

## ORIGINAL RESEARCH

# Natural variation of temperature acclimation of *Arabidopsis thaliana*

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**Abstract**

Acclimation is a multigenic trait by which plants adjust photosynthesis and metabolism to cope with a changing environment. Here, natural variations of photosynthetic efficiency and acclimation of the central carbohydrate metabolism were analyzed in response to low and elevated temperatures. For this, 18 natural accessions of *Arabidopsis thaliana*, originating from Cape Verde Islands and Europe, were grown at 22°C before being exposed to 4°C and 34°C for cold and heat acclimation, respectively. Absolute amounts of carbohydrates were quantified together with their subcellular distribution across plastids, cytosol and vacuole. Linear electron transport rates (ETRs) were determined together with the maximum quantum efficiency of photosystem II (Fv/Fm) for all growth conditions and under temperature fluctuation. Under elevated temperature, ETR residuals under increasing photosynthetic photon flux densities significantly correlated with the degree of temperature fluctuation at the original habitat of accessions, indicating a geographical east/west gradient of photosynthetic acclimation capacities. Plastidial sucrose concentrations positively correlated with maximal ETRs under fluctuating temperature, indicating a stabilizing role within the chloroplast. Our findings revealed specific subcellular carbohydrate distributions that contribute differentially to the photosynthetic efficiency of natural *Arabidopsis thaliana* accessions across a longitudinal gradient. This sheds light on the relevance of subcellular metabolic regulation for photosynthetic performance in a fluctuating environment and supports the physiological interpretation of naturally occurring genetic variation of temperature tolerance and acclimation.

**1 | INTRODUCTION**

Environmental dynamics have direct effects on plant metabolism and performance. Plant stress response and acclimation to environmental changes stabilize physiological homeostasis and prevent irreversible tissue damage or adverse effects on development and growth. Both

stress response and acclimation are multigenic traits and typically comprise many physiological and molecular changes, including significant reprogramming of photosynthesis, primary and secondary metabolisms (Hannah et al., 2006; Garcia-Molina et al., 2020; Schwenkert et al., 2022; Seydel et al., 2022). For example, a changing temperature regime affects the properties of membrane systems and enzyme

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kinetics, resulting in shifts in membrane fluidity, transmembrane concentration gradients and reaction rates (Elias et al., 2014; Cano-Ramirez et al., 2021). Metabolic pathways and signaling cascades need to be reprogrammed in order to sustain and stabilize metabolism (Herrmann et al., 2019). If environmentally driven deflection from a metabolic state exceeds a certain threshold, this might lead to the formation of reactive oxygen species (ROS) and irreversible tissue damage (Choudhury et al., 2017). Below this threshold, plants are able to prevent irreversible damage by adjusting metabolism, membrane structure and the composition of the photosynthetic apparatus. For example, low temperatures result in an increase of cytochrome *b6f* complex, ATP synthase, Rubisco and other Calvin-Benson-Bassham cycle (CBBC) enzymes, whereas heat leads to increased proportions of light-harvesting complex II (LHCII) and photosystem I (PSI), while CBBC activity is downregulated (Gjindali and Johnson, 2023). This shows that changes in environmental temperature regimes adjust various processes on a molecular and physiological scale, which challenges our understanding and experimental analysis of pathways involved in temperature stress response and acclimation. Further complication of experimental and theoretical analysis is added by the interaction effects of different environmental stimuli, e.g., effects different light intensities have on temperature response (Huner et al., 1998). Although light has been found to be essential for full acclimation to low temperatures (Wanner and Junttila, 1999), too high intensities result in photoinhibition and photooxidative damage (Gray et al., 2003).

When exposed to low temperatures, sucrose metabolism was observed to play a key role in stabilizing photosynthetic efficiency, CO<sub>2</sub> fixation and carbon allocation (Kitashova et al., 2023). It was previously shown that carbon uptake in a low-temperature regime is limited by the capacity to synthesize sucrose (Strand et al., 2003). Cold-sensitive natural accessions of *Arabidopsis thaliana* were restricted in their maximal sucrose phosphate synthase (SPS) activity, which might result in ROS generation (Nägele et al., 2012). Furthermore, photosynthesis was found to be stabilized by vacuolar sucrose cleavage as metabolic control is preserved under cold stress (Weizmann et al., 2018; Nägele, 2022). These findings suggest a central role of subcellular sucrose metabolism in temperature stress response and acclimation. Previously, it has been suggested that carbon partitioning plays a crucial role in enhancing photosynthesis at low temperatures. Specifically, a higher flux of carbon directed towards sucrose, rather than starch, can result in this improvement (Lundmark et al., 2006). A theoretical model has demonstrated that a shift in carbon partitioning towards sucrose can stabilize metabolism in the face of fluctuating environmental conditions (Hernandez and Nägele, 2022). Sucrose, together with galactinol, is a substrate for raffinose biosynthesis, which has previously been found to stabilize photosystem II during freeze-thaw cycles (Knaupp et al., 2011). Hence, carbon partitioning into sucrose might also stabilize downstream biosynthetic pathways, which play a central role for temperature acclimation.

Increased starch degradation results in a drop of starch amount during the first 24 h of cold exposure. This was suggested to serve as carbon resource for hexose biosynthesis, which has the capacity to act as cryoprotectants and as a source for rapid energy supply

(Sicher, 2011). Further, soluble sugars were discussed to effectively scavenge ROS (Morelli et al., 2003) and simultaneously represent substrates for sugar alcohol biosynthesis, which supplies, e.g., raffinose family oligosaccharide (RFO) biosynthesis (Peterbauer and Richter, 2001). Following the initial decrease in starch amount, plants then accumulate starch to higher levels than before cold exposure (Guy et al., 2008). It was reported earlier that natural variation of starch accumulation during cold acclimation might be explained by a differential regulation of the starch degradation pathway resulting in higher starch amount in the freezing sensitive accession Cvi-0 (Cape Verde Islands), originating from Africa, than in the freezing tolerant accession Rsch (Rschew), originating from Russia (Nagler et al., 2015). Comparing Rsch under elevated temperature to the Sicilian accession Ct-1 (Catania) revealed that, similar to cold, Rsch accumulated a lower starch amount than Ct-1 (Atanasov et al., 2020). However, in contrast to cold, the total starch amount decreased under heat compared to control conditions, and the decrease was stronger in Rsch than in Ct-1. Further, decreased starch amount under heat is rather due to inhibition of ADP-glucose pyrophosphorylase and starch synthesis than increased capacities of starch degradation (Geigenberger et al., 1998; Awasthi et al., 2014).

Recently, the availability of sugars has been shown to be essential for plant survival under heat (Olas et al., 2021). While this demonstrates the crucial role of carbohydrate metabolism, its regulation under such conditions is less well understood. It has become evident that, to comprehensively address questions about metabolic regulation, subcellular and compartment-specific data is required (Nägele and Heyer, 2013; Hoermiller et al., 2017; Fürtauer et al., 2019; Höhner et al., 2021). A suitable method for addressing such questions is non-aqueous fractionation, which allows metabolites to be proportionally attributed to the plastid, cytosol, vacuole, and mitochondria (Gerhardt and Heldt, 1984; Fürtauer et al., 2019). Using such an approach, it was, for example, found that substantial reorganization of soluble sugar concentrations occurs at low temperatures (Hoermiller et al., 2017; Weizmann et al., 2018; Patzke et al., 2019). This subcellular reorganization is hypothesized to be both necessary to distribute cryoprotective sugars to key cellular structures, e.g., raffinose to thylakoid membranes, and to stabilize the cellular metabolic homeostasis (Knaupp et al., 2011; Nägele and Heyer, 2013). Also, under elevated temperature, subcellular compartmentation and reprogramming of metabolism plays an important role, e.g., for the regulation of signaling cascades and ROS production (Kohli et al., 2019). Changes in subcellular signaling might explain the previously observed downregulation of transcripts of photosynthesis and carbohydrate metabolism under heat stress (Prasch and Sonnewald, 2013). While transcripts are downregulated, sugar amounts were found to either stabilize or increase during heat acclimation (Atanasov et al., 2020; Garcia-Molina et al., 2020). Simultaneously, a heat-induced increase of neutral and cell wall-associated invertase activities may explain an observed hexose accumulation, which was more pronounced in a natural *Arabidopsis* accession originating from southern Europe than in a northern accession (Atanasov et al., 2020).

