Integrative Biology

PAPER

Identifying core features of adaptive metabolic mechanisms for chronic heat stress attenuation contributing to systems robustness†

Jenny Gu,*[a] Katrin Weber,[b] Elisabeth Klemp,[b] Gidon Winters,[a] Susanne U. Franssen,[a] Isabell Wienpahl,[a] Ann-Kathrin Huylmans,[a] Karsten Zecher,[a] Thorsten B. H. Reusch,[c] Erich Bornberg-Bauer[a] and Andreas P. M. Weber[b]

The contribution of metabolism to heat stress may play a significant role in defining robustness and recovery of systems; either by providing the energy and metabolites required for cellular homeostasis, or through the generation of protective osmolytes. However, the mechanisms by which heat stress attenuation could be adapted through metabolic processes as a stabilizing strategy against thermal stress are still largely unclear. We address this issue through metabolomic and transcriptomic profiles for populations along a thermal cline where two seagrass species, *Zostera marina* and *Zostera noltii*, were found in close proximity. Significant changes captured by these profile comparisons could be detected, with a larger response magnitude observed in northern populations to heat stress. Sucrose, fructose, and *myo*-inositol were identified to be the most responsive of the 29 analyzed organic metabolites. Many key enzymes in the Calvin cycle, glycolysis and pentose phosphate pathways also showed significant differential expression. The reported comparison suggests that adaptive mechanisms are involved through metabolic pathways to dampen the impacts of heat stress, and interactions between the metabolome and proteome should be further investigated in systems biology to understand robust design features against abiotic stress.

Introduction

Cellular metabolism may play a significant role in defining robustness and recovery of systems in response to heat stress; either through the provision of energy and metabolites for achieving a new state of cellular homeostasis,[1] or contributing to systems robustness against heat stress with the production of protective organic osmolytes observed in both animals and plants.[2,3] Understanding the thermodynamic contributions of these osmolytes, and possible synergistic interactions between mixed contributors, to systems stabilization is a growing field of investigation.[3,4] Furthermore, osmolytes have been shown to act as chemical chaperones influencing the conformational state and stability of heat shock molecular chaperones,[5] thus underscoring the need to understand the solvent context of proteomes and contributions to robustness from the holistic perspective of systems biology.

[a] *Institute for Evolution and Biodiversity, University of Muenster, Germany. E-mail: j.gu@uni-muenster.de*
[b] *Plant Biochemistry, Heinrich-Heine-University, Duesseldorf, Germany*
[c] *Leibniz-Institute for Marine Sciences, Marine Ecology – Evolutionary Ecology, Kiel, Germany*
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2ib00109h

Insight, innovation, integration

Core features of metabolic heat stress attenuation important for robustness against heat stress were identified through a parallel comparison of two closely related seagrass species, *Zostera marina* and *Zostera noltii*, sampled from populations along a thermal cline. Seagrasses enable investigations to understand responses of systems to chronic heat stress in marine plants. This investigation is the first to leverage metabolomic and transcriptomic data to identify shared and diverged features to understand adaptive properties of metabolic responses in the context of global warming. Key enzymes contributing to attenuation of heat stress were found to be involved in the Calvin cycle, glycolysis and pentose phosphate pathways. The results also suggested potential interactions between the metabolome and proteome as an adaptive feature for systems robustness against abiotic stress facilitated through the use of organic osmolytes acting as chemical chaperones.

To this end, a number of metabolomic investigations have been conducted in recent years on plant abiotic stress as well as plant–pathogen interactions.[6–8] Metabolites also have important regulatory roles in signaling and contribute to posttranslational modifications of proteins such as glycosylation. Generally, the contributions of metabolic responses have been observed to respond more quickly to abiotic stress than adjustments of gene expression regulation.[9] Previous investigations of the plant heat stress response using nonspecific metabolite profiling showed that 23% of 497 analyzed metabolites were heat responsive, which also overlapped with the significantly larger number of identified cold responsive metabolites.[7] Changes in transcript abundance from different biosynthetic pathways were also found to correlate with the changing metabolite levels.

However, still unclear is an understanding of how heat shock mitigation through metabolic mechanisms could be evolved and adapted to chronic thermal stress. Since energetic costs are associated with the continued overexpression of heat shock proteins (HSPs) as a primary response to dampen heat stress effects,[10] further investigations to understand alternative mechanisms to achieve new states of homeostasis would be important for our mechanistic understanding of systems robustness to changing environments. An evolutionary study using the fish species *Fundulus heteroclitus* along a thermal cline suggested that metabolic genes contribute to the majority of significant adaptive variations in gene expression that correlated with habitat temperature.[11] Significant metabolic reprogramming in dealing with thermal stress has also long been recognized to be important contributor to thermo-tolerance and adaptation in plants.[12] In physics, attenuation consequently results in the observed dampened effect on the intensity of energy, *e.g.* in the form of heat, light, and sound, based on properties of a medium. Here, we aim to understand how changes in the osmolyte solvent medium could contribute to heat attenuation and use seagrasses as the study system.

Seagrasses have recently been subjected to a series of research efforts to understand their molecular response to heat stress,[13–15] and evolution to an aquatic environment.[16] Investigations using this study species are important for several reasons. First, the seagrass species *Zostera marina* is poised to serve as a model organism to understand molecular evolution and responses of marine plants because of the growing transcriptomic resources and anticipated genome sequencing. Second, seagrasses are widely distributed foundational species[17] supporting and defining marine ecosystems with a large commercial value.[18] The two species used in this investigation are *Zostera marina* and *Zostera noltii*, both spanning a wide biogeographic range along the northern hemisphere shorelines. The distribution of these species along the Atlantic European coast from Norway to the Mediterranean seas, including the collection sites for this investigation, has been well studied.[19,20] The difference between the estimated maximum temperature for the northern (21 °C) and southern (29 °C) collection sites is approximately 8 °C based on available data obtained through remote sensing and monitoring efforts of seagrass beds (data not shown). Lastly, seagrass populations are in decline due to challenges associated with increasing thermal stress at the ocean surface resulting from global warming.[20,21] An initial study using expressed sequence tags identified 62 responsive transcripts

that suggested significant photosynthetic adjustments.[14] Some candidate heat shock genes and oxygen scavengers contributing to the stress response were also identified. More recently, a deeper transcriptomic investigation using 454 mRNA-seq demonstrated that the adaptive benefits of seagrass populations with respect to heat are more likely to be revealed during the recovery phase post-heat stress rather than the immediate stress response.[22]

Extending on these previous studies investigating the response of a single seagrass species, we present here the first in-depth metabolomic and transcriptomic investigation conducted on the thermal stress response of two seagrass species, *Zostera marina* and *Zostera noltii*, sampled along a thermal cline where southern populations experience an estimated average maximum temperature up to 29 °C compared to northern populations at 21 °C (data not shown). The responses of 29 organic metabolites were profiled across a 5-week heat stress experiment and observations were integrated with transcriptome profiles sampled at the peak of the heat stress treatment. The detected molecular responses were also considered in the framework defined by physiological measurements to make an integrated inference across different levels of biological organization. This study design supports an investigation of metabolic responses to chronic heat stress using an ecologically important founder species threatened by changing thermal patterns resulting from global warming. The results also highlighted potential adaptive strategies through metabolic processes for heat attenuation to achieve a new state of homeostasis. Through this investigation, we propose a new paradigm for an HSP independent strategy that is complementary to heat stress attenuation evolved by protection of misfolding *via* organic osmolytes as chemical chaperones.

Results

Several fitness-related physiological parameters such as growth and photosynthesis were monitored to detect potential differences in the temperature response of seagrasses at weekly timepoints (Fig. 1B) and to complement the molecular interpretations obtained during downstream analysis. Shoot count, growth rate, and photosynthetic measurements of the maximum electron transport rate (ETR_{max}) and 10 second dark-adapted effective quantum yield (Y_0) of photosystem II were conducted. These physiological parameters were used to measure only immediate response to heat stress. Statistical significance of responses was tested with ANOVA ($\alpha < 0.001$). The growth rate and shoot counts for all populations (Fig. S1 and S2, ESI†) show significant differences in stress treatment with respect to timepoint ($F = 98.89$, $p < 2.2 \times 10^{-16}$), location ($F = 284.41$, $p < 2.2 \times 10^{-16}$), and species ($F = 3226.40$, $p < 2.2 \times 10^{-16}$). Significant interactions between the parameters of timepoint ($F = 23.35$, $p = 1.75 \times 10^{-6}$), and location ($F = 68.69$, $p = 8.548 \times 10^{-16}$) with species were detected. Interestingly, stress treatment effects for pulse amplitude modulated (PAM) fluorometry results showed only weak significance ($F = 9.55$, $p = 0.002$) and did not exceed our significance threshold of $\alpha < 0.001$. Similarly, ANOVA analysis of Y_0 over the time course also showed no significant treatment effects ($F = 4$, $p = 0.046$, $\alpha < 0.001$). Overall, the fitness indicators of heat stress treated samples appeared to remain relatively similar to
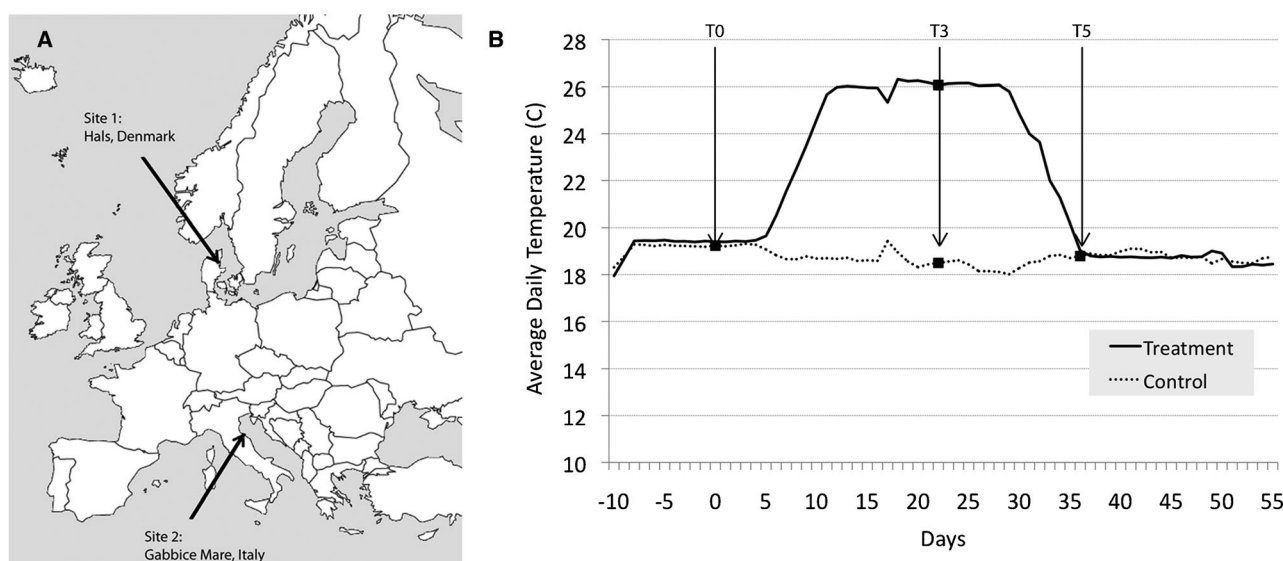
Fig. 1 Collection site of seagrass populations subjected to a controlled heat stress regime. (A) Collection sites where the study species *Zostera marina* and *Zostera noltii* were found co-localized within approximately 100 m were used. Cold adapted seagrasses were collected from Hals (Denmark; 56°50′ N, 10°1′ E; early May 2009) and warm adapted species from Gabbice mare (Italy; Adriatic Sea; 43°50′ N, 12°45′ E; late April 2009). (B) A heat stress regime was applied to the collected samples after acclimation for a minimum of 4 weeks. Metabolomic profiles representing 29 metabolites were analyzed for T0, T3, and T5, and this dataset was complemented with a transcriptomic profile at T3 to identify key treatment responsive contributions.

that of the control group, slowly declining over time. An accelerated decay during post-stress recovery after T5 was observed in the physiological measurements for some populations.

