

Article

# Indications for a central role of hexokinase activity in natural variation of heat acclimation in *Arabidopsis thaliana*

Vasil Atanasov <sup>§</sup>, Lisa Fürtauer <sup>§</sup> and Thomas Nägele <sup>\*</sup>

LMU Munich, Plant Evolutionary Cell Biology, Großhaderner Str. 2-4, 82152 Planegg, Germany

<sup>§</sup> Authors contributed equally

<sup>\*</sup> Correspondence: thomas.naegle@lmu.de

**Abstract:** Diurnal and seasonal changes of abiotic environmental factors shape plant performance and distribution. Changes of growth temperature and light intensity may vary significantly on a diurnal, but also on a weekly or seasonal scale. Hence, acclimation to a changing temperature and light regime is essential for plant survival and propagation. In the present study, we analyzed photosynthetic CO<sub>2</sub> assimilation and metabolic regulation of the central carbohydrate metabolism in two natural accessions of *Arabidopsis thaliana* originating from Russia and south Italy during exposure to heat and a combination of heat and high light. Our findings indicate that it is hardly possible to predict photosynthetic capacities to fix CO<sub>2</sub> under combined stress from single stress experiments. Further, capacities of hexose phosphorylation were found to be significantly lower in the Italian than in the Russian accession which could explain an inverted sucrose-to-hexose ratio. Together with the finding of significantly stronger accumulation of anthocyanins under heat/high light these observations indicate a central role of hexokinase activity in stabilization of photosynthetic capacities within a changing environment.

**Keywords:** photosynthesis; carbohydrate metabolism; hexokinase; heat acclimation; environmental changes; natural variation; high light; combined stress.

---

## 1. Introduction

Changes of growth temperature and light intensity broadly affect plant molecular, physiological and developmental processes. Hence, plant acclimation to changing environmental conditions is essential for stabilizing growth, development, and yield production. The genetic model plant *Arabidopsis thaliana* represents an attractive system to study acclimation capacities to environmental conditions in context of geographical origin. The availability of numerous natural accessions from a wide range of geographical origin enables the comparison of acclimation strategies and stress-responses on a genetic, biochemical, physiological and ecological level [1-4]. Such comparative approaches potentially provide detailed molecular insight into complex regulation of plant metabolism which promotes our understanding of how global climatic changes affect plant communities and ecosystems, and might indicate breeding strategies to increase stress tolerance of crop plants. As outlined before, it is necessary to study plant acclimation and response to an abiotic stress combination because it remains hardly possible to reliably predict specific plant responses from single stress experiments [5,6].

Estimates of how global warming will affect ecosystems is a central task of current (plant) biological research. Elevated temperature immediately affects plant metabolism and metabolic regulation by its impact on enzyme activities, membrane composition and rigidity, and signaling cascades. Heat stress typically results in protein misfolding and accumulation of reactive oxygen species (ROS) which can cause membrane damage, induce cytotoxic free-radical reactions and impact on redox regulation and cellular signaling [7]. Subjecting plants to heat stress induces a series of *heat stress-responsive* (HSR)

genes, encoding proteins like chaperones and ROS scavengers which stabilize cellular processes and are critical for plant thermotolerance [7].

Photosynthetic activity has been reported over a wide range of temperatures, and photosynthetic acclimation capacity to changing temperature regimes may vary substantially between species [8,9]. For example, comparison of *Arabidopsis thaliana*, accession Columbia, to *Brassica oleracea* var. *acephala* revealed a larger capacity of *B. oleracea* for acclimation of photosynthesis to moderate changes in growth temperature than of *A. thaliana* [8]. Under moderately elevated temperatures, i.e. between 35 and 40°C, net photosynthetic rates typically decrease which can be explained by deactivation of Rubisco while PSII function is not affected [10,11]. Enzyme activities involved in regeneration of ribulose biphosphate, e.g. phosphoribulokinase, glyceraldehyde 3-phosphate dehydrogenase and sedoheptulose-1,7-bisphosphatase were found to be decreased under heat which implied a direct effect on the central carbohydrate metabolism [12-15]. Metabolome analysis in *Arabidopsis* showed that a majority of heat-responsive metabolites are shared with cold shock response [16]. Comparing diverse abiotic stress responses revealed that sucrose, the central transport sugar in many plant species and an important energy and signaling molecule [17], accumulated under many stress conditions [18]. However, although sucrose accumulation represents a well-known and conserved stress response, many aspects of underlying metabolic regulation remain unclear. Among others, this is due to the central metabolic and regulatory role of sucrose metabolism in plants which causes many dependencies of biochemical and physiological functions [19].