In summary, these findings indicate that it is necessary to study carbohydrate metabolism on a subcellular level, which is hardly

predictable from the genome or transcriptome. With such an approach, regulatory patterns that stabilize photosynthesis in a changing temperature regime might be identified. Comparing natural accessions of *Arabidopsis thaliana* has been shown before to be a promising strategy to unravel conserved and specialized stress response and acclimation mechanisms (Maloof et al., 2001; Hannah et al., 2006; Weiszmann et al., 2023). Here, we analyzed the role of subcellular sugar compartmentation in stabilizing photosynthetic efficiency by comparing the natural variations of metabolic acclimation in *Arabidopsis* to both a high- and a low-temperature regime. Natural *Arabidopsis* accessions, originating from a wide geographical range, were acclimated to low and high temperatures before being exposed to a fluctuating temperature regime. Carbohydrates were quantified on a cellular and subcellular level. Finally, we developed an app to estimate subcellular compound distribution from experimental data using Monte Carlo simulations.

## 2 | MATERIALS AND METHODS

### 2.1 | Growth conditions and plant material

Plants of 18 natural accessions of *Arabidopsis thaliana* were grown for 5 weeks in a climate chamber under short-day conditions (8 h/16 h light/dark; photosynthetically active radiation (PAR): 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; 22°C/16°C; 60% relative humidity; see Table S1 for a full list of natural accessions). Then, with same daylength, PAR and relative humidity, plants were transferred to 4°C, 34°C, or left at 22°C for 4 days. Plant material was sampled after 4 hours in the light, i.e., at the middle of the diurnal light period. Leaf rosettes were cut with a scalpel at the hypocotyl and immediately transferred to liquid nitrogen to quench metabolism. Under constant supply of liquid nitrogen, plant material was ground to a fine powder and subsequently lyophilized.

### 2.2 | Chlorophyll fluorescence measurements

Light response curves were recorded using a pulse-amplitude-modulation (PAM) protocol on single leaves using a WALZ JUNIOR-PAM® ([www.walz.com](http://www.walz.com)) in which photosynthetic photon flux density (PPFD,  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was stepwise increased every 20 sec after 15 min of dark incubation at 4°C, 22°C or 34°C for all acclimation conditions (PPFD list: 0, 40, 72, 104, 144, 200, 304, 456, 672, 1000, 1312, 1840, 2400  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). This achieved quantifying photochemical energy conversion efficiency in acclimated plants (growth temperature = measurement temperature) and under temperature stress (growth temperature  $\neq$  measurement temperature). The linear electron transport rate (ETR) was calculated as follows (Equation 1):

$$ETR = Y(II) \cdot E \cdot A \cdot 0.5, \quad (\text{Eq.1})$$

with  $Y(II)$  being the effective photochemical quantum yield of PS II,  $E$  the incident irradiance, and  $A$  the absorptance.

### 2.3 | Quantification of carbohydrates

Starch and soluble sugars were extracted and determined as described before (Kitashova et al., 2023). In brief, approx. 3 mg of lyophilized leaf material was incubated twice with 80% ethanol at 80°C for 30 min to extract soluble sugars. The pellet contained leaf starch granules, which were hydrolyzed, digested with amyloglucosidase, and quantified in a photometric assay using a coupled reaction of glucose oxidase, peroxidase and o-dianisidine. Extracted supernatants were pooled, dried in a desiccator and sugars in the pellet were dissolved in  $\text{H}_2\text{O}_{\text{dd}}$ . Amounts of the soluble carbohydrates sucrose, glucose and fructose were determined photometrically. Sucrose was determined after incubation with 30% KOH at 95°C using an anthrone reagent composed of 14.6 M  $\text{H}_2\text{SO}_4$  and 0.14% (w/v) anthrone, yielding a complex with a specific absorbance maximum at 620 nm. Glucose and fructose amounts were determined within a coupled hexokinase/glucose 6-phosphate dehydrogenase assay, which yielded  $\text{NADPH} + \text{H}^+$  detectable at 340 nm.

### 2.4 | Non-aqueous fractionation (NAF)

Subcellular fractionation followed a NAF protocol described earlier (Fürtauer et al., 2016). After the fractionation of lyophilized leaf material in mixtures of tetrachlorethylene and n-heptane with differential densities, marker enzyme activities were determined for plastids (alkaline pyrophosphatase activity), cytosol (UDP-glucose pyrophosphorylase activity) and vacuole (acidic phosphatase activity). Subcellular amounts of sucrose, glucose and fructose were determined as described in the previous paragraph.

To calculate the relative distributions of metabolites present in each subcellular compartment, an approach based on linear regression and a Monte Carlo simulation was used. This approach is based on the fact that metabolite distributions are made from a linear combination of the compartment-specific gradients (Equation 2):

$$Ax = b \quad (\text{Eq.2})$$

With  $A$  being the matrix containing relative values of the marker metabolites or enzymes. Each row  $m$  represents a density fraction and each column  $n$  a subcellular compartment. The vector  $x$  consists of the relative distribution of our target metabolite present in each compartment  $n$  and is the unknown vector of interest. Finally, the vector  $b$  consists of the relative values of target metabolites in each fraction  $m$ .

Thus, to obtain the relative distribution of a target metabolite across the subcellular compartments, (Equation 2) needs to be solved for  $x$ . For this, linear least-squares fitting constrained to the interval  $[0, 1]$  was applied, as a relative distribution within a compartment cannot be negative or greater than 1.

However, experimental data has shown that a technical error of approx. 10% cannot be excluded when performing NAF (Fürtauer et al., 2016). To account for this, random noise was added to each measurement following a normal distribution with a standard deviation of 5%. This resulted in more robust results and negates infeasible relative distributions such as  $x = (0, 0, 1)^T$ .

This method has been converted into an R Shiny App for ease of use and can be found in a GitHub repository (<https://github.com/cellbiomaths/NAFalyzer>). The app also contains a detailed explanation of how to apply and validate NAF data.

## 2.5 | Enzyme activities

Enzyme activities of the central carbohydrate metabolism were quantified photometrically as described before with slight modifications (Kitashova et al., 2023). All enzymes were quantified from the same extract, which consisted of 50 mM HEPES-KOH (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM EDTA, 1 mM DTT, 1% (v/v) glycerol, 0.1% (v/v) Triton, and protease inhibitor cocktail for plant cell and tissue extracts in DMSO solution (Sigma-Aldrich®).

Following incubation on ice and centrifugation at 4°C, neutral and acidic invertase activities were determined at 30°C by incubation of supernatants in neutral reaction buffer (20 mM HEPES-KOH pH 7.5, 100 mM sucrose) and acidic reaction buffer (20 mM sodium acetate pH 4.7, 100 mM sucrose), respectively. The reaction was stopped by incubation at 95°C before glucose equivalents were determined photometrically using a coupled glucose oxidase/peroxidase/o-dianisidine assay.

Glucokinase and fructokinase activities were quantified from supernatants using a reaction buffer consisting of 100 mM HEPES-KOH (pH 7.5), 10 mM MgCl<sub>2</sub>, 2 mM ATP, 1 mM NADP<sup>+</sup>, 0.5 U glucose 6-phosphate dehydrogenase, and either 5 mM glucose for glucokinase measurement or 5 mM fructose for fructokinase measurement. Production of NADPH was recorded photometrically for 20 min at 30°C and activities were derived from slopes in a linear range of the kinetic.

## 2.6 | Climate data

Climate data was obtained from a database (<https://climatecharts.net/>) and comprises values from 1950 until 2019. Temperature mean of a month is given in [°C] and precipitation in [mm month<sup>-1</sup>].

