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Heat stress response in the closest algal relatives of land plants reveals conserved stress signaling circuits

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SUMMARY

All land plants (embryophytes) share a common ancestor that likely evolved from a filamentous freshwater alga. Elucidating the transition from algae to embryophytes – and the eventual conquering of Earth’s surface – is one of the most fundamental questions in plant evolutionary biology. Here, we investigated one of the organismal properties that might have enabled this transition: resistance to drastic temperature shifts. We explored the effect of heat stress in Mougeotia and Spirogyra, two representatives of Zygnematophyceae – the closest known algal sister lineage to land plants. Heat stress induced pronounced phenotypic alterations in their plastids, and high-performance liquid chromatography-tandem mass spectrometry-based profiling of 565 transitions for the analysis of main central metabolites revealed significant shifts in 43 compounds. We also analyzed the global differential gene expression responses triggered by heat, generating 92.8 Gbp of sequence data and assembling a combined set of 8905 well-expressed genes. Each organism had its own distinct gene expression profile; less than one-half of their shared genes showed concordant gene expression trends. We nevertheless detected common signature responses to heat such as elevated transcript levels for molecular chaperones, thylakoid components, and – corroborating our metabolomic data – amino acid metabolism. We also uncovered the heat-stress responsiveness of genes for phosphorylation-based signal transduction that links environmental cues, calcium signatures and plastid biology. Our data allow us to infer the molecular heat stress response that the earliest land plants might have used when facing the rapidly shifting temperature conditions of the terrestrial habitat.

Keywords: early plant evolution, stress physiology, streptophyte algae, plant terrestrialization, signal transduction, charophytes, heat stress, RNA-seq, metabolomics.
INTRODUCTION

Land plants evolved from streptophyte algae. Comparing data gleaned from streptophyte algae and land plants allows for the inference of properties of the progenitors of embryophytes – from which organisms that eventually gave rise to the entire terrestrial macroflora evolved (Delwiche and Cooper, 2015; de Vries and Archibald, 2018). Yet, there are several classes of streptophyte algae that live in freshwater and terrestrial habitats (Lewis and McCourt, 2004), the habitats in which the land plant progenitors likely dwelled (Becker and Marin, 2009; Harholt et al., 2016). Furthermore, trait evolution across the streptophyte algae paints a complicated picture (Delwiche and Cooper, 2015; Delwiche, 2016; de Vries and Archibald, 2018), and which specific streptophyte algal lineages have the potential to be the most informative is hence a key question. Major phyllogenetic analyses have thus been devoted to clarifying the relationships among streptophytes. Phylogenetic analyses based on chloroplast (Turmel et al., 2006; Ruhtfel et al., 2014) and nuclear genomic data (Wodniok et al., 2011; Wickett et al., 2014; Puttick et al., 2018; Leebens-Mack et al., 2019) have consistently recovered the Zygnematophyceae as the closest algal relatives to land plants. This has important implications for inferring the nature of the last common ancestor of land plants and algae – and hence the earliest land plants themselves.

The streptophyte algae can be split into two grades: the Mesostigmatophyceae, Chlorokybophyceae and Klebsormidiophyceae (KCM) (de Vries et al., 2016) and the Zygnematophyceae, Coleochaetophyceae and Charophyceae (ZCC) (de Vries et al., 2016). The ZCC grade and land plants form the monophyletic Phragmoplastophyta (Lecointre and Le Guyader, 2006). Among all phragmoplastophytic streptophyte algae, the Zygnematophyceae exhibit the least morphological complexity (see also discussions in Wodniok et al., 2011 and Wickett et al., 2014). This illustrates some of the challenges in inferring the early evolution of plants (recently reviewed in Cooper and Delwiche [2015] as well as de Vries and Archibald, 2018). Yet, as previously highlighted (de Vries et al., 2018), Zygnematophyceae possess specific physiological properties that appear to tie them to land plants. These include, for example, a complete set of genes that is homologous to a signaling cascade required for transducing the stress phytohormone ABA of land plants (de Vries et al., 2018). Zygnematophyceae physiology is further relevant in the context of a successful establishment on land because they exhibit, among other noteworthy properties, astounding levels of desiccation tolerance (Holzinger and Karsten, 2013; Pichrová et al., 2016; Rippin et al., 2017; Herburger et al., 2019).

The first land plants must have successfully overcome a barrage of terrestrial stressors in order to rise above their substrate and establish the terrestrial flora as we know it (Becker and Marin, 2009; Delwiche and Cooper, 2015; de Vries and Archibald, 2018; Rensing, 2018; Fürst-Jansen et al., 2020). One of these challenges was rapid but drastic shifts in temperature (de Vries and Archibald, 2018). Land plants face these challenges on a frequent basis and evolved an elaborate temperature stress response (Chinnusamy et al., 2007; Ohama et al., 2017). They process these environmental inputs using the plant perceptron, which integrates external cues into a complex array of signaling molecules – such as phytohormones – and regulatory switches (Scheres and van der Putten, 2017). One of these environmental cues is heat.

Heat stress has several biophysical consequences that affect the biology of all cells. These include increased membrane fluidity and the accumulation of misfolded proteins (Murata and Los, 1997; Kotak et al., 2007). Cells respond to these heat-induced maladies using the appropriate counter-action. Membrane fluidity is counteracted using fatty acid desaturases (Falcone et al., 2004) and misfolded proteins are dealt with by molecular chaperones, many of which are also known as heat shock proteins (HSPs) (Nakamoto and Vigh, 2007; Kotak et al., 2007). Mounting the molecular response to temperature stress is based on intricate signaling pathways. These include the transcription factor (TF) network of heat shock factors (Ohama et al., 2017); such generic responses are hardened into the molecular biology of all cellular life (Feder and Hofmann, 1999). In land plant and algal cells, the plastid (chloroplast) must also be considered. Indeed, a recent study highlighted that the cyclopentenones dinor-12-oxo-10,15(Z)-phytodienoic acid (dn-OPDA) and OPDA, which are synthesized in the plastid, provide tolerance to heat stress in the land plants Arabidopsis thaliana and Marchantia polymorpha, as well as the streptophyte alga Klebsormidium nitens (Monte et al., 2020).

Plastids are a major site of abiotic stress response (Fernández and Strand, 2008; Chan et al., 2016). Because most of the proteins found in green plastids are encoded in the nucleus (Timmis et al., 2004; Ferro et al., 2010; Terashima et al., 2011), the plastid communicates with the nucleus to adequately respond to external cues (Chan et al., 2016). The molecular biological chassis underpinning plastid retrograde signaling has been the subject of much recent debate and conceptual reordering (Page et al., 2017; Song et al., 2018; Wu et al., 2018; Zhao et al., 2018; Kacprzak et al., 2019; Shimizu et al., 2019). These discussions nevertheless revolve around a (relatively) robust core set of proteins including those that are tetrapyrrrole biosynthesis-associated (Chan et al., 2016; Hernández-Verdeja and Strand, 2018). Several carotenoid-derived metabolites (Ramal et al., 2012; Xiao et al., 2012; Avendaño-Vázquez et al., 2014; Kleine and Leister, 2016) and the phosphonucleotide 3′-phosphoadenosine 5′-phosphate (Estavillo et al., 2020). The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd, The Plant Journal, (2020), doi: 10.1111/tpj.14782 © 2020 The Authors.
et al., 2011) have also been added. Recent genomic and transcriptomic data suggest that (i) most of the genes required for canonical land plant-like retrograde signaling were already present in ZCC-grade streptophyte algae (Nishiyama et al., 2018; Zhao et al., 2019) and (ii) these genes respond to high light and cold stress (de Vries et al., 2018).

Heat stress also impairs plastid physiology. Studies in land plants and the green alga *Chlamydomonas reinhardtii* show that heat-induced perturbations of plastid physiology include damage to reactions at the thylakoid membrane (Berry and Björkman, 1980; Sharkey, 2005), as well as reduced abundance (Hemme et al., 2014) and inhibition (Tewari and Tripathy, 1998) of chlorophyll biosynthesis enzymes. It is hence not surprising that heat stress is connected to adjustments of plastid physiology on multiple levels (Lin et al., 2014; Wang et al., 2014). Interestingly, heat induces calcium signatures in the plastid (Stael et al., 2011; Lenzoni and Knight, 2018); indeed, among the prime signaling components triggered by heat are Ca\(^{2+}\)-mediated processes.

Experiments with angiosperms and moss show that heat stress induces an influx of calcium (Ca\(^{2+}\)) into the cytoplasm that generates a characteristic Ca\(^{2+}\) profile (Klein and Ferguson, 1987; Gong et al., 1998; Saïdi et al., 2009; Finka et al., 2012); experimental tampering with these Ca\(^{2+}\) signatures by inhibiting the action of calcium channels decreases the thermotolerance of plants (Larkindale and Knight, 2002). Plant cells interpret Ca\(^{2+}\) signatures using a core set of well-defined gene families (Edel and Kudla, 2015; Edel et al., 2017). Among these is the CALCIUM-DEPENDENT PROTEIN KINASE (CPK/CDPK) family (Hrabak et al., 2003; Reddy et al., 2012), whose members are involved in responses to a variety of biotic and abiotic stressors (Schulz et al., 2013). CDPKs consist of a Ca\(^{2+}\)-sensing domain with a kinase output domain (Harper et al., 1994; Harmon et al., 2000). Upon Ca\(^{2+}\)-dependent activation, they phosphorylate downstream targets such as TFs that control stress response and interact with hormone-mediated signaling, such as by ABA (Kaplan et al., 2006; Lynch et al., 2012; Edel and Kudla, 2016). Land plant genomes usually encode numerous CDPKs (e.g., 10 in the lycophyte *Selaginella moellendorffii*; 34 in *A. italiana*) (Edel and Kudla, 2015); streptophyte algae also harbor multiple CDPK-encoding genes (Edel and Kudla, 2015; Nishiyama et al., 2018).