Samples for metabolomics analysis were also taken during these weekly fitness measurements to measure the response profiles of organic metabolites. Samples for metabolomic profiles were taken at the timepoints of T0, T3, and T5 (Fig. 1). After removal of weak signals and outliers, the profiles of 29 metabolites were analyzed in response to heat stress treatment over the time course of the experiment (Fig. 2). Significant differences were identified in the metabolomic response between the two species (ANOVA; $F = 28.37$, $p = 4.799 \times 10^{-7}$), temperature treatment ($F = 26.015$, $p = 1.295 \times 10^{-6}$) and metabolites ($F = 99.0368$, $p < 2.2 \times 10^{-16}$, $\alpha < 0.001$). Significant interactions between metabolites with treatment ($F = 30.56$, $p < 2.2 \times 10^{-16}$), species ($F = 30.56$, $p < 2.2 \times 10^{-16}$), and all three variables at the same time ($F = 4.10$, $p = 3.310 \times 10^{-12}$) were identified ($\alpha < 0.001$).

ANOVA analysis of the metabolomic response of only *Z. marina* populations shows significant differences between the northern and southern populations ($F = 32.013$, $p = 4.539 \times 10^{-7}$, $\alpha < 0.001$) with interactions between metabolites for both treatment and species variables ($\alpha < 0.001$). Significant differences between the metabolites were also detected ($F = 61.3922$, $p < 2.2 \times 10^{-16}$), as can be expected. Finally, interactions between the metabolites and the variables of timepoint ($F = 3.6194$, $p < 2.2 \times 10^{-16}$), treatment ($F = 33.70$, $p < 2.2 \times 10^{-16}$), and population from which the *Z. marina* plants were sampled could be detected ($F = 5.5880$, $p < 2.2 \times 10^{-16}$). Interactions identified between the three factors of metabolites, treatment, and population ($F = 2.4240$, $p = 4.76 \times 10^{-5}$) suggested that the measured metabolomic response to heat stress depends upon population affiliation. *Z. noltii* shows

significant differences with treatment effects ($F = 12.41$, $p = 0.0008$) and metabolites ($F = 62.6088$, $p < 2 \times 10^{-16}$). There was also significant interaction between the treatment response and metabolites ($F = 15.6278$, $p < 2 \times 10^{-16}$), however the difference between populations was not as strongly detected compared to the *Z. marina* populations.

Hierarchical clustering of the averaged metabolomic profiles for *Z. marina* (Fig. 3A) shows all profiles from control conditions to cluster together. This cluster also included samples designated for heat treatment at timepoint T0; with the exception of the *Z. marina* northern population, where the averaged profile showed more similarity to those observed for southern *Z. marina* population under heat stress. The observed deviation from the control cluster for the *Z. marina* northern population at T0 may be an observable heat stress response during the acclimation phase which involved a slow gradual temperature increase from 14 °C to the final baseline temperature of 19 °C. Otherwise there appears to be a population specific metabolomic response to the heat stress regime with profiles of southern populations showing more similarity to the control conditions than the response of northern populations to heat stress. Furthermore, the metabolomic profiles do not return immediately to the control state post-heat stress at T5. This distinction between populations was less evident for *Z. noltii* (Fig. 3B) where results only showed a clear separation between control and treatment groups instead. The results for *Z. noltii* also suggested that metabolomic profiles of the treated group returned to the initial profiled state at T0 prior to treatment since samples from T0 and T5 clustered together for each respective population (Fig. 3B).

Of the analyzed metabolites, principal component analysis (PCA) conducted on the averaged replicate samples identified
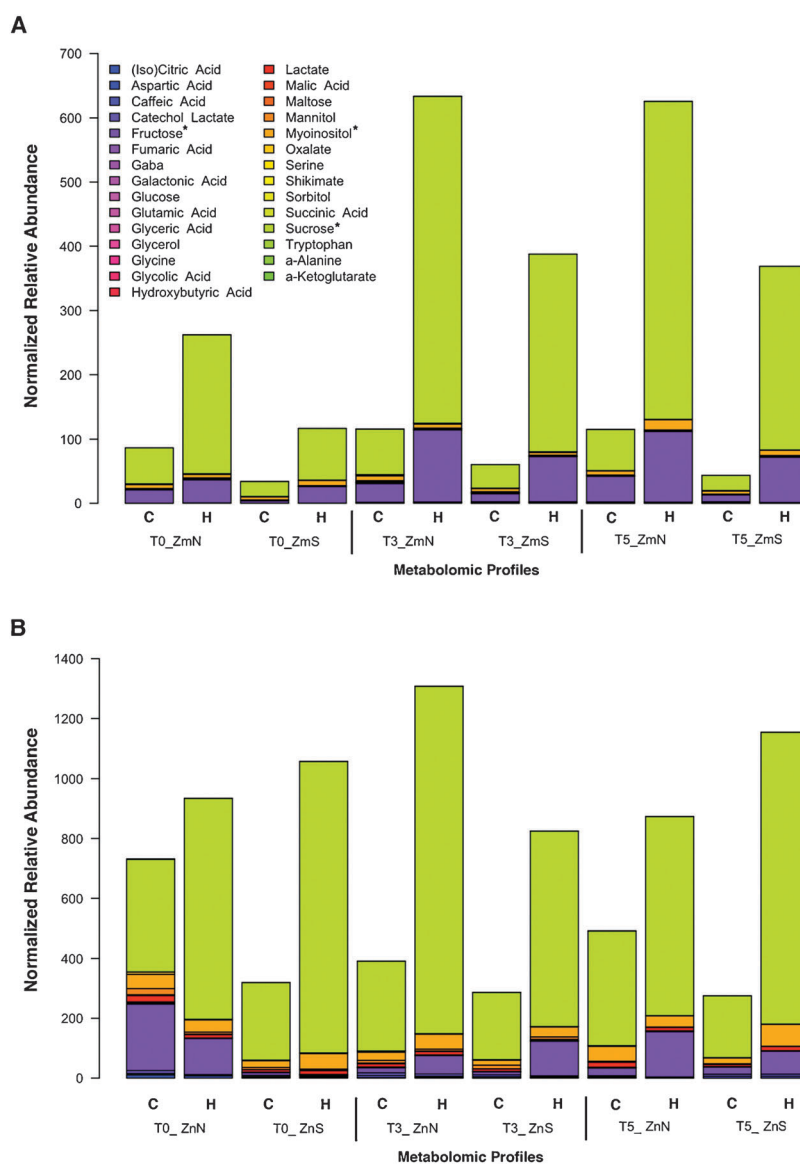
Fig. 2 Metabolomics response profile of seagrasses to heat stress treatment. (A) The averaged response across 6 replicates for 29 analyzed metabolites is shown for both the northern and southern populations of *Z. marina* and (B) *Z. noltii*. The top three most responsive metabolites identified using principal component analysis are sucrose, fructose, and *myo*-inositol (*).

sucrose to be most responsive to treatment and accounted for most of the observed variance in the first principal component (93.3%), followed by fructose and then *myo*-inositol in *Z. marina*. Similar results were also observed for *Z. noltii* where the first principal component accounted for 78.01% of the variance. PCA showed that the averaged control samples from treated populations taken from different timepoints tended to cluster based on the first two principal components, similar to what was observed for the hierarchical clustering analysis. On the non-averaged dataset, PCA identified the same dominant contributions of sucrose and fructose. However, the replicates did not form distinct clusters to show clear separation of treatment, population, and control groups, further underscoring the high amount of variance between replicate samples in the metabolomics analysis (Fig. S5, ESI†).

The transcriptomic responses to heat stress treatment, one representing each population and experimental conditions for

both species, were sampled and sequenced for only T3. *De novo* assembly of the transcriptome resulted in a total of 7460 tentative unigenes, which we now refer to as genes, shared by the two species that have been successfully annotated using *A. thaliana* and *O. sativa* data resources (Table S1, ESI†). Within this gene set, 2636 (35.34%) were successfully annotated to be involved in metabolic processes using the METACYC database.[23] Since a representative genome for the *Zostera* family is not yet available, the transcriptome was analyzed for the under-representation of GO terms based on GOSLIM for plants. Results show no significantly underrepresented biological processes in the transcriptome, as annotated by using the GO terms, thus indirectly suggesting that sufficient gene coverage has been achieved for an unbiased investigation of changes in differential expression responses.

