibrium with 60% glycerol (62% RH) or saturated solutions of potassium nitrate (95% RH), potassium chloride (85% RH), or magnesium chloride (33.5% RH) [Johnson 1949; Winston & Bates 1969]. After preconditioning, the animals were exposed to 10% RH in the presence of activated silica gel for 2 days to dehydrate. The dehydrated animals were humidified in 95% RH for 1 day and rehydrated with 2 ml of stMQ water, while the dehydrated *R. varieornatus* was rehydrated without pre-exposure to 95% RH when they were desiccated without preconditioning. The number of recovered animals was counted at 1 h and 24 h after rehydration. I defined recovered animals as those exhibiting spontaneous movements or at least responding to touch stimuli. Recovery rates were calculated as the percentage of recovered animals in each population. As a criterion for successful anhydrobiosis, I defined the recovery rates 60% or more at both 1h and 24h in control or untreated animals. When the recovery rates did not meet this criterion, the trials were excluded. When necessary, the shapes of the dehydrated tardigrades were observed, and the number of tun-shaped individuals was counted for calculation of the tun formation rates. Animals that were markedly stretched or twisted after desiccation were considered to have non-tun shapes.

Chemical treatment

The stock solutions were diluted to an appropriate concentration with stMQ water with an adjustment of the DMSO concentration to 1% just prior to chemical treatment. Tardigrades were collectively transferred with 3 µl stMQ water into 50 µl chemical solution of the defined concentration and thus, tardigrades were exposed to slightly diluted concentration of the chemicals (94% of the defined concentration). Tardigrades were incubated in chemical solution

for 5 h, except incubation in okadaic acid was for 10 h. Tardigrades in 53 µl solution were then dropped onto a nylon filter placed on the filter paper, and lightly washed with an additional 72 µl stMQ water (total volume: 125 µl) and subjected to desiccation tolerance assays.

Chemical screening

I screened 81 chemicals that were provided to us by Drug Discovery Initiative, The University of Tokyo (Japan) as a numbered list of compounds. I was blinded to the identification of the compounds at the time of screening. Because it was difficult to analyze all 81 chemicals simultaneously, due to limitation in animal supply and technical difficulties, I divided 81 chemicals into 20 groups and each group was separately assayed with control treatment (1% DMSO). Ten to 30 *H. dujardini* were treated with each chemical solution as described above and subjected to a desiccation series of 95% RH for 2 days, 10% RH for 2 days, and 95% RH for 1 day. Three or four dishes of 10 to 30 tardigrades/dish were assayed for each chemical. The detailed conditions for each group are listed in Table 1. The recovery rates for each chemical were normalized to the mean recovery rates of tardigrades treated with 1% DMSO (control) in the same group.

Statistics

Statistical tests were performed using Statcel 3 (OMS, Japan). Specific inhibitory effects on anhydrobiotic survival were examined using the Tukey-Kramer test, comparing four conditions for each chemical. To detect significant inhibition in the chemical screening, the Student's *t*-test or Dunnett's test were performed against the DMSO control in the same group.

Results

H. dujardini Z151 strain requires preconditioning to tolerate low humidity exposure

In a previous study, Wright (1989) examined the tolerability of the tardigrade identified as *H. dujardini* with an uncertain origin and reported that this species requires pre-incubation at 85% RH for 150 to 200 min to tolerate exposure to a low RH environment (25-31% RH). The Z151 strain of *H. dujardini* was established in 1987 [Gabriel et al. 2007] and recently used for various studies, including evo-devo analyses [Tenlen et al. 2013] and an expressed sequence tag/genome project, and is thus one of the most suitable tardigrade strains for analyzing the molecular mechanisms activated during preconditioning. Whether the Z151 strain has a similar tolerability as described for *H. dujardini* in the previous work, however, is unclear. To address this, we examined the desiccation tolerance of the Z151 strain in a low humidity environment with varying lengths of preconditioning periods (Fig. 2A). Without preconditioning, the Z151 strain cannot tolerate exposure to 10% RH (Fig. 2B and C). A 1-day preconditioning at 95% RH dramatically improved recovery rates to more than 90% and no further differences were detected for longer preconditioning periods of up to 4 days (Fig. 2B and C). I further examined the effect of varying levels of humidity during the preconditioning period and found that tardigrades preconditioned at 33.5% or 62% RH had no or very low survival after exposure to 10% RH (Fig. 2D and E). In contrast, tardigrades preconditioned at 85% RH could tolerate 10% RH at levels similar to those at 95% RH (Fig. 2D and E), though we observed an occasional decrease in the recovery rates when they were preconditioned at 85% RH (data not shown). These findings indicated that the Z151 strain of *H. dujardini* requires preconditioning at high humidity, ≥85% RH, to tolerate severe desiccation (10% RH for 2 days). Preconditioning at 95% RH for 1 day was more reliably sufficient to confer tolerance to exposure to 10% RH for this strain.

De novo* gene expression is required for anhydrobiotic survival in *H. dujardini* but not in *R. varieornatus

Many anhydrobiotic animals induce the expression of putative protection molecules upon desiccation, such as late embryogenesis abundant proteins [Browne et al. 2002; Erkut et al. 2013]. Accordingly, I hypothesized that *H. dujardini* would also induce the expression of protection molecules necessary for transition to anhydrobiosis during the preconditioning period. To examine this possibility, I pre-treated the tardigrades with the transcription inhibitor α-amanitin [Lindell et al. 1970] for 5 h prior to preconditioning (Fig. 3A). Treatment with the transcription inhibitor dramatically reduced the recovery rates from severe desiccation (Fig. 3B and C). To confirm whether the inhibitor specifically impaired anhydrobiotic survival rather than having general toxic effects, I also examined the effect of the transcription inhibitor on survival after high-humidity treatment, in which exposure to low humidity (10% RH) was replaced with exposure to high humidity (95% RH; Fig. 3A). When the tardigrades were exposed to high humidity only, the transcription inhibitor hardly affected their recovery rates, suggesting that α-amanitin specifically impaired anhydrobiotic survival (Fig. 3B and C). I also examined the effect of the translation inhibitor cycloheximide [Schneider et al. 2010], which showed similar results (Fig. 3D and E). Taken together, these findings suggest that *H. dujardini* requires *de novo* transcription and translation for successful transition to anhydrobiosis. This tardigrade likely produces gene products necessary for survival in a low humidity environment during preconditioning periods.

In addition, I also examined the effects of these inhibitors on anhydrobiotic survival in *R. varieornatus*, which does not require preconditioning to withstand severe desiccation. Unlike *H. dujardini*, both inhibitors had no effects on anhydrobiotic survival of *R. varieornatus* even at higher concentrations of the inhibitors regardless of preconditioning (1h, Fig. 4B-E; 24h, data not shown), suggesting that *R. varieornatus* does not require *de novo* gene expression to enter anhydrobiosis and this is consistent with their tolerant ability against desiccation without

preconditioning.

Pre-incubation for 5 h with chemicals was sufficient for both inhibitors against transcription and translation to interfere with anhydrobiotic survival in *H. dujardini*. Thus, I conclude that this experimental scheme is useful for evaluating the effects of various chemicals on anhydrobiotic survival in *H. dujardini*.

Identification of inhibitors of anhydrobiotic survival in *H. dujardini*

The requirement of *de novo* gene expression for anhydrobiotic survival prompted me to postulate the presence of signal transduction pathways connecting desiccation stimuli to responsive expression of genes related to desiccation tolerance. To elucidate such signaling pathways, I conducted chemical screening to identify chemicals that inhibit anhydrobiotic survival in *H. dujardini*. I selected 81 chemicals as candidates from available inhibitory chemicals whose target molecules were known, based on knowledge of the stress-responsive pathways in other organisms, general signaling molecules, and the gene repertoire encoded in the tardigrade genome (Table 1). Chemical treatment was performed mostly at predefined concentrations such as 100 μM or 20 μM, depending on the solubility of the chemicals in DMSO. When the animals appeared immotile after chemical treatment, the concentration was decreased to avoid immobility of the animals. After screening the 81 chemicals, I identified 5 chemicals that significantly inhibited anhydrobiotic survival (Fig. 5 and Table 1). These five chemicals included a calmodulin antagonist (J-8) [MacNeil et al. 1980], an immunosuppressive agent (triptolide) [Liu 2011], a Syk and Src kinase inhibitor (MNS) [Wang et al. 2006], a PP1 and PP2A inhibitor (cantharidic acid) [Li et al. 1993], and a calcium release modulator (2-APB) [Bootman et al. 2002]. Triptolide and cantharidic acid exhibited much stronger inhibitory effects than the other three chemicals at 1 h as well as 24 h after rehydration (Fig. 5B, C, F, and G), suggesting that their target molecules play an important role in the transition to anhydrobiosis. For MNS and 2-APB, significant inhibition of the recovery rates was detected only at 1 h after

rehydration (Fig. 5D, E, H, and I).

The identified chemicals specifically impaired anhydrobiotic survival in *H. dujardini*

To elucidate whether the identified chemicals specifically impaired anhydrobiotic survival rather than having nonspecific toxicity, I examined the effects of the chemicals on survival of tardigrades exposed only to high humidity (95% RH) instead of exposure to low humidity (10% RH). I tested plural concentrations for each chemical and at the appropriate concentrations, all five chemicals exhibited significant inhibitory effects specifically on survival after exposure to low humidity, with little effect on survival after exposure to only high humidity (Fig. 6 and Fig. 7; only the most effective concentration is shown for each chemical). These findings suggest that all five identified chemicals have specific inhibitory effects on anhydrobiotic survival.

Inhibition of PP1/PP2A activity impaired anhydrobiotic survival in *H. dujardini*.

Among the five identified chemicals, cantharidic acid and triptolide exhibited the most profound inhibitory effects on anhydrobiotic survival (Fig. 6A-D and Fig. 7A-D). Cantharidic acid is a highly selective inhibitor against two serine/threonine phosphatases, PP1 and PP2A (PP1/PP2A) [Li et al. 1993], while triptolide interacts with various biologic molecules such as calcium channel polycyctin-2 and thus it is difficult to determine the important target for anhydrobiosis [Leuenroth et al. 2007; McCalluma et al. 2007; Soundararajan et al. 2009; Titov et al. 2011]. Therefore, I focused on PP1/PP2A, the target of cantharidic acid. To confirm whether PP1/PP2A activity is required for anhydrobiosis, I examined the effect of other potent and selective PP1/PP2A inhibitors, okadaic acid [Haystead et al. 1989] and tautomycetin [Mitsuhashi et al. 2001]. Either chemical also significantly inhibited recovery from severe desiccation, whereas no toxic effects were observed when the tardigrades were exposed to high humidity only, suggesting that okadaic acid and tautomycitin also specifically impaired anhydrobiotic survival (Fig. 8). Among three PP1/PP2A inhibitors tested in this study, okadaic acid is the best studied

and the interacting residues of the catalytic subunit of PP2A have been clarified [Xing et al. 2006]. There is one gene encoding PP2A catalytic subunit (g05338) in the *H. dujardini* genome. To check the possible interaction of okadaic acid with PP2A even in the tardigrade, I compared the amino acid sequences of g05338 with the human ortholog PPP2CA by sequence alignment, and found that all residues that recognize the okadaic acid are also conserved in g05338 (Fig. 9). Taken together with the effect of cantharidic acid, these findings suggest that PP1/PP2A activity plays an important role in anhydrobiotic survival in *H. dujardini.*

Discussion

The findings in this chapter indicate that inhibiting either transcription or translation severely impaired anhydrobiotic survival of *H. dujardini* (Fig. 3). To my knowledge, this is the first report to clarify the requirement of *de novo* gene expression for successful transition to anhydrobiosis in tardigrades. In contrast, anhydrobiotic survival in *R. varieornatus* was not affected by these inhibitors (Fig. 4), supporting the previous observation that *R. varieornatus* does not need preconditioning for anhydrobiosis and consistently exhibits the desiccation tolerance. In addition, I identified seven additional chemicals that specifically inhibited survival from severe desiccation (Figs. 5-8 and Table 1), and three distinct inhibitors against PP1/PP2A exhibited particularly profound inhibitory effects, suggesting an important role of PP1/PP2A activity in anhydrobiosis of *H. dujardini*.

Requirement of *de novo* gene expression suggests that *H. dujardini* sense mild desiccation during preconditioning and synthesize new transcripts to prepare the necessary molecules to tolerate severe dehydration. This implies that newly synthesized transcripts during preconditioning are candidate molecules involved in the transition to anhydrobiosis, and thus, based on this evidence, comparative analyses of gene expression during preconditioning appears to be a promising strategy for identifying anhydrobiosis-related genes using *H. dujardini*. In the present study, I used α-amanitin, a selective inhibitor of RNA polymerase II [Lindell et al. 1970], and thus synthesized messenger RNA is a primary candidate molecule involved in anhydrobiosis. This does not rule out the possible roles of other RNA molecules transcribed by other polymerases, such as some non-coding RNAs transcribed by RNA polymerase III [White 2011]. Chemical inhibition of RNA polymerase III, however, also affects the synthesis of transfer RNA, making it difficult to clarify the role of such non-coding RNA in anhydrobiosis because inhibition of translation itself affected anhyrobiotic survival (Fig. 3D and E). Therefore, total RNA-sequencing analyses would be the most suitable strategy for identifying differentially

expressed genes, including noncoding RNA. These analyses will be described in the next chapter.

In addition to transcriptional regulation, my chemical screening suggests that multiple signaling pathways are also required for successful anhydrobiosis, possibly in the signal transduction of desiccation stimuli to appropriate gene expression. In particular, dephosphorylation by PP1/PP2A could play an important role in acquiring tolerance against severe desiccation in *H. dujardini*, because three different PP1/PP2A inhibitors, cantharidic acid, okadaic acid and tautomycetin, exhibited profound inhibitory effects on anhydrobiotic survival (Figs. 6, 7 and 8). Although these inhibitors suppressed survival only when animals were exposed to low humidity condition, suggesting their specific inhibitory effects, I could not exclude the possibility that nonspecific toxic action of these inhibitors might reach lethality only when animals were exposed to severe desiccation in low humidity condition. This is the limitation of chemical approaches and other loss of function experiments, such as knock down by RNAi, will consolidate the requirement of corresponding signaling molecules, including PP1/PP2A. PP1/PP2A is multimeric serine/threonine protein phosphatases composed of a highly conserved catalytic subunit and various regulatory subunits, and PP2A also contains a scaffolding subunit [Janssens & Goris 2001; Shi 2009]. Cantharidic acid and okadaic acid inhibit both catalytic subunits of PP1 and PP2A [Shi 2009]. Regulatory subunits determine the substrate specificity of PP1/PP2A holoenzymes and are thus involved in specific biologic processes. In budding yeast, *Saccharomyces cerevisiae*, one regulatory subunit of PP2A, Cdc55, is required for full activation of osmotic stress response genes via indirect regulation of stress-responsive transcription factors Msn2/Msn4 [Reiter et al. 2013]. Because mild dehydration during preconditioning likely causes osmotic stress, *H. dujardini* might possess a similar pathway contributing to anhydrobiosis. In various animals, PP2A is involved in the positive regulation of forkhead transcription factor, FOXO, via inactivation of Akt kinase or direct dephosphorylation [Vereshchagina et al. 2008; Yan et al. 2008; Narasimhan et al. 2009;

Padmanabhan et al. 2009; Singh et al. 2010]. FOXO is an important transcription factor involved in longevity, stress-resistance, and regulation of metabolism downstream of insulin/insulin-like growth factor signaling [Van der Horst & Burgering 2007]. In particular, FOXO upregulates the transcription of antioxidant enzymes such as manganese superoxide dismutase, and increases resistance to oxidative stress [Kops et al. 2002; Ambrogini et al 2010]. Desiccation stress enhances the accumulation of reactive oxygen species [de Jesus Pereira et al. 2003; Contreras et al. 2011], and thus FOXO activation through PP2A might be involved in the anhydrobiotic response during preconditioning in tardigrades.

In addition to PP2A, PP1 activity may also be involved in anhydrobiosis. In dauer larvae of *Caenorhabditis elegans*, myosin light chain (MLC) is clearly dephosphorylated during the preconditioning period [Erkut et al. 2013], although its significance in desiccation tolerance remains elusive. Dephosphorylation of MLC is mostly catalyzed by MLC phosphatase, whose catalytic subunit is the same as that of PP1, the target of cantharidic acid and okadaic acid [Shi 2009]. Thus, dephosphorylation by PP1 could be a common process during preconditioning in *H. dujardini* and dauer larvae of *C. elegans*.

In addition to phosphatase inhibitors, MNS, a protein tyrosine kinase inhibitor, also significantly inhibited anhydrobiotic survival (Fig. 6G, H and Fig. 7G, H). MNS is a selective inhibitor of Syk and Src kinases [Wang et al. 2006]. In vertebrates, Syk and Src are involved in the response to oxidative stress [Abe et al. 1997; Ding et al. 2006; Pal et al. 2014], and Syk is also involved in the response to osmotic stress in chicken B cells [Qin et al. 1997]. As described above, oxidative and osmotic stresses likely occur during desiccation stress; thus, Syk and Src might be involved in mitigation of these stresses during preconditioning in *H. dujardini*. Their contributions to anhydrobiosis, however, might only be partial, because the inhibitory effect of MNS was rather moderate compared to that of cantharidic acid (Fig. 6 and Fig. 7).

Mitogen-activated protein kinase (MAPK) pathways are representative phosphorylation signaling pathways involved in various environmental stress responses [Manning et al. 2002;

Cowan & Storey 2003]. These pathways are highly conserved in eukaryotic organisms and are well-studied. In an anhydrobiotic nematode, *Panagrolaimus superbus*, pre-treatment with MAPK inhibitors against JNK (SP600125; 50 μM or 100 μM) and p38 (SB239063; 50 μM or 100 μM) partially inhibits anhydrobiotic survival only when nematodes were exposed to 10% RH without preconditioning [Banton & Tunnacliffe 2012]. In the same report, however, anhydrobiotic survival after preconditioning was almost unaffected by these MAPK inhibitors. Correspondingly, I detected no significant effects of the same MAPK inhibitors, such as SP600125 (JNK; Chemical ID 1) and SB239063 (p38; Chemical ID 50), and also no significant effect by U0126 (MEK1/MEK2; Chemical ID 47) [DeSilva et al. 1998] at the same concentration of 100 μM of these inhibitors (Table 1). Thus, MAPK signaling is likely not essential for anhydrobiosis in preconditioned tardigrades as well as preconditioned nematodes. I cannot, however, exclude the possibilities that in the tardigrade, these chemicals could not effectively penetrate or inhibit the target kinases or concentrations used in this study were not appropriate.

Triptolide was another chemical with strong inhibitory effects (Fig. 6 C, D and Fig. 7C, D). This compound was originally identified as an immunosuppressive agent. Triptolide indirectly interferes with the NF-κB pathway and suppresses immune responses [Liu 2011]. After its discovery, many other target molecules were identified for triptolide, e.g., calcium channel polycystin-2 [Leuenroth et al. 2007; McCalluma et al. 2007; Soundararajan et al. 2009; Titov et al. 2011]. One of the target proteins is XPB, a subunit of the core transcription factor TF-II H, and triptolide also inhibits general transcription [Bensaude 2011; Titov et al. 2011]. Therefore, the strong suppression of triptolide on anhydrobiotic survival could be due to its inhibitory effect on transcription, which is consistent with the effects of α-amanitin (Fig. 3B and C). Inhibitory effects on other targets could also contribute to suppression of anhydrobiotic survival.