Calcium-mediated signaling is not only important upon heat stress, it is also one of the classical broad-spectrum responses towards a variety of stressors (Reddy et al., 2012). Another such broad-spectrum response is signaling processes mediated by the stress phytohormone ABA (Zhu, 2016). In land plants, ABA is synthesized from carotenoids; most of these key steps occur in the plastid, with the finishing touches occurring in the cytosol (Nambara and Marion-Poll, 2005). The presence of ABA in the cytosol is perceived by the PYRABACTIN RESISTANCE(-LIKE)/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR receptor) (Ma et al., 2009; Park et al., 2009). Once ABA is bound by this receptor, it inhibits the activity of the phosphatases of the PROTEIN PHOSPHATASE 2C (PP2C) family, including ABA INSENSITIVE 1 (ABI1) and HYPERSENSITIVE TO ABA1 (HAB1) (Rubio et al., 2009; Nishimura et al., 2010). The inhibition of the PP2Cs then results in the release of the SUCCROSE NONFERMENTING 1-RELATED PROTEIN KINASEs (SnRKs), including the well-known protein OPEN STOMATA 1 (OST1, i.e. SnRK2.6) (Vlad et al., 2009; Umezawa et al., 2009). The SnRK kinases are then free to phosphorylate various downstream targets, including TFs such as ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTORS (ABFs) and ion channels such as SLOW ANION CHANNEL 1 (SLAC1) (Furihata et al., 2006; Soon et al., 2012).

The core set of genes that constitutes the ABA-triggered signaling loop appears conserved across land plants (Umezawa et al., 2010; Hauser et al., 2011; Eklund et al., 2018; Sussmilch et al., 2019). Recently, we have noticed that the zygnematoophycean alga *Zygmena circumcarinatum* has all the genes to potentially use this signaling cascade – including a gene homologous to the PYR/PYL/RCAR genes of land plants (de Vries et al., 2018). The presence of PYR/PYL/RCAR genes in *Zygnematoophyceae* was confirmed through sequencing of the nuclear genome of *Mesotaenium endlicherianum* (Cheng et al., 2019). Zygnematoophyceae are hence the first algal group shown to have the complete gene set for ABA-mediated signaling. Our most recent functional data on *Z. circumcarinatum*, however, show that, although zygnematoophycean PYL does regulate the downstream phosphatases, it does so in an ABA-independent manner (Sun et al., 2019). Hence, the cascade likely acts in the modulation of stress physiology, although the regulating agent is unknown. Nonetheless, the presence and partially shared functionality makes the capacities of the molecular stress physiology of Zygnematoophyceae of particular relevance for the evolution of the physiology of land plants (see also discussions in Fürst-Jansen et al., 2020).

Here, we investigated the molecular stress response of *Zygnematoophyceae* by analyzing two representative species that likely diverged at an early time point, thus spanning close to 500 million years of zygnematoophycean evolution (dating based on Morris et al., 2018). We uncovered ubiquitous signature responses to heat, including metabolomic changes in amino acid levels and the induction of global gene expression patterns associated with biomolecule homeostasis. Importantly, we found the responsiveness of putative components in signal transduction to be conserved between land plants and their closest algal relatives.
RESULTS AND DISCUSSION

Heat stress induces changes in plastid morphology in two Zygnematales

The Zygnematales represents the closest algal sister group of land plants (Wickett et al., 2014). We used two representative species of filamentous Zygnematales, Mougeotia sp. (MZCH240) and Spirogyra pratensis (MZCH10213); these two species span some of the deepest branches of the phylogeny of Zygnematales (Gontcharov and Melkonian, 2010; Wickett et al., 2014; Leebens-Mack et al., 2019). Mougeotia is characterized by a plate-like plastid that takes up most of the cell; S. pratensis instead has a twisted, helical plastid. Both species grow as predominantly unbranched filaments – despite the fact that branching can occur – and are ubiquitous in most freshwater habitats.

We cultivated both species at 22°C and 120 μmol quanta m−2 sec−1 from a light-emitting diode (LED) light source (photosynthetically active radiance), which is referred to as the control condition. Drastic shifts in temperature are a known terrestrial stressor (de Vries and Archibald, 2018). To investigate the effect of temperature stress, we used heat stress. We shifted the algae from the control condition to 37°C (but same light regime, i.e. 120 μmol quanta m−2 sec−1) for 24 h and used microscopy to assess their phenotypic responses (Figure 1).

Mougeotia sp. and S. pratensis both showed phenotypic alterations upon heat stress treatment (Figure 1a). In Mougeotia sp., the plastids appeared more clumped together and changed significantly (P = 2.2 × 10−16) in length from an average of 48.1 ± 9.2 μm to 24.7 ± 7.5 μm (Figure 1b); the length of the cells themselves did not change significantly from an average of 64.4 ± 12.8 μm to 60.6 ± 14.3 μm (P = 0.063). Because we obtained tractable (and thus comparable) measures for each given cell and plastid exposed to control (n = 40) and heat (n = 56) conditions, we calculated the relative length the plastid occupied in relation to the total length of the cell. The relative length of the plastids decreased significantly from an average of 74.8 ± 4.8% to 41.9 ± 13.0% (P < 2.2 × 10−16). The width of the plastids showed pronounced heat-induced fluctuations. On average, however, plastid width merely, but significantly, decreased from 10.7 ± 1.2 μm to 9.3 ± 1.8 μm (P = 7.2 × 10−8), whereas cell width stayed exactly the same (12.6 ± 1.2 μm and 12.6 ± 1.2 μm in control and heat-treated cells, respectively; P = 0.99). For the relative width of the plastid (in relation to the width of the cell), this meant a decrease from 85.5 ± 7.0% to 74.0 ± 11.7% (P = 4.8 × 10−7). Having obtained length and width measures, we calculated the area of the plastid, assuming a non-square rectangular geometry. As expected from the width and length measurements, the area of the plastid decreased significantly from 518.5 ± 120.6 μm2 to 228.6 ± 82.9 μm2 (P = 3.9 × 10−15), whereas the change in area of the cell was less pronounced, from 813.1 ± 180.6 μm2 to 763.1 ± 196.4 μm2 (P = 0.063). In relative terms, this means a change in plastid area from 63.9 ± 5.8% to 30.8 ± 10.6% (P < 2.2 × 10−16). Furthermore, Mougeotia’s plastids were often visibly contorted, giving them a fringed and jagged appearance.

In S. pratensis, heat stress induced a variety of drastic changes of the overall morphology of any given plastid. More than 95% (n = 67) of the plastids of heat-treated Spirogyra filaments had lost their characteristic spiral arrangement (Figure 1a). Among these 95%, the spiral plastid was sometimes vaguely apparent, although it was less tightly spiraled, yet often the plastid had an entirely irregular shape. Concomitantly, the pigmentation of the plastid often changed from grass green towards orange. Furthermore, the entire cellular content of heat-treated Spirogyra cells appeared more granular.

Because both species showed alterations in plastid morphology, we made use of the autofluorescence of the plastid and used confocal microscopy to more finely pinpoint the structural changes of the plastid associated with heat stress treatment. For Mougeotia sp. the confocal micrographs confirmed the huddled and jagged appearance of the plastids. In the case of S. pratensis, this revealed that the pronounced changes upon heat stress in gross morphology were indeed a result of plastid ‘unwinding’. This looser corkscrew shape (i.e. loss of some twists and turns) was even more apparent under fluorescence. Moreover, S. pratensis plastids often had (granular) specks of high fluorescence and a more jagged surface upon heat treatment. Overall, our observations reveal that heat stress impacted both species and morphological alterations were most obvious in their plastids.

Mougeotia and Spirogyra show reduced differential transcript abundance of house-keeping genes upon heat stress

To determine how these two Zygnematales respond to heat on a molecular level, we performed comparative transcriptome sequencing using the NovaSeq 6000 platform (Illumina, San Diego, CA, USA; operated by Génome Québec, Montréal, Canada). RNA was extracted for each species and condition from three (and in the case of Mougeotia sp. control samples six) biological replicates. In total, we obtained 232 911 820 and 226 275 398 paired-end reads (47.05 and 45.71 Gbp) with a length of 100 bp for Mougeotia sp. and S. pratensis, respectively (Data S1). After (i) filtering for sequence quality, (ii) de novo assembly, and (iii) filtering for high expression level as well as taxonomic affiliation (for details, see Material and Methods), we obtained 4955 contigs for Mougeotia sp. and 3950 contigs for S. pratensis. For each gene (determined by the TRINITY clustering algorithm) (Haas et al., 2013), we
defined the transcript with the highest gene expression as the representative isoform. Additionally, we applied an expression-based cut-off by retaining only those genes that had an average transcripts per million (TPM) over all the libraries that was higher than the number of libraries sequenced. The representative isoforms were in silico translated using a custom perl script that extracted the best protein alignments obtained by BLASTX (Altschul et al., 1990) screening against protein data from selected chlorophyte and streptophyte genomes; protein data were used to link annotation with the differential gene expression values that were calculated via EDGER (Robinson et al., 2010) (Data S2).

To obtain a global perspective on the influence of heat on the two Zygnematales, we used the pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) to contextualize our expression values. For this, we first identified KEGG orthologs among our de novo assembled protein-coding transcripts using BLASTKOALA (Kanehisa et al., 2016); if multiple transcripts were annotated as the same KEGG ortholog, we added up their gene expression values. In total, we identified 1178 and 1000 unique KEGG orthologs in Mougeotia sp. and S. pratensis that mapped onto plant KEGG pathways. In Mougeotia sp., heat induced an up-regulation (log$_2$[fc] ≥ 1) of 116 and down-regulation (log$_2$[fc] ≤ −1) of 439 KEGG orthologs; in S. pratensis, a mere 22 KEGG orthologs showed up-regulation, whereas 625 displayed down-regulation (Figure 2a). Of the KEGG pathways that exhibited differential regulation, 32 and 43 had 10 or more differentially regulated orthologs (up or down regulation) in Mougeotia sp. and S. pratensis, respectively (Figure 2b). The KEGG pathway with the most differentially regulated orthologs included information processing such as ‘Ribosome’ (three and two up-regulated, 47 and 29 down-regulated in Mougeotia sp. and S. pratensis, respectively), ‘Spliceosome’ (nine and zero up, 15 and 41 down) and ‘RNA transport’ (six and zero up, 16 and 35 down). We interpret these as signatures of the necessity to implement major shifts in molecular/cell biology and physiology in response to heat.

Heat stress perturbs protein homeostasis. A common theme across all three domains of life, thus, is an up-regulation of molecular chaperones: the HSPs (Lindquist and Craig, 1988). In KEGG, many HSPs fall into the umbrella category of ‘Protein processing in the ER’, which hence showed the second-most and most up-regulated orthologs in Mougeotia sp. (seven up-regulated and six down-regulated) and S. pratensis (six up-regulated and 26 down-regulated), respectively (Figure 2c). The finding of ‘Protein processing in the ER’ as a differentially responding KEGG category also makes sense in a broader context. As in other eukaryotes, in plants and algae there is a connection between heat stress, ER response and an accumulation of unfolded proteins (Deng et al., 2011; Mittler et al., 2012; Pérez-Martín et al., 2014; Schröda et al., 2015). In the context of misfolded protein accumulation, it is thus not
surprising to find the pathway ‘Ubiquitin-mediated proteolysis’ to be responsive; indeed, ubiquitin ligases have even been found to directly contribute to plants’ resistance to heat stress (Liu et al., 2014).