## 2.7 | Statistics and data evaluation

Statistics and data evaluation was performed in R (Version 4.2.2) using RStudio, Version 2022.07.2 Build 576 (R Core Team, 2021). Correlation analysis and p-value computation were performed via the *rcorr* function of the *Hmisc* package (Version 5.1-0). Principle component analysis was performed via the *prcomp* function of the base stats package (Version 4.2.2).

# 3 | RESULTS

## 3.1 | Fluctuation of linear ETR reveals a longitudinal gradient of natural accessions

After 5 weeks of growth under short-day conditions, plants of 18 natural accessions were acclimated to low (4°C) or elevated (34°C)

temperatures. Maximum quantum yield of PSII (Fv/Fm) and linear ETR were quantified to reveal photosynthetic efficiencies. Detailed information about the accessions is provided in Table S1. Measurements were conducted at the temperature to which plants were acclimated to (i.e., 4°C, 22°C and 34°C). Additionally, plants acclimated at each temperature were exposed to both other temperature regimes for 15 minutes in the dark before, also under these cross-conditions, Fv/Fm and ETR were determined. These cross-condition measurements revealed the effect of a sudden change in temperature regimes on photosynthesis (Figure S1). Linear ETR was found to be both dependent on the measuring and the acclimation temperature. Plants acclimated and measured under 34°C showed the highest mean rates, while plants acclimated at 4°C and measured at 4°C demonstrated the lowest mean rates of ETR.

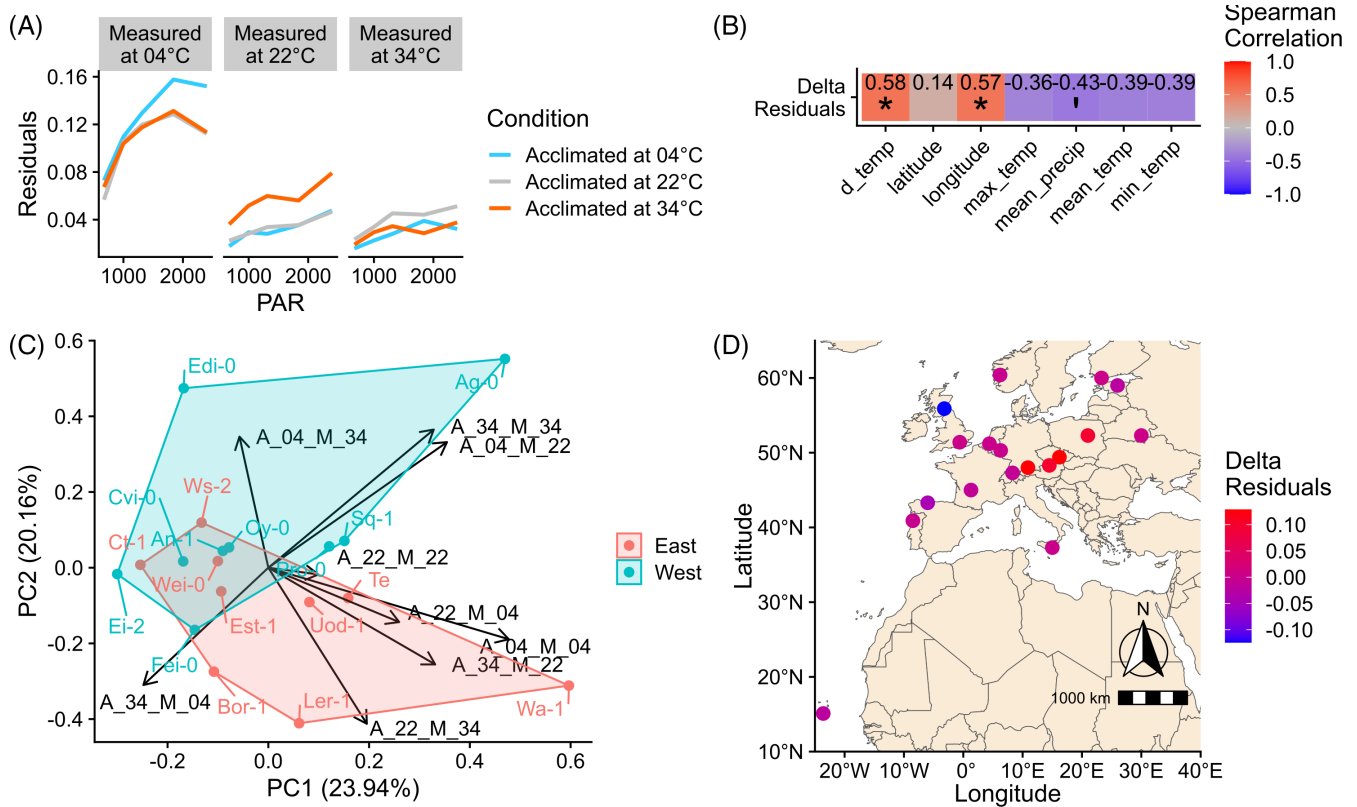
Particularly, yet not exclusively, from cross-conditional measurements, fluctuations of ETR curves were recognized, which indicate instability in photosynthesis over increasing light intensities. To quantify these fluctuations, a curve following a basic saturation kinetic was fitted to the data (Equation 3):

$$ETR = (PAR \cdot p1) / (PAR + p2), \quad (\text{Eq. 3})$$

with *p1* and *p2* being optimized parameters. The resulting residuals between the fit and experimental data then represent a measure for the degree of fluctuation of ETR. Strongest fluctuations were observed at PAR > 500 μmol photons m<sup>-2</sup> s<sup>-1</sup>, while only a weak signal-to-noise ratio was identified for PAR < 500 μmol photons m<sup>-2</sup> s<sup>-1</sup> (Figure S1). Due to this, residual analysis focused on ETR for PAR > 500 μmol photons m<sup>-2</sup> s<sup>-1</sup>. Further, for scaling across measurements, all residuals were normalized to the maximum ETR observed in the respective measurement.

Mean scaled residuals across all accessions became highest for plants acclimated and measured at 4°C (Figure 1A). In general, residuals were observed to be higher for measurements at 4°C than at 22°C and 34°C. Yet, interestingly, cold acclimation resulted in higher residuals than in non-cold or heat-acclimated plants when measured at 4°C. In contrast, heat and cold acclimation resulted in a lower fluctuation level of ETR compared to non-acclimated plants (22°C) when measured at 34°C (Figure 1A, right panel). To test whether scaled residuals can provide information about geographic origin and habitat of natural accessions, they were Spearman-correlated with latitude, longitude, temperature, and precipitation of the first quarter of the year (Figure 1B). This part of the year has recently been found to be indicative of the primary metabolism's regulation of natural *Arabidopsis* accessions (Weiszmann et al., 2023).

When measured at 22°C, plants acclimated to 4°C and 22°C showed similar residuals of ETR, while plants acclimated at 34°C showed the highest fluctuations. When measured at 34°C, plants acclimated at 4°C and 34°C showed the most stable ETR, while 22°C showed higher scaled residuals. This observation indicated a conserved mechanism of temperature acclimation and stabilization of photosynthetic efficiency. Hence, for further temperature acclimation analysis, the difference between the mean scaled residuals of plants acclimated at 22°C and 4°C, both measured at 34°C, was calculated



**FIGURE 1** Fluctuations of electron transport rates across natural *Arabidopsis* accessions. (A) Overall trends of residuals in each combination of growth and measurement temperatures across all natural accessions. Residuals were normalized to the maximum ETR. (B) Spearman correlation between geographical and climate data to the difference between the mean scaled residuals of plants measured at 34°C after being acclimated at 22°C and 4°C. Climate data comprise the first quarter of the year (i.e., Jan – Mar). *d\_temp*: maximum temperature difference; *max\_temp*: highest temperature; *mean\_precip*: mean precipitation; *mean\_temp*: mean temperature; *min\_temp*: minimal temperature. Details are provided in Table S1. Asterisks indicate significance (Spearman,  $n \geq 5$ ): \*  $p < 0.05$ . Mean precipitation was significantly correlated with residuals to a significance level of 10% (i.e.,  $p < 0.1$  indicated as (!)). Red colour indicates positive correlation, purple colour indicates negative correlation. (C) PCA of the mean scaled residuals of all conditions. Accessions labeled in blue represent the accessions classified as ‘western’, while the red label represents ‘eastern’ accessions. Loadings indicate mean scaled residuals at a certain acclimation (A) and measurement (M) temperature, e.g.: A\_04\_M\_34: acclimated at 4°C, measured at 34°C. (D) Geographic origins of accessions and the delta in residuals of plants acclimated at 22°C and 4°C, when measured at 34°C.

