Towards establishment of model organisms for conjugating green algae – Zygnematophyceae

and studies on the evolution of the regulatory network of the plant hormone cytokinin

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Abstract

The cytokinins (CKs) are important signaling molecules regulating plant growth and development as well as responses to environmental stresses. Their actions in land plants are executed *via* a complex regulatory network of CK biosynthesis, metabolism, transport, signal perception, and transduction. In land plants this regulatory network has been well studied for the model organisms *Arabidopsis thaliana* and the bryophyte *Physcomitrella patens*. However, knowledge about the origin and evolutionary trajectory of the CK regulatory system is rather limited. In this thesis, the occurrence of CKs and their regulatory network in charophyte green algae (CGA) have been investigated.

The CK profiles of five selected CGA species (*Cosmarium crenatum* var. *boldtianum*, *Micrasterias radians* var. *evoluta*, *Spirogyra pratensis*, *Klebsormidium nitens*, and *Mesostigma viride*) showed a predominance of isopentenyladenine (iP)- and *cis*-zeatin (cZ)- type CKs in free base, riboside, and phosphate forms, whereas glucosides did not contribute to the algal CK pools. Species from CGA were found to release endogenously produced CKs into the culture medium over the growth period. Correspondingly, BLAST searches of algal and plant genomic and transcriptomic databases indicated the presence of CK biosynthesis and transport across the green lineage, while the CK oxidase/dehydrogenase (CKX) mediated CK breakdown is restricted to land plants. I also found that a complete set of components involved in CK signaling can only be found in CGA and land plants. The CHASE-containing histidine kinases (CHKs) and type-A response regulators seem to first appear in CGA. A domain-based phylogenetic analysis revealed that the CGA genomes encode CHKs related to both, canonical and noncanonical CK receptors. Functional analysis was carried out for two CHKs from *Spirogyra pratensis* (Transeau) (Zygnematales) (SpCHKs). The SpCHK2, which is closely related to canonical CK receptors, showed weak affinity to iP in a bacterial CK binding assay. However, both SpCHKs were not able to activate a CK response when expressed in a CHK receptor-deficient mutant of the moss *Physcomitrella patens*. Our study provided hints that the two-component CK signaling pathway has first been acquired in CGA but it is likely to function in a manner independent or less dependent on the CK ligand when compared to land plants.

The importance of Zygnematophyceae, the direct sister group of land plants, is discussed with respect to evolutionary and physiological studies, where the urgent need for the establishment of model organisms within this class becomes evident. The current development of model organisms in Zygnematophyceae is reviewed. By own work, two candidates (*Micrasterias radian*s var. *evoluta* (W.B.Turner) and *Spirogyra pratensis*) were selected from the Microalgae and Zygnematophyceae Collection Hamburg (MZCH). Both organisms are able to perform their complete life cycle *in vitro*. Efficient transient transgene expression in *M. radian*s var. *evoluta via* particle bombardment and polyethylene glycol (PEG)-mediated protoplast transformation is presented. Via protoplast transformation the influence of various promoters on transformation efficiency was compared. Further establishment of transgenesis protocols and genetic resources together with the *in vitro* control of the life cycle will help to elucidate the processes which enabled the ancestors of extant Zygnematophyceae to adapt to terrestrial environments.

Zusammenfassung

Cytokinine (CK) sind wichtige endogene Signalmoleküle, die das Wachstum und die Entwicklung von Pflanzen regulieren und ebenfalls Bedeutung für die Anpassung an Umweltbedingungen besitzen. Die Wirkung von CK wird bei Landpflanzen durch ein komplexes regulatorisches Netzwerk bestehend aus CK-Biosynthese, Metabolismus, Transport, Signalperzeption und Signalweiterleitung realisiert. Dieses regulatorische System wurde ausgiebig bei den Modellorganismen für Landpflanzen, (v.a. *Arabidopsis thaliana* sowie ebenfalls für das Laubmoos *Physcomitrella patens*) untersucht.

Das drzeitige Verständnis des evolutionären Ursprungs sowie der evolutionären Entwicklung des CK-Systems ist lediglich fragmentarisch. Im Rahmen dieser Arbeit wurde das Vorkommen von CK Metaboliten sowie das regulatorische Netzwerk der CK bei charophytischen Grünalgen (CGA) untersucht.

Das CK Profil der ausgewählten CGA Species (*Cosmarium crenatum* var. *boldtianum*, *Micrasterias radians* var. *evoluta*, *Spirogyra pratensis*, *Klebsormidium nitens*, and *Mesostigma viride*) zeigten eine Dominanz von Metaboliten aus den Isopentenyladenin (iP)- und *cis*-Zeatin (cZ) Familien, die als freie Basen, Riboside und Nukleotide vorlagen. CK-Glykoside wurden nicht bzw. nur im Spurenbereich detektiert. Im Kulturverlauf wurde festgestellt, dass die untersuchten Vertreter der CGAs endogen produzierte CK ins Kulturmedium abgeben. BLAST Analysen in Transkriptomen und Genomen von Algen und Landpflanzen zeigten die durchgehende Anwesenheit von Genen für Biosynthese und Transport in der gesamten grünen Linie der Pflanzen. Hingegen waren Gene, die für den metabolischen Weg der Cytokinindeaktivierung mittels Cytokininoxidase/dehydrogenase kodieren, ausschließlich bei Landpflanzen nachweisbar. Die bioinformatischen Analysen ergaben weiterhin, dass die CGA Vertreter, vergleichbar mit den Landpflanzen, komplette Sets von Genen für CK-Signalperzeption und Weiterleitung besitzen.

CHASE-Domäne enthaltende Histidinkinasen (CHKs) sowie Typ-A Response Regulatoren scheinen erstmals bei CGAs vorzukommen. Eine Domänen-basierte phylogenetische Analyse ergab, dass die CGA-Genome sowohl Vertreter von kanonischen als auch von nicht-kanonischen CHKs als potentielle CK Rezeptoren enthalten. Für zwei CHKs der Schraubenalge *Spirogyra pratensis* (Transeau) (Zygnematales) (SpCHK1 und -2) wurden funktionelle Untersuchungen durchgeführt. SpCHK2, welches Ähnlichkeiten zu den kanonischen CHKs aufweist, zeigte eine leichte Affinität gegenüber iP in einem bakteriellen CK-Bindunsgstest. Beide SpCHKs konnten in Komplementationsversuchen mittels Überexpression in Rezeptormutanten des Laubmooses *Physcomitrella patens* keine Reversion des Phänotyps auslösen. Zusammenfassend ergaben die Untersuchungen, dass das Zweikomponenten-CK-Signaltransduktionssystem erstmals in der CGA Linie etabliert wurde. Es ist jedoch sehr

wahrscheinlich, dass seine Funktionalität im Gegensatz zu Landpflanzen nicht oder nur zu einem geringen Teil an die Bindung von CK Liganden gekoppelt ist.

Die Bedeutung von Zygnematophyceen (Jochalgen) als direkte Schwestergruppe der Landpflanzen wurde hinsichtlich evolutionärer und physiologischer Studien erörtert, wobei die Notwendigkeit der Etablierung von CGA Modellorganismen deutlich wird. Der derzeitige Forschungsstand zum Thema Modellorganismen für Zygnematophyceae wird zusammenfassend dargestellt. Im Rahmen dieser Arbeit wurden zwei Organismen (*Micrasterias radians* var. *evoluta* (W.B.Turner) and *Spirogyra pratensis*) aus der Algensammlung MZCH (Microalgae and Zygnematophyceae Collection Hamburg) für Transgeneseexperimente ausgewählt. Für *M. radians* var. *evoluta* wird die effiziente transiente Transformation mittels via Gen-Kanone und Polyethylenglycol (PEG)-vermittelter Protoplastentransformation dargelegt. Mittels Protoplastentransfromation wurde der Einfluss verschiedener Promotoren auf die Transformationseffizienz untersucht. Die weitere Standardisierung von Kulturverfahren von Zygnematophyceen incl. des in-vitro Vollzugs des Lebenszyklus, die Etablierung von Transgenese-Protokollen und Sequenzresourcen werden es künftig ermöglichen, unser evolutionäres Verständnis zur Anpassung von Pflanzen an terrestrische Lebensbedingungen zu erweitern.

Abbreviations

ABCG14	ATP-binding cassette transporter
Ade	adenine
ADK	adenosine kinase
Ado	adenosine
ADP	adenosine 5'-dihosphate
AMP	adenosine 5'-monophosphate
APRT	adenine phosphoribosyltransferase
ATP	adenosine 5'-triphosphate
BA	N^6-benzyladenine
CGA	charophyte green algae
CHASE(-domain)	cyclase/histidine kinase associated sensory extracellular(-domain)
CHK	CHASE-domain containing histidine kinase
CK	cytokinin
CKX	CK oxidase/dehydrogenase
CRF	cytokinin response factors
CYP735A	cytochrome p450 monooxygenase
cZ	*cis*-zeatin
DHZ	dihydrozeatin
ENT	euilibrative nucleoside transporter
ER	endoplasmic reticulum
EST	expressed sequence tags
HGT	horizontal gene transfer
HPT	histidine phosphotransferase
iP	N^6-(2-isopentenyl) adenine
IPT	isopentenyltransferase
LCA	last common ancestor
LOG	CK riboside 5´-monophosphate phosphoribohydrolase
Mya	million years ago
NRH	nucleoside ribohydrolase
PM	plasma membrane
PNP	purine-nucleoside phosphorylase
PUP	purine permease
REC	response regulator receiver domain
RR	response regulator
TCS	two-component system
T-DNA	transfer deoxyribonucleic acid
tRNA	transfer-ribonucleic acid
tZ	*trans*-zeatin
UGT	UDP-glucosyltransferase
UPLC	ultra-performance liquid chromatography

1. Introduction

1.1 The occurrence of cytokinins in plants

Kinetin was the first synthetic cytokinin (CK) characterized from autoclaved herring sperm DNA and described as a cell division factor that promotes cell proliferation in cultured plant cells (Miller *et al.*, 1955). With the following identification of natural CKs from *Zea mays* (Letham, 1963; Miller, 1961), CKs have been linked to many processes related to plant growth and development, such as regulation of meristematic activity in shoot and root development (Kyozuka, 2007; Raines *et al.*, 2016), vascular development (Mähönen *et al.*, 2006), seed germination (Khan, 1971), leaf senescence (Riefler *et al.*, 2006) and nodule organogenesis (Tirichine *et al.*, 2007). For detailed information of the regulation mechanism, see the recent comprehensive review by Wybouw and De Rybel (2019). It has also been reported that CKs play an important role in biotic and abiotic stress responses including pathogen resistance and adaptive stress responses in sub-optimal environmental conditions (Choi *et al.*, 2011; Cortleven *et al.*, 2019; Tran *et al.*, 2007). Chemically, the naturally occurring CKs are adenine derivatives, of which the N^6 position carries certain substituents and the biological activities are mainly affected by the structure of the N^6 side chain (Auer, 1997; Schmitz *et al.*, 1972). Thus, CKs are classified into two types based on the side chain structures: aromatic/furfuryl and isoprenoid CKs (Fig. 1). The furfuryl CK kinetin is the best known synthetic CKs while the aromatic N^6-benzyladenine (BA) and its hydroxy derivatives *ortho*- and *meta*-topolin (*o*T and *m*T) have only been found in a few plant species from Populus and Corylus (Gentile *et al.*, 2017; Strnad, 1997; Strnad *et al.*, 1997). The isoprenoid type CKs, which are most prevalent in land plants, algae, and cyanobacteria (Yokoya *et al.*, 2010; Žižková *et al.*, 2017), mainly include the N^6-(2-isopentenyl) adenine (iP) and zeatin-type CKs possessing a hydroxylated isopentenyl side chain. The zeatin-type CKs comprise the *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), and dihydrozeatin (DHZ), which vary in stereoisomeric configuration or saturation of the hydroxylated side chain, and their abundance is reported to vary between organisms (Gajdošová *et al.*, 2011). The *c*Z-type CKs are usually much less active than the *trans* forms in plant bioassays (Schäfer *et al.*, 2015). The modification at the purine ring or the side-chain hydroxyl group leads to the generation of different CK forms (Mok, 2001). Conjugation at the adenine moiety at the N^7- or N^9-position with different sugar

moieties results in a variety of CK conjugates including nucleotides, nucleosides, N^7- and N^9-glucosides etc. While the glucosylation at the side-chain hydroxyl group forms the CK-*O*-glucosides. The hydroxyl group of nucleosides or nucleotides of zeatin can also be O-glucosylated. Normally, the CK free bases and corresponding nucleosides are the physiologically active forms in flowering plants whereas the CK-*O*- and CK-*N*-glucosides seem to be non-active or weakly active forms (Romanov *et al.*, 2005).

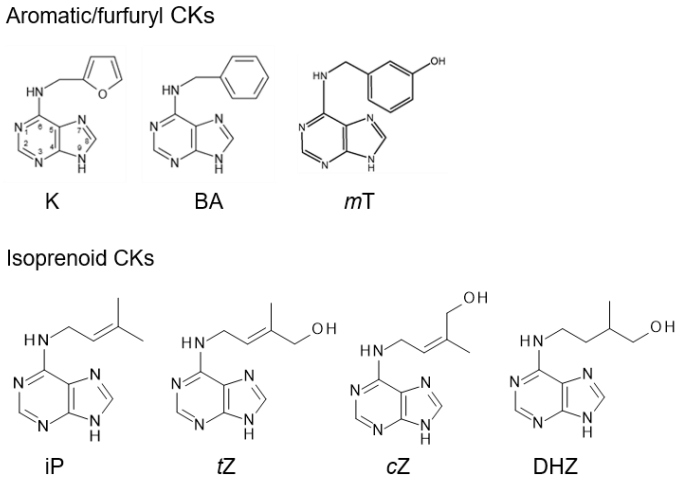

Fig. 1: Chemical structures of some aromatic/furfuryl and isoprenoid CKs. Abbreviations: K, kinetin (N^6-furfurylaminopurine); mT, meta-Topolin; BA, N^6-benzyladenine; iP, N^6-(2-isopentenyl) adenine; *t*Z, *trans*-Zeatin; *c*Z, *cis*-Zeatin; DHZ, Dihydrozeatin. The figure is modified from Schmülling (2004).

To date, great improvements have been made for CK analytical technology to identify and quantify the endogenous CKs, which are usually present in very low concentrations (pmol/g dry weight, DW) in plant tissues. Knowing the naturally occurring CK profiles is of great help for understanding the metabolism, transport, and function of different types of CKs *in planta* (Auer, 1997). A commonly used protocol for detecting the endogenous CKs is a combination of the following techniques: the ultra-performance liquid chromatography (UPLC) is used for separation of CKs from samples extracted with immunoextraction (IAE) and micropurification procedure, and a tandem quadrupole mass spectrometer (MS/MS) equipped with an electrospray interface (ESI) for the identification and quantification of CKs (Novák *et al*., 2008). These techniques will also be applied for the CK profiling of different charophyte algae species in this thesis.

The CK distribution patterns have been identified for a variety of plant species as well as for many algae and cyanobacteria strains. It has been shown that the endogenous CK

pools vary greatly in quantities and CK forms among different species (Stirk *et al.*, 2013; Yokoya *et al.*, 2010; Žižková *et al.*, 2017). Even within the same species, the composition of the endogenous CK pools differs between tissues and developmental stages and is affected by multiple factors such as culture conditions, exogenous supplied CKs, and pathogen infection (Sakakibara, 2006). For example, the N^7-glucoside forms of the iP and *t*Z are the most abundant CKs in *Arabidopsis thaliana*. The root produces a similar amount of iP- and *t*Z- types while the shoot contains ca. 2-fold higher amounts of *t*Z-type than iP-type CKs (Zhang *et al.*, 2014). Analyses of the endogenous CKs in the buds of *Pinus radiata* revealed that juvenile buds have a relatively high level of iP base and corresponding nucleoside, while the adult buds are rich in contents of nucleotides and glucosides (Zhang *et al.*, 2003). The seedlings of maize turn to produce more bioactive forms of CKs during acclimation to salt and osmotic stresses (Vyroubalová *et al.*, 2009). The infection of *Phaseolus vulgaris* with white clover mosaic potexvirus leads to a decrease of the CK free bases, accompanied by an increase in the N^9-glucosides and nucleotides (Clarke *et al.*, 1999). Therefore, it might be difficult to compare the compositions of the CK pools of different species, which are determined by different approaches and analytical techniques. However, CK profiles established in the same laboratory by the same techniques help to get comparative insights into the occurrence of endogenous CKs in different organisms and can show clues regarding homeostasis and metabolism of CKs from an evolutionary sight.

1.2 Cytokinin metabolism and transport

CK activity and homeostasis in land plants are regulated on various levels including biosynthesis, interconversion between different forms, and inactivation by degradation or conjugation reactions, as well as local and long-distance transport (Kakimoto, 2001; Sakakibara, 2006). The levels of homeostatic regulation are described in this chapter and summarized in Fig. 2. The rate-limiting step in the biosynthesis of the isoprenoid CKs is catalyzed by isopentenyltransferases (IPTs) transferring the isopentenyl group from dimethylallyl diphosphate (DMAPP) to the N^6-position of the adenine moiety. IPTs are classified into two types namely the adenylate IPTs and the tRNA-IPTs (Lindner *et al.*, 2014; Miyawaki *et al.*, 2006). The adenylate-IPTs use adenosine 5'-mono-, di-, or tri-phosphate (AMP, ADP, or ATP) as substrates for the biosynthesis of iP- and *t*Z-type CKs. The first characterized adenylate IPT belongs to the pathogen adenylate IPTs which is

encoded by the T-DNA of *Agrobacterium tumefaciens* and preferentially utilizes AMP as substrate (Akiyoshi *et al.*, 1984; Lindner *et al.*, 2014). The plant adenylate-IPTs (DMAPP: ATP/ADP-IPTs) are mainly encoded by the genomes of vascular plants (Kakimoto, 2001; Li *et al.*, 2019; Miyawaki *et al.*, 2006). The tRNA-IPTs widely exist in all organisms except Archeae. These enzymes catalyze the isopentenylation of an adenine at the position A^{37} called isopentenyladenosine ($i6A^{37}$) of tRNA recognizing UNN-codons to enhance the precision of the codon-anticodon binding (Golovko *et al.*, 2002; Yevdakova and von Schwartzenberg, 2007). The decay of such prenylated tRNA leads to the release of the nucleotide form of *c*Z. There are two types of tRNA-IPTs, among which the class I tRNA-IPTs are closely related to bacterial tRNA-IPTs and the class II tRNA-IPTs to adenylate-IPTs (Lindner *et al.*, 2014; Nishii *et al.*, 2018).

Biologically active CK bases are generated from the corresponding nucleotides either through a one-step or a two-step CK activation pathway (Kamada-Nobusada and Sakakibara, 2009). In the one-step activation pathway, the CK nucleotides are directly converted to their corresponding free bases by 5'-monophosphate phosphoribohydrolases (lonely guy, LOGs), which belong to a multigene family with redundant functions in plants and act specifically on CK nucleotides (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009; Tokunaga *et al.*, 2012). The constitutively expressed adenine phosphoribosyltransferases (APRTs) mainly catalyze the one-step salvage of adenine to AMP and utilize CK free bases as substrates as well (Allen *et al.*, 2002; Craig and Eakin, 2000). It has been confirmed *in planta* that the APRT is involved in CK metabolism and functions opposite to LOG (Zhang *et al.*, 2013). In the two-step activation pathway, the monophosphate nucleotides are firstly dephosphorylated to the nucleosides by phosphatases (5'-nucleotidases) (Chen and Kristopeit, 1981a, b) and subsequently converted to free bases by nucleoside ribohydrolases (NRHs) which accept purine ribosides such as inosine and xanthosine as well as the pyrimidine, uridine, and CK ribosides as substrates (Kopečná *et al.*, 2013). The opposite reactions of the two-step activation pathway are the ribosylation of the purines as well as of the CK free bases by the purine nucleoside phosphorylases (PNPs) (Bromley *et al.*, 2014; Chen and Petschow, 1978) followed by the phosphorylation of the nucleosides catalyzed by the adenosine kinases (ADK) (Schoor *et al.*, 2011; von Schwartzenberg *et al.*, 1998). The enzymes catalyzing interconversions between corresponding nucleotides, nucleosides, and free bases of different types of CKs, except for LOGs, are not CK specific but accept also members of the purine metabolic pathway (Chen, 1997). In flowering plants, the majority of *t*Z comes from the trans-hydroxylation of

the prenyl side chain of iP ribonucleotides by a cytochrome P450 monooxygenase 735A (CYP735A), which shows a substrate preference in the following order: iPRMP>iPRDP>iPRTP (Takei *et al.*, 2004). The conversion of *t*Z to its *cis*-counterpart was only documented through *in vitro* experiment whereas the presence of *cis-trans* isomerase in plants has never been confirmed (Bassil *et al.*, 1993; Hošek *et al.*, 2020). The same situation happens to the irreversible conversion of *t*Z to DHZ, for which the zeatin reductase activity was detected in protein extracts from bean embryos (Martin *et al.*, 1989), but with no *in planta* activity and no corresponding coding genes have been identified (Hluska *et al.*, 2016).

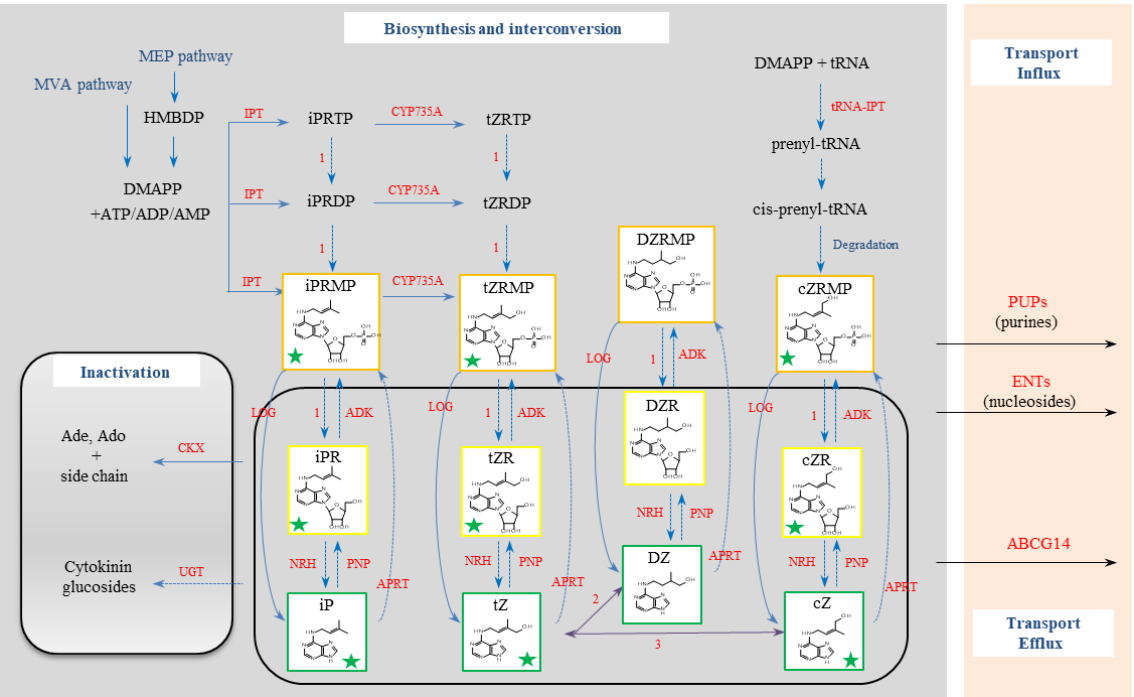

Fig. 2: Integrated model of the CK metabolism and transport in plants. The figure is modified from Hwang and Sakakibara (2006); Sakakibara (2006); Werner and Schmülling (2009); Kieber and Schaller (2018). Metabolites are marked in black while the enzymes are in red. Metabolites in dark orange, yellow and green boxes are CK nucleotides, nucleosides, and free bases. The black box outlines the active forms of CKs. Substrates involved in the inactivation pathway are indicated with green stars. Abbreviations: IPT, isopentenyltransferase; CYP735A, Cytochrome p450 monooxygenase; 1, 5'-nucleotidase (alkaline phosphatases and acid phosphatase); ADK, adenosine kinase; NRH, nucleoside ribohydrolase; PNP, purine-nucleoside phosphorylase; LOG, CK riboside 5´-monophosphate phosphoribohydrolase, APRT, adenine phosphoribosyltransferase; CKX, CK oxidase/dehydrogenase; UGT, UDP-glucosyltransferase; 2, zeatin reductase; 3, zeatin isomerase; Ade, adenine; Ado, adenosine; PUPs, purine permeases; ENTs, euilibrative nucleoside

transporters; ABCG14, ATP-binding cassette transporter. Blue flows indicated by dotted lines are not specific in CK metabolism; Purple arrows indicate enzymatic conversions confirmed only by *in vitro* assays; Black arrows indicate the transportation pathways.

Inactivation of the bioactive CKs is achieved *via* two pathways: the irreversible degradation of CKs and the conjugation to a sugar moiety, most commonly glucose. The CK degradation is catalyzed by CK oxidase/dehydrogenases (CKXs), which catalyze the cleavage of the N^6-isopenoid side chains from a series of CKs (Avalbaev *et al.*, 2012; Galuszka *et al.*, 2001). CKs with an unsaturated side chain (iP, *t*Z, *c*Z) can be cleaved while DHZ and BA are resistant to CKX mediated breakdown. CKXs function with substrate preference for CK free bases and their ribosides. Compounds with substitutions of additional groups on the purine ring are not substrates for the CKXs (Werner *et al.*, 2006). CKs can also be converted to inactive forms by the irreversible *N*-glucosylation of the adenine moiety at N^7- or N^9- position (Hou *et al.*, 2004) or to storage forms by the *O*-glycosylation at the side chains of zeatin types, which can be reversed by *β*-glucosidases (Mok *et al.*, 2000; Veach *et al.*, 2003). Five members of glucosyltransferases are found in *Arabidopsis*: two of them (UGT76C1 and UGT76C2) are *N*-glucosyltransferases while the left three catalyze *O*-glucosylation (Jin *et al.*, 2013; Wang *et al.*, 2013; Wang *et al.*, 2011).