Focusing only on METACYC subset of annotated genes (35.34%), genes with significant differential expression, adjusted
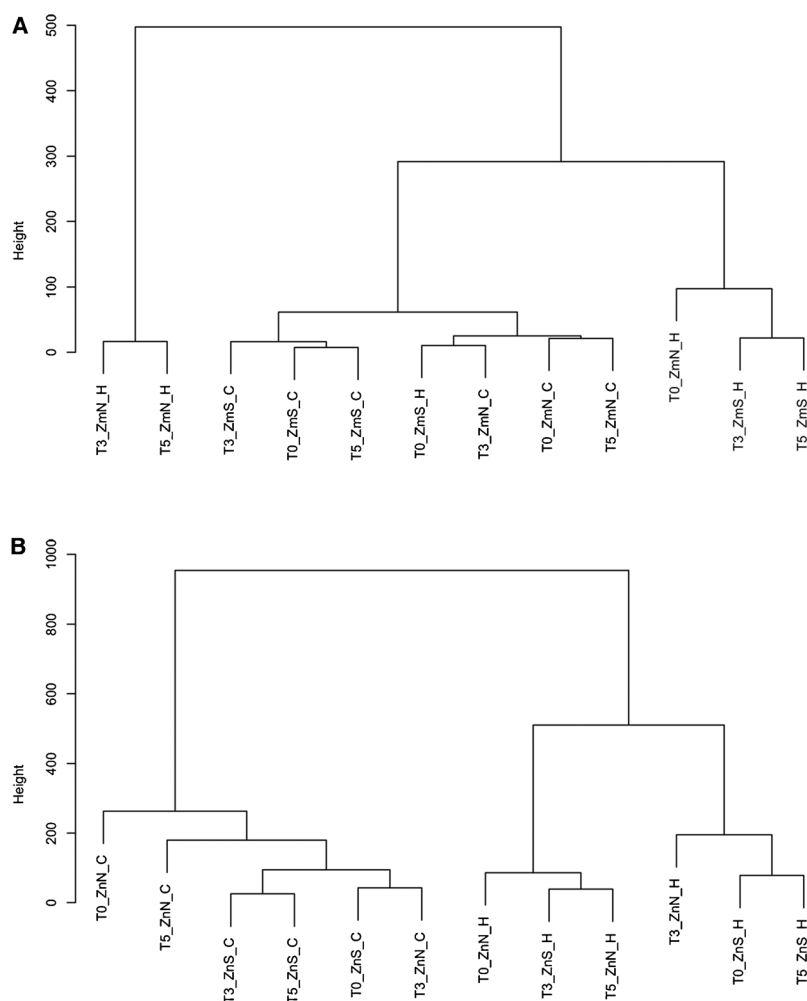
Fig. 3 The response of populations clusters closely by treatment conditions. (A) Hierarchical clustering of averaged metabolomic profiles representing the respective population response of *Z. marina* and (B) *Z. noltii*. Results highlight the specificity and magnitude of population response to temperature.

for problems of multiple testing (*q*-values, α = 0.001), were identified using a bootstrapping strategy (see Materials and Methods). For *Z. marina*, 1136 and 1342 significant differentially expressed genes were identified for the northern and southern populations, respectively. In total, 1638 genes were differentially expressed in *Z. marina*, with 840 genes shared between both populations. For *Z. noltii*, 1594 and 1433 genes were differentially expressed in the northern and southern populations, respectively. This resulted in a total of 2064 nonredundant genes for *Z. noltii* with 963 shared between the two populations. Merging the results of all libraries, a consensus of 398 metabolic genes was detected to be significantly responsive to treatment effects for both species and populations.

Analyzing the shared list of 398 tentative unigenes responding significantly to heat stress treatment, 30 of these have been annotated to have ATPase activity (7.5%), many of which are heat shock responsive proteins. Putative genes responding similarly across the species and populations within this subset were further analyzed (Table 1). 74 genes (18.6%) were upregulated for both species across all populations while 32 were down-regulated, with remaining genes showing mixed responses. A large number of upregulated genes corresponded to HSPs,

proteases, protein isomerases, and DNA maintenance and repair. Proteins involved in oxidation–reduction processes were also upregulated such as glutathione transferase (P46422),[24] glutathione-disulfide reductase (P42770) and superoxide dismutase (O81235). Key components of electron transport and photosynthesis were observed to be significantly upregulated. For example, the chloroplastic isoform of ATP-dependent zinc metalloprotease FTSH 5 involved in thylakoid formation[25] which has also been annotated to be involved in the removal of the D1 subunit of photosystem II to prevent cell death under high-intensity light conditions.[26] Lastly, heat treatment responsive immune defense associated proteins were also observed. These include the downregulation of putative protein phosphatase 2C 59 (Q8RXV3) used in the defense to pathogenic bacteria.[27] A number of ubiquitin-related proteins were also downregulated, and members of this family contribute to targeted protein recycling and degradation through posttranslational modification. Ubiquitination is also important for resistance against pathogenic attacks.[28]

Interestingly, despite the large treatment response observed with sucrose and fructose, only a small fraction of putative genes involved in carbohydrate metabolism were upregulated. Identified upregulated genes included ribose-5-phosphate

Table 1 Universal response in differential gene expression to treatment across all populations. Subset of genes identified with significant differential expression ($\alpha = 0.001$) with the same directionality observed, upregulated (top) or downregulated (bottom), to heat stress across all populations for both species

++++	Significantly upregulated in both species and populations
O23044	GO:0004601 (peroxidase)
O23627	Glycyl-tRNA synthetase 1, mitochondrial
O49081	ATP-dependent Clp protease proteolytic subunit
O60869	GO:0008168 (methyltransferase)
O65572	GO:0045549 (9-cis-epoxycarotenoid dioxygenase)
O80013	Ribulose-1,5-bisphosphate carboxylase/oxygenase N-methyltransferase
O81235	GO:0004784 (superoxide dismutase)
O82413	Histidyl-tRNA synthetase
P02829	GO:0042623 (ATPase activity, coupled), Heat shock protein Hsp90 heat-inducible isoform
P07639	3-dehydroquinate synthase, Shikimate Pathway
P0A3C9	GO:0009055 (electron carrier), Ferrodoxin I
P10591	GO:0016887 (ATPase), Heat shock protein SSA1
P12398	GO:0016887 (ATPase), Heat shock protein SSC1, mitochondrial
P19456	GO:0008553 (hydrogen-exporting ATPase activity, phosphorylative mechanism)
P19882	GO:0016887 (ATPase), Heat shock protein 60, mitochondrial
P20081	GO:0003755 (peptidyl-prolyl cis-trans isomerase)
P23321	GO:0010242 (oxygen evolving),33 kDa subunit of oxygen evolving system of photosystem II
P25857	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic
P28331	GO:0008137 (NADH dehydrogenase (ubiquinone))
P34791	GO:0003755 (peptidyl-prolyl cis-trans isomerase)
P35055	Coproporphyrinogen-III oxidase, chloroplastic, heme biosynthesis
P38989	GO:0016887 (ATPase) Structural maintenance of chromosomes protein 2
P39206	Carnitine operon protein CaiE
P42770	GO:0004362 (glutathione-disulfide reductase)
P42804	GO:0008883 (glutamyl-tRNA reductase)
P46422	GO:0004364 (glutathione transferase)
P46643	GO:0004069 (aspartate transaminase)
P47122	GO:0016887 (ATPase), GTPase NPA3
P47735	Receptor-like protein kinase 5
P47968	GO:0004751 (ribose-5-phosphate isomerase)
P48047	GO:0016887 (ATPase), ATP synthase subunit O, mitochondrial
P52701	GO:0016887 (ATPase), DNA mismatch repair protein Msh6
P92983	GO:0004657 (proline dehydrogenase)
P99999	GO:0045155 (electron transporter, transferring electrons from CoQH2-cytochrome c reductase complex and cytochrome c oxidase complex)
Q05924	GO:0042578 (phosphoric ester hydrolase)
Q06205	GO:0003755 (peptidyl-prolyl cis-trans isomerase), FK506-binding protein 4
Q08162	GO:0000175 (3'-5'-exoribonuclease), Exosome complex exonuclease DIS3
Q0WRJ7	GO:0016491 (oxidoreductase), FKBP-type peptidyl-prolyl cis-trans isomerase 6, chloroplastic
Q1H537	GO:0051744 (3,8-divinyl protochlorophyllide a 8-vinyl reductase)
Q40677	GO:0004332 (fructose-bisphosphate aldolase)
Q43870	GO:0004781 (sulfate adenylyltransferase (ATP))
Q52T38	GO:0016417 (S-acyltransferase)
Q56Y11	GO:0045547 (dehydrodolichyl diphosphate synthase)
Q56YN3	GO:0042736 (NADH kinase)
Q5SGD2	Protein phosphatase 1L
Q6ZFH6	Putative beta 1,2-xylosyltransferase
Q78J03	GO:0000318 (protein-methionine-R-oxide reductase)
Q7XJR6	Rhodanese-like
Q84UH6	glutathione dehydrogenase (ascorbate)
Q8GXX3	GO:0016682 (oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor)
Q8GY89	GO:0016667 (oxidoreductase activity, acting on sulfur group of donors)
Q8H0P5	Serine acetyltransferase 7
Q8IRW8	GO:0042800 (histone lysine N-methyltransferase activity (H3-K4 specific))
Q8KTI8	Tungsten-containing formate dehydrogenase beta subunit
Q8LK04	Glyceraldehyde 3-phosphate dehydrogenase
Q8LSZ3	NADPH:protochlorophyllide oxidoreductase
Q8RWF1	GO:0030267 (glyoxylate reductase (NADP))
Q8RWZ3	GO:0003995 (acyl-CoA dehydrogenase)
Q8VYJ1	GO:0008756 (o-succinylbenzoate-CoA ligase)
Q93KB8	ABC ATPase
Q963A7	Co-chaperonin CPN10
Q96533	GO:0080007 (S-nitrosoglutathione reductase)
Q99LX0	GO:0051920 (peroxiredoxin)
Q9FV53	GO:0042586 (peptide deformylase)
Q9LDC0	GO:0003755 (peptidyl-prolyl cis-trans isomerase)
Q9LZV4	GO:0004672 (protein kinase)
Q9M2E2	GO:0047504 ((-)-menthol dehydrogenase)
Q9M591	GO:0048529 (magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase)
Q9MA43	GO:0042800 (histone lysine N-methyltransferase activity (H3-K4 specific))
Q9MBA1	GO:0010277 (chlorophyllide a oxygenase)
Q9SD85	GO:0016711 (flavonoid 3'-monooxygenase)
Q9SL79	GO:0031202 (RNA splicing factor activity, transesterification mechanism)
Q9SW18	GO:0046406 (magnesium protoporphyrin IX methyltransferase)
Q9XIL5	GO:0031202 (RNA splicing factor activity, transesterification mechanism)

----	Significantly down-regulated in both species and populations
A9LDD9	GO:0004372 (glycine hydroxymethyltransferase)
O64867	GO:0004842 (ubiquitin-protein ligase)
O75251	GO:0003954 (NADH dehydrogenase)
P05314	Ferredoxin--nitrite reductase, chloroplastic
P14654	Glutamine synthetase cytosolic isozyme 1-2
P23686	GO:0004478 (methionine adenosyltransferase)
P40024	GO:0042624 (ATPase activity, uncoupled)
P49598	GO:0004722 (protein serine/threonine phosphatase) ABA-HYPERSENSITIVE GERMINATION 3
P51996	GO:0003924 (GTPase)GTP-binding protein YPT32/YPT11
P60662	GO:0030898 (actin-dependent ATPase)
Q03071	GO:0004842 (ubiquitin-protein ligase)
Q38869	GO:0004672 (protein kinase), Calcium-dependent protein kinase 4
Q39152	GO:0080019 (fatty acyl-CoA reductase (alcohol-forming))
Q39227	GO:0008757 (S-adenosylmethionine-dependent methyltransferase)
Q3EDK8	GO:0009924 (octadecanal decarboxylase)
Q4G338	Cyclophilin
Q6SJP5	Sucrose synthase
Q8BGM2	GO:0004349 (glutamate 5-kinase)
Q8RXV3	GO:0004722 (protein serine/threonine phosphatase), Probable protein phosphatase 2C 59
Q94F47	GO:0004842 (ubiquitin-protein ligase), Ubiquitin-conjugating enzyme E2 28
Q9FH02	GO:0016887 (ATPase), ATP-dependent zinc metalloprotease FTSH 5, chloroplastic
Q9FUB7	GO:0016210 (naringenin-chalcone synthase)
Q9FUC2	Nitrate reductase
Q9H0N0	GO:0003924 (GTPase)
Q9LQ04	GO:0008831 (dTDP-4-dehydrorhamnose reductase)
Q9S816	GO:0004629 (phospholipase C)
Q9SI64	Arginine decarboxylase 1
Q9SQJ1	UDP-glucose dehydrogenase
Q9SRQ7	GO:0004629 (phospholipase C)
Q9X0Y1	Phosphorylated carbohydrates phosphatase TM_1254
Q9ZTP4	Zeta-carotene desaturase, chloroplastic/chromoplastic
Q9ZUY3	GO:0047769 (arogenate dehydratase)