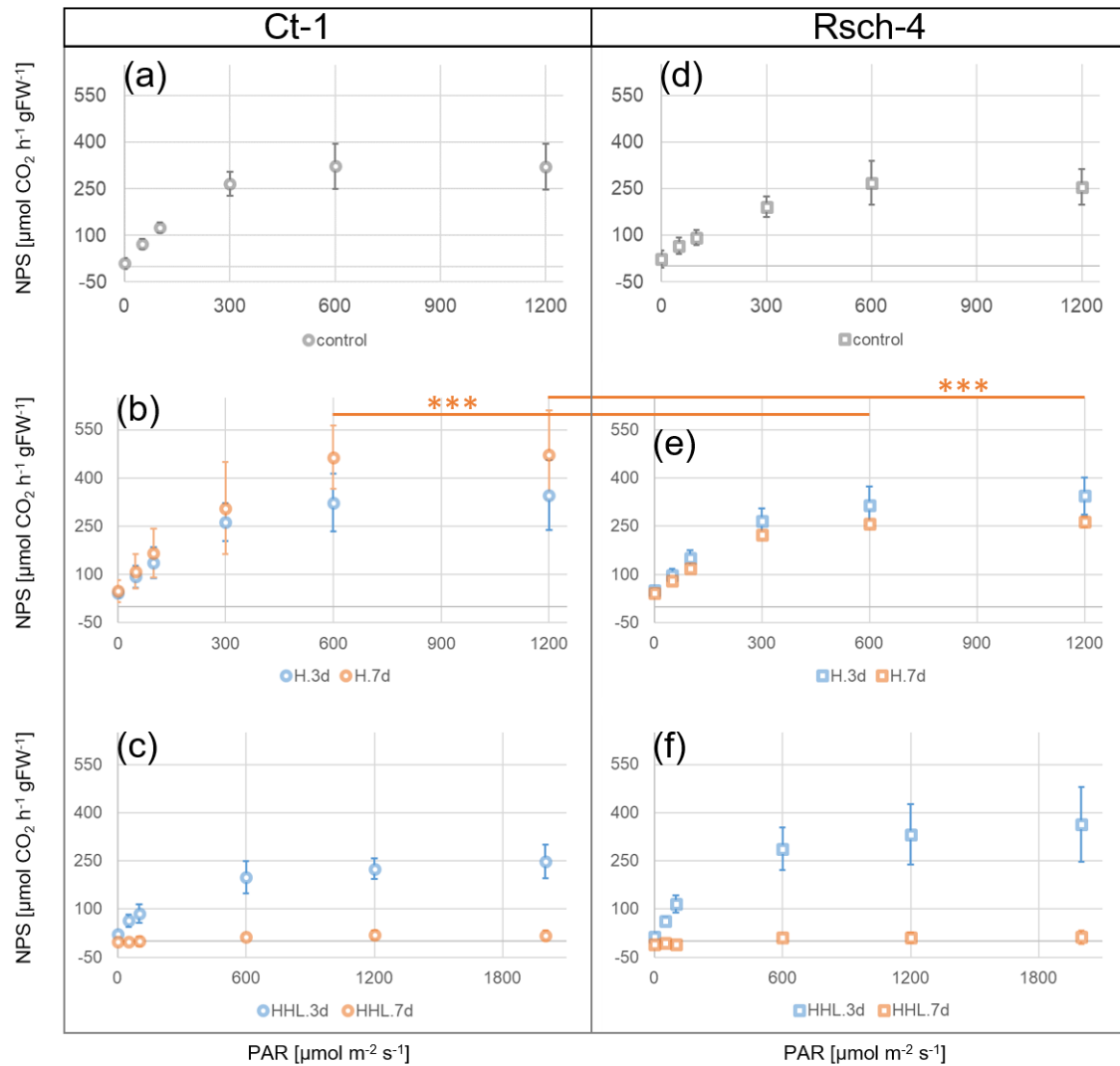
To elucidate effects and regulation of photosynthetic CO<sub>2</sub> assimilation and carbohydrate metabolism under changing environmental conditions, we compared response of two natural accessions with geographical origin from Sicily/Italy (Ct-1) and Russia (Rsch-4) to moderate heat (32°C). Based on the finding that Ct-1 had transiently higher capacity to fix CO<sub>2</sub> under heat and high light intensities, we further combined moderate heat with high light exposure and monitored dynamics of the central carbohydrate metabolism.

## 2. Results

Photosynthesis and carbohydrate metabolism are immediately affected by changes in temperature and/or light intensity. Hence, to estimate effects of elevated temperature and high light intensities on natural *Arabidopsis* accessions Rsch-4 (Russia) and Ct-1 (Italy), rates of net photosynthesis (NPS) were recorded together with relative changes of carbohydrate, protein, and anthocyanin amount and enzyme activities of the central carbohydrate metabolism.

### 2.1. Light response curves of net photosynthesis indicate differential heat acclimation capacities in Ct-1 and Rsch-4

Comparing light response curves of NPS rates under control (22°C/100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), heat (H, 32°C/100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and heat/high light (HHL, 32°C/600  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) revealed significant differences between Ct-1 and Rsch-4 after 7d at 32°C (Figure 1). Rates of NPS were significantly higher in Ct-1 than in Rsch-4 under high PAR intensity, i.e. 600 and 1200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Figure 1 (b), (e)). This effect was only observed after 7d at 32°C, not yet after 3d. Under all other tested conditions, i.e. control and HHL, no significant effects were observed. However, although not significant, NPS rates in Rsch-4 were ~1.5-fold higher after 3d HHL and this effect was most pronounced under high PAR, i.e. >600  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Figure 1 (c), (f)). After 7d of HHL, leaves became senescent (not shown) and NPS rates of both accessions dropped below 25  $\mu\text{mol CO}_2 \text{ h}^{-1} \text{ gFW}^{-1}$  across all PAR intensities (Figure 1 (c), (f)).

