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ARTICLE ADDENDUM

Distinct light-, stress-, and nutrient-dependent regulation of multiple tryptophan-rich sensory protein (*TSPO*) genes in the cyanobacterium *Fremyella diplosiphon*

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ABSTRACT

The cyanobacterium *Fremyella diplosiphon* possesses 3 genes encoding homologs of the tryptophan-rich sensory protein (TSPO). TSPO proteins are membrane proteins implicated in stress responses across a range of organisms from bacteria to humans. Diverse TSPO proteins appear to generally bind tetrapyrrole ligands. Previously, we reported that one of these homologs, *Fd*TSPO1, is involved in salt-, osmotic- and oxidative stress responses in *F. diplosiphon*. Here, we show distinct regulation of cellular mRNA levels of all 3 *FdTSPO* homologs by different abiotic stresses. Given the prior finding that all 3 *Fd*TSPO proteins are capable of binding tetrapyrroles of functional relevance in *F. diplosiphon* and the observation of a ligand-dependent functional role for *Fd*TSPO1 *in vivo*, *Fd*TSPO1, *Fd*TSPO2, and *Fd*TSPO3 appear to have distinct, yet overlapping, roles *in vivo*. We propose that these proteins regulate tetrapyrrole homeostasis and/or tetrapyrrole-modulated functions in *F. diplosiphon* in response to multiple environmental stresses.

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Introduction

Fremyella diplosiphon is a filamentous, freshwater cyanobacterium that serves as a model organism for complementary chromatic acclimation (CCA). CCA is a process by which the organism exhibits distinct physiologic and morphological changes primarily in response to green light (GL) and red light (RL), an acclimation that allows maximum light absorption and promotes productivity in different depths where either RL or GL is more abundant.[1-3] *F. diplosiphon* carries 3 genes encoding proteins with homology to the nearly ubiquitous tryptophan-rich sensory protein/translocator protein 18kDa (TSPO).[4] TSPO homologs have largely been studied in mammals and non-oxygenic photosynthetic bacteria, and the general role of TSPO proteins appears to be in organismal responses to stress where determined.[5] We previously established a role for *TSPO1* in *F. diplosiphon* (*FdTSPO1*) in stress- and light-regulated processes.[6] We confirmed GL-dependent upregulation of *FdTSPO1* and established physiologic roles for FdTSPO1 in stress-regulated processes including salt-, oxidative- and osmotic-dependent stresses[6] and in ligand-dependent organismal responses.[4] Based on quantitative real-time polymerase chain reaction (qRT-PCR) analyses, *FdTSPO1* transcript was more abundant transiently after treatment with the salt sodium chloride, the osmoticum sorbitol, and the oxidative stress-inducing agent methyl viologen (MV).[6] Recently, we reported the presence of 2 additional *F. diplosiphon* TSPO genes (*FdTSPO2* and *FdTSPO3*) that are more similar in sequence to each other and smaller in size than the first identified TSPO in *F. diplosiphon*.[4] Such a diverse complement of *TSPO* genes is relatively rare and among cyanobacteria appears to occur primarily in filamentous, nitrogen-fixing strains.[4] All 3 encoded *Fd*TSPO proteins bind functionally relevant tetrapyrroles, although with apparently distinct binding efficiencies.[4] This observation, together with the observed disparate light-dependent regulation of the expression of *FdTSPO* genes, suggests potentially distinct, if partially overlapping, functions of these 3 homologs *in vivo*. Here, we describe novel stresses, including abiotic and nutrient stresses, that influence *TSPO* mRNA abundance in growing cells and describe distinctions in the impact of these factors on the regulation of the 3 distinct *FdTSPO* genes in *F. diplosiphon*. This survey of the environmental factors that impact *FdTSPO* expression is a first appraisal of the potentially distinct roles these genes may play in fine-tuning organismal responses to stress.