Legend:
- ■ (green) Photosynthesis/Sugar/Myoinositol Metabolism
- ■ (blue) Protein Folding
- ■ (yellow) Oxidative/Reductive
- ■ (orange) Heat Shock Responsive

isomerase (P47968) which catalyzes the interconversion between ribose-5-phosphate (R5P) and ribulose-5-phosphate (Ru5P); both are substrates used in the pentose phosphate pathway and the Calvin cycle, respectively. Fructose-6P is generated during the pentose phosphate pathway while glyceraldehyde-3-phosphates generated during the Calvin cycle are substrates used to synthesize other carbohydrates such as starch, sucrose, and cellulose. Fructose-bisphosphate aldolase (Q40677) contributes to fructose metabolism where products are then later shuttled down the glycolysis pathway. Glyceraldehyde 3-phosphate dehydrogenase (Q8LK04), a key enzyme involved in glycolysis and the Calvin cycle, depending on the isoform, was observed to be upregulated. Lastly, UDP-glucose dehydrogenase (Q9SQJ1) was observed to be downregulated.

With respect to the large treatment response in sucrose levels observed in the metabolomic profiles, sucrose synthase (Q6SJP5) gene expression was also downregulated in all four populations, along with phosphorylated carbohydrates phosphatase TM_1254 (Q9X0Y1), an enzyme involved in the intracellular metabolism of many phosphorylated carbohydrates such as fructose 6-phosphate. Sucrose-phosphate synthase (Q94BT0) was observed to be significantly upregulated only in both *Z. noltii* populations while downregulated in *Z. marina* populations. Putative genes involved in starch metabolism such as ADP glucose pyrophosphorylase, starch synthase, and starch branching enzyme were not identified to be differentially expressed in the sampled tissues with the exception of granule-bound starch synthase (O64925) which

Table 2 Population specific response in differential gene expression. Subset of genes identified with significant differential expression ($\alpha = 0.001$) responding inversely between the northern and southern populations for both species

+-+-	Parallel up-regulation in northern populations and down-regulation in southern populations	
O64437	Inositol-3-phosphate synthase, Myo-inositol-1-phosphate synthase	
P0A8F4	Uridine kinase	
P29448	GO:0016671 (oxidoreductase activity, acting on sulfur group of donors, disulfide as acceptor)	
P46644	GO:0004069 (aspartate transaminase)	
P52920	GO:0016887 (ATPase)	
Q09154	GO:0008121 (ubiquinol-cytochrome-c reductase)	
Q43157	GO:0004750 (ribulose-phosphate 3-epimerase)	
Q43295	GO:0004020 (adenylylsulfate kinase)	
Q5U3Z7	GO:0008732 (L-allo-threonine aldolase)	
Q949X0	GO:0009979 (16:0 monogalactosyldiacylglycerol desaturase)	
Q9BZC7	GO:0016887 (ATPase)	
Q9C5J7	GO:0003872 (6-phosphofructokinase)	
Q9LQF2	GO:0004564 (beta-fructofuranosidase)	
-+-+	Parallel down-regulation in northern populations and up-regulation in southern populations	
O50008	GO:0008705 (methionine synthase)	
O55234	GO:0008233 (peptidase)	
P34099	cAMP-dependent protein kinase catalytic subunit	
Q05957	Petal death protein	
Q39241	GO:0016671 (oxidoreductase activity, acting on sulfur group of donors, disulfide as acceptor)	
Q3U462	GO:0004568 (chitinase)	
Q8W4K6	GO:0003924 (GTPase)	
Q9LRB0	GO:0017050 (D-erythro-sphingosine kinase)	
Q9LSQ5	GO:0016655 (oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor)	
Q9NY43	BarH-like 2 homeobox protein	

NORTH (rows O64437–Q9LQF2), SOUTH (rows O50008–Q9NY43)

Legend:
- ■ Photosynthesis/Sugar/Myoinositol Metabolism (green)
- ■ Protein Folding (blue)
- ■ Oxidative/Reductive (yellow)
- ■ Heat Shock Responsive (orange)

was upregulated in the northern and southern populations of *Z. marina* and *Z. noltii*, respectively, while downregulated in the other two populations.

Finally, genes observed to have inverse response in differential expression between the northern and southern populations were investigated (Table 2). Genes up-regulated in the southern but down-regulated in the northern populations for both species ($-+-+$: 10 genes) appear to be largely associated with developmental processes. Conversely, analyzing the inverse relationship, up-regulated in the northern populations but down-regulated in the southern populations, shows an interesting set for further investigation to understand the role of sugars and other metabolites during heat stress response ($+-+-$: 13 genes). For example, inositol-3-phosphate synthase (O64437), an enzyme involved in *myo*-inositol synthesis, was identified to be upregulated in the northern populations. *Myo*-inositol was identified through PCA to be the third most responsive metabolite to the heat stress treatment. Significant increase in *myo*-inositol levels was observed post-treatment during the recovery phase (T5) for treated samples from northern populations. Other enzymes involved in sugar metabolism identified in this subset of genes were ribulose-phosphate 3-epimerase (Q43157) in the Calvin cycle, 6-phosphofructokinase (Q9C5J7) from glycolysis, and β-fructofuranosidase (Q9LQF2), an invertase catalyzing the breakdown of sucrose to fructose and glucose.[29]

Discussion

Although previous metabolomics analyses on plant response to thermal stress have been conducted,[7,8,30] comparisons between different thermally adapted wild strain populations subjected to chronic heat stress, such as that can be observed in nature for marine plants, have not yet been investigated (Fig. 1). The metabolomic and transcriptomic responses of seagrass populations collected along a thermal cline were explored to understand potential stabilizing metabolic contributions that would complement the well documented HSP based heat shock response to maintain cellular homeostasis.[31] The metabolomic and transcriptomic datasets allow inspection of fluctuating changes in response to heat stress between the populations for both gene expression and metabolites. Taken together, the combined datasets also permit analysis to distinguish between two alternative mechanisms for changes in steady state metabolite levels. For example, increasing steady states in metabolite levels can be accounted for by an (i) increase in the rate of biosynthesis and (ii) decrease in the rate of breakdown. As will be demonstrated through this analysis using seagrasses, the data indicate that the latter mechanism is the main reason for the observed heat responsive increase in sucrose levels, whereas the increase in *myo*-inositol is apparently driven by increased rates of biosynthesis.

Two closely related seagrass species found in near proximity (~ 100 m) along a thermal cline were used in this investigation to identify shared features within the metabolic response to heat stress treatment using a stringent criterion ($\alpha = 0.001$) to reduce the possibility of detecting correlations in heat responsive changes between false positive observations. The aim of this investigation was not to identify all key players that may be contributing to metabolic heat stress attenuation, but to identify core-shared as well as population-specific features. Comparing the responses of two species along a thermal cline would be suggestive of a heat stress response strategy through metabolic responses evolved in parallel. It should be noted that investigations using only two species can be insufficient in establishing definitive adaptive features,[32] but the findings are compelling enough

to warrant further investigations in understanding metabolic contributions to heat attenuation. Furthermore, an investigation at different levels of biological organization using metabolites, gene expression, and physiological measurements can help identify possible gene candidates and pathways yielding the observed global systemic response.

As can be observed through physiological measurements (Fig. S1–S3, ESI†), a significant response to treatment can be detected in the growth rate and shoot counts. However, treatment response with respect to photosynthesis is less evident since statistical significance was not established. Taken together, these measurements suggest that the treated groups relative to the control were not under extreme stress in the mesocosm and were observed to decline slowly in health over the duration of the experiment. A small increase in growth at the peak of the heat wave (T3) was observed through shoot count and growth rates for the southern populations of both species. While Y_0 appears to be maintained in one species, the relative ETR_{max} in photosynthesis appeared to be more treatment responsive, thus suggesting an increase in photosynthetic activity. The data also showed differences in these measurements between treatment and control samples prior to the application of the heat stress regime for the southern Z. marina population. A proper explanation for this discrepancy, an increase in the growth rate and Y_0 cannot be rationalized. However the remaining measurements reflect expected outcomes based on previous investigations conducted on seagrass populations using similar experimental setups.[13] Therefore detected heat stress responses in this experiment would be fairly reasonable and indicative of what may be observed in nature.

The metabolomic profiles of 6 individuals were averaged to represent the response to the given experimental treatment conditions for each respective population (Fig. 2). Treatment response can be clearly observed in both species, with a larger magnitude of response in the northern populations. Although increases in metabolite variations to heat treatment between replicates were observed (Fig. S4, ESI†), it should be noted that the observations were from samples of non-identical genotypes. To further illustrate the variations between the individuals, replicates do not show a tendency for clustering when conducting PCA on the individual metabolomic profiles (Fig. S5, ESI†). Therefore, the averaged profiles included also the individual variations that would be observed within populations. Since metabolite variations appear relatively stable in the control group across the time course of the experiment, particularly for Z. marina, this suggests that observations and interpretation of trends can be inferred. Furthermore, the observed variations within populations in response to heat stress are most likely a necessary requirement conferring beneficial advantages for the long-term survival of the species.[33] For Z. noltii, metabolomic profiling had been more difficult due to the much smaller quantities of the biological sample for this species, thus requiring adjustments to protocols that pushed the technical limits of metabolite profiling. The consequence of this limitation could be evidenced by the large increase in variation between replicates compared to Z. marina (Fig. S4, ESI†). Therefore conclusions specific for Z. noltii could benefit from follow-up experiments for more accuracy with improvements in technological sensitivity.