Next to protein homeostasis, other biomolecule maintenance processes were highlighted in our KEGG-based analysis. RNA stability is known to be influenced by heat (Su et al., 2018), which is consistent with finding ‘mRNA surveillance’ and ‘RNA degradation’ among the top 30 most responsive KEGG pathways. Alterations in lipid composition are a typical response to heat stress, including in plants (Narayanan et al., 2016), and we found that the KEGG pathway ‘Glycerolipid metabolism’ (two and two up-regulated, 11 and 14 down-regulated in Mougeotia sp. and S. pratensis, respectively) and ‘Glycerophospholipid metabolism’ (one and one up-regulated, 12 and 13 down-regulated) were heat responsive.

The effects of heat stress on molecular biology have been studied across the tree of life (Feder and Hofmann, 1999), and our observed gene expression patterns speak to the occurrence of some of the classical responses to heat in the Zygnematales investigated in the present study. These include, among others, the up-regulation of chaperones, response to ER-stress, and changes in lipid metabolism. This confirms that our differential RNA-sequencing (RNA-seq) analysis approach captures signature responses that occur upon heat. Various amino acid metabolism pathways were also found to be highly responsive. KEGG

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**Figure 2.** Responsiveness of metabolic pathways to heat stress in Mougeotia sp. and Spirogyra pratensis. Per species, de novo assembled contigs from RNA-seq data were mapped against the Kyoto Encyclopedia of Genes and Genomes (KEGG). For each obtained KEGG ortholog, all gene expression data (TPM TMM-normalized) were summed up and set relative to control (heat/control). A KEGG ortholog was considered up- and down-regulated if it had a \( \geq 2 \)-fold change in gene expression levels. TMM, trimmed mean of M values; TPM, transcripts per million.

(a) A heat map visualizing the number of up- (green to blue gradient colored) and down-regulated (blue to purple gradient) KEGG orthologs in each of the 118 detected KEGG plant pathways; each color tick represents one pathway. All data were sorted by the responsiveness (sum of the number of occurrences of \( \geq 2 \)-fold up- and down-regulation) of the KEGG pathway in Mougeotia sp. The stacked bar plot to the right shows the total occurrence of up- and down-regulation, as well as no change, for each of the 1178 and 1000 unique KEGG orthologs in Mougeotia sp. (top) and S. pratensis (bottom).

(b) A bar diagram highlighting the 32 KEGG pathways labeled on the x-axis that had at least 10 responsive KEGG orthologs in Mougeotia sp.

(c) A heat map of the gene expression response of KEGG orthologs of the KEGG pathway ‘Protein processing in endoplasmic reticulum (ER)’. Data are calculated as a \( \log_2 \) (fold change \( \text{heat/control} \)) and visualized by a gradient color from green (up-regulation) to blue (no change) to purple (down-regulation); white color means that a KEGG ortholog was not detected (likely below the expression cut-off) in that respective species.
pathways associated with the metabolism of 14 different proteinogenic amino acids had more than ten KEGG orthologs responsive (up- and down-regulation) in either of the two species. This suggests a pronounced impact of heat stress on the general metabolism of both organisms. We therefore performed a targeted metabolomic approach to explore this possibility further.

**Targeted metabolomic profiling of heat-stressed Mougeotia and Spirogyra highlights differential changes in amino acid abundance**

The shaping of metabolite profiles by heat stress has been investigated in land plants such as Arabidopsis (Kaplan et al., 2004) as well as chlorophytic green algae such as C. reinhardtii (Hemme et al., 2014). Heat stress has been shown to affect the level of various polar metabolites across the breadth of green organisms, from Chlamydomonas to Arabidopsis. The KEGG analysis outlined in the previous section suggests that levels of similar metabolites were being modified upon heat stress in the two species of Zygmenatophyceae. We thus used a targeted tandem mass spectrometry approach to identify changes in the metabolism of Mougeotia sp. and S. pratensis in response to heat stress. The analysis comprised 565 parent ion to daughter ion transitions for the significant elevation of a few signature metabolites of heat stress. These include a few additional amino acids – and potentially Zygmenatophyceae and land plants. Both Mougeotia and Spirogyra also showed elevated levels of Ser in response to heat (adjusted \( P < 0.01 \)), as in heat-stressed C. reinhardtii (Hemme et al., 2014), whereas changes in this amino acid was associated with a cold stress-specific response in Arabidopsis (cf. Kaplan et al., 2004). The aromatic amino acids Phe and Tyr are key compounds for the production of secondary metabolites, including phenylpropanoid-derived ones (Vogt, 2010) – for which the genetic basis is present in many streptophyte algae (de Vries et al., 2017; see also Figure S1). Furthermore, flavonoids, which branch off the same route, have been detected in the zygmenatophycean alga *Penium margaritaceum* (Jiao et al., 2019). It is tempting to speculate that these aromatic amino acids are funneled into (phenylpropanoid-derived) secondary metabolite production under heat stress, which is known to occur in land plants, too (Guy et al., 2008), also recognizing that the elevation of Phe was not significant. Such claims await further investigations.

The non-proteinogenic amino acid \( \gamma \)-aminobutyric acid (GABA) was found to increase in different land plants upon a variety of stressors – including temperature stress (Kinnersley and Turano, 2000). In Mougeotia sp. and S. pratensis, GABA was concordantly and significantly (adjusted \( P < 0.01 \)) induced upon heat stress (Figure 3). In the metabolome of Arabidopsis, GABA is induced upon cold and heat (Kaplan et al., 2004). The exact role of GABA as a signaling molecule in the stress response of land plants is still unclear. GABA can result from the catabolism of glutamate (Hildebrandt et al., 2015); hence, it could simply be the result of protein degradation and reallocation of resources under heat stress. That said, GABA has been proposed to act as a signaling molecule via the regulation of aluminum-activated malate transporter membrane channels (Ramesh et al., 2015). Furthermore, application of exogenous GABA has been shown to increase tolerance; for example, towards heat stress in the grass *Agrostis stolonifera* (Li et al., 2016). GABA is hence a putative signaling molecule that might have acted in the molecular physiology of the earliest land plants.

The metabolome of *S. pratensis* is noteworthy in light of the significant elevation of a few signature metabolites of heat stress. These include a few additional amino acids (Leu, Ile, His and Lys) and sucrose, a common stress responsive metabolite in plants (Kaplan et al., 2004; Rizhsky et al., 2004). Importantly, sucrose can also act as a signaling molecule in land plants (e.g. Rolland et al., 2006). Furthermore, the amino acids Leu and Ile both increased significantly in *S. pratensis* in response to heat stress, which aligns with data on Arabidopsis (Kaplan et al., 2004) and *C. reinhardtii* Hemme et al. (2014), where steady heat-associated increases of Leu and Ile were found.
Overall, there were few shared responses between *Mougeotia* sp. and *S. pratensis*. Of the 43 significantly changed features (adjusted \( P < 0.01 \), determined by an ANOVA), 34 could be attributed to species-specific differences alone. Altogether, we observed metabolomic changes that were quite distinct between the two streptophyte algae, many of which are nevertheless in line with what has been observed in heat metabolome studies of other ‘green’ organisms. Next, we turned again to our RNA-seq data and investigated which transcriptional responses were shared between the two organisms.

**More than one-half of the genes shared by *Mougeotia* and *Spirogyra* respond differently to heat stress**

Heat stress responses that (i) are shared by the two species and (ii) can be linked to relevant biology in land plants are likely to have played a role in stress response in the last common ancestor shared by land plants and Zygnematophyceae, allowing for inferences with regard to the heat stress signaling network of the earliest land plants. To pinpoint these responses, we analyzed those protein-coding transcripts that are likely orthologous to one another. For this, we performed a reciprocal best blast hit (RBBH) approach in which we queried the protein datasets of the two Zygnematales against one another. In total, we detected 2134 orthologs shared between the two species. Of those 2134 orthologs, *Mougeotia* sp. up-regulated 305 (log\(_2\) [fold change] ≥ 1) and down-regulated 567 (log\(_2\) [fold change] ≤ −1), whereas *S. pratensis* up-regulated 197 and down-regulated 789; 1262 and 1148 orthologs showed no regulation in *Mougeotia* sp. and *S. pratensis*, respectively (Figure 4a).

How do the expression patterns of these 2134 orthologs in *Mougeotia* sp. and *S. pratensis* compare? To evaluate this, we quantified how frequently both orthologs showed up-regulation (log\(_2\) [fold change] ≥ 1), non-regulation...
Figure 4. Differential gene expression patterns of putative orthologs shared between Mougeotia sp. and Spirogyra pratensis. Using a reciprocal best BLAST hit (RBBH) approach, we determined orthologs shared between Mougeotia sp. and Spirogyra pratensis and analyzed their differential gene expression changes (log2[fold change heat/control], calculated using EDGER).

(a) A heat map depicting the differential gene expression changes of all detected orthologs shared between Mougeotia sp. and Spirogyra pratensis, sorted by their average expression change. Each colored tick represents the log2(fold change), gradient colored from green (up-regulation) to blue (unchanged) to purple (down-regulation). The stacked bar plot to the right shows the total occurrence of up-regulation (log2[fc] ≥ 1), down-regulation (log2[fc] ≤ −1) and unchanged gene expression (|log2[fc]| ≲ 1) among the 2134 shared orthologs for each of the two species.

(b) A quantification of the concordant and discordant differential gene expression patterns among the 2134 shared orthologs. We defined concordant trends as both orthologs being up-regulated (both had a log2[fc] ≥ 1), down-regulated (both had a log2[fc] ≤ −1) and unchanged (both had a |log2[fc]| ≲ 1). Discordant trends means that one ortholog was up-regulated in species A at the same time as unchanged in species B ('one up'), down-regulated in species A at the same time as unchanged in species B ('one down'), and up-regulated in species A at the same time as down-regulated in species B ('opposite'). Fanned out below the 269 'one up' and 789 'one down' is an illustration of how often this was caused by orthologs being up/down-regulated in Spirogyra pratensis (S.p.) and Mougeotia sp. (M).