As signaling molecules, CKs are synthesized tissue- and cell-specifically and cause responses locally as well as in long-distance tissues. Potential efflux and influx transporters for nucleosides and nucleotides (and other phytohormones) are also involved in the translocation of CKs (Hirose *et al.*, 2008). The efflux of CKs is carried out through two transporter families including the purine permease (PUP) family and the equilibrative nucleoside transporter (ENT) family (Girke *et al.*, 2014). The PUPs mediate the uptake of purines such as adenine, as well as the transport of CK bases with relatively lower affinity (Bürkle *et al.*, 2003). The PUP14 protein is localized to the plasma membrane and functions in the uptake of bioactive CKs (Zürcher *et al.*, 2016). The ENTs can transport adenosine and riboside forms of iP- and *t*Z-type CKs (Sun *et al.*, 2005; Wormit *et al.*, 2004). CKs efflux across the plasma membrane is carried out with ATP-binding cassette (ABC) transporters. Transporter ABCG14 is confirmed to be essential for the root-to-shoot translocation of *t*Z-type CKs (Ko *et al.*, 2014; Zhang *et al.*, 2014). More detailed information is reviewed by Kang *et al.* (2017) and Liu *et al.* (2019).

1.3 Cytokinin signaling pathway

Generally, the response pathways of plant cells to signal molecules include a series of biochemical reactions (signal initiation and transduction) and finally result in the stimulation or repression of the transcription of target genes. The CK molecules are perceived and the signal is transduced by multistep phosphorelay system (also called two-component systems, TCS). The basic TCS systems are prevalent signaling pathways in bacteria for sensing and responding to a variety of environmental stimuli (Chang and Stewart, 1998; Krell *et al.*, 2010). The bacterial TCS comprises two major components including a membrane-localized sensor histidine kinase (HK) for input signal perception and a response regulator (RR) for signal propagation. This signal transduction relies on the ATP-dependent autophosphorylation at a conserved histidine (His) residue of the HK protein and the phosphotransfer to a conserved aspartate (Asp) residue in the receiver domain of the RR protein, which results in activation of the RR and generation of the output responses (Casino *et al.*, 2010). Extended versions of the basic TCS systems are utilized by some prokaryotes and are prevalent in eukaryotes for signal perception and transduction. Plant TCS involves hybrid HKs (HK protein fused with an RR domain) and multiple phosphotransfer steps among more than two proteins (Lohrmann and Harter, 2002; Schaller *et al.*, 2008). The CK signaling in plants is mediated by such an extended multistep TCS system that includes four sequential phosphorylation reactions alternating between His and Asp residues (Fig. 3). The multistep phosphorelay involves three proteins: hybrid HK receptors for CK perception and signal initiation, histidine phosphotransferases (HPTs) for signal transduction and separate RRs that contain a conserved Asp residue in the RR domain and a DNA-binding domain mediating the signal output (Argueso *et al.*, 2010; Heyl *et al.*, 2013; Hwang *et al.*, 2002; Hwang *et al.*, 2012; Kieber and Schaller, 2018).

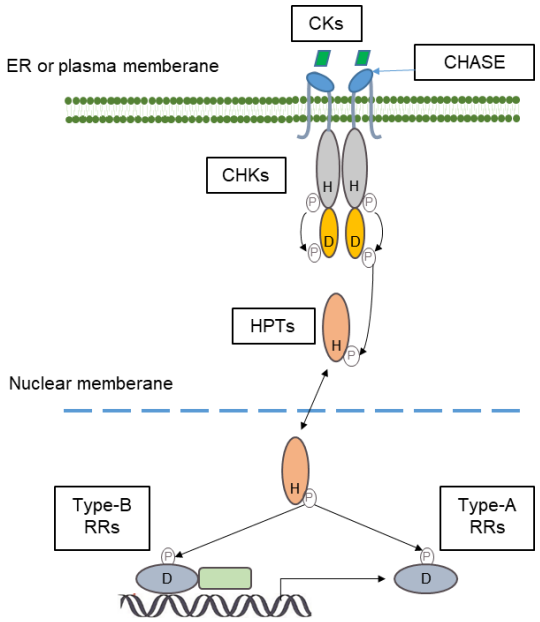

Fig. 3: Diagram of the basic CK signaling pathway in flowering plants. H, histidine; D, aspartate; P, phosphoryl group. The figure is modified from Kieber and Schaller (2018) and Hwang *et al.* (2012). This multistep phosphorelay makes use of a 'hybrid' HK receptor that contains both histidine kinase and receiver domains, a histidine phosphotransferases (HPT) and separate response regulators (RRs) with type-B RRs serving to regulate the transcriptional output from the phosphorelay, and type-A RRs serving as negative feedback regulators to desensitize plants to CK.

Cytokinin receptors (CHASE-domain containing histidine kinases, CHKs)

In flowering plants and the basal land plant *P. patens*, the CK receptors are encoded by a family of genes with redundant functions (Pils and Heyl, 2009; von Schwartzenberg *et al.*, 2016). Three functional CK receptor HKs are confirmed in the *A. thaliana* genome namely AHK2, AHK3, AHK4 (CRE1) (Higuchi *et al.*, 2004; Inoue *et al.*, 2001; Nishimura *et al.*, 2004; Yamada *et al.*, 2001). CKI1 was firstly found to activate CK signaling, but no further results confirmed the function of CK receptor activity (Higuchi *et al.*, 2004; Kakimoto, 1996). The three hybrid HKs are highly conserved in the amino acid sequence level and share similar multi-domain structures. The extracytosolic region of these CK receptors contains at least two transmembrane domains flanking an extracellular sensory domain in the *N*-terminal, called cyclase/histidine kinase associated sensory extracellular domain (CHASE, PF03924) (Hwang *et al.*, 2002), which binds CKs with affinity in the nanomolar range (Anantharaman and Aravind, 2001; Romanov *et al.*, 2006). The cytosolic region contains a histidine-kinase domain (HisKA, PF00512), a HATPase domain (PF02518), a canonical response regulator receiver domain (REC, PF00072) and a diverged receiver domain

unlikely to function in phosphotransfer (Inoue *et al.*, 2001; Schaller *et al.*, 2011). The CHK proteins were first thought to localize in the plasma membrane (PM) (Kim *et al.*, 2006). However, the results of less CK binding efficiency at acidic pH, which is typical for the apoplast, pointed to a receptor function inside the plant cell (Romanov *et al.*, 2006). Later studies of subcellular localization and biochemical analyses of membrane fractions harboring high-affinity CK-binding sites confirmed that the CHKs are mainly localized in endoplasmic reticulum (ER) membranes with CK binding in the lumen of the ER (Caesar *et al.*, 2011; Lomin *et al.*, 2018; Lomin *et al.*, 2011; Wulfetange *et al.*, 2011). A recent report shows that extracellular CKs binding to the sensing domain of PM-localized receptors helps to initiate the CK signaling (Zürcher *et al.*, 2016). But no evidence for the presence of CK receptors in the PM and their CK-dependent activity have been shown by the analytic strategy utilized for the ER, meaning isolation of pure PM free of ER contamination (Romanov *et al.*, 2018).

The CHASE domain is the sensor domain and is responsible for CK binding. The CHASE domain of AHK4 comprises a long α-helix and two PAS (Per-Arnt-Sim)-like domains forming β-strands connected by a helical linker (Hothorn *et al.*, 2011). It recognizes the CKs with both the adenine and the isoprene moiety through the binding pocket. The Asp262 and Leu284 are important hydrophobic amino acid residues for the formation of the hydrogen bonds with the adenine moiety of CK molecules while the Thr294 residue for binding the tail hydroxyl group of *t*Z (Hothorn *et al.*, 2011; Steklov *et al.*, 2013). They are highly conserved in *Arabidopsis* CK receptors and substitution of them resulted in a reduction of ligand binding affinity (Hothorn *et al.*, 2011). The CHKs show affinities to various types of CKs with a substrate preference for biologically active CKs since the conjugated forms of CKs (glucosides or nucleotides) do not fit the binding pocket of the CHASE domain due to their molecular structure and size (Romanov *et al.*, 2006). The ligand specificity of receptors to CK types also differs from each other. AHK3 and AHK4 both show the highest affinity to *t*Z but AHK4 recognizes 10-fold more iP compared to AHK3 (Romanov *et al.*, 2006). ZmHK1 prefers iP and shows similar affinities to *t*Z and *c*Z while ZmHK3a prefers iP (Lomin *et al.*, 2011). The bacterial PcrK receptor specifically binds iP (Wang *et al.*, 2017). The formation of the CK-receptor complex leads to a conformation change of the hybrid HK and triggers autophosphorylation at the conserved His residue in HisKA domain (Hwang *et al.*, 2002; Schaller *et al.*, 2008). The phosphoryl group is then intracellularly transferred to the Asp residue of the C-terminal REC domain containing a highly conserved DDK motif as the first REC domain following the HisK lacks

conserved phosphoryl accepting residues and does not function as a real receiver (Hwang *et al.*, 2002; Hwang *et al.*, 2012; Kieber and Schaller, 2014).

Histidine phosphotransferases (HPTs)

As part of the CK signaling pathway, the HPTs contain the Hpt domain (PF01627) including the canonical His residue for transferring the phosphoryl group from the response regulator receiver domain of CHKs to the downstream RRs (Hwang *et al.*, 2012). A multigene family including five HPT genes with the highly conserved XHQXKGSSX motif is encoded in *A. thaliana* (namely AHPs) and function as redundant positive regulators of CK signaling (Hutchison *et al.*, 2006; Hwang and Sheen, 2001; Suzuki *et al.*, 1998). The phosphorelay is regulated by S-nitrosylation of AHPs at a conserved cysteine residue by nitric oxide, which results in repression of receiving a phosphate from upstream HKs and subsequent transferring of the phosphoryl group to RRs (Feng *et al.*, 2013). The AHPs are also the major factors acting downstream of other plant HKs (such as CKI1) in TCS pathways (Deng *et al.*, 2010; Liu *et al.*, 2017). Labeling the AHPs with the green fluorescent protein (GFP) has shown a constant nuclear/cytosolic distribution of the AHPs in a CK-independent manner (Punwani *et al.*, 2010). The AHP6, which is a pseudo-HPT, does not contain the canonical His residue and is not able to receive a phosphate (Mähönen *et al.*, 2006). It acts as a negative regulator of CK signaling and regulates the proliferation and differentiation of cell lineages during vascular development and lateral root initiation (Mähönen *et al.*, 2006; Moreira *et al.*, 2013).

Response Regulators (RRs)

The plant RRs involved in CK signaling are classified into two types: the type-A RRs (RRAs) and type-B RRs (RRBs). Both of them possess the REC domain containing the canonical Asp residue, where the phosphorylation by HPTs happens.

The RRBs share the domain architecture comprising of a REC domain and a *C*-terminal extension including Myb-like DNA binding domain as well as sequences involved in activation and nuclear localization (Hosoda *et al.*, 2002; Sakai *et al.*, 2001). They function as transcription factors that regulate target gene expression stimulated by the CK signal (Argyros *et al.*, 2008; Sakai *et al.*, 2001). The target genes include the RRAs and the CK response factors (CRFs), which typically contain a short (A/G)GAT core DNA sequence for RRBs binding (Hosoda *et al.*, 2002). A recent analysis of the *Arabidopsis* RRBs using protein-binding microarrays shows that RRBs bind to their target genes with extended

DNA motifs containing the 4-base core sequence in a CK-dependent manner (Xie *et al.*, 2018; Zubo *et al.*, 2017). The domain architecture of the RRBs is important for regulating their activity since the REC domain inhibits the DNA binding in its non-phosphorylated state whereas the phosphorylation of its conserved Asp residue relieves the inhibition and activates the binding to target genes to initiate transcription. The *Arabidopsis* genome contains 11 genes encoding RRBs showing redundant function in CK signal transduction but not all of them contribute equally, among which the ARR1, ARR10, and ARR12 play predominant roles in regulating transcriptional and physiological responses to CKs (Mason *et al.*, 2005). The loss function of the three RRBs results in almost complete insensitivity to high levels of exogenously applied CK as well as stunted growth and abnormality in vascular development (Argyros *et al.*, 2008; Ishida *et al.*, 2008).

The RRAs are single-domain proteins that contain a REC domain and a short *C*-terminal extension. Unlike the RRBs, the RRAs do not contain a DNA binding domain and serve for specific downstream effects. Like other members of the CK signaling pathway, RRAs occur in *A. thaliana* as a multigene family. *Arabidopsis* RRAs (ARRs in the *Arabidopsis* terminology) comprise 10 proteins (ARR3-9, 15-17), which are divided into five closely related pairs (To *et al.*, 2004). They are rapidly transcriptionally induced by CKs, although with varying kinetics, and function as negative-feedback regulators of CK signaling by repressing RRBs (D'Agostino *et al.*, 2000; Kiba *et al.*, 2003; To *et al.*, 2004). The RRA turnover is determined by the phosphorylation state of the REC domain and CKs regulate the function of RRAs in part by the control of protein stability *via* phosphorylation (To *et al.*, 2007). It is hypothesized that the RRAs negatively regulate CK signaling possibly through competition for phosphotransfer from RRBs as well as phospho-dependent interactions with downstream targets (To *et al.*, 2007). However, no experimental evidence has been shown yet. Transcript levels of some RRAs are also responsive to various other signal inputs. For example, the ARR7 and ARR15 genes are activated by auxin to antagonize CK output in the root apical meristem (Müller and Sheen, 2008).

1.4 Charophytes: the key aquatic organisms which evolved to land plants

As showed in Fig. 4, the Viridiplantae ("green linage"), which is one of the major groups of oxygenic photosynthetic eukaryotes, includes the embryophytes (land plants) and the green algae, which inhabit primarily aquatic ecosystems (Becker and Marin, 2009; Leliaert *et al.*, 2012). It is widely accepted that the green algae originated (about 800-1200 Mya)

from a hypothetical ancestral green flagellate following the primary endosymbiosis event and diverged early into two clades: the Chlorophytes and the charophyte green alga (CGA) (Leliaert *et al.*, 2012). The Chlorophytes include the majority of what has been traditionally called green algae with a wide variety of morphologies, ranging from planktonic unicellular organisms to colony forming, multicellular, and siphonous forms (Lewis and McCourt, 2004). There are more than 6700 described species of chlorophyte green algae inhabiting marine, freshwater, and terrestrial environments (Guiry and Guiry, 2019). The CGA is a monophyletic group of unicellular and multicellular green algae from freshwater environments that share the last common ancestor (LCA) with land plants which has initiated the transition from water to land (Harholt *et al.*, 2016; Wickett *et al.*, 2014).

There are six groups within the CGA, including the earlier-diverging classes KCM (Klebsormidiophyceae, Chlorokybophyceae, and Mesostigmatophyceae) and the later-diverging classes ZCC (Zygnematophyceae, Coleochaetophyceae, and Charophyceae) (de Vries and Archibald, 2018; de Vries *et al.*, 2016). The KCM algae undergo cell division by furrowing, the ZCC group, however, evolved a peculiar mode of cell division involving the production of a phragmoplast and cell-walls with plasmodesmata, which was kept by the land plants for intercellular cytoplasmic communication and development of complex tissues (Buschmann and Zachgo, 2016; Leliaert *et al.*, 2012). Thus, the ZCC along with land plants, constitute the monophyletic group Phragmoplastophyta and contain the closest living relative of land plants. In older publications, the evolution from Charophyceae to land plants was regarded as a stepwise increase in complexity of plant body plans (from unicellular Desmids to simple filamentous forms such as Zygnema, to pseudoparenchymatous growth such as Coleochaete, and, finally, to a complex multicellular plant-like Chara) (Karol *et al.*, 2001). However, according to both nuclear and plastid sequence-based molecular phylogenetic analysis, several studies unexpectedly placed the Zygnematophyceae as the group most closely related to land plants, what is now widely accepted (Timme *et al.*, 2012; Wickett *et al.*, 2014; Zhong *et al.*, 2015; Zhong *et al.*, 2014). Even though the Zygnematophyceae represent a divergent group, all the species within this class possess simple body plans (either unicellular or filamentous) and lack flagella. The recent evolutionary pattern of the green linage reflects a secondary loss of previous morphological complexity (larger size, branching, oogamous reproduction, and plasmodesmata) in the evolutionary process (Delwiche and Cooper, 2015). The morphological simplicity of Zygnematophyceae may be advantageous for adaptation to semi-aquatic environments since the generation periods of these small organisms are

short and not much water is needed for the completion of their life cycles (Delwiche and Cooper, 2015).

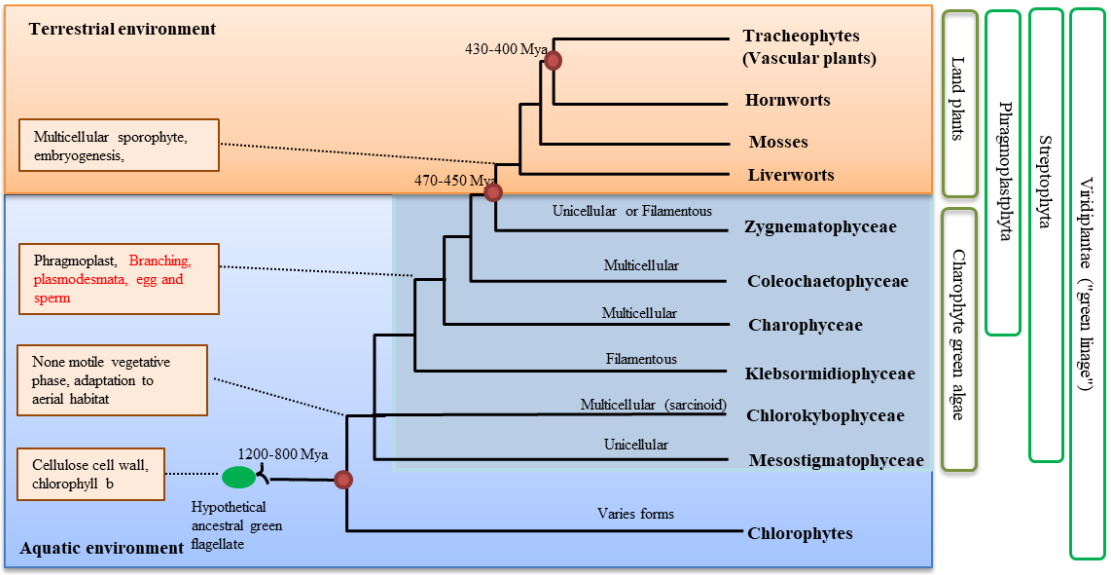

Fig. 4: Cladogram of the Viridiplantae (green lineage) rooted in a branch descending from a hypothetical ancestral green flagellate. Some nodes are marked with orange dots and their estimated dates in million years ago (Mya). Gained features are described in the boxes (left); the features marked with red font were lost in Zygnematophyceae. The figure is modified after Leliaert *et al.* (2012), Bowman (2013), Wickett *et al.* (2014) and Nishiyama *et al.* (2018).

The water-to-land transition of green plants represented a biological keystone event that changed the atmospheric and terrestrial conditions and was a prerequisite for the emergence of more and complex lifestyles. The first fundamental adaptation for preceding terrestrialization might have been the freshwater lifestyle (Becker and Marin, 2009). Although there are a few CGA species adapted to semi-terrestrial or 'subaerial' environments, most species of the CGA ancestrally inhabited freshwater. This allowed the green plants slowly and gradually to move towards moist habitats in the proximity of water, and ultimately the colonization of land, where water is supplied by rainfall (Delwiche and Cooper, 2015). Meanwhile, this transition to terrestrial environments is often coupled with exposure to a variety of naturally occurring biotic and abiotic stress conditions such as desiccation, varying temperatures, increased CO_2 concentrations, and high irradiance (Holzinger and Karsten, 2013; Holzinger and Pichrtova, 2016). To cope with this harsh environment, the CGA required a variety of physiological and biochemical adaptations including desiccation-tolerance strategies (formation of dormant spores, aggregation of

cells, flexible cell walls, mucilage production and accumulation of osmotically active compound) and protective strategies in conditions with high irradiation (accumulation of UV-screening compounds such as mycosporine-like amino acids and phenolic substances) (detailed review by Holzinger and Pichrtova (2016)).

Given their special evolutionary position, CGA hold the key to get insights into the origin, diversification and fundamental properties of land plants in the context of evolutionary history and the relationships among the organisms, as well as the development of adaptive traits relevant to the transition from aquatic to terrestrial environments (de Vries and Archibald, 2018; Delwiche and Cooper, 2015). With the accessibility of more and more DNA sequence information of different algae species, it becomes possible to perform comparative genomic and phylogenetic analysis within the CGA organisms, which will be of great help for future studies of the genetic basis of the evolution and adaptation process within the green lineage (Delwiche, 2016). The first genomic sequence in CGA was from the early-diverging *Klebsormidium nitens* (Klebsormidiophyceae), which possesses Streptophyta-specific intermediates involved in biological process categories such as response to various stress conditions, cell wall biogenesis and phytohormone-related functions (Hori *et al.*, 2014). Attention has been especially paid to investigate the origin of phytohormones which are involved in the regulation of plant growth and development processes, and play important roles in mediating plant defense response against biotic and abiotic stresses (Verma *et al.*, 2016). The phytohormones have been shown to be present in diverse algae species (Stirk *et al.*, 2013; Žižková *et al.*, 2017), but the functional roles of these endogenous phytohormones are unclear in the ancestral algae (Lu and Xu, 2015). For example, *K. nitens* produces endogenous phytohormones including, abscisic acid (ABAs), CKs, jasmonic acid (JAs) and salicylic acid (SA) (Hori *et al.*, 2014). Furthermore, its genome encodes some counterparts but not a complete set of homologs of the signaling pathways of the auxin, ABA, CK, gibberellin, ethylene, JA, and SA， which are essential for the responses to environmental stimuli in land plants (Hori *et al.*, 2014). The similar distribution of proteins involved in the signaling pathways of these phytohormones is also found in other charophyte species including *Chara braunii*, *Penium margaritacium* and *Spirogloea muscicola* (all three species belong to the ZCC clade) (Cheng *et al.*, 2019; Jiao *et al.*, 2020; Nishiyama *et al.*, 2018).

Phytohormone responses have been reported in *K. nitens* and *Spirogyra pratensis* (Ohtaka et al. 2017; Mutte et al. 2018). *K. nitens* was shown to react with extensive

transcriptional responses to exogenously supplied auxin. For *S. pratensis*, a cell elongation response to ethylene was reported (Ju *et al.*, 2015). The components of three classical phytohormone signaling pathways (CK, ABA, and ethylene signaling) are responsive to abiotic stress in the form of severe desiccation in *K. crenulatum* (Holzinger and Becker, 2015). These findings suggest that as the ancestors of the land plants, the early-diverging CGA likely had already established some of the important signaling pathways essential for terristrialization. However, details of the origin and evolution of each phytohormone have to be elucidated, as well as the functions of the components of the hormone regulatory system. In this project, focus has been given to the study of the CK regulatory system.

1. 5 Zygnematophyceae: the sister group to land plants

Among the ZCC clade of Charophytes, the Zygnematophyceae is the most species-rich lineage and the group most closely related to land plants (Leliaert *et al.*, 2012; Zhou and von Schwartzenberg, 2020). This group of algae is characterized by a peculiar mode of sexual reproduction (conjugation), involving a syngamic fusion of non-flagellate gametes formed by two neighboring vegetative cells (Guiry, 2013). They morphologically consist of unicellular and filamentous forms in the vegetative phase and possess no flagellate stages and centrioles (Gontcharov, 2008). According to a recent revision of the taxonomy of the Zygnematophyceae, there are two subclasses (Spirogloeophycidae and Zygnematophycidae) and three orders (Desmidiales, Zygnematales and Spirogloeales) within this class containing more than 4000 described species (Cheng *et al.*, 2019; Guiry and Guiry, 2019).

The morphological details and modes of both, vegetative growth and sexual reproduction of species belonging to different orders have been observed and documented (Blackburn and Tyler, 1980; Coesel and Teixeira, 1974; Fowke and Pickett‐Heaps, 1971; Pickett-Heaps and Fowke, 1971; Zhou and von Schwartzenberg, 2020). In most cases, the Zygnematophyceae algae perform vegetative growth *via* cell division whereas sexual reproduction happens when the growth condition turns to be unfavorable. Sexual conjugation happens either between different mating types in heterothallic strains or between clonal cells in homothallic strains and finally form zygospores (Coesel and Teixeira, 1974). The intercellular communication and the determination of mating type for

sexual reproduction in the genus *Closterium* (Desmidiales) have been reviewed in detail (Sekimoto *et al.*, 2014; Tsuchikane and Sekimoto, 2019). The matured zygospores are dormant and resistant to harsh environmental conditions, such as drought. Meiosis and meiotic cell division were reported to take place during germination of zygospores of *Micrasterias thomasiana* var. *notata* and *Closterium ehrenbergii* Meneghini under favorable conditions (Blackduran and Tyler, 2007; Kasai and Ichimura, 1983). The environmental conditions required for the germination of zygospores have been well discussed by Agrawal and Chaudhary (1994) and Agrawal (2009).

Many algal species belonging to the filamentous Zygnematales can asexually form specialized cells like akinetes, aplanospores, and azygospores (parthenospores) allowing to survive extreme stress conditions (Holzinger and Pichrtova, 2016). The akinetes and aplanospores develop within individual vegetative cells by thickening of the cell wall or shrinking coupled with new wall formation whereas the azygospores result from failure of the protoplasts fusion post conjugation (Holzinger and Pichrtova, 2016; Stancheva *et al.*, 2014). These specialized cells are not dormant and able to continue to form new vegetative cells under favorable conditions (Agrawal, 2009).