The population profiles were successfully grouped into three main clusters for Z. marina and Z. noltii, matching expectancy with a few minor exceptions detailed in results (Fig. 3). First, the control groups across the time course of the experiment were clustered into one group, including samples not yet subjected to the heat stress regime for Z. marina. The remaining two clusters were based on population rather than treatment specific variables. The results also suggested that metabolomic profiles recovered to the initial state prior to the heat stress treatment for Z. noltii, which was not observed for Z. marina. Due to the difficulties mentioned for measurements for the Z. noltii samples, this observation needs to be revisited. Clearly observed, however, between the two species was a population distinctive response with northern populations showing a larger magnitude and sensitivity to heat stress.

The novelty of this investigation is a comparison of the metabolic responses of closely related species sampled from different thermal habitats to heat stress through the combined use of metabolomic and transcriptomic data. The most responsive metabolites accounting for the majority of the variations over the course of the experiment were sucrose and fructose, with myo-inositol as the third most responsive (Fig. 4). Sugar responses, particularly sucrose and fructose, have already been extensively observed in previous temperature stress experiments to have protective effects.[34] Myo-inositol is a necessary substrate for the galactinol and raffinose biosynthetic pathways which has been identified to have osmoprotective properties against oxidative stress.[35] A number of ATPases, HSPs, and carbohydrate metabolic enzymes were identified to be upregulated in all populations exposed to heat stress treatment. Some immunological genes were also affected by the heat stress treatment.

Focusing on the subset of genes involved in carbohydrate metabolism (Fig. 5), glyceraldehyde 3-phosphate dehydrogenase (Q8LK04) was one of the upregulated genes (Table 1). Depending on the cellular location and isoform, this is a key enzyme involved either in glycolysis (cytosolic) or in the Calvin cycle (plastidic). Ascertaining the correct isoforms of genes was difficult for two reasons. First, no reference genome is available. Second, high levels of heterogeneity and redundancy for putative genes within the assembled transcriptome were present. This problem was addressed by clustering transcripts based on METACYC BLAST annotations to obtain read counts at the expense of distinguishing isoforms at a high resolution. However, the annotation suggests that the cytosolic isoform of GAPDH is differentially expressed, possibly due to a higher energy demand during heat stress that could also be linked to the observed upregulation of a number of ATPases. Alternatively, if further studies instead identified the plastid isoform of GAPDH to be the contributing gene, this interpretation could also account for the observed increase in sucrose levels.

Other identified candidate genes impacting sucrose in response to heat stress were sucrose synthase (Q6SJP5), UDP-glucose dehydrogenase (Q9SQJ1), and sucrose-phosphate synthase (Q94BT0). Sucrose synthase catalyzes the reversible conversion of sucrose and UDP into UDP-glucose and fructose[36] where downregulation of this enzyme would result in less breakdown of sucrose. Following this reaction, the substrate UDP-glucose is later oxidized by UDP-glucose dehydrogenase (Q9SQJ1) to UDP-glucuronate, which is a precursor for downstream cell
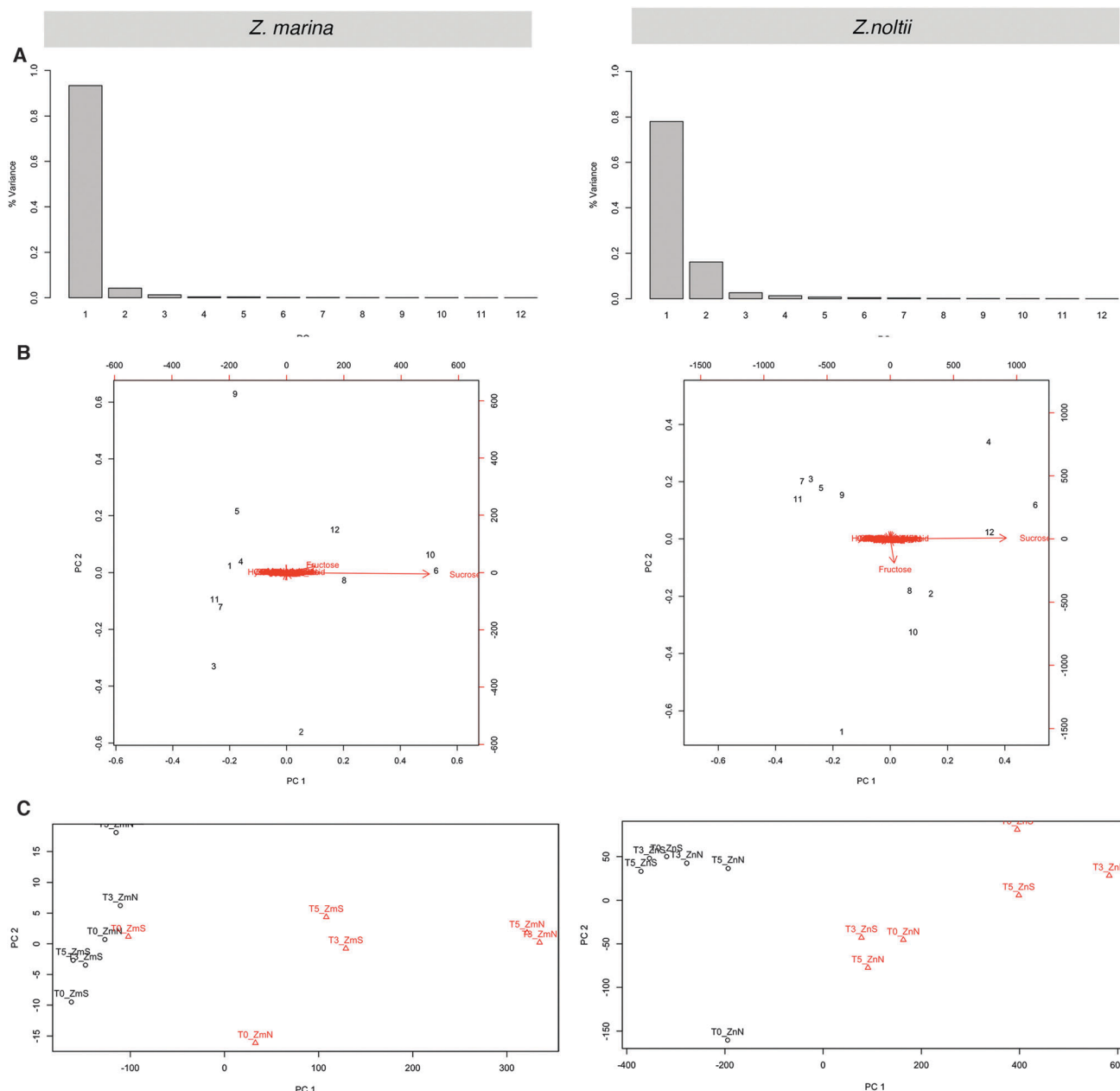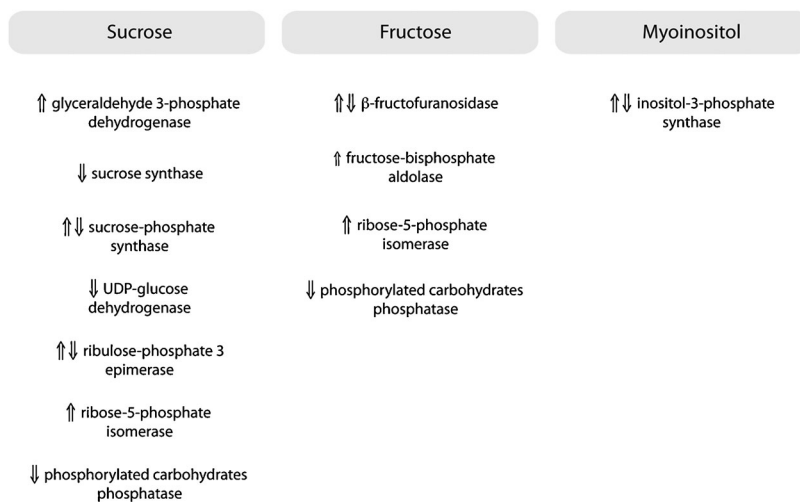
Fig. 4 Sucrose, fructose, and *myo*-inositol found to be the most treatment responsive over the time course of the heat stress regime. The results of principal component analysis (PCA) conducted on the metabolomic response over the time course of the heat stress regime for *Z. marina* and *Z. noltii* are shown. A large portion of the (A) variance in responses was attributed to (B) the two principal components represented by sucrose and fructose, with the third principal component represented by *myo*-inositol (not shown). (C) Population response can be well distinguished based on both the applied thermal stress and time course of the experiment.

wall biosynthesis.[37] Again, downregulation of this gene may also lead to the observed increase, or accumulation, of sucrose since there is less diversion of carbon for use in cell wall synthesis. Sucrose-phosphate synthase, while observed only to be upregulated in both *Z. noltii* populations and downregulated in *Z. marina* (data not shown), is also involved in the breakdown of sucrose[38] although its predominant role is in sucrose synthesis. This glycosyltransferase catalyzes the conversion of UDP-glucose and D-fructose 6-phosphate to UDP and sucrose 6-phosphate. Upregulation of sucrose-phosphate synthase has also been suggested to be one contributor to improved heat stress tolerance in chrysanthemums.[39]

Enzymes involved in starch metabolism, such as β-amylase,[34,40] have been previously identified to contribute significantly during heat stress, but were not identified within this universal subset of treatment responsive metabolic enzymes. Putative genes such as ADP glucose pyrophosphorylase, starch synthase, and starch branching enzyme were not identified to be differentially expressed in the sampled tissues with the exception of granule-bound starch synthase (O64925) which was upregulated in the northern and southern populations of *Z. marina* and *Z. noltii*, respectively, while downregulated in the other two populations. Although these genes were not differentially expressed, the catalytic activities

Sucrose	Fructose	Myoinositol
⇑ glyceraldehyde 3-phosphate dehydrogenase	⇑⇓ β-fructofuranosidase	⇑⇓ inositol-3-phosphate synthase
⇓ sucrose synthase	⇑ fructose-bisphosphate aldolase	
⇑⇓ sucrose-phosphate synthase	⇑ ribose-5-phosphate isomerase	
⇓ UDP-glucose dehydrogenase	⇓ phosphorylated carbohydrates phosphatase	
⇑⇓ ribulose-phosphate 3 epimerase		
⇑ ribose-5-phosphate isomerase		
⇓ phosphorylated carbohydrates phosphatase		

Fig. 5 Core heat attenuation gene candidates for sucrose, fructose, and *myo*-inositol. Summary of identified gene candidates with impact on sucrose, fructose, and *myo*-inositol levels in response to heat stress response shared by the two species is provided. Observed directionalities of differential gene expression between the investigated populations are also indicated.

may nevertheless be modulated in a thermal dependent manner based on the intrinsic properties of the protein stability. For example, starch synthase was characterized to have a low optimum temperature and therefore became less active at higher temperatures.[41]

Genes potentially affecting fructose adjustments include ribose-5-phosphate isomerase (P47968), fructose-bisphosphate aldolase (Q40677), and phosphorylated carbohydrates phosphatase TM_1254 (Q9X0Y1). Ribose-5-phosphate isomerase (P47968) is a highly conserved enzyme with a pivotal role in both the pentose phosphate pathway and the Calvin cycle, catalyzing the conversion between ribose-5-phosphate, the shared substrate between the two pathways, and ribulose-5-phosphate. Due to having roles in carbohydrate anabolism and catabolism, the immediate impacts on fructose and sucrose levels are not clearly evident in the upregulation of this gene, particularly since this isomerase does not directly catalyze the reactions involving these sugars. Fructose-bisphosphate aldolase was also upregulated and previously identified to be heat and drought responsive.[42] Lastly, phosphorylated carbohydrates phosphatase was identified to be significantly downregulated. Incidentally, the annotation is derived from sequence similarity to an enzyme identified in *Thermotoga maritima,* an extremophilic organism found in high thermal environments. This enzyme displays high phosphatase activity towards a number of phosphorylated carbohydrates,[43] however mechanistic details of contributions to heat stress robustness are not yet known.