(c) The precise differential gene expression (as log2[fold change heat/control]) of the 40 concordantly up-regulated, shared orthologs; the data in the diagram are sorted by the between species average log2[fc] values for each given ortholog.
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(\log_{2}\text{fold change}) \geq 1$, and down-regulation ($\log_{2}\text{fold change} \leq -1$). 923 orthologs showed the same (concordant) gene expression trends and 1211 showed discordant trends (Figure 4b). Discordance was most frequently a result of orthologs showing non-regulation in one species and down-regulation in the other; we observed this in 789 cases, with 318 being caused by down-regulation in Mougeotia sp. and 471 in S. pratensis. We counted 269 cases of discordance as a result of orthologs with non-regulation in one species and up-regulation in the other; such cases roughly occurred in an equal distribution across the two species (154 for Mougeotia sp. and 115 for S. pratensis). Only 153 showed the opposite regulation (i.e. up in one species and down in the other). The concordant differential gene expression trends of orthologs was dominated by non-regulation: 676 cases of ‘unchanged’ out of all 923 concordantly regulated orthologs (Figure 4b). There were 207 orthologs that exhibited concordant down-regulation (Figure S2) and only 40 displayed concordant up-regulation.

The 40 concordantly up-regulated orthologs likely include key candidates for the zygnematophycean response to heat. To explore their molecular role, we assigned functional annotations to the orthologs by manually comparing their respective best hits to Arabidopsis. As expected, the concordantly up-regulated orthologs included HSPs (e.g. a HSP20-like gene that was 100.1- and 54.8-fold up-regulated in Mougeotia sp. and S. pratensis, respectively); in total, seven of the 40 concordantly up-regulated orthologs could be associated with HSP function. This further included an ortholog of the heat-inducible HSP70-HSP90 ORGANIZING PROTEINS (HOP). Proteins of the HOP family are involved in various aspects of plant heat tolerance, including protein quality control (Fernández-Bautista et al., 2018).

The ortholog with the second highest (average) concordant up-regulation was a poly (ADP ribose) polymerase (PARP) (8.7-fold in Mougeotia sp. and 39.6-fold in S. pratensis). These PARP orthologs recovered as RBBHs the RADICAL-INDUCED CELL DEATH1 (RCD1) proteins, which are land plant specific proteins that contain an RST (RCD-SRO-TAF4) domain and sometimes a WWE domain (Jaspers et al. 2010). Plants whose PARPs are knocked out exhibit altered stress tolerance; both increased as well as decreased, depending on the stressors (Ahlfors et al., 2004; Fujibe et al., 2004). The presence of RCD homologs in streptophyte algae might speak to an earlier origin of these plant-specific SOR proteins than previously thought. Yet, the domain composition of the RCD found in the two species could not easily be compared because the S. pratensis sequence was fragmented. The Mougeotia sp. ortholog (Moug13104_c0.g1) had an additional ankyrin repeat (ANK), having a domain composition in the order ANK::WWE::PARP. Indeed, pHMMER searches recovered only two species that had PARPs that match the domain composition of Moug13104_c0.g1: K. nitens (KFL_000210040; genome published by Hori et al., 2014) and Chara braunii (CBR_g19059 and CBR_g16983; genome published by Nishiyama et al., 2018). Our previous RNA-seq analyses (de Vries et al., 2018) uncovered a transcript coding for a similar protein in Coleochaete scutata (Csc-tata_DN35021_c1.g1). These ANK::WWE::PARP might represent streptophyte algae-specific DNA repair proteins.

The third and fourth highest (average) concordant up-regulated orthologs were two FK506-binding proteins (FKBP). FKBP s are known for their involvement in stress tolerance, including tolerance to temperature stress as a result of the assurance of correct HSP accumulation (Meiri and Breiman, 2009). Chloroplastidial FKBP s are important for the correct assembly of the photosystem II components at the thylakoid membrane (Lima et al., 2006) and overexpression of FKBP16 in Arabidopsis resulted in an increased tolerance to abiotic stressors such as drought and high light because of elevated photosystem stability (Seok et al., 2014). The targeting prediction tool GTP (Fuss et al., 2013) suggests that three of them might be targeted to the plastid. Hence, our data on the concordant up-regulation of FKBP s highlight the importance of photosystem stability in Zygmatophyceae under heat stress.

Mougeotia sp. and S. pratensis concordantly up-regulated a gene that likely codes for a protein with homology to an Arabidopsis protein justly called BIG. The Arabidopsis BIG protein is 5098 amino acids in length and links auxin transport and light signaling (Gil et al., 2001). High concentrations of auxin were shown to elicit changes in the growth of the streptophyte alga K. nitens (Ohtaka et al., 2017). Furthermore, how auxin links (via intracellular auxin transport) temperature response and growth was recently elaborated for Arabidopsis (Feraru et al., 2019).

Yet, although streptophyte algae do harbor many of the components of auxin signaling, their action in a bona fide auxin response is still unclear (Nishiyama et al., 2018; Mutte et al., 2018; Bowman et al., 2019). Instead of (or in addition to) a role in auxinmediate processes, it is possible that the BIG protein(s) of Mougeotia sp. and/or S. pratensis are woven into Ca\textsuperscript{2+}/calmodulin-mediated regulation, as is the case for the animal homolog calossin (Xu et al., 1998).

Cinnamyl-alcohol dehydrogenase (CAD) was among the concomitantly up-regulated genes. We recently showed that many streptophyte algae have an almost complete gene set corresponding to the land plant-like phenylpropanoid biosynthesis pathway (de Vries et al., 2017). CAD is a key enzyme in the biosynthesis of lignin and lignin-like compounds. To analyze this further, we conducted a phylogenetic analysis of putative CAD sequences from major lineages of land plants and streptophyte algae. Most streptophyte algae-derived sequences cluster with other
dehydrogenases, such as zinc-binding or glutathione-dependent formaldehyde dehydrogenases (annotation based on BLASTP against TAIR10) (Berardini et al., 2015) (Figure S1). The monophyletic CAD-like and CAD cluster included twelve streptophyte algal sequences. We recovered CAD groups I and V with high support (Figure S1). CAD I likely emerged in the ancestor of tracheophytes because it includes a CAD I sequence from S. moellendorffii, yet none from bryophytes or streptophyte algae. CAD V includes sequences from bryophytes (M. polymorpha and Physcomitrella patens) and tracheophytes, indicating that it may have been present in the ancestor of land plants. We were unable to recover the CAD group ‘II&III’. It is split into several sub-clades and interspersed by CAD groups I and V (Figure S1). Most of the 12 streptophyte algal CAD-like sequences cluster with sequences that fell in CAD group II&III in previous analyses (de Vries et al., 2017). The current position suggests that the sequences are CAD-like sequences that do not belong to CAD group II&III, but rather form their own clades, within the cluster of canonical CAD sequences. Based on our metabolome data, it is tempting to speculate that the changes in the pool of aromatic amino acids might indicate the activity of a pathway that features CAD.

Conserved and functionally diverse orthologs highlight signature responses to heat

We next addressed the question of which putative orthologs that are shared by one of the two Zygnematales and land plants showed the highest gene expression changes. For this, we again made use of an RBBH approach, this time against the best-annotated land plant genome, that of Physcomitrella patens. We focused on the top 100 strongest up-regulated orthologs (Figure S1). The data featured many proteins that have been pinpointed by the 100 strongest up-regulated orthologs (Figure 5). The data included twelve streptophyte algal sequences. We recovered CAD groups I and V with high support (Figure S1). CAD I likely emerged in the ancestor of tracheophytes because it includes a CAD I sequence from S. moellendorffii, yet none from bryophytes or streptophyte algae. CAD V includes sequences from bryophytes (M. polymorpha and Physcomitrella patens) and tracheophytes, indicating that it may have been present in the ancestor of land plants. We were unable to recover the CAD group ‘II&III’. It is split into several sub-clades and interspersed by CAD groups I and V (Figure S1). Most of the 12 streptophyte algal CAD-like sequences cluster with sequences that fell in CAD group II&III in previous analyses (de Vries et al., 2017). The current position suggests that the sequences are CAD-like sequences that do not belong to CAD group II&III, but rather form their own clades, within the cluster of canonical CAD sequences. Based on our metabolome data, it is tempting to speculate that the changes in the pool of aromatic amino acids might indicate the activity of a pathway that features CAD.

Conserved and functionally diverse orthologs highlight signature responses to heat

We next addressed the question of which putative orthologs that are shared by one of the two Zygnematales and land plants showed the highest gene expression changes. For this, we again made use of an RBBH approach, this time against the best-annotated land plant genome, that of A. thaliana. We focused on the top 100 strongest up-regulated orthologs (Figure S1). The data featured many proteins that have been pinpointed by the independent analyses that we described above, including orthologs likely involved in information processing, orthologs associated with plastid biology, and chaperones/HSPs. Indeed, in both species, the highest induced ortholog was an HSP, being significantly up-regulated by 685.6-fold in Mougeotia sp. and 100.1-fold in S. pratensis (Benjamini–Hochberg-adjusted P < 0.001). In terms of organellar biology, Spirogyra, for example, up-regulated two genes coding for Rossman-fold dehydrogenases that were predicted to be targeted to the chloroplast [significantly (adjusted P < 0.001) 24.1- and 13.7-fold up]; one additional protein that was significantly induced (5.6-fold; adjusted P < 0.001) is likely mitochondria-localized.

The third-highest up-regulated putative Arabidopsis ortholog (89.4-fold, adjusted P = 5.2 × 10^-45) in Mougeotia sp. coded for an aquaporin (returning NOD26-LIKE INTRINSIC PROTEIN as RBBH). Aquaporins act as channels that transport water as well as small molecules, including reactive oxygen species (Maurel et al., 2015); the repertoire of aquaporins in the membrane is known to be dynamically adjusted under stress conditions (Maurel et al., 2015). In addition, an iron transporter ortholog to IRON REGULATED 1 was 41.6-fold up-regulated (adjusted P = 7.8 × 10^-26). Hence, Mougeotia sp. appeared to undergo pronounced adjustment of its small molecule transporter profile.

The fifth most up-regulated putative Arabidopsis ortholog in S. pratensis (37.4-fold, adjusted P = 2.0 × 10^-15) coded for a RING/U-box-containing protein (with likely E3 ubiquitin ligase activity) that returned as RBBH the protein ABA INSENSITIVE RING PROTEIN 3 (AIRP3). Although AIRP3 has obviously been tied to ABA and stress response in land plants (Kim and Kim, 2013), its role (and targets) in S. pratensis needs to be clarified. It is noteworthy that another transcript that coded for a RING/U-box-containing protein was also in S. pratensis’ top 100 (up-regulated 12.1-fold, adjusted P = 3.8 × 10^-7). Furthermore, a putative ortholog of KOBITO1 (KOBI; aka ABA INSENSITIVE 8 (ABI8)) was 6.7-fold up-regulated (adjusted P = 8.6 × 10^-13) in S. pratensis. KOBI effects plant growth and is a mediator of ABA signaling, possibly integrating stress signals (Pagant et al., 2002; Brocard-Gifford et al., 2004). The putative KOBI ortholog in Spirogyra is hence a promising candidate that might link stress input to the cell and the plastid morphological changes that we have observed.