The typical classification of Zygnematophyceae has generally been based on differences in cell morphology especially on the cell wall ultrastructure and cellular organization (Brook, 1981). The Desmidiales comprise ca. 30-33 genera belonging to four families namely Closteriaceae, Desmidiaceae, Gonatozygaceae, and Peniaceae. The distinct features of Desmidiaceae are that each cell consists of two half cells (semicells) attached at a central isthmus zone and possesses ornamented and porous cell walls (Brook, 1981). All algae in Desmidiales have a complex extracellular matrix, which is typically composed of a multilayered cell wall, a network of “pores”, and an external sheath of an extracellular polymeric substance (mucilage or slime) (Domozych *et al.*, 2007). During the cell division, the morphological features of the parental semicells are maintained while the secondary cell wall, as well as the symmetry and specific shape, will be re-established by the new daughter semicell (Domozych *et al.*, 2009; Lacalli, 1975; Pickett‐Heaps, 1972; Zhou and von Schwartzenberg, 2020). The algae belonging to Zygnematales are characterized by a smooth cell wall in one whole piece which is lacking pores and other ornamentations (Guiry, 2013). Zygnematales are divided into two families: the filamentous Zygnemataceae and the unicellular Mesotaeniaceae which share some common features with Desmidiaceae and formerly known as ‘‘saccoderm desmids’’ (Brook, 1981; Jarman

and Pickett-Heaps, 1990). The vegetative growth is achieved by cell division involving the formation of a phragmoplast and cell wall formation *via* centripetal encroachment of a peripheral septum (Fowke and Pickett‐Heaps, 1971; Galway and Hardham, 1991). Multiple conjugation bridges are often observed in the process of conjugation (scalariform conjugation) in species from the filamentous Zygnematales (Ikegaya *et al.*, 2012; Novis, 2004).

1.6 Cytokinin in land plant evolution

The evolution of land plants from their algal ancestors was achieved through a variety of morphological and physiological adaptations driven by increased regulatory networks including the intricate signaling pathways mediating communication within the organism as well as between the organism and the surrounding environment. The phytohormones and their signaling pathways are indispensable parts of the regulatory networks. They have been shown to occur and function in organisms beyond land plants and enables activation of appropriate and effective defense responses against biotic and abiotic stresses (Holzinger and Becker, 2015; Ju *et al.*, 2015; Ohtaka *et al.*, 2017; Žižková *et al.*, 2017). Although knowledge about the conservation of the phytohormones is fragmented, studying the regulatory networks opens a new perspective on the understanding of the single-to-multicellular and water-to-land transition of plants. In the last couple of years, the evolution of several phytohormone regulatory systems has been investigated and discussed. Attention has been especially paid to the evolution of the signaling pathways of auxin (Mutte *et al.*, 2018; Ohtaka *et al.*, 2017), abscisic acid (Hanada *et al.*, 2011; Sun *et al.*, 2019), gibberellin (Wang *et al.*, 2015), strigolactones (Waters *et al.*, 2017), jasmonic acid (Han, 2017), ethylene (Ju *et al.*, 2015; Papon and Binder, 2019) and CK (Gruhn and Heyl, 2013; Kabbara *et al.*, 2018). Here, we focus on the occurrence and evolution of the CK regulatory system.

CKs are ancient molecules in the form of prenylated nucleotides in tRNA and exist in a wide range of prokaryotic and eukaryotic organisms and play a role in assuring translation accuracy (Persson *et al.*, 1994). However, as described in chapter 1.1, natural occurring CKs are present in plant cells in a variety of types and forms and function as signaling molecules regulating plant growth and development through a complex system including the CK metabolism, transport, and signaling pathways (see chapter 1.2). This raises great

interest in tracing back the evolution of the complexity of the CK regulatory system which is based on the employment and diversification of genes of bacterial origin. Up to date, the acquisition of these bacterial genes in plants is supposed to have occurred either *via* horizontal gene transfer (HGT) or *via* gene duplication in the evolutionary process (Spíchal, 2012). But detailed information about the time point and the function of the HGT or duplication events has to be revealed and specified individually for each gene involved in the different pathways. Since many steps of CK interconversion and transport pathways are not CK specific, we will in this study mainly focus on CK biosynthesis and signaling pathways.

In the evolution of land plants, the endosymbiotic uptake of cyanobacteria was one of the most important processes as it gave rise to plastids. The endosymbiont had undergone considerable gene losses and HGT from the endosymbiont genome to the host genome (Leliaert *et al.*, 2012). The IPTs, which are responsible for CK biosynthesis, have diversified into tRNA-IPTs and the adenylate IPTs, might have been acquired as a result of the HGT since orthologs of IPTs have been found in the genome of cyanobacteria (Nishii *et al.*, 2018). The two cyanobacterial IPTs have been experimentally confirmed for prenylation activity in a biochemical assay (Frébortová *et al.*, 2015; Frébortová *et al.*, 2017), which offered evidence for the hypothesis of the bacterial origin of the CK biosynthesis pathway.

As shown in chapter 1.2, the CK signaling pathway is an extended version of the TCS system that is the most widely used signal transduction system in bacterial responses to environmental stimuli. The number of multiple TCS signaling proteins in individual genome scales with genome size and diversity of the environment. The expansion of the TCS components is achieved through a combination of gene duplication and horizontal gene transfer (Capra and Laub, 2012). Generation of new TCS by gene duplication requires the following changes: modification of the sensory domain of the HK for a new input signal and adaptation of the regulatory domain for signal output (Capra and Laub, 2012). Generally, the signal transduction domains of the HKs are highly conserved while the ligand sensory domains and the transmembrane domains vary to allow the perception of different ligands. The domain shuffling of signal outputs of the TCS often results in diverse transcriptional responses (Capra and Laub, 2012). The coevolution of cognate HKs and RRs is essential to maintain their interaction and to avoid cross-talk with other pathways (Capra and Laub, 2012). As a typical TCS pathway, the CK signaling uses the CHASE-

domain containing HKs as CK receptor proteins and the RRs as signal outputs (see chapter 1.2). The two protein families are CK specific and are widely addressed in evolutionary studies of the modular CK signaling system. Recent findings showing CK sensing in phytopathogenic bacteria (*Xanthomonas campestris*) and cyanobacteria (Nostoc sp. 7120) help to get insight into the origin of the CK signaling system and its evolutionary path(s) (possibly involving HGT) to form specialized CK receptors in prokaryotes and the green lineage (Frébortová *et al.*, 2017; Wang *et al.*, 2017). Phylogenetic studies have shown that the full plant-like CK signaling module does not exist in chlorophyte green algae (Lu *et al.*, 2014; Wang *et al.*, 2015), but is present with a partial or complete set in several genomes of CGA (Cheng *et al.*, 2019; Hori *et al.*, 2014; Jiao *et al.*, 2020; Nishiyama *et al.*, 2018). However, no CK response has so far been reported for any CGA species.

1.7 Cytokinin studies in the basal model organism *Physcomitrella patens*

P. patens belongs to the early divergent land plant lineage of Bryophytes, which shares the common ancestor with vascular plant and evolved from the CGA (Wickett *et al.*, 2014). It has been used as the model organism for evolutionary and developmental studies in the non-flowering plants for several decades mainly due to its relatively simple morphology and a low number of cell types (Cove *et al.*, 2006; Cove *et al.*, 2009). The axenic culture was established by the germination of a single spore and cultures can be kept in a mineral (minimal) medium allowing rapid completion of the life cycle (Reski and Abel, 1985). The whole genome has been sequenced and is publicly accessible *via* online platforms including NCBI, JGI, and Plant GDB (Rensing *et al.*, 2008). The genetic transformation of *P. patens* has been achieved via PEG-mediated transformation of protoplasts (Schaefer *et al.*, 1991). Details on promoters and reporter genes as well as other DNA delivery methods are reviewed by Kamisugi and Cuming (2009). Gene targeting *via* efficient homologous recombination and CRISPR-Cas9 techniques have been well established and allow manipulation of genes for reverse genetic analyses (Collonnier *et al.*, 2017; Schaefer and Zrÿd, 1997).

The life cycle of *P. patens* includes alternation of generations: the haploid gametophyte generation and the diploid sporophyte generation, where the haploid gametophyte is the predominant generation (Cove, 2005; Reski, 1998). The gametophytic stage starts by tip growth during the germination of an individual spore under favorable conditions, which

leads to the formation of a branched filamentous protonema. Protonemata comprises two types of cells including chloronema (many chloroplasts and slow growth) and caulonema (fewer chloroplasts and fast growth) (Thelander *et al.*, 2005). Buds form at side branch initials and develop into leafy shoots known as gametophores, which are composed of a stem, numerous leaflets, and rhizoids at the base. After culturing under sporophyte inductive conditions (8h light /16h dark and 15-17°C), both antheridia and archegonia are produced on the same leafy shoot (Hohe *et al.*, 2002). After fertilization by motile male gametes (or spermatozoids, produced in antheridia), the egg cell develops into a small diploid sporophyte. The sporangium undergoes series of maturation steps indicated by the colorations of the spore capsule, and the formation of a short seta as a connection between the gametophytic apex and the sporophyte (Hohe *et al.*, 2002). The mature sporophyte contains about 4000 spores in the spore capsule (Cove, 2005).

P. patens is responsive to external applications of low concentrations of CKs which cause the formation of buds and gametophores (Bopp and Diekmann, 1967; Hahn and Bopp, 1968; Reski and Abel, 1985). The nucleotide forms of CKs and *c*Z have no detectable bud-inducing effects in *P. patens* while the inducing activities of other forms are listed in the following order: iP>tZ>BA>BAR>iPR>tZR>mT>DHZ>oT (von Schwartzenberg *et al.*, 2007). However, an excessive dose of CKs provokes callus-like growth of buds which do not further differentiate to leafy gametophores and finally results in inhibition even termination of development and growth (Reski and Abel, 1985; von Schwartzenberg *et al.*, 2016). The reduction of extracellular CKs in *P. patens via* overexpression of the AtCKX not only showed a reduction of budding formation but also showed an irregular growth of chloronemal filaments and cells, which confirmed the function of CKs in the regulation of protonemal development (von Schwartzenberg *et al.*, 2007).

CK profiling of whole-culture extracts shows that iP and *c*Z-types are predominant CKs in *P. patens* (von Schwartzenberg *et al.*, 2007). Only homologs of tRNA-IPTs are found in *P. patens*. The PpIPT1, PpIPT4, and PpIPT5 enzymes were confirmed as functional tRNA-IPTs by a yeast complementation assay (Patil and Nicander, 2013; Yevdakova and von Schwartzenberg, 2007). The chloroplast-bound PpIPT1 gene is responsible for the A^{37} prenylation of tRNA and a considerable reduction of *c*Z in the tissue of PpIPT1 knockout mutants was shown (Lindner *et al.*, 2014).

The PpNRH enzymes being to some extent involved in CK conversion and activation have also been functionally characterized by knockout mutation (Kopečná *et al.*, 2013).

Considering the CK signaling, the genome analysis has revealed that the whole TCS signaling machinery is encoded by *P. patens* (Pils and Heyl, 2009). Mutations of three classical CHKs (PpCHK1, PpCHK2, and PpCHK3) which share high similarity with AHK4, demonstrate that these receptors respond to CKs and play an essential role in bud induction and gametophore development (von Schwartzenberg *et al.*, 2016). A subfamily of eight additional PpCHKs that share less identity with AHK4 was uncovered and it was shown that PpCHK4 binds CK and is capable of activating a two-component signaling chain (Gruhn *et al.*, 2014; Gruhn *et al.*, 2015). However, the biological role of these receptors remains to date unresolved.

1.8 Model organism need to be established in Charophytes

As introduced in chapter 1.4, the charophyte algae share the LCA with land plants and hold the key to the understanding of the water-to-land transition and the mechanism of adaptation to terrestrial environments (Domozych *et al.*, 2016). While several model organisms have been established in the clade of Chlorophytes (Volvox and *Chlamydomonas reinhardtii*) and land plants (*Arabidopsis*, maize, rice, and *Physcomitrella*), comparable model systems are also urgently needed for Charophytes, which is an extremely species-rich and highly divergent clade. Fortunately, the past few years have seen attempts to fill in these gaps through various approaches. Several charophyte genera have been proposed as model organisms for the study of different biological processes.

In Zygnematophyceae, owning to their typical symmetrical cell architectures, the unicellular *Micrasterias*, *Penium*, and *Closterium* were chosen as models to represent the Desmidiales in the research fields concerning cell morphogenesis, cell wall biology and sexual reproduction (Domozych *et al.*, 2014; Lutz-Meindl, 2016; Tsuchikane and Sekimoto, 2019). The filamentous *Mougeotia* and *Spirogyra* from the Zygnematales were proposed as possible models for evolutionary studies on the cell division pattern and plant-like metabolic and signaling pathways (Buschmann and Zachgo, 2016; Van de Poel *et al.*, 2016). They fulfill several but not all requirements for serving as model organisms, which will be detailed in chapter 2 and chapter 3.

The earlier diverging Coleochaetophyceae are morphologically more complex than the Zygnematophyceae. The thallus of *Coleochaete orbicicularis* comprises a disc that is

formed by monoplastidic single-layer vegetative cells and sheathed hairs (setae) that develop from dorsal surfaces of particular cells having C-shaped plastids (seta cells) (Graham *et al.*, 2012). The radial and circumferential growth of the thallus is achieved through precise division of the peripheral cells in either anticlinal or periclinal directions, which is determined by a series of autonomous rules based on the cell position, size and shape (Cook, 2004; Dupuy *et al.*, 2010). The simplified two-dimensional growth of the thallus and the ease of culture make *C. orbicicularis* an ideal model strain for studies about the developmental processes (Domozych *et al.*, 2016; Dupuy *et al.*, 2010).

The body plan of *Chara* (Characeae) is more complex than that of any other charophyte algae. It comprises a shoot-like axis consisting of nodes with whorls, multinucleate internodes, a simplex apical meristem, and root-like rhizoids. This morphology makes Characeae good experimental systems on different levels: (I) Cellular studies dealing with the mechanism of cytoplasmic streaming, dynamics of endomembrane trafficking and cell wall expansion of the giant intermodal cells (Foissner *et al.*, 2015; Staves, 1997). (II) Cell-to-cell transmembrane transport through plasmodesmata and the electrophysiology of membrane transporters, which are involved in the transportation of nutrients from rhizoids to growing parts of the thallus (Beilby, 2016). The genome of *C. braunii* has been sequenced, which will help to get insight into the relevant mechanisms in an evolutionary context (Beilby, 2019; Nishiyama *et al.*, 2018).

1.9 Strains and genetic information available for Charophytes

The uni-algal strains of CGA are available from several international algal collections including the University of Texas Algal Collection (UTEX; USA), the University of Duisburg-Essen Algal Culture Collection (CCAC; Germany), the NIES Microbial Culture Collection (NIES, Japan), the Sammlung von Algenkulturen (SAG; Germany) Coimbra Collection of Algae (ACOI; Portugal) and some other algae collections. These collections are harboring thousands of strains covering a variety of freshwater algae and seaweed species. In contrast to the mentioned collections, the Microalgae and Zygnematophyceae Collection Hamburg (MZCH; Germany) is specialized on Zygnematophyceae. The details about the coverage of Zygnematophyceae species by algae collections worldwide have been reviewed by Zhou and von Schwartzenberg (2020). Most of the algal strains kept in public collections are either originally unaxenic or were contaminated with bacteria/fungi during the process of culture maintenance. Further establishment of new reference cultures and

their phylogenomic characterization will be of great help for the molecular research of the CGA.

Unlike the well-established model organisms, sequence information for the CGA came up late and is not abundant yet. For example, the whole genome sequences of *A. thaliana* and *C. reinhardtii* were released, well annotated, and publicly available at the beginning of this century (Merchant *et al.*, 2007; The Arabidopsis Genome Initiative, 2000). In the same period, only the chloroplast/mitochondrial genomes and some ESTs (expressed sequence tags) were sequenced for some representative strains of the CGA for phylogenetic analysis of the land plant origin (Lewis and McCourt, 2004; Turmel *et al.*, 2002). With the development of sequencing technology and the increasing interest in CGA research, more and more sequence information is getting available. Transcriptomes have been reported for many CGA species in recent years (e.g. transcriptomes of Zygnematophyceae algae: *Mougeotia scalaris*, *Spirogyra pratensis, Cosmarium crenatum*, and *Penium margaritaceum*) (Cooper and Delwiche, 2016; Mundt *et al.*, 2019). The genomic data of *K. nitens NIES-2285*, *S. muscicola* gen. nov., *M. endlicherianum,* and *C. braunii* have been released and are publicly available for genome browsing as well as BLAST search in online webservers (Cheng *et al.*, 2019; Hori *et al.*, 2014; Nishiyama *et al.*, 2018). The genomic sequencing projects have been accomplished with some algal species (e.g. *P. margaritaceum* and *Closterium psl. complex*) (Jiao *et al.*, 2020; Tsuchikane and Sekimoto, 2019), but the access to these databases is still limited. More charophyte genome sequencing projects are currently underway and will offer more resources for deepening our understanding of this group of algae.

1.10 Molecular tools established in Zygnematophyceae

During the past decade, some effort has been taken to achieve genetic transformation algae in Zygnematophycea. The first successful transient genetic transformation was achieved in the unicellular Desmid strain *Closterium psl. complex via* biolistic bombardment with gold particles (Abe *et al.*, 2008). The GFP protein is proved to be a convenient and sensitive reporter for the direct visualization of transformants and when fused to open reading frames of genes of interest, allows subcellular localization in living algal cells. The endogenous CpHSP70 (heat shock protein 70) and CpCAB1 (chlorophyll a/b-binding protein) promoters function efficiently in driving gene expression. Based on the transient transformation approach, stable nuclear gene transformation was achieved

two years later by using the same promoters and the same gene delivery techniques. The phleomycin resistance gene (*ble*) was used as selective maker and was shown to be randomly inserted into the genome of the transgenic *C. psl* complex with one or more copies (Abe *et al.*, 2011). It was also confirmed that CpCAB1 used as a heterologous promoter in *Micrasterias denticulata*, can effectively drive the transient expression of the fusion of cellulose synthase (MdCesA1) with GFP protein in a study of desmid cell wall biology and morphology. However, the expression level was much lower than that in *C. psl* complex (Vannerum *et al.*, 2010). Recently, the filamentous *Mougeotia scalaris* was also transiently transformed by using constructs bearing its native a-tubulin1 promoter through biolistic bombardment (Regensdorff et al., 2018). An *Agrobacterium*-mediated transformation technique for introduction of transgenes into *Penium margaritaceum* was reported by Sørensen *et al.* (2014), for the RNA interference (RNAi) mediated suppression of cell wall biosynthesis. Unfortunately, detailed information on the transformation efficiency and follow-up reports are lacking to date. Successful targeting of genes of interest, like knockdown and CRISPR/Cas9-based knockout of receptor-like protein kinase (RLK) in *C. psl* complex, were firstly and only reported by the working group of Hiroyuki Sekimoto (Hirano *et al.*, 2015; Kanda *et al.*, 2017).

1.11 Aim of the project

In this project, two major questions were tackled.

Firstly, does the plant-like CK regulatory system exist and function in the sister group of land plants, the Zygnematophyceae?

Secondly, is it possible to select suitable axenic algal strains to serve as model organisms in Zygnematophyceae allowing genetic modification and physiological studies?

Does the plant-like CK regulation system exist and function in the sister group of land plants-Zygnematophyceae?

CKs are widely produced by various organisms and have been well described for their metabolism, transport, signaling, and action in land plant species. However, with the

deepened understanding of the evolution of the green lineage, the studies of the CK regulatory system in organisms ancestral to land plants attract increasing interests. With the increasing accessibility of axenic CGA species and sequence information, it is possible to search for the answers to the following questions:

What is the CK distribution pattern like in the charophyte algae?

Do the CGA and land plants share the same CK biosynthesis and metabolic mechanisms? If not, what are the differences?

It has been shown that some components of the plant CK signaling pathway are also encoded in the CGA genomes (Cheng *et al.*, 2019; Hori *et al.*, 2014; Nishiyama *et al.*, 2018; Wang *et al.*, 2015). Is there a complete plant-like CK signaling machinery existing already in the aquatic ancestors before conquering the land? Are the algal putative CHK proteins functioning as real CK receptors initiating CK response? Contents related to all these questions above will be addressed in the first chapter:

Zhou H, von Schwartzenberg K. (in prep.): Searching for the role of cytokinin regulation system in charophyte green algae.

Is it possible to find suitable axenic algal strains to serve as a model organism in Zygnematophyceae which allows reverse genetic and physiological study?

Due to its remarkable evolutionary position, Zygnematophyceae attract more and more interest in performing functional studies on algal metabolic and regulatory pathways *in planta*. However, it is still far to achieve such aims since model organisms are not well established yet for the Zygnematophyceae and several technical barriers for reverse genetic approaches still exist. To search for solutions, we will screen the MZCH and worldwide algae collections for suitable representative strains that meet the requirements of potential model organisms. Details will be shown in the second chapter:

Zhou H, von Schwartzenberg K. (2020): Zygnematophyceae - From living Algae collections to the establishment of future models. Journal of Experimental Botany 71, 3296–3304.

Transient genetic transformation of Micrasterias radians var. evoluta

As possible model organisms selected (and purified) from the algae collection MZCH, *Spirogyra pratensis,* and *Micrasterias radians* var. *evoluta* meet all the criteria except that they are lacking the genetic transformation enabling reverse genetics studies. For the transformation of the candidate algal strains, specific issues concerning promoters, transgenic technology, selectable marker, etc will be addressed in the third chapter:

Zhou H, Wilkens A, Hanelt D, von Schwartzenberg K. (2020): Expanding the molecular toolbox for Zygnematophyceae – transient genetic transformation of the desmid *Micrasterias radians* var. evoluta. European Journal of Phycology, https://doi.org/10.1080/09670262.2020.1768298

2. Discussion

Huge progress has been made in understanding the occurrence of CKs and their regulatory system in land plants in the last decades. Up to date, most components involved in the CK regulatory system are well described in *Arabidopsis thaliana* and *Physcomitrella patens* for their functions due to the accessibility of the genomic sequences and the subsequent characterization of CK regulatory players *via* a combination of genetic tools and physiological analysis. Although there is CK related information reported for other plant species, our understanding of the origin and the development of the CK regulatory system is still limited. Previous studies showed that the phytohormone ability of CKs derived from the reassignment of a bacterial signaling regulatory system of the metabolites that occurred as a degradation product of modified tRNAs, where CK play a role in improving the accuracy of the translation process (Persson *et al.*, 1994). However, two additional aspects of this evolutionary process garnered significant interest. Firstly, how has the CK metabolic system evolved to maintain the various endogenous CK pools at a certain level that meets the requirements for plant growth and development? Secondly, how was the response system for CK molecules first established and by which steps has the complexity of this signaling system increased during the plant evolution? To address these two points, it is essential to investigate the metabolism, signaling, and functions in organisms ancestral to land plants especially in the charophyte green algae, which form a monophyletic group together with land plants and diverged from the chlorophyte green algae more than 800 million years ago (Leliaert *et al.*, 2012).

Recent genome analyses of several Charophytes species reveal that this group of algae seems to be the most basal group of the sequenced organisms within the green lineage, which contains a complete set of the CK regulatory system (Cheng *et al.*, 2019; Hori *et al.*, 2014). However, more information has to be collected from Charophytes aiming at understanding the evolutionary progress forming the plant-like CK regulatory system. On the one hand, the distribution patterns of CKs, which has been well reported for various chlorophyte algae and plant species (Stirk *et al.*, 2013; Žižková *et al.*, 2017) need to be investigated in the charophyte algae to get an overview on CK metabolism in Charophytes. This was achieved in this study (Zhou *et al*., in prep.) by the quantitation of extracellular and intracellular CK contents of different charophyte algal species over a growth period of three weeks. With the currently available transcriptomic and genomic data of Charophytes,

a phylogenetic analysis was performed to investigate the occurrence of the components of the regulatory mechanisms for both CK metabolites and signaling in organisms representing the evolutionary status prior to the conquest of land (Zhou *et al*., in prep.). On the other hand, as the Charophytes, especially the Zygnematophyceae, possess various typical properties and hold a key position in the evolution of the green lineage, and therefore are ideal models for different research purposes including functional characterization of plant-like signaling pathways (Zhou and von Schwartzenberg 2020). It is essential to establish experimentally easy to handle models within the clade of Zygnematophyceae harboring features such as easy manipulation in an axenic status, closed life cycle, accessibility of genomic sequence, and efficient genetic transformation (Zhou and von Schwartzenberg 2020). Especially, a genetic transformation protocol has been established for one of the candidate model organisms- *Micrasterias radians* ver. evoluta (Zhou *et al.*, 2020) and *Spirogyra pratensis* (Zhou, unpublished).

2.1 The occurrence of cytokinin within the green lineage

CKs are known to be intimately involved in many physiological processes associated with plant growth and development as well as stress responses since the endogenous zeatin and its riboside were purified and identified as plant growth regulators (Letham, 1963; Letham, 1973). With the development of the techniques for isolation and quantitation of the phytohormones, a variety of CK types including isoprenoid or aromatic CKs have been determined for their occurrence in many organisms within the green lineage, such as land plants, Chlorophytes and cyanobacteria (Ördög *et al.*, 2004; von Schwartzenberg *et al.*, 2007; Žižková *et al.*, 2017). Generally, the majority of naturally occurring CKs are in isoprenoid type while the aromatic CKs occur either in trace amounts or are hardly detectable in most organisms except few plant species of the genera Corylus, Pinus, and Populus (Cuesta *et al.*, 2012; Gentile *et al.*, 2017; Jaworek *et al.*, 2019; Strnad *et al.*, 1997). The minor role of aromatic CKs is also evident for Charophytes, in the CK profiles of which, almost no aromatic CKs were detected (Zhou *et al*., in prep.). Therefore, in this thesis, only the distribution of different types of isoprenoid CKs in organisms along the green lineage will be discussed. To ensure a certain level of CK activity for growth and development, the endogenous CK pools comprise different CK forms including free bases, ribosides, nucleotides, and glucosides. The conversions between different CK forms are

regulated by various metabolic enzymes, which have been well characterized in the model organism *A. thaliana* (see Fig. 2 in chapter 1) (Frébort *et al.*, 2011).