Myo-inositol, on the other hand, may be used as a substrate to generate protein stabilizing osmolytes such as di-*myo*-inositol phosphate which has been observed to accumulate in thermal extremophiles in response to heat stress.[44] *Myo*-inositol alone has been found to increase the midpoint denaturation temperature of proteins by ∼1.5 °C.[45] Heat attenuation using *myo*-inositol in seagrasses may be achieved through inositol-3-phosphate synthase (O64437) identified to be upregulated in the northern population of both species. This isomerase catalyzes the conversion between D-glucose 6-phosphate and 1D-*myo*-inositol 3-phosphate, thus converting carbohydrates for inositol pathways.

Overexpression of this enzyme has resulted in elevated levels of free inositol in a transgenic study using *A. thaliana*.[46]

Since additional enzymes involved in sugar metabolism were also observed to be upregulated in northern populations of both species while down-regulated in the southern populations, the findings further suggest the importance of metabolic heat attenuation *via* modulating carbohydrates for heat stress response, coupled with previous findings showing that sugars have osmoprotective properties.[8,34] Also observed in the metabolic heat attenuation of northern populations was ribulose-phosphate 3-epimerase (Q43157) in the Calvin cycle which may subsequently lead to sucrose synthesis, and β-fructofuranosidase (Q9LQF2), a cytosolic invertase that may contribute significantly to the increase of fructose levels through cleavage of sucrose to fructose and glucose. Cumulatively, between the physiological, metabolomic, and transcriptomic datasets, findings suggest that the Calvin cycle, glycolysis, pentose phosphate and neighboring associated pathways are the main candidate metabolic processes for heat attenuation with increased productivity in response to thermal stress.

There are several limitations for this study warranting further investigation in order to dissect the detailed mechanistic contribution of genes to the metabolomic profiles and identify absent candidates previously identified to be thermally responsive metabolic enzymes in *Arabidopsis thaliana*. First, the resolution of the transcriptomic and metabolomic datasets obtained in this investigation afforded only an overview of the observed holistic systemic response with insight into possible molecular explanations. Direct linkage of gene transcripts and metabolic pathways to heat responsive metabolites can only be drawn from inferences of previous findings and future investigations. Second, although the systemic response was investigated with good coverage, it was by no means comprehensive. Therefore, the response of many metabolites and gene transcripts may still be unknown. Lastly, a number of expected candidates may not be identified due to the stringency of the statistical tests, tissue specific expression, or alternative mechanisms of heat attenuation *via* other strategies and mechanisms of molecular evolution

not investigated in this study. For example, enzymatic activity can be thermally responsive based on the innate properties of protein thermostability, as mentioned earlier for starch synthase, and therefore the rate of metabolite catalysis is not dependent on gene expression levels.[47] Protein stability modulations as an adaptive mechanism have been observed within the photosynthetic complex for thermal acclimation.[48] Metabolic heat attenuation should be further investigated in these other additional molecular dimensions, such as alternative splicing, and expanded to include different tissues to gain insight into the holistic systemic response.

The consensus universal subset presented here is an overlap between sampled leaves from different thermally adapted seagrass populations of two species, and therefore may potentially serve as core features of metabolic heat attenuation *via* differential gene expression. The contributions of sugar metabolites and pathways involved in heat attenuation to thermal response need to be further understood as the processes are complex, particularly with respect to thermal acclimation and tolerance.[12] Although seagrasses were used as the study species in this investigation, the observed metabolic heat attenuation and putative key candidates identified could be a fundamental contributor to global heat stress response for stabilization. The role of these candidate genes, often viewed as stable housekeeping genes, in heat stress response should be reconsidered. They may be additional key players for adaptation to changing environments. The variance at the genotypic levels for metabolic heat attenuation in the ecological context is worthy of further investigation in the field of molecular ecology.[49] While carbohydrate metabolism has dominated the central discussion in this investigation, largely due to the strong inferences afforded by the resolution of the combined metabolomic and transcriptomic analysis, significant contributions from other pathways contributing to heat attenuation are also of equal interest. With improved resolution, specific contributions of isoforms and other metabolic pathways should be further detailed to understand mechanisms of abiotic stress attenuation through metabolic pathways in future investigations. Finally, the potential synergistic effects between metabolome and proteome, for which current investigations are lacking, as a necessary component within the heat stress response repertoire should be further explored in the field of systems biology.

Materials and methods

Experimental setup

The study species used in this investigation were *Zostera marina* and *Zostera noltii*. Seagrasses were collected from Hals (Denmark; 56°50′ N, 10°1′ E; early May 2009) and Gabbice mare (Italy; Adriatic Sea; 43°50′ N, 12°45′ E; late April 2009) representing high- (northern) and low (southern) latitude adapted subtidal populations, respectively (Fig. 1A). Sufficient amounts of ramets were harvested from within in an area of 0.25 m^2 to raise the likelihood of belonging to the same genet. The distance between sampling of plants within the meadows was at least 10 m to ensure genetic diversity. Genotyping using microsatellite markers and four primer pairs confirmed that at least 34 genotypes from the southern *Z. marina* population were sampled compared to 14 for the northern population

(data not shown). A minimum of 9 genotypes was confirmed for both *Z. noltii* populations.

Ramets, individual shoot with roots, were transplanted within 36 hours to the AQUATRON culturing facility (Münster) where seagrasses were then monitored and acclimated for a minimum of 4 weeks prior to the heat stress treatment regime. The starting temperature used for the acclimation phase was the midpoint at 15 °C between the observed field temperatures of the two populations. This temperature was then incrementally increased to a final control baseline of 19 °C (Fig. 1B). The AQUATRON consists of 12 mesocosms (tank capacity: 700 L) connected in two closed seawater circuits with a flow rate of 1200 L h^{-1}. Each mesocosm served as a replication block containing 8 planting boxes filled with sediments to a height of 10 cm and was illuminated by a pair of 400 W bulbs (Philips Master SON-T PIA Green Power, Philips Master HPI-T Plus) fixed 60 cm above the water surface. Species from each population and location were planted in two boxes per mesocosm. The sandy sediment used in the AQUATRON was procured from a coastline of the Baltic Sea in Kiel, Germany and seawater was formulated from Instant Ocean® Sea Salt (Aquarium Systems), deionized water, and nutrients (15 μM NaNO$_3$, 1 μM NaH$_2$PO$_4$, and 6 μM Na$_2$SiO$_4$·9H$_2$0).

Heat stress treatment was initiated at T0 after the four week plant acclimation period with increments of 1 °C per day until the final target temperature (26 °C) for the treated samples while the control group was maintained at the baseline temperature of 19 °C. Fitness measurements of shoot count, growth rates, and pulse amplitude modulated (PAM) fluorometry were conducted weekly to monitor the health of the seagrasses. Shoot tissues were sampled at T3 for RNA extraction and subsequent transcriptomic sequencing and analysis. At this timepoint (T3), seagrasses had been subjected to 2 weeks of applied heat stress, during which an exposure of constant heat dosage for one week at 26 °C was applied.

Metabolomics

Metabolomic investigations were conducted for timepoints T0, T3, and T5. Metabolites were extracted using an adapted protocol from Lee and Fiehn,[50] as described previously.[51] Six replicates of ∼15 mg and ∼5 mg of *Z. marina* and *Z. noltii* sampled plant leaves were used and kept frozen at all times until extraction. Frozen powdered plant materials were extracted using a 50 μM ribitol solution with a mixture of water, methanol, and chloroform in a volume proportion of 1:2.5:1 and centrifugation for 2 min at 20 000×g.

Extracts were then derivatized for analysis by GC/TOF mass spectrometry. 20–50 μl of the supernatant were concentrated to dryness in a vacuum concentrator. 10 μl of a solution of 20 mg ml^{-1} of 98% pure methoxyamine hydrochloride in pyridine was added and shaken at 30 °C for 90 min to protect aldehyde and ketone groups. 90 μl of MSTFA was added for trimethylsilylation of acidic protons and shaken at 37 °C for 30 min. After 2 h incubation time, samples were injected.

A Gerstel automatic liner exchange system with multipurpose sample MPS2 dual rail and two derivatization stations was used in conjunction with a Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany). For every 10–12 samples, a fresh multi-baffled liner was inserted.

Chromatography was performed using the Agilent 7890A GC. Metabolites were separated on an Agilent HP-5MS column (30 m × 0.25 mm), oven temperature was ramped at 12.5 °C min^{-1} from 70 °C (initial temp for 2 min) to 320 °C (final temp hold 5 min). Metabolites were ionized in an EI source (70 V, 200 °C source temp) and detected using Waters GCT Premier TOF-MS.

Normalization of data for analysis was achieved based on the calculated surface area of respective leaf samples.

Zostera transcriptome and gene expression profiling

Total RNA was extracted from seagrass leaf tissue samples using the Invisorb® RNA Plant HTS 96 Kit/C (Invitek). A controlled pooling of 8 libraries representing ∼7 genotypes was sequenced for comparative transcriptomic analysis. The eight libraries included two populations, two species, and two treatment conditions capturing the transcriptomic response at timepoint T3 which corresponds to the midpoint of the heat stress treatment. 3′ UTR sequencing was performed using the Genome Analyzer II (Illumina/Solexa) with a depth of ∼5 million reads per library. A reference transcriptome backbone was also sequenced only for *Z. noltii* using 454 Titanium (Roche) since a reference transcriptome for *Z. marina* is already available through prior experiments[22] and the Joint Genome Institute (USA). All cDNA library construction and sequencing were conducted by GATC Biotech AG.