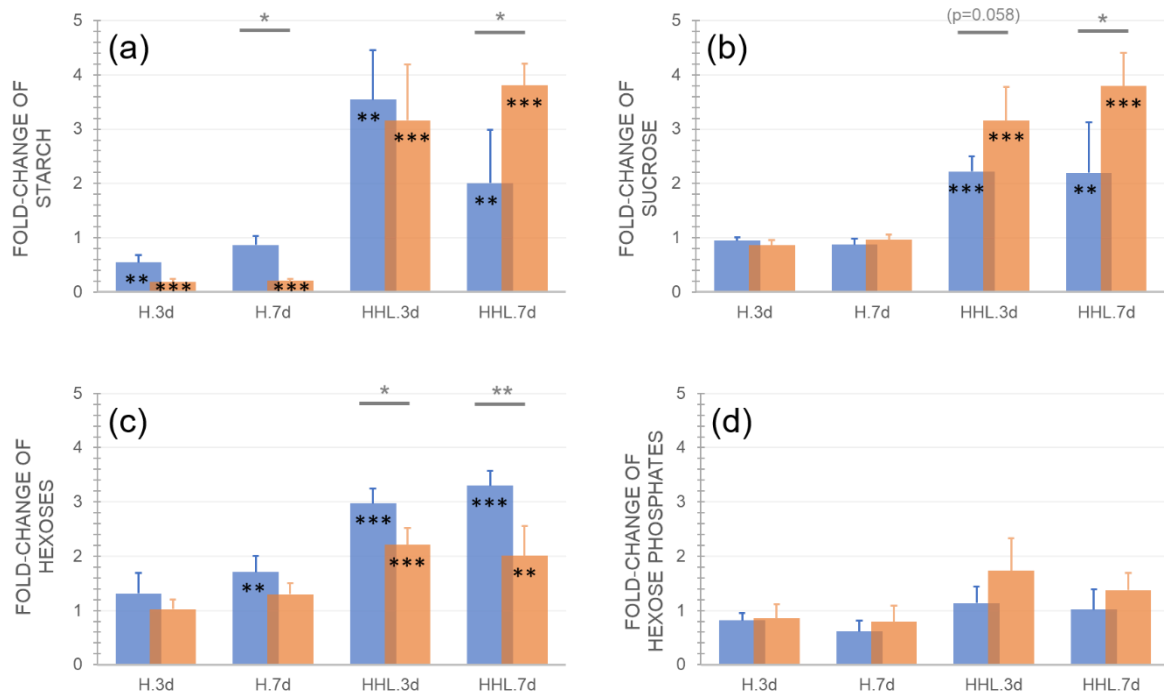


**Figure 1.** Light response curved of net photosynthesis (NPS) after exposure to heat (H) and heat/high light (HHL) for 3 days (blue) and 7 days (orange). (a) – (c) NPS of Ct-1 under (a) control (22°C/100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), (b) heat (32°C/100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and (c) heat/high light (32°C/600  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). (d) – (f) NPS of Rsch-4 under (d) control (22°C/100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), (e) heat (32°C/100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and (f) heat/high light (32°C/600  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Asterisks indicate significance (ANOVA): \*\*\*  $p < 0.001$ . Circles (Ct-1) and squares (Rsch-4) represent mean values  $\pm$  SD ( $n=3-4$ ).

## 2.2. Sucrose-hexose dynamics indicate differential metabolic regulation in Ct-1 and Rsch-4 during HHL exposure

Exposure to H and HHL had significantly different impact on the central carbohydrate metabolism of Ct-1 and Rsch-4. Starch amount was significantly decreased in both accessions under heat and this effect was stronger in Rsch-4 than in Ct-1 (Figure 2 (a)). After 7d at 32°C, starch amount in Ct-1 did not differ significantly compared to control condition while starch amount in Rsch-4 remained significantly lower than under 22°C. Under HHL, both accessions accumulated significantly more starch than under control, and after 7d starch amount in Rsch-4 was significantly higher than in Ct-1. Sucrose amount remained unaffected in both accessions under H while it accumulated 2-fold in Ct-1 and 3-fold in Rsch-4 under HHL (Figure 2 (b)). Rsch-4 accumulated significantly more sucrose than Ct-1 if heat was combined with high light. Reciprocally, hexoses, i.e.

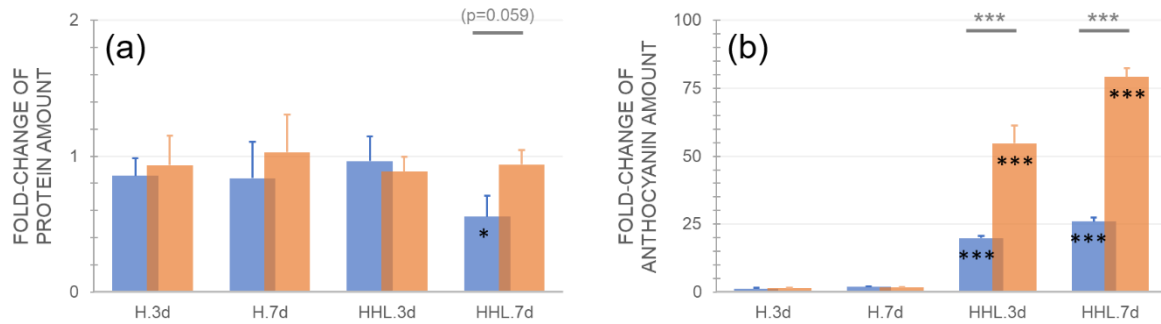
glucose and fructose, accumulated to a significantly higher amount in Ct-1 than in Rsch-4 under HHL (Figure 2 (c)). For hexoses phosphates no significant accumulation was observed in neither tested condition (Figure 2 (d)).



**Figure 2.** Relative changes of central carbohydrates after exposure to heat (H) and heat/high light (HHL). Fold-change of (a) starch amount, (b) sucrose amount, (c) hexoses amount (glucose + fructose), and (d) hexose phosphate amount (glucose 6-P + fructose 6-P) compared to control (i.e. a fold-change of 1 indicates no change compared to control). Blue bars: Ct-1, orange bars: Rsch-4. Asterisks indicate significance (ANOVA), grey asterisks for comparison between accessions, black asterisks for comparison of treatment to control: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . Bars represent mean values  $\pm$  SD (n=5).

### 2.3. Accessions differ in their protein and anthocyanin amount under HHL

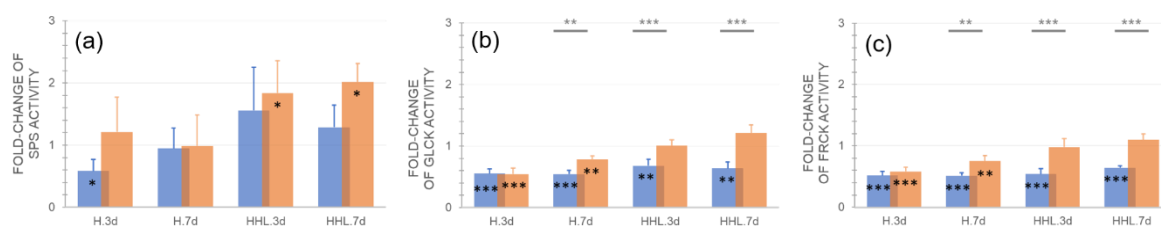
Protein amount did not differ between control and treatment samples except for Ct-1 after 7d HHL where protein amount was significantly decreased (Figure 3 (a)). While anthocyanin amount neither significantly changed under heat, combination with high light (HHL) resulted in a strong (>20-fold) and significant increase in both accessions (Figure 3 (b);  $p < 0.001$ , ANOVA). Already after 3d HHL, anthocyanin amount increased more than 2-fold higher in Rsch-4 compared to Ct-1 and more than 3-fold after 7d HHL (Figure 3 (b)).