As reviewed in chapter 1.4, the Charophytes occupy the key node in the singe-to-multicellular and water-to-land transition processes of land plants, in which the phytohormones especially the CKs may play important regulatory roles. However, compared to the earlier divergent Chlorophytes and the land plants, reports concerning the occurrence of endogenous CKs in Charophytes are rare and scattered, which represents a huge obstacle for our understanding of the CK metabolic system from an evolutionary point of view. Therefore, experiments were carried out to monitor the production and distribution of CKs of five charophyte algal species over a growth period of three weeks (Fig. 1 in Zhou *et al*., in prep.). As the endogenous CK compositions and contents are affected by various factors such as cell types, developmental stages, and other external factors, the algal strains were selected under consideration of the following aspects: First, each of these algae has a simple body plan (either unicellular or filamentous) and the cultures were easy to be synchronized. Second, the analyzed algal strains were all free of contamination with microorganisms thus excluding that the CK pools of both, the culture medium and the algae cells, were affected by the the presence of microorganisms. Finally, all samples of the five algae were collected during the light period of their growth as endogenous CK concentrations were reported to be higher during the light period compared to the dark period (Stirk *et al.*, 2011). The typical analytic technique combining the immunoaffinity purification and the UHPLC-MS/MS quantification (Novák *et al.*, 2008) was applied in this project for the determination of the intracellular and extracellular CK profiles, which was the same as that used for the CK profiling of species from Chlorophytes and cyanobacteria (Stirk *et al.*, 2013; Žižková *et al.*, 2017).

Our identification and quantification analysis of the CK profiles in Charophytes showed an extensive variation in the CKs contents (from pmol g^{-1} DW to nmol g^{-1} DW) within organisms (Fig. 1 and Tab. S1 in Zhou *et al*., in prep.). However, the distribution patterns of the CK types and forms of the five charophyte algae were found to be similar to each other (Zhou *et al*., in prep.), also with few exceptions resembling the patterns previously reported for the chlorophyte algae and cyanobacterial species (Stirk *et al.*, 2013; Žižková *et al.*, 2017). For most of the algal species, the CK pools mainly comprised iP- and *c*Z-types, whereas the content of the *t*Z- and DHZ-types were very low or even hardly detectable (Fig. 1 and Tab. S1 in Zhou *et al*., in prep.). The predominance of iP- and *c*Z-

types was also found in the CK profiles of the basal land plants such as the moss *Physcomitrella patens* (von Schwartzenberg *et al.*, 2007) and the liverwort *Marchantia polymorpha* (Aki *et al.*, 2019). In contrast, seed plants are characterized by a more complex array of CK pools which contain all the four types of isoprenoid CKs varying in ratios depending on species and tissues (Jaworek *et al.*, 2019; Ko *et al.*, 2014; Zhang *et al.*, 2014). Although different types of CKs in seed plants as well as in *P. patens* occur in both, active and storage forms (*N*- or *O*-glucosides), the latter forms appear to be the predominant components (von Schwartzenberg *et al.*, 2007; Zhang *et al.*, 2014). On the contrary, the most abundant CK forms in all analyzed CK profiles of the charophyte green algae are free bases, ribosides, and nucleotides (Fig. 1 and Tab. S1 in Zhou *et al*., in prep.), and the same distribution pattern was also found in Chlorophytes (Stirk *et al.*, 2013) and in *M. polymorpha* (Aki *et al.*, 2019). These early divergent organisms hardly produce CK-*O*- and *N*-glucosides. Taken together, within the green lineage, the early divergent organisms including Chlorophytes, Charophytes, and the most basal land plants turned to produce iP- and cZ-type CKs in forms of bases, ribosides and nucleotides (Zhou *et al*., in prep.). Apparently, the homeostasis of these pools is controlled on the biosynthesis level instead of using breakdown or conjugation pathways. Therefore, the functional deactivation pathway of CKs *via* conjugation with sugar moieties seems to arise for the first time in basal land plants. Furthermore, since *c*Z-type of CKs have been shown to be biosynthesized by tRNA-IPTs (Lindner *et al.*, 2014; Miyawaki *et al.*, 2006), the richness of *c*Z in all the algae species and basal land plants suggested a high conservation of this CK biosynthesis pathway in the plant evolution.

The extracellular and intracellular CK levels of these Charophytes species are in dynamic changes over different growth period of the culture (0-, 13- and 22-day), but with minor changes in the distribution patterns (Fig. 1 and Tab. S1 in Zhou *et al*., in prep.), which is similar to the monitored CK profiles of *P. patens* (von Schwartzenberg *et al.*, 2007). Only the intracellular CK content of *Mesostigma viride* shows a continuous increase over time, while *K. nitens* and *S. pratensis* only accumulate the CKs at the beginning of the growth and maintain the CK levels in certain range in the following cultivation process (Fig. 1 in Zhou *et al*., in prep.). This suggests that the biosynthesis pathway is active in the fast growth stage of these charophyte algae. The extracellular CK levels of all investigated algae kept increasing over the growth period (Fig. S1 in Zhou *et al*., in prep.). This increase has resulted from the release of the CKs from the large intracellular CK pools to the medium. The release of CKs from *K. nitens* and *S. pratensis* cells against the

concentration gradient indicated the possibility of the existence of an active transportation mechanism (Fig. 1 and Fig. S1 in Zhou *et al*., in prep.). In the analyzed charophyte algae, the major components of extracellular CK pools are free bases, ribosides, and nucleotides of iP- and cZ-type CKs, the proportion of free bases being bigger than that of nucleotides and ribosides (Fig. 1 in Zhou *et al*., in prep.). Although the transport of *t*Z-type CKs from the root to the shoot of *Arabidopsis* has been proved to be mediated by the ABC transporters (Ko *et al.*, 2014; Zhang *et al.*, 2014), the transportation mechanisms for the *c*Z- and iP-type CKs remain undetermined.

2.2 Understanding the evolution of the regulatory machinery of cytokinin metabolites

As previously outlined, the endogenous CK levels in flowering plants are regulated by the rates of CK biosynthesis, interconversion, transportation, and inactivation, which can be achieved either by the formation of inactive conjugates or irreversible degradation (Frébort *et al.*, 2011). However, relatively little was known about the regulatory mechanism of the endogenous CK pools of green algae particularly Charophytes. The recent expansion of genomic and transcriptomic sequence information of some Charophytes allows a detailed bioinformatic analysis of the putative components involved in the metabolic pathways regulating the size of CK pools. Together with the CK profiling analysis, this might shed new light on the possible evolutionary steps leading to the establishment of the regulatory machinery tuning the endogenous CK levels in land plants. Since the pathways of interconversion between different CK forms and CK transportation are shared with other metabolites such as purines and pyrimidines, we focus on CK biosynthesis and inactivation pathways.

The present study in Zhou *et al*. (in prep.) showed that the IPTs, which catalyze the rate-limiting step of CK biosynthesis, occur only as tRNA-IPTs in basal land plants and ancestral algae. Both, Chlorophytes and Charophytes species encode a single-copy IPT gene individually while each of the land plants contains a multigene family for the IPTs (Fig. 2 in Zhou *et al*., in prep.). Our phylogenetic analysis of the isopentenylpyrophosphate transfer domain (IPPT, PF01715) within the green lineage showed a cluster of the single-domain IPT proteins from all investigated algal species with all IPTs from *P. patens* and *M. polymorpha* as well as IPT9 from *A. thaliana* (Fig. S1 in Zhou *et al*., in prep.), which

have been classified as class I tRNA-IPTs. These IPTs originated from bacterial miaA-like genes and evolved independently from the plant class II tRNA-IPTs and adenylate IPTs (Frébortová *et al.*, 2015; Lindner *et al.*, 2014; Nishii *et al.*, 2018). Due to the limitation of sampling in the non-green lineages, the evolutionary steps of the green lineage acquiring class I tRNA-IPTs are not yet clear. It is hypothesized that the algae obtained class I tRNA-IPT genes from bacteria through the initial endosymbiotic events leading to mitochondria in the last eukaryotic common ancestor followed by gene losses in some lineages like fungi or acquired the class I tRNA-IPTs from cyanobacteria *via* independent HGT events (Nishii *et al.*, 2018). As the closest living relatives to land plants, the charophyte green algae including the multicellular species maintained only single-copy class I tRNA-IPT (Fig. 1 in Zhou *et al*., in prep.). The gene number is increased in the last common ancestor (LCA) of land plants by gene duplication and followed by gene loss events in LCA of flowering plants which results in the predominance of class II tRNA-IPTs and adenylate IPTs in flowering plants for biosynthesis of various types of CKs. The class II tRNA-IPT (AtIPT2) is probably acquired by LCA of flowering plants through secondary HGT events (Nishii *et al.*, 2018) or duplication event of the class I tRNA-IPT (Lindner *et al.*, 2014). Duplication of class II tRNA-IPTs and functional diversification resulted in the extant adenylate IPTs in flowering plants. This evolutionary pattern could be part of the explanation of the preferred production of cZ-type CKs by the charophyte algal species (Zhou *et al*., in prep.) as well as basal land plants (Aki *et al.*, 2019; von Schwartzenberg *et al.*, 2007) and more diverse distribution patterns of the endogenous CK profiles in flowering plants. However, it is insecure to deduce the CK biosynthesis mechanism in early-diverging organisms within the green lineage purely based on sequence prediction and without functional characterization. This has been demonstrated in Physcomitrella by the analysis of Ppipt1 mutants which suggested the presence of a tRNA-independent CK biosynthesis mechanism although only class I tRNA-IPTs are encoded by the moss genome (Lindner *et al.*, 2014).

In land plants, the oxidative cleavage of the side chain by the CK dehydrogenase/oxidases (CKXs) leads to an irreversible breakdown and reduction of the endogenous CK levels (Kollmer *et al.*, 2014). Our investigation of the occurrence of CKX genes in the available transcriptomes/genomes of organisms from the green lineage demonstrated that the CKX proteins exist ubiquitously in land plants including the most basal *M. polymorpha*, but they are absent in other earlier clades including the chlorophyte and charophyte green algae (Fig. 2 in Zhou *et al*., in prep.). These CKXs were previously thought to be acquired through

HGT from cyanobacteria (Frébort *et al.*, 2011). However, the confirmation of the lack of CKX activity of the NoCKX1 contradicted this hypothesis (Frébortová *et al.*, 2015). Recently, homologs to CKX have been found in some prokaryotic species including Actinobacteria, Proteobacteria, and Cyanobacteria, plus a few from Chlamydiae, Chloroflexi, and unclassified bacteria (Wang *et al.*, 2020). The extended phylogenetic analysis also indicated a close relationship of the CKX genes between land plants and Chlamydiae species, which suggested that plants likely acquired the CKXs from an ancient chlamydial endosymbiont during primary endosymbiosis (Wang *et al.*, 2020).

Besides irreversible degradation, the plant CKs can be inactivated by conjugation with a sugar moiety, mostly glucose, at the *N*-position of the adenine moiety and the *O*-position of the zeatin side chain. This is catalyzed by the uridine diphosphate glycosyltransferases (UGTs), which are encoded by a large multi-gene family in plants that are involved in a wide range of pathways including post-translational protein modifications, biosynthesis of plant natural products and regulation of plant hormones (Keegstra and Raikhel, 2001). An obvious gene expansion of UGTs has occurred during the evolution of land plants (Yonekura-Sakakibara and Hanada, 2011). I found the gene families encoding multiple members of UGTs in the charophyte algae genome (e.g. *K. nitens*) and transcriptomes. However, the algal UGTs share only low sequence similarities with the five previously functionally characterized CK-specific UGTs from *Arabidopsis* (for example the KnUGTs share 30 – 35 % identities with the Arabidopsis CK-*N*-UGTs). Apparently, algal UGTs probably prefer glucosylation of other substrates more than CKs since the CK-*N*- or *O*-glucosides were hardly detectable in all algal species tested (see Fig. 1 and Tab. S1 in Zhou *et al*., in prep.). However, further evidence has to be given by biochemical analysis of the catalytic activities of these algal UGTs.

2.3 Understanding the evolution of the regulatory machinery of cytokinin signaling

A complete set of components of CK signaling pathway has been well characterized in model organisms and supposed to be restricted to land plants (Pils and Heyl, 2009). This was challenged by the increasing accessibility of genomic information from charophyte algae species ancestral to land plants (Cheng *et al.*, 2019; Hori *et al.*, 2014; Jiao *et al.*, 2020; Nishiyama *et al.*, 2018). Some components involved in CK signaling pathway have been predicted for their occurrence in Charophytes, which makes this clade of algae an important node for investigation of the acquisition of the CK signal regulatory machinery.

The presented study by Zhou *et al*. (in prep.) employed currently available genomic/transcriptomic data of seven algal species from different charophyte classes including the early diverging ones to gain insights into the establishment of the CK signaling system. As the depth and quality of RNA-seq-derived transcriptomes are restricted by the quality of RNA samples prepared for sequencing and the expression of genes at a certain growth stage or under certain growth conditions, the number of homologs identified for the investigated multigene families might be underestimated. Underestimation of genes could also happen to the species with draft genome assemblies, as their quality may not be as high as the well-established genomic database of model organisms. For example, the genome of *Chara braunii* was supposed to encode more proteins to underpin its complex morphology than other charophyte algal lineages (Nishiyama *et al.*, 2018), but fewer counterparts of CK signaling proteins have been found in this genome database (see Fig. 2 in Zhou *et al*., in prep.), which might result from gene loss events during evolution or low coverage of the whole genome sequence. However, the inclusion of these databases offered a chance to get a first view on the occurrence of CK signaling proteins across the charophyte algal lineage. For phylogenetic analysis, conserved domains of predicted proteins were utilized since some of the homologs retrieved from algal transcriptomes represented only fragments of the open reading frames. Moreover, functionally conserved domains were used because the interconnecting regions of multi-domain proteins are diverged and induce gaps in the alignment, therefore lowering the quality of the phylogeny.

These genome/transcriptome databases were screened for CK signaling components though either BLASTP or tBLASTn searches. As BLAST may not be stringent enough to distinguish orthology groups in large families such as histidine kinases and response regulator proteins, protein structure analysis has been performed to identify putative proteins involved in CK signaling system (Zhou *et al*., in prep.). Complete sets of homologs of the typical CK signaling proteins, CHASE-containing HKs (CHKs), Histidine phosphotransferases (HPTs) and type-B response regulators (RRBs) were shown to be encoded by genomes of all land plants and their investigated charophyte ancestors except for *C. braunii* (no RRBs are found in its genome), which may due to gene loss event in the evolution or the poor quality of the genome dataset (Fig. 2 in Zhou *et al*., in prep.).

However, in other investigated non-streptophyte species, only parts of the two-component system (TCS) were found (Fig. 2 in Zhou *et al*., in prep.). The CHASE-containing proteins

in Chlorophyte species did not appear as hybrid HKs (Gruhn *et al.*, 2014) and all their hybrid HKs contain no conserved CHASE domains, while the single-copy homologs to HPTs and RRBs responsible for signal transduction and output are present (Fig. 2 in Zhou *et al*., in prep.). The signaling proteins appear as multidomain proteins in cyanobacteria, with a complex domain arrangement including both hybrid HK region and HPT region (Fig. S4 in Zhou *et al*., in prep.). This indicated that the functional CK signaling machinery has been established in the charophyte algae or later in land plants, but the different players constituting the CK signaling pathway apparently evolved independently.

The eukaryotic HPTs contain a single Hpt domain (PF01627) including the canonical His residue and shuttle between the cytosolic REC domain (PF00072) of the membrane-bound receptors and the downstream nuclear RRs. The plant HPTs probably were acquired by the last common eukaryotic ancestor *via* HGT resulting from endosymbiosis event and since then used by various eukaryotic TCS signal pathways (Schaller *et al.*, 2011). The RRBs comprising a REC domain and a myeloblastosis (Myb)-like DNA-binding domain (PF00249) were found throughout the green lineage except *C. bruanii*, but completely missing in other eukaryotic lineages (Fig. 2 in Zhou *et al*., in prep.). It is supposed that the recombining and shuffling of the two evolutionary ancient domains that occurred in the early diverging species of the green lineage resulted in the typical RRBs possibly functioning in the algal TCS pathway. However, they are doubtful to function in CK signaling in Chlorophytes as no CHKs are encoded by organisms from this evolutionary clade. Duplication of RRBs happened in later-diverging charophyte algal species, where they might add regulatory options for more complex responsive programs triggered by new signals. In seed plants, the type-A response regulators (RRAs) are rapidly induced following the phosphorylation of RRBs and represent negative regulators of CK signaling (Kiba *et al.*, 2003; To *et al.*, 2004). They comprise a single REC domain and a short C-terminal extension, which is similar to the protein structure of type-C response regulators (RRCs), but can be clearly distinguished based on phylogenetic analysis. As shown in Fig. S3 (Zhou *et al*., in prep.), RRAs are clearly distinct from other types of RRs and are only found in land plants and some charophyte algae. I hypothesize that the early ancestor of charophyte algae acquired RRAs *via* gene duplication and mutation, but they underwent gene loss events in some algal species. However, whether these RRAs acquired a function as regulators in the CK signaling pathway in charophyte algae remains unknown.

The CHKs sharing the similar domain arrangement (CHASE (PF03924)—HisKA (PF07730)—HATPase_C (PF02518)—REC (PF00072)) with the characterized CK receptors in land plants, have been shown to be established in the last common ancestor of charophyte green algae and land plants (Fig. 2 and Fig. S4 in Zhou *et al*., in prep.). The HisKA, HATPase_C and REC domains of HKs are phosphorylation-dependent signal transduction domains and highly conserved among eukaryotic TCS pathways, whereas the *N*-terminal sensory domain (CHASE) and its flanking transmembrane regions of CHKs differ from the ligand perception domains of other histidine kinases. Therefore, phylogenetic analysis and homology modeling were mainly performed with the CHASE domains (Zhou *et al*., in prep.). The results suggest that the putative CHKs from the charophyte green algae diverged into two groups including the characterized CK receptors (Inoue *et al.*, 2001; von Schwartzenberg *et al.*, 2016) and the less conserved subfamily of non-canonical CHKs from *Physcomitrella patens* and *Marchantia polymorpha* (Aki *et al.*, 2019; Gruhn *et al.*, 2014) (see Fig. 3 in Zhou *et al*., in prep.). Structural homology models of CHKs from both clades displayed that a high level of conservation of the important residues for CK binding only occurs among the canonical CHK receptors, and all the other CHKs possess divergent residues at the same positions (see Fig. 4 in Zhou *et al*., in prep.). This suggests that the CHKs from early diverging organisms could exhibit different properties in CK perception and signaling. This was confirmed by the live-cell CK-binding assays, which showed that the PpCHK4 and one of the MpCHKs bind tZ with relatively low affinity (Gruhn *et al.*, 2014) while the cyanobacterial CHK only weakly binds iP (Frébortová *et al.*, 2017). The same experiment has been performed with two CHKs from *Spirogyra pratensis*. It was shown that the SpCHK2 only binds iP, although with much lower affinity than that of AHK4, whereas the SpCHK1 possesses no ligand affinity to iP or *t*Z (see Fig. 5 in Zhou *et al*., in prep.). Taken together, this indicates that divergent amino acids in the CHASE domains of some non-canonical CK receptors still enable the binding of CKs, although the affinities are low (Zhou *et al*., in prep.).

To test whether they are able to activate CK responses in moss, the two SpCHK proteins were expressed *via* knock-in into the PpCHK3 gene locus of the CK receptor double mutant of *Physcomitrella patens* (PpΔchk1,2) (Zhou *et al*., in prep.). The obtained SpCHKs expressing moss lines showed a similar phenotype as the CK receptor triple-mutant and no developmental responses to exogenously applied CKs, even though the transcripts of the SpCHKs were detected (see Fig. 7 in Zhou *et al*., in prep.). This suggests again that

the algal CHKs are functionally different from the classical CK receptors. They are either not able to respond to CKs, or they exhibited a very low response, which leads to an insensitivity to exogenously applied CKs and a disability for CK induced developmental changes in moss. However, this does not mean a complete absence of a functional CK signaling system in charophyte algae as the SpCHK2 specifically binds iP, which is one of the predominant endogenous CKs. A similar situation seems to exist in *Marchantia polymorpha*, whose MpCHK (although it is unclear which one) exhibits a poor response to all tested CKs (Gruhn *et al.*, 2014) (Gruhn et al., 2014). In contrast to *Physcomitrella patens*, other mosses are not sensitive to exogenous CK treatment (Aki *et al.*, 2019). But it has been shown to deploy the negative feedback loop of CK signaling through analyzing the overexpression lines of MpRRA, and the knockout lines of MpRRB (Aki *et al.*, 2019). Thus, the question whether the charophyte algal CHKs function *in planta* as typical CK receptors is still an open question. Given that there is a complete set of CK-signaling related components in Charophytes, we can only speculate that the SpCHKs possibly already function in the TCS pathway, but their low ligand binding affinity may render them less restricted to CKs signaling compared to the canonical CK receptors of land plants. They might only have become CK-dependent after having evolved to *bona fide* CK receptor proteins. Nevertheless, our results indicate a stepwise evolution of CK signaling mechanism, of which the recombination and shuffling of domains of the TSC system have been accomplished in the last common ancestor of charophyte algae. Gene duplication and mutation is likely to have resulted in specification of the TCS to CK signal transduction. However, to clarify the biological function of the ancestral CHKs, more *in planta* studies such as mutation analysis of the downstream components of CK signaling pathway, have to be performed in the charophyte algae in the future.

2.4 The development of model organisms in Zygenematophyceae

Since the discovery of their close relationship to land plants, as mentioned in chapter 1.8, the Zygnematophyceae have drawn tremendously increased interest in research fields of physiology, cell biology, developmental biology, and plant evolutionary studies (Zhou and von Schwartzenberg, 2020). However, the lack of universal model organisms that meet the experimental requirements in the Zygnematophyceaen algae is one of the major limiting factors for studies in the mentioned fields. In the last decade, great efforts have been made to fill in this blank and several algal genera representing different families of

Zygnematophyceae (including *Closterium, Penium, Micrasterias, Mougeotia,* and *Spirogyra*) have been suggested as model systems fulfilling different research requirements (Domozych *et al.*, 2016; Zhou and von Schwartzenberg, 2020). As algal species from the mentioned genera have been relatively poorly studied, the establishment of model strains remains important and is currently underway in several laboratories.

Up to date, with the anticipated genome release for the *Closterium peracerosum-strigosum-littorale* complex belonging to Closteriaceae (Tsuchikane and Sekimoto, 2019) and the *Penium margaritacium* belonging to Peniaceae (Jiao *et al.*, 2020), these two algal strains seem to be promising to be the first two well established model organisms in Zygnematophyceae in near future. Comprehensive reviews have been published already to outline the feasibility of using the *C. psl* complex as the model strain for studying the mechanisms of sexual reproduction (Tsuchikane and Sekimoto, 2019) and *P. margaritacium* as the model strain for understanding cell expansion and cell wall biochemistry (Rydahl *et al.*, 2015). However, both species are still far from being widely used as universal model organisms when compared to other well established ones like *A. thaliana* and *C. reinhardtii*, since the genomic information and/or the successful transformation of these species are restricted to the laboratories of origin (Zhou and von Schwartzenberg, 2020).

Another well-known unicellular algal genus belonging to the family of Desmidiaceae is Micrasterias, which exhibits extremely differentiated symmetric cell architecture and has been used in cell biology research especially for cell pattern formation related studies (see review by Lutz-Meindl, 2016 and Zhou and von Schwartzenberg, 2020). This algal genus includes about 80 morphospecies according to AlgaeBase and one of the most prominent morphological characteristics varying between species is the branching of lateral lobes of the semicell (Škaloud *et al.*, 2011). Among them, the disk-like *M. denticulata* had been regarded as a model strain for investigations on ultrastructural details during cytomorphogenesis and under influence of varying environmental conditions (Lutz-Meindl, 2016). This species was taken as a model because it was the first one, of which the developmental stages were well defined and its big cell size enables convenient visualization on subcellular features using simple microscopy techniques (Kiermayer, 1964; Meindl, 1993). However, the big cell size turned to be a disadvantage in the establishment of genetic experimental platforms allowing to elucidate the molecular basis of interesting cytological processes. On one hand, a significant positive correlation

between nuclear DNA content and both, average cell length and number of terminal lobes has been shown within the *Micrasterias* genus (Poulickova *et al.*, 2014). Therefore, *M. denticulata* could possess a large and complex genome, which makes it difficult for sequencing and future reverse genetic studies. On the other hand, the transient genetic transformation protocol has been established for *M. denticulata* using biolistic bombardment with relatively low transformation efficiency (Vannerum *et al.*, 2010). One of the reasons for the limitation of this approach is that the number of bombarded cells is limited due to their big cell size so that the chance to successfully select rare stable transformation events is relatively low. The *M. radians* var. *evoluta*, which shows smaller-sized, deeply divided cells with narrow polar lobes (Škaloud *et al.*, 2011) and less DNA content than the *M. denticulata* (Poulickova *et al.*, 2014), has recently been proposed as a candidate model strain for this genus by Zhou and von Schwartzenberg (2020). Other important arguments for choosing this species are its ease of cultivation under axenic conditions and the fact that vegetative growth, as well as sexual reproduction process can be achieved in the laboratory (see Fig. 1 in Zhou and von Schwartzenberg, 2020). Efficient transient genetic transformation has been achieved with different approaches including transformation of protoplasts and intact vegetative cells (see Fig. 2 and 3 in Zhou *et al.,* 2020). Further sequencing of genome or transcriptomes and establishment of stable genetic transformation protocols enabling manipulation of genes of interests are still required in order to make *M. radians* var. *evoluta* a universal model.

Generally, the Zygnematales are thought to represent an early divergent group of algae within Zygnematophyceae and are therefore more closely related to land plants than the Desmidiales, although the classification of certain species might change with the revision of the taxonomy based on phylogenetic analysis (Gontcharov, 2008). A recent genome study of subaerial *Spirogloea muscicola* gen. nov. placed this species in the new subclass of Spirogloeophycidae within Zygnematophyceae, which is at the basis of the class and therefore closer related to land plants (Cheng *et al.*, 2019). However, the species of the family Zygnemataceae are still taken as models for investigation of plant-like features and signaling regulatory networks. Because the reconstruction of phylogeny within the class of Zygnematophyceae might change with extended taxon sampling, it seems to date impossible to determine extant algal taxa being most closely related to the last common ancestor of Zygnematophyceae and land plants. A major reason for this lack of knowledge is the extremely low coverage of the described species diversity of Zygnmatophyceae with

by accessions in living culture collections as described in detail in Zhou and von Schwartzenberg (2020).