Reference transcriptomes were assembled using MIRA v.3.2.0.[52] Gene expression read counts were obtained with the mapping of 3′UTR reads onto these reference transcriptomes using BWA v0.5.8 c[53] and Sequence Alignment/Map (SAM) tools v0.1.8.[54] Transcriptomes were annotated with BLAST using the *Arabidopsis thaliana* (TAIR9)[55] and *Oryza sativa* v6.1 (Rice Genome Annotation Project)[56] databases. Tentative unigenes involved in metabolic processes were annotated using the Metacyc database.[23,57] GO annotations and statistical determination for over- and under-representation of genes were achieved with BiNGO v 2.44[58] using the GOSLIM PLANTS and the full database of GO gene ontology.[59]

Bootstrapping to identify differential gene expression

Statistically significant differential gene expression was identified using a bootstrapping strategy adapted for this experiment.[60] Only the successful METACYC annotated genes using BLAST (*e*-value = 1×10^{-4}) were used for this analysis. Null models of gene expression were generated for each unigene to test statistical significance in differential expression to treatment response. Bootstrapping was achieved through sampling with replacement from pooled reads of both the control and treatment groups. Null model libraries are of equal size to the treatment library tested. Multiple testing corrections were applied to control the family wise error rate (FWER) and the false discover rate (FDR).[61]

Statistical analysis

ANOVA, principal component analysis, and hierarchical clustering were conducted using *R*.

Financial disclosure

Competing interests

None

Author contributions

Conception, implementation, and writing of the manuscript for this publication were guided by JG, THBR, EBB, AW. Fieldwork sampling and monitoring of seagrasses in the AQUATRON through various fitness measurements were conducted by a team effort of JG, GW, IW, KH, and SF. Photosynthetic measurements were conducted by GW, IW, and JG. Photosynthetic analysis and statistics on fitness measurements were done by GW. Metabolomics sampling and analysis were conducted by JG, IW, KH, KZ, EK, and KW. Transcriptomic extraction was performed by SF, GW, and JG. Bioinformatic analysis of metabolomic and transcriptomic was conducted by JG.

Acknowledgements

References

1 M. Ralser, M. M. Wamelink, A. Kowald, B. Gerisch, G. Heeren, E. A. Struys, E. Klipp, C. Jakobs, M. Breitenbach, H. Lehrach and S. Krobitsch, Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress, *J. Biol.*, 2007, **6**, 10.

2 T. Arakawa and S. N. Timasheff, The stabilization of proteins by osmolytes, *Biophys. J.*, 1985, **47**, 411–414; O. Fernandez, L. Bethencourt, A. Quero, R. S. Sangwan and C. Clement, Trehalose and plant stress responses: friend or foe?, *Trends Plant Sci.*, 2010, **15**, 409–417; P. H. Yancey, Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses, *J. Exp. Biol.*, 2005, **208**, 2819–2830; M. F. Thomashow, PLANT COLD ACCLIMATION: Freez, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1999, **50**, 571–599.

3 L. M. Holthauzen and D. W. Bolen, Mixed osmolytes: the degree to which one osmolyte affects the protein stabilizing ability of another, *Protein Sci.*, 2007, **16**, 293–298.

4 M. Auton and D. W. Bolen, Application of the transfer model to understand how naturally occurring osmolytes affect protein stability, *Methods Enzymol.*, 2007, **428**, 397–418; J. Rosgen, B. M. Pettitt and D. W. Bolen, An analysis of the molecular origin of osmolyte-dependent protein stability, *Protein Sci.*, 2007, **16**, 733–743; L. M. F. Holthauzen, M. Auton, M. Sinev and J. Rosgen, Protein Stability in the Presence of Cosolutes, *Methods in Enzymology: Biothermodynamics, Vol. 492, Pt Da*, 2011, **492**, 61–125.

5 S. Diamant, N. Eliahu, D. Rosenthal and P. Goloubinoff, Chemical chaperones regulate molecular chaperones *in vitro* and in cells under combined salt and heat stresses, *J. Biol. Chem.*, 2001, **276**, 39586–39591; T. O. Street, K. A. Krukenberg, J. Rosgen, D. W. Bolen and D. A. Agard, Osmolyte-induced conformational changes in the Hsp90 molecular chaperone, *Protein Sci.*, 2010, **19**, 57–65.

6 J. W. Allwood, D. I. Ellis and R. Goodacre, Metabolomic technologies and their application to the study of plants and plant-host interactions, *Physiol. Plant.*, 2008, **132**, 117–135; C. Guy, J. Kopka and T. Moritz, Plant metabolomics coming of age, *Physiol. Plant.*, 2008, **132**, 113–116; N. Schauer and A. R. Fernie, Plant metabolomics: towards biological function and mechanism, *Trends Plant Sci.*, 2006, **11**, 508–516; V. Shulaev, D. Cortes, G. Miller and R. Mittler, Metabolomics for plant stress response, *Physiol. Plant.*, 2008, **132**, 199–208.

7 C. Guy, F. Kaplan, J. Kopka, J. Selbig and D. K. Hincha, Metabolomics of temperature stress, *Physiol. Plant.*, 2008, **132**, 220–235.

8 F. Kaplan, J. Kopka, D. W. Haskell, W. Zhao, K. C. Schiller, N. Gatzke, D. Y. Sung and C. L. Guy, Exploring the temperature-stress metabolome of Arabidopsis, *Plant Physiol.*, 2004, **136**, 4159–4168.

9 G. Chechik, E. Oh, O. Rando, J. Weissman, A. Regev and D. Koller, Activity motifs reveal principles of timing in transcriptional control of the yeast metabolic network, *Nat. Biotechnol.*, 2008, **26**, 1251–1259; M. Ralser, M. M. Wamelink, S. Latkolik, E. E. Jansen, H. Lehrach and C. Jakobs, Metabolic reconfiguration precedes transcriptional regulation in the antioxidant response, *Nat. Biotechnol.*, 2009, **27**, 604–605.

10 J. G. Sorensen, T. N. Kristensen and V. Loeschcke, The evolutionary and ecological role of heat shock proteins, *Ecol.Lett.*, 2003, **6**, 1025–1037.

11 A. Whitehead and D. L. Crawford, Neutral and adaptive variation in gene expression, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 5425–5430.

12 H. K. Lichtenthaler, The stress concept in plants: an introduction, *Ann. N. Y. Acad. Sci.*, 1998, **851**, 187–198.

13 G. Winters, P. Nelle, B. Fricke, G. Rauch and T. B. H. Reusch, The effects of a simulated heat wave on the photophysiology and gene expression of high and low-latitude populations of *Zostera marina*, *Mar. Ecol.: Prog. Ser.*, 2011, in press. N. Bergmann, G. Winters, G. Rauch, C. Eizaguirre, J. Gu, P. Nelle, B. Fricke and T. B. H. Reusch, Population-specificity of heat stress gene induction in northern and southern eelgrass Zostera marina populations under simulated global warming, *Mol. Ecol.*, 2010, **19**, 2870–2883.

14 T. B. Reusch, A. S. Veron, C. Preuss, J. Weiner, L. Wissler, A. Beck, S. Klages, M. Kube, R. Reinhardt and E. Bornberg-Bauer, Comparative analysis of expressed sequence tag (EST) libraries in the seagrass Zostera marina subjected to temperature stress, *Mar. Biotechnol.*, 2008, **10**, 297–309.

15 S. U. Franssen, J. Gu, N. Bergmann, G. Winters, U. C. Klostermeier, P. Rosenstiel, E. Bornberg-Bauer and T. B. Reusch, Transcriptomic resilience to global warming in the seagrass Zostera marina, a marine foundation species, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, in press.

16 L. Wissler, F. M. Codoner, J. Gu, T. B. H. Reusch, J. L. Olsen, G. Procaccini and E. Bornberg-Bauer, Back to the sea twice: identifying candidate plant genes for molecular evolution to marine life, *BMC Evol. Biol.*, 2011, **11**, DOI: 10.1186/1471-2148-11-8.

17 F. Short, T. Carruthers, W. Dennison and M. Waycott, Global seagrass distribution and diversity: A bioregional model, *J. Exp. Mar. Biol. Ecol.*, 2007, **350**, 3–20.

18 R. Costanza, R. dArge, R. deGroot, S. Farber, M. Grasso, B. Hannon, K. Limburg, S. Naeem, R. V. ONeill, J. Paruelo, R. G. Raskin, P. Sutton and M. vandenBelt, The value of the world's ecosystem services and natural capital, *Nature*, 1997, **387**, 253–260.

19 J. A. Coyer, O. E. Diekmann, E. A. Serrao, G. Procaccini, N. Milchakova, G. A. Pearson, W. T. Stam and J. L. Olsen, Population genetics of dwarf eelgrass Zostera noltii throughout its biogeographic range, *Mar. Ecol.: Prog. Ser.*, 2004, **291**, 51–62; E. P. Green and F. T. Short, *World atlas of seagrasses*, UNEP World Conservation Monitoring Centre University of California Press, Berkeley, 2003.

20 R. J. Orth, T. J. B. Carruthers, W. C. Dennison, C. M. Duarte, J. W. Fourqurean, K. L. Heck, A. R. Hughes, G. A. Kendrick, W. J. Kenworthy, S. Olyarnik, F. T. Short, M. Waycott and S. L. Williams, A Global Crisis for Seagrass Ecosystems, *BioScience*, 2006, **56**, 987–996.

21 F. T. Short and H. A. Neckles, The effects of global climate change on seagrasses, *Aquat. Bot.*, 1999, **63**, 169–196.

22 S. U. Franssen, J. Gu, N. Bergmann, G. Winters, U. C. Klostermeier, P. Rosenstiel, E. Bornberg-Bauer and T. B. Reusch, Transcriptomic resilience to global warming in the seagrass Zostera marina, a marine foundation species, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 19276–19281.

23 R. Caspi, T. Altman, J. M. Dale, K. Dreher, C. A. Fulcher, F. Gilham, P. Kaipa, A. S. Karthikeyan, A. Kothari, M. Krummenacker, M. Latendresse, L. A. Mueller, S. Paley, L. Popescu, A. Pujar, A. G. Shearer, P. Zhang and P. D. Karp, The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases, *Nucleic Acids Res.*, 2010, **38**, D473–D479.

24 P. G. Sappl, A. J. Carroll, R. Clifton, R. Lister, J. Whelan, A. Harvey Millar and K. B. Singh, The Arabidopsis glutathione transferase gene family displays complex stress regulation and co-silencing multiple genes results in altered metabolic sensitivity to oxidative stress, *Plant J.*, 2009, **58**–63.

25 R. A. Rodrigues, M. C. Silva-Filho and K. Cline, FtsH2 and FtsH5: two homologous subunits use different integration mechanisms leading to the same thylakoid multimeric complex, *Plant J.*, 2011, **65**, 600–609.

26 M. Yoshioka and Y. Yamamoto, Quality control of Photosystem II: where and how does the degradation of the D1 protein by FtsH proteases start under light stress?–Facts and hypotheses, *J. Photochem. Photobiol., B*, 2011, **104**, 229–235; R. Wagner, H. Aigner, A. Pruzinska, H. J. Jankanpaa, S. Jansson and C. Funk, Fitness analyses of Arabidopsis thaliana mutants depleted of FtsH metalloproteases and characterization of three FtsH6 deletion mutants exposed to high light stress, senescence and chilling, *New Phytol.*, 2011, **191**, 449–458.