In addition to KOBI, we identified other genes that might be associated with the phenotypic alterations observed in heat-stressed S. pratensis (Figure 1). The second-highest induced transcript (59.8-fold elevated by heat, adjusted P = 3.1 × 10^-23) coded for a protein likely involved in microtubule dynamics (containing a predicted LisH motif); it returned as RBBH TONNEAU 1B (TON1B), which is critical for microtubule organization and hence cell division and morphogenesis (Azimzadeh et al., 2008). Reorganization of the cytoskeleton might underpin the elaborate changes in intracellular organization of Spirogyra’s cells under heat stress. Using a unidirectional BLAST against the well-annotated genome of Arabidopsis thaliana, we thus screened for other up-regulated homologs of proteins known to be relevant for (re-)organization of the cytoskeleton. In both species, we detected a few up-regulated candidates for motor and additional microtubule-associated proteins (Figure S3); these homologs are interesting candidates for underpinning the changes in plastid morphology observed in both species.

Oleosin was the 19th highest up-regulated putative ortholog of Arabidopsis proteins in Spirogyra. Oleosins are oil body-associated proteins (Frandsen et al., 2001). From other Zygmematophyceae, such as many species of Zygnema, we know that lipid body accumulation is induced upon stressors, including during lipid accumulation that happens upon the formation of the more stress-resilient pro-akinetes (Pichrtová et al., 2016). This aligns with our
morphological observations in which *S. pratensis* cells accumulated granular contents upon heat exposure (Figure 1).

A few TFs were among the top 100 strongest up-regulated genes. This is noteworthy because TFs are situated upstream in genetic regulatory hierarchies and hence influence the expression of various downstream genes. In *Mougeotia* sp., a putative ortholog of KNOTTED1-LIKE HOMEOBOX GENE 3 (KNAT3) TF was 8.0-fold up-regulated. In Arabidopsis, KNAT3 modulates response to environmental factors and ABA signaling (Kim *et al.*, 2013). In *Spirogyra*, a PLATZ TF was 20.0-fold up-regulated; this PLATZ TF returned as RBBH AT1G76590, for which a homolog was found to be responsive to drought in the halophyte *Thellungiella* (Wong *et al.*, 2006). These TFs are thus key candidates for future work on how the molecular response to stress is regulated in Zygnematophyceae.

The blue-light signaling component cryptochrome (retrieving as RBBH CRY3) was 11.1-fold up-regulated in *Mougeotia* sp.; we had already identified cryptochrome as being very stress responsive in *Coleochaete scutata* (de Vries *et al.*, 2018). Indeed, the blue-light photoreceptor CRY1 was shown to intersect with red light signaling under heat stress and *Mougeotia* sp. was found to 3.3-fold down-regulate (adjusted *P* < 0.001) a putative ortholog of PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (*Moups17599_c0_g1*).

Considering that Zygnematophyceae stand out by harboring the red- and blue-light receptor fusion proteins called neochromes (Li *et al.*, 2015), our data further highlight the photoreceptors in streptophyte algae.

Quite a few carotenoid-derived metabolites have recently been brought into focus by linking the plastid and cell physiology of plants (Ramel *et al.*, 2012; Xiao *et al.*, 2012; Avendaño-Vázquez *et al.*, 2014; Kleine and Leister, 2016; Hou *et al.*, 2016). *Mougeotia* sp. up-regulated a transcript 17.5-fold that returned CAROTENOID CLEAVAGE DIOXYGENASE 1 (CCD1) as its RBBH. CCDs, and especially CCD1 (Vogel *et al.*, 2008), produce a variety of apocarotenoids. Apocarotenoids have been proposed to play a role in stress response of land plants (Hou *et al.*, 2016). For example, CCD4 of saffron crocus is induced by heat stress (Rubio *et al.*, 2008) and potato RNAi lines of CCD4 display a heat-stressed phenotype (Campbell *et al.*, 2010). The phytohormones ABA and strigolactones are both carotenoid-derived (Nambara and Marion-Poll, 2005; Al-Babili and Bouwmeester, 2015). Our observation of the regulation of zygnematophycean enzymes likely involved in the production of apocarotenoids further indicates that such proteins play a significant role in the physiology of the closest algal relatives of land plants.

Among the top 100 most highly up-regulated proteins summarized in the present study are key factors of the...
molecular stress physiology that these specific Zygnemato-
phyceae employ during heat stress and acclimation. We
next aimed to integrate this information into a com-
parative plant physiological perspective.

The closest algal relatives of land plants respond to heat
via calcium signaling, histidine kinases and chloroplast–
nucleus communication

Our recent work in *Z. circumcarinatum* (SAG 698-1a) has
highlighted the existence of homologous signaling circuits
that in land plants unite environmental inputs, ABA signal-
ing and plastid physiology (de Vries *et al.*, 2018). Addition-
ally, in a recent transcriptomic study, Rippin *et al.* (2017)
found that CPKs/CDPKs were among the most responsive
signaling components in desiccation stressed *Z. circum-
carinatum* (SAG 2419). Indeed, in land plants, there is a large
overlap of ABA-mediated and Ca\(^{2+}\)-mediated signaling that
occurs under stress (Edel and Kudla, 2016). The plastid is
among the most heat stress-responsive sub-cellular com-
partment in plant cells (Schroda *et al.*, 2015; Sun and Guo,
2016). In particular, the components of the light reaction at
the thylakoid membrane have long been considered a pri-
mary target (Berry and Björkman, 1980). Furthermore, plas-
tid signaling under heat is assumed to involve calcium
signaling (Sun and Guo, 2016; Lenzoni and Knight, 2018),
which we highlighted above as being heat responsive in
our zygnematalean algae.

Ca\(^{2+}\)-mediated signaling is a key aspect of the stress
physiology of land plants. CPDs unite Ca\(^{2+}\) sensing and
response in a single protein (Kudla *et al.*, 2018). Further-
more, CPKs have been shown to be key for orchestrating
the signal transduction upon heat-shock in land plants (Davletova *et al.*, 2001; Liu *et al.*, 2008; Dubrovina *et al.*, 2017).
Based on a query against the *A. thaliana* protein database, we detected 32 putative CPK homologs in
*Mougeotia* sp. and 21 in *S. pratensis*. A few of these CPKs
showed up-regulation [six in *Mougeotia* sp. (three signifi-
cant, Benjamini–Hochberg corrected \(P < 0.05\)] and three in
*S. pratensis* (two with \(P < 0.05\)), whereas many were
down-regulated (14 in *Mougeotia* sp. [all with \(P < 0.05\)
and 15 in *S. pratensis* (10 with \(P < 0.05\)]) (Figure 6). Land
plant CPKs have a well-defined domain organization.
They consist of an N-terminal Ser/Thr kinase domain and a
series of C-terminal calcium-binding EF-hands (typically
four) (Cheng *et al.*, 2002). Using INTERPROSCAN (Quevillon
*et al.*, 2005), we screened the putative zygnematalean
CPK sequences for this architecture; seven *Mougeotia* sp.
and eight *S. pratensis* CDPK homologs showed this
arrangement. Four and seven were down-regulated (four
and five with \(P > 0.05\)); one homolog in each species
showed significant up-regulation. Our previous transcrip-
tome of *Z. circumcarinatum* (strain SAG698-1a) (de Vries
*et al.*, 2018) identified four CDPK sequences with a canoni-
cal S/T-kinase-EF-hand structure, one of which was down-
regulated upon cold stress. Because Rippin *et al.* (2017)
found strong up- and down-regulation of CDPKs in a differ-
ent strain of *Z. circumcarinatum* (SAG 2419), CDPKs are
hence potential general regulators of the response to stres-
sors in Zygnematoxyceae. Although we are unaware of
any functional studies in Zygnematoxyceae, McCurdy
and Harmon (1992) demonstrated the Ca\(^{2+}\)-dependent pro-
tein-kinase activity of a CDPK homolog in the Charo-
phyceae *Chara corralina*. It is hence conceivable that the
closest algal relatives of land plants use CDPKs when deal-
ing with a variety of stressors. We can thus further infer
that the earliest land plants likely used CDPK-mediated sig-
signaling when they faced the barrage of terrestrial stressors.
Future research will need to clarify the details of Ca\(^{2+}\)-signa-
tures under abiotic stress in the Zygnematoxyceae and
where in the cell these changes in Ca\(^{2+}\)-signatures occur.

The thylakoid, the site of photosynthetic light reactions,
is at the frontline of the cellular abiotic stress response
(Foyer *et al.*, 1994). Therefore, components directly woven
into the photosynthetic light reactions are known to be reg-
ulated under stress. With a stress exposure time of 24 h,
the two Zygnematoxyceae examined in the present study
will likely have started long-term acclimation processes
that involve reorganization of the antenna proteins and,
hence, transcriptional changes (Eberhard *et al.*, 2008).
Indeed, the light-harvesting chlorophyll-\(a/b\) proteins of
photosystem II (LHCB) showed especially pronounced tran-
scriptional changes. *Mougeotia* sp. regulated four of the
eight detected LHCB2.1 homologs significantly (corrected
\(P < 0.001\)) up (98.4-, 7.4-, 3.6- and 3.5-fold); no LHCB2
homolog experienced significant down-regulation. In
*S. pratensis*, none of the three LHCB2.1 homologs showed
differential regulation; one of the three LHCB2.3 homologs
was significantly (corrected \(P < 0.05\)) 2.1-fold up-regulated
(none of *Mougeotia*’s three LHCB2.3 homologs exhibited
significant regulation).