Various environmental cues have different impacts on distinct FdTSPO *homologs*

Light-dependent regulation of TSPOs

*FdTSPO*1 is upregulated under GL compared with RL conditions.[4,6,7] A ~6.5-fold induction under GL compared with RL

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Busch AWU, WareJoncas Z, Montgomery BL. Tryptophan-Rich Sensory Protein/Translocator Protein (TSPO) from Cyanobacterium *Fremyella diplosiphon* Binds a Broad Range of Functionally Relevant Tetrapyrroles. (2017). Biochemistry 56:73–84. doi:10.1021/acs.biochem.6b01019.

was observed in microarray analyses,[7] a ~2.5-fold induction under GL was reported in RNA-sequencing based analysis[6] and a ~2-fold induction under GL observed by qRT-PCR.[6] We recently determined that neither *FdTSPO2* nor *FdTSPO3* appear to be significantly regulated by these light qualities, as levels of mRNA accumulating in GL vs. RL was not significantly different for either gene.[4] However, cellular levels of *FdTSPO2* and *FdTSPO3* mRNA were significantly higher than those observed for *FdTSPO1*,[4] potentially indicating distinct regulation and functions for *Fd*TSPO1 vs. *Fd*TSPO2/*Fd*TSPO3.

Recent research in *Synechocystis* sp. PCC 6803, a single-celled non-chromatically acclimating cyanobacterium, reported oscillation of the expression of the *Synechocystis TSPO* homolog with peak expression from very early (i.e., from 1 to 3 h) to near the middle (i.e., at 5 h) of the light phase under a 12 h diurnal cycle.[8] We, thus, grew *F. diplosiphon* cells in diurnal cycles (12 h of white light [WL] at ~10 μmol m^{-2} s^{-1} and 12 h of dark) and tested *TSPO* transcript accumulation. As an initial survey of the potentially distinct regulation of these genes under diurnal conditions, we determined *TSPO* mRNA levels 12 h into the light period (L12), 12 h into the dark period (D12) and 3 h into the light period (L3) (for schematic see Fig. 1A). *FdTSPO1* mRNA levels increased (~2-fold) at L3 compared with L12 or D12. *FdTSPO2* was slightly, yet significantly, increased (~1.5-fold) at D12 compared with L12, but was not significantly different from either L12 or D12 at L3 (Fig. 1B). *FdTSPO3* exhibited the strongest response of all 3 homologs. Whereas there was no significant difference between mRNA levels for L3 vs. L12, *FdTSPO3* transcript abundance was over 12-fold increased at the end of the dark period (D12) compared with light conditions (Fig 1). Although a more extensive analysis is necessary to confirm diurnal regulation of *TSPO*, together these results show that *TSPO* mRNA levels peak early during the light period (*Fd*TSPO1) or directly before the onset of light (*Fd*TSPO2/*Fd*TSPO3), suggesting a role for these proteins during dark/light transitions. A role for TSPO in the switch from oxygenic to anaerobic photosynthetic growth in *Rhodobacter sphaeroides* has been reported previously.[9] Together, these data support roles for TSPO proteins in temporal or transitory responses to changing environmental conditions.

Additional abiotic stresses alter TSPO transcript abundance

In addition to a role for light in regulation of *FdTSPO* expression, we previously assessed effects of several abiotic stresses on *FdTSPO1* levels and found that transcript levels are transiently upregulated under multiple stress conditions, including salt stress, osmotic stress and oxidative stress.[6] We therefore chose to further analyze the impact of select abiotic stresses on the family of *FdTSPO* genes. We first assessed salt stress-induced expression of *FdTSPO2* and *FdTSPO3* (Fig. 2). The mRNA levels of both *FdTSPO2* and *FdTSPO3* were upregulated in the presence of salt, with the highest average transcript levels observed 3 h after adding 200 mM salt (fold change: ~66 in GL, ~176 in RL for *FdTSPO2* and ~36 in GL and ~47 in RL