The other two identified inhibitors, J-8 and 2-APB, are a calmodulin antagonist and

calcium release modulator, respectively [MacNeil et al. 1980; Bootman et al. 2002]. These findings suggest that calcium signaling is involved in anhydrobiotic survival in *H. dujardini* in part, because their inhibitory effects were relatively weak (Fig. 6 and Fig. 7). Calcium is a general signaling molecule that regulates the activities of various enzymes, mostly through binding with a partner protein, calmodulin (CaM). Recent work revealed that CaM regulates c-Src kinase activity, and the CaM antagonist W-7 inhibits the activation of c-Src in response to hydrogen peroxide-induced oxidative stress [Stateva et al. 2015]. As described above, the Src inhibitor MNS also inhibited anhydrobiotic survival at a level comparable to that of the CaM antagonist J-8 (Fig. 6 and Fig. 7). Calcium signaling and c-Src might play a role in the same pathway in anhydrobiosis in the tardigrade. I could not detect significant effects of various inhibitors against other CaM-regulated enzymes (Table 1), such as CaMK2 (Chemical ID 15) [Hashimoto et al. 1991], calcineurin (Chemical ID 29) [Waters et al. 2005], nitric oxide synthase (Chemical ID 55) [Raman et al. 2001], phosphodiesterase type 1 (Chemical ID 43) [Ahn et al. 1989], and MLC kinase (Chemical ID 80) [Ito et al. 2004], nor inhibitors against other calcium-related proteins, like calcium channels (e.g., Chemical ID 5) [Harper et al. 2003], possibly due to their small contribution to anhydrobiosis or to insufficient inhibition of target proteins in *H. dujardini*.

The effects on tun formation are generally much smaller than those on anhydrobiotic survival, and the significance of the effects varied among experiments, suggesting that these chemicals have subtle inhibitory effects on tun formation, if any (Fig. 10).

In summary, the findings in this chapter suggest the requirement of *de novo* gene expression in anhydrobiosis of *H. dujardini*, and provide evidence that comparative transcriptome analyses are a promising strategy for identifying anhydrobiosis-related genes. Furthermore, the newly identified seven inhibitory chemicals, including three PP1/PP2A inhibitors, could become powerful tools for the molecular dissection of the regulatory mechanisms of anhydrobiosis in *H. dujardini* and possibly other tardigrades as well.

Chapter 2

Investigation of transcriptional response to desiccation stress during preconditioning in *Hypsibius dujardini*.

Introduction

In Chapter 1, I discovered that *de novo* gene expression during preconditioning is required for anhydrobiotic survival in *H. dujardini*. Based on this finding, I emphasized the importance of investigating the transcriptional response during preconditioning to identify the essential molecules as well as the molecular mechanisms regulating anhydrobiosis in this tardigrade. As trials to uncover the mechanisms underlying tardigrade anhydrobiosis, we and some other groups have previously conducted transcriptome analyses by comparing gene expression levels between the active hydrated state and the inactive dehydrated state [Mali et al. 2010; Wang et al. 2014]. Our previous transcriptome analysis using *R. varieornatus*, a tardigrade that does not require preconditioning for anhydrobiosis, revealed that gene expression levels were not remarkably altered between pre- and post-dehydration in this tardigrade species (unpublished data). This finding suggests that *R. varieornatus* constitutively synthesizes and holds essential molecules for anhydrobiosis, which is consistent with the results obtained in Chapter 1 indicating that anhydrobiotic survival of *R. varieornatus* was not affected by either a transcription or translation inhibitor (Fig. 4). All other transcriptome studies so far published were performed using another anhydrobiotic tardigrade, *Milnesium tardigradum*, which tolerates direct exposure from a hydrated state to a low humidity condition [Wright 1989]. Those studies demonstrated that some genes, including mainly ribosomal proteins, are remarkably downregulated, consistent with the common perception that global metabolic arrest occurs during desiccation stress [Mali et al. 2010; Wang et al. 2014]. In contrast, upregulated genes were hardly detected, except for some small heat shock protein genes [Wang et al. 2014]. Based on these findings, Wang et al. (2014) concluded that *M. tardigradum* can achieve desiccation tolerance by a constitutive cellular protection system. Therefore, in the previous transcriptome analyses, it was difficult to identify candidate genes related to tardigrade anhydrobiosis.

The finding in Chapter 1 that *H. dujardini* requires *de novo* gene expression for successful transition to anhydrobiosis suggests that genes essential for anhydrobiosis in this species should be upregulated during preconditioning. The desiccation-induced genes and their transcriptional regulatory mechanisms, however, were unknown.

In this chapter, therefore, I investigated the transcriptional response during preconditioning using RNA-sequencing analysis. First, I compared the gene expression profiles between the active hydrated state and the 6-h preconditioned state, and identified 146 upregulated genes in the 6-h preconditioned state. More than 70% of these genes were unannotated, suggesting the presence of unique molecular mechanisms of anhydrobiosis in this tardigrade. I classified these 146 upregulated genes as primary response genes or secondary response genes based on their sensitivity to pre-treatment with a translation inhibitor. Consistent upregulation, regardless of translation inhibition, was verified by qRT-PCR for at least four putative primary response genes. As primary response genes are induced without *de novo* translation, these genes are thought to be the first to be transcribed upon desiccation stress. The upregulation of these primary response genes was partly impaired by pre-treatment with the PP1/PP2A inhibitor cantharidic acid, suggesting that PP1/PP2A could be involved in a signaling pathway transducing desiccation stimuli to the initial transcriptional response during preconditioning. The identified primary response genes could be powerful tools for investigating the initial transcriptional regulation of anhydrobiosis through the identification of cis-regulatory elements responsive to desiccation and corresponding transcription factors.

Materials and Methods

RNA-sequencing analysis

Seven hundred fifty *H. dujardini* were used for each sample (N=1). Active hydrated tardigrades were used as the pre-desiccation control (Active). Tardigrades preconditioned at 95% RH for 6 h after 5-h incubation in solutions of 1% DMSO (D6H) or 500 μM cycloheximide (C6H) were used as post-desiccation samples. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and chloroform (Wako, Japan) with PureLink RNA Mini Kit (Life Technologies, USA). Illumina cDNA libraries were constructed using Truseq Stranded Total RNA with a Ribo-Zero Gold rRNA Removal Kit (Human/Mouse/Rat; Illumina, USA) according to the manufacturer's instructions and sequenced with a Hiseq 2500 (Illumina). Approximately 75 million paired-end reads (101 bp x2, insert size 145 bp) were produced from each sample. The sequenced reads were mapped to *H. dujardini* draft genome version 2.3 (http://badger.bio.ed.ac.uk/H_dujardini/home/download) using TopHat (with the following options: -p 4 --max-intron-length 10000) [Langmead et al. 2009; Trapnell et al. 2012]. Gene models (version 2.3.1) and Annotation Information (version 2.3.1) were obtained from the same database (http://badger.bio.ed.ac.uk/H_dujardini/home/download). The model identified 23,021 predicted protein-coding genes on the *H. dujardini* draft genome, and 12,002 (52.1%) of these are annotated [Koutsovoulos et al. 2015; a preprint uploaded in bioRxiv server]. Estimation of expression levels and subsequent comparative analysis were performed using Cufflinks (with the following options: -p 4 -u -b -no-update-check –max-intron-length 10000 -overlap-radius 20) and Cuffdiff (with the following options: -p 4 -u -b –no-update-check –M annotated ribosomal RNA [rRNA] file) programs, respectively, based on the provided gene model [Trapnell et al. 2012]. Putative orthologous genes of *R. varieornatus* and several model organisms, including *Homo sapiens*, *Drosophila melanogaster,* and *C. elegans,* were assigned to all genes of *H. dujardini* by reciprocal BLAST (e-value < 1e-3; T. Kunieda, unpublished data).

Quantitative RT-PCR analysis

Total RNA was extracted as described above from active hydrated tardigrades (Active) as a pre-desiccation control, 6-h preconditioned tardigrades after 5-h incubation in 1% DMSO (D6H) or 250 μM cycloheximide (C6H), and 3-h or 6-h preconditioned tardigrades after 5-h incubation in 1% DMSO or 1 μM CA. The cDNA was synthesized using a PrimeScript RT reagent Kit with a gDNA eraser (Perfect Real Time; Takara, Japan). Quantitative RT-PCR (qRT-PCR) was performed using SYBR premix ExTaq Ⅱ (Tli RNaseH plus; Takara, Japan) with LightCycler (Roche, Switzerland). Sample variance was normalized based on *ef1α* levels. The primers used in this analysis are listed in Table 2.

Gene ontology term enrichment analysis

Gene ontology (GO) term enrichment analysis was performed using GOrilla [Eden et al. 2009]. To investigate GO term enrichment in annotated upregulated genes, a whole gene set (fragments per kilobase of exon model per million mapped reads [FPKM]≧1) of *H. dujardini* and selected gene sets of upregulated genes were converted to corresponding human orthologous genes and submitted to the web service of GOrilla as background and target sets, respectively. The GO terms with both P-value and FDR q-value less than 5e-2 were considered to be significantly enriched.

Statistics

Statistical tests were performed using Statcel 3 (OMS, Japan). The differences in gene expression levels quantified by qRT-PCR were examined using Student's *t*-test, Dunnett's test, or the Tukey-Kramer test, depending on the experiments.

Results

High proportion of unannotated genes in upregulated genes

Because the chemical genetic approach suggested that *H. dujardini* requires *de novo* gene expression for successful transition to anhydrobiosis, newly transcribed genes during preconditioning should contain genes essential for anhydrobiosis. Therefore, I searched for the upregulated genes during preconditioning by comparing gene expression profiles using RNA-seq. I sequenced three RNA-seq libraries constructed from active tardigrades (Active) and 6h-preconditioned tardigrades after 5h-incubation in solution of 1% DMSO (D6H) or 500 μM cycloheximide (C6H). A total of ~75 million paired-end reads of 101 nucleotides were obtained from each sample (Table 3). The overall read mapping rate exceeded 85% in the three libraries, indicating that most reads were successfully mapped. These mapped reads contained multiple aligned reads that might consist mainly of rRNA, as most of the detected overrepresented sequences were likely a part of rRNA sequences that have low similarities with other organisms' rRNA (data not shown). These findings indicate that removal of rRNA by Ribo-Zero (Human/Mouse/Rat; Illumina, USA) was not fully achieved in *H. dujardini*.

The reference genome contains 23,021 protein-coding genes: 12,002 (52.1%) annotated genes and 11,019 (47.9%) unannotated genes. To exclude insignificant genes, I focused on the 14,593 genes whose FPKM was higher than 1 in both Active and D6H; 9641 (66.1%) annotated genes and 4952 (33.9%) unannotated genes (Table 4). Among these, I identified 146 genes (1.0%) that were upregulated by more than 4-fold in D6H compared to Active (Table 4). In these upregulated genes, only 42 genes (28.8%) were annotated and the other 104 genes (71.2%) were unannotated (Table 4). The proportion of unannotated genes was clearly higher in the upregulated genes than in the total gene repertoire, suggesting that the tardigrade utilizes its unique genes for transition to anhydrobiosis.

Xenobiotic metabolic process, as an enriched GO term in upregulated genes

To further characterize the upregulated genes, I performed GO term enrichment analysis for the annotated upregulated genes using corresponding human orthologous genes. As a result, the "xenobiotic metabolic process" was the only GO term that was significantly enriched (Table 5). The genes categorized in this GO term included glutathione S-transferase and cytochrome P450. These genes have roles in the detoxification of not only xenobiotics, but also endobiotic toxins, such as lipophilic or electrophilic molecules [Leaver & George 1998; McElwee et al. 2004; Lindblom & Dodd 2006], suggesting that these genes could be involved in the removal of biologic molecules damaged by desiccation stress.

Classification of upregulated genes as primary response genes and secondary response genes

Various environmental stressors induce responsive transcriptional cascades [Shinozaki et al. 2003; Yoshida et al. 2011]. If this is also the case for the desiccation stress response in *H. dujardini*, desiccation-induced genes could be classified into two groups: primary response genes and secondary response genes. The primary response genes are transcribed first upon exposure to the initial desiccation stimuli without translation of other gene products, and are thus located at the top of the transcriptional cascade (Fig. 11A). The secondary response genes are transcribed after synthesis of the gene products, typically transcription factors possibly included in the primary response genes, and are thus considered to be sensitive to inhibition of translation (Fig. 11A). These two types of genes can therefore be distinguished by examining their sensitivity to translation inhibitor treatment. Based on this logic, I examined the expression levels of upregulated genes in C6H (tardigrades treated with the translation inhibitor cycloheximide prior to the 6-h preconditioning treatment). Among the 146 upregulated genes, 102 (69.9%) were upregulated by at least 4-fold in C6H compared with those in the Active sample. I classified these genes as candidate primary response genes (Fig. 11B). The remaining

44 genes (30.1%) were candidate secondary response genes whose upregulation was less than 4-fold in C6H (Fig. 11B). Most of the upregulated genes were classified as primary response genes (69.9%), possibly because gene expression profiles were analyzed in a relatively early phase of preconditioning (~6 h), in which the transcriptional cascade might have just begun. Because primary response genes are thought to be transcribed by pre-existing desiccation-activated transcription factors, these genes could be regulated by cis-elements responsive to desiccation and are thus useful for exploring such desiccation responsive cis-elements involved in anhydrobiosis. Therefore, I focused on the primary response genes and validated the expression patterns of four selected genes by qRT-PCR. All four unannotated genes were significantly upregulated during preconditioning regardless of translation inhibition (Fig. 11C). This finding suggests that these selected genes are primary response genes that could be first transcribed upon desiccation without *de novo* translation and located at the top of the transcriptional cascade.

Upregulation of selected primary response genes was impaired by treatment with CA, a selective PP1/PP2A inhibitor

In Chapter 1, I revealed that CA specifically inhibited anhydrobiotic survival in *H. dujardini* (Fig. 6A and B, Fig. 7A and B). Because its inhibitory effect was much stronger than that of other identified chemicals, I hypothesized that CA inhibits the signaling pathways activated at the initial step during preconditioning. Accordingly, transcription of primary response genes could be regulated downstream of the signaling pathways inhibited by CA. To test this hypothesis, I examined the effect of CA treatment on the expression levels of the four primary response genes identified above. The upregulation of g00176 and g03577 was significantly inhibited at 3 h and 6 h during preconditioning by treatment with 1 μM CA (Fig. 12A and B), while upregulation of g11085 and g16517 was significantly inhibited only at 3 h (Fig. 12C and D). These inhibitory effects of CA treatment were reproduced only partly in another trial, in

which expression levels of g00176 and g16517 were significantly impaired (Fig. 12E and H), but those of g03577 and g11085 were not (Fig. 12F and G). These findings suggest that the signaling pathways inhibited by CA treatment are involved in the transcriptional regulation of at least two selected primary response genes, g00176 and g16517.

Expression of some *cahs* genes was upregulated during preconditioning

Some anhydrobiotic animals, such as the nematode *Aphelenchus avenae* accumulate late embryogenesis abundant proteins upon desiccation [Browne et al. 2002]. Late embryogenesis abundant proteins are heat-soluble proteins and putative protective molecules that may function as ion scavengers and protect other proteins from aggregation caused by desiccation stress [Hand 2011]. Previous analyses of heat-soluble proteins in *R.* varieornatus, however, revealed that the tardigrade does not mainly express late embryogenesis abundant proteins but does express heat-soluble proteins unique to tardigrades, such as CAHS, SAHS, and MAHS [Yamaguchi et al. 2012; Tanaka et al. 2015]. These unique heat-soluble proteins could play important roles in desiccation tolerance in tardigrades. *H. dujardini* has 9 genes encoding *cahs*, 10 genes encoding *sahs*, and 1 gene encoding *mahs*, and all genes except for 2 *sahs* genes, g17779 and g19251, were expressed at levels greater than 1 FPKM in both the Active and D6H samples (Table 6). Transcriptome analysis revealed that 3 *cahs* genes, g00353, g03020, and g04939, were upregulated more than 4-fold during preconditioning, while no *sahs* or *mahs* genes exhibited such remarkable upregulation (Table 6). I examined the expression levels of three upregulated *cahs* genes in C6H, and determined that g03020 could be classified as a primary response gene and g00353 and g04939 could be classified as secondary response genes (Table 6). I validated the expression patterns of these genes using qRT-PCR. Consistent with the RNA-seq results, g03020 was significantly upregulated in both D6H and C6H (Fig. 13A), while inhibiting translation significantly decreased the upregulation of g00353 and g04939 in C6H (Fig. 13B and C). These findings suggest that three *cahs* genes were induced during

preconditioning, but their transcription was regulated in a different manner in *H. dujardini*.

Upregulation of three induced-*cahs* genes was impaired by treatment with CA

I examined the effect of CA treatment on the expression levels of the three *cahs* genes identified above. Regardless of primary or secondary response genes, the upregulation of three *cahs* genes was significantly inhibited at both 3h and 6h during preconditioning by treatment with 1 μM CA (Fig. 14). These results suggest that the signaling pathways inhibited by CA treatment are also involved in the transcriptional regulation of upregulated *cahs* genes during preconditioning.

Discussion

In this chapter, I explored genes that were upregulated during preconditioning in *H. dujardini* using RNA-seq. In previous transcriptome studies using *R. varieornatus* and *M. tardigradum*, which require no preconditioning to enter anhydrobiosis, few upregulated genes were detected upon desiccation stimuli, and it was thus nearly impossible to identify the genes related to anhydrobiosis by comparing transcriptomes between pre- and post-desiccation [Mali et al. 2010; Wang et al. 2014]. In the current study, I used *H. dujardini*, which requires preconditioning, and found 146 genes that were upregulated more than 4-fold during preconditioning (Table 4). To exclude false-positives, I applied a relatively high threshold of 4-fold or more, because I could not replicate the RNA-seq due to the difficulty in preparing the large number of tardigrades required for the experiment. The expression levels and patterns of the selected genes that were used for further analyses were confirmed by qRT-PCR. The identified upregulated genes exhibited a much higher proportion of unannotated genes compared to the whole gene repertoire whose FPKM was greater than 1 (Table 4), indicating that tardigrades have unique molecular mechanisms of anhydrobiosis. This notion is also supported by the findings of several previous studies that tardigrades do not accumulate huge amounts of trehalose upon desiccation stress, in contrast to many other anhydrobiotic animals, including the sleeping chironomid *Polypedilum vanderplanki* and the anhydrobiotic nematode *Aphelenchus avenae* [Westh & Ramløy 1991; Higa & Womersley 1993; Watanabe et al. 2002]. Tardigrades are thought to have developed alternative anhydrobiotic mechanisms that are independent of trehalose.

GO enrichment analysis revealed a ‘xenobiotic metabolic process’ as only one GO term was enriched among the annotated upregulated genes (Table 5). Corresponding *H. dujardini* genes are expected to be involved in the elimination of biomolecules damaged by desiccation stress. Among these genes, glutathione S-transferase (*gst*) and cytochrome P450 (*cyp450*) were remarkably increased in my transcriptome analysis (*gst*, 85.2-fold increase; *cyp450 2j2*,

24.4-fold increase). An antarctic nematode, *Plectus murrayi*, accumulates expressed sequence tags encoding *gst* under gradual desiccation stress at 97% RH for 3 days and at 87% RH for 2 days [Adhikari et al. 2009]. In dauer larvae of *C. elegans*, *gst* and *cyp450* are increased during preconditioning at 98% RH [Erkut et al. 2013]. Thus, the contribution of these genes to desiccation stress could be shared among various anhydrobiotic animals.

By evaluating their sensitivity to a translation inhibitor, I was able to classify the upregulated genes as primary and secondary response genes (Fig. 11A and B), indicating the presence of a transcriptional cascade in response to exposure to desiccation stimuli in *H. dujardini*. Because secondary response genes require *de novo* translation for transcription, some primary response genes likely encode transcription factors that regulate the transcription of secondary response genes. In fact, I found two primary response genes encoding a transcription factor and a component of another transcription factor (Table 7). One is cell death specification protein 2 (*ces-2*), which is involved in the positive regulation of apoptosis in certain neurons in *C. elegans* [Metzstein et al. 1996]. The other is upstream activation factor subunit (*uaf30*), which is a nonessential component of yeast RNA polymerase Ⅰ, and together with other components UAF30 likely stimulates basal transcription of rRNA to a fully activated level [Siddiqi et al. 2001]. This gene might be related to the observation in Chapter 1 that pre-treatment with a translation inhibitor impaired anhydrobiotic survival in *H. dujardini*.

Transcription of primary response genes is likely promoted by pre-existing transcription factors activated by desiccation stress. Therefore, primary response genes might have desiccation-responsive elements in their loci, and identification of such elements will allow the identification of corresponding transcription factors. Detailed analyses are necessary to reveal the molecular mechanisms governing the initial transcriptional response to desiccation in tardigrades.