As mentioned in chapter 2.3, the components of biosynthesis and signaling pathways for many phytohormones are already encoded in species throughout the Charophytes. However, hormone effects on growth and morphology of algae have only been reported in *S. pratensis*, which reacts exogenous application of ethylene by cell elongation (Ju *et al.*, 2015). Since then, efforts have been made in our laborotary to make the filamentous *S. pratensis* experimentally operable for investigation of hormone functions and regulation system. An axenic strain has been made by the author from a single filament and maintained in the Microalgae and Zygnematophyceae collection Hamburg (MZCH) under the number 10213. The closed life cycle of *S. pratensis* under laboratory cultivation conditions has been described by Zhou and von Schwartzenberg (2020). Unlike the easy and standardized vegetative growth, the sexual reproduction including the conjugation, formation of zygospores, and germination of the spores has been shown to be affected by many different factors, such as certain nutrient ratios, light component and intensity, and drought stress caused by aeration, etc. (Schwab, 2017; Zwirn, 2013). No general induction procedure similar to that has been reported for *C. psl* complex (Tsuchikane and Sekimoto, 2019) has been established so far for promoting both, the conjugation and the germination process in *S. pratensis.* The molecular tool kit including bioinformatic resources and genetic manipulation tools is still limited for this species. However, some progress has been made by the author for the establishment of the genetic transformation system (see Fig. 2 in Zhou and von Schwartzenberg, 2020). Even though, the preliminary results obtained currently only showed transient gene expression in protoplasts of *S. pratensis* (Zhou and von Schwartzenberg, 2020), the relatively high transformation rate (1 % of the survival cells expressing GFP) and the ability for protoplast regeneration (Zhou, unpublished) makes this alga promising for the further development of protocols for stable manipulation of genes. Considering the bioinformatic resources, transcriptomes for the same strain of *S. pratensis* under a variety of stress conditions have been available (Van de Poel *et al.*, 2016).

Another Zygnemataceae genus is *Mougeotia*, which has attracted interests for being a candidate models for cell division related research (Buschmann and Zachgo, 2016) and transient genetic transformation has been published (Regensdorff *et al.*, 2018).

2.5 The genetic transformation of the potential Zygnematophyceae model strains

The establishment of genetic transformation protocols is the basic step for developing strategies for modifying organisms to meet specific needs and for functional studies of genes involved in pathways of interest (Hansen and Wright, 1999). Successful genetic transformation has been achieved for several Zygnematophyceaen strains (reviewed in chapter 1.10). In this thesis, transient transformation protocols have been established for the proposed potential model strains *S. pratensis* (Zhou. unpublished) and *M. radians* var. *evoluta* (Zhou *et al*., 2020) through different approaches. The success of these transformations mainly depends on two important aspects, which include the DNA delivery techniques and the expression of the cloned genes.

The Zygnematophyceaen cells possess a complex extracellular matrix, which is typically composed of a multilayered cell wall and an external mucilage layer with polysaccharides as the major components (Domozych *et al.*, 2007). This leads to difficulties in the introduction of exogenous genes into the nucleus of these algae. Up to now, most of the reported transformation protocols for Zygnematophyceae algae (such as *Closterium*, *Micrasterias,* and *Mougeotia*) are based on particle bombardment, which employs high acceleration pressure to directly deliver the DNA into intact cells. This ballistic technique enables the transformation of all kinds of plant species without host-range limitations or the requirement of special vector systems which are characteristics of *Agrobacterium*-mediated transgenesis. The transformation efficiencies varied between species and greatly depended on factors such as the coating of the microcarrier, the parameters used for shooting, and the target cell properties. Normally gold particles are widely used as microcarriers for algae transformation and yield optimal efficiency at a size of 0.6 µm (Abe *et al.*, 2008; Vannerum *et al.*, 2010). The PDS-1000/He System (Bio-Rad) has been used for introducing the DNA coated particles into the target cells with setting parameters specified for each species (see Table 2 in Zhou *et al*., 2020). The pre-culture of the algal cells on the solid medium to mid-logarithmic phase is essential for gaining more transformants (Abe *et al.*, 2008; Regensdorff *et al.*, 2018; Zhou *et al.*, 2020). However, even though after optimization of the mentioned factors, the particle bombardment is still less efficient than other gene delivery methods (Zhou *et al.*, 2020). Another perceived disadvantage of this technique is that it tends to generate stable transgenic lines with the integration of foreign DNA at multiple sites or a single site with multiple copies (Abe *et al.*,

2011). This makes it essential to analyze the transgene loci structure in detail prior to phenotypic interpretation of transformants.

The *Agrobacterium*-mediated gene transfer has been successfully employed for the stable transformation of Zygnematophyceae algae (Sørensen *et al.*, 2014). The advantages of the *Agrobacterium* system are that the protocol is easy to perform with minimal costs for equipment and transgenic plants obtained by this method often contain simple copy insertions. This system depends on the function of Vir proteins, which target specifically to the nuclear genome (Li *et al.*, 2000). The efficiency of the T-DNA transfer and its integration into the host genome is affected by both *Agrobacterium* and host-specific properties as well as cultural conditions (vir-gene inducers, culture media composition, suppression and elimination of *Agrobacterium* infection after co-cultivation) (Cheng *et al.*, 2004). The efficient transformation of *Penium margaritacium* was only obtained by infection cells undergoing conjugation, which were thought to be susceptible to the entry of the *Agrobacterium* during the wall loosening associated with the formation of the conjugation papillae (Sørensen *et al.*, 2014). However, no reports followed on *Agrobacterium*-mediated transformation, neither for the *P. margaritacium* nor for other Zygnematophyceae species. Limiting factors probably are that the culture conditions for the induction of sexual reproduction has yet not been standardized for most of the Zygnematophyceaen species and that it is difficult to eliminate the *Agrobacterium* contamination post-transformation.

Protoplast transfection is another very efficient tool for the transformation of different organisms including the recalcitrant Zygnematophyceae. The cell walls, as well as the extracellular layers of the target cells, are removed by enzymatic processes to generate protoplast suspensions in very high density, which helps to increase the chance of transfection. Standard protocols for protoplast isolation and transient transformation by polyethylene glycol (PEG) mediated DNA delivery have been established in this thesis for *M. radians* var. *evoluta* (Zhou *et al.*, 2020) and *S. pratensis* (Zhou, unpublished). Even though the transformation efficiency of the two algae is much lower compared to that of *A. thaliana,* where about 90 % of the treated cells were transformed (Yoo *et al.*, 2007), thousands of algal transgenic cells can be obtained with this protocol under optimal transfection conditions (Zhou *et al.*, 2020). The utilization of proper starting material and the transfection parameters were found critical for the whole transformation procedure (Zhou, unpublished). Since the algae are cultured as unicellular or filamentous cell

suspensions, cells at the mid-logarithmic phase yielded a large number of viable protoplasts for efficient transformation (Zhou, unpublished). Protoplasts prepared from younger or older algal cultures may be similar to those from a mid-logarithmic-phase culture in amount and appearance, but they are more fragile during the PEG transfection. Due to the complexity and difficulty in the regeneration of their original symmetric cell structures, the Desmid algae including *M. radians* var. *evoluta* are excluded from being stably transformed using protoplasts (Zhou *et al*., 2020). However, the protoplast transformation system still has robust applications in transient gene expression for analysis of gene function, protein localization, and interaction between proteins without the requirement of regeneration. The stable transformation is promising for the filamentous Zygenemataceae species like *S. pratensis* with this approach since the fast and efficient regeneration of protoplasts has been achieved (Ohiwa, 1977) (Zhou, unpublished).

The constructs generally used for direct gene transfer include a promoter, a reporter, and a terminator. The cGFP protein has been widely used as a stable visual reporter for the transformation of the potential Zygnematophyceae models, because this allows the detection of transient expression lines in a short period post transformation (see Fig. 2 and 3 in Zhou *et al*., 2020) and the confirmation of stable expression after antibiotic selection of the transformed lines when it is fused with the selective maker (Abe *et al.*, 2008; Abe *et al.*, 2011). Selective markers have to be specified for each species based on their sensitivity to suitable antibiotics which allow killing or suppressing the large excess of non-transformed cells. The detection of the GFP signal within the cells relies on reporter signal detection techniques and its expression levels, which are mainly affected by the promoter activity and the intrinsic background level within cells (de Ruijter *et al.*, 2003). Several endogenous promoters have been isolated from Zygnematophyceae species including *Closterium psl* complex (Abe *et al.*, 2008), *Mougeotia scalaris* (Regensdorff *et al.*, 2018), *Micrasterias radians* var. *evoluta* (see Fig. 1 in Zhou *et al*., 2020) and *Spirogyra pratensis* (Zhou, unpublished) and shown to be more efficient in driving the GFP expression compared to the exogenous ones (see Table 1 in Zhou *et al*., 2020). Among them, the CpCAB and CpHSP70 promoters from *Closterium* are inducible promoters, of which the expression activity can be enhanced by applying either light or thermal stress (Abe *et al.*, 2008). The promoters isolated from the other three species are expected to drive constitutive expression of actin and tubulin genes (Zhou *et al*., 2020; Zhou, unpblished). Only the 3-kb MsTUA1 promoter is confirmed to facilitate strong transgene expression in plant organisms other than charophyte algae (Regensdorff *et al.*, 2018). The failure to

achieve satisfactory gene expression driven by the other algal promoters in other organisms (such as *S. pratensis*) could result from codon bias or lack of *cis*-elements in their relatively short promoter sequences or absence of proper transcription factors in heterologous systems.

2.6 Conclusion and future perspective

Investigations of the occurrence of CKs and their regulatory systems, conducted in this thesis, in the earlier diverging organisms within the green lineage, have brought the following facts in light: First, CKs are actively produced by the charophyte green algae, however, their distribution patterns differ greatly from that of land plants. The regulatory network of the algal CK pools seems to mainly comprise archaic biosynthesis and metabolism pathways but lacks deactivation mechanisms, which is a major player assuring CK homeostasis in flowering plants. Second, the stepwise assembly of plant CK signaling-like pathway from pre-existing components, such as the combination of the ligand-binding CHASE domain and the histidine kinase that may result in neofunctionalization of the TCS pathway, is accomplished in charophyte green algae. However, functional analysis of two algal CHKs points to a low CK dependency of the newly established TCS pathway. These findings provide insights into a functional evolutionary path of the CK metabolic and signaling mechanisms, and emphasize the importance of charophyte green algae, especially species belonging to Zygnematophyceae, for studing the origin of CK regulatory system as well as other plant processes.

Although CK effects on growth and development as well as on responses to biotic and abiotic stressors of flowering plants were described through the years, the CK-specific characteristics in organisms possessing a very simple body plan remain unclear. In this project, I have investigated the effects on the sexual reproduction of Zygnematophyceae by exogenous CK applications, but no specific and sensitive phenotypic interactions have been found with relatively high CK concentrations (data not shown). Studies on CK actions on growth and growth-related parameters as well as on the development and activity of chloroplast of algae is underway. Whether the newly developed CK-signaling-like TCS pathway is responsible for sensing CKs in Zygnematophyceae is still an intriguing question. It is noteworthy that, there was no indication for transcriptional regulation of the TCS components (CHKs and RRs) in the wild type of *Spirogyra pratensis* upon CK treatment

as measured by qRT-PCR analyses (Fig. S5 in de Vries *et al.*, 2020). Monitoring the changes in gene expression in response to a CK treatment through sequencing-based techniques would be helpful to gain broad insights into the molecular CK responses in the algae. Further functions of the CK-signaling-like TCS pathway in algae might be identified by generating mutants of the signaling components. For example, single knockout mutation of algal HPT or RRB would possibly abolish the TCS-mediated CK signaling in algae, thus providing important clues for the elucidation of TCS-mediated CK responses. For these genetic studies, a better understanding of the biological features of charophyte green algae, and versatile platforms in this clade of organisms is becoming urgent. The unicellular *Micrasterias radians* var. *evoluta* and the filamentous *Spirogyra pratensis* are proposed as candidates for model organisms as they fulfill several criteria including easy cultivation in axenic state and closed life cycles under laboratory conditions, while molecular toolkits enabling genetic manipulation are still the major obstacles for functional studies. These are not insurmountable, but a lot of work has to be accomplished. Although successful transient transformation has been achieved for *M. radians* var. *evoluta* (Zhou *et al.*, 2020) and *S. pratensis* (Zhou, unpublished), work on stable transformation is still undergoing in our laboratory. Based on the transformation protocols, homologous recombination or gene-specific nucleases (e.g. CRISPR/Cas9) mediated gene targeting systems could be established in the future. Transcriptomes have been described and easily accessible for *S. pratensis* (Cooper and Delwiche, 2016; Van de Poel *et al.*, 2016) and genome sequencing has been initiated in the framework of the DFG project “CharMod”. For *M. radians* var. *evoluta,* although the sequence information is limited due to a small community working on it, its comparatively small genome size should enable relatively easy sequencing.

3. References

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4. Manuscripts

4.1 Searching for the roles of cytokinin regulation in charophyte green algae

Hong Zhou, Jan de Vries, David Kopecny, Zusana Pekna, Onrdej Novak, Miroslav Strnad, Klaus von Schwartzenberg* (prepared for submission)

Contribution of Zhou H.

Lab work: Preparation of axenic strains of *Cosmarium crenatum* and *Spirogyra pratensis*; Cloning of SpCHK1 and SpCHK2; Bioinformatic analysis (Fig. 2, Fig. 3, Fig. 4, Fig. S2, Fig. S3, Fig. S4); Expression of the two SpCHKs in *E. coli* for CK binding assays; Generation and characterization of the SpCHK expression lines in CK receptor deficient mutant of *Physcomitrella patens* (Fig. 6); Phenotypic characterization and CK response assays (Fig. 7).

Manuscript: Participation in conceptualization and designing of the research; Data curation, validation, and visualization; Writing the original draft and working on the review and editing.

Searching for the roles of cytokinin regulation in charophyte green algae

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Abstract

The plant hormone cytokinins (CKs) regulate numerous developmental and physiological processes in land plants. However, their occurrence and ancestral roles in charophyte green algae remain enigmatic. Here we present CK profiling data of five axenic charophyte green algae (*Cosmarium crenatum* var. *boldtianum*, *Micrasterias radians* var. *evoluta*, *Spirogyra pratensis*, *Klebsormidium nitens*, and *Mesostigma viride*) monitored over a 22-day growth period by UHPLC-MS/MS. The CK pools of algae showed a predominance of iP- and cZ- type CKs in forms of bases, nucleosides, and nucleotides whereas glucosides were almost absent. The accumulation of intracellular and extracellular CKs some species over time suggested an active production and a possible transport mechanism in

Charophytes. Bioinformatic analysis using available genomic and transcriptomic resources of seven charophyte algae as well as of two chlorophyte algae has proved that the biosynthetic and transport pathways of CKs are widespread within the green lineage, while the inactivation mechanisms of CKs seem to be missing in the ancestral algal clades. The complete set of genes constituting the CK sensing and signaling pathways are encoded by *Klebsormidium nitens* and *Spirogyra pratensis*, suggesting an early establishment of CK-signaling-like mechanisms in Charophytes. We investigated on two CHASE (Cyclases/Histidine kinases Associated Sensory Extracellular) domain-containing histidine kinases (CHKs) from *Spirogyra pratensis* (Zygnematophyceae). Although they share a similar domain arrangement with the classical CK receptors and are highly conserved in the response regulator receiver domain, their CHASE domains showed divergent residues at the positions essential for CK binding. For functional analysis, SpCHK1 and SpCHK2 were cloned and expressed in both, *Escherichia coli* and a CK receptor mutant of the moss *Physcomitrella patens*. The SpCHK2 exhibited a very weak affinity to iP-type CKs and no detectable affinity to tZ-type CKs. The SpCHKs overexpression moss lines also did not show detectable responses to exogenous CKs on a phenotypic level. Therefore, it seems possible that the CK signaling-like two-component signaling system functions in a CK-independent manner in the ancestral charophyte algae clade.

Key words: cytokinin; charophyte green algae; phytohormone profile; biosynthesis; signaling; CHK; *Physcomitrella patens*.

1. Introduction

The plant hormone cytokinins (CKs) are a class of N^6-substituted adenine derivatives with either an isoprenoid or an aromatic side chain (Frébort *et al.*, 2011; Mok, 2001). They are involved in multiple physiological processes including plant growth regulation, development as well as response to biotic and abiotic stress (Kieber and Schaller, 2018; Riefler *et al.*, 2006; Tran *et al.*, 2007). A wide spectrum of CKs occurs ubiquitously in the plant kingdom. The most abundant, naturally occurring CKs are isoprenoid types such as N^6-(Δ^2-isopentenyl) adenine (iP), trans-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DHZ) appearing as free bases, ribosides, nucleotides, and *O*-/*N*- glucosides. However, the naturally occurring aromatic CK types have only been detected in few plant species of genera such as Corylus and Populus (Gentile *et al.*, 2017; Jaworek *et al.*, 2019a; Strnad *et al.*, 1997). CKs function at very low concentrations (10^{-6} to 10^{-9} M) in plants (Kieber and Schaller, 2014). The free base and nucleoside CKs are considered as the bioactive forms with high affinities to CK receptors (Romanov *et al.*, 2005; Sakakibara, 2006). The composition of the CK pools varies greatly between species, tissues, and growth stages (Frébort *et al.*, 2011). For example, iP and tZ are the major CK types in the CK pools of vascular plants while the group of cZ type CKs is one of the most predominant compounds in some chlorophyte green algae (Stirk *et al.*, 2013; von Schwartzenberg *et al.*, 2007; Zhang *et al.*, 2014; Žižková *et al.*, 2017). The CK profiles in plants are affected by several aspects including biosynthesis, interconversion, inactivation, degradation, and transport (Mok, 2001; Sakakibara, 2006).

The rate-limiting step in the biosynthesis pathway of CKs is catalyzed by isopentenyltransferases (IPTs). IPTs diversify into two types namely adenylate IPTs and tRNA-IPTs, based on their nucleotide substrates (Kakimoto, 2001; Miyawaki *et al.*, 2006). The former enzymes are responsible for the synthesis of iP- and *t*Z-type CKs while the latter for *c*Z type. In *Arabidopsis thaliana*, the genes encoding both types of IPTs are expressed in almost every part of the plant although with different tissue preferences (Miyawaki *et al.*, 2004). Similarly, the riboside 5'-monophosphate phosphoribohydrolases (LONELY GUYs, LOGs) for CKs activation and the CK oxidase/dehydrogenases (CKXs) for CKs degradation are encoded by multiple gene families and expressed in plants (Kurakawa *et al.*, 2007; Werner *et al.*, 2003). As signal molecules, CKs are transported intercellularly or translocated between different tissues in long distance. To date, the purine permeases (PUPs) and equilibrative nucleoside transporters (ENTs) have been

characterized as influx transporters whereas the ATP-binding cassette transporter G subfamily member, ABCG14 as an efflux transporter of CKs (Bürkle *et al.*, 2003; Ko *et al.*, 2014; Sun *et al.*, 2005; Zhang *et al.*, 2014). The CK signal perception and transduction pathways in land plants are similar to the bacterial two-component regulatory system (TCS). It relies on the multistep phosphorylation among three proteins: the histidine kinase receptors with a sensory extracellular (CHASE) domain for binding the ligand, and two downstream members including the histidine phosphotransfer proteins (HPTs) and type-B response regulators (RRBs) (Hwang *et al.*, 2012; Kieber and Schaller, 2018; To and Kieber, 2008). The RRBs are transcription factors regulating the transcription of CK responsive genes, among which the type-A RRs (RRAs) act as negative regulators in the CK signal transduction pathway (Kiba *et al.*, 2003; Yokoyama *et al.*, 2007).

As ancient molecules existing ubiquitously, CKs have drawn more and more interest in evolutionary studies, which are seeking for the evolutionary path of the regulatory machinery of these signaling molecules as well as for their possible roles in the transition from single-cellular to multicellular growth and the adaptation from aquatic habits to land. The biology of CKs and the regulatory system are studied in meticulous detail in the flowering plant model *A. thaliana.* In other models such as the bryophytes *Physcomitrella patens*, and *Marchantia polymorpha* our understanding is advancing but still fragmented (Aki *et al.*, 2019; Pils and Heyl, 2009; von Schwartzenberg *et al.*, 2016).

Although important conclusions can be drawn from bioinformatic analysis based on comparative transcriptomics and genomics, detailed functional confirmations are still essential. Regarding metabolism, it is noteworthy that several enzymes involved in the interconversions among different CK metabolites are not only CK specific but also have important roles in purine metabolic pathways. The enzymes such as nucleoside ribohydrolase (NRH), purine-nucleoside phosphorylase (PNP), adenosine kinase and adenosine phosphoribosyl transferase mainly catalyze the conversion between purine bases, nucleosides and nucleotides (Chen and Petschow, 1978; Kopečná *et al.*, 2013). Here, we focus on the enzymes involved in CK biosynthesis and signaling pathways, especially IPTs and CHKs, of which the evolution has frequently been investigated. Studies on seed plants revealed two classes of tRNA-IPTs, one is closely related to bacteria tRNA-IPTs (AtIPT2 and 9) and the other to adenylate-IPTs (AtIPT1, 3–8), were possibly acquired by the plant *via* horizontal gene transfer (HGT) from cyanobacteria

(Frébort *et al.*, 2011; Lindner *et al.*, 2014; Nishii *et al.*, 2018). Functional adenylate-IPTs have so far only been described for seed plants.

Concerning the origin of the plant CK signaling pathway, it was originally thought to be restricted to land plants, since no canonical CHASE domain-containing HK receptors were found in some chlorophyte green algae (Gruhn *et al.*, 2014; Lu *et al.*, 2014). However, the newly published genomes of charophyte green algae (Cheng *et al.*, 2019; Hori *et al.*, 2014; Nishiyama *et al.*, 2018) revealed the occurrence of CHK homologs. Moreover, the identification of CK receptors in phytopathogenic bacteria (Wang *et al.*, 2017) indicates the possibility of a broader origin of the CK signaling machinery (Kabbara *et al.*, 2018). Taken together, investigations on the phytohormone-like CK system in species representing an evolutionary situation prior to transition to land will expand our knowledge about this phytohormone and its evolution.

In this study, we focus on the charophyte green algae, which possess a key position with respect to land plant evolution. Firstly, the intracellular and extracellular profiles of CKs in several charophyte algal species were monitored over different growth stages providing the first insight into the existence of putative CK metabolic and transport pathways. Then we performed a deep bioinformatics analysis of the major proteins involved in CK metabolism, transport, and signaling by using genome/transcriptome datasets with plant and algal species from three major evolutionary clades, thus inferring evolutionary patterns of each protein. Based on the findings that the plant-like two-component signaling system might first have appeared as a complete archetype in charophyte green algae, conservation of the CK receptor was investigated through phylogenetic trees and domain analysis. Finally, two CHASE domain-containing histidine kinases (CHKs) were cloned from *Spirogyra pratensis,* which belongs to the closest evolutionary clade of land plants (Zygnematophyceae). For functional studies based on overexpression, two SpCHKs were introduced into a CHK deficient mutant of *Physcomitrella patens* (von Schwartzenberg *et al.*, 2016) by means of gene replacement. Our work provides further understanding of the complexity of the origin and evolution of the CK system and indicates a possible multifunctionality of the CHASE domain-containing histidine kinases.

2. Results

2.1 Charophyte green algae produce cytokinins

To investigate the occurrence of CKs in the charophyte green algae, the lineage from which the monophyletic clade of land plants is nested (Leebens-Mack *et al.*, 2019), we utilized five axenic algal strains from the Microalgae and Zygneatophyceae Collection Hamburg (MZCH) (von Schwartzenberg *et al.*, 2013; Zhou and von Schwartzenberg, 2020) for the establishment of CK profiles. From the class of Zygnematophyceae, which is phylogenetically closest to land plants (Leebens-Mack *et al.*, 2019; Wickett *et al.*, 2014), the unicellular *Cosmarium crenatum* var. *boldtianum* (Cc), *Micrasterias radians* var. *evoluta* (Mr) (both belonging to Desmidiales) and the filamentous species *Spirogyra pratensis* (Sp) belonging to the class of Zygnematales, were chosen. From the earlier diverging classes, we chose *Klebsormidium nitens* (Kn) (Klebsormidiophyceae) and *Mesostigma viride* (Mv) (Mesostigmophyceae).

The profiles of intracellular and extracellular CK metabolites were measured over a growth period of 22 days by ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-MS/MS).

Total amount and distribution of intracellular CKs

The hormone profiling revealed a wide spectrum of isoprenoid CKs in the five algal species. The total amount of intracellular CKs varied with the age of the culture and ranged from 19 to 7168 pmol g^{-1} dry weight (DW) (Tab. S1). The intracellular content of CKs in *C. crenatum* was the highest at the beginning of cultivation but dropped significantly in the following growth stages. On the contrary, the CK content in *M. radians* var. *evoluta* was relatively stable throughout the growth period. The levels of total CKs in the other three algal species increased after cultivation for 13 days. However, ongoing cultivation led to different courses of the content of total intracellular CKs in the three strains: 1) in *S. pratensis*, the total CK content remained nearly unchanged; 2) in *K. nitens,* it decreased to a level similar to that of the initial stage; 3) in *M. viride*, it increased over time (Tab. S1).