and described as  $\Delta(\text{delta})$  residuals, indicating the acclimation capacity of photosynthetic electron transport under maximal temperature fluctuation. The  $\Delta$ residuals were correlated to geographical and climate data from the first quarter of the year at the original habitat of the accessions. The analysis revealed a significant correlation between the  $\Delta$ residuals and the longitude, as well as the difference in monthly mean temperatures recorded within the first quarter of the year, i.e.,  $d\_temp = \text{maximum mean temperature} - \text{minimum mean temperature}$ . Due to the significant positive correlation of  $\Delta$ residuals with longitude, accessions were classified as western (50%, i.e., 9 out of 18 accessions) or eastern (50%; Figure 1D). Principal component analysis (PCA) showed that variance on PC1 was explained by measurements at 4°C, while PC2 was explained by measurements at 34°C (Figure 1C). Most distant accessions on PC1 were Ei-2 (Eifel, Germany) and Wa-1 (Warsaw, Poland). Most distant accessions on PC2 were Ag-0 (Argentat, France) and Ler-1 (Landsberg am Lech, Germany).

Maximum quantum yield of PSII (Fv/Fm) did not significantly differ between eastern and western accessions under any of the

analyzed conditions (Figure S1). In general, it was lowest for plants acclimated to 4°C (Fv/Fm ~0.7–0.8) and highest for non-acclimated plants grown and analyzed at 22°C (Fv/Fm  $\geq 0.8$ ). Variance of Fv/Fm was highest for cold acclimated plants, suggesting higher variability of acclimation of PSII among natural accessions than under elevated temperature. The Fv/Fm showed a significant negative correlation with ETR residuals, except for non-acclimated plants or heat-acclimated plants when measured at 4°C (Figure S2). Finally, short-term cold treatment induced higher photosynthetic fluctuations than both long-term and short-term heat exposure.

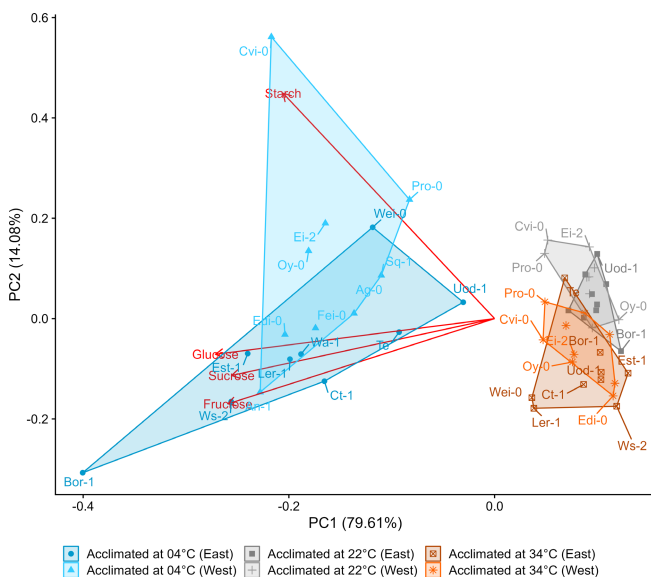
### 3.2 | Differential carbohydrate compartmentation during heat and cold acclimation relates to a longitudinal gradient

Amounts of starch, sucrose, glucose, and fructose were quantified across all 18 natural accessions under each acclimation condition.



Soluble carbohydrates significantly increased during cold acclimation, but total amounts varied strongly between accessions (Figure S3). In heat-acclimated plants, sucrose amounts also increased but to a lower extent than in the cold, whereas hexose amounts decreased compared to non-acclimated plants. The sucrose-to-starch ratios, calculated from C6 equivalents, showed distinct patterns across the natural accessions under low and high temperatures. For most accessions, the ratio of sucrose to starch was higher when plants were exposed to heat rather than cold. In Pro-0, Wei-0, and Cvi-0, the sucrose-to-starch ratio in cold-treated plants remained similar to that of non-acclimated plants.

In a PCA, temperature-induced carbohydrate dynamics separated cold-acclimated plants from non-acclimated (22°C) and heat-acclimated plants on PC1, explaining almost 80% of the total variance (Figure 2). On PC2, a separation of eastern and western accessions was observed, which was strongest for cold-acclimated plants and weakest for non-acclimated plants. A differential ratio of starch-to-soluble carbohydrates explained the observed separation on PC2 (~14%). In general, starch accumulation was stronger in western accessions, while soluble carbohydrates were accumulated stronger in eastern accessions. Carbohydrate accumulation was significantly stronger under 4°C than under 34°C, which resulted in a stronger separation of 22°C samples from cold samples than from heat samples (Figures 2 and S3). In average, exposure to elevated temperature resulted in a stronger increase of sucrose-to-starch ratios than under low temperature (Figure S3).



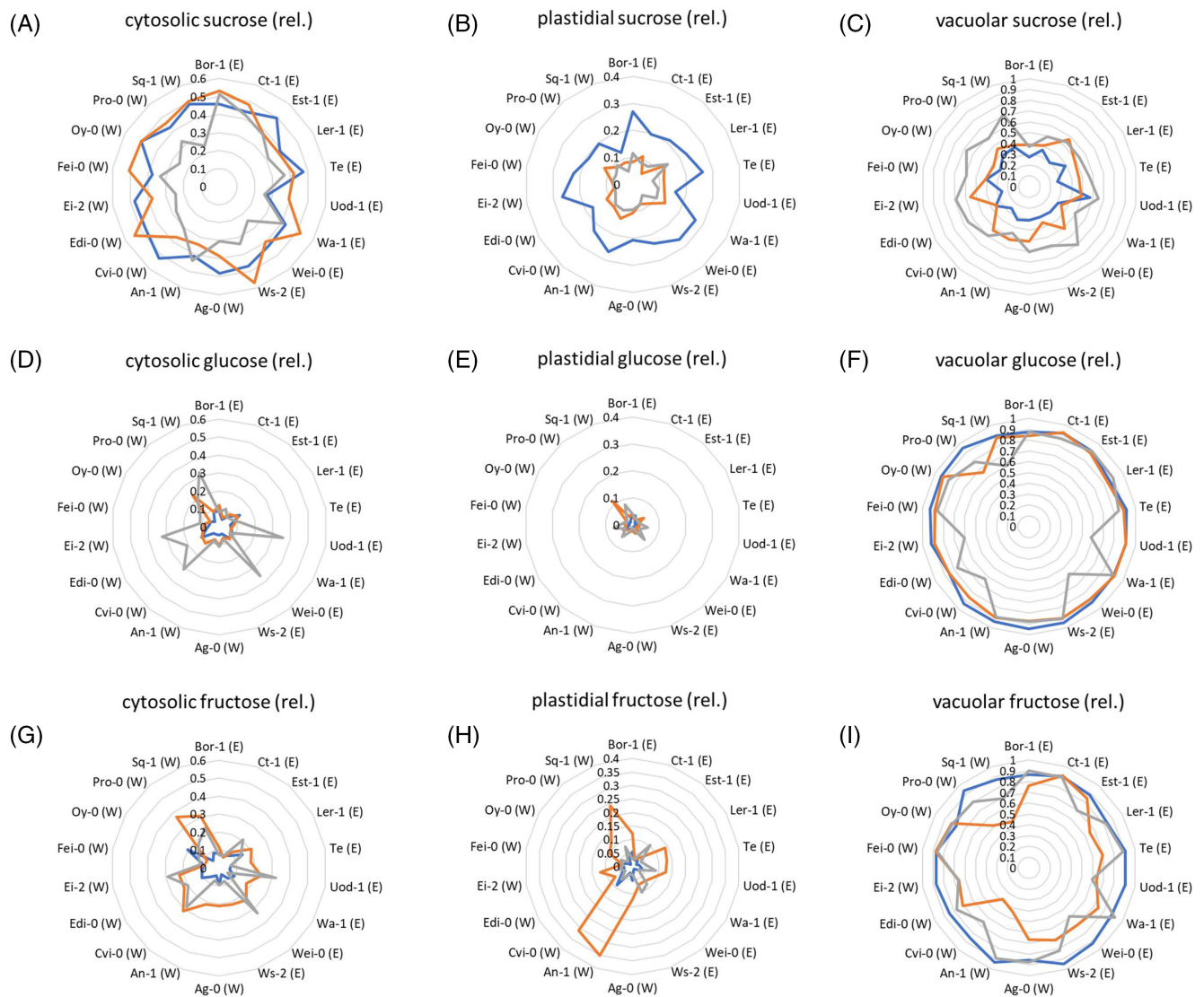
**FIGURE 2** Differential carbohydrate accumulation during heat and cold acclimation in eastern and western accessions. Dots represent scores of accessions acclimated at different temperatures as indicated by the color (grey: 22°C, blue: 4°C, orange: 34°C). Loadings, i.e., coefficients of the linear combination of carbohydrate concentrations, are shown in red ( $n \geq 5$ ). Classification of eastern and western accessions refers to scaled ETR residuals of heat-acclimated plants (see Figure 1).