In Chapter 1, I suggested the involvement of PP1/PP2A activity in anhydrobiosis in *H. dujardini*, but the roles of PP1/PP2A remain unclear. In this chapter, I revealed that CA partly

inhibited the upregulation of selected primary response genes (Fig. 12), indicating that PP1/PP2A is involved in the signaling pathway connecting desiccation stimuli to the initial transcriptional response, because primary response genes are the first to be transcribed upon desiccation stimuli Investigation of the upstream pathway regulating PP1/PP2A activity could lead to the identification of a molecule that serves as a desiccation sensor. Known activators of PP1/PP2A, such as protein kinase A and protein kinase C, might be primary candidates involved in the desiccation response in *H. dujardini* [Kitatani et al. 2006; Ahn et al. 2007]. Although CA treatment drastically impaired anhydrobiotic survival (Fig. 15), it only had a partial effect on the transcription of the selected primary response genes (Fig. 12). One possible explanation is that an inadequate concentration of CA was used in the experiment. Another possibility is that other signaling pathways also contribute to the transcriptional regulation of some primary response genes, especially g03577 and g11085, whose upregulation is relatively insensitive to CA treatment (Fig. 12F and G).

PP2A is known to activate the FOXO transcription factor in the context of oxidative stress, suggesting the possible involvement of FOXO in the regulation of the initial transcriptional response to desiccation in *H. dujardini*. Two FOXO-like transcription factors were found in the available genome. Knockdown of these FOXO genes using recently available RNA interference techniques will clarify the involvement of FOXO in anhydrobiosis in *H. dujardini*.

Previously identified heat-soluble proteins unique to tardigrades, such as CAHS, SAHS, and MAHS, are proposed to contribute to desiccation tolerance through the protection of biomolecules against desiccation stress. A recent study demonstrated that introducing the MAHS protein of *R. varieornatus* to human cultured cells increased their tolerance to osmotic stress induced by sucrose supplementation [Tanaka et al. 2015]. Osmotic stress likely occurs at the initial phase of desiccation, and therefore these tardigrade-unique heat-soluble proteins could be involved in anhydrobiosis. There are 9 *cahs* genes, 10 *sahs* genes, and 1 *mahs* gene in

the genome. Transcriptome analysis and subsequent validation by qRT-PCR revealed that three *cahs* genes were upregulated during preconditioning (Fig. 13 and Table 6). Why were only three of the genes in the *cahs* gene family upregulated upon desiccation stimuli? There are two possible explanations for this. One possibility is that only three desiccation-induced gene members are involved in anhydrobiosis and the other members have different roles. The other possibility is that the constitutive expression levels of the non-induced gene members are sufficient to enable anhydrobiosis. These two possibilities could be examined by loss of function analyses of each gene using RNA interference. Elucidation of the involvement of tardigrade-unique genes and characterization of each gene in anhydrobiosis will clarify the anhydrobiotic mechanisms unique to tardigrades. Curiously, the regulatory mechanisms of transcription seem to vary among the three upregulated *cahs* genes, as only one gene member was classified as a primary response gene and the other two were classified as secondary response genes (Fig. 13 and Table 6). Comparison of upstream sequences among differently regulated *cahs* genes will provide valuable information for identifying the cis-regulatory elements.

In summary, I identified 146 upregulated genes during preconditioning with classification as primary and secondary response genes. The upregulation of some primary response genes was inhibited by treatment with CA, a selective inhibitor against PP1/PP2A, suggesting that PP1/PP2A is involved in the signaling pathway that transduces desiccation stimuli to the initial transcriptional response. The identified primary response genes could be useful for elucidating initial regulatory mechanisms of anhydrobiosis through identification of desiccation-responsive cis-elements and corresponding transcription factors.

Conclusion and future perspectives

In this thesis, I investigated the molecular mechanisms regulating anhydrobiosis in *H. dujardini*. Because this species requires preconditioning to acquire tolerability against severe dehydration in a low humidity environment, there could be signal transduction mechanisms to sense desiccation stress and prepare for the upcoming severe dehydration. In Chapter 1, I used a chemical genetic approach and suggested that *de novo* gene expression is required for successful transition to anhydrobiosis, and multiple signaling pathways are involved in the process, including the protein phosphorylation/dephosphorylation and calcium signaling pathways (Fig. 16). In particular, PP1/PP2A activity could play an important role. These findings emphasize the importance of future studies to investigate the regulatory subunits of PP1/PP2A that are required for anhydrobiosis and other signaling molecules involved in the signaling pathway.

Although previous studies suggest that molecular protective mechanisms in anhydrobiotic tardigrades are different from those in other anhydrobiotic animals, whether the signaling mechanisms are different among anhydrobiotic animals was unknown. The PP1 activity might be also involved in the nematode desiccation tolerance, because dauer larvae of *C. elegans* exhibit the dephosphorylation of myosin light chain protein which is the substrate of the core enzyme of PP1 during preconditioning [Erkut et al. 2013]. On the other hand, the mechanisms of sensing desiccation would be species dependent. The brain-deprived larvae of *P. vanderplanki* exhibit similar recovery rates to those of normal larvae after almost complete dehydration, suggesting that the central nervous system plays no role in sensing desiccation in this animal [Watanabe et al. 2002]. In contrast, desiccation tolerance of dauer larvae of *C. elegans* likely depends on two genes, osm11 and osm9, which are expressed in head neurons and required for osmotic avoidance, suggesting that certain head neurons participate in their desiccation tolerance [Erkut et al. 2013]. Based on these notions, it is hypothesized that the

sensory mechanisms vary but the downstream signaling pathways are shared among anhydrobiotic animals. Therefore, it would be interesting to investigate the involvement of PP1/PP2A activity in other anhydrobiotic animals.

In Chapter 2, I performed comparative transcriptome analyses, and identified 146 upregulated genes during preconditioning. Surprisingly, more than 70% of these upregulated genes were unannotated, indicating that tardigrades developed their own anhydrobiotic mechanisms. Previous studies suggest that multiple factors are required to enable anhydrobiosis [Oliver et al. 2001; Shannon et al. 2005]. In fact, introduction of only single putative protective molecule such as trehalose and LEA protein is not sufficient to confer tolerance against desiccation and/or osmotic stresses on desiccation-sensitive cultured cells [García et al. 2000; Guo et al. 2000; Tanaka et al. 2015]. To develop the applied technologies for the preservation of desiccation-sensitive cells at ambient temperature, it would be necessary to identify the complete gene set essential for anhydrobiosis. The identified upregulated genes in this study are good candidates for such molecules. Additional transcriptome data on preconditioning from tardigrades pre-treated with CA or other chemicals identified in this study will be also helpful. Because pre-treatment with CA significantly impaired anhydrobiotic survival and upregulation of some primary response genes, the transcription of some essential genes could be collectively regulated downstream of the signaling pathway inhibited by CA.

I classified the upregulated genes as primary response genes and secondary response genes. Quantitative RT-PCR analyses of selected primary response genes revealed that the upregulation of some primary response genes was impaired by pre-treatment with a PP1/PP2A inhibitor, cantharidic acid. This finding suggests that PP1/PP2A is involved in the signaling pathway connecting desiccation stimuli and the initial transcriptional response (Fig. 16). The identified primary response genes could become powerful tools for investigating transcriptional regulation at the initial phase of desiccation stress in *H. dujardini*. To identify

cis-regulatory elements responsive to desiccation in primary response gene loci, gene editing techniques such as CRISPR/Cas9 should be a suitable method [Mali et al. 2013]. The transcription factor, FOXO, and its binding elements are the primary candidates for the future detailed studies. As FOXO is involved in the protection against oxidative stress, molecular mechanisms regulating anhydrobiosis may have a commonality to those regulating oxidative stress. One experiment to test this possibility is to investigate the anhydrobiotic survival in a tardigrade pre-treated with an anti-oxidant agent. If reactive oxygen species have some roles in the induction of anhydrobiosis, the tardigrade pre-treated with an anti-oxidant agent would show decreased recovery rates after desiccation exposure. The identified upregulated genes can be used as a desiccation marker. Thus, examining the responsiveness of such a marker gene to various environmental stresses will clarify the common or different features of the molecular mechanisms regulating anhydrobiosis in the tardigrade from those regulating other stress responses.

This is the first study suggesting the involvement of PP1/PP2A activity in anhydrobiotic survival and particularly in the initial transcriptional response to desiccation stimuli during preconditioning in tardigrades. Future detailed studies will lead to a more comprehensive understanding of the molecular mechanisms regulating anhydrobiosis in tardigrades.

In contrast to *H. dujardini*, *R. varieornatus* does not require preconditioning prior to severe dehydration. In the present study, I revealed that anhydrobiotic survival in *R. varieornatus* was unaffected by a transcription inhibitor, suggesting that *de novo* gene expression is not necessary for anhydrobiosis in this species. These findings together with the previous transcriptome analysis revealing no significant change during desiccation indicate that *R. varieornatus* constitutively synthesizes and holds essential molecules involved in desiccation tolerance. Because the different preconditioning requirements between *H. dujardini* and *R. varieornatus* are well correlated with the water availability of their habitats

(Fig. 1B), *R. varieornatus* could inhabit moss where the animals frequently encounter severe dehydration, due to its constitutive anhydrobiotic ability. As revealed in this study, *H. dujardini* has desiccation-responsive mechanisms including transcriptional regulation, but the molecular mechanisms regulating constitutive expression of protective molecules in *R. varieornatus* remain totally unknown. The underlying molecular mechanisms could be found in the promoter sequences of required protective molecules and/or upstream signaling molecules which are constitutive active without desiccation stimuli. To clarify molecular mechanisms achieving constitutive protection in *R. varieornatus*, molecular regulatory mechanisms found in *H. dujardini* should provide effective strategies. The comparison of molecular mechanisms regulating anhydrobiosis between *H. dujardini* and *R. varieornatus* will enhance our understanding of how organisms extend their habitats.

Tables

Table 1. List of chemicals assayed in the screening and results.

Group ID	Chemical ID	Chemicals	Concentration	N	Number of tardigrades	Recovery rates (%) 1 hour after rehydration		Recovery rates (%) 24 hours after rehydration	
						Raw Data	% of DMSO	Raw Data	% of DMSO
1	Control	DMSO	1%	3	10	76 ± 8	100 ± 11	89 ± 11	100 ± 12
	1	SP600125	100 µM	3	10	87 ± 6	115 ± 8	93 ± 13	104 ± 14
	2	Furafylline	100 µM	3	10	63 ± 21	84 ± 28	90 ± 10	101 ± 11
	3	Lithium Chloride	100 µM	3	10	83 ± 5	110 ± 7	97 ± 6	110 ± 7
2	Control	DMSO	1%	3	10	87 ± 6	100 ± 7	83 ± 6	100 ± 7
	4	Nocodazole	100 µM	3	10	93 ± 6	107 ± 7	96 ± 6	116 ± 8
	5	MRS 1845	100 µM	3	10	60 ± 17	69 ± 20	80 ± 20	96 ± 24
	6	Propafenone hydrochloride	100 µM	3	10	90 ± 10	104 ± 12	100 ± 0	120 ± 0
3	Control	DMSO	1%	3	10	93 ± 6	100 ± 6	96 ± 6	100 ± 6
	7	Taxol	100 µM	3	10	83 ± 12	90 ± 12	83 ± 6	87 ± 6
	8	Zonisamide sodium	100 µM	3	10	97 ± 6	104 ± 6	100 ±0	104 ± 0
	9	1400W dihydrochloride	100 µM	3	10	87 ± 6	93 ± 6	87 ± 6	90 ± 6
	10	Acetazolamide	100 µM	3	10	97± 6	104 ± 6	100 ± 0	104 ± 0
4	Control	DMSO	1%	3	10	71 ± 14	100 ± 1	89 ± 10	100 ± 11
	11	Artemisinin	100 µM	3	10	97 ± 6	135 ± 8	100 ± 0	112 ± 0
	12	Deguelin	100 µM	3	10	73 ± 6	103 ± 8	90 ± 10	101 ± 11
	13	Novobiocin, Na salt	100 µM	3	10	93 ± 12	131 ± 16	100 ± 0	112 ± 0
	14	Sulindac Sulfide	100 µM	3	10	63 ± 40	89 ± 57	63 ± 40	71 ± 45
5	Control	DMSO	1%	3	10	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	15	K-252a	100 µM	3	10	97 ± 6	97 ± 6	100 ± 0	100 ± 0
	16	5-azacytidine	100 µM	3	10	94 ± 10	94 ± 10	100 ± 0	100 ± 0
	17	CBIQ	100 µM	3	10	83 ± 19	83 ± 19	83 ± 19	83 ± 19
	18	Chelerythrine chloride	100 µM	3	10	79 ± 1	79 ± 1	97 ± 6	97 ± 6
6	Control	DMSO	1%	3	10	77 ± 12	100 ± 16	83 ± 6	100 ± 7
	19	Me-3,4-dephostatin	100 µM	3	10	97 ± 6	126 ± 8	93 ± 12	112 ± 14
	20	SU 6656	100 µM	3	10	80 ± 17	104 ± 23	90 ± 0	108 ± 0
	21	(-)-Perillic acid	100 µM	3	10	68 ± 21	89 ± 27	75 ± 23	90 ± 27
	22	Phloretin	100 µM	3	10	69 ± 20	89 ± 25	78 ± 19	93 ± 23
	23	SMER28	100 µM	3	10	87 ± 14	113 ± 19	90 ± 17	109 ± 20
7	Control	DMSO	1%	3	10	82 ± 17	100 ± 21	94 ± 10	100 ± 11
	24	Rottlerin	100 µM	3	10	97 ± 6	118 ± 7	97 ± 6	102 ± 6
	25	Ruthenium red	100 µM	3	10	73 ± 21	89 ± 25	81 ± 1	85 ± 1
	26	SCH-202676 hydrobromide	100 µM	3	10	77 ± 12	93 ± 14	87 ± 15	92 ± 16
	27	SKF 96365	100 µM	3	10	100 ± 0	122 ± 0	97 ± 6	102 ± 6
	28	BIX 01294 trihydrochloride hydrate	100 µM	3	10	90 ± 17	109 ± 21	100 ± 0	106 ± 0
8	Control	DMSO	1%	3	10	97 ± 6	100 ± 6	92 ± 7	100 ± 8
	29	Cyclosporin A	20 µM	3	10	97 ± 17	93 ± 18	93 ± 12	102 ± 13
	30	Althiazide	20 µM	3	10	81 ± 19	84 ± 19	89 ± 10	97 ± 11
	31	Decamethonium bromide	20 µM	3	10	100 ± 0	103 ± 0	100 ± 0	109 ± 0
	32	L-Buthionine-sulfoximine	100 µM	3	10	92 ± 7	95 ± 7	96 ± 7	104 ± 8
	33	Diltiazem hydrochloride	100 µM	3	10	93 ± 12	97 ± 12	97 ± 6	105 ± 6
9	Control	DMSO	1%	3	10	83 ± 6	100 ± 13	90 ± 10	100 ± 1
	34	WS-12	100 µM	3	10	88 ± 11	105 ± 14	92 ± 8	102 ± 8
	35	Paxilline	100 µM	3	10	97 ± 6	116 ± 7	97 ± 6	107 ± 6
10	Control	DMSO	1%	3	10	92 ± 7	100 ± 8	92 ± 7	100 ± 8
	36	Dantrolene sodium	100 µM	3	10	80 ± 16	88 ± 17	84 ± 19	91 ± 21
	37	GW5074	100 µM	3	10	83 ± 19	91 ± 21	92 ± 14	100 ± 16
11	control	DMSO	1%	4	30	83 ± 11	100 ± 13	87 ± 8	100 ± 9
	38	5-(N,N-Dimethyl)amiloride hydrochloride	100 µM	4	30	72 ± 11	87 ± 13	78 ± 10	90 ± 12
	39	Bromoenol lactone	100 µM	4	30	61 ± 15	73 ± 18	71 ± 5	82 ± 6
	40	Diacylglycerol Kinase Inhibitor II	100 µM	4	30	77 ± 12	92 ± 14	90 ± 12	103 ± 13
	41	Furosemide	100 µM	4	30	64 ± 20	76 ± 24	76 ± 15	88 ± 17
	42	LY-294,002 hydrochloride	100 µM	4	30	80 ± 8	95 ± 9	88 ± 4	102 ± 5
	43	8-Methoxymethyl-3-isobutyl-1-methylxanthine	100 µM	4	30	88 ± 8	106 ± 9	90 ± 5	104 ± 5
	44	MJ33	100 µM	4	30	74 ± 12	88 ± 15	84 ± 7	96 ± 9
	45	NS 2028	100 µM	4	30	74 ± 26	88 ± 31	85 ± 15	97 ± 17
12	control	DMSO	1%	4	30	80 ± 14	100 ± 18	83 ± 12	100 ± 18
	46	T-0156	100 µM	4	30	71 ± 18	89 ± 22	80 ± 10	97 ± 12
	47	U0126	100 µM	4	30	60 ± 34	75 ± 43	79 ± 21	95 ± 26
	48	Caffeic acid	100 µM	4	30	85 ± 15	107 ± 19	89 ± 11	108 ± 13
	49	J-8, HCl	100 µM	4	30	27 ± 26	34 ± 33	38 ± 31	46 ± 38
	50	SB239063	100 µM	4	30	79 ± 8	99 ± 10	85 ± 6	103 ± 7
	51	CTPB	100 µM	4	30	87 ± 10	109 ± 12	88 ± 9	107 ± 11
	52	Triptolide;PG490	100 µM	4	30	3 ± 3	3 ± 4	13 ± 16	16 ± 20
	53	Evodiamine	100 µM	4	30	90 ± 5	113 ± 6	93 ± 3	113 ± 4

13	control	DMSO	1%	4	30	89 ± 12	100 ± 13	90 ± 10	100 ± 11
	54	H-89, 2HCl	100 μM	4	30	63 ± 26	71 ± 29	75 ± 27	83 ± 30
	55	3-Bromo-7-nitroindazole	100 μM	4	30	58 ± 12	65 ± 13	65 ± 22	72 ± 24
	56	Benzamil hydrochloride	100 μM	4	30	79 ± 8	88 ± 9	89 ± 9	99 ± 10
	57	Supercinnamaldehyde	100 μM	4	30	64 ± 28	71 ± 31	78 ± 20	87 ± 22
	58	9-cyclopentyladenine	100 μM	4	30	77 ± 16	86 ± 17	90 ± 13	99 ± 15
	59	Y-27632 dihydrochloride	100 μM	4	30	83 ± 9	93 ± 10	89 ± 6	78 ± 42
	60	MNS	100 μM	4	30	50 ± 20	56 ± 23	58 ± 18	64 ± 20
	61	PD 98,059	100 μM	4	30	89 ± 4	100 ± 5	97 ± 4	107 ± 4
14	control	DMSO	1%	4	30	73 ± 6	100 ± 8	81 ± 7	100 ± 9
	62	Rutaecarpine	100 μM	4	30	57 ± 27	78 ± 37	67 ± 21	83 ± 26
	63	Thio-NADP sodium	100 μM	4	30	53 ± 25	73 ± 35	74 ± 21	92 ± 26
	64	Thapsigargin	100 μM	4	30	72 ± 20	99 ± 28	82 ± 17	101 ± 22
	65	Farnesylthiosalicylic acid	100 μM	4	30	64 ± 20	87 ± 28	84 ± 19	92 ± 23
	66	AGGC	100 μM	4	30	69 ± 14	95 ± 20	79 ± 12	98 ± 14
	67	Aurantimycin A	100 μM	4	30	49 ± 33	67 ± 46	65 ± 27	80 ± 34
	68	ML 141	100 μM	4	30	65 ± 21	88 ± 29	79 ± 6	99 ± 7
	69	Rac1 Inhibitor W56	100 μM	4	30	84 ± 7	115 ± 10	91 ± 5	113 ± 6
15	control	DMSO	1%	4	30	77 ± 12	100 ± 16	85 ± 7	100 ± 8
	70	PF-573228	100 μM	4	30	66 ± 19	85 ± 25	84 ± 9	98 ± 10
	71	Astaxanthin	20 μM	4	30	71 ± 12	93 ± 24	81 ± 5	95 ± 6
	72	Procaine hydrochloride	20 μM	4	30	77 ± 19	100 ± 24	85 ± 15	100 ± 18
	73	Epicatechin-(-)	20 μM	4	30	81 ± 12	106 ± 15	86 ± 10	101 ± 12
	74	Niflumic acid	50 μM	4	30	49 ± 34	64 ± 45	60 ± 39	68 ± 47
	75	D-609 potassium	50 μM	4	30	61 ± 26	79 ± 33	58 ± 32	69 ± 39
	76	BAY 11-7082	50 μM	4	30	45 ± 30	59 ± 39	59 ± 25	66 ± 30
16	control	DMSO	1%	4	30	74 ± 22	100 ± 30	74 ± 24	100 ± 32
	77	Cantharidic Acid	5 μM	4	30	10 ± 7	14 ± 10	12 ± 10	16 ± 13
17	control	DMSO	1%	4	30	75 ± 14	100 ± 19	83 ± 8	100 ± 10
	78	2-APB	10 μM	4	30	16 ± 17	21 ± 23	70 ± 22	84 ± 26
18	Control	DMSO	1%	3	10	73 ± 17	100 ± 23	75 ± 13	100 ± 17
	79	4-Aminopyridine	100 μM	3	10	83 ± 6	114 ± 8	80 ± 10	107 ± 13
19	Control	DMSO	1%	3	10	90 ± 10	100 ± 1	93 ± 6	100 ± 6
	80	ML-9	100 μM	3	10	90 ± 10	100 ± 11	97 ± 6	104 ± 6
20	Control	DMSO	1%	3	10	77 ± 25	100 ± 1	90 ± 10	100 ± 1
	81	Trichostatin A	100 μM	3	10	69 ± 12	89 ± 16	72 ± 7	80 ± 8

The effects of 81 chemicals on anhydrobiotic survival were examined. The 81 chemicals were divided into 20 groups and each group was assayed independently with a separate DMSO-treated control for each group. Tardigrades were treated with chemical solution or 1% DMSO solution (control; blue highlighting) for 5 h prior to the desiccation tolerance assay. After chemical treatment, they were preconditioned at 95% RH for 2 days, followed by dehydration at 10% RH for 2 days, and humidification at 95% RH for 1 day. Recovery was examined at 1 h and 24 h after rehydration. In the list, recovery rates are shown as raw data as well as relative values normalized with that of DMSO-treated control (% of DMSO) in the same group (N=3 or 4; 10 to 30 tardigrades each). For each group, statistically significant differences compared with the DMSO-treated control were determined using Dunnett's test (Groups 1 - 15) or Student's *t*-test (Groups 16 - 20). Pink highlighting indicates five chemicals that exhibited significant inhibitory effects ($P<0.01$). With regard to the other chemicals that did not exhibit significant inhibitory effects, we cannot exclude the possibility that concentrations used in the screening were not appropriate and/or insufficient effects on the tardigrade.