In general, iP- and *c*Z-type CKs were the predominant components of the CK pools of all investigated algae while *t*Z- and DHZ-types occurred only at very low or undetectable levels (Fig. 1A; Tab. S1). All types of CKs mainly presented in the forms of free bases and nucleosides as well as CK nucleotides in all samples at different growth stages. The most

abundant CK forms in *C. crenatum* and *M. radians* var. *evoluta* cells were the *c*Z nucleotides (*c*ZRMP) while the iP-type CKs were detected mainly in free bases and nucleosides in all samples of the two algae except for the *C. crenatum* at T0 stage (Fig. 1 A1-A2). The *S. pratensis* and *M. viride* cells contained the similar levels of free bases and nucleosides at the beginning, the early growth phase saw an acumination of all three forms of iPs and cZs but mainly in the forms of iP-bases and *c*Z-nucleotides. Both strains produced a huge amount of free bases of either iP-type (*S. pratensis*) or both iP- and *c*Z-types (*M. viride*) during the growth (Fig. 1 A3, A5). The CK conjugates such as glucosides were not detectable or detected only in trace amounts among the five analyzed algal species. For example, the CK-*N*-glucosides of DHZ were found in the *C. crenatum* samples at T0 stage at a concentration as low as 7 pmol g^{-1} DW (Tab. S1).

Distribution of extracellular CKs

The CK profiling of the algal culture medium at different sampling times for all five species revealed that the CK metabolites steadily accumulated over the cultivation period (Fig. 1B; Tab. S1). The composition patterns of the extracellular isoprenoid CK pools were similar to that of the corresponding algal cells (Fig. 1 B1-B5). In the culture medium of *C. crenatum* (Fig. 1 B1) and *M. radians* var. *evoluta* (Fig. 1 B2), both the free bases and nucleotides of iP- and cZ-type CKs accumulated over time. However, in the medium of *S. pratensis* (Fig. 1 B3), only the bioactive forms especially the free base of iP- and *c*Z-type CKs were found increasing during the algal growth. Different from its intracellular CK profile, *S. pratensis* accumulated a huge amount of *c*Z bases in its extracellular CK pool. In *K. nitens* (Fig. 1 B4) and *M. viride* (Fig. 1 B5), the cZ bases of was dominating while the nucleosides of iP-type CKs occurred also at significant amounts, for example, the culture medium of *K. nitens* contained a higher concentration of the iPR than the iP bases. The contents of extracellular tZ- and DHZ-type CKs were close to the detection limit in all five algae and generally remained unchanged throughout the sampling period in the medium, except for *S. pratensis* and *M. viride*, where an increase of *t*Z bases was found (Fig. 1 B1-B5).

Taken together, the iP- and cZ-type CKs were the predominant components in both the intracellular and extracellular CK pools of the investigated charophyte green algae whereas a very low amount of tZ-type CKs was presented. The free base, nucleoside and nucleotide forms of the two CK types accumulated in the culture medium of *S. pratensis* and *K.nitens* to a level that is higher than the estimated intracellular CK concentrations (Fig. S1), suggesting the possible existence of an active efflux transportation mechanism

in these species. The increase of the CK bases and nucleosides especially the iP and cZ bases, in the CK pools of *S. pratensis, K.nitens* and *M. viride* pointed to a highly active CK biosynthesis mechanism.

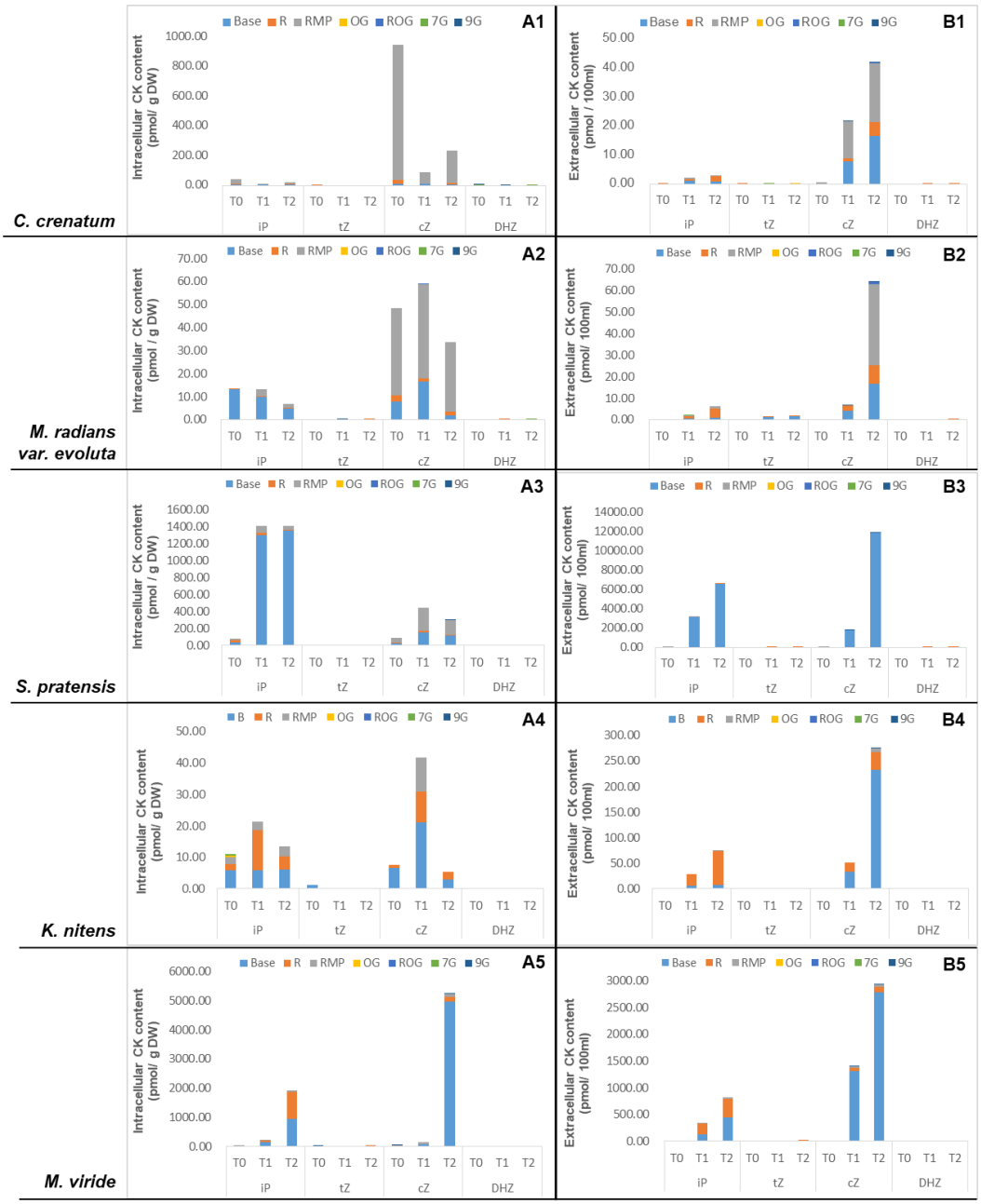


Fig. 1: The CK profiles within the cells (A1-5) and in corresponding culture medium (B1-5) of five axenic charophyte green algal species (*C. crenatum*, *M. radians* var. *evoluta*, *S. pratensis*, *K. nitens* and *M. viride*) at three time points (T0– starting point, T1– 13 days, T2– 22 days). Intracellular levels of CKs (A1-5) are shown in pmol g^{-1} DW and CK concentrations in the medium (B1-5) are shown

in pmol per 100 ml. CK types and conjugates are labeled according to the following abbreviations: Base, free bases; R, ribosides; RMP, nucleotides; OG, O-glucosides; ROG, riboside O-glucosides;7G,N^7-glucosides; 9G, N^9-glucosides.

2.2 Charophyte green algae have a lean homologous genetic chassis for cytokinin metabolism, transport, and signaling

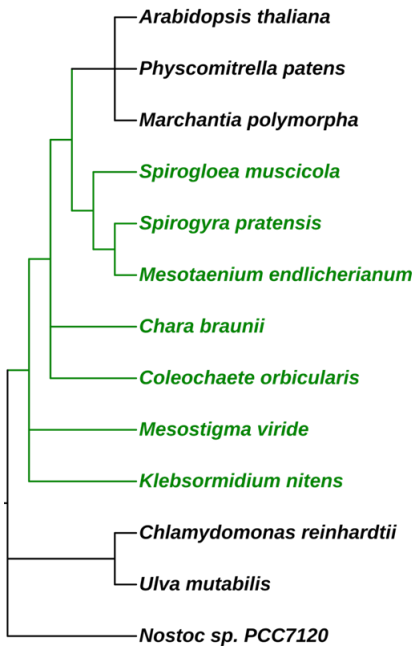
To understand the evolution of the chassis that is required for utilizing CK as a phytohormone, we explored its occurrence across the green lineage. For this, we mined genomic/transcriptomic data of 12 representative species of Chloroplastida (Adl *et al.*, 2019; Leliaert *et al.*, 2012) and *Nostoc* as an outgroup (Kaneko *et al.*, 2001). Given our focus on charophyte green algae, the publically accessible genome data of the early diverging species *Klebsormidium nitens* (Hori *et al.*, 2014) as well as the later diverging *Chara braunii* (Nishiyama *et al.*, 2018), *Spirogloea muscicola* and *Mesotaenium endlicherianum* (Cheng *et al.*, 2019) were used for analysis. The transcriptome data of *Spirogyra pratensis*, *Coleochaete orbicularis* and *Mesostigma viride* (Cooper and Delwiche, 2016) were additionally included. The model species *Arabidopsis thaliana*, *Marchantia polymorpha,* and *Physcomitrella patens* were included as reference points (Berger *et al.*, 2016; Mok, 2001; Rensing *et al.*, 2008). The multicellular *Ulva mutabilis* and the unicellular *Chlamydomonas reinhardtii* represented strains for the chlorophyte green algae (De Clerck *et al.*, 2018; Harris, 2001). Similarity searches were carried out by using the *A. thaliana* proteins involved in CK metabolism, transport, and signaling pathways as BLAST queries. The BLAST hits were identified for conserved domain arrangements with the Pfam database.

Two enzymes are key to the biosynthesis and activation of CKs: isopentenyltransferases (IPTs) and riboside 5'-monophosphate phosphoribohydrolase (LOG). The IPTs catalyze the first rate-limiting step, adding an isopentenyl group to the adenine ring (Kakimoto, 2001) whereas the LOGs catalyze the conversion of inactive CK nucleotides to hormonally active free bases (Kurakawa *et al.*, 2007). There are two types of IPTs, ATP/ADP IPTs (AtIPT1, 3–8) and tRNA IPT (AtIPT2 and 9) in Arabidopsis. Homologs of tRNA-IPTs were found in all species being investigated except for *S. muscicola* (Fig. 2), whereas the adenylate IPTs limited to flowering plants and cyanobacteria which aligns with previous findings (Frébort *et al.*, 2011; Lindner *et al.*, 2014; Nishii *et al.*, 2018). Unlike land plants, the tRNA-IPT appeared to be encoded by a single-copy gene in both charophyte and chlorophyte green

algae. The phylogenetic analysis indicated that all the algal tRNA-IPTs grouped with the previously functionally identified PpIPT1 (Lindner *et al.*, 2014) and cyanobacterial alr5266 (Frébortová *et al.*, 2017), which are classified to the class I tRNA-IPTs (Fig. S2) (Nishii *et al.*, 2018). Putative LOGs were ubiquitously present, although at a low level of gene copies in green algae and the most basal land plant. The CK oxidase/dehydrogenases (CKXs) catalyze the irreversible inactivation of CKs *via* oxidative cleavage of the side chain. We found no match for a CKX through BLAST searches in any DNA data from both green algae lineages (Fig. 2). The *N.* sp 7120 genome encodes a CKX-like protein, which has been shown none functional in CK degradation (Frébortová *et al.*, 2015). For CK transport, proteins from both ENT and PUP families are functional for CK influx while the AtABCG14 has been shown functional for CK efflux (Bürkle *et al.*, 2003; Hirose *et al.*, 2008; Sun *et al.*, 2005; Zhang *et al.*, 2014). We found ENTs homologs in all other investigated organisms but with fewer gene duplications compared to land plants. However, the PUP transporters seem to only occur in *A. thaliana* and *M. polymorpha* (Fig. 2). The AtABCG14 belongs to the conserved superfamily of ATP-dependent binding cassette (ABC) transporters, which widely exist in all lineages (Dean and Annilo, 2005) including the green algae (data not shown).

Previous analysis of genomes of several charophyte green algae revealed the existence of some putative CK signaling components (Cheng *et al.*, 2019; Hori *et al.*, 2014; Nishiyama *et al.*, 2018). Here, we included more species for the investigation of the presence of all the four members of the CK signaling pathway. As shown in Fig. 2, CHKs (CHASE domain-containing HKs), which are homologous to the functional CK receptors (von Schwartzenberg *et al.*, 2016), were found in all investigated species except for the two chlorophyte green algal species that encode the CHASE domain-containing proteins but not in the form of hybrid HKs. All the charophyte algal species except for *M. viride* contain at least two genes encoding CHKs. Putative HPTs have been found in *M. polymorpha* and all the green algal species and to be encoded only by a single-copy gene. This finding points to the first expansion of the HPTs in the basal land plants. Similarly, the RR homologs seem to present in all the investigated algae except for the later diverging *C. braunii* (Fig. 2). Most of the putative algal RRs clustered to the functional clade of RRBs while two single-copy homologs from *S. pratensis* and *K. nitens* appeared as members of the RRA clade (Fig. S3). It is noteworthy that the *S. muscicola*, *M. endlicherianum,* and *C. orbicularis*, which are considered as earlier evolutionary organisms than the *M. polymorpha* contain one more gene individually for encoding the

RRBs. Taken together, the results showed the possible occurrence of a complete CK signal regulation system in the charophyte green algae.



	Biosynthesis		Degradation	Transport		Signaling			
	IPT	LOG	CKX	ENT	PUP	CHK	HPT	RRB	RRA
Arabidopsis thaliana	9	9	7	8	3	3	5	11	11
Physcomitrella patens	7	9	6	4	0	11	2	5	7
Marchantia polymorpha	2	1	2	6	1	2	1	1	1
Spirogloea muscicola	0	1	0	3	0	4	1	2	0
Spirogyra pratensis	1	1	0	1	0	2	1	1	1
Mesotaenium endlicherianum	1	1	0	3	0	3	1	2	0
Chara braunii	1	1	0	2	0	2	1	0	0
Coleochaete orbicularis	1	1	0	1	0	2	1	2	0
Mesostigma viride	1	1	0	2	0	1	1	1	0
Klebsormidium nitens	1	1	0	2	0	7	1	1	1
Chlamydomonas reinhardtii	1	1	0	2	0	0	1	1	0
Ulva mutabilis	1	1	0	1	0	0	1	1	0
Nostoc sp. PCC7120	2	1	1	0	0	1	0	0	0

Fig. 2: Distribution of genes coding for proteins involved in CK biosynthesis, degradation, transport, and signaling pathway in the green lineage. The number of putative protein-coding sequences is shown. The green font marked the species belonging to charophyte green algae. Abbreviations used: IPT, adenine or tRNA isopentenyltransferase; LOG, riboside 5'-monophosphate phosphoribohydrolase; ENT, equilibrative nucleoside transporter; PUP, purine permease; CKX, CK oxidase/dehydrogenase; CHK, CHASE- containing histidine kinase; HPT, His containing phosphotransfer; RRA, type-A response regulator; RRB, type-B response regulator. All the gene identifiers as well as their reciprocal BLASTP e-values are listed in Tab. S2.

2.3 The algal CHKs are phylogenetically related to CK receptors but divergent in residues for CK binding

Homologs of the CHASE-containing HKs present in all investigated plants and charophyte green algae (Fig. 2 and Tab. S2). These algal CHKs showed the similar domain

architectures as the plant CK receptors, which comprise the extracellular ligand-binding CHASE domain (PF03924), the HisKA domain (PF07730), HATPase C domain (PF02518) and the response regulator receiver domain (REC, PF00072) (Fig. S4). The only exceptions were Co26630 from *C. orbicularis* and Mvcomp38356_c2_seq1 from *M.* viride, of which the C-terminal REC domain is missing. This can be explained by the fact that the corresponding sequence was retrieved from transcriptome not covering the full length of all genes. In contrast, the deduced Nsall2875 protein form *N.* sp. 70120 possesses not only the four mentioned domains, but also multiple PAS domains (PF00989) and one Hpt domain (PF01627). The Nsalr3761 shares a similar domain arrangement as the Nsall2875 but contains a CHASE3 domain (PF05227) instead of the CHASE domain (PF03924) in its *N*-terminus.

To get insight into the phylogenetic relationship of these algal CHKs, the CHASE and REC domain sequences have been employed for phylogenetic analysis. The maximum likelihood tree for the CHASE domain showed a distribution into three major clades (Fig. 3A). The distribution of the land plant CHASE domains shows the same topology as published previously by Gruhn *et al.* (2014) with one clade for the classical CK receptors from *A. thaliana* and *P. patens*, and the other clade with the new subfamily of eight CHKs from *P. patens* as well as two CHKs from *M. polymorpha*. The latter displays a low level of conservation compared with the CHASE domain of AHK4.

The CHASE domains from the algal CHKs are found in all clades. The later diverging charophyte algae, including *C. braunii*, *S. muscicola*, *M. endlicherianum*, *S. pratensis,* and *C. orbicularis*, contain at least one CHASE domain closely related two the classical CK receptors (Fig. 3A). Most of the CHASE domains from the CHKs of the earlier diverging alga *K. nitens* clustered with the less conserved subfamily of the basal-plant CHASEs as well as CHASE domains from SM000010S04334 of *S. muscicola* and Sp1900 of *S. pratensis*. The third clade contained CHASE domains from CHKs of cyanobacteria and charophyte algae (Fig. 3A). The CHASE3 domain from Nsalr3761 of *N.* sp. 7120 was shown as outgroup.

The maximum likelihood tree for the REC domains exhibited a different topology than that for the CHASE domains (Fig. 3B). The main difference is that nearly all REC domains from charophyte algae are clustering with those from plant land plants, except for Mvcomp38356c2 seq1 (*M. viride*) and kfl000150240 (*K. nitens*).

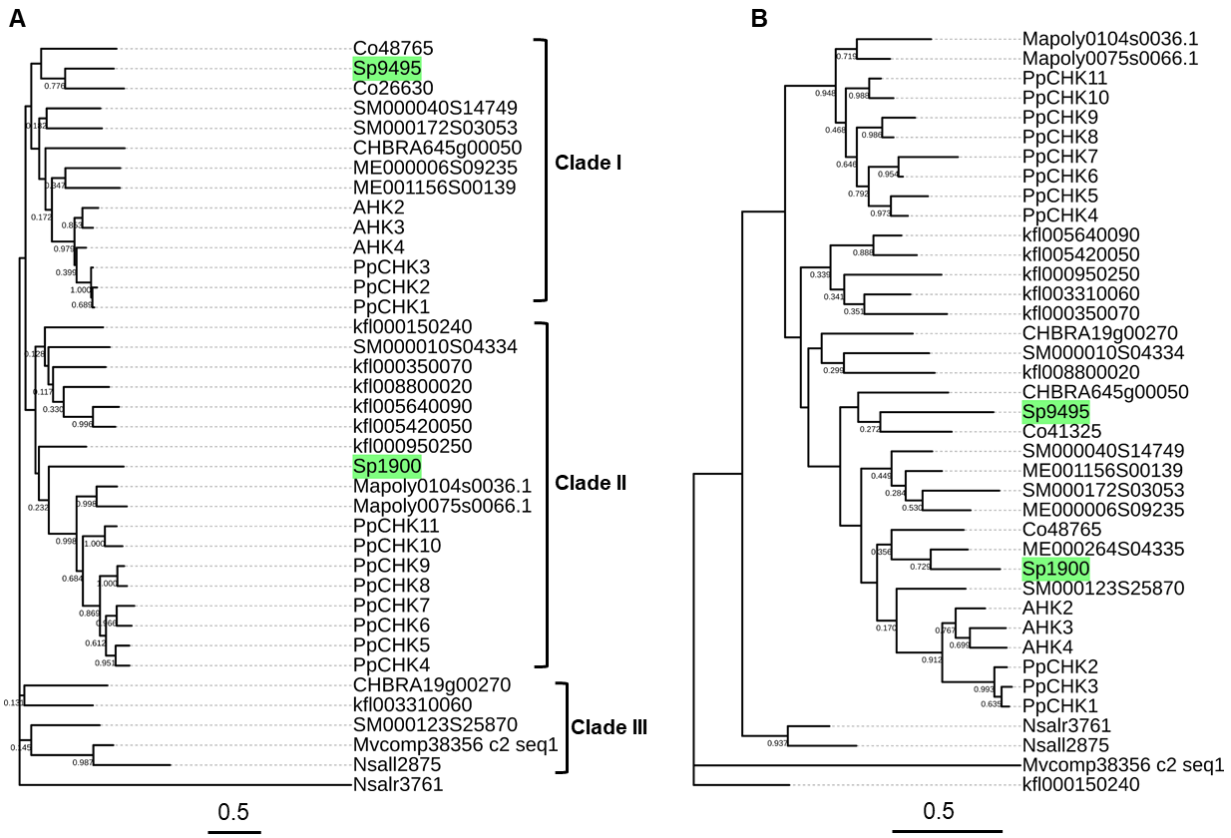


Fig. 3: Phylogenetic trees constructed based on the CHASE domain (A) and the REC domain (B) of CHK proteins. All the sequences were aligned with MAFFT and the maximum likelihood trees were inferred with the MEGAX program. The labels with green background marked the CHKs from *S. pratensis*. Abbreviations: Pp, *Physcomitrella patens*; Mapoly, *Marchantia polymorpha*; Sm, *Spirogloea muscicola*; Me, *Mesotaenium endlicherianum*; Sp, *Spirogyra pratensis*; Chbra, *Chara braunii*; Co, *Coleochaete orbicularis*; Kf, *Klebsormidium nitens*; Mv, *Mesostigma viride*; Ns, *Nostoc* sp. PCC7120.

Interestingly, the two CHKs from *S. pratensis* share similar REC domains (Fig. 3B), but diverge in their CHASE domains, which clustered in different clades (Fig. 3A). In the following, focus was set on *S. pratensis* CHKs as among the organisms studied, this is the species most closely related to land plants encoding for the complete set of putative components of the CK signaling pathway (Fig. 2). To investigate the conservation of the SpCHKs, homology models were generated based on the available CHASE domain crystal structures of AHK4 protein (Hothorn *et al.*, 2011). The CHKs from the basal land plants *P. patens* and *M. polymorpha* were also included for comparison. The results indicated that the CHASE domains share a similar structural topology with that of AHK4 (Fig. 4A). All CHASE domains contain AHK4-like ligand-binding pockets. On the sequence

level, the CK-binding residues of the classical CK receptors showed a high degree of conservation to AHK4, while the CHK sequences of *M. polymorpha* and *S. pratensis* are found to be more divergent at several positions relevant for CK binding (Fig. 4B). Among the four less conserved CHKs, the Sp9495 contains the conserved hydrophobic Leucine residue (Leu284 of AHK4), which is critical for interactions with the adenine moiety of CK molecule mediated by water molecules (Hothorn *et al.*, 2011).

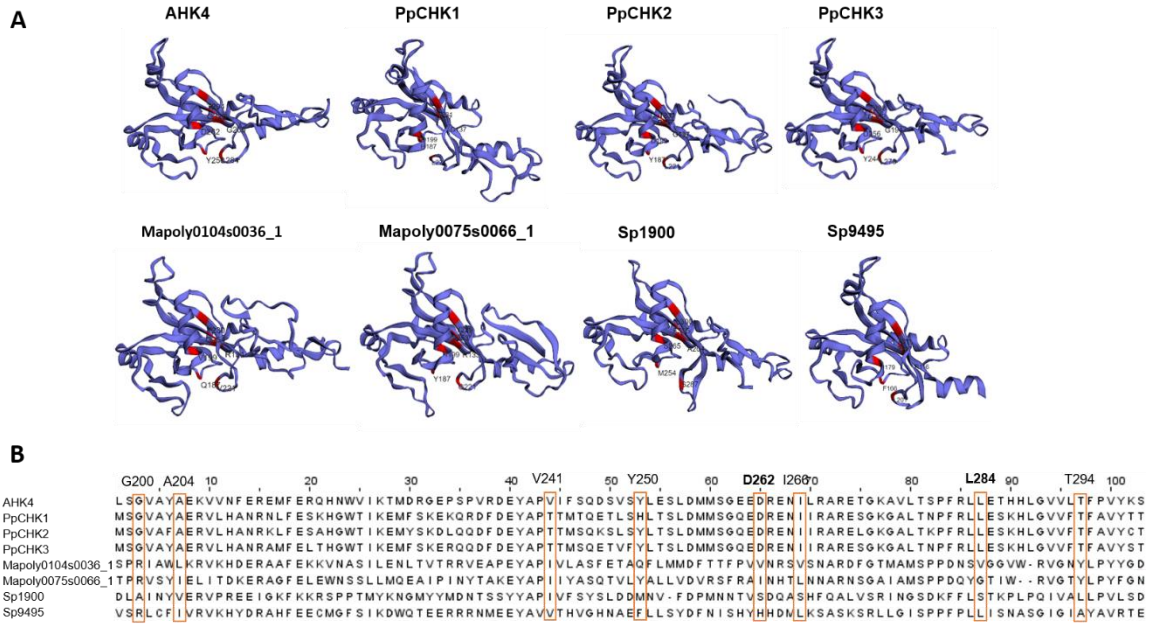


Fig. 4: (A) Homology models for CHASE domains of CHKs based on the AHK4 crystal structure (Hothorn *et al*. 2011). The 3D structures are shown with important residues for cytokinin binding (red marks). Abbreviations: Pp, *Physcometrella patens*; Mapoly, *Marchantia polymorpha*; Sp, *Spirogyra pratensis*. (B) Alignment of CHASE domains of CHKs. Different CHASE sequences were pairwise aligned with that of AHK4 separately. Orange boxes indicate reference residues of AHK4 interacting with iP in crystal structure.