Subcellular distribution of sucrose, glucose and fructose was determined experimentally by applying a non-aqueous fractionation protocol, which enables the separation of plastidial, cytosolic and vacuolar fractions while metabolism is efficiently and continuously quenched (Fürtauer et al., 2016). A conserved cold response across accessions was an increased proportion of sucrose in plastids and cytosol, while the vacuolar proportion decreased compared to plants at 22°C (Figure 3 A-C). At 34°C, only cytosolic portions increased, while vacuolar sucrose content decreased. The plastidial relative sucrose content remained similar compared to 22°C plants. Relative glucose fractions were highest in the vacuole across all conditions and accessions, and both low and elevated temperatures resulted in an even stronger vacuolar accumulation (Figure 3 F). For fructose, a similar distribution was observed except for plants acclimated to 34°C (Figure 3 G-I). It was observed that the mean fructose portion increased in the plastids and decreased in the vacuole. Further, fructose compartmentation at 34°C was the most variable across accessions, ranging from a similar distribution across all compartments in Cvi-0 to a very strong vacuolar compartmentation in Ct-1, Est-1 and Fei-0 (Figure 3, lower panel).

While relative fructose amount in the cytosol and plastids increased in heat-acclimated plants, vacuolar hexose compartmentation and plastidial sucrose allocation separated cold acclimated accessions from heat and non-acclimated plants (Figure 4). Plastidial sucrose allocation was found to be a conserved cold response across western and eastern accessions. In contrast, vacuolar glucose accumulated stronger in western accessions, while an increase of vacuolar fructose portions was indicative for cold-acclimated eastern accessions. Vacuolar sucrose fractions together with relative amounts of glucose in plastids and cytosol were correlated with plants at 22°C.

Based on the observation of general and specific temperature responses in carbohydrate metabolism, central enzyme activities of carbohydrate metabolism were quantified to reveal if they can explain the observed metabolic output. Enzyme activities were quantified in accessions Cvi-0 and Wa-1, which covered a large geographical range of analysed accessions from southwest to northeast (approx. 5,500 km). Further, subcellular carbohydrate metabolism in both accessions explained a large proportion of PC 1 and PC 2 when comparing temperature acclimation (see Figure 4). In both accessions, neutral invertase activities were found to be significantly increased under elevated temperature (Figure 5A). Acidic invertase activity was significantly reduced at low temperature in Wa-1, while activity significantly increased in Cvi-0 under elevated temperatures (Figure 5B). Fructokinase activity significantly increased during cold acclimation of Wa-1, while no significant temperature effect was observed for Cvi-0, which had lower activities than Wa-1 under all conditions (Figure 5C). Also, mean glucokinase activities were lower in Cvi-0 than in Wa-1, and no significant temperature effect was observed in either accession (Figure 5D).

To estimate the impact of physiologically relevant subcellular carbohydrate concentrations on the stability of photosynthesis, effective subcellular concentrations were calculated from relative metabolite distributions, their absolute amounts and assumptions about



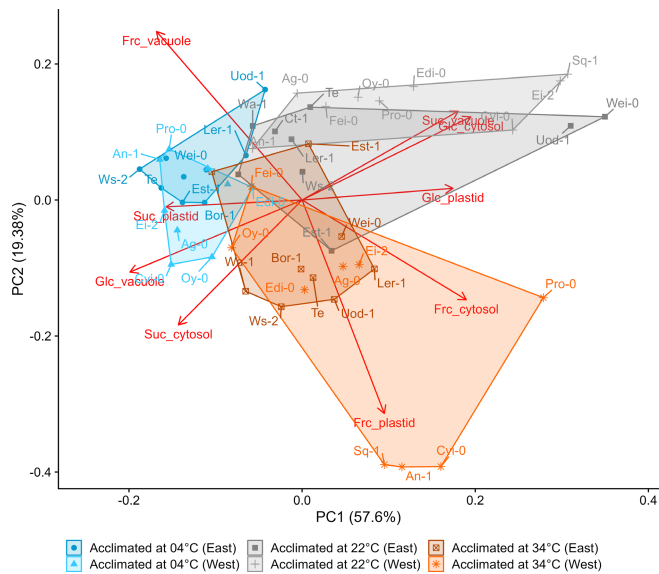
**FIGURE 3** Subcellular carbohydrate compartmentation of cold- and heat-acclimated natural accessions. Accessions and classification into eastern I or western (W) are indicated in each diagram. Relative amounts are indicated by circle lines corresponding to values between 0 (0%) and 1 (100%). Grey lines: 22°C; blue lines: 4°C; orange lines: 34°C. (A – C) sucrose proportions in cytosol, plastid and vacuole. (D – F) glucose proportions in cytosol, plastid and vacuole. (G – I) fructose proportions in cytosol, plastid and vacuole. Lines represent mean values ( $n = 3$ ). All data, including SD, is provided in Table S4.

compartment volumes (Nägele and Heyer, 2013). The volume of plastids was estimated as 20% of the total cellular volume. For cytosol, it was estimated to be 5% and 75% for the vacuole. The estimated compartment-specific concentration was then correlated to Fv/Fm, maximum ETR and residuals of scaled ETR (Figure 6; Tables S3 and S4).

The strongest positive and most significant correlations were observed between the maximum ETR and the effective plastidial sucrose concentration for plants acclimated at 4°C and measured at 34°C, and *vice versa*. Also, vacuolar sucrose concentration correlated significantly with maximum ETR and Fv/Fm for heat-acclimated plants, measured at 4°C and 34°C, respectively. A significant positive correlation was found for cytosolic sucrose concentration and Fv/Fm in plants acclimated to 4°C and measured at 34°C. In plants

acclimated at 22°C, vacuolar fructose concentration was found to be significantly positively correlated to Fv/Fm.