Heat stress induced the expression of genes for proteins
that are involved in photoprotection. These photoprotect-
ion components primarily shield proteins integrated in the
light reaction chain and are hence generally stress-associ-
ad. This included the significant (corrected \(P < 0.001\)) 4.7-,
4.1- and 3.6-fold up-regulation of three homologs of
PHOTOSYSTEM II SUBUNIT S (PSBS) in *Mougeotia* sp.;
PSBS is a major player in non-photochemical quenching
(NPQ) including of Zygnematoxyceae (Gerotto and Moro-
sinotto, 2013). Furthermore, the NPQ-relevant violaxanthin
de-epoxidase (NPQ1) participating in the xanthophyll cycle
(Johns and Holzwarth, 2012) was significantly (corrected
\(P < 0.001\)) up-regulated in *Mougeotia* sp. by 4.2-fold.
EARLY LIGHT-INDUCABLE PROTEIN (ELIP) is a photoprot-
rective chlorophyll-\(a/b\)-binding protein that accumulates
under stress—especially cold and high light (Montané
*et al.*, 1997; Hutin *et al.*, 2003). Indeed, in previous work,
we observed that cold treatment strongly induced the
expression of ELIP-encoding genes in various streptophyte algae, including *Z. circumcarinatum* (de Vries et al., 2018); in a differential transcriptomic analysis, Rippin et al. (2019) found that the exposed upper layer of mats of *Zygnema* sp. growing in the High Arctic exhibit elevated ELIP gene expression levels. Of the three ELIP homologs detected in both *Mougeotia* sp. and *S. pratensis*, only one *Mougeotia* ELIP was significantly up-regulated (corrected *P* < 0.001; 8.4-fold). The nuclear-encoded photosystem II component PSBW was significantly up-regulated (corrected *P* < 0.05) in both *Mougeotia* sp. (2.3-fold) and *S. pratensis* (6.9-fold); this is noteworthy because PSBW has been linked to photosystem II turnover (Hagman et al., 1997) and stability (Garcia-Cerdán et al., 2011). Altogether, these data reveal that heat stress induces processes tied to photoprotection and thylakoid reorganization in our two Zygnematophyceae.

Next to these classical components of the light reaction in the thylakoids, some peripheral light reaction-associated genes showed interesting differential gene expression patterns. LIGHT-HARVESTING-LIKE 3 (LIL3) proteins are localized in the thylakoids (Takahashi et al., 2014) and are associated with chlorophyll biosynthesis (Tanaka et al., 2010). Although a LIL3 homolog was present in *Mougeotia* sp. and *S. pratensis* each, only *S. pratensis* induced LIL3 (4.2-fold), albeit not significantly (corrected *P* = 0.077). It is nonetheless tempting to speculate that this is tied to heat-induced phenotypic alterations (Figure 1). FERRITIN (FER) homologs, potentially targeted to the chloroplast (GTP score of 0.664 and 0.503 for *Mougeotia* sp. and *S. pratensis*, respectively) were significantly (adjusted *P* < 0.05) down-regulated by 3.9-fold in *Mougeotia* sp. and up-regulated by 13.5-fold in *S. pratensis*. Long and Merchant (2008) reported that the model green alga *C. reinhardtii* down-regulates FER1 upon high light stress; yet, based on data from rice (Murchie et al., 2005), Arabidopsis (Rossel et al., 2002) and barley (Atiena et al., 2004), it was also noted that some land plants up-regulate FER, whereas others down-regulate FER under high light stress. The responsiveness of FER in both directions that we observe in our two Zygnematophyceae hence adds to this intricate picture.

Various pentatricopeptide repeat (PPR) proteins act in chloroplast gene expression and RNA metabolism (Schmitz-Linneweber and Small, 2008). A likely plastid/mitochondrion dual-localized (ATP2 score of 0.566) *Mougeotia* sp. homolog encoding the PPR protein WHAT’S THIS FACTOR 1 (WTF1) was significantly up-regulated by 7.1-fold upon heat (adjusted *P* < 0.001); *S. pratensis* down-regulated its WTF1 homolog non-significantly (adjusted *P* = 0.15) by 4.4-fold. Also, the NAR5 proteins (GENES NECESSARY FOR THE ACHIEVEMENT OF RUBISCO ACCUMULATION 5) play a role in plastid gene expression (Ogawa et al., 2009). A homolog of NAR5 was significantly (adjusted *P* < 0.05) up-regulated by 2.3-fold *Mougeotia* sp. and significantly (adjust *P* < 0.05) down-regulated in *S. pratensis* by 2.8-fold; in each species, the homologs were likely plastid/mitochondrion dual localized, with ATP2 scores of 0.664 and 0.653. The poly(A)-specific ribonuclease ABA-HYPERSENSITIVE GERMINATION 2 affects mRNA stability under stress (Nishimura et al., 2005). A likely dual-localized homolog of *Mougeotia* sp. AHG2 was significantly 5.3-fold up-regulated upon heat (ATP2 score of 0.533; adjusted *P* < 0.001); in cold-treated *Z. circumcarinatum* (de Vries et al., 2018), the putative AHG2 ortholog *Zcicumcarinatum_DN45859_c2_g1* was significantly (adjusted *P* < 0.05) 3.0-fold up-regulated and also likely plastid/mitochondrion dual-localized (ATP2 score of 0.577). Altogether, these data highlight the integration of organellar gene expression and response in the zygnematophyceaean reaction to heat.

RELATIVE-SPOT HOMOLOG (RSH) proteins synthesize guanosine pent- and tetraphosphate (ppGpp), which is a signaling nucleotide that has been shown to impact various aspects of chloroplast function (Sugliani et al., 2016) and, importantly, abiotic stress (Takahashi et al., 2004). In *Mougeotia* sp., a homolog of RSH3 was non-significantly induced by 2.0-fold (adjusted *P* = 0.07; likely plastid/mitochondrion dual localized with an ATP2 score of 0.649). In *S. pratensis*, an RSH3 homolog was significantly 7.9-fold down-regulated (adjusted *P* < 0.01; likely plastid/mitochondrion dual localized with an ATP2 score of 0.677); one homolog of RSH1 found in *Spirogyra* was non-significantly reduced by 4.0-fold (adjusted *P* = 0.07; partial sequence). RSH-based synthesis of ppGpp was also detected in the chlorophyte *C. reinhardtii* (Kasai et al., 2002). This suggests that ppGpp is a signaling molecule used in stress response across the green tree of life, including in streptophyte algae.

Streptophyte algae harbor an elaborate set of genes homologous to those involved in land plant retrograde signaling (de Vries et al., 2018; Nishiyama et al., 2018). Many of these proved transcriptionally responsive under stress (de Vries et al., 2018). Given the importance of retrograde signaling in response to physiological challenges such as abiotic stress (Nott et al., 2006; Chan et al., 2016), we screened our transcriptome for 17 components known from Arabidopsis to be involved in retrograde signaling; we found at least one homolog for ten (13 genes in total) and six (eight in total) of these proteins in *Mougeotia* sp. and *S. pratensis*, respectively. Upon heat stress, *Mougeotia* sp. significantly up-regulated four and down-regulated three of the 13 homologs detected (adjusted *P* < 0.05); *S. pratensis* significantly down-regulated two (adjusted *P* < 0.05). Importantly, we detected a GUN1 homolog in *Mougeotia* sp., which corroborates our previous inference that GUN1 is part of the plastid-nucleus communication chassis in ZCC streptophyte algae (de Vries et al., 2018);
Nishiyama et al., 2018). Altogether, plastid–nucleus communication likely plays a role in zygnematophycean responses to a variety of stressors.

Both Mougeotia sp. and S. pratensis encode histidine kinase AHK homologs in their transcriptomes. AHKs are well known for their involvement in the canonical cytokinin signaling pathway of land plants (Kieber and Schaller, 2018). Additionally, certain AHKs are recognized as being responsive to a variety of abiotic stressors (Tran et al., 2007; Jeon et al., 2010; Pham et al., 2012).

AHKs that are involved in cytokinin signaling contain a CHASE domain (Cyclases/Histidine kinases Associated Sensory Extracellular) (Heyl et al., 2007), known to bind cytokinins in seed plants and thereby inhibit the downstream phosphorelay chain that leads to the phosphorylation of type B response regulators. For the non-CHASE domain-containing AHKs, such as AHK1, knowledge on the ligand remains obscure. Based on a Pfam search, we identified CHASE domains in two sequences from Mougeotia sp. (Mosp15483_c1_g3 and Mosp17224_c0_g1) and in four sequences from S. pratensis (Spipra3165_c2_g1, Spipra3977_c2_g2, Spipra3130_c0_g4 and Spipra3862_c3_g8). We performed phylogenetic analysis of all detected AHK homologs as well as Arabidopsis and P. patens AHK sequences; we recovered an AHK1 clade, with one ortholog in Mougeotia and two to three orthologs in Spirogyra (Figure S4). All AHK homologs with the best BLAST hit against AHK2, 3 and 4 did not cluster with the expected groups in land plants, which can be interpreted as lineage-specific expansions in these streptophyte algeae.

As in Arabidopsis (Brenner and Schmülling, 2015), the expression of AHK (homologs) did not significantly change under the influence of exogenous cytokinin in S. pratensis (Figure S5). However, a strong differential gene expression response occurred under heat stress in our transcriptome data. In Mougeotia sp., five AHK homologs were significantly (Benjamini–Hochberg corrected \( P < 0.05 \)) up-regulated (4.2, 3.0, 2.8, 2.7 and 2.7-fold) upon heat stress, two of them being CHASE domain-containing ones. In S. pratensis, one CHASE domain-containing AHK homolog was significantly (adjusted \( P < 0.001 \)) 3.2-fold up-regulated and two were significantly (adjusted \( P < 0.05 \)) 5.8-fold and 22.4-fold down-regulated. Of the down-regulated AHK homologs, one contained a CHASE domain (Figure 6). Both Mougeotia CHASE domain-containing AHK homologs (best BLAST hit: AHK4) were significantly up-regulated (corrected \( P < 0.05 \)) upon heat (2.8- and 2.7-fold). Also, both Spirogyra CHASE domain-containing AHK homologs (best BLAST hit: AHK4) showed induction, although this was not statistically significant. Hence, it is conceivable that, in both species, AHK homologs cause a modulation of phosphorelay signal transduction contributing to the regulation of stress response.

In a previous study (de Vries et al., 2018), we noted the responsiveness of AHK to cold stress and thus speculated that interconnections between the AHKs, cytokinin and stress (and possibly ABA) were already present in the closest relatives of land plants. For example, Zcicircumcarinatum_DN47719_c0_g1 shows putative orthology to AHK4, has a chase domain, and is 1.9-fold up-regulated upon cold stress. The present data on heat stress further corroborate this notion. Histidine kinases, potentially even CK-sensing ones, are hence key candidates for future research into the early evolution of the molecular physiology of plants. That said, the cytokinin-mediated induction of certain ARR homologs (a key feature of cytokinin signaling in seed plants) (Brenner and Schmülling, 2015) is either missing or very low in S. pratensis (maximum 1.5-fold) (Figure S5). Additional physiological and reverse genetic studies are necessary to complete the picture.