2.4 The two CHKs from *Spirogyra pratensis* showed different affinities to cytokinin and did not activate CK response in the CK receptor mutant of *Physcomitrella patens*

In vivo *CK binding assay with SpCHK1 and SpCHK2*

The functionality of the CHKs from *S. pratensis* was further analyzed in depth. Open reading frames (ORFs) of the two SpCHKs, namely *SpCHK1* (Sp1900) and *SpCHK2*

(Sp9495), were amplified from *S. pratensis* cDNA by PCR. A single pair of primers has been used for the amplification of *SpCHK1* and two pairs of nested primers for *SpCHK2*. The *SpCHK1* and *SpCHK2* were cloned to the vector pINIII-4xMyc and expressed in *E. coli* for the cytokinin binding assay (Romanov *et al.*, 2005; Yamada *et al.*, 2001). The AHK4 and the empty vector were respectively employed as positive and negative controls. Bacterial cultures were first incubated with 50 nM tritium labeled *t*Z ([3H]*t*Z; 1.3 TBq mmol^{-1}) alone and in the presence of a saturating concentration (10 µM) of unlabeled *t*Z that compete for the binding site of CHKs and effectively reduce the binding of labeled tZ (see Fig. 5A). Unlike the positive control, the AHK4, which showed a high affinity to tZ, the SpCHK1 and SpCHK2 were not detected for any tZ binding activities since they showed similar signal intensities with the background. Similar experiments were done with different concentrations of tritium labeled iP alone and competition with a saturating concentration (50 µM) of unlabeled iP. The calculated specific binding curves have been shown in Fig. 5B. The results indicated that the radiolabeled iP did not specifically associate with SpCHK1 as it displayed a similar level of [^{3}H]iP-binding affinities with the negative control. In contrast, the SpCHK2 was found to bind iP at high concentrations in a dose-dependent manner, but its affinity to iP is much lower than that of AHK4 (disassociation constant K_D = 6.88 nM).

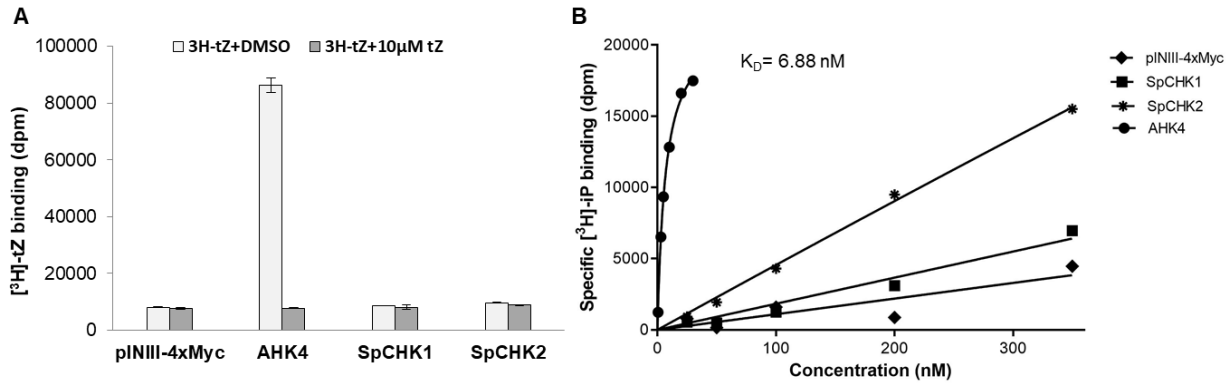


Fig. 5: Bacterial *in vivo* binding assay of SpCHK1 and SpCHK2 with [^{3}H]*t*Z (A) and [^{3}H]iP (B) as ligand. *E. coli* harboring the empty vector, or constructs expressing AHK4, SpCHK1 and SpCHK2 were analyzed in presence of DMSO (grey bar) or unlabeled10 µM *t*Z (dark grey bar) or 50 µM iP as competitors.

Generation of SpCHK1 and SpCHK2 overexpression lines with the PpΔchk1,2 mutant of moss

Due to the lack of genetic tools in charophyte algae, CHK mutants of moss *P. patens* were chosen for complementation studies in order to investigate the *in planta* function of *Spirogyra* CHKs (SpCHK1 and SpCHK2). The moss lines were generated in a previous study (von Schwartzenberg *et al.*, 2016) and are partially or completely affected in CK perception. Complementation assays of the CK receptors on the background of the double mutant PpΔchk1,2 were carried out by a knock-in expression of the SpCHKs at the *Ppchk3* locus. The *SpCHK1* and *SpCHK2* genes were expressed separately under control of the constitutive *Actin1* promoter from *Oryza sativa* in the gene replacement construct, harboring the expression cassette with the zeocin resistance gene (*ble*), which was flanked by the 5' and 3' sequences of the *Ppchk3* gene for targeted recombination into the *Ppchk3* gene locus (von Schwartzenberg *et al.*, 2016). Detailed information about the generation of the constructs is shown in Fig. S4. The constructs were introduced into the PpΔchk1,2 strain by the PEG mediated protoplast transformation. Stable transformants were selected with zeocin and characterized by PCR-based approaches following the strategy described in material and methods. The results (see Fig. 6) showed that the *Ppchk3* gene was knocked out and no transcript could be detected meanwhile the *SpCHK1* and *SpCHK2* were respectively integrated into the *Ppchk3* gene locus and their corresponding transcripts were detectable in the obtained SpCHKs expression lines (PpΔchk1,2,3-SpCHK1$_{ox}$ and PpΔchk1,2,3-SpCHK2$_{ox}$). The transcriptional levels of the two introduced *SpCHK* genes were similar to that of the original *PpCHK3* gene.

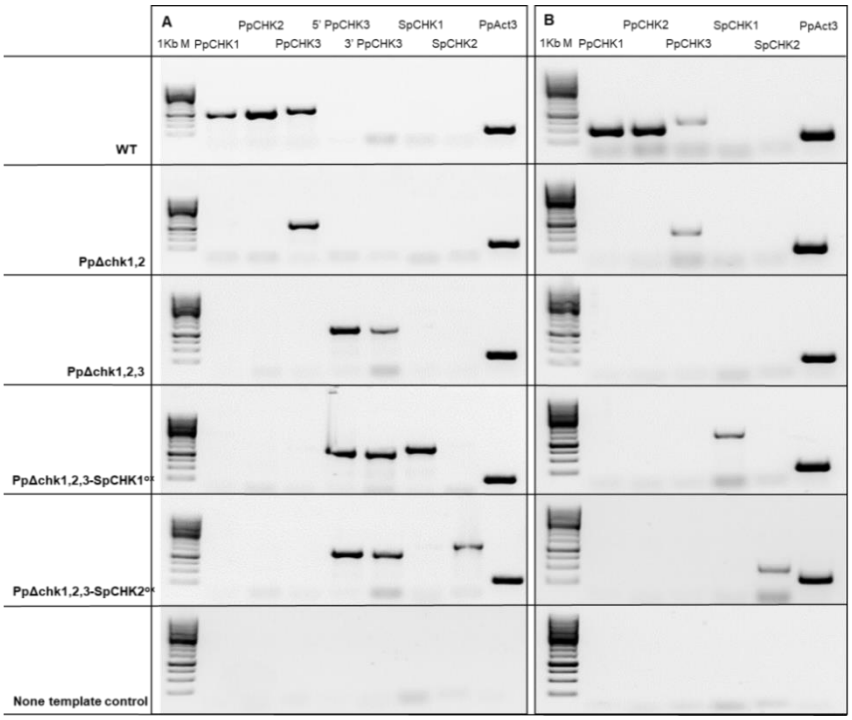


Fig. 6: PCR (A) and RT-PCR (B) characterization of the SpCHKs expression lines. Genomic DNA from the colonies and cDNA generated from total RNA from liquid protonema culture of moss lines were used as templates. Primers employed for testing of the presence/absence of the PpCHK genes as well as the recombination at both sides of PpCHK3 (PpCHK1: P505/P590; PpCHK2: P346/P591; PpCHK3: P316/P317; 5´ PpCHK3: P316/P361, 3´ PpCHK3: P364/P365) were from von Schwartzenberg *et al.* (2016). Primers for amplifying the two SpCHK transcripts (SpCHK1-P-F/SpCHK1-P-R and SpCHK2-P-F/SpCHK2-P-R) were designed within the ORF regions. Sequence information of all primers is listed in Tab. S3.

Phenotype and cytokinin responses of the SpCHK1 and SpCHK2 expression lines

Phenotype and the CK responses were analyzed in the new moss lines PpΔchk1,2,3-SpCHK1$_{ox}$ and PpΔchk1,2,3-SpCHK2$_{ox}$, as well as in controls including the WT, PpΔchk1,2, and PpΔchk1,2,3. Protonemata of the five moss lines were inoculated on KNOP agar medium in presence or absence of benzyladenine (BA) and iP individually. As shown in Fig. 7A, after three-week growth in the absence of external CKs, all the five moss lines developed into colonies with similar size. However, the colonies of WT and PpΔchk1,2 displayed buds and gametophores in the inner parts and undifferentiated protonema in the outer parts. The PpΔchk1,2,3-SpCHK1$_{ox}$ and PpΔchk1,2,3-SpCHK2$_{ox}$ colonies displayed mainly protonemal growth, which was similar to that of the triple mutant PpΔchk1,2,3. The WT and PpΔchk1,2 showed obvious CK response which led to the

reduction of the protonemal growth upon cultivation with 1 µM BA or 1 µM iP. In contrast, the growth of the moss lines expressing the SpCHKs as well as the PpΔchk1,2,3 triple mutant was not affected by the high dose of CKs, indicating that these lines were insensitive to the CK signals. Since bud formation of WT, PpΔchk1,2, and PpΔchk1,2,3 differed to exogenously applied CKs (von Schwartzenberg *et al.*, 2016), we further carried out the same bioassay to assess whether the *Spirogyra* CHKs expression affected the budding response. After 10 days of growth with 400 nM BA or iP, the samples were observed with the microscope and the number of buds was counted (Fig. 7B). No buds found in the SpCHKs expression lines as well as the PpΔchk1,2,3 triple mutant. In summary it is concluded that neither phenotype analysis nor budding assay pointed to a CK receptor function of SpCHK1 and SpCHK2 in the *Physcomitrella* complementation assay.

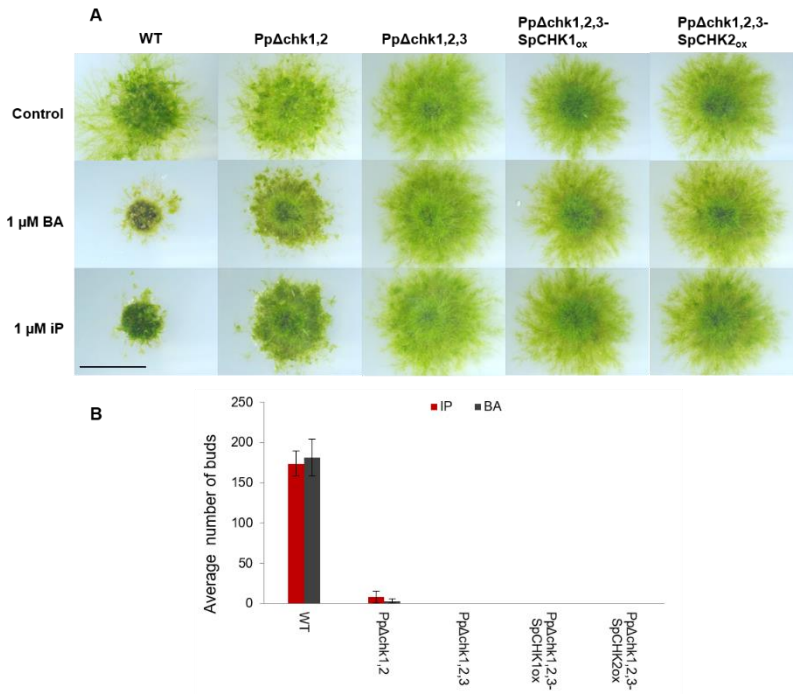


Fig. 7: Functional analysis of SpCHK1 and SpCHK2 in CK receptor deficient mutant of moss. (A) Growth response of the wild type (WT), double, and triple mutants of *P. patens* together with the PpΔchk1,2,3-SpCHK1$_{ox}$ and PpΔchk1,2,3-SpCHK2$_{ox}$ lines. These strains were cultured on KNOP agar plates with no exogenous CK (control), 1 µM BA or 1 µM iP, respectively. The scale bar is 1 cm. (B) Budding response of the five different genotypes to CKs (mean values and SD). Protonema were evenly distributed and cultured on KNOP agar plates supplemented with 400 nM iP or BA for 10 days. The number of buds was counted from 10 microscopic view fields. Three different biological replicates were counted for each strain.

3. Discussion

Since the first purification of natural occurring zeatin from *Zea mays* (Letham, 1963), dozens of CK metabolites were quantified for their occurrence within a variety of organisms including land plants, algal species and cyanobacteria by HPLC combined with mass spectral identification (Jiskrová *et al.*, 2016; Stirk *et al.*, 2013; Zhang *et al.*, 2014; Žižková *et al.*, 2017). That said, information on CK metabolites and regulatory system for the grade of charophyte green algae, the algal relatives of land plants (Leliaert *et al.*, 2012) is scarce. Understanding of the roles of phytohomones in these algae is thus key information for the inference of the evolution of these regulatory systems. Some gaps with regard to the role of CKs were filled by this study.

The CK profiles of five fast-growing algal species belonging to three classes within Charophyta including Zygnematophyceae (3 strains), Klebsormidiaceae (1 strain) and Mesostigmatophyceae (1 strain) were analyzed and monitored for the production of endogenous CK over a period of three weeks. The CK content varied significantly among species ranging from tens to thousands of pmol g^{-1} DW and also differed in the samples cultured for different growth periods (Tab. S1) which is in line with fluctuations known from other groups of organisms (Stirk *et al.*, 2013; Žižková *et al.*, 2017).

The cells used for inoculation were washed and cultivated with fresh medium. Therefore, at the beginning of the cultivation process, the concentrations of both extracellular and intracellular CKs was low except for *C. crenatum*. Further cultivation led to the accumulation of CK bases and ribosides in *S. pratensis* and *M. viride* cells, while the intracellular CK levels of *M. radians* and *K. nitens* were relatively stable over time. The CKs accumulation in the culture medium might reflect the release and transport of excessive amounts of endogenous CKs, since the compositions of extracellular and intracellular CK pools were alike. Taken together, this speaks to an active biosynthesis of CKs in the investigated algae (except for *C. crenatum*) during the exponential growth stage as well as a functional CK transport across the cell membrane in *S. pratensis* and *K. nitens*, which was essential to release the intracellular CKs against the concentration gradient (Fig. S1).

In vascular plants, the majority of active CKs occurs as iP- and zeatin-types. The ratio between the two zeatin isomers varies between species and growth stages (Gajdošová *et al.*, 2011). For example, the young green leaves of *Arabidopsis* contain dominantly tZ-type

CKs while the leaves during senescence contain a higher level of cZ-type CKs (Gajdošová *et al.*, 2011). However, in the intracellular and extracellular CK pools of the five algal strains investigated in this study, the iP- and *c*Z-type CKs were the predominant components and only a small portion or trace amounts of *t*Zs were detected. A dominance of *c*Z-type CKs was also found in the basal land plant *P. patens* (von Schwartzenberg *et al.*, 2007), and most of the cyanobacteria and with few exceptions chlorophyte green algae (Stirk et al. 2013; Žižková et al. 2017). Moreover, within the CK pools of the five charophyte algae, more than 95 % of the different iP- and *c*Z-type CKs were found in form of free bases, ribosides, and nucleotides while the conjugated forms (glucosides) were close to the detection limit indicating an extremely low activity of corresponding enzymes. This represents an obvious difference to the land plants *A. thaliana* and *P. patens*, where the inactive CK conjugates occurr as dominant components in the profiles of endogenous CKs (von Schwartzenberg *et al.*, 2007; Zhang *et al.*, 2014).

In flowering plants, the level of active free CKs is regulated by rates of biosynthesis, conversion into storage forms, and degradation. The occurrences of proteins involved in these pathways in the green lineage would provide insights into the origin and fate of CKs in the earlier divergent species representing the evolutionary status before the conquest of land.

Homologous genes encoding proteins involved in the CK biosynthesis pathways, such as IPT and LOG, were found in cyanobacteria. It has been suggested that these proteins were acquired from the prokaryotic ancestors *via* horizontal gene transfer (Frébort *et al.*, 2011; Lindner *et al.*, 2014), which was confirmed by the recent studies showing the functional adenylate IPT and tRNA-IPT homologs in *N.* sp. PCC 7120 (Frébortová *et al.*, 2015; Frébortová *et al.*, 2017). All the investigated charophyte green algae contained a single gene encoding the IPT catalyzing the rate-limiting step in CK biosynthesis. These algal IPTs are exclusively homologs of tRNA-IPTs, which catalyze the addition of an isopentenyl moiety to the adenine at the position A37 of tRNA in the process of *cZ* biosynthesis in *A. thaliana* (Miyawaki et al. 2006) and *P. patens* (Lindner *et al.*, 2014). The dominance of cZ-type CKs might reflect the exclusive occurrence of tRNA-IPTs.

The LOG proteins also exist across the green lineage and rise in the number of genes encoded per gene family after the conquest of land (Frébort *et al.*, 2011). Genes encoding CKXs, which catalyze the irreversible inactivation of CKs, widely exist in genomes of land plants including earlier divergent species such as *P. patens* and *M. polymorpha*, and was

also detected in the cyanobacterium Nostoc sp. 7120. However, the *NoCKX1* gene does not encode an active protein functional in CK degradation (Frébortová *et al.*, 2015). Both the Chrarophyte and Chlorophyte green algae genomes do not contain any CKX homologs. Based on the predominance of *cZ* and the low content of CK glucosides in the algal CK pools, we interpret that the algae probably maintain a relatively stable intracellular CK activity, which is not tuned by degradation and glucolysation. When the cells accumulate an excess amount of CKs, these are efficiently released to the medium *via* selective transport mechanisms. In Arabidopsis, the AtABCG14 functions as an efflux transporter of CKs (Ko *et al.*, 2014; Zhang *et al.*, 2014) and belongs to the plasma membrane-localized ABC transporter superfamily. These transporter superfamilies widely exist in both eukaryotes and prokaryotes. It is hard to identify the exact CK specific transporter in the algae only based on the sequence information without experimental evidence. Uptake of exogenous supplied CKs was only observed by treatments of algae cells with a high dose of radiolabeled *t*Z (Žižková *et al.*, 2017). The ENT homologs, which function as CK influx transporters with broad substrate specificities (Sun *et al.*, 2005; Wormit *et al.*, 2004) are found throughout the green lineage. Further functional characterization of the algal putative transport proteins will provide insight into the origins of the CK influx translocation system. The response of algae to CKs and selective transportation of CKs into the algal cytosol remains speculative.

Investigation of the genomes or transcriptomes of charophyte algae revealed that all the four members of CK signaling pathway are encoded by *S. pratensis* and *K. nitens,* the latter has been described by Hori *et al.* (2014). All the investigated eukaryotic species contain genes encoding HPTs and RRBs with the only exception that *C. braunii* genome encodes no complete RRBs probably due to the complexity of its genome bearing the risk of gaps during sequencing or the evolutionary loss of this gene (Nishiyama *et al.*, 2018). An early acquisition of the HPTs and RRBs in the green lineage *via* endosymbiosis process from prokaryotes can be assumed. The RRAs are similar to the *N*-termini receiver domain of RRBs and function as primary CK response factors and negative regulators in CK signaling by competing with RRBs for phosphate transfer (Kiba *et al.*, 2003; To *et al.*, 2007). These proteins appeared to be newly acquired since they occurred only in land plants and charophyte green algae (Hori *et al.*, 2014; Jiao *et al.*, 2020). The absence of RRAs in genomes of several later divergent Charophytes including *C. braunii, S. muscicola,* and *M. endlicherianum* could be either due to the quality of the used DNA databases or substitution of function by other genes.

Similar to the RRAs, the CHASE-containing HKs seem to be acquired in the Charophytes. Although the cyanobacterial CHASE-containing protein (Nsall2875) has been reported to have a weak affinity to CK but unable to activate the phosphorelay pathway in *E. coli* (Frébortová *et al.*, 2017), it possesses a more complex domain structure compared to the classical CK receptors. This suggests that the plant-like CK sensing and transduction mechanisms might be established in Charophytes by functional domain recruitment, shuffling or loss. Homologs of CHKs were found to be encoded by multiple gene numbers in most investigated Charophytes. The phylogenetic analysis of CHASE domains placing them in three different clades and detailed residue analysis showing divergent CK binding residues indicated that the duplication of CHK gene and mutations in the CHASE domain must have preceded the deployment of these proteins as classical and functional CK receptors. The fact that CHKs from later diverged charophyte species are present in both, the classical CK receptor clade and its sister clade, among which the CHKs were shown to bind to CKs *in vitro* but the *in planta* function was unknown (Gruhn *et al.*, 2014; von Schwartzenberg *et al.*, 2016), points to an early separation of the CHKs in the evolution of charophyte algae and a possibility of the emergence of receptor-like CHKs in the algal ancestor of land plants. The existence of the plant-like CK regulatory system in basal Charophytes *K. nitens* and *M. viride* is doubtful as their CHKs show a low similarity with classical CK receptors. These CHKs may function differently from CK receptors of land plants. Therefore, studies revealing the functions of CHKs in the later diverged Charophytes are essential for investigating the origin of CK as a plant hormone.

Our phylogenetic analysis indicated that there are two divergent CHKs in *S. pratensis* (SpCHK1 and SpCHK2). The SpCHK2 possesses a similar domain arrangement as the classical CK receptors while the SpCHK1 protein contains no 5’ flanking sequence encoding the transmembrane helix of the CHASE domain (Fig. S4). To test the functionality of the two SpCHKs, they were cloned and expressed both in *E. coli* for analysis in a CK binding assay (Romanov *et al.*, 2005) and in a CK receptor mutant of *P. patens* for CK response assay (von Schwartzenberg *et al.*, 2016). The CK binding assay revealed that both SpCHKs have no affinity to *t*Z, which is in agreement with the lack of tZ-type CKs in Spirogyra as well as in the other tested charophyte algae (see Fig. 1). The absence of iP binding activity of SpCHK1 and a very weak affinity of SpCHK2 to iP suggest

that both SpCHKs are fundamentally different from classical CK receptors. This is in line with the results of the expression of SpCHKs in the CK receptor-deficient mutant of *P. patens*, which did not rescue the mutant phenotype although transcripts of SpCHKs were detected. Moreover, treatment with either a high dose or an inductive amount of CKs yielded no increased CK responses compared with the triple CK receptor mutants of moss. This is identical to the results that the transcription of AHK4 homologs and downstream response regulators were not significantly affected by exogenous CK in *S. pratensis* (Vries *et al.*, 2020). Thus, it appears that SpCHKs probably do not respond to CKs and function in the two-component signaling system *in planta* in a CK-independent manner or possibly in response to other environmental stimuli as *Spirogyra* CHASE domain-containing HKs showed induction in response to heat stress (Vries *et al.*, 2020).

4. Material and methods

Cultures and growth condition

Axenic cultures of five representative charophyte green algae (Tab. S4) were employed for the CK profiling. The storage algal cultures were refreshed regularly and cultured in corresponding medium in standard growth condition (23 °C, 80 µM photons m^{-2} s^{-1} with a light:dark cycle of 16:8 h) with aeration (sterile air *ca.* 500 ml min^{-1}). For CK profiling assays, the algal cells at late log phase or stationary phase were washed with fresh medium and transferred to new flasks containing 1 liter fresh medium. The cultures were further cultivated in the same growth condition for a growth period of 22 days.

The wild type, as well as two CK receptor mutants of the moss *P. patens,* was used for the SpCHKs expression and complementation assays. The moss liquid culture for protoplast isolation was disintegrated every 5 days and cultured in modified Knop medium as described by von Schwartzenberg *et al.* (2016). For phenotyping and budding assays, the moss cultures were cultivated in petri dishes, which contained Knop medium without ammonium tartrate dibasic solidified with 1.5 % plant agar (Duchefa).

Cytokinin profiling

For CK identification and quantification, both cell and culture medium samples were taken from the algal cultures at three time points during the cultivation (T0- starting point, T1-13 days, T2- 22 days) and freeze-dried until the dry weight turned stable. They were stored at -80°C until CK profile analysis. Each algal species was cultured with three independent

biological replicates for the sampling. The cells from *S. pratensis* were harvested by filtering through Whatman paper filters (Ø 9 cm) while the other four algae were harvested by centrifugation (300*g*, 5 min). The extraction and purification of the CKs was performed following the protocol by (Novák *et al.*, 2003). The quantification of CKs was carried out with ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) according to Novák *et al.* (2008).

Bioinformatics and phylogenic analysis

The sequence data of the organisms used in this project were either genomic or transcriptomic sequences of three land plants, seven charophyte algae, two chlorophyte algae, and one cyanobacterium, which can be retrieved from sources in Tab. S5. Local nucleotide databases were created using algal transcriptomes with the BioEdit7.2 software. Protein sequences of *A. thaliana* were used to query each database independently *via* BLASTp or tBLASTn. All the scaffolds with the BLAST hits were extracted from the respective transcriptomes and translated into amino acid sequences using OFR finder (https://www.ncbi.nlm.nih.gov/orffinder/). The protein sequences were run through the NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd) and the Pfam search (https://pfam.xfam.org/) to look for conserved domains. Filtered sequences were further tested by BLASTp against the *Arabidopsis* genome in JGI (https://phytozome.jgi.doe.gov/pz/portal.html) to confirm orthology inferences.

Multiple sequence alignment at the amino acid level was performed with the program Jalview 2.11.0 (Waterhouse *et al.*, 2009) using its own web service MAFFT (Katoh and Standley, 2013). Alignment positions with more than 50% gaps were removed manually. The maximum likelihood algorithm implemented in MEGA X with Jones-Taylir-Thornton (JTT) model was used for the phylogenetic analysis. Bootstrap method using 1000 replicates was employed for all phylogenetic trees. Obtained trees were visualized and edited using the iTOL (https://itol.embl.de/) phylogeny visualization program.