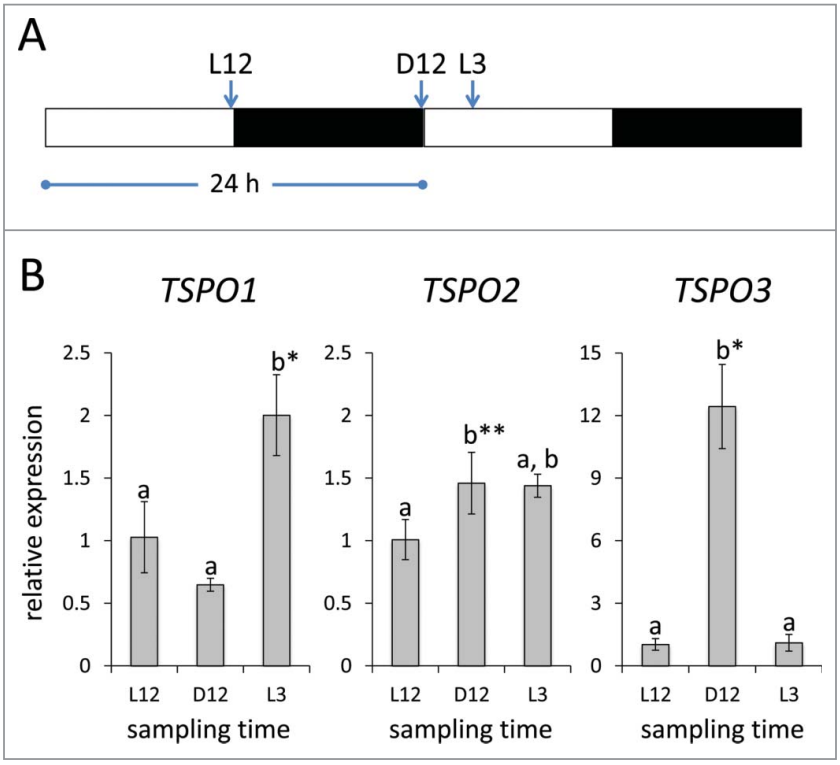

Figure 1. Light-dependent regulation of *FdTSPO* transcript levels under diurnal cycles. Samples for quantitative real-time reverse transcription PCR (qRT-PCR) were taken from white light (WL; ~10 μ mol^{-2} s^{-1}) grown wild-type *Fremyella diplosiphon* cells acclimated to a 12 h diurnal cycle at 12 h into the light (L12) or dark period (D12) and 3 h into the light period (L3) [see schematic in (A)]. (B) qRT-PCR analysis for *FdTSPO* genes, including primers used, was as described in Busch et al, 2016[4]. The relative expression level compared with *ORF10B* gene is shown as average values ($\pm$ SD, $n = 3$). Expression of each *FdTSPO* is shown relative to expression levels at L12 sampling time. Identical letters over bars represent homogenous mean groups ($p > 0.05$), whereas asterisks indicate a significant difference ($^{*}p < 0.01$, $^{**}p < 0.05$) between expression level at 0 h relative to each time point, as determined by ANOVA and Tukey test.