**Figure 3.** Relative changes of protein and anthocyanin amount after exposure to heat (H) and heat/high light (HHL). Fold-change of (a) protein amount, (b) anthocyanin amount compared to control (i.e. a fold-change of 1 indicates no change compared to control). Blue bars: Ct-1, orange bars: Rsch-4. Asterisks indicate significance (ANOVA), grey asterisks for comparison between accessions, black asterisks for comparison of treatment to control: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . Bars represent mean values  $\pm$  SD ( $n=5$ ).

#### 2.4. Effects of H and HHL on enzyme activities of the central carbohydrate metabolism

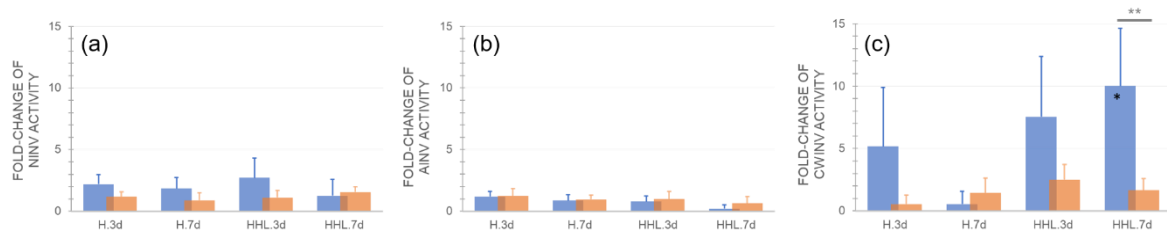
In Rsch-4, HHL resulted in a significant increase of SPS activity (Figure 4 (a)). Also, in Ct-1 SPS activity increased under HHL compared to control conditions but less significantly. After 7d HHL, Rsch-4 had significantly doubled its SPS activity while no significant change was observed for Ct-1. Exposure to 3d H resulted in a significant decrease of SPS activity in Ct-1 (~50% of control) and this effect disappeared after 7d H. SPS activity of Rsch-4 was not significantly affected by heat exposure. Gluco- and fructokinase activity was significantly reduced in Ct-1 by 40-50% under H and HHL (Figure 4 (b) and (c)). In Rsch-4, both gluco- and fructokinase activity was significantly reduced under H, yet not under HHL, when compared to control.



**Figure 4.** Relative changes of enzyme activities after exposure to heat (H) and heat/high light (HHL). Fold-change of (a) sucrose phosphate synthase (SPS) activity, (b) glucokinase (Glck) activity, and (c) fructokinase (Frck) activity compared to control (i.e. a fold-change of 1 indicates no change compared to control). Blue bars: Ct-1, orange bars: Rsch-4. Asterisks indicate significance (ANOVA), grey asterisks for comparison between accessions, black asterisks for comparison of treatment to control: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . Bars represent mean values  $\pm$  SD ( $n=5$ ).

Activity of neutral invertase (nInv) doubled in Ct-1 when exposed to H and HHL but this effect was not significant (Figure 5 (a)). Acidic invertase (aInv) was least affected by H and HHL in both accessions (Figure 5 (b)) while cell wall invertase (cwInv) was increased up to 10-fold in Ct-1 under HHL (Figure 5 (c)). Due to relatively high variance of cwInv activities in Ct-1 most differences between control and treatment and between both accessions were not significant although mean

values indicated a strong treatment effect in Ct-1. In general, nInv and cwInv activity in Rsch-4 was less affected by H and HHL than in Ct-1.



**Figure 5.** Relative changes of invertase activities after exposure to heat (H) and heat/high light (HHL). Fold-change of **(a)** neutral invertase (nInv) activity, **(b)** acidic invertase (aInv) activity, and **(c)** cell wall invertase (cwInv) activity compared to control (i.e. a fold-change of 1 indicates no change compared to control). Blue bars: Ct-1, orange bars: Rsch-4. Asterisks indicate significance (ANOVA), grey asterisks for comparison between accessions, black asterisks for comparison of treatment to control: \*  $p < 0.05$ ; \*\*  $p < 0.01$ . Bars represent mean values  $\pm$  SD ( $n=5$ ).

### 3. Discussion

Regulation of plant metabolism under a changing temperature regime comprises and affects numerous pathways, enzymatic reactions and signaling cascades. Previous studies have shown that many compounds of primary metabolism show similar dynamics under heat and cold stress. For example, coordinate increases of amino acids derived from pyruvic acid or oxaloacetate, precursors of polyamines and compatible solutes were observed under both high and low temperatures [16]. Stabilization of a metabolic homeostasis under changing temperature regimes essentially depends on tight regulation of enzyme activities in biosynthesis and degradation pathways. Although temperature was only moderately increased in the present study from 22°C to 32°C, velocities of enzyme-catalyzed reactions typically increase 2 to 3-fold per each 10°C [20,21]. As a consequence, transcriptional, translational, post-translational and metabolic regulation needs to be reprogrammed to stabilize metabolism and prevent over-accumulation of ROS under elevated temperature [7]. In the present study, heat acclimation of two natural accessions, Ct-1 and Rsch-4, was analyzed after 3d and 7d exposure to 32°C. Light response curves of NPS rates under 32°C were found to be similar, or even slightly elevated, compared to control measurements under 22°C. This indicates that, independent of geographical origin, *Arabidopsis* plants are able to photosynthetically acclimate to moderate heat at least within 3 days. These findings are similar to a previous study which showed a slight increase of NPS rates in *Arabidopsis thaliana*, accessions Columbia, between 20°C and 30°C [8]. Yet, under high PAR within light response curves, Ct-1 was observed to have significantly higher NPS rates than Rsch-4 after 7 days of heat acclimation (see Figure 1 (b) and (e)). The accession Ct-1 originates from Sicily/Italy where transient high PAR intensities under high temperature may occur more often than in western Russia which represents the geographical origin of Rsch-4. However, after exposure to high temperature and high light for 3d and 7d, no significant difference was observed anymore between NPS rates of both accessions indicating that acclimation capacity to such environmental conditions cannot be reliably predicted from transient measurements. Such limited predictability of combined stress and acclimation response has been reported before and has also been shown for combination of abiotic and biotic stress [22,23]. After 7d of exposure to HHL plants became senescent which was indicated by yellow leaves (not shown) and NPS rates dropped dramatically in both accessions. Hence, although Rsch-4 and Ct-1 both could stabilize NPS rates until 3d of HHL to a similar extent like under control and heat, 7d of combined stress resulted in severe reduction of photosynthetic CO<sub>2</sub> fixation which was accompanied by strong accumulation of anthocyanins in both accessions. Interestingly, although we could not detect any significant difference in NPS rates (normalized to leaf fresh weight) after 7d HHL between both accessions, only