To reveal how subcellular carbohydrate concentrations relate to the ETR residual-based classification of western and eastern accessions, Spearman's rank correlation coefficients were also determined for both accession groups separately (Figure 7). Vacuolar sucrose concentrations were positively correlated with maximum ETR at 22°C in eastern accessions while, under fluctuating temperature (acclimated at 34°C → measured at 4°C, and *vice versa*), plastidial sucrose concentrations significantly correlated with maximum ETR. This was also observed for cold-acclimated western accessions. However, heat-acclimated western accessions measured at 4°C showed a significant negative correlation of plastidial sucrose concentration with Fv/Fm, which contrasted with eastern accessions. Also, plastidial fructose

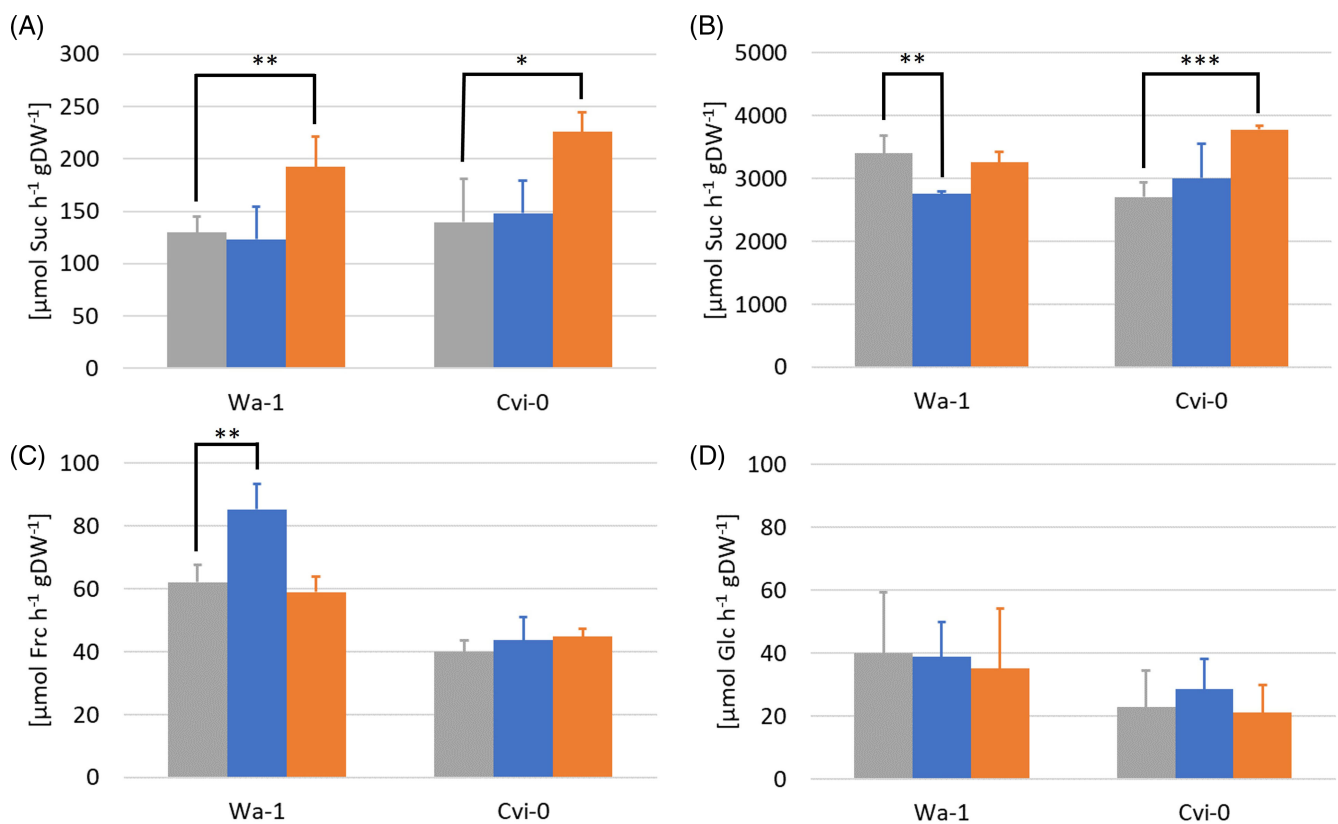


**FIGURE 4** Natural variation of subcellular carbohydrate compartmentation during heat and cold acclimation. Dots represent scores of accessions acclimated at different temperatures as indicated by the color (grey: 22°C, blue: 4°C, orange: 34°C). Loadings represent relative carbohydrate distribution across plastids, cytosol and vacuole ( $n = 3$ ). Classification of eastern and western accessions refers to scaled ETR residuals of heat-acclimated plants (see Figure 1).

concentrations were negatively correlated with Fv/Fm in western accessions when heat-acclimated plants were measured at 4°C. For heat-acclimated western accessions, analysed at 34°C, it was found that vacuolar sucrose concentration was negatively correlated with ETR residuals, indicating a stabilizing function of photosynthesis. In general, those observations suggested that not only photosynthetic efficiencies in a fluctuating environment but also subcellular metabolic acclimation strategies differed between accessions with distinct longitudinal origins.

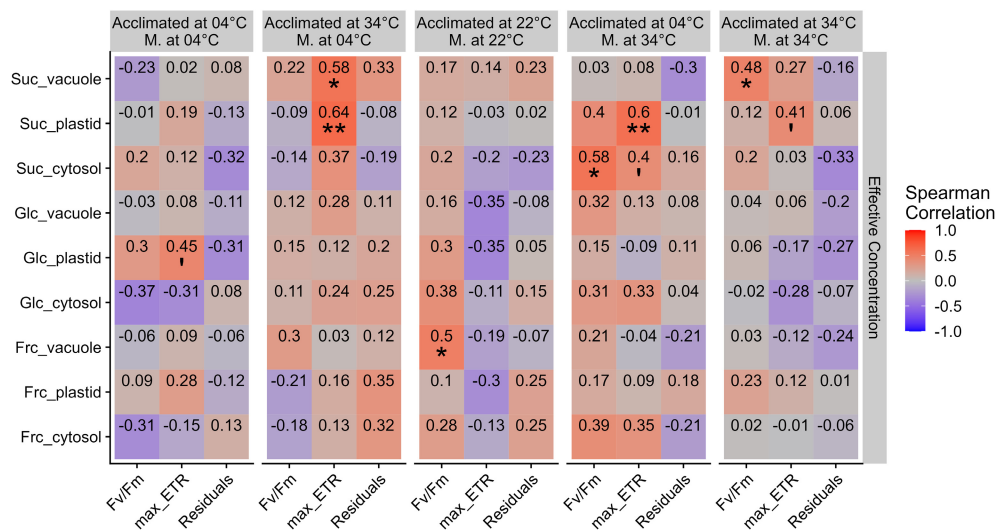
## 4 | DISCUSSION

Changing temperature regimes belong to typical environmental dynamics to which plants are exposed on different time scales. Already within a diurnal cycle, temperature changes might comprise large amplitudes. Comparing temperature amplitudes over recent decades has revealed an upward trend of minimum and maximum temperatures and an increased probability of extreme events in the temperate climate zone (Ouyang et al., 2023). Plant physiological and molecular consequences of such a dynamic environment can be estimated by analyzing temperature stress response and acclimation output. Immediate adjustment of photosynthesis and metabolism to



**FIGURE 5** Enzyme activities of the central carbohydrate metabolism compared in the two geographically distant *Arabidopsis* accessions Wa-1 and Cvi-0. (A) Activity of cytosolic/neutral invertase, (B) activity of vacuolar/acidic invertase, (C) activity of fructokinase, (D) activity of glucokinase. Bars represent means  $\pm$  SD ( $n = 6$ ). Asterisks indicate significance (ANOVA, \*  $p > 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). Grey bars: 22°C; blue bars: 04°C; orange bars: 34°C.