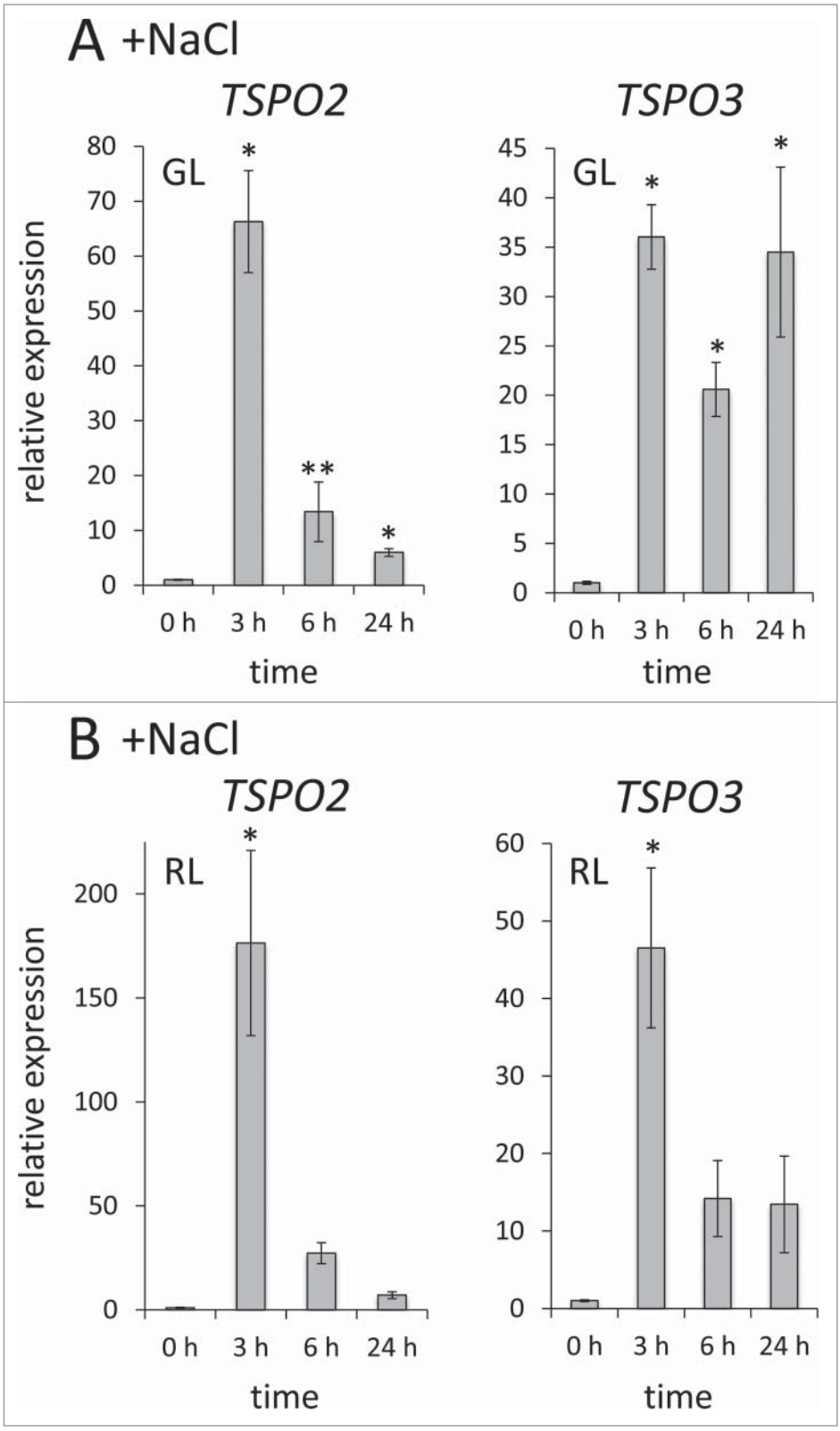

Figure 2. Salt-dependent regulation of *FdTSPO2* and *FdTSPO3* transcript levels. Samples for quantitative real-time reverse transcription PCR (qRT-PCR) were taken before (0 h) and after (3 h, 6 h, 24 h) addition of 200 mM NaCl (w/v) in either (A) green light (GL) or (B) red light (RL) from wild-type *F. diplosiphon* cells adapted to the respective light color to assess *FdTSPO*2 and *FdTSPO*3 transcript accumulation in response to salt stress. Relative expression level compared with *ORF10B* gene is shown as average values ($\pm$ SD, n = 3). Expression of *FdTSPO2* or *FdTSPO3* is shown relative to expression levels in WT cells at time 0 h in the respective light condition. Asterisk indicates a significant difference ($^{*}p < 0.01$, $^{**}p < 0.05$) between expression level at 0 h relative to each time point, as determined by ANOVA and Tukey test.