Ct-1 was significantly affected in protein amount compared to control which might indicate a more advanced level of senescence than in Rsch-4 which is typically accompanied by protein degradation [24]. Thus, due to differential capabilities of inducing secondary metabolism and stabilization of protein amount, comparing differential acclimation response of Rsch-4 and Ct-1 might also be of interest for further molecular studies focusing, e.g., on regulation of phytohormones, anthocyanin metabolism, and photoreceptors [25].

Although light response curves revealed similar NPS rates in both accessions, metabolism of starch and soluble carbohydrates, i.e. sucrose and hexoses, was found to significantly differ between Ct-1 and Rsch-4. While sucrose amount did not vary significantly under heat in both accessions, starch was significantly decreased after 3d and 7d in Rsch-4, and it reached control amount again after 7d in Ct-1. Starch amount has previously been reported to decrease in accession Columbia-0 during heat exposure which is also confirmed by our data [26]. However, differential starch dynamics between both accessions after 7d at 32°C indicate that heat acclimation in Ct-1 results in recovery of starch metabolism while it remains affected in Rsch-4. From the presented data it remains to be elucidated whether also diurnal starch dynamics in Ct-1 are similar to control or if diurnal rates of starch synthesis and degradation are dampened or enhanced after 7d at 32°C.

Neither sucrose nor hexose amount was significantly affected during heat exposure, except for Ct-1 which accumulated ~1.5-fold hexose amount after 7d at 32°C. This finding contrasts previous reports which have shown a significant doubling of sucrose under heat [22]. This discrepancy might be explained by differential growth conditions which were long day (16/8h) in the present study and 12/12h in the study of Prasch and colleagues [22]. Further, Prasch et al. used *Arabidopsis* accession Columbia for their analyses which might induce further accession-specific variance in heat acclimation mechanisms.

Most significant differences between Ct-1 and Rsch-4 were detected for gluco- and fructokinase activities during of H and HHL exposure. Particularly under HHL, both hexose phosphorylating activities remained significantly lower in Ct-1 than in Rsch-4. Simultaneously, hexoses accumulated to significantly higher amount in Ct-1 than in Rsch-4 which rather accumulated sucrose under HHL. These findings indicate differential and accession-specific regulatory strategies of carbohydrate metabolism under such conditions. Although fold-change of SPS activity did not significantly differ between both accessions, Rsch-4 increased its activity under 32°C slightly more than Ct-1 which supports the hypothesis of two different heat acclimation strategies, which are either sucrose accumulating (Rsch-4) or hexose accumulating (Ct-1). Further, aInv and cwInv activity was higher in Ct-1 than in Rsch-4, and this difference became most significant for cwInv activity after 7d HHL. In summary, these observations suggest that higher amounts of hexoses in Ct-1 occurred due to (i) lower hexose phosphorylation rates, and (ii) higher vacuolar and apoplastic sucrose cleavage rates. Enzymatic reactions catalyzed by SPS, GlcK and FrcK, and invertases constitute a futile cycle of sucrose biosynthesis and degradation which has previously been discussed in context of metabolic stabilization under environmental changes, e.g. low temperature [27-29]. In context of recent findings which provided evidence for central metabolic role of hexokinase 1 in response to high light stress [30], it remains tempting to speculate that differential GlcK and FrcK activities in Ct-1 and Rsch-4 explain different subcellular sucrose cycling capacities resulting in different stress acclimation capacities. However, in the present study no significant difference was detected between NPS rates of Ct-1 and Rsch-4 which suggests similar capacities of both accessions to photosynthetically acclimate to H and HHL. It remains to be tested in future studies whether different acclimation capacities can be detected between 3d and 7d of HHL before NPS rates drop dramatically, or if other measures for heat acclimation capacity, e.g. leakage or survival assays, may correlate with sucrose cycling capacity. Finally, resolving plant metabolism to a subcellular level and estimation of enzymatic reaction rates by kinetic modelling will reveal whether sucrose cycling represents a conserved mechanism of stabilization of plant metabolism in a changing environment

## 4. Materials and Methods

### 4.1 Plant material and growth conditions

Seeds of natural *Arabidopsis thaliana* accessions Ct-1 (Sicily/Italy; latitude 37.3°, longitude 15°; 1001 Genomes Id: 7067) and Rsch-4 (Russia; latitude 56.3°, longitude 34°; 1001 Genomes Id: 7322) were incubated in tap water in darkness at 4°C for 2 days before sowing. Plants for molecular analysis were grown in 9x9 cm pots on a 1:1 mixture of GS90 soil and vermiculite with 4 plants per pot. For photosynthesis measurements, plants were grown in round pots with diameter 7cm. All plants were grown for 28 days under short day conditions (8h/16h light/dark, 22°C/18°C, 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 60% humidity) before transfer to long day growth conditions for further 14 days (16h/8h light/dark). Leaf material was sampled at midday, i.e. after 8h in the light. Heat samples (H) were exposed to 32°C/100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 16h and 28°C during the night (8h). Heat/high light samples (HHL) were exposed to 32°C/600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 16h and 28°C during the night (8h). Plants of all conditions were grown in the same growth cabinet (Conviron®, Adaptis A1000-AR, www.conviron.com). One sample consisted of three complete leaf rosettes which were immediately snap frozen in liquid nitrogen and stored at -80°C until further use.