Table 2. Primers used for quantification of gene expression by qRT-PCR

Gene ID	Primers		Product size (bp)
	Left	Right	
g00176	GGGAGACGAAGGTGTCCAG	GAAAAAGCATCCTTGGCATC	118
g03577	GTCGGAGTGGGATCAGTGTC	TCAATCCTTCCAGGTTGGTC	103
g11085	AGCTTTTCAGCAGGGAATCA	GACTCTTCCAGTTCGGCTTG	171
g16517	AGATGGGGAGTGTTTTCACG	CACCCGAAAAGGCATTAGAA	144
g03020	TCAAGAATATCGGCGAGGAC	ACTCGATACGGTTGGGAATG	117
g00353	GGTCAACAGCAAGAGCAACA	AGCTGATGAAAGGTGCGATT	106
g04939	TCGGAAGGACTTGCTAAGGA	CCTGGGCTAGTTGTTCTTGC	166

Table 3. Mapping summary of RNA-seq reads

		Active[#]	D6H[#]	C6H[#]
Left reads	input	72474446	78144503	70520824
	mapped	62219257 (85.8%)	68554795 (87.7%)	63537599 (90.1%)
	multiple aligned	10179803 (16.4%)	12077222 (17.6%)	11298175 (17.8%)
Right reads	input	72474446	78144503	70520824
	mapped	61582111 (85.0%)	67972072 (87.0%)	62667350 (88.9%)
	multiple aligned	9908386 (16.1%)	11711294 (17.2%)	11020141 (17.6%)
Overall read mapping rate[+]		85.40%	87.40%	89.50%
Aligned pairs		57170118	63136781	58440521
	multiple aligned	9484900 (16.6%)	11187555 (17.7%)	10492734 (18.0%)
	discordant alignments	745896 (1.3%)	914657 (1.4%)	764324 (1.3%)
Concordant pair alignment rate[++]		77.90%	76.90%	81.80%
Uniquely mapped pairs rate[+++]		64.80%	65.30%	66.90%

[#] Transcriptomes were analyzed by RNA-seq in active hydrated tardigrades (Active), 6h-preconditioned tardigrades after 5h-incubation in solution of 1% DMSO (D6H) or 500 μM cycloheximide (C6H).

[+] Calculated as follows; (Left reads mapped + Right reads mapped)/2*100.

[++] Calculated as follows; (Aligned pairs - discordant alignments) / input*100.

[+++] Calculated as follows; (Aligned pairs - multiple aligned of Aligned pairs - discordant alignments) / input*100.

Table 4. Upregulated genes during preconditioning

	Total	Annotated	Unannotated
All protein-coding genes	23021	12002 (52.1%)	11019 (47.9%)
FPKM≧1 (in both Active and D6H)[#]	14593	9641 (66.1%)	4952 (33.9%)
Upregulated genes (≧4-fold)[+]	146	42 (28.8%)	104 (71.2%)

[#] Only genes with more than 1 FPKM in both Active and D6H were targets for further analyses.

[+] Expression levels of D6H were compared with those of Active, and the genes showing more than 4-fold upregulation in D6H were defined as upregulated genes.

Table 5. GO term enrichment in annotated upregulated genes

GO term	Description	P-value	FDR q-value	Corresponding human orthologous Genes[+]
GO:0006805[#]	xenobiotic metabolic process	2.52E-06	2.76E-02	HPGDS - hematopoietic prostaglandin d synthase (Glutathione S-transferase)
				CYP2D7P1 - cytochrome p450, family 2, subfamily d, polypeptide 7 pseudogene 1
				UGT1A6 - udp glucuronosyltransferase 1 family, polypeptide a6
				CRYZ - crystallin, zeta (quinone reductase)
				CYP2J2 - cytochrome p450, family 2, subfamily j, polypeptide 2
GO:0042738	exogenous drug catabolic process	3.72E-04	1.00E+00	CYP2D7P1 - cytochrome p450, family 2, subfamily d, polypeptide 7 pseudogene 1
				CYP2J2 - cytochrome p450, family 2, subfamily j, polypeptide 2
GO:0042737	drug catabolic process	5.44E-04	1.00E+00	CYP2D7P1 - cytochrome p450, family 2, subfamily d, polypeptide 7 pseudogene 1
				CYP2J2 - cytochrome p450, family 2, subfamily j, polypeptide 2

[#] Only significantly enriched GO term in biological process. The GO terms with both P-value and FDR q-value less than 5.00E-02 were considered to be significantly enriched.

[+] Corresponding to upregulated *H. dujardini* genes

Table 6. Expression levels of *cahs*, *sahs* and *mahs* genes estimated in RNA-seq analysis

	Gene ID	FPKM		Fold change(log2)*	FPKM		Fold change(log2)*
		Active	D6H		Active	C6H	
cahs#	g00353+	99.5205	477.652	2.26289	84.3914	128.649	0.608268
	g00541	95.8308	203.372	1.08556	81.2633	176.811	1.12153
	g00542	306.199	333.447	0.122987	259.561	1533.32	2.56252
	g03020+	871.837	4427.37	2.34432	739.005	8575.88	3.53663
	g03640	230.416	683.212	1.56809	195.344	1354.93	2.79413
	g04939+	223.172	1425.01	2.67475	189.237	338.357	0.838351
	g09023	598.857	1419.89	1.2455	507.816	1731.69	1.7698
	g15389	2401.36	8290.42	1.78759	2035.82	24533.7	3.59108
	g16095	31.6437	37.1105	0.229909	26.8292	17.5073	-0.615848
sahs#	g01000	304.986	295.346	-0.0463385	258.7	72.3317	-1.83858
	g05537	21.6436	15.3596	-0.494795	18.3651	9.10151	-1.01279
	g11961	29.6288	10.9251	-1.43935	25.1313	2.49038	-3.33505
	g17779++	0	70.739	Inf†	0	0	0
	g17780	6802.39	22811	1.74561	5769.06	7683.64	0.413454
	g17781	95.4535	77.8091	-0.294859	80.9524	140.949	0.800026
	g17782	12547.5	20323.1	0.69572	10639.7	1423.01	-2.90244
	g18074	400.098	303.447	-0.398907	339.203	69.2899	-2.29143
	g19251++	2.61171	0.993277	-1.39473	2.2146	0.267486	-3.04951
	g20778	6986.21	13419.1	0.941704	5924.96	4114.37	-0.526136
mahs#	g05570	1211.04	1023.48	-0.242756	1027.04	570.749	-0.847565

Expression levels of *cahs*, *sahs* and *mahs* genes resulted from RNA-seq were analyzed in active hydrated tardigrades (Active), 6h-preconditioned tardigrades after 5h-incubation in solution of 1% DMSO (D6H) or 500 µM cycloheximide (C6H).

+ Upregulated *cahs* genes.

++ *sahs* genes with FPKM less than 1 in Active or D6H, therefore excluded from the analysis.

* Calculated as Log_2(D6H/Active) or Log_2(C6H/Active)

† infinite

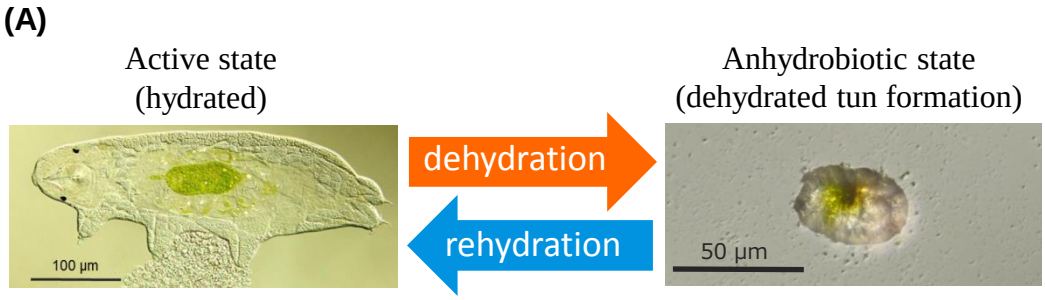
Table 7. A transcription factor and a transcription factor- related gene in annotated upregulated genes

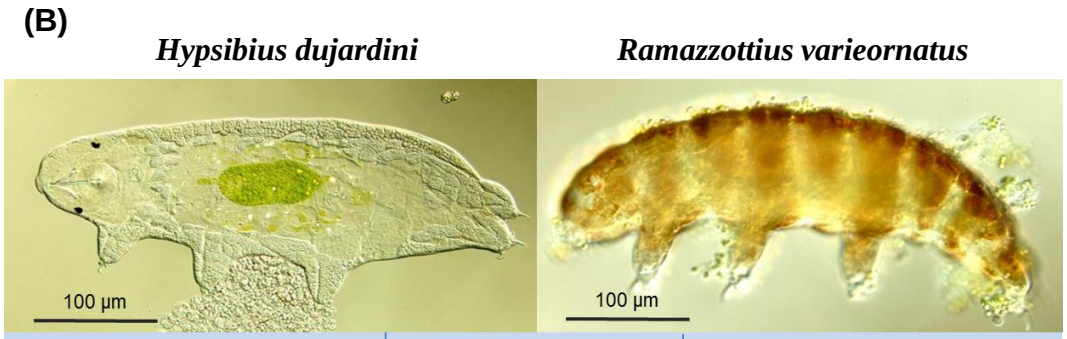
Gene ID	FPKM		Fold change(log2)[+]	FPKM		Fold change(log2)[+]	Annotation
	Active	D6H		Active	C6H		
G01624[#]	4.53015	18.4083	2.02272	3.84153	36.3887	3.24374	Cell death specification protein 2 (ces-2)
G08286[#]	53.0317	255.219	2.26681	44.9891	396.347	3.13912	Upstream activation factor subunit UAF30; AltName: Full=Upstream activation factor 30 KDa subunit; Short=p30

[#] RNA-seq results of the genes annotated as a transcription factor or a transcription factor-related gene in active hydrated tardigrades (Active), 6h-preconditioned tardigrades after 5h-incubation in solution of 1% DMSO (D6H) or 500 µM cycloheximide (C6H).

[+] Calculated as Log_2(D6H/Active) or Log_2(C6H/Active)

Figures





Yes	**Requirement of preconditioning**	No
Benthic sediments in a pond etc.	**Habitats**	Easy to dry moss
Rare	**Frequency of desiccation stress in a habitat**	Frequent
Yes (Revealed in this study)	**Changes in transcriptomes between pre- and post-desiccation**	Not significant (Unpublished data)

Fig 1. Features of anhydrobiotic tardigrades.

(A) Active hydrated state (left) and dehydrated state in tun formation (right) of *Hypsibius dujardin*i. (B) Two distinct anhydrobiotic tardigrades, *H. dujardini and Ramazzottius Varieornatus.*

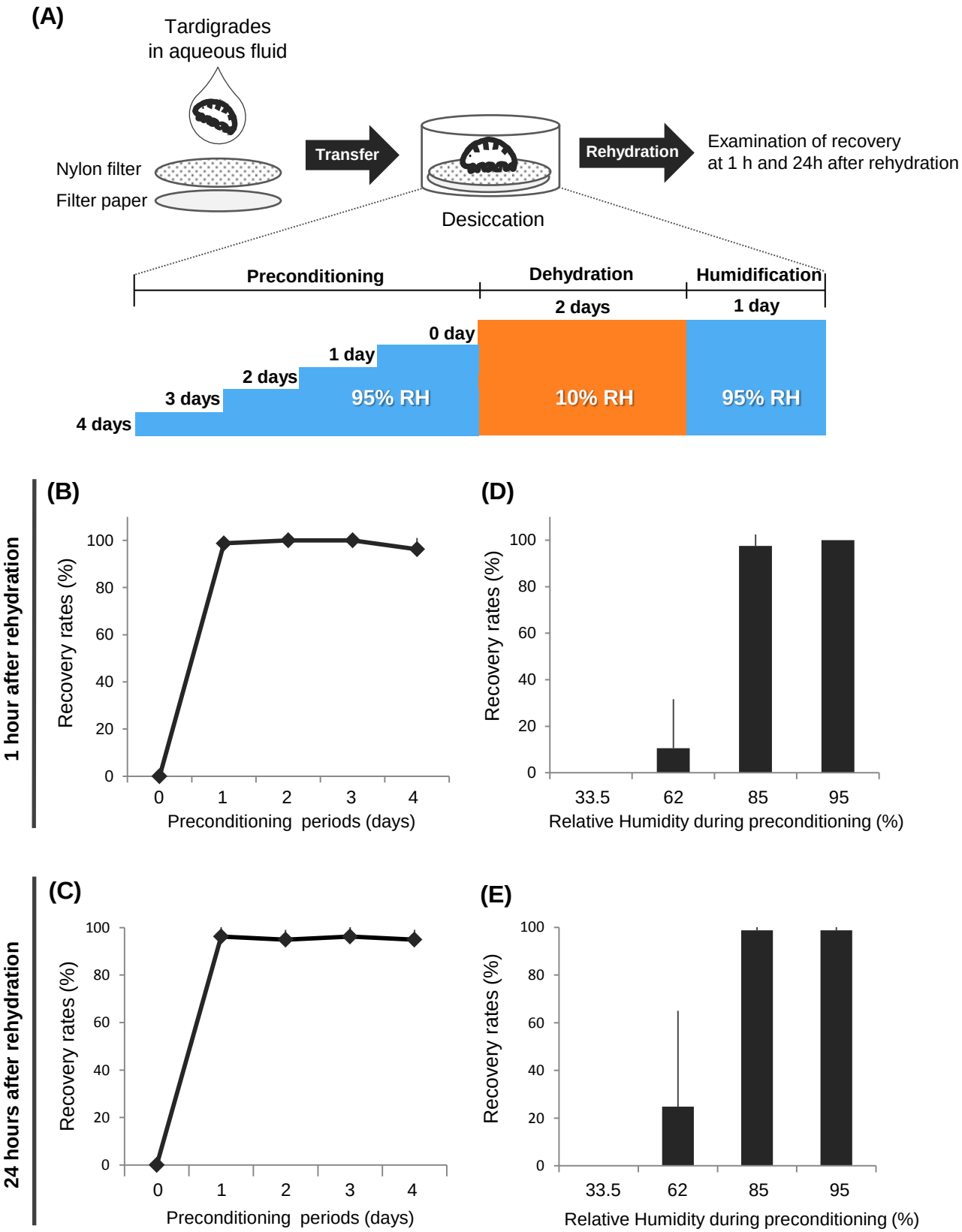
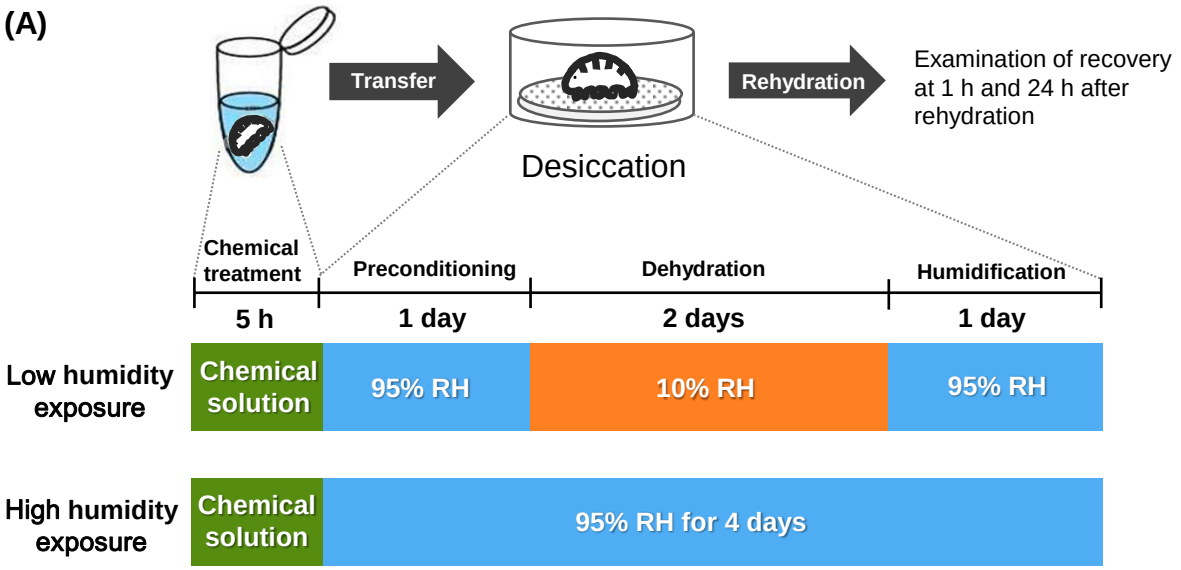

(A)
Tardigrades
in aqueous fluid
Nylon filter
Filter paper
Transfer
Desiccation
Rehydration
Examination of recovery
at 1 h and 24h after rehydration
Preconditioning
Dehydration
Humidification
2 days
1 day
0 day
1 day
2 days
3 days
4 days
95% RH
10% RH
95% RH
1 hour after rehydration
(B)
Recovery rates (%)
Preconditioning periods (days)
(D)
Recovery rates (%)
Relative Humidity during preconditioning (%)
24 hours after rehydration
(C)
Recovery rates (%)
Preconditioning periods (days)
(E)
Recovery rates (%)
Relative Humidity during preconditioning (%)

Fig. 2

Fig 2. Effects of preconditioning conditions on anhydrobiotic survival in Z151 strain of *H. dujardini*.