Our previous analyses on the zygnematophycean alga Z. circumbinatum highlighted the presence and transcriptional responsiveness of homologs of the ABA signaling components of land plants (de Vries et al., 2018); the detection of a putative PYR/PYL/RCAR ortholog was particularly unexpected. Here, we detected a PP2C (best hit against Arabidopsis was AtABI2) and one SnRK2 (best hit against Arabidopsis was AtSnRK2.6/AtOST1) in the transcriptomes of Mougeotia sp. and S. pratensis, respectively. Spirogyra pratensis significantly (corrected \( P < 0.01 \)) down-regulated the homologs of PP2C 5.5-fold and SnRK2 2.2-fold. We detected no PYR/PYL/RCAR homolog in either alga. Given that PYR/PYL/RCAR homologs are present in other Zygmenatophyceae (Sun et al., 2019), including the genome of Mesotaenium endlicherianum (Cheng et al., 2019), there are several possible explanations for this, the most likely being that it was simply below the expression cutoff. Yet, (i) not all Zygmenatophyceae have PYR/PYL/RCAR encoded in their genome (as in the case of Spirogyra muscicola) (Cheng et al., 2019) and (ii) the PYR/PYL/RCAR proteins of Zygmenatophyceae likely do not function as bona fide ABA receptors (Sun et al., 2019). Thus, streptophyte algal homologs of genes that in land plants are known to form the foundation of stress-regulatory networks might be regulated through different components, and even used for divergent means (see also discussions in Fürst-Jansen et al., 2020). Nonetheless, the mere presence of such homologs sheds light on the genetic material from which the stress-regulatory cascades of land plants were molded.

**Pinpointing conserved patterns in two divergent Zygmenatophyceae**

In the present study, we have investigated two divergent species, both of which thrive in freshwater systems such as streams or springs. It is prudent to note that a rapid shift from 22°C to 37°C likely does not occur in the
freshwater environment in which *Mougeotia* and *Spirogyra* naturally occur. We have used a very drastic shift in temperature to ensure that we trigger a pronounced gene expression response; information that is not aimed at illuminating (eco-)physiological properties of the organisms but, instead, the responsiveness of genes in a conserved stress-regulatory response network (see also Fürst-Jansen et al., 2020). Irrespective of the habitat in which these extant algae live, inferences made from the stress-responsiveness observed here shed light on the homologous genetic chassis that might have existed in their last common ancestor.

We have detected a variety of signaling components (such as those for phosphorelay systems) in Zygnematophyceae that are known to play important roles in the stress response system of land plants. The networks regulating plant physiology are interwoven on multiple levels. This is well-illustrated by the interconnections of ABA- and Ca²⁺-mediated signaling, comprising a convergence that likely occurs at PP2Cs in land plants (Edel and Kudla, 2016). Regarding their role in ABA-mediated signal transduction processes, the protein-protein interactions upstream and downstream of PP2Cs appear to be conserved between streptophyte algae and land plants (Lind et al., 2015; Sun et al., 2019). This demonstrates that the PP2Cs are exciting candidates for acting as master switches in stress signaling since before the dawn of land plants. That said, during the course of evolution, these regulatory networks will have been rewired multiple times (Fürst-Jansen et al., 2020). Through comparative approaches, we can pinpoint which parts of the wires (such as protein–protein interactions, phosphorylation, TF-binding, and many more) are conserved. Yet, the mere fact that the required components (e.g. CDPKs, PP2Cs) are present is already instructive; it highlights the genetic potential residing within the Zygnematophyceae (de Vries and Archibald, 2018). It is this potential that served as a hotbed for the evolution of the plant perceptron that is so critical for the molecular stress physiology of land plants.

**CONCLUSIONS**

The earliest land plants must have had the means to respond to and ultimately overcome terrestrial stressors (de Vries and Archibald, 2018; Fürst-Jansen et al., 2020). A key terrestrial stressor is drastic temperature elevation. We have shown that the zygnematophycean algae...
Mougeotia sp. and S. pratensis have distinct responses to heat. Differences in the response were visible phenotypically, as well as at the molecular level (metabolome, transcriptome). These differences likely reflect that the two algae diverged from a common ancestor about 500 mya (Morris et al., 2018). That said, their shared responses to heat stress are instructive: they reveal a set of genes encoding proteins that are known as regulators of the stress response in land plants. The responsiveness of these regulators to heat not only tells us about how both embryophytes and their algal relatives deal with elevated temperatures, but also pinpoints those components that form the perceiving of land plants (Scheres and van der Putten, 2017). Our data suggest that the perceiving of the earliest land plants featured the responsiveness of a relay system of calcium and histidine kinases that may have tied environmental inputs (and their effects on cell and plastid physiology) to the appropriate responses. Genetic dissection of the cornerstones in this relay system will highlight the degree of functional conservation. The most promising candidates for such an approach have been outlined in the present study.

EXPERIMENTAL PROCEDURES

Culturing and treatments

Cultures of Mougeotia sp. (MZCH240; originally collected in Byron’s Pool, Trumpington, Cambridge, UK, and deposited by E. A. George in 1949) and Spirogyra pratensis (MZCH10213; originally deposited by M. A. Allen in 1958) were provided by the Microalgae and Zygnematophyceae Collection, Hamburg, Germany (Schwartzenberg et al., 2013). We used 7-day-old cultures of Mougeotia sp. and 12-day-old Spirogyra pratensis for every experiment. The algae were grown on modified freshwater F/2 (Gul-lard, 1975) with 1% agar at 22°C. An LED light source provided photosynthetically active radiance at 120 μmol quanta m⁻² sec⁻¹ under a 12:12 h light/dark photocycle. Heat stress treatments (in a 37°C culture room) were performed at the same light regime and photoperiod. All experiments were carried out 6 h after the light was turned on; for the heat-treated algae this means that, after 6 days (in the case of Mougeotia sp.) and 11 days (Spirogyra pratensis) at 22°C, the algae experienced 6 h light, 12 h darkness and 6 h light at 37°C.

Microscopy

Light microscopy was performed using Nomarski interference contrast on an Axioplan II system (Carl Zeiss, Oberkochen, Germany) with an Axiocam HRC colour camera (Zeiss). Specimens were mounted in liquid freshwater F/2. For each individual, fully imaged cell (i.e. where a whole cell was in the field of view), measurements of cell as well as plastid lengths and widths were taken using Fiji (Schindelin et al., 2012). R studio (RStudio Team, 2016) was used to plot the data and perform the statistics. A Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to determine normality of the data. If normally distributed, equal variance was tested and a two-sample t-test or Welch’s two sample t-test (Gosset, 1908) was performed accordingly. If not normally distributed, a Mann-Whitney U-test was performed (Mann and Whitney, 1947).

For confocal laser scanning microscopy, algae were incubated for 10 min in liquid freshwater F/2 containing 1% calcfluor white (Fluorescent Brightener 28; Sigma-Aldrich, St Louis, MO, USA). Specimens were washed with and mounted in liquid freshwater F/2. Imaging of the samples was done on a LSM710 system (Carl Zeiss), ZEN software (Carl Zeiss) was used for the process of image capturing. Final images were generated using FIJ (Schindelin et al., 2012).

RNA extraction and sequencing

Algal material was scraped off the plates and immediately homogenized in a Tenbroek tissue homogenizer. RNA was extracted using the Trizol reagent (Thermo Fisher, Walth, MA, USA) in accordance with the manufacturer’s instructions. For Mougeotia sp., we isolated RNA from six control and three heat samples; for S. pratensis, we isolated RNA from three control samples and three heat samples. RNA samples were treated with DNase I (Thermo Fisher); we assessed RNA quality using a formamide agarose gel and quantity using a Nanodrop spectrometer (Thermo Fisher). RNA of adequate quality was shipped on dry ice to Genome Québec (Montreal, Canada). After an additional round of quality assessment using a Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA), libraries were constructed using the NEB mRNA stranded Library preparation kit (New England Biolabs, Beverly, MA, USA) and sequenced on the NovaSeq 6000 platform (Illumina). In total, we obtained 459 187 218 reads (92.76 Gbp).

RNA-seq analysis

All raw RNA-seq read data were quality assessed using FASTQC, version 0.11.7 (FASTQC, 2018), and trimmed using TRIMMOMATIC, version 0.36 (Bolger et al., 2014), with the settings: ILLUMINACLIP:TruSeq3-PE- 2:fa:2:30:10:2:TRUE HEADCROP:10 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36. All trimmed data were uploaded to the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) (accession numbers SRR9083881 to SRR9083701). Trimmed data were quality assessed again (Bolger et al., 2014). For each species, we pooled all the trimmed read data (to obtain the best possible assembly for Mougeotia sp., we added additional reads from the same isolate that are however not part of this study and the downstream differential analyses) and computed a de novo assembly using the TRINITY pipeline (Haas et al., 2013), which included various isoforms and transcripts with low abundance. We, hence, next performed a series of steps to refine and filter the data.

Using RSEM (RNA-Seq by Expectation Maximization) (Li and Dewey, 2011) we mapped the trimmed reads onto the contigs and calculated expression values as TPM that we normalized using the trimmed mean of M values (TMM) procedure (Robinson and Osh-luck, 2010). Making use of our biological replicates, we calculated differential gene expression changes comparing heat versus control using DEGGER (Robinson et al., 2010). The TRINITY pipeline clusters de novo assembled transcripts into genes and isoforms; here, we worked only with the ‘genes’ datasets and filtered out lowly expressed genes by retaining only those that had an average TPM over all the libraries that were higher than the number of libraries sequenced. The highest expressed isoform was chosen as the repre-sentative for each gene. Querying all the representative sequences against the NCBI nr database (https://www.ncbi.nlm.nih.gov/protein) via DIAMOND (Buchfink et al., 2015), we assigned a taxonomic affiliation to our transcripts and retained only those sequences with the best hit against Chloroplastida. We thus obtained 4955 contigs for Mougeotia sp. and 3950 contigs for

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Spirogyra pratensis (all sequence data have been deposited under the NCBI BioProject PR-JNA543475). Next, we in silico translated our transcripts into proteins using the EMBOSS getorf tool (Rice et al., 2000) and a custom perl script that evaluated all possible protein sequences and selected the best option; this process was based on the BLASTX alignment obtained when querying the transcripts against a database of well-curated Chloroplastida genomes. Any work with the sequence data was based on the protein sequences.