### 4.2 Quantification of CO<sub>2</sub> assimilation rates (NPS)

Plants were dark incubated for 15 minutes before intensity of photosynthetically active radiation (PAR) was stepwise increased in intervals of 5 minutes from 0 to 50, 100, 300, 600 and 1200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . For plants grown under HHL, PAR intensities were 0, 50, 100, 600, 1200 and 2000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . All measurements were performed within the growth cabinet (Conviron®, Adaptis A1000-AR, www.conviron.com) under 22°C (control) or 32°C (H and HHL). Ambient air CO<sub>2</sub> concentration was 410 ppm. All measurements were performed using a WALZ® GFS-3000FL system equipped with measurement head 3010-S including an LED-array to set PAR intensities within light response curves (Heinz Walz GmbH, Effeltrich, Germany, <https://www.walz.com/>).

### 4.3 Quantification of carbohydrate, anthocyanin and protein amounts

Relative starch amount was determined as described earlier with slight modification [31]. Frozen and ground plant material was incubated with 400 $\mu\text{l}$  of 80% EtOH at 80°C for 30min. After centrifugation, the supernatant was transferred into a new tube and extraction was repeated. Supernatants were unified and dried in a desiccator. The resulting dried pellet was used for relative quantification of soluble carbohydrates. Starch containing pellets were incubated with 500 $\mu\text{l}$  0.5N NaOH at 95°C for 60min before slight acidification with 500 $\mu\text{l}$  1M CH<sub>3</sub>COOH. Starch hydrolysate was digested with amyloglucosidase for 2h at 55°C. The amount of glucose which resulted from starch digestion was determined photometrically by a glucose oxidase/oxidase/o-dianisidine reaction at 540nm.

Soluble sugars, i.e. glucose, fructose and sucrose, were determined from dried EtOH extracts. Sucrose was quantified using an anthrone assay after incubation with 30% KOH at 95°C. For the assay, anthrone was dissolved in 14.6M H<sub>2</sub>SO<sub>4</sub> (0.14% w/v), incubated with the samples for 30min at 40°C before absorbance was determined at 620nm.

Glucose amount was determined using a coupled hexokinase/glucose 6-phosphate dehydrogenase assay resulting in NADPH which was determined photometrically at 340nm. For fructose quantification, phosphoglucosomerase was added to the reaction mixture after glucose determination.

Hexose phosphates, i.e. glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P), were extracted and photometrically quantified using an enzyme cycling-based assay [32]. Hexose phosphates were



extracted using trichloroacetic acid (TCA) in diethyl ether (16% w/v), washed with 16% (w/v) TCA in 5mM EGTA and neutralised with 5M KOH/1M triethanolamine. Enzymatic reactions catalysed the equimolar interconversion of G6P and F6P into NADPH which was finally detected photometrically from slopes of a cyclic reaction yielding a formazan dye from thiazolyl blue (MTT) at 570nm.

Anthocyanins were extracted using 1M HCl, first at 25°C and a second time at 80°C. Supernatants were pooled and relative anthocyanin amount was determined photometrically at 520nm.

Protein were extracted from plant material in 8M urea and 50mM HEPES-KOH (pH=7.8). Overnight, proteins were precipitated in acetone with 2mM DTT, subsequently washed with methanol and acetone and again solubilized in 8M urea, 50mM HEPES-KOH (pH=7.8). Protein amount was determined photometrically using the Bradford assay [33].

#### 4.4 Quantification of enzyme activities

Activity of sucrose phosphate synthase (SPS) was determined using the anthrone assay as described earlier [31]. Frozen leaf tissue was suspended in extraction buffer containing 50mM HEPES-KOH (pH 7.5), 10mM MgCl<sub>2</sub>, 1mM EDTA, 2.5mM DTT, 10% (v/v) glycerine and 0.1% (v/v) Triton-X-100. After incubation on ice, extracts were incubated for 30min at 25°C with reaction buffer containing 50mM HEPES-KOH (pH 7.5), 15mM MgCl<sub>2</sub>, 2.5mM DTT, 35mM UDP-glucose, 35mM F6P and 140mM G6P. Reactions were stopped by adding 30%KOH and heating the samples to 95°C. Sucrose was determined photometrically after incubation with anthrone in H<sub>2</sub>SO<sub>4</sub> as described above and blanks (stopped before reaction) were subtracted.

Gluc- and fructokinase activities were determined photometrically at 340nm recording the slopes of NADP<sup>+</sup> reduction to NADPH+H<sup>+</sup> [34]. In brief, frozen leaf tissue was suspended in extraction buffer containing 50mM Tris-HCl (pH 8.0), 0.5mM MgCl<sub>2</sub>, 1mM EDTA, 1mM DTT and 1% (v/v) Triton-X-100. Following incubation on ice, reaction was started with a reaction buffer containing 100mM HEPES-KOH (pH 7.5), 10mM MgCl<sub>2</sub>, 2mM ATP, 1mM NADP, 0.5U G6PDH and either 5mM glucose for glucokinase measurement or 5mM fructose and 0.5U PGI for fructokinase measurement.

Neutral (nInv), acidic (aInv) and cell wall-bound (cwInv) invertase activities were determined as described before with slight modifications [35]. Frozen leaf tissue was homogenized in extraction buffer containing 50mM HEPES-KOH (pH 7.5), 5mM MgCl<sub>2</sub>, 2mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM DTT, 10% (v/v) glycerine and 0.1% (v/v) Triton-X-100. After incubation on ice, samples were centrifuged at 4°C and supernatants were transferred to a new tube. Pellets contained cell wall-bound invertase and were re-suspended in extraction buffer. Soluble, i.e. nInv and aInv, invertase activities were determined from supernatants, cwInv activity was determined from resuspended pellets. Activity of nInv was determined using a reaction buffer with pH 7.5 (20mM HEPES-KOH, 100mM sucrose), aInv and cwInv activities were determined at pH 4.7 (20mM sodium acetate, 100mM sucrose). After incubation of extract with reaction buffers reactions were stopped at 95°C and glucose built from invertase reactions was photometrically determined by a glucose oxidase/peroxidase/o-dianisidine assay at 540nm.