(A) Experimental scheme of the desiccation tolerance assay. Tardigrades in aqueous fluid were dropped onto a nylon filter placed on filter paper, and preconditioned at 95% RH for 0 to 4 days. After preconditioning, they were desiccated at 10% RH for 2 days and humidified at 95% RH for 1 day prior to rehydration. Recovery rates were examined at 1 h and 24h after rehydration. (B, C) Effects of various preconditioning periods on anhydrobiotic survival at 1h (B) and 24h (C). Data are shown as mean ± SD (N=4; 20 tardigrades each). (D, E) Effects of relative humidity exposed during preconditioning on anhydrobiotic survival at 1h (D) and 24h (E). The preconditioning period was fixed as 1 day, and tardigrades were exposed to 33.5%, 62%, 85%, or 95% RH as preconditioning. Data are shown as mean ± SD (N=4; 20 tardigrades each).


(A)
Transfer
Desiccation
Rehydration
Examination of recovery at 1 h and 24 h after rehydration
Chemical treatment
Preconditioning
Dehydration
Humidification
5 h
1 day
2 days
1 day
Low humidity exposure
Chemical solution
95% RH
10% RH
95% RH
High humidity exposure
Chemical solution
95% RH for 4 days

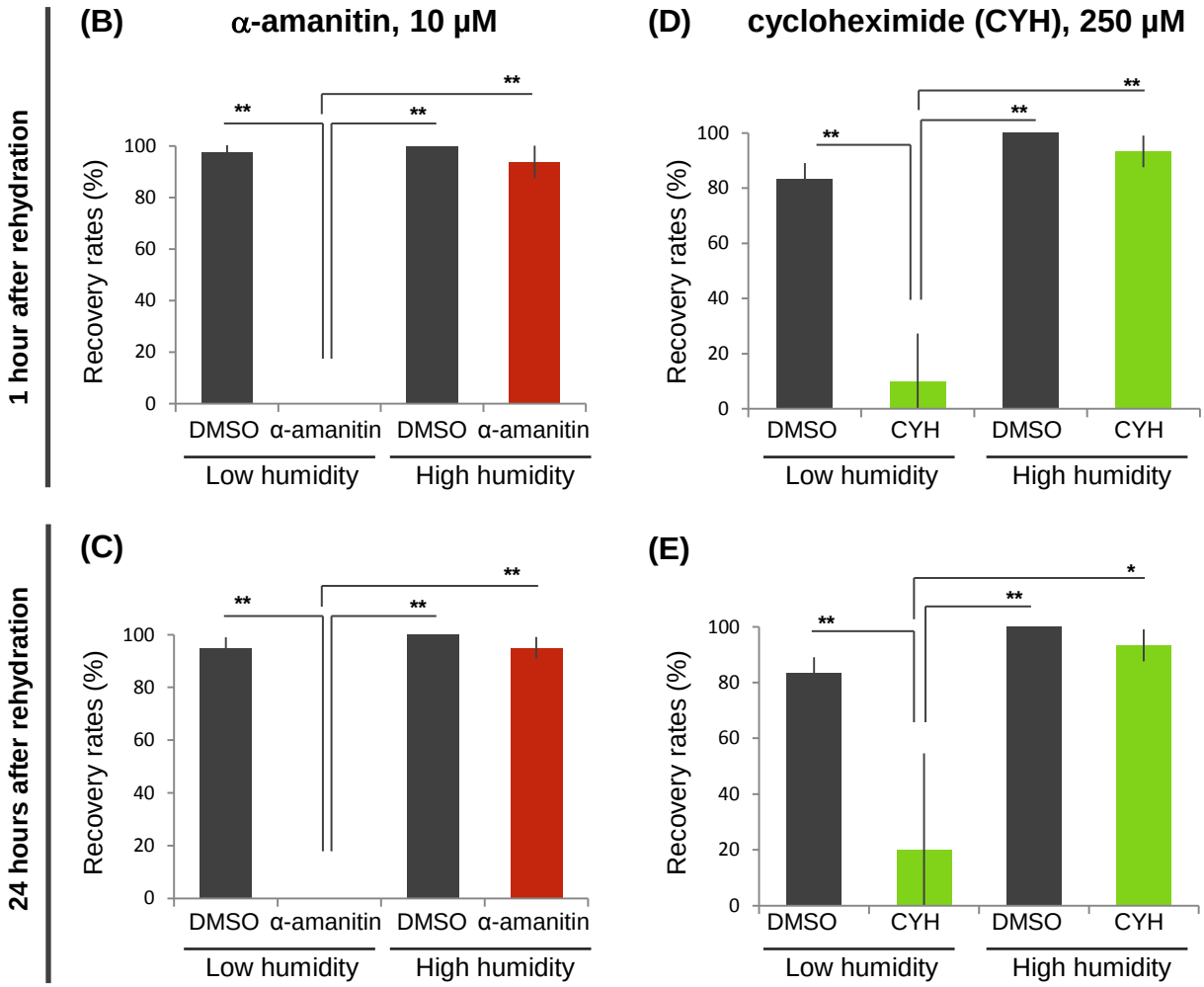

1 hour after rehydration
24 hours after rehydration
(B) α-amanitin, 10 μM
(C)
(D) cycloheximide (CYH), 250 μM
(E)
Recovery rates (%)
DMSO
α-amanitin
CYH
Low humidity
High humidity

Fig. 3

Fig 3. Effects of inhibition of transcription and translation on anhydrobiotic survival in *H. dujardini.*

(A) Scheme of chemical treatment and subsequent desiccation tolerance assay. Tardigrades were incubated in a chemical solution or 1% DMSO solution (control) for 5 h. After chemical treatment and 1 day preconditioning at 95% RH, one group was exposed to low relative humidity (10% RH) for 2 days (Low humidity exposure). The other group was exposed to high relative humidity (95% RH) for same period (High humidity exposure). (B, C) Effects of a transcription inhibitor (10 μM α-amanitin) on recovery rates at 1 h (B) and 24 h (C) after rehydration. Mean ± SD (N=4; 20 tardigrades each). (D, E) Effects of translation inhibitor (250 μM cycloheximide) on recovery rates at 1 h (D) and 24 h (E) after rehydration. Mean ± SD (N=3; 10 tardigrades each). Statistically significant differences among samples were determined by Tukey-Kramer test (*, $P<0.05$; **, $P<0.01$). Low humidity, low humidity exposure; High humidity, high humidity exposure.

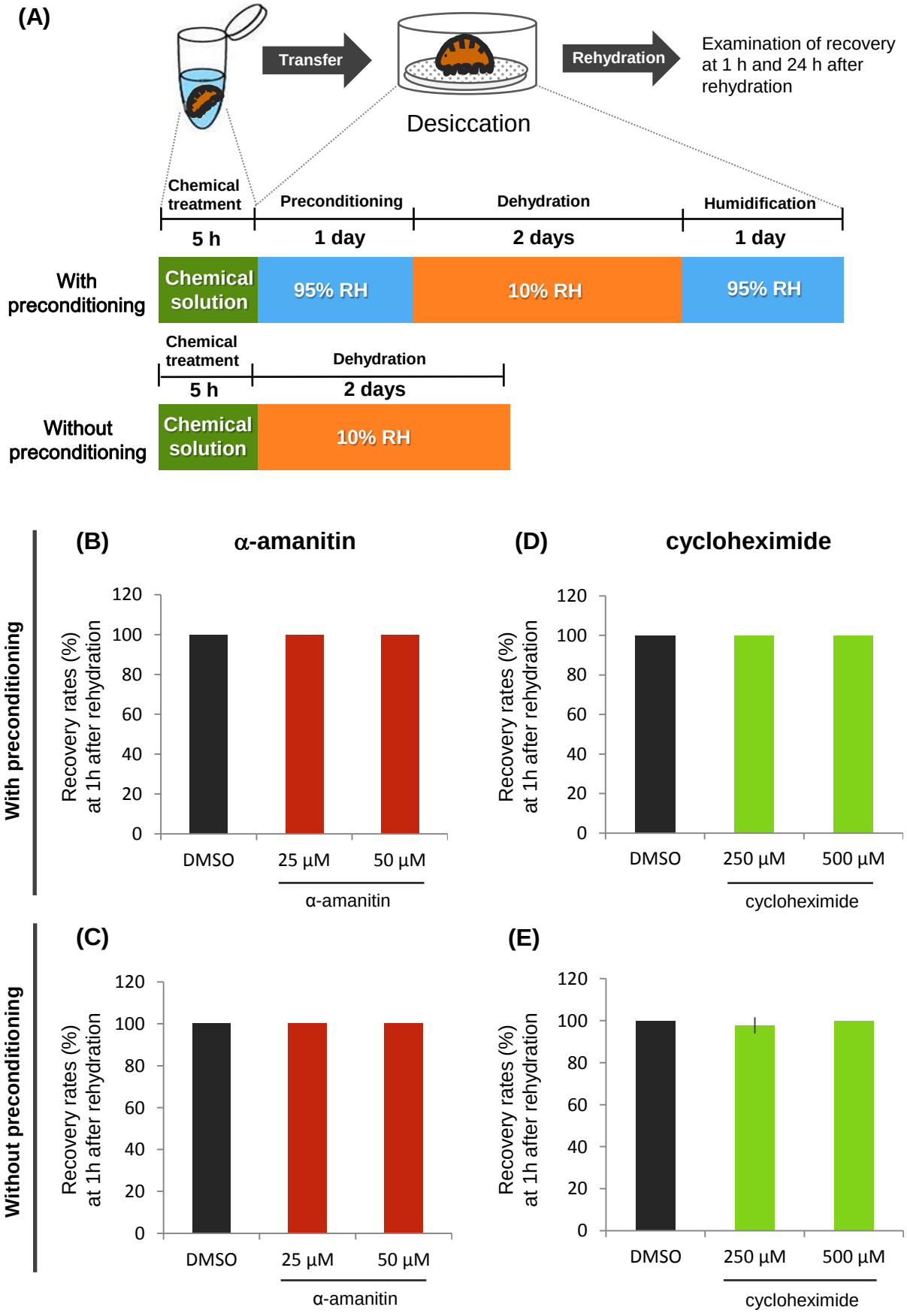

(A)
Transfer
Desiccation
Rehydration
Examination of recovery at 1 h and 24 h after rehydration
Chemical treatment
Preconditioning
Dehydration
Humidification
5 h
1 day
2 days
1 day
With preconditioning
Chemical solution
95% RH
10% RH
95% RH
Chemical treatment
Dehydration
5 h
2 days
Without preconditioning
Chemical solution
10% RH
(B)
α-amanitin
(D)
cycloheximide
With preconditioning
Recovery rates (%) at 1h after rehydration
120
100
80
60
40
20
0
DMSO
25 μM
50 μM
α-amanitin
DMSO
250 μM
500 μM
cycloheximide
(C)
(E)
Without preconditioning

Fig. 4

Fig 4. Effects of inhibition of transcription and translation on anhydrobiotic survival in *R. varieornatus*.

(A) Scheme of chemical treatment and subsequent desiccation tolerance assay. Tardigrades were incubated in a chemical solution or 1% DMSO solution (control) for 5 h. After chemical treatment, one group was preconditioned at 95% RH for 1 day, followed by exposure to 10% RH for 2 days and 95% RH for 1 day (With preconditioning). The other group was exposed to 10% RH for 2 days (Without preconditioning). (B, C) Effects of a transcription inhibitor (25 or 50 μM α-amanitin) on recovery rates at 1 h after rehydration in the desiccation tolerance assay with preconditioning (B) and without preconditioning (C). Mean ± SD (N=3; 10 tardigrades each). (D, E) Effects of translation inhibitor (250 or 500 μM cycloheximide) on recovery rates at 1 h after rehydration in the desiccation tolerance assay with preconditioning (D) and without preconditioning (E). Mean ± SD (N=3; 10 tardigrades each). Statistically significant differences among samples were determined by Dunnett's test, and no significant differences were detected.

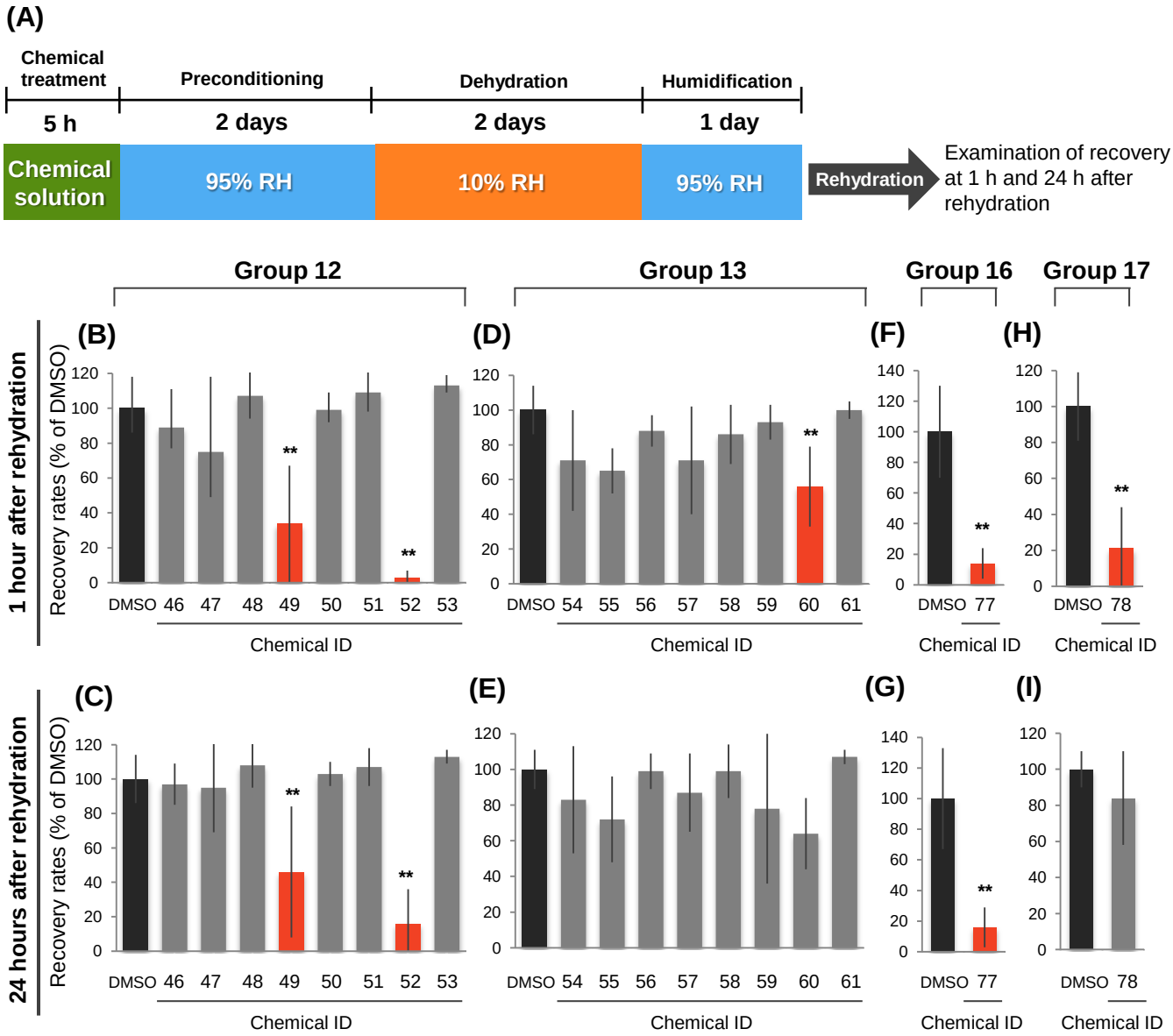

(A)
Chemical treatment
Preconditioning
Dehydration
Humidification
5 h
2 days
2 days
1 day
Chemical solution
95% RH
10% RH
95% RH
Rehydration
Examination of recovery at 1 h and 24 h after rehydration
Group 12
Group 13
Group 16
Group 17
1 hour after rehydration
24 hours after rehydration
Recovery rates (% of DMSO)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
DMSO 46 47 48 49 50 51 52 53
DMSO 54 55 56 57 58 59 60 61
DMSO 77
DMSO 78
Chemical ID
**

Fig. 5

Fig 5. Screening of chemicals inhibiting anhydrobiotic survival.

(A) Experimental scheme used for chemical screening. (B-I) The effects of various chemicals on anhydrobiotic survival in the four experimental groups that contained the chemicals that significantly inhibited anhydrobiotic survival. Significant inhibitory effects were detected for chemicals #49 (J-8) and #52 (triptolide) of Group 12 (B, C); chemical #60 (MNS) of Group 13 (D); chemical #77 (cantharidic acid) of Group 16 (F, G); and chemical #78 (2-APB) of Group 17 (H). At 24 h after rehydration, no significant inhibitory effects of chemical #60 of Group 13 and chemical #78 of Group 17 were detected (E, I). Recovery rates are shown as percent of DMSO control. Mean ± SD (N=4; 30 tardigrades each). In each group, statistically significant differences from the DMSO control were determined by Dunnett's test (Groups 12 and 13) or Student's *t*-test (Groups 16 and 17), **, $P<0.01$.