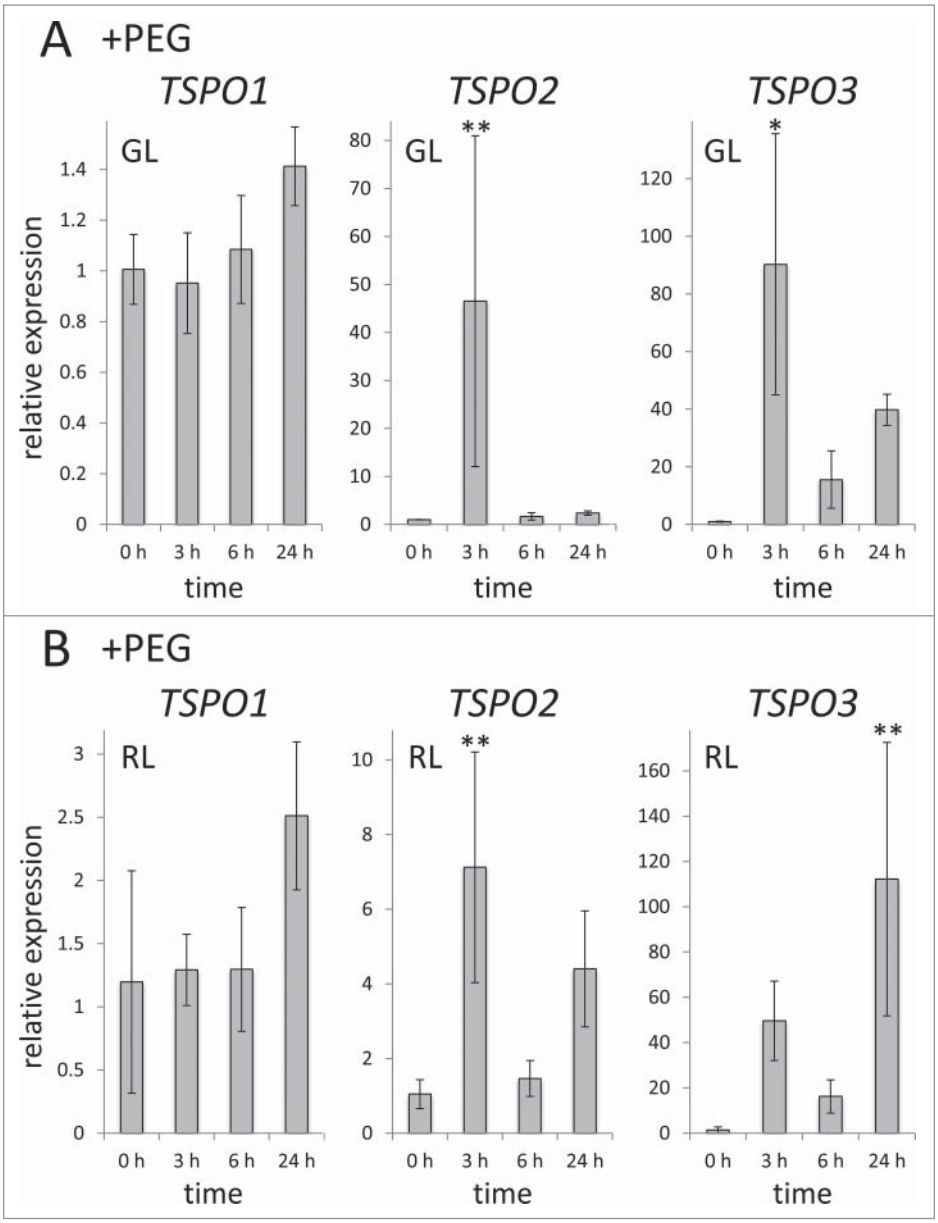

Figure 3. Effect of PEG-induced drought on *FdTSPO* transcript abundance. Samples for quantitative real-time reverse transcription PCR (qRT-PCR) were taken before (0 h) and after (3 h, 6 h, 24 h) addition of 20% PEG6000 (w/v) in (A) green light (GL) and (B) red light (RL) to assess *FdTSPO* transcript accumulation in response to induced stress. Relative expression level compared with *ORF10B* gene is shown as average values (± SD, n = 3). Expression of each *FdTSPO* is shown relative to expression levels in wild-type *F. diplosiphon* cells at time 0 h in the respective light condition. Asterisks indicate a significant difference ($^{*}p < 0.01$, $^{**}p < 0.05$) between expression level at 0 h relative to each time point after treatment with PEG, as determined by ANOVA and Tukey test.

for *FdTSPO3*). As observed for *FdTSPO1*, this upregulation was transient in all cases except for *FdTSPO*3 where levels at 24 h in GL are similar to those at 3 h (Fig. 2A). The overall increase of transcript levels upon salt stress for *FdTSPO2* and *FdTSPO3* were substantially higher than those observed for *FdTSPO1* (compare Fig. 2 with results for *TSPO1* in ref. 6 in which a 2-fold induction of *FdTSPO* is reported), implying potentially distinct roles for these 2 groups of TSPOs in response to a common stressor, i.e., salt.

As salt stress involves both ionic stress and osmotic stress[10] and TSPO has a role in salt stress in plants and cyanobacteria and has been also been implicated in drought stress in plants,[6,11,12] we investigated the role of drought stress on *FdTSPO* mRNA accumulation. To mimick drought stress, we treated *F. diplosiphon* cells with the osmoticum PEG6000 (20% w/v) (Fig 3). A similar level of PEG (i.e., 20%) has been previously used to induce osmotic or drought stress in filamentous cyanobacteria.[13,14] We observed distinct impacts of induced drought on the 3 *FdTSPO* homologs. *FdTSPO1* mRNA levels exhibited a limited response to induced drought in GL and a ~2.5-fold induction at 24 h in RL (Fig. 3). By contrast, both *FdTSPO2* and *FdTSPO3* were transiently upregulated at 3 h in GL (Fig. 3A), whereas the 2 genes showed distinct time points of significantly higher mRNA accumulation, i.e., early (3 h) for *FdTSPO2* and late (24 h) for *FdTSPO3*, compared with 0 h in RL (Fig. 3B). These results distinctly implicate *Fd*TSPO2 and *Fd*TSPO3 in temporal cellular responses to induced drought stress in *F. diplosiphon*.