#### 4.5 Statistics

For statistics, each replicate of a measurement under stress (H or HHL) was divided by the corresponding mean value measured under control condition to determine fold-changes. All (relative) metabolite amounts, protein amounts and enzyme activities were normalized to fresh weight before calculation of fold-changes. Statistical data analysis was performed using the free software environment R Version 3.6.1 (www.r-project.org/) (R Core Team, 2019) and RStudio Version 1.2.5019

(<https://rstudio.com/>) (RStudio Team, 2019). Statistics comprised analysis of variance (ANOVA) and Tukey's honestly significant difference post-hoc test. Data were visualized using Microsoft Excel ([www.microsoft.com](http://www.microsoft.com)).

**Author Contributions:** Conceptualization, L.F. and T.N.; methodology, V.A., L.F.; validation, V.A., L.F. and T.N.; formal analysis, V.A., L.F., T.N.; resources, T.N.; writing—original draft preparation, T.N.; writing—review and editing, V.A., L.F.; supervision, L.F., T.N.; funding acquisition, T.N. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the LMUexcellent Junior Researcher Fund and by the SFB/TR175, funded by Deutsche Forschungsgemeinschaft (DFG).

**Acknowledgments:** We thank all members of Plant Evolutionary Cell Biology at LMU Munich for constructive advice and fruitful discussions.

**Conflicts of Interest:** The authors declare no conflict of interest.



© 2020 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

## References

1. Exposito-Alonso, M., Gómez Rodríguez, R., Barragán, C., Capovilla, G., Chae, E., Devos, J., Dogan, E.S., Friedemann, C., Gross, C., Lang, P., Lundberg, D., Middendorf, V., Kageyama, J., Karasov, T., Kersten, S., Petersen, S., Rabbani, L., Regalado, J., Reinelt, L., Rowan, B., Seymour, D.K., Symeonidi, E., Schwab, R., Tran, Diep T.N., Venkataramani, K., Van de Weyer, A.-L., Vasseur, F., Wang, G., Wedegärtner, R., Weiss, F., Wu, R., Xi, W., Zaidem, M., Zhu, W., García-Arenal, F., Burbano, H.A., Bossdorf, O., Nielsen, R., Weigel, D. Natural selection on the *Arabidopsis thaliana* genome in present and future climates. *Nature* **2019**, *573*, 126-129, doi:10.1038/s41586-019-1520-9.
2. Zuther, E.; Lee, Y.P.; Erban, A.; Kopka, J.; Hinch, D.K. Natural Variation in Freezing Tolerance and Cold Acclimation Response in *Arabidopsis thaliana* and Related Species. In *Survival Strategies in Extreme Cold and Desiccation*, Springer: 2018; pp. 81-98.
3. Hannah, M.A.; Wiese, D.; Freund, S.; Fiehn, O.; Heyer, A.G.; Hinch, D.K. Natural genetic variation of freezing tolerance in *Arabidopsis*. *Plant Physiol* **2006**, *142*, 98-112, doi:10.1104/pp.106.081141.
4. Monroe, J.G.; McGovern, C.; Lasky, J.R.; Grogan, K.; Beck, J.; McKay, J.K. Adaptation to warmer climates by parallel functional evolution of CBF genes in *Arabidopsis thaliana*. *Mol Ecol* **2016**, *25*, 3632-3644, doi:10.1111/mec.13711.
5. Zandalinas, S.I.; Mittler, R.; Balfagón, D.; Arbona, V.; Gómez-Cadenas, A. Plant adaptations to the combination of drought and high temperatures. *Physiol Plantarum* **2018**, *162*, 2-12, doi:10.1111/ppl.12540.
6. Birami, B.; Nägele, T.; Gattmann, M.; Preisler, Y.; Gast, A.; Arneith, A.; Ruehr, N.K. Hot drought reduces the effects of elevated CO<sub>2</sub> on tree water-use efficiency and carbon metabolism. *New Phytol* **2020**, *226*, 1607-1621, doi:10.1111/nph.16471.