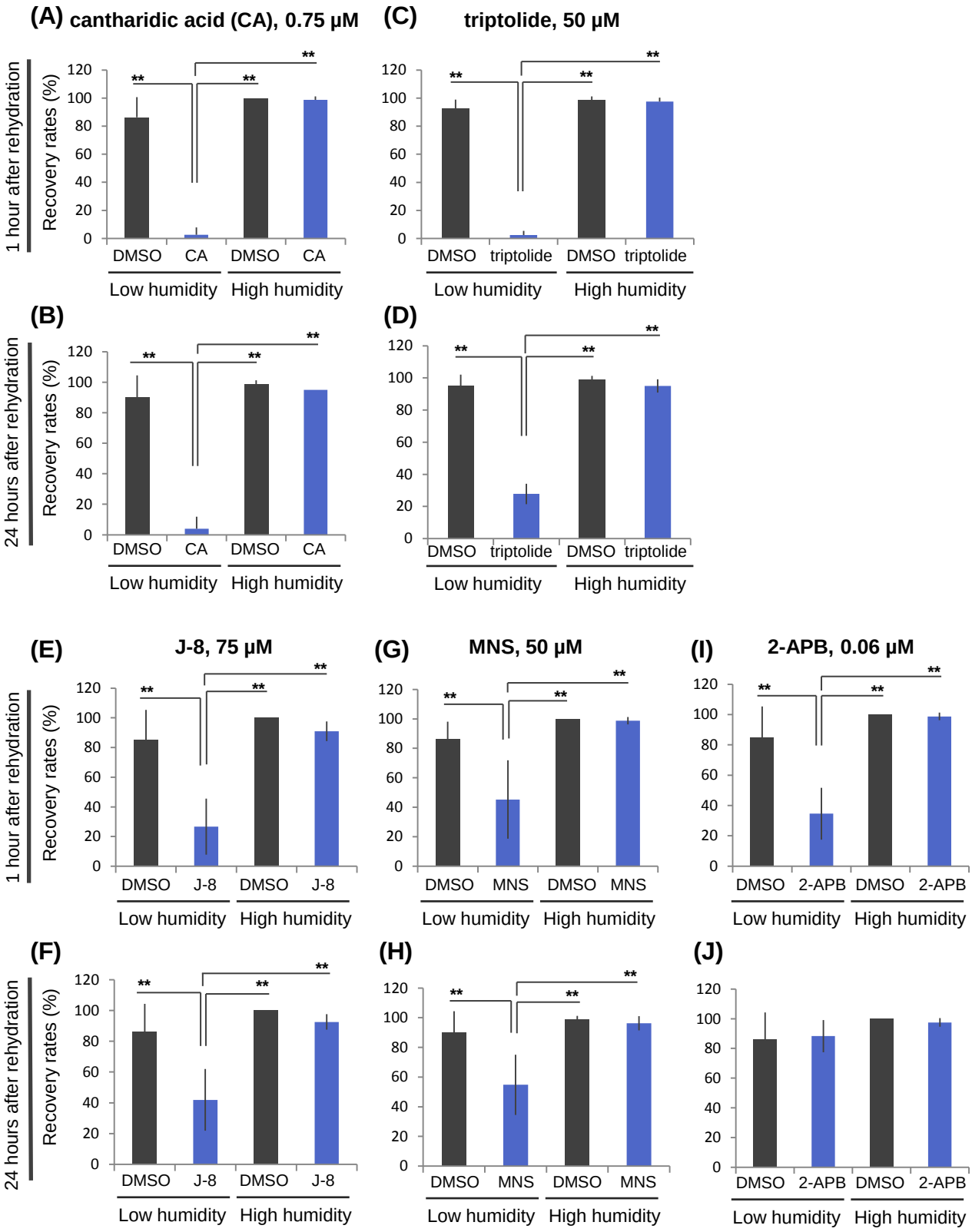
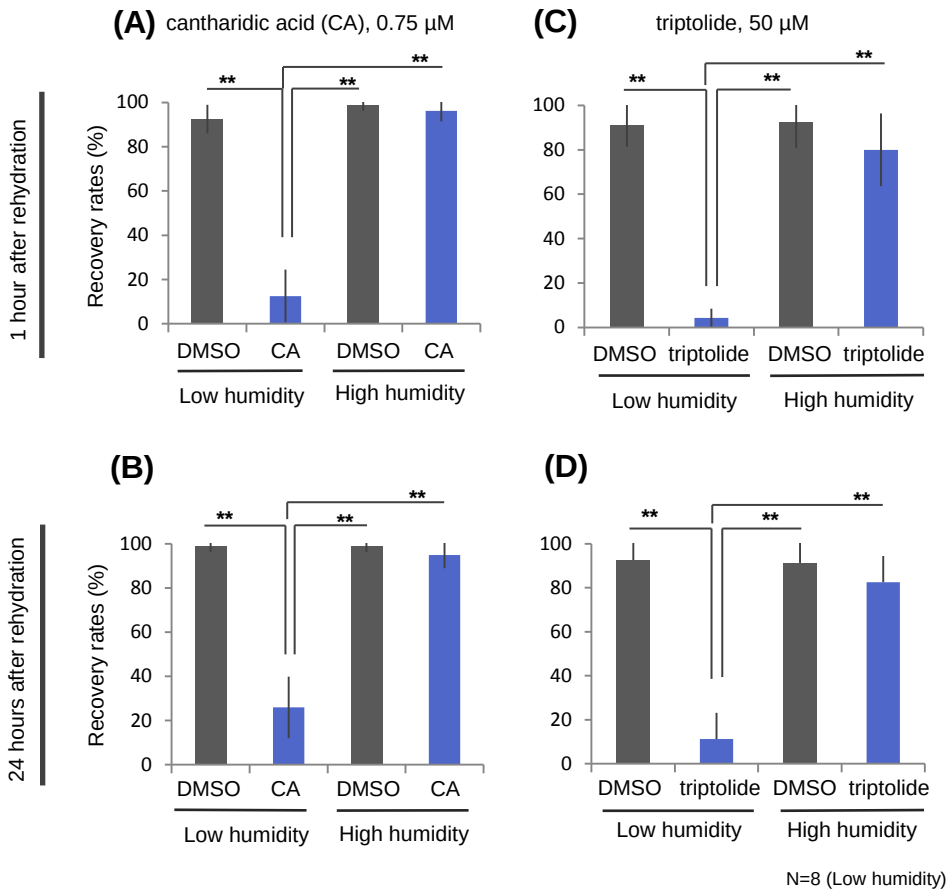

(A) cantharidic acid (CA), 0.75 μM
(C) triptolide, 50 μM
(B)
(D)
(E) J-8, 75 μM
(G) MNS, 50 μM
(I) 2-APB, 0.06 μM
(F)
(H)
(J)
1 hour after rehydration
24 hours after rehydration
Recovery rates (%)
DMSO
CA
triptolide
J-8
MNS
2-APB
Low humidity
High humidity
**

Fig. 6

Fig 6. Specific inhibition of anhydrobiotic survival by identified chemicals.

The specificity of the inhibitory effects on anhydrobiotic survival was examined for the five identified chemicals using essentially the same experimental scheme shown in Fig. 3A. Effects on recovery rates are shown for 0.75 μM cantharidic acid (CA) (A, B); 50 μM triptolide (C, D); 75 μM J-8 (E, F); 50 μM MNS (G, H); and 0.06 μM 2-APB (I, J). Recovery rates were examined at both 1 h (A, C, E, G, I) and 24 h (B, D, F, H, J) after rehydration. Mean ± SD (N=4; 20 tardigrades each). Statistically significant differences among samples were determined by Tukey-Kramer test (**, $P<0.01$). Low humidity, low humidity exposure; High humidity, high humidity exposure.


(A) cantharidic acid (CA), 0.75 μM
(C) triptolide, 50 μM
(B)
(D)
1 hour after rehydration
24 hours after rehydration
Recovery rates (%)
DMSO
CA
triptolide
Low humidity
High humidity
N=8 (Low humidity)

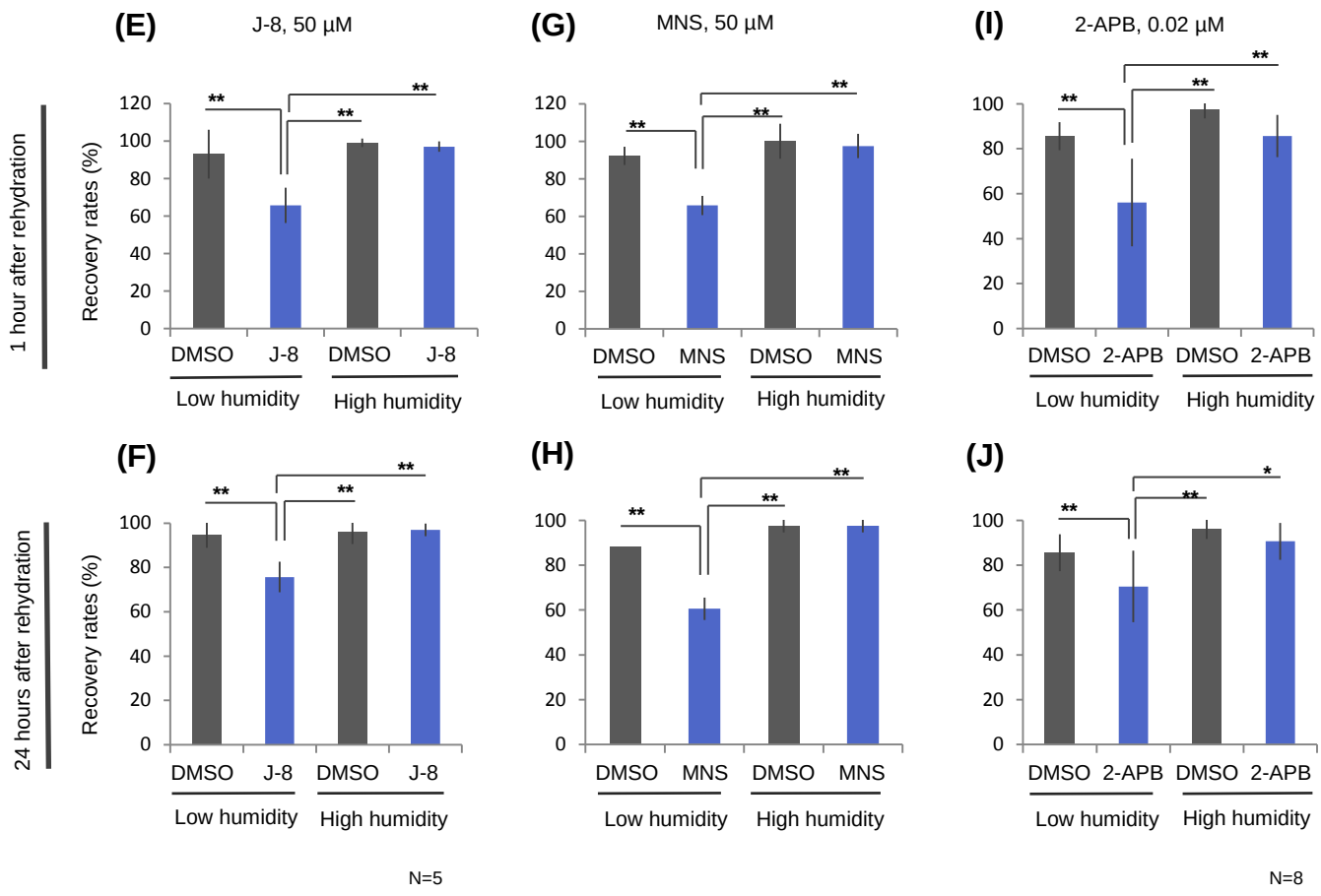

(E) J-8, 50 μM
(G) MNS, 50 μM
(I) 2-APB, 0.02 μM
(F)
(H)
(J)
1 hour after rehydration
24 hours after rehydration
Recovery rates (%)
DMSO
J-8
MNS
2-APB
Low humidity
High humidity
N=5
N=8

Fig. 7

Fig 7. Specific inhibition of anhydrobiotic survival by identified chemicals.

Specific inhibitory effects on anhydrobiotic survival were reproduced for all five identified chemicals. The optimal concentration varied slightly for J-8 and 2-APB (see Fig. 6), possibly due to the differences in the physiological conditions of tardigrades. Effects on recovery rates are shown as mean ± SD for 0.75 μM cantharidic acid (CA) (A, B); 50 μM triptolide (C, D); 50 μM J-8 (E, F); 50 μM MNS (G, H); and 0.02 μM 2-APB (I, J). Recovery rates were examined at both 1 h (A, C, E, G, I) and 24 h (B, D, F, H, J) after rehydration. N=4 unless otherwise stated; 20 tardigrades each. Statistically significant differences among samples were determined by Tukey-Kramer test (*, $P<0.05$; **, $P<0.01$). Low humidity, low humidity exposure; High humidity, high humidity exposure.

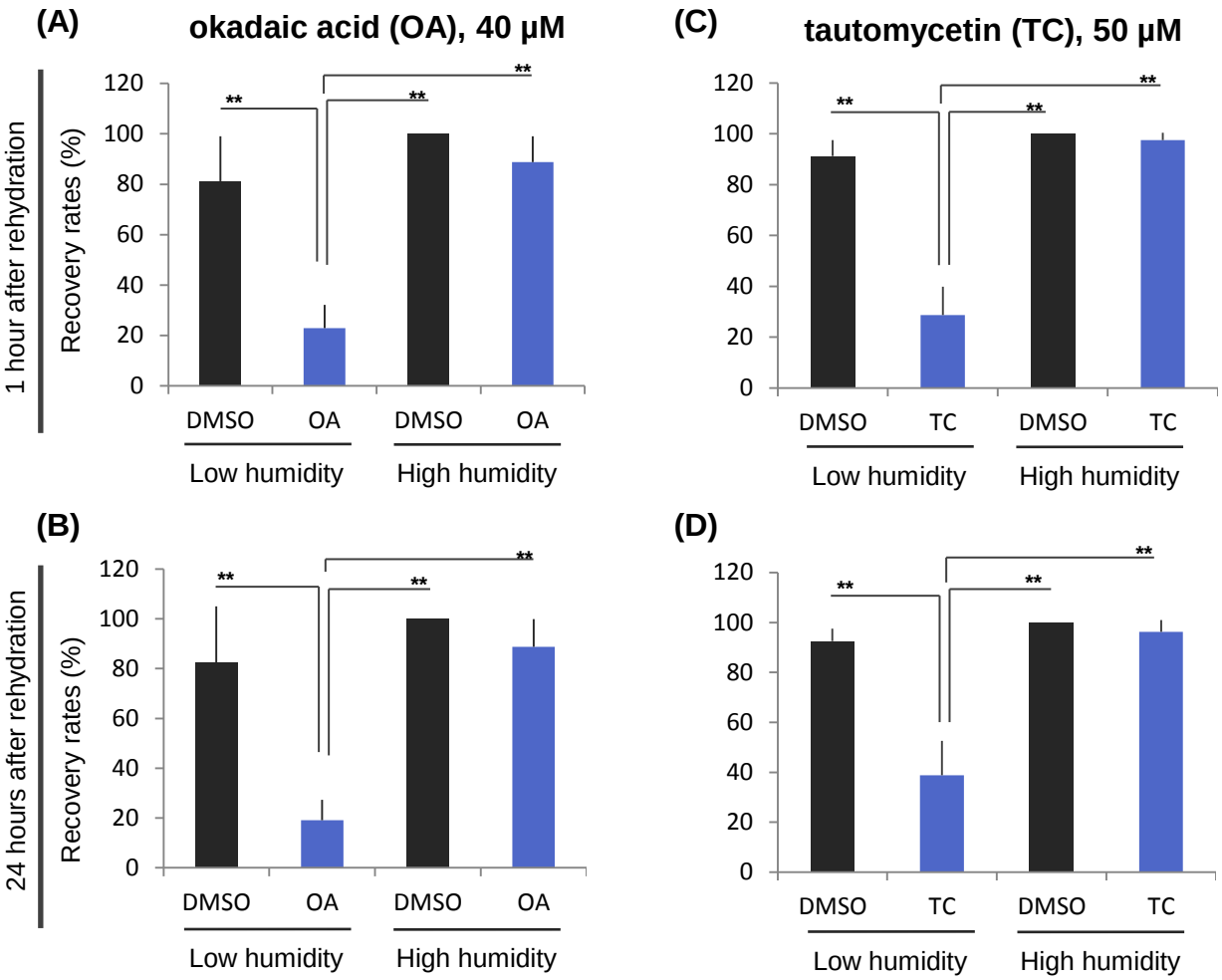

(A)
okadaic acid (OA), 40 μM
1 hour after rehydration
Recovery rates (%)
120
100
80
60
40
20
0
**
**
**
DMSO
OA
DMSO
OA
Low humidity
High humidity
(C)
tautomycetin (TC), 50 μM
**
**
**
DMSO
TC
DMSO
TC
Low humidity
High humidity
(B)
24 hours after rehydration
Recovery rates (%)
**
**
**
DMSO
OA
DMSO
OA
Low humidity
High humidity
(D)
**
**
**
DMSO
TC
DMSO
TC
Low humidity
High humidity

Fig. 8

Fig 8. Inhibition of PP1/PP2A activity impaired anhydrobiotic survival.

The effects of other selective PP1/PP2A inhibitor, okadaic acid (OA) and tautomycetin (TC), on anhydrobiotic survival were examined using essentially the same scheme shown in Fig. 3A, with a longer chemical treatment period for OA (10 h). Forty μM OA specifically inhibited recovery rates after low humidity exposure at both 1 h (A) and 24 h (B) after rehydration. In a similar fashion, 50 μM TC specifically inhibited recovery rates after low humidity exposure at both 1 h (C) and 24 h (D) after rehydration. Mean ± SD (N=4; 20 tardigrades each). Statistically significant differences among samples were determined by Tukey-Kramer test (**, $P<0.01$). Low humidity, low humidity exposure; High humidity, high humidity exposure.

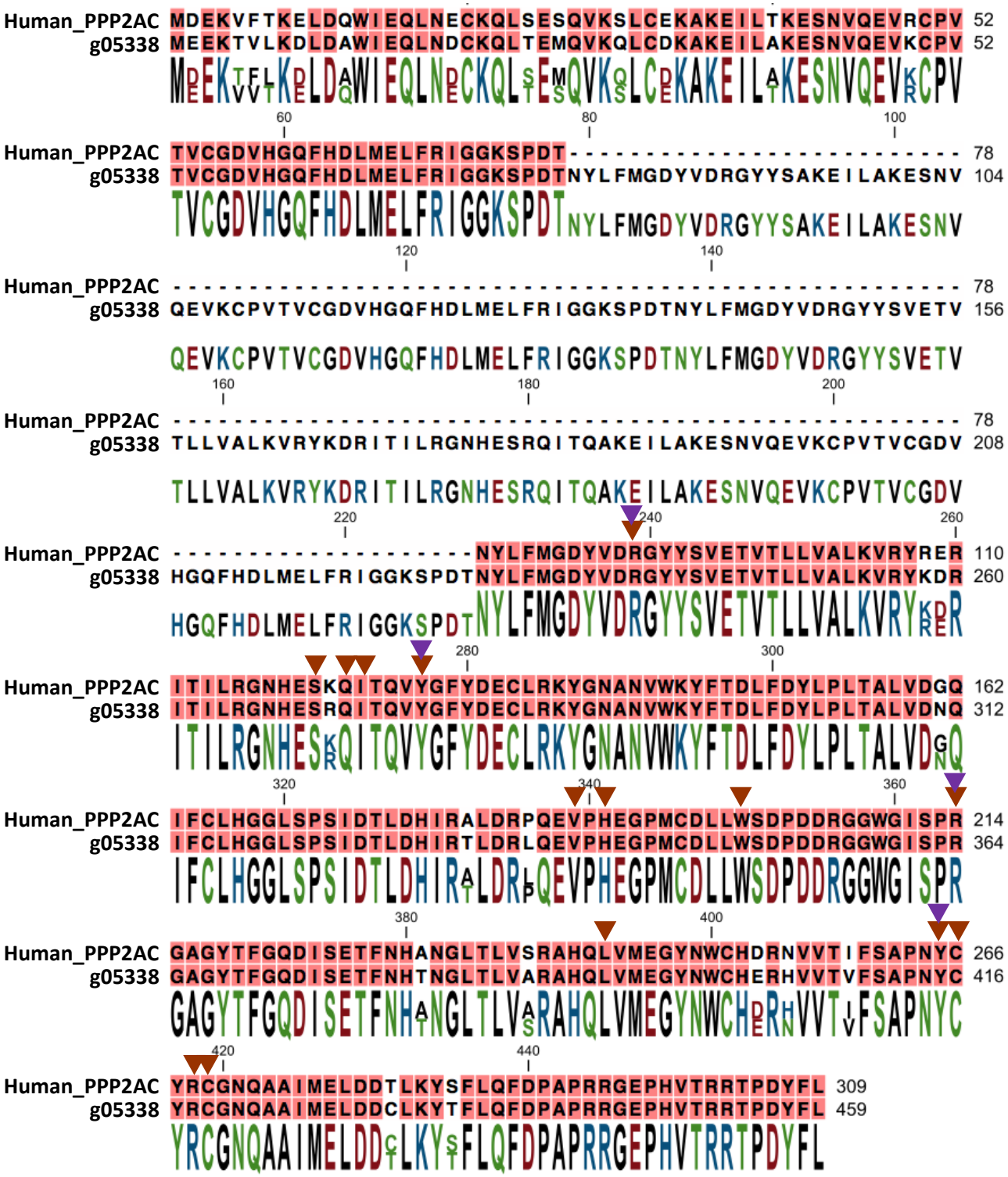
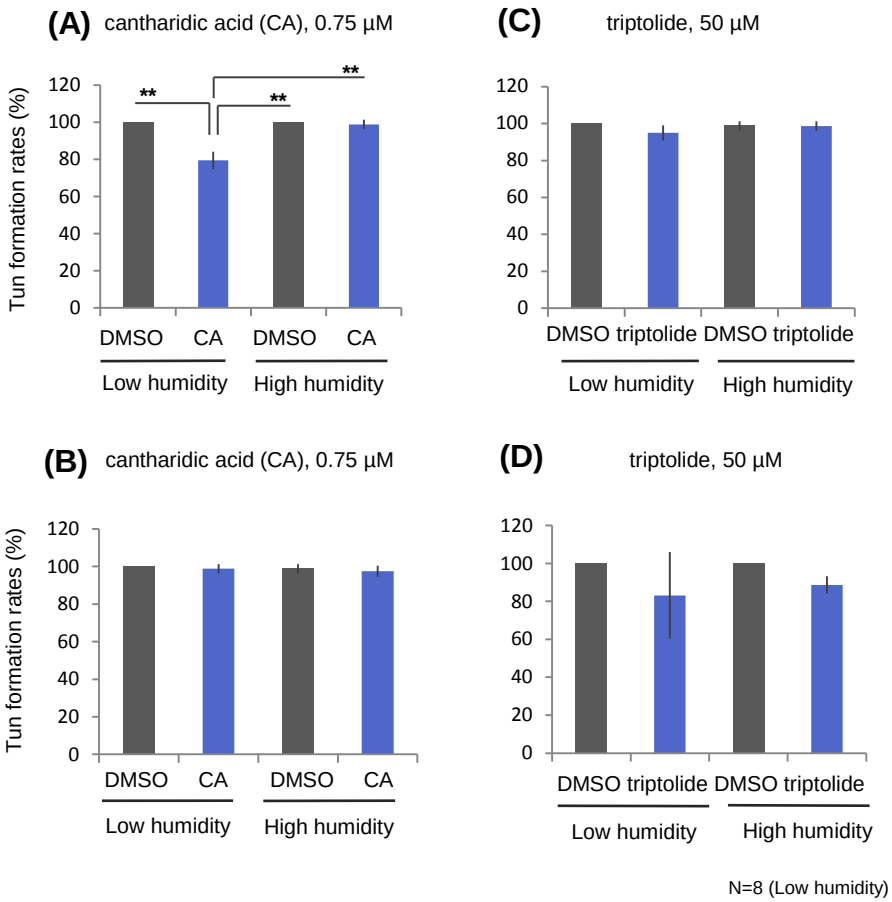