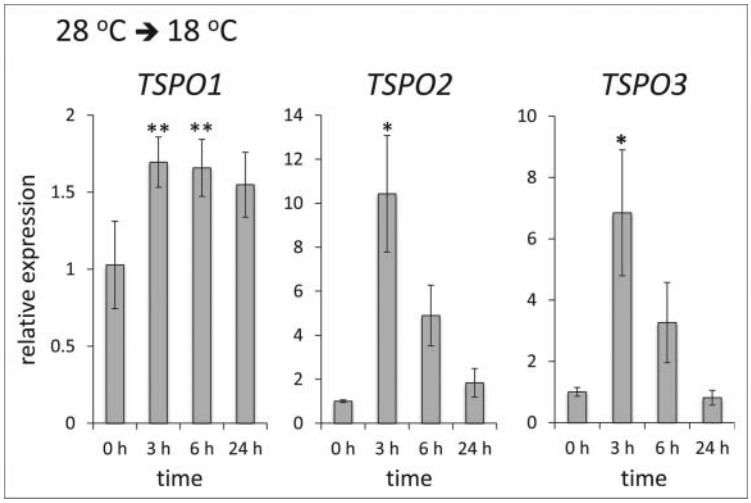

Figure 4. Temperature-dependent regulation of *FdTSPO* transcript levels. Samples for quantitative real-time reverse transcription PCR (qRT-PCR) were taken before (0 h) and after (3 h, 6 h, or 24 h) transferring cells acclimated to 28°C in white light (WL) to 18°C to assess transcript accumulation in response to cold stress for *FdTSPO1*, *FdTSPO2* and *FdTSPO3*. The relative expression level compared with *ORF10B* gene is shown as average values (± SD, $n = 3$). Expression of each *FdTSPO* is shown relative to expression levels in wild-type *F. diplosiphon* cells at time 0 h before the temperature shift. Asterisks indicate a significant difference ($^{*}p < 0.01$, $^{**}p < 0.05$) between expression level at 0 h relative to each time point after the shift from 28°C to 18°C, as determined by ANOVA and Tukey test.