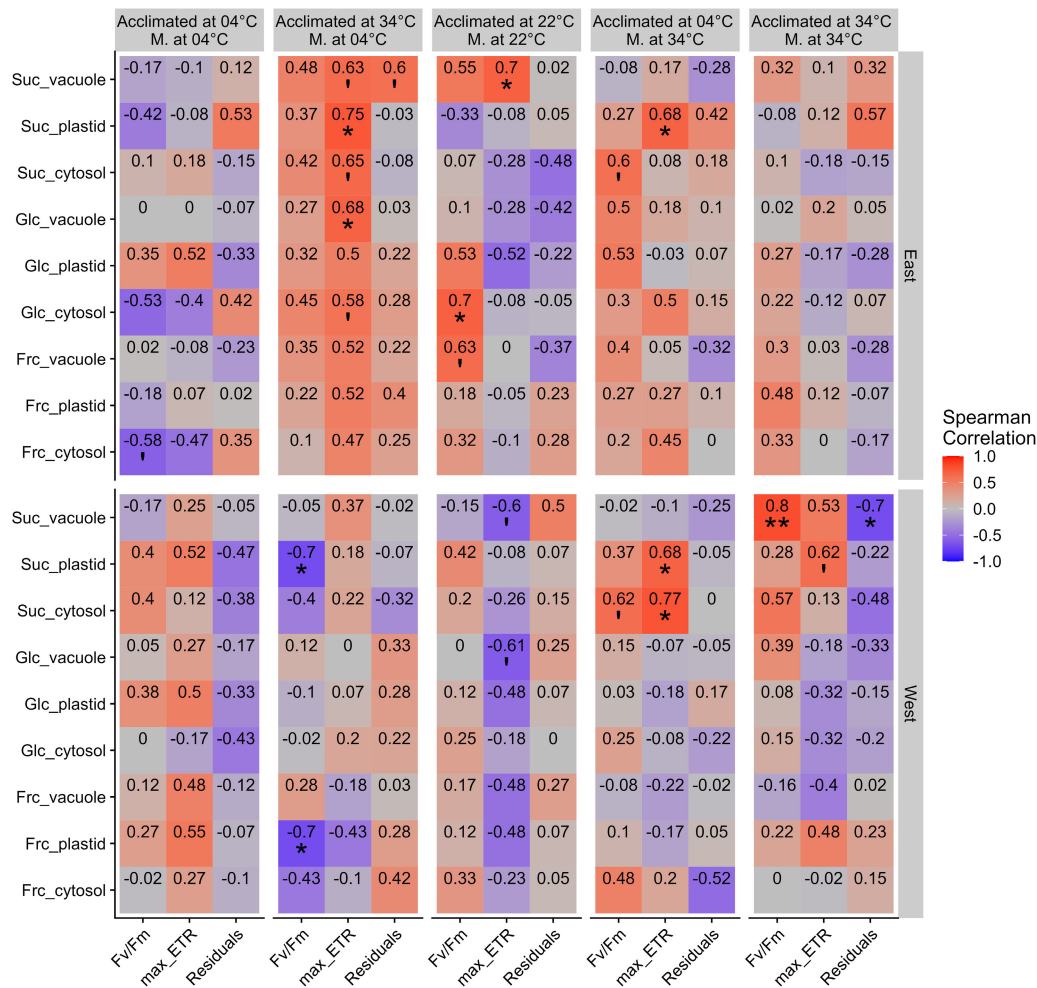
**FIGURE 6** Correlation between photosynthetic efficiencies and subcellular carbohydrate concentrations. Numbers represent Spearman's rank correlation coefficients. Columns relate to experiments to determine  $F_v/F_m$ , maximal ETR ( $max\_ETR$ ) and residuals of scaled ETR ( $Residuals$ ), from left to right: Acclimated at 4°C, Measured at 4°C; Acclimated at 34°C, Measured at 4°C; Acclimated at 22°C, Measured at 22°C; Acclimated at 4°C, Measured at 34°C; Acclimated at 34°C, Measured at 34°C. Red color: positive correlation; purple color: negative correlation. Significance codes (Spearman,  $n \geq 5$ ): \*\* 0.001 <  $p$  < 0.01; \* 0.01 <  $p$  < 0.05;  $\dagger$  0.05 <  $p$  < 0.1.

environmental stressors, e.g., significant temperature fluctuation, represents a prerequisite for plant survival and long-term acclimation responses (Kosova et al., 2011). While forward genetic screens, QTL mappings and reverse genetic approaches have essentially contributed to the understanding of plant-environment interactions (Chinnusamy et al., 2003; Panter et al., 2019), the study of natural variation of traits has further contributed important insights. For example, comparing freezing-tolerant to freezing-sensitive accessions revealed a central role of the C-repeat binding factors (CBFs) in adjusting a low-temperature metabolome (Cook et al., 2004). The study of transcriptomes and metabolomes of natural accessions has further revealed negative regulators of freezing tolerance, e.g., auxin- and cytokinin-induced response regulator genes (Hannah et al., 2006). Simulating naturally occurring low-temperature profiles during early vegetative growth showed that seedling growth differs significantly between *Arabidopsis* accessions originating from northern and southern latitudes, which was suggested to reflect local adaptation mechanisms (Clauw et al., 2022). This was supported by substantial reprogramming of the transcriptome and primary metabolome, resulting in differential metabolome plasticity that was found to be negatively correlated to the maximum temperature of the first quarter of the year at the respective natural habitat (Weismann et al., 2023). Additionally, in the present study, climate data of the first quarter of the year over almost seven decades was found to correlate negatively with fluctuations of photosynthetic (linear) ETR. Here, measurements at elevated temperatures were considered because they showed the least ETR residuals under temperature fluctuations, suggesting the highest photosynthetic acclimation output across all experiments. Hence, the observed correlation output with climate data suggests that plants originating from habitats with high temperature fluctuation during the first quarter of the year show more efficient photosynthetic

acclimation than accessions from habitats with low temperature fluctuation. This fell together with a longitudinal gradient of the chosen accessions. However, the maximum quantum yield of PSII was found to be stable across the full longitudinal range, which indicates that these conditions are not selective enough to either prevent the growth of eastern accessions in western habitats or *vice versa*.

The ratio of the insoluble storage compound starch to soluble carbohydrates has been found earlier to indicate differential metabolic reprogramming due to temperature changes across natural accessions (Klotke et al., 2004; Guy et al., 2008; Nagler et al., 2015). Here, starch was observed to accumulate stronger in cold-acclimated western accessions, while soluble carbohydrates were associated with eastern accessions. Although neither cold, freezing, nor heat tolerance levels were quantified in the present study due to differential ETR residuals under heat, it might be speculated that western accessions tendentially represent more heat tolerant accessions while eastern accessions might be cold-adapted. If this assumption pertained, reduced starch accumulation in eastern accessions might be due to increased starch degradation capacities to support accumulation of soluble carbohydrates and other cryoprotective compounds (Kaplan et al., 2006; Guy et al., 2008; Sicher, 2011). During heat exposure, starch amounts were found to decrease in most accessions, which is in line with previous findings, and a reasonable explanation might be the inhibition of ADP-glucose pyrophosphorylase and starch synthesis rather than increased capacities of starch degradation (Geigenberger et al., 1998; Awasthi et al., 2014). Interestingly, some accessions showed less heat-sensitive starch metabolism (e.g., Fei-0, Oy-0 or Sq-1) and it might be interesting to analyze differences in starch degradation pathways among these accessions in future studies.

As a result of increased starch degradation and sucrose accumulation under heat, many accessions displayed an increased sucrose-



**FIGURE 7** Correlation between photosynthetic efficiencies and subcellular carbohydrate concentrations of eastern (top panel) and western (lower panel) accessions. Numbers represent Spearman's rank correlation coefficients. Columns relate to experiments to determine  $F_v/F_m$ , maximal ETR ( $max\_ETR$ ) and residuals of scaled ETR ( $Residuals$ ), from left to right: Acclimated at 4°C, Measured at 4°C; Acclimated at 34°C, Measured at 4°C; Acclimated at 22°C, Measured at 22°C; Acclimated at 4°C, Measured at 34°C; Acclimated at 34°C, Measured at 34°C. Red color: positive correlation; purple color: negative correlation. Significance codes (Spearman,  $n \geq 5$ ): \*\* 0.001 <  $p$  < 0.01; \* 0.01 <  $p$  < 0.05; † 0.05 <  $p$  < 0.1.

to-starch ratio (Figure 8). Enzyme activity measurements in the two accessions Wa-1 and Cvi-0 revealed a significant heat-induced increase of neutral invertase activity in both accessions while, in Cvi-0, also acidic invertase activity increased significantly compared to ambient conditions. This finding proves that increasing sucrose-to-starch ratios can also be observed under increased sucrose cleavage capacities. Previous findings demonstrated that changes in invertase activities hardly predict cleavage rates or fluxes due to efficient feedback-inhibition by cleavage products, i.e., glucose and fructose (Nägele et al., 2010). Hence, the observed increased partitioning of glucose into the vacuole and fructose into the cytosol might crucially repress invertase activities and stabilize sucrose concentrations, while oxidative stress defense is promoted (Xiang et al., 2011).