Human_PPP2AC MDEKVFTKELDQWIEQLNECKQLSESQVKSLCEKAKEILTKESNVQEVRCPV 52
g05338 MEEKTVLKDLDAWIEQLNDCKQLTEMQVKQLCDKAKEILAKESNVQEVKCPV 52
60
80
100
Human_PPP2AC TVCGDVHGQFHDLMELFRIGGKSPDT---------- 78
g05338 TVCGDVHGQFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSAKEILAKESNV 104
120
140
Human_PPP2AC ---------- 78
g05338 QEVKCPVTVCGDVHGQFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETV 156
160
180
200
Human_PPP2AC ---------- 78
g05338 TLLVALKVRYKDRITILRGNHESRQITQAKEILAKESNVQEVKCPVTVCGDV 208
220
240
260
Human_PPP2AC ----------NYLFMGDYVDRGYYSVETVTLLVALKVRYRER 110
g05338 HGQFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETVTLLVALKVRYKDR 260
280
300
Human_PPP2AC ITILRGNHESKQITQVYGFYDECLRKYGNANVWKYFTDLFDYLPLTALVDGQ 162
g05338 ITILRGNHESRQITQVYGFYDECLRKYGNANVWKYFTDLFDYLPLTALVDNQ 312
320
340
360
Human_PPP2AC IFCLHGGLSPSIDTLDHIRALDRPQEVPHEGPMCDLLWSDPDDRGGWGISPR 214
g05338 IFCLHGGLSPSIDTLDHIRTLDRLQEVPHEGPMCDLLWSDPDDRGGWGISPR 364
380
400
Human_PPP2AC GAGYTFGQDISETFNHANGLTLVSRAHQLVMEGYNWCHDRNVVTIFSAPNYC 266
g05338 GAGYTFGQDISETFNHTNGLTLVARAHQLVMEGYNWCHERHVVTVFSAPNYC 416
420
440
Human_PPP2AC YRCGNQAAIMELDDTLKYSFLQFDPAPRRGEPHVTRRTPDYFL 309
g05338 YRCGNQAAIMELDDCLKYTFLQFDPAPRRGEPHVTRRTPDYFL 459

Fig. 9

Fig 9. Pairwise alignment of human PPP2C and g05338.

Pairwise alignment of amino acid sequences of human PPP2CA (PP2A catalytic subunit α) and *H. dujardini* ortholog, g05338. Conserved amino acids are indicated in pink background. Purple and brown triangles indicate the residues that recognize the okadaic acid via hydrogen bonds and van der Waals contacts, respectively [Xing et al. 2006].


(A) cantharidic acid (CA), 0.75 μM
Tun formation rates (%)
120
100
80
60
40
20
0
**
**
**
DMSO
CA
DMSO
CA
Low humidity
High humidity
(C) triptolide, 50 μM
DMSO triptolide
DMSO triptolide
Low humidity
High humidity
(B) cantharidic acid (CA), 0.75 μM
(D) triptolide, 50 μM
N=8 (Low humidity)

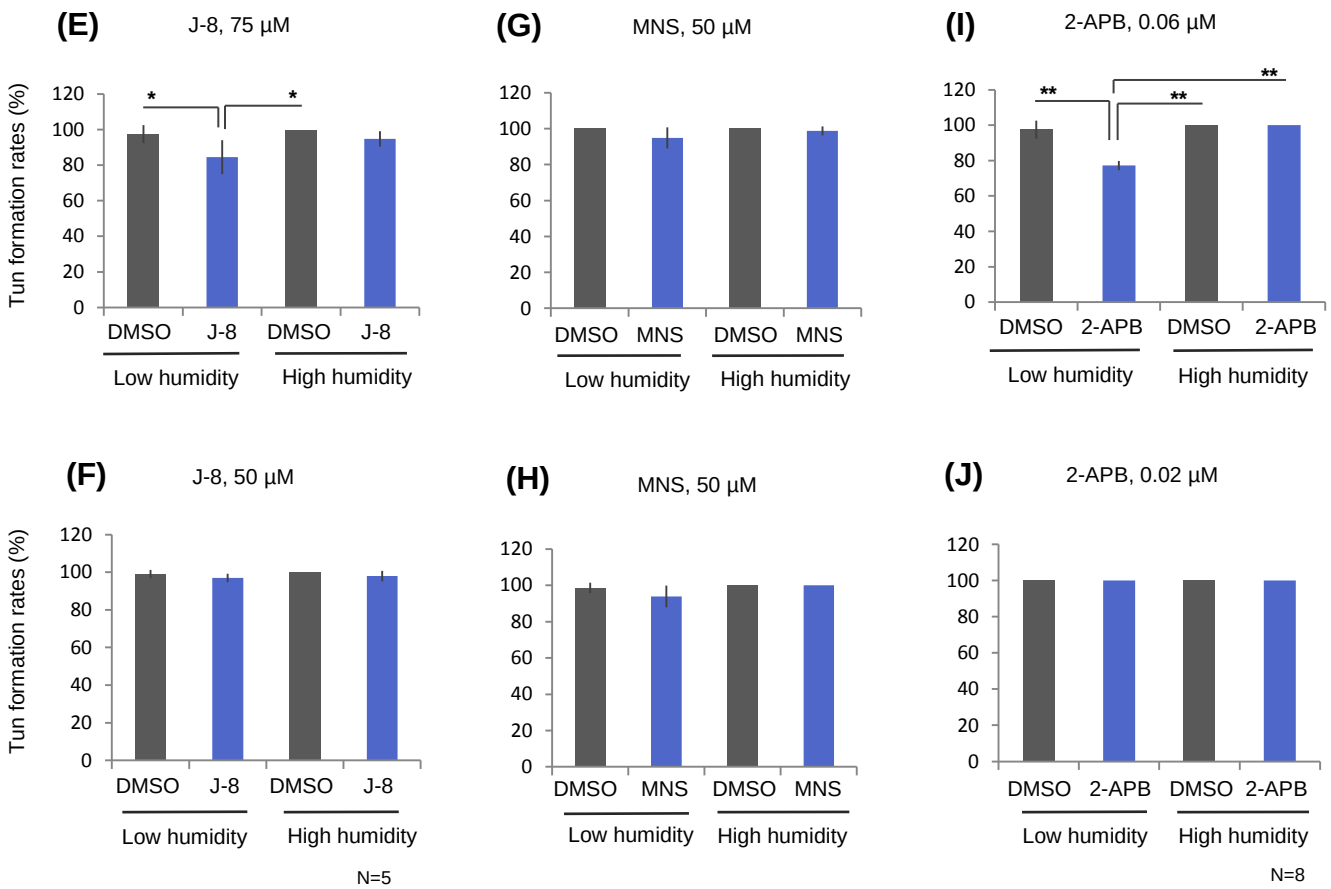
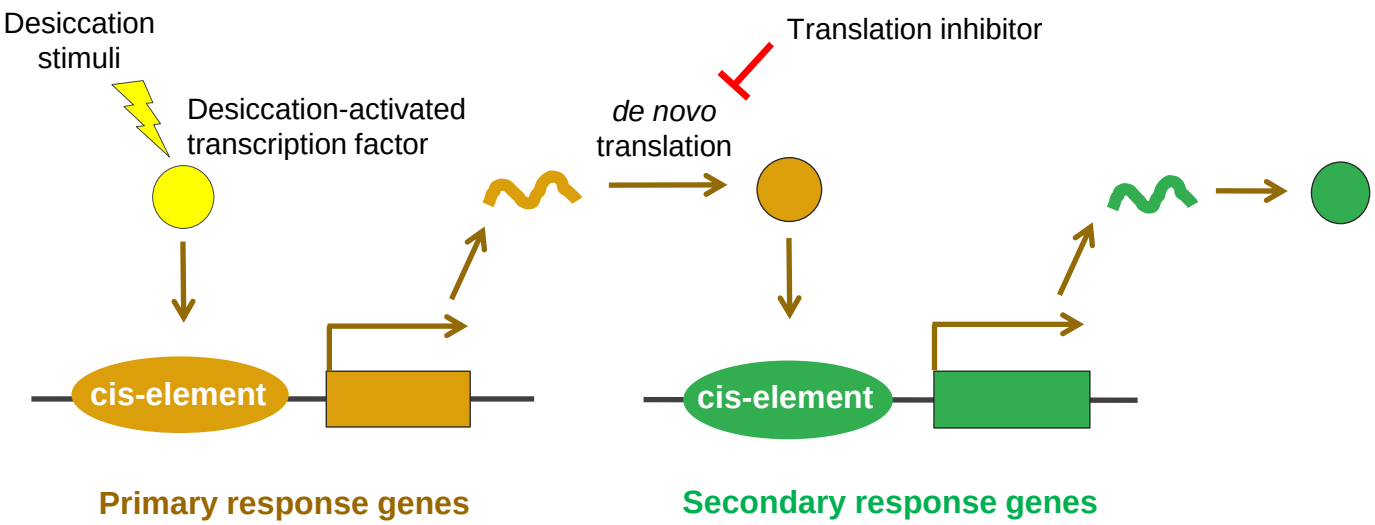

(E) J-8, 75 μM
(G) MNS, 50 μM
(I) 2-APB, 0.06 μM
Tun formation rates (%)
*
*
**
**
**
DMSO
J-8
MNS
2-APB
Low humidity
High humidity
(F) J-8, 50 μM
(H) MNS, 50 μM
(J) 2-APB, 0.02 μM
N=5
N=8

Fig. 10

Fig 10. Variable effects of the identified chemicals on tun formation

The effects of identified chemicals on tun formation were examined. Tun formation rates are shown for experiments corresponding to Fig. 6 (A, C, E, G, I) and Fig. 7 (B, D, F, H, J). (A, B) cantharidic acid (CA), (C, D) triptolide, (E, F) J-8, (G, H) MNS, and (I, J) 2-APB. N=4 unless otherwise stated; 20 tardigrades each. Statistically significant differences among samples were determined by Tukey-Kramer test (*, $P<0.05$; **, $P<0.01$). Low humidity, low humidity exposure; High humidity, high humidity exposure.

(A)



(B)

	Total	Annotated	Unannotated
Upregulated genes	146 (100%)	42	104
Primary response genes	102 (69.9%)	30	72
Secondary response genes	44 (30.1%)	12	32

(C)

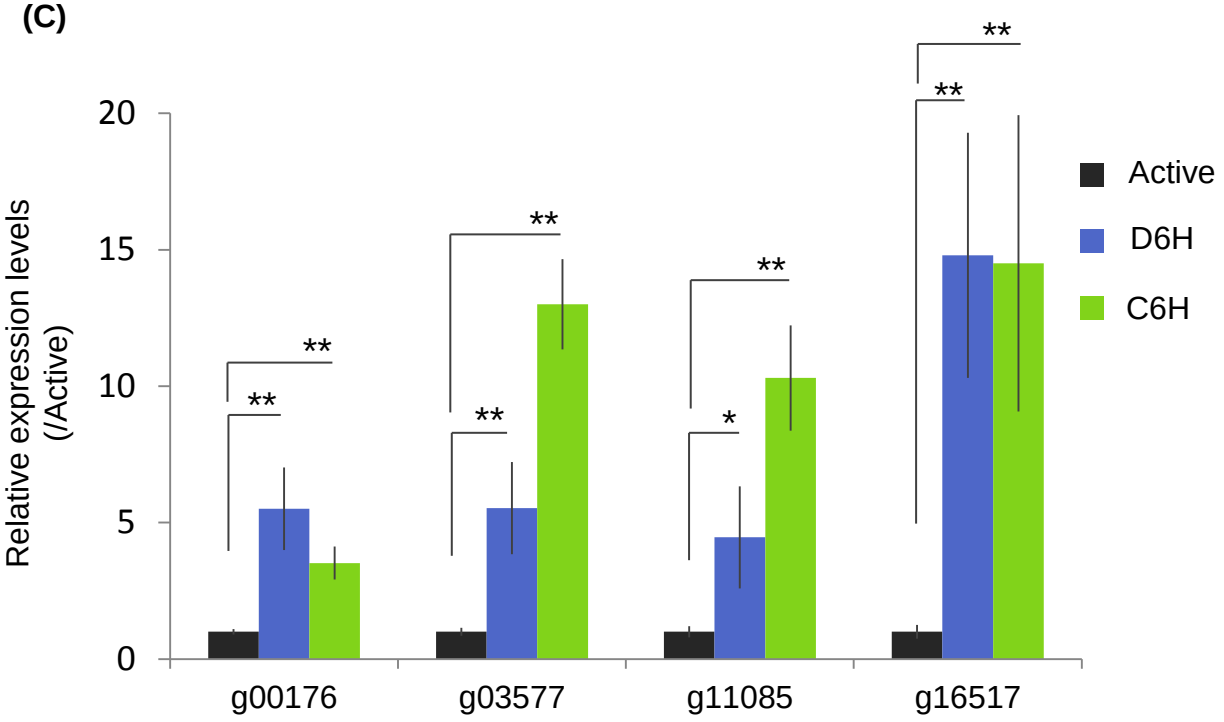


Fig. 11

Fig 11. Classification of upregulated genes as primary or secondary response genes.

(A) Strategy for classifying upregulated genes. Primary response genes are assumed to be first transcribed upon desiccation stimuli by a desiccation-activated transcription factor. Secondary response genes are assumed to be transcribed only after gene products possibly included in primary response genes are translated and become functional. A translation inhibitor could impair the transcription of secondary response genes. (B) Summary of classification of 146 upregulated genes as primary or secondary response genes. Expression levels of candidate primary response genes were upregulated more than 4-fold during preconditioning even after translation inhibitor treatment, while upregulation of candidate secondary response genes was suppressed less by the translation inhibitor treatment. (C) Verification of upregulation of selected candidate primary response genes by qRT-PCR. Total-RNA was extracted from active hydrated tardigrades (Active), 6h-preconditioned tardigrades after 5h-incubation in solution of 1% DMSO (D6H) or 250 μM cycloheximide (C6H). Relative expression levels are normalized with those of Active. Mean ± SD (N=4; 20 tardigrades each). For each gene, statistically significant differences compared with Active were determined by Dunnett's test (*, $P<0.05$; **, $P<0.01$).

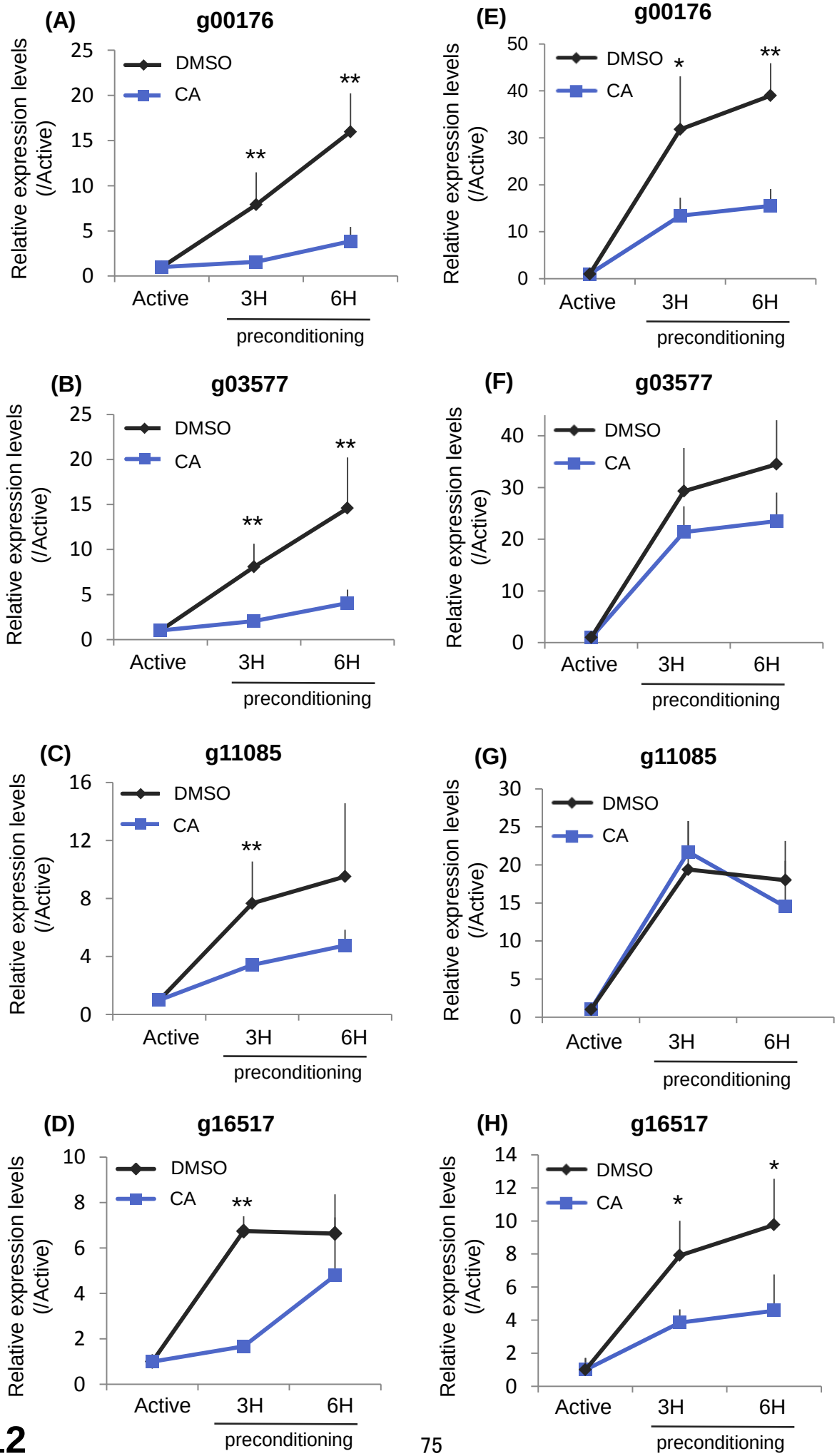
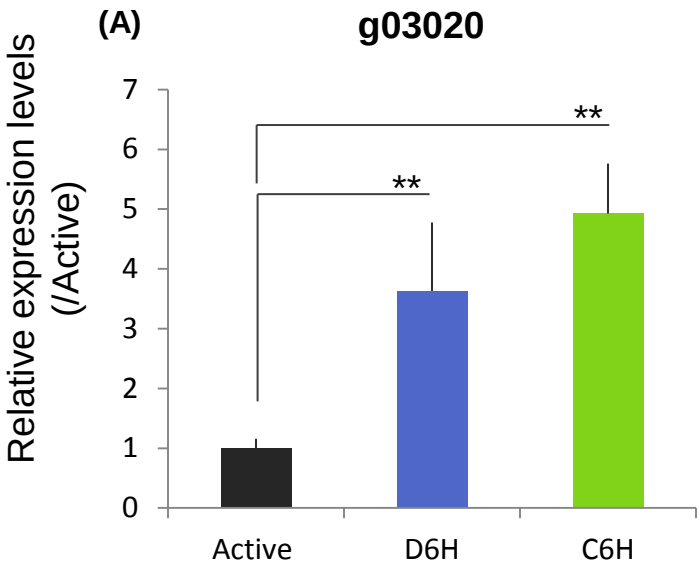
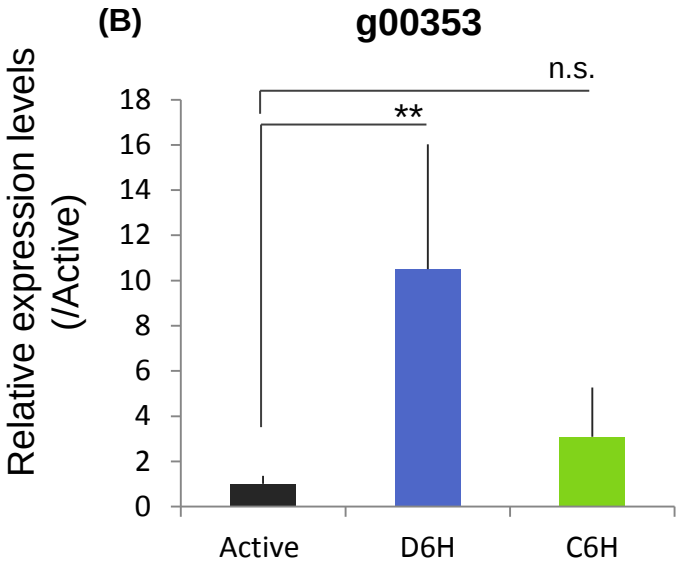

(A)
g00176
Relative expression levels (/Active)
DMSO
CA
**
**
Active
3H
6H
preconditioning
(E)
g00176
Relative expression levels (/Active)
DMSO
CA
*
**
Active
3H
6H
preconditioning
(B)
g03577
Relative expression levels (/Active)
DMSO
CA
**
**
Active
3H
6H
preconditioning
(F)
g03577
Relative expression levels (/Active)
DMSO
CA
Active
3H
6H
preconditioning
(C)
g11085
Relative expression levels (/Active)
DMSO
CA
**
Active
3H
6H
preconditioning
(G)
g11085
Relative expression levels (/Active)
DMSO
CA
Active
3H
6H
preconditioning
(D)
g16517
Relative expression levels (/Active)
DMSO
CA
**
Active
3H
6H
preconditioning
(H)
g16517
Relative expression levels (/Active)
DMSO
CA
*
*
Active
3H
6H
preconditioning

Fig. 12

Fig 12. Effects of cantharidic acid treatment on expression levels of selected primary response genes.