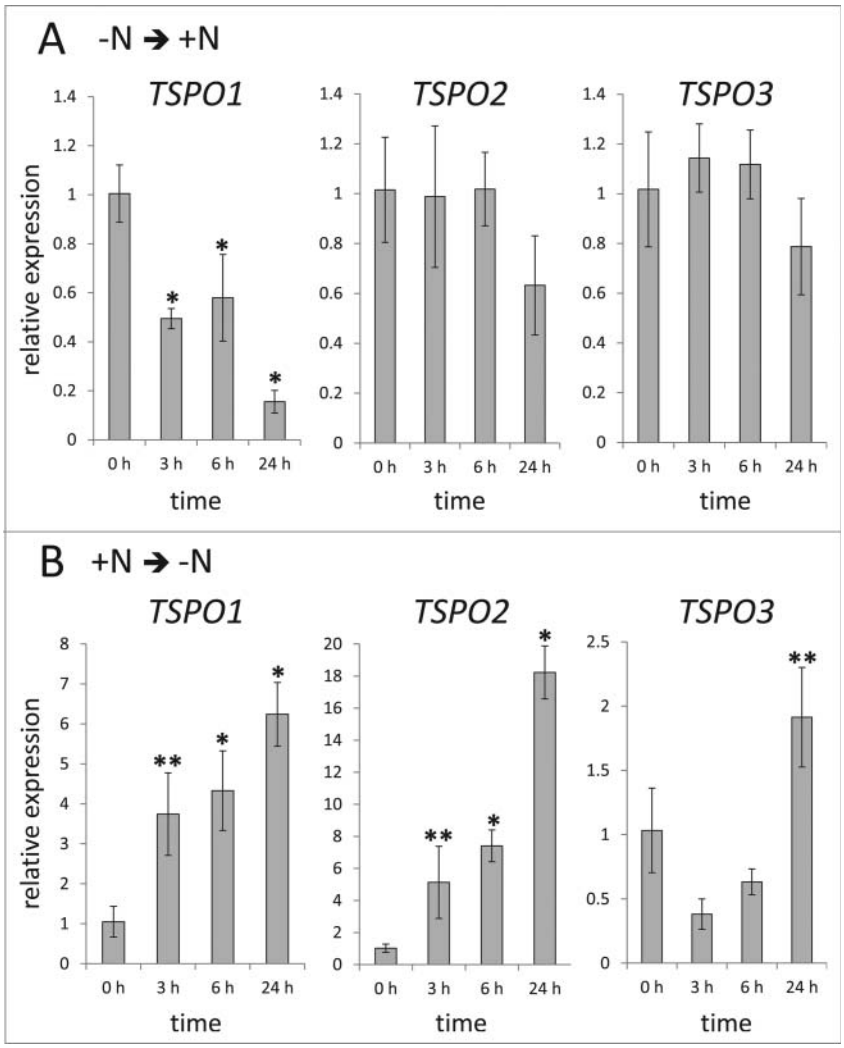

Figure 5. Nitrogen-dependent regulation of *FdTSPO* transcript levels. Samples for quantitative real-time reverse transcription PCR (qRT-PCR) were taken before (0 h) and after (3 h, 6 h, or 24 h) exposing white light (WL) grown cells acclimated to either (A) nitrogen-limited (-N) conditions to nitrogen-replete (+N) medium [-N ➔ +N] or (B) grown under nitrogen-replete conditions and transferred to medium lacking added nitrogen [+N ➔ -N]. The relative expression level compared with *ORF10B* gene is shown as average values (± SD, $n = 3$). Expression of each *FdTSPO* is shown relative to expression levels in wild-type *F. diplosiphon* cells at time 0 h before changing nitrogen availability. Asterisks indicate a significant difference ($^{*}p < 0.01$, $^{**}p < 0.05$) between expression level at 0 h relative to each time point before the growth medium exchange, as determined by ANOVA and Tukey test.

Given that changes in temperature impacts organismal fitness and are associated with diurnal environmental conditions, we investigated temperature effects on the regulation of *FdTSPO* homologs. To test the effect of a temperature drop, *F. diplosiphon* cells were initially acclimated under white light (WL) in standard temperature conditions of 28°C. Acclimated cells were then switched to a lower temperature of 18°C (Fig. 4). *FdTSPO*1 was ~1.7-fold upregulated after 3 h of the temperature shift. Of the measured time points, *TSPO* mRNA levels were highest after 3 h for all 3 *TSPO* homologs. Notably, the more similar *TSPO*s, i.e., *FdTSPO*2 and *FdTSPO*3, showed a stronger increase than *FdTSPO*1 at ~10.4-fold and ~6.8-fold increases in mRNA levels at 3 h for *FdTSPO*2 and *FdTSPO*3, respectively. The increase in transcript abundance was transient, especially for *FdTSPO2* and *FdTSPO3* (Fig. 4).

Nutrient stress affects TSPO *transcript abundance*