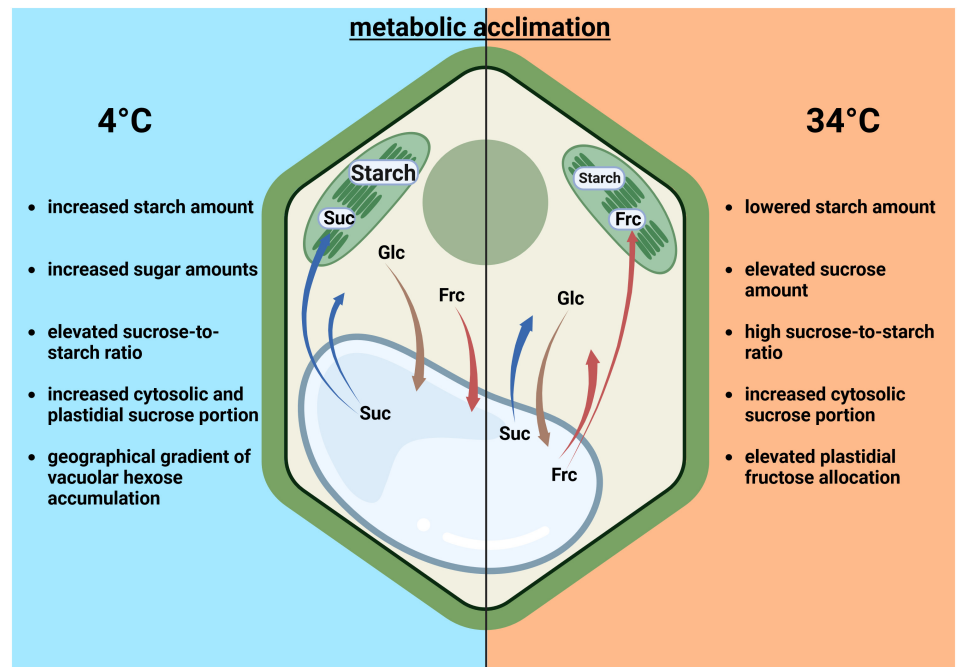
Subcellular analysis revealed that, at low temperatures, a significant fraction of sucrose was accumulating in the plastids, which would preserve it from being cleaved by cytosolic and/or vacuolar invertases (Klotke et al., 2004; Weiszmann et al., 2018). While invertases have

also been shown to occur in plastids (Vargas et al., 2008), their activity might be much lower than in other compartments. We have shown earlier that plastidial accumulation of sucrose represents a conserved cold acclimation strategy in *Arabidopsis* (Nägele and Heyer, 2013), maybe to support stabilization of thylakoid membranes and photosynthesis similar to raffinose (Knaupp et al., 2011) or to balance carbon allocation throughout the cell (Patzke et al., 2019).

Sucrose accumulation in plastids was observed for all accessions, but eastern accessions accumulated fructose, while western accessions rather accumulated glucose in the vacuole. This finding indicates that not only sucrose biosynthesis and cleavage but also the subsequent metabolism of cleavage products glucose and fructose shows significant natural variation. Indeed, activity measurements of fructo- and glucokinase showed that both selected accessions differed in both absolute activities and temperature-induced dynamics. HEXOKINASE 1 (AtHXK1) catalyzes the phosphorylation of glucose and has recently been shown to improve drought and heat tolerance when

### FIGURE 8 Comparing natural variation of metabolic acclimation to low and elevated temperature.

Comparisons relate to non-acclimated plants (22°C). Arrow sizes indicate effect strength, arrow color refers to different sugars. Suc – sucrose (blue arrows); Glc – glucose (brown arrows); Frc – fructose (red arrows). Created with [BioRender.com](https://BioRender.com).



overexpressed in potato (Lehretz et al., 2021). Further, AtHXK1 is a central conserved sugar sensor that might directly affect photosynthesis and transpiration by controlling stomatal aperture (Moore et al., 2003; Granot and Kelly, 2019). Fructokinase catalyzes the carbon flow into the glycolytic pathway and the tricarboxylic acid cycle to provide substrates for amino acid biosynthesis and mitochondrial respiration (Pego and Smeekens, 2000). Hence, natural variation of cold and heat acclimation capacities may, among others, be due to differential hexose phosphorylation capacities, which would also have an impact on respiratory acclimation to a changing temperature regime (Talts et al., 2004; Atanasov et al., 2020).

An increasing body of evidence has revealed that, to comprehensively understand the regulation of photosynthesis and metabolic processes involved in plant temperature response, subcellular data needs to be considered (Hoermiller et al., 2017; Hurry, 2017; Höhner et al., 2021). Yet, estimating effective *in vivo* metabolite concentrations remains challenging not only because of laborious sample fractionation but also due to a lack of information about organellar volumes and their temperature-dependent dynamics. Particularly, such information becomes essential for quantitative analysis of compartment-specific reaction rates and metabolic fluxes because compartmentalization of metabolism directly affects enzymatic activity and function, thus altering regulation and fluxes within a metabolic network (Szecowka et al., 2013; Herrmann et al., 2021; Nägele, 2022). Data of the present study provides further evidence for the importance of estimating subcellular effective concentrations, which enabled the interpretation of physiological effects on photosynthesis. Comparing correlations between subcellular sugar concentrations and photosynthetic efficiencies indicated a split of accessions into east and west. Even under ambient temperature, such a split revealed positive and negative correlation of vacuolar sucrose concentrations with Fv/Fm and maximum ETR in eastern

and western accessions, respectively. Hence, uncovering such effects supports our hypothesis of a physiologically relevant classification due to geographical origin and habitat information.

Finally, many significant correlations between subcellular sugar concentrations and photosynthetic efficiencies were only observed under fluctuating temperatures, which suggests that a subcellular metabolic adjustment during temperature acclimation results in a metabolic state which stabilizes photosynthesis against further environmental deflections. This hints towards the necessity of rethinking the definition of plant (temperature) acclimation, which might rather consider the plasticity of metabolism towards environmental changes than its absolute configuration. It is tempting to speculate that metabolite transport across membranes like the tonoplast or the chloroplast envelope may significantly affect and determine such metabolic plasticity and, by this, temperature acclimation capacities. For example, sucrose transport between chloroplast and cytosol has been shown recently to affect the cold acclimation capacities of *Arabidopsis thaliana* (Patzke et al., 2019). Although the exact *in vivo* function of the pSuT transporter of the inner plastid envelope is still under debate, its activity was found to be essential to maximize freezing tolerance. In the present study, natural variations of subcellular fructose partitioning were observed. Besides differential fructokinase activities, this might also be due to a naturally occurring variation of SWEET17 regulation, which is a homolog of SUGARS WILL EVENTUALLY BE EXPORTED (SWEET) proteins being located to the tonoplast (Guo et al., 2014; Keller et al., 2021). Future studies might decipher the contribution of these transporters and metabolic enzymes to the natural variation of metabolic regulation. In conclusion, using analytical platforms to quantify subcellular primary and secondary metabolism together with studies on metabolite transport promises to promote our understanding of naturally occurring temperature acclimation output.

## AUTHOR CONTRIBUTIONS

J.H. performed experiments and data evaluation, developed the R app (NAFalyzer) and wrote the paper. D.D. performed experiments and supported data evaluation. L.S., C.S. and A.K. performed experiments. V.B. developed the R app (NAFalyzer). T.N. conceived the study, supported data evaluation and wrote the paper.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

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