7. Ding, Y.; Shi, Y.; Yang, S. Molecular Regulation of Plant Responses to Environmental Temperatures. *Mol Plant* **2020**, *13*, 544-564, doi:10.1016/j.molp.2020.02.004.
8. Bunce, J.A. Acclimation of photosynthesis to temperature in *Arabidopsis thaliana* and *Brassica oleracea*. *Photosynthetica* **2008**, *46*, 517-524, doi:10.1007/s11099-008-0088-7.
9. Sage, R.F.; Kubien, D.S. The temperature response of C(3) and C(4) photosynthesis. *Plant Cell Environ* **2007**, *30*, 1086-1106, doi:10.1111/j.1365-3040.2007.01682.x.
10. Haldimann, P.; Feller, U. Inhibition of photosynthesis by high temperature in oak (*Quercus pubescens* L.) leaves grown under natural conditions closely correlates with a reversible heat-dependent reduction of the activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Cell Environ* **2004**, *27*, 1169-1183, doi:10.1111/j.1365-3040.2004.01222.x.
11. Sharkey, T.D. Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. *Plant Cell Environ* **2005**, *28*, 269-277, doi:10.1111/j.1365-3040.2005.01324.x.
12. Ahsan, N.; Donnart, T.; Nouri, M.-Z.; Komatsu, S. Tissue-specific defense and thermo-adaptive mechanisms of soybean seedlings under heat stress revealed by proteomic approach. *J Proteome Res* **2010**, *9*, 4189-4204, doi:10.1021/pr100504j.
13. Sharmin, S.A.; Alam, I.; Rahman, M.A.; Kim, K.-H.; Kim, Y.-G.; Lee, B.-H. Mapping the leaf proteome of *Miscanthus sinensis* and its application to the identification of heat-responsive proteins. *Planta* **2013**, *238*, 459-474, doi:10.1007/s00425-013-1900-6.
14. Lee, D.-G.; Ahsan, N.; Lee, S.-H.; Kang, K.Y.; Bahk, J.D.; Lee, I.-J.; Lee, B.-H. A proteomic approach in analyzing heat-responsive proteins in rice leaves. *Proteomics* **2007**, *7*, 3369-3383, doi:10.1002/pmic.200700266.
15. Wang, X.; Xu, C.; Cai, X.; Wang, Q.; Dai, S. Heat-responsive photosynthetic and signaling pathways in plants: insight from proteomics. *Int J Mol Sci* **2017**, *18*, 2191, doi:10.3390/ijms18102191.
16. Kaplan, F.; Kopka, J.; Haskell, D.W.; Zhao, W.; Schiller, K.C.; Gatzke, N.; Sung, D.Y.; Guy, C.L. Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiol* **2004**, *136*, 4159-4168, doi:10.1104/pp.104.052142.
17. Rolland, F.; Baena-Gonzalez, E.; Sheen, J. Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu Rev Plant Biol* **2006**, *57*, 675-709, doi:10.1146/annurev.arplant.57.032905.105441.
18. Obata, T.; Fernie, A.R. The use of metabolomics to dissect plant responses to abiotic stresses. *Cell Mol Life Sci* **2012**, *69*, 3225-3243, doi:10.1007/s00018-012-1091-5.
19. Koch, K. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Curr Opin Plant Biol* **2004**, *7*, 235-246, doi:10.1016/j.pbi.2004.03.014.

20. van't Hoff, M.J.H. Etudes de dynamique chimique. *Recueil des Travaux Chimiques des Pays-Bas* **1884**, *3*, 333-336, doi:10.1002/recl.18840031003.
21. Bisswanger, H. pH and Temperature Dependence of Enzymes. In *Enzyme Kinetics*, Wiley-VCH: 2017; pp. 145-152. doi:10.1002/9783527806461.ch6
22. Prasch, C.M.; Sonnewald, U. Simultaneous application of heat, drought, and virus to Arabidopsis plants reveals significant shifts in signaling networks. *Plant Physiol* **2013**, *162*, 1849-1866, doi:10.1104/pp.113.221044.
23. Prasch, C.M.; Sonnewald, U. Signaling events in plants: Stress factors in combination change the picture. *Environ Exp Bot* **2015**, *114*, 4-14, doi:10.1016/j.envexpbot.2014.06.020.
24. Buet, A.; Costa, M.L.; Martínez, D.E.; Guiamet, J.J. Chloroplast protein degradation in senescing leaves: proteases and lytic compartments. *Front Plant Sci* **2019**, *10*, doi:10.3389/fpls.2019.00747.
25. Huang, J.; Zhao, X.; Chory, J. The Arabidopsis transcriptome responds specifically and dynamically to high light stress. *Cell Reports* **2019**, *29*, 4186-4199.e4183, doi: 10.1016/j.celrep.2019.11.051.
26. Vasseur, F.; Pantin, F.; Vile, D. Changes in light intensity reveal a major role for carbon balance in Arabidopsis responses to high temperature. *Plant Cell Environ* **2011**, *34*, 1563-1576, doi:10.1111/j.1365-3040.2011.02353.x.
27. Geigenberger, P.; Stitt, M. A futile cycle of sucrose synthesis and degradation is involved in regulating partitioning between sucrose, starch and respiration in cotyledons of germinating *Ricinus-Communis* L seedlings when phloem transport is inhibited. *Planta* **1991**, *185*, 81-90, doi:10.1007/bf00194518.
28. Fürtauer, L.; Nägele, T. Approximating the stabilization of cellular metabolism by compartmentalization. *Theory Biosci* **2016**, *135*, 73-87, doi:10.1007/s12064-016-0225-y.
29. Weizmann, J.; Fürtauer, L.; Weckwerth, W.; Nägele, T. Vacuolar sucrose cleavage prevents limitation of cytosolic carbohydrate metabolism and stabilizes photosynthesis under abiotic stress. *FEBS J* **2018**, *285*, 4082-4098, doi:10.1111/febs.14656.
30. Küstner, L.; Fürtauer, L.; Weckwerth, W.; Nägele, T.; Heyer, A.G. Subcellular dynamics of proteins and metabolites under abiotic stress reveal deferred response of the Arabidopsis thaliana hexokinase-1 mutant gin2-1 to high light. *Plant J* **2019**, *100*, 456-472, doi:10.1111/tbj.14491.
31. Nägele, T.; Stutz, S.; Hörmiller, II; Heyer, A.G. Identification of a metabolic bottleneck for cold acclimation in *Arabidopsis thaliana*. *Plant J* **2012**, *72*, 102-114, doi:10.1111/j.1365-313X.2012.05064.x.
32. Gibon, Y.; Vigeolas, H.; Tiessen, A.; Geigenberger, P.; Stitt, M. Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. *Plant J* **2002**, *30*, 221-235.

33. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **1976**, *72*, 248-254, doi:[https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
34. Wiese, A.; Gröner, F.; Sonnewald, U.; Deppner, H.; Lerchl, J.; Hebbeker, U.; Flügge, U.; Weber, A. Spinach hexokinase I is located in the outer envelope membrane of plastids. *FEBS Letters* **1999**, *461*, 13-18.
35. Nägele, T.; Henkel, S.; Hörmiller, I.; Sauter, T.; Sawodny, O.; Ederer, M.; Heyer, A.G. Mathematical modeling of the central carbohydrate metabolism in Arabidopsis reveals a substantial regulatory influence of vacuolar invertase on whole plant carbon metabolism. *Plant Physiol* **2010**, *153*, 260-272, doi:10.1104/pp.110.154443.