Changes in nitrogen availability previously have been shown to affect the abundance of a putative *F. diplosiphon* (aka *Tolypothrix* sp PCC 7601[15]) *TSPO*-containing cotranscript.[16] Therefore, the effect of changes in nitrogen availability on *TSPO* mRNA levels was assessed before and after exposing *F. diplosiphon* cells to changes in nitrogen availability (Fig. 5). Nitrogen-limited cells switched to nitrogen-replete medium exhibited a decrease in *TSPO* transcript abundance over the course of a day, albeit results were only significant for *FdTSPO*1 among the 3 homologs (Fig. 5A). When cells grown in nitrogen-replete medium were moved into nitrogen-deficient medium *TSPO* abundance was highest after 24 h for all 3 homologs (Fig. 5B). The highest fold change was observed for *FdTSPO*2 (~18-fold), followed by *FdTSPO*1 (~6-fold) and then *FdTSPO*3 (~2-fold). Notably, *FdTSPO1* and *FdTSPO2* exhibited significant upregulation early (i.e., at 3 h) compared with *FdTSPO3*, which was only significantly higher at 24 h.

Conclusion

Multiple abiotic stresses impact mRNA levels for all 3 *TSPO* genes found in *F. diplosiphon*. In most cases, *FdTSPO1, FdTSPO2*, and *FdTSPO3* respond to similar stresses, but with distinct patterns and/or magnitudes. Frequently, *FdTSPO2* and *FdTSPO3* show the more similar stress-induced responses (e.g., induced drought, temperature shift, nitrogen stress), which is not surprising given their higher sequence similarity.[4] Distinct responses for each is observed in some cases, including growth in diurnal light cycles and light-dependent responses to salt. Together with apparently distinct tetrapyrrole-binding profiles, these results indicate potentially distinct regulation and functions for these 3 *FdTSPO* homologs. Furthermore, distinct functions may be supported by the distinct location of these genes in 3 distinct phylogenetic clades.[4] Definitive confirmation of the distinct roles of these homologs will require additional genetic experiments.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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