Expression levels of four primary response genes were determined by qRT-PCR in active hydrated tardigrades (Active), 3h- (3H) or 6h-preconditioned (6H) tardigrades after 5h-incubation in solution of 1% DMSO (DMSO; black diamond) or 1 μM cantharidic acid (CA; blue square). Temporal expression levels of g00176 (A, E), g03577 (B, F), g11085 (C, G) and g16517 (D, H) were examined in two independent experiments. (A, B, C, D) One experimental set. (E, F, G, H) The other experimental set. Mean ± SD (N=4; 20 tardigrades each). For each gene, statistically significant differences between DMSO and CA in 3H or 6H were determined by Student's *t*-test respectively (*, $P<0.05$; **, $P<0.01$).


(A)
g03020
Relative expression levels (/Active)
7
6
5
4
3
2
1
0
**
**
Active
D6H
C6H


(B)
g00353
Relative expression levels (/Active)
18
16
14
12
10
8
6
4
2
0
n.s.
**
Active
D6H
C6H

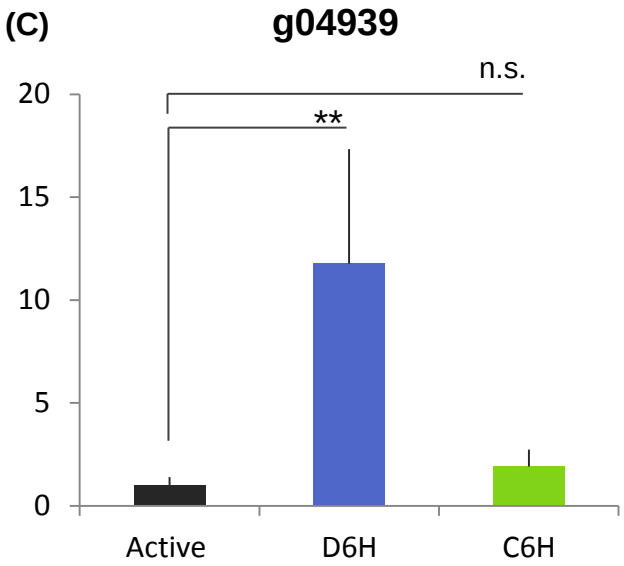
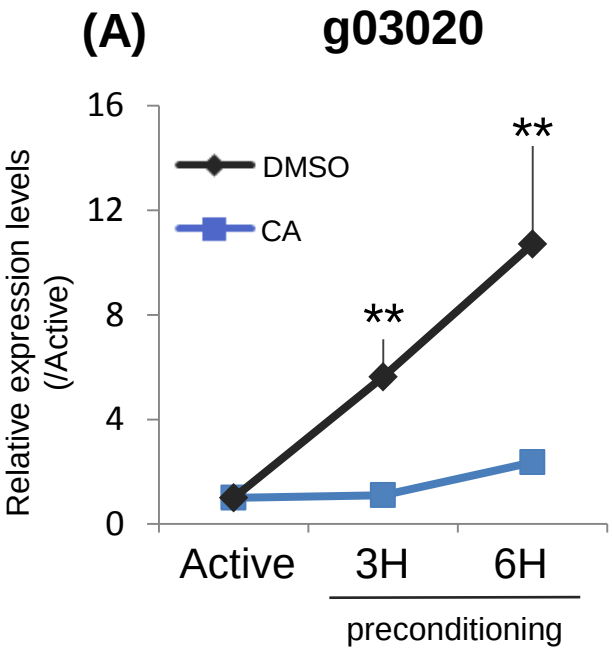

(C)
g04939
20
15
10
5
0
n.s.
**
Active
D6H
C6H

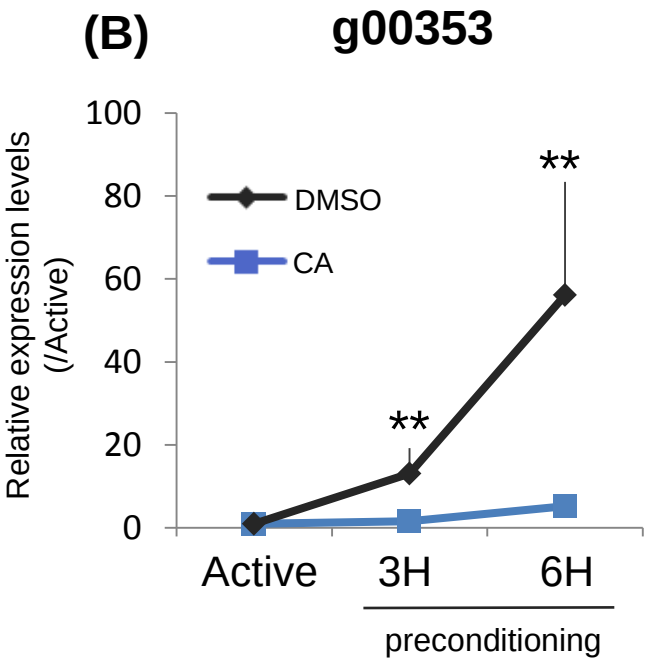
Fig. 13

Fig 13. Verification of upregulation of *cahs* genes by qRT-PCR.

Expression levels of three *cahs* genes, whose upregulation was detected in RNA-seq analysis, were determined by qRT-PCR in active hydrated tardigrades (Active), 6h-preconditioned tardigrades after 5h-incubation in solution of 1% DMSO (D6H) or 250 μM cycloheximide (C6H). Relative expression levels of g03020 (A), g00353 (B) and g04939 (C) are shown. Mean ± SD (N=4; 20 tardigrades each). For each gene, statistically significant differences compared with Active were determined by Dunnett's test (**, $P<0.01$).

n.s., not significantly different.


(A)
g03020
Relative expression levels (/Active)
16
12
8
4
0
DMSO
CA
**
**
Active
3H
6H
preconditioning


(B)
g00353
Relative expression levels (/Active)
100
80
60
40
20
0
DMSO
CA
**
**
Active
3H
6H
preconditioning

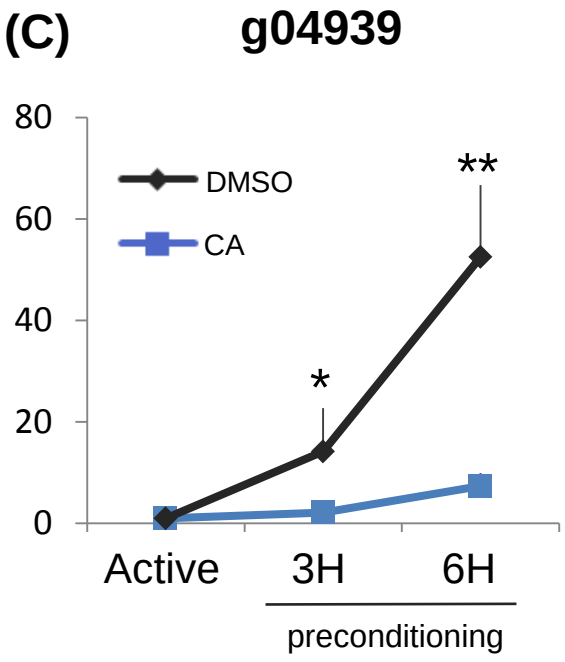

(C)
g04939
80
60
40
20
0
DMSO
CA
*
**
Active
3H
6H
preconditioning

Fig. 14

Fig 14. Effects of cantharidic acid treatment on expression levels of three induced-*cahs* genes.

Expression levels of three induced-*cahs* genes were determined by qRT-PCR in active hydrated tardigrades (Active), 3h- (3H) or 6h-preconditioned (6H) tardigrades after 5h-incubation in solution of 1% DMSO (DMSO; black diamond) or 1 μM cantharidic acid (CA; blue square). Temporal expression levels of g03020 (A), g00353 (B) and g04939 (C). Mean ± SD (N=4; 20 tardigrades each). For each gene, statistically significant differences between DMSO and CA in 3H or 6H were determined by Student's *t*-test respectively (*, $P<0.05$; **, $P<0.01$).

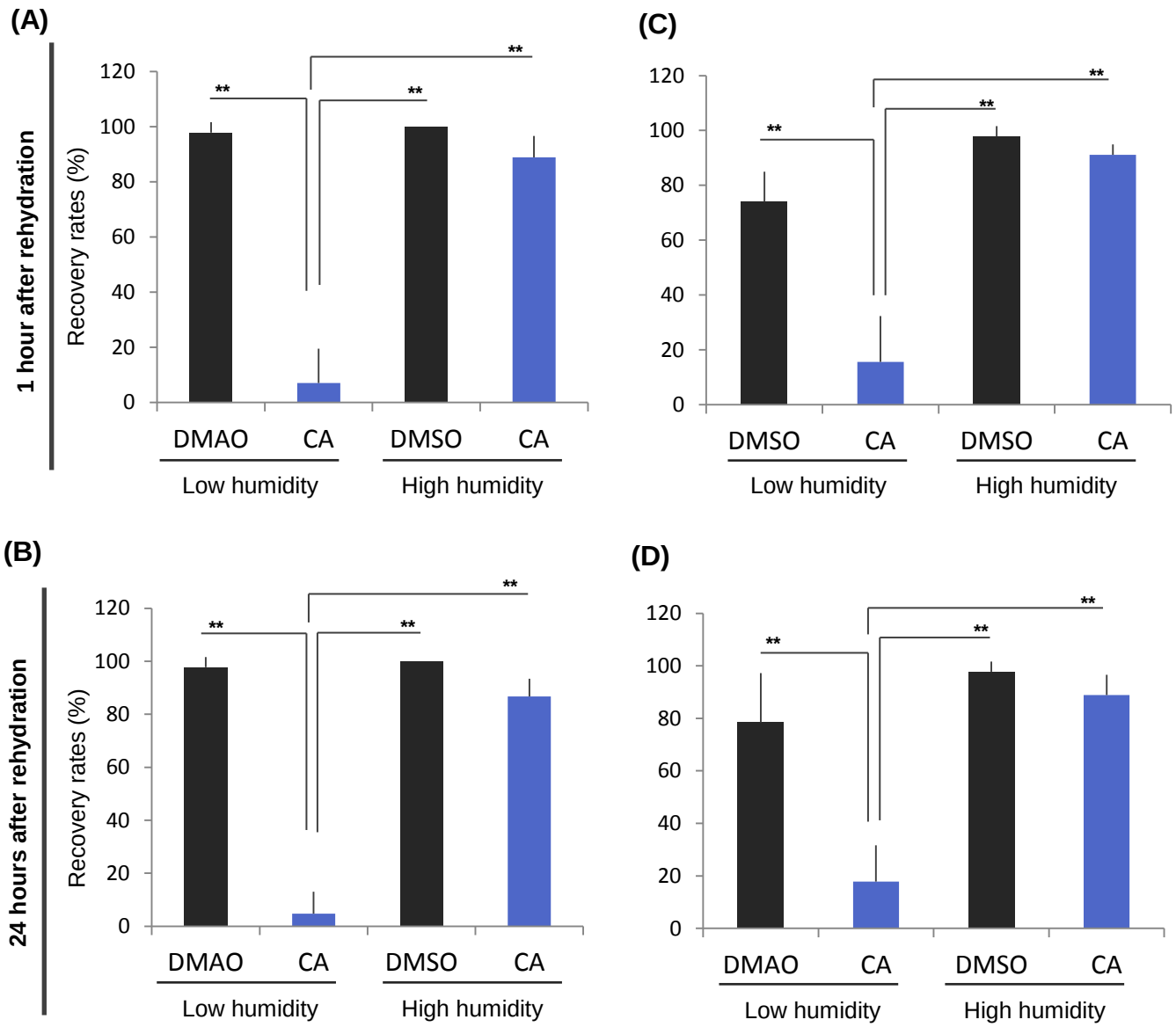

(A)
1 hour after rehydration
Recovery rates (%)
120
100
80
60
40
20
0
**
**
**
DMAO
CA
DMSO
CA
Low humidity
High humidity
(C)
DMSO
CA
DMSO
CA
Low humidity
High humidity
(B)
24 hours after rehydration
(D)

Fig. 15

Fig 15. Verification of inhibitory effects of cantharidic acid on anhydrobiotic survival

The inhibitory effects of cantharidic acid (CA) on anhydrobiotic survival were checked at the same time when samples were collected for examination of expression levels of selected primary response genes by qRT-PCR (See Fig. 12). Tardigrades were incubated in 1% DMSO solution (DMSO) or 1 μM CA (CA) for 5 h. After chemical treatment and 1 day preconditioning at 95% RH, one group was exposed to low relative humidity (10% RH) for 2 days (Low humidity exposure). The other group was exposed to high relative humidity (95% RH) for the same period (High humidity exposure). Recovery rates at 1 h (A, C) and 24 h (B, D) after rehydration. (A, B) Verification results corresponding to Fig. 12A, B, C and D. (C, D) Verification results corresponding to Fig. 12E, F, G and H. Mean ± SD (N=3; 15 tardigrades each). Statistically significant differences among samples were determined by Tukey-Kramer test (**, $P<0.01$). Low humidity, low humidity exposure; High humidity, high humidity exposure.

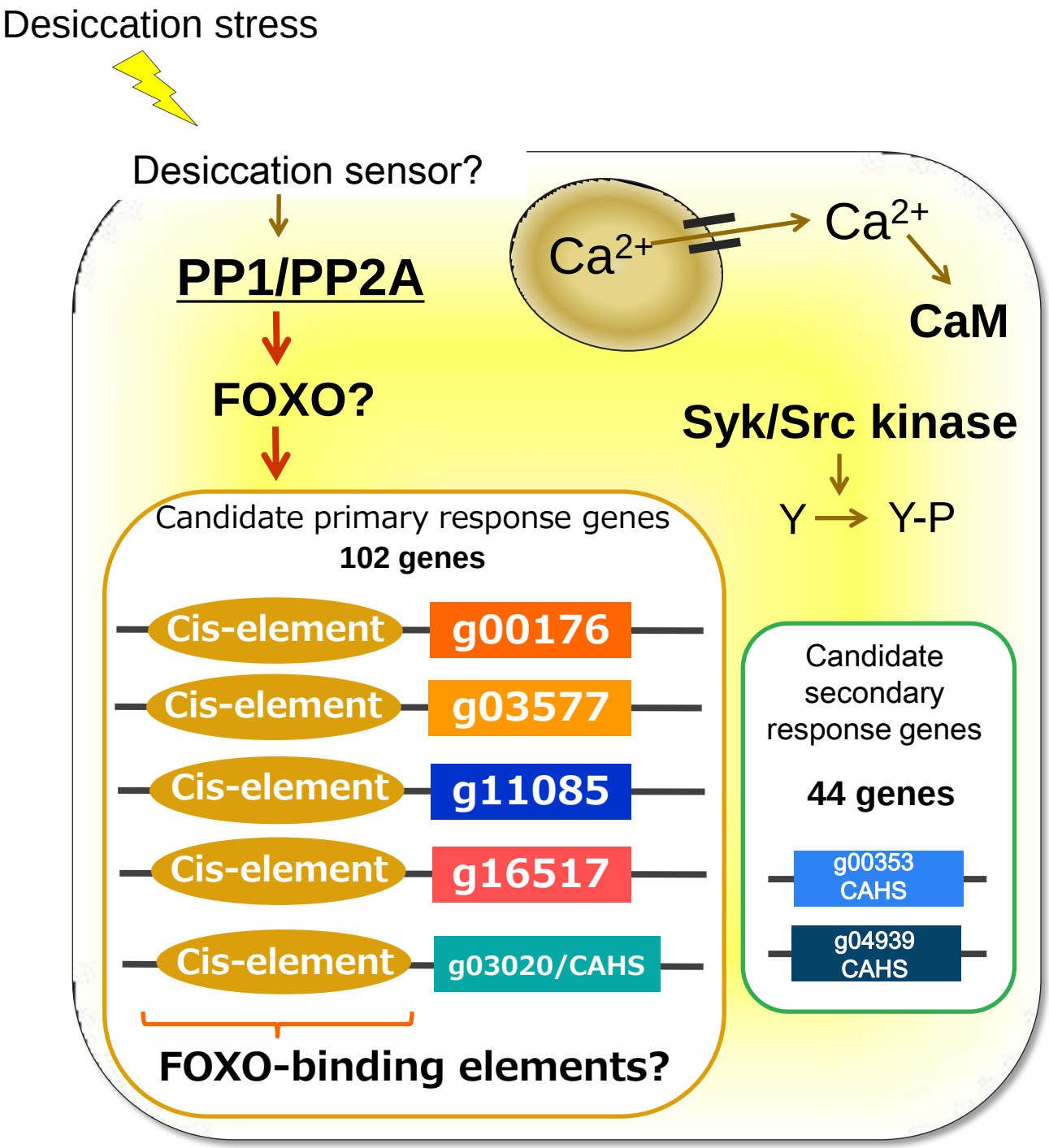

Desiccation stress
Desiccation sensor?
PP1/PP2A
FOXO?
Ca2+
Ca2+
CaM
Syk/Src kinase
Y
Y-P
Candidate primary response genes
102 genes
Cis-element
g00176
Cis-element
g03577
Cis-element
g11085
Cis-element
g16517
Cis-element
g03020/CAHS
FOXO-binding elements?
Candidate secondary response genes
44 genes
g00353 CAHS
g04939 CAHS

Fig. 16

Fig 16 Suggested scheme of molecular regulation of anhydrobiosis in *H. dujardini*.

Protein phosphorylation/dephosphorylation by Syk/Src and PP1/PP2A and calcium signaling via calmodulin (CaM) and intracellular calcium release are suggested to be involved in the process during preconditioning. Upregulated genes during preconditioning contain primary and secondary response genes. PP1/PP2A could be involved in the signaling pathway starting from desiccation sensing to transcriptional regulation of primary response genes.

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