27 I. Widjaja, I. Lassowskat, G. Bethke, L. Eschen-Lippold, H. H. Long, K. Naumann, J. L. Dangl, D. Scheel and J. Lee, A protein phosphatase 2C, responsive to the bacterial effector AvrRpm1 but not to the AvrB effector, regulates defense responses in Arabidopsis, *Plant J.*, 2010, **61**, 249–258; X. Hu, H. Zhang, G. Li, Y. Yang, Z. Zheng and F. Song, Ectopic expression of a rice protein phosphatase 2C gene OsBIPP2C2 in tobacco improves disease resistance, *Plant Cell Rep.*, 2009, **28**, 985–995.

28 A. Devoto, P. R. Muskett and K. Shirasu, Role of ubiquitination in the regulation of plant defence against pathogens, *Curr. Opin. Plant Biol.*, 2003, **6**, 307–311; I. J. Stulemeijer and M. H. Joosten, Post-translational modification of host proteins in pathogen-triggered defence signalling in plants, *Mol. Plant Pathol.*, 2008, **9**, 545–560; A. Craig, R. Ewan, J. Mesmar, V. Gudipati and A. Sadanandom, E3 ubiquitin ligases and plant innate immunity, *J. Exp. Bot.*, 2009, **60**, 1123–1132.

29 Y. L. Ruan, Y. Jin, Y. J. Yang, G. J. Li and J. S. Boyer, Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat, *Mol. Plant*, 2010, **3**, 942–955; A. Sturm, Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning, *Plant Physiol.*, 1999, **121**, 1–8.

30 L. Rizhsky, H. Liang, J. Shuman, V. Shulaev, S. Davletova and R. Mittler, When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress, *Plant Physiol.*, 2004, **134**, 1683–1696.

31 K. Richter, M. Haslbeck and J. Buchner, The heat shock response: life on the verge of death, *Mol. Cell*, 2010, **40**, 253–266.

32 T. Garland and S. C. Adolph, Why Not to Do Two-Species Comparative Studies: Limitations on Inferring Adaptation, *Physiol. Zool.*, 1994, **67**, 797–828.

33 T. B. Reusch, A. Ehlers, A. Hammerli and B. Worm, Ecosystem recovery after climatic extremes enhanced by genotypic diversity, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 2826–2831.

34 F. Kaplan and C. L. Guy, beta-Amylase induction and the protective role of maltose during temperature shock, *Plant Physiol.*, 2004, **135**, 1674–1684.

35 A. Nishizawa, Y. Yabuta and S. Shigeoka, Galactinol and raffinose constitute a novel function to protect plants from oxidative damage, *Plant Physiol.*, 2008, **147**, 1251–1263.

36 S. Matic, H. E. Akerlund, E. Everitt and S. Widell, Sucrose synthase isoforms in cultured tobacco cells, *Plant Physiol. Biochem.*, 2004, **42**, 299–306.

37 M. Klinghammer and R. Tenhaken, Genome-wide analysis of the UDP-glucose dehydrogenase gene family in Arabidopsis, a key enzyme for matrix polysaccharides in cell walls, *J. Exp. Bot.*, 2007, **58**, 3609–3621; A. Karkonen, A. Murigneux, J. P. Martinant, E. Pepey, C. Tatout, B. J. Dudley and S. C. Fry, UDP-glucose dehydrogenases of maize: a role in cell wall pentose biosynthesis, *Biochem. J.*, 2005, **391**, 409–415.

38 A. K. Verma, S. K. Upadhyay, P. C. Verma, S. Solomon and S. B. Singh, Functional analysis of sucrose phosphate synthase (SPS) and sucrose synthase (SS) in sugarcane (Saccharum) cultivars, *Plant Biol.*, 2011, **13**, 325–332.

39 B. Hong, C. Ma, Y. Yang, T. Wang, K. Yamaguchi-Shinozaki and J. Gao, Over-expression of AtDREB1A in chrysanthemum enhances tolerance to heat stress, *Plant Mol. Biol.*, 2009, **70**, 231–240.

40 F. Kaplan, D. Y. Sung and C. L. Guy, Roles of beta-amylase and starch breakdown during temperatures stress, *Physiol. Plant.*, 2006, **126**, 120–128.

41 K. Denyer, C. M. Hylton and A. M. Smith, Effect of High-Temperature on Starch Synthesis and the Activity of Starch Synthase, *Aust. J. Plant Physiol.*, 1994, **21**, 783–789.

42 S. P. Zhou, R. J. Sauve, Z. Liu, S. Reddy, S. Bhatti, S. D. Hucko, Y. Yong, T. Fish and T. W. Thannhauser, Heat-induced Proteome Changes in Tomato Leaves, *J. Am. Soc. Hortic. Sci.*, 2011, **136**, 219–226; Z. B. Ye, P. J. Gong, J. H. Zhang, H. X. Li, C. X. Yang, C. J. Zhang, X. H. Zhang, Z. Khurram, Y. Y. Zhang, T. T. Wang and Z. J. Fei, Transcriptional profiles of drought-responsive genes in modulating transcription signal transduction, and biochemical pathways in tomato, *J. Exp. Bot.*, 2010, **61**, 3563–3575.

43 E. Kuznetsova, M. Proudfoot, S. A. Sanders, J. Reinking, A. Savchenko, C. H. Arrowsmith, A. M. Edwards and A. F. Yakunin, Enzyme genomics: Application of general enzymatic screens to discover new enzymes, *FEMS Microbiol. Rev.*, 2005, **29**, 263–279.

44 J. A. Brito, N. Borges, C. Vonrhein, H. Santos and M. Archer, Crystal structure of Archaeoglobus fulgidus CTP:inositol-1-phosphate cytidylyltransferase, a key enzyme for di-*myo*-inositol-phosphate synthesis in (hyper)thermophiles, *J. Bacteriol.*, 2011, **193**, 2177–2185; A. M. Keese, G. J. Schut, M. Ouhammouch, M. W. Adams and M. Thomm, Genome-wide identification of targets for the archaeal heat shock regulator phr by cell-free transcription of genomic DNA, *J. Bacteriol.*, 2010, **192**, 1292–1298; L. G. Goncalves, P. Lamosa, R. Huber and H. Santos, Di-*myo*-inositol phosphate and novel UDP-sugars accumulate in the extreme hyperthermophile *Pyrolobus fumarii*, *Extremophiles*, 2008, **12**, 383–389.

45 M. Ortbauer and M. Popp, Functional role of polyhydroxy compounds on protein structure and thermal stability studied by circular dichroism spectroscopy, *Plant Physiol. Biochem.*, 2008, **46**, 428–434.

46 C. C. Smart and S. Flores, Overexpression of D-*myo*-inositol-3-phosphate synthase leads to elevated levels of inositol in Arabidopsis, *Plant Mol. Biol.*, 1997, **33**, 811–820.

47 J. Gu and V. J. Hilser, Sequence-based analysis of protein energy landscapes reveals nonuniform thermal adaptation withinthe proteome, *Mol. Biol. Evol.*, 2009, **26**, 2217–2227.

48 O. Shlyk-Kerner, I. Samish, D. Kaftan, N. Holland, P. S. Sai, H. Kless and A. Scherz, Protein flexibility acclimatizes photosynthetic energy conversion to the ambient temperature, *Nature*, 2006, **442**, 827–830.

49 A. C. Dalziel, S. M. Rogers and P. M. Schulte, Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology, *Mol. Ecol.*, 2009, **18**, 4997–5017.

50 D. Y. Lee and O. Fiehn, High quality metabolomic data for *Chlamydomonas reinhardtii*, *Plant Methods*, 2008, **4**, 7.

51 A. Brautigam and A. P. Weber, Do metabolite transport processes limit photosynthesis?, *Plant Physiol.*, 2011, **155**, 43–48; A. Brautigam, K. Kajala, J. Wullenweber, M. Sommer, D. Gagneul, K. L. Weber, K. M. Carr, U. Gowik, J. Mass, M. J. Lercher, P. Westhoff, J. M. Hibberd and A. P. Weber, An mRNA blueprint for C4 photosynthesis derived from comparative transcriptomics of closely related C3 and C4 species, *Plant Physiol.*, 2011, **155**, 142–156.

52 B. Chevreux, T. Pfisterer, B. Drescher, A. J. Driesel, W. E. Muller, T. Wetter and S. Suhai, Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs, *Genome Res.*, 2004, **14**, 1147–1159.

53 H. Li and R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform, *Bioinformatics*, 2009, **25**, 1754–1760.

54 H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis and R. Durbin, The Sequence Alignment/Map format and SAMtools, *Bioinformatics*, 2009, **25**, 2078–2079.

55 D. Swarbreck, C. Wilks, P. Lamesch, T. Z. Berardini, M. Garcia-Hernandez, H. Foerster, D. Li, T. Meyer, R. Muller, L. Ploetz, A. Radenbaugh, S. Singh, V. Swing, C. Tissier, P. Zhang and E. Huala, The Arabidopsis Information Resource (TAIR): gene structure and function annotation, *Nucleic Acids Res.*, 2008, **36**, D1009–D1014.

56 S. Ouyang, W. Zhu, J. Hamilton, H. Lin, M. Campbell, K. Childs, F. Thibaud-Nissen, R. L. Malek, Y. Lee, L. Zheng, J. Orvis, B. Haas, J. Wortman and C. R. Buell, The TIGR Rice Genome Annotation Resource: improvements and new features, *Nucleic Acids Res.*, 2007, **35**, D883–D887.

57 P. Zhang, H. Foerster, C. P. Tissier, L. Mueller, S. Paley, P. D. Karp and S. Y. Rhee, MetaCyc and AraCyc. Metabolic pathway databases for plant research, *Plant Physiol.*, 2005, **138**, 27–37.

58 S. Maere, K. Heymans and M. Kuiper, BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks, *Bioinformatics*, 2005, **21**, 3448–3449.

59 M. Ashburner, C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, J. T. Eppig, M. A. Harris, D. P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J. C. Matese, J. E. Richardson, M. Ringwald, G. M. Rubin and G. Sherlock, Gene ontology: tool for the unification of biology. The Gene Ontology Consortium, *Nat. Genet.*, 2000, **25**, 25–29.

60 A. C. Davison and D. Hinkley, *Bootstrap Methods and their Application*, Cambridge Series in Statistical and Probabilistic Mathematics, Cambridge, 8 edn, 2006; B. Efron and R. J. Tibshirani, *An Introduction to the Bootstrap*, CRC Press, Boca Raton, 1994.

61 Y. Benjamini and Y. Hochberg, Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing, *J. R. Stat. Soc. Ser. B-Methodol.*, 1995, **57**, 289–300.