**Metabolite extraction and metabolomics using liquid chromatography-tandem mass spectrometry**

Algal material was scraped off the agar plates and immediately frozen in liquid nitrogen. The fresh weight of the frozen tissue was determined. For extraction, we transferred the frozen tissue into a 4°C cold MeOH:ddH₂O mixture (7:3 v/v) and homogenized, on ice, in a Tenbroek tissue homogenizer. Debris was removed from the homogenate by (cooled) centrifugation. The supernatant was dried using a vacuum centrifuge (SPD111V SpeedVac; Thermo Electron Corporation, Waltham, MA, USA) and resuspended in 1 ml of buffer containing 95% acetonitrile, 5% 20 m mol ammonium carbonate (pH 9.8) and 1.25 µM metabolomics amino acid mix standard (PN MSK-A2-1.2; Cambridge Isotope Laboratories, Tewksbury, MA, USA), the latter used as internal standard. Then, 100 µl of each sample were pooled together for quality controls (QCs). All samples including QCs, where then analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using selected reaction monitoring (SRM) (also known as multiple reaction monitoring) acquisitions on an Agilent 1100 liquid chromatograph coupled in-line to a QTRAP 5500 (Sciex, Framingham, MA, USA) triple-quadrupole linear ion trap tandem mass spectrometer. Chromatographic separation was achieved by injecting 50 µl of sample on an XBridge Amide 3.5 µm particle, 1.0 × 50 mm column (PN 186004971; Waters, Milford, MA, USA) heated to 37°C across an 8 min gradient starting at 5% 20 µmol ammonium carbonate (pH 9.8) and 95% acetonitrile, ending at 98% 20 µmol ammonium carbonate pH 9.8 and 2% acetonitrile (for details on the gradient, see Data S4). The LC flow rate was set at 200 µl min⁻¹. A 475°C heated-assisted electrospray source (TurbolonSpray; Sciex) was used for ionization; curtain gas was kept at 20 units and gas 1 and 2 at 33 units (arbitrary units in MS peak integration was performed using SKYLINE VERSION 19.1 (MacLean et al., 2010; Pino et al., 2017).

**Phylogenetic analysis**

Sequences for phylogenetic analyses were retrieved from the protein data of the in silico translated transcripts of Mougeotia sp. and S. pratensis, published transcriptomes of streptophyte algae (Ju et al., 2015; de Vries et al., 2018), and the genomes of K. nitens (Hori et al., 2014), C. braunii (Nishiyama et al., 2018), M. polymorpha (Bowman et al., 2017), P. patens (Lang et al., 2018), S. moellendorfii (Banks et al., 2011), Picea abies (Nystedt et al., 2013) and A. thaliana TAIR10 (Berardini et al., 2015). Sequences were aligned using MAFFT (Katoh et al., 2002; Katoh and Standley, 2013) and maximum likelihood phylogenies were computed using IQ-TREE (Nguyen et al., 2015).

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of cytokinin treated S. pratensis

One-week-old S. pratensis cultures were harvested and washed with fresh medium. Approximately 0.2 g fresh weight of algal filaments was suspended in 20 ml of medium and treated with different cytokinins for the indicated times. The total RNA was isolated as described in Onate-Sanchez and Vicente-Carbajosa (2008) and the concentration and purity of the RNA were measured spectrophotometrically. RNA samples were treated with RNase free DNase I (Thermo Scientific) and cDNA synthesis was performed using the RevertAid Reverse Transcriptase (Thermo Scientific). Real-time PCR was performed using a StepOnePlus™ Real-Time PCR System (#4376800; Applied Biosystems, Foster City, CA, USA). SYBR Fast qPCR master mix (#KM4102; KAPA Biosystems, Wilmington, MA, USA) was used in accordance with the manufacturer’s instructions. A test for contamination with genomic DNA was carried out by PCR using the SpUBI-F/R primers on control samples prepared without reverse transcriptase. The temperature profile was set as: 3 min of initial denaturation at 95°C; then 40 cycles of 30 sec at 95°C, 15 sec at 58°C and 30 sec at 72°C. Data were acquired at 72°C. A melt curve was run at the end of the 40 cycles to test for a homogenous PCR product. Amplification efficiency was determined using a standard curve and expressions of target genes were calculated by the 2⁻ΔΔCt method (Livak andSchmittgen, 2001) relative to expression of Ubiquitin gene as reference. The primers sequences were: SpUBI-F 5’-GAGTCCACCCTTCCACCTTGTT-3’; SpUBI-R 5’-CTTGCGCCCTAATTGGTTG-3’; SpCHK1-F 5’-CTCGAGAGAGAAGAGGC-3’; SpCHK1-R 5’-CGGACTTCCAAGCGAAGATG-3’; SpCHK2-F 5’-TGACCCACCGATCTTACCTT-3’; SpCHK2-R 5’-AAAGCGGGAGAGAAAGGCATT-3’; SpCHK3-F 5’-GTGCAAACTCTGTTGTC-3’; SpCHK3-R 5’-TTTCCTCCGTTGCAACTCC-3’; SpRRA-F 5’-TGCGCCGTGCTTCAAACAG-3’; SpRRA-R 5’-TTAGCATTGTCATCTTACGGA-3’; SpRRA-R 5’-AGCTCAGGTCTGATGG-3’.

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**CONFLICT OF INTERESTS**

The authors declare that they have no competing interests.

**AUTHOR CONTRIBUTIONS**

JdV designed the study. JdV, SDv and HZ performed experiments. Algae were provided by KvS, JdV, SDv and...
AMC carried out the metabolite extraction. AMC and SP generated and processed the mass spectrometry data. AMC, SP, KF and DMP analyzed the metabolome. JdV, SdV and BAC performed the bioinformatic analyses. JdV and SdV performed statistical analysis. JdV and JMA acquired funding for the study. JMA, AMC and MS provided research infrastructure. JdV wrote the initial draft with JMA and SdV. All authors contributed to writing the final manuscript. All authors discussed the results.

**DATA AVAILABILITY STATEMENT**

All sequence data have been deposited under the NCBI BioProject PRJNA543475. The Transcriptome Shotgun Assembly project for *Spirogyra pratensis* MZCH-SVCK 10213 has been deposited at DDBJ/EMBL/GenBank under the accession GICF00000000. The version described in this paper is the first version, GICF01000000. The Transcriptome Shotgun Assembly project for *Mougeotia* sp. MZCH-SVCK 240 has been deposited at DDBJ/EMBL/GenBank under the accession GHUK00000000. The version described in this paper is the first version, GHUK01000000. The corresponding reads are deposited in the NCBI Sequence Read Archive (SRA) under the accessions SRX585892 to SRX5858912. Additional light micrographs regarding the quantification of the stress phenotypes are deposited at Zenodo under http://doi.org/10.5281/zenodo.3769748.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Phylogeny of CAD-related sequences. Sequences were identified based on a BLASTP/BLASTN search with CAD sequences from *Arabidopsis thaliana* as query against a sequence database of genomic and transcriptomic data from bryophytes and streptophyte algae. Additional sequences that were identified in de Vries et al. (2017) were also included. Transcriptomic data from this study and de Vries et al. (2018) were filtered and only sequences with a TPM ≥ 1 in at least one condition were included. The phylogeny is based on full-length sequences, which were aligned using MAFFT (option G-INS-I). Annotation for CAD and CAD-like sequences is based on de Vries et al., 2017 and annotation of the other sequences is based on a BLASTP search using the sequences as query and the genome of the *Arabidopsis thaliana* TAIR10 release as the database. The e-value cut-off for all BLASTP searches was 10. SOR-like sequences were used as an outgroup.

**Figure S2.** A bar diagram showing the differential gene expression (as log2[fold change] relative to control) of the 207 concordantly down-regulated, shared orthologs depicted in Figure 4B. The diagram is sorted by the between species average log2(fc) values for each given ortholog.

**Figure S3.** Up-regulated homologs of *Arabidopsis thaliana* proteins that are associated with cytoskeletal reorganization detected in the Zygnematophyceae *Mougeotia* sp. and *Spirogyra pratensis*. Protein domains found in the (in silico translated) protein candidates were predicted via InterProScan. On the left, gradient-colored circles indicate the associated gene expression data (log2[fold change heat/control], calculated using edgeR) from green (up-regulation) to blue (unchanged).

**Figure S4.** Unrooted phylogeny of histidine kinases. A phylogeny was computed based on a maximum likelihood (ML) WAG+F + I+G4 method using IQ-tree (bootstrap values < 50 are not shown) AHK homologs of (a) the land plants *Arabidopsis thaliana* and *Physcomitrella patens* and (b) the Zygnematophyceae *Mougeotia* sp. and *Spirogyra pratensis*.

**Figure S5.** (a) Relative expression levels of SpCHK1, SpCHK2, SpCHK3, SpARR and SpRBR affected by cytokinin (400 nm IP for 4 h). Three biological replicates were measured with two technical replicates. A ubiquitin gene in *S. pratensis* served as the endogenous control, and gene expression was normalized to levels of untreated samples (0 m). (b) Relative expression levels of SpCHK1, SpCHK2 and SpRRA affected by different types of cytokinin (1 μm IP, 12 and 24 h).

**Data S1.** Sequencing statistics.

**Data S2.** For the two species *Mougeotia* sp. (*Mousp*) and *Spirogyra pratensis* (*Spipra*) all transcripts for ‘genes’ (as defined by TRINITY) that were retained after filtering are shown. The columns under ‘TPM’ show the expression levels for all biological replicates (control and heat) in trimmed mean of M values (TMM)-normalized TPM values. Based on in silico-translation of the representative transcript, a BLASTp was performed against protein data from the genomes of *Arabidopsis thaliana* (TAIR10; At), *Physcomitrella patens* (V3; Pp), *Chara braunii* (Cb) and *Klebsormidium nitens* (Kn). The column to the right of the respective best BLASTp hits indicates whether this hit was recovered reciprocally. The last two columns show the results of the differential gene expression analysis (heat versus control) in log2(fold change) and the Benjamini–Hochberg-corrected P values.

**Data S3.** Relative metabolite abundance normalized on fresh-weight of the samples. On the top, the MiZ transition (precursor → product; black) and the tentative metabolites (dark grey) are given. On the left, the biological replicates, the species, and the condition under which the sample was taken (control or after 24 h of heat stress) are labeled. All data are based on technical duplicates.

**Data S4.** Top: gradient of solvent system A, 5% acetonitrile. Of 20 μM ammonium carbonate pH 9.8, and solvent system B, 100% acetonitrile. Bottom: pump details.

**Data S5.** The differential gene expression data plotted in Figure 6 for the two Zygnematophyceae, *Mougeotia* sp. (*Mousp*) and *Spirogyra pratensis* (*Spipra*). For each transcript, the best BLASTp hit against protein data from the genomes of *Arabidopsis thaliana* (TAIR10; At) is shown. The last two columns show the results of the differential gene expression analyses (heat versus control) in log2(fold change) and the Benjamini–Hochberg-corrected P values.

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