Isolation of full length SpCHKs and construction of expression vectors

Full-length ORFs of two *SpCHK* genes (3183bp *SpCHK1*, Seq. 1900 and 3648bp SpCHK2, Seq. 9495) were isolated from the cDNA *S. pratensis*. Total RNA was isolated according to Oñate-Sánchez and Vicente-Carbajosa (2008). The cDNA was synthesized by using the RevertAid Reverse Transcriptase (Thermo Scientific) with oligo $(dT)_{18}$ primer. The

SpCHK1 was amplified by PCR with Phusion High Fidelity DNA polymerase (Thermo Scientific) with the primer pair SpCHK1-MluI-F/SpCHK1-SgfI-R. The *SpCHK2* was amplified by nested PCR. The primer pair SpCHK2-F-I/SpCHK2-R-I was used in the first round of PCR for the accumulation of the transcripts. The primer pair SpCHK2-MluI-F/SpCHK2-BsrGI-R was used in the second round for amplifying the ORF by using the first-round product as a template. The primer sequences are listed in Tab. S3.

For the expression of SpCHKs in the CK receptor mutant of moss, the plasmids pBZR-SpCHK1 and pBZR-SpCHK2 were constructed as follows: The vector pBAS-GFP (Zeidler et al., 1999) containing the rice (Oryza sativa) *Actin1* promoter (accession no. S44221) was modified by substituting the GFP gene with multiple cloning site (MCS) between the restriction sites *NcoI* and *BsrGI*. By digestion of the generated pBAS-MCS with *ApaI* and *SacII*, the 1816 bp Pactin1-MCS-Tnos cassette was isolated and ligated into the same restriction site of the construct pBZR-PpCHK3-ko (von Schwartzenberg *et al.*, 2016), which targets to the native PpCHK3 locus in *P. patens* by carrying 5' and 3' homologous genomic fragments. Finally, PCR products of full-length ORF sequences of *SpCHK1* and *SpCHK2* were digested with corresponding restriction endonucleases (*MluI* and *SgfI* for *SpCHK1, MluI* and *BsrGI* for *SpCHK2*), the recognition sites of which were included in the primers and ligated to the same site of the intermediate plasmid pBZR-PpCHK3-MCS. The diagram of the cloning strategy is shown in Fig. S5. All the resulting vectors were checked by PCR, restriction analysis, and full-length sequencing to exclude mutations.

Cytokinin binding assay

For CK binding assay, the vector pINIII-4xMyc gifted by David Zalabák (Palacký University, CZ) (Yamada *et al.*, 2001), was used as backbone for SpCHKs expression. The ORF of *SpCHK1* containing the GATT motif in the 5' end was subcloned into restriction sites *BamHI* and *SpeI* to generate plasmid pINIII-SpCHK1 while the ORF of *SpCHK2* was cloned to the *BsrGI* and *SpeI* sites to generate plasmid pINIII-SpCHK2. The AtAHK4 sequence from *A. thaliana* was cloned to the *BamHI* and *SpeI* sites to generate plasmid pINIII-AHK4 to serve as a positive control. These plasmids were transformed into the Top10 *E. coli* strain (Invitrogen, G) for live-cell competitive binding assays as previously described by Romanov *et al.* (2005) and Jaworek *et al.* (2019b). The bacteria bearing the empty vector was used as the negative control. Tritium labeled *t*Z ([^{3}H]tZ; 1.3 TBq mmol^{-1}) and iP ([^{3}H]iP; 1.3 TBq mmol^{-1}) obtained from the Isotope Laboratory of the Institute of Experimental Botany (Prague, Czech Republic) were utilized in the experiment.

Generation of SpCHKs expression lines in the Δchk1,2 double mutant of P. patens

Linearized plasmids pBZR-SpCHK1 and pBZR-SpCHK2 were introduced to *PpΔchk1,2* double mutant of *Physcomitrella* by PEG mediated protoplast transformation according to (D. Schaefer, 1991). The stable transgenic lines were screened *via* three cycles of survival test on the Knop medium agar plate (1.5 % plant agar, Duchefa) supplemented with or without 100 µg ml^{-1} Zeocin. The stable lines *PpΔchk1,2,3-SpCHK1*$_{ox}$ and *PpΔchk1,2,3-SpCHK2*$_{ox}$ were characterized by PCR and reverse transcription-PCR (RT-PCR). For the confirmation of locus disruption and transcript loss of the PpCHK3 gene, primers were used following the strategy described by von Schwartzenberg *et al.* (2016). For the test of insertion and overexpression of SpCHKs genes, primer pairs SpCHK1-P-F/SpCHK1-P-R and SpCHK2-P-F/SpCHK2-P-R were employed. The wild type (WT), *PpΔchk1,2* double mutant, and PpΔchk1,2,3 triple mutant (von Schwartzenberg *et al.*, 2016) were taken as controls. Sequences of all primers are listed in Tab. S3.

Phenotype, CK tolerance and budding assays of SpCHKs expression lines

For the phenotypic analysis of the SpCHKs expression lines, undifferentiated protonema from liquid cultures was rinsed thoroughly with an excess of fresh medium and point inoculated in the center of Knop agar plates supplemented either with or without 1µM BA or 1µM iP. The samples were cultured under standard growth condition and after three-week of growth, pictures of buds formed on each colony were taken with an inverse microscope (Olympus, Germany). The budding bioassays were carried out according to von Schwartzenberg *et al.* (2007). Ten days after addition of CKs and growth under standard condition, the number of buds of the different lines was recorded by microscopic observation using the inverse microscope.

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Supplementary figures:

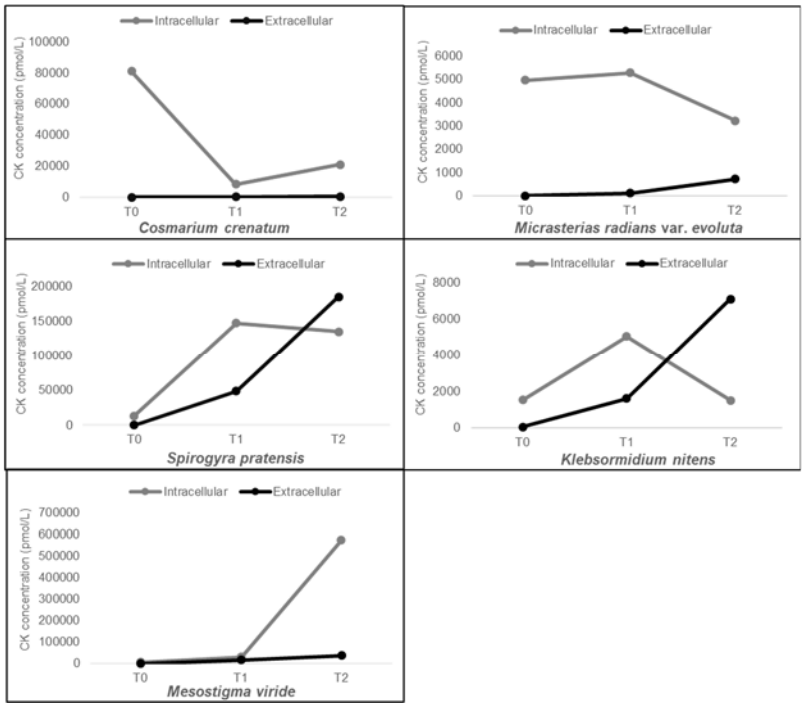

Fig. S1: Intracellular (pmol per liter estimated cell volume) and extracellular (pmol per liter culture supernatant) concentrations of cytokinins. 1g fresh weight was considered equal to 1ml volume. The relationship between dry weight and fresh weight of algal cells was regarded as 8 % according to Sládeček and Sládečková (1963). Data derived from Tab. S1.

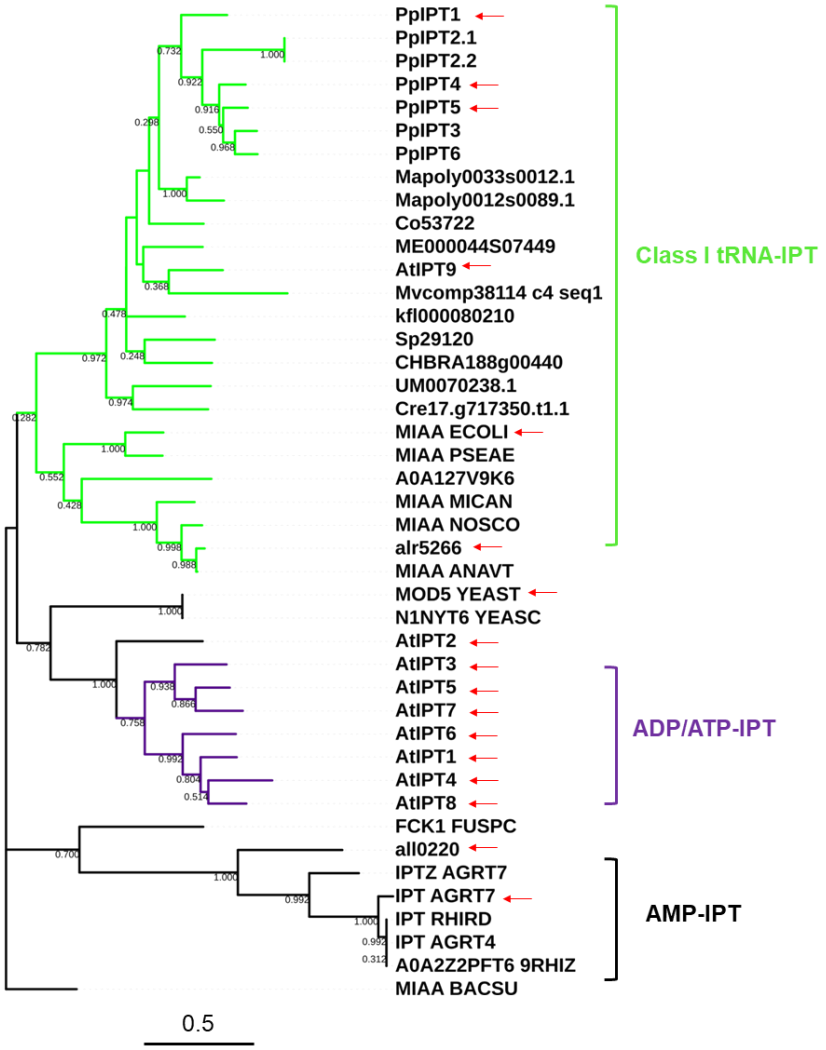

Fig. S2: Maximum likelihood tree of IPT proteins inferred using MEGAX program with 1000 bootstraps. The amino acid sequences were aligned using MAFFT. Gene identifiers and species names are available in Supplementary Tab. S2. Based on the functional characterized IPT- domain containing proteins (marked with red arrow, PpIPT1 (Lindner *et al.*, 2014); PpIPT4,5 (Patil and Nicander, 2013); MiaA_ECOLI (Caillet and Droogmans, 1988); alr5266 (Frébortová *et al.*, 2017); MOD5_YEAST (Dihanich *et al.*, 1987); AtIPT1,4 (Kakimoto, 2001); AtIPT2 (Golovko *et al.*, 2002); AtIPT3,5,6,7,8,9 (Miyawaki *et al.*, 2006) IPTZ_AGRT7 (Akiyoshi *et al.*, 1984); all0220 (Frébortová *et al.*, 2015)), the proposed function (homology-based) clades are marked by coloring the branches.

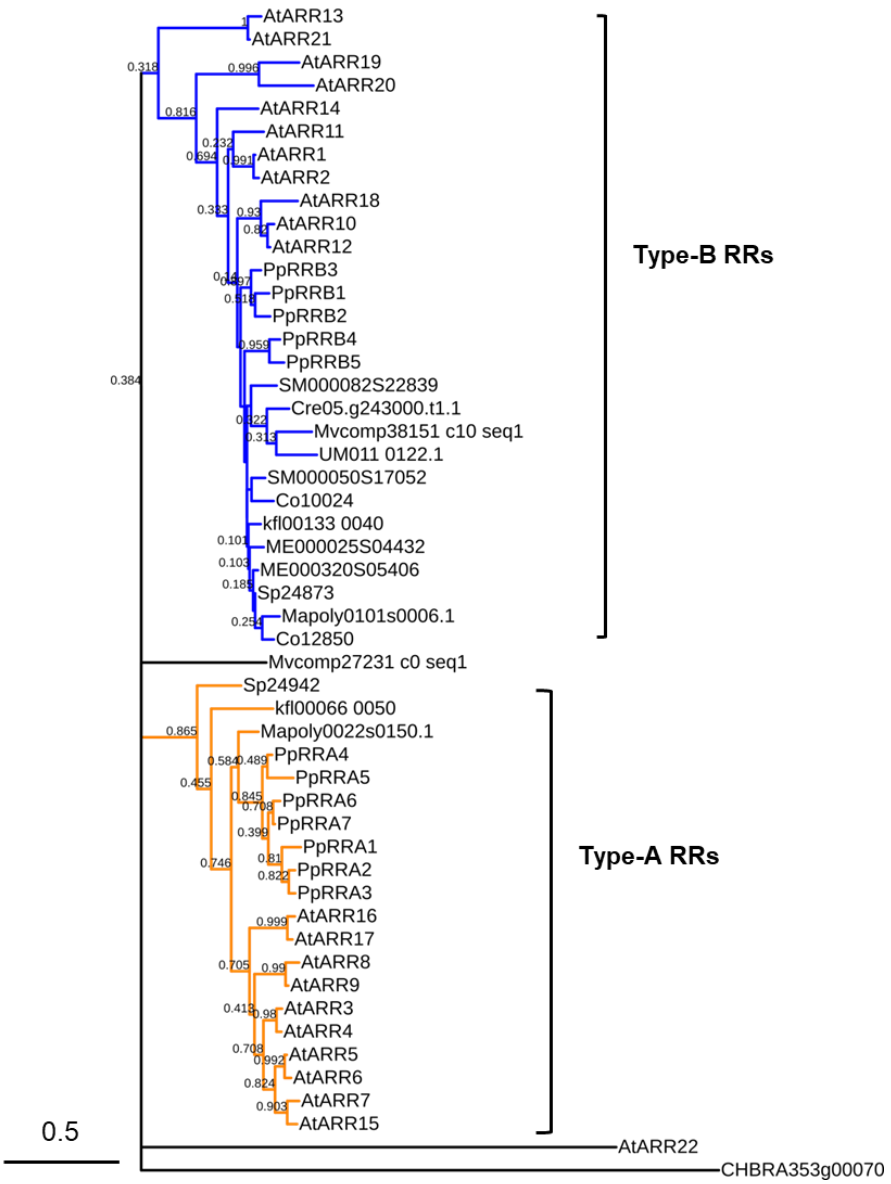

Fig. S3: Maximum likelihood tree of REG domains of response regulators inferred using MEGAX program with 1000 bootstraps. The amino acid sequences were aligned using MAFFT. Gene identifiers and species names are available in Supplementary Tab. S2.

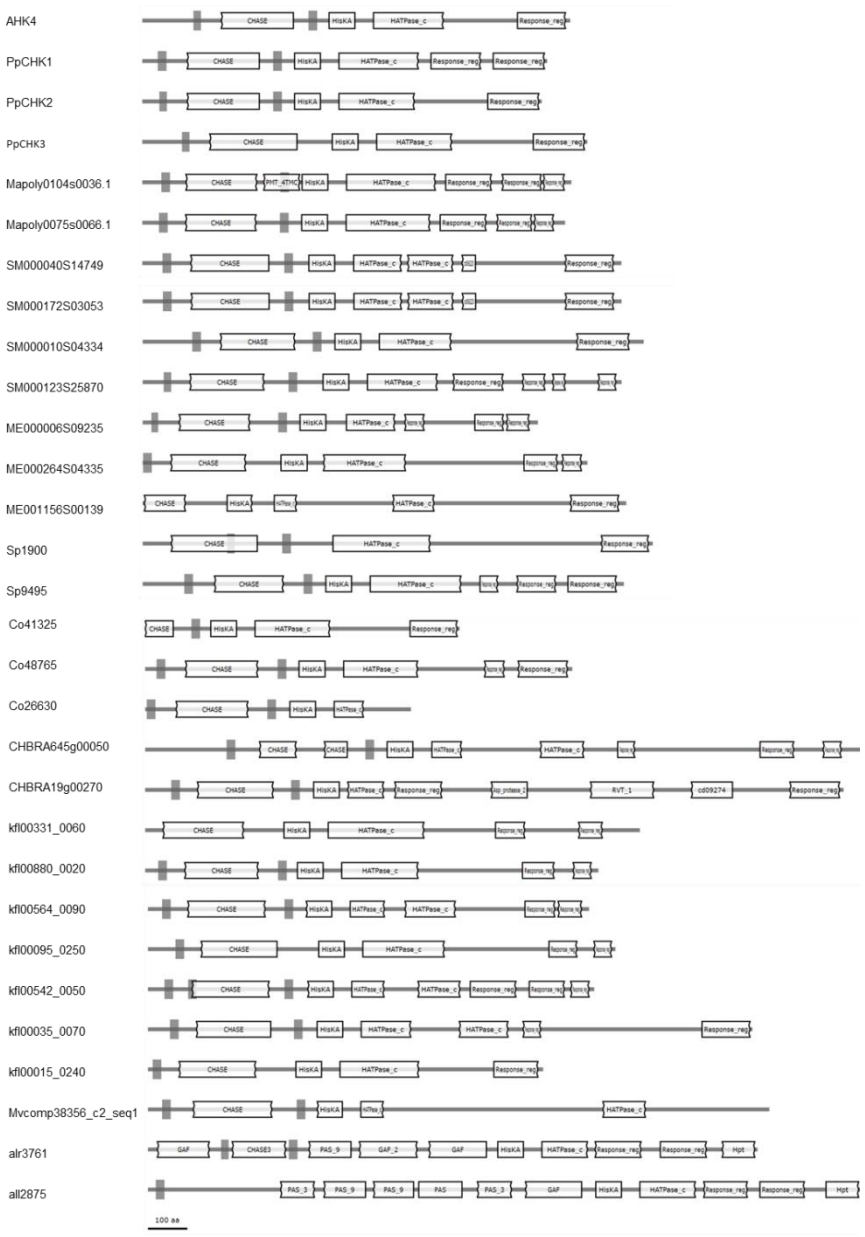

Fig. S4: Domain structures of the CHASE-containing proteins generated with the online server CDvist (Comprehensive Domain Visualization Tool). N-terminal grey vertical bars represent transmembrane helixes. Domains: CHASE (PF03924), HisKA (PF07730), HATPase_ C (PF02518), Response reg (REC, PF00072), CHASE3 (PF05227), GAF (PF01590), GAF_2 (PF13185), HPT (PF01627); PAS (PF00989), PAS_3 (PF08447), PAS_9 (PF13426), RVT_1 (PF00078).

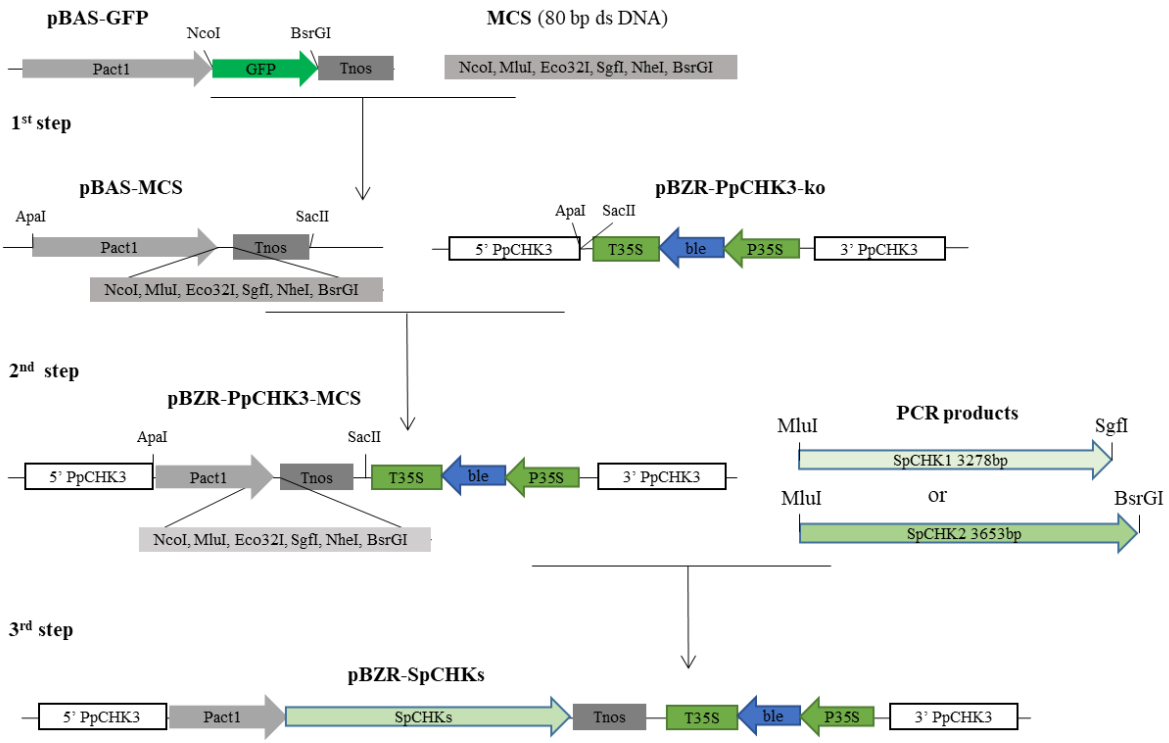

Fig. S5: Diagram of the cloning strategy of transformation vectors pBZR-SpCHK1 and pBZR-SpCHK2 for complementary in double mutant PpΔchk1,2 of moss. The plasmid pBAS-GFP was kindly given by Dr. Mathias Zeidler (University of Giessen). The DNA sequence of multiple cloning site (MCS) was synthesized by Metabion International AG (Planegg, Germany)

Supplementary tables:

Tab. S1: Isoprenoid cytokinin levels in the cells and corresponding culture medium of the five investigated charophyte green algae at three sampling periods (T0– starting point, T1– 13 days, T2– 22 days). Data is shown by mean ± SD. ND, not detectable. Abbreviations: iP, N^6-(Δ^2-isopentenyl) adenine; *c*Z, *cis*-zeatin; *t*Z, *trans*-zeatin; R, riboside; RMP, riboside-5'-monophosphate; OG, O-glucoside; ROG, riboside-O-glucoside; 7G, N^7-glucoside; 9G, N^9-glucoside; Kn, *Klebsormidium nitens*; Cc, *Cosmarium crenatum* var. *boldtianum*; Mr, *Micrasterias radians* var. *evoluta*; Sp, *Spirogyra pratensis*; Mv, *Mesostigma viride*.

	Species	Samples	iP	iPR	iPRMP	ΣiP-types	tZ	tZR	tZRMP	tZOG	tZROG	tZ7G	tZ9G	ΣtZ-types
CK in cells (pmol g^{-1} DW)	Kn	T0	5.88 ± 0.24	1.98 ± 0.36	2.23 ± 0.37	10.54 ± 0.13	1.28 ± 0.14	ND	ND	ND	ND	ND	ND	1.44 ± 0.07
		T1	5.77 ± 1.32	12.75 ± 3.55	2.73 ± 0.60	21.25 ± 5.37	ND	0.08 ± 0.01	ND	ND	ND	0.01 ± 0.00	ND	0.05 ± 0.03
		T2	6.06 ± 1.38	4.13 ± 0.71	3.21 ± 0.12	13.40 ± 1.90	ND	ND	ND	ND	ND	ND	ND	ND
	Cc	T0	8.73 ± 0.44	4.96 ± 0.84	32.08 ± 2.62	45.80 ± 3.62	2.75 ± 0.87	0.33 ± 0.09	ND	ND	ND	ND	ND	3.56 ± 0.77
		T1	6.49 ± 1.25	ND	4.01 ± 0.67	10.50 ± 0.77	ND	0.04 ± 0.01	ND	ND	ND	ND	ND	0.02 ± 0.00
		T2	9.99 ± 0.49	3.04 ± 0.72	10.64 ± 2.94	23.67 ± 3.54	ND	0.14 ± 0.04	ND	ND	ND	ND	ND	0.14 ± 0.04
	Mr	T0	13.24 ± 1.91	0.42 ± 0.10	ND	13.67 ± 1.94	ND	ND	ND	ND	ND	ND	ND	ND
		T1	9.70 ± 2.10	0.52 ± 0.14	2.87 ± 0.21	13.09 ± 2.08	ND	ND	ND	ND	ND	0.04 ± 0.01	0.34 ± 0.03	0.27 ± 0.11
		T2	4.83 ± 0.12	0.41 ± 0.07	1.45 ± 0.09	6.69 ± 0.21	ND	0.04 ± 0.01	ND	ND	ND	ND	ND	0.04 ± 0.01
	Sp	T0	35.07 ± 0.72	24.89 ± 3.73	20.09 ± 4.28	80.06 ± 7.00	ND	ND	ND	ND	ND	ND	ND	ND
		T1	1292.42 ± 101.47	30.72 ± 4.89	78.82 ± 19.64	1401.96 ± 113.03	1.14 ± 0.36	0.10 ± 0.03	ND	ND	ND	ND	ND	0.86 ± 0.61
		T2	1318.81 ± 395.67	7.59 ± 2.16	45.02 ± 10.82	1401.42 ± 395.99	0.31 ± 0.07	0.04 ± 0.02	ND	ND	ND	ND	ND	0.40 ± 0.07
	Mv	T0	18.71 ± 2.85	6.96 ± 0.82	9.22 ± 0.57	34.89 ± 2.75	5.39 ± 1.48	ND	ND	ND	ND	0.37 ± 0.03	0.50 ± 0.11	6.33 ± 1.35
		T1	156.83 ± 12.03	41.55 ± 7.60	37.27 ± 3.15	235.65 ± 8.38	ND	0.01 ± 0.00	ND	ND	ND	ND	ND	0.01 ± 0.00
		T2	953.05 ± 290.62	923.48 ± 248.44	55.58 ± 14.65	1932.11 ± 530.29	14.17 ± 1.43	0.27 ± 0.08	ND	ND	ND	ND	ND	14.49 ± 1.49
	Species	Sample	iP	iPR	iPRMP	ΣiP-types	tZ	tZR	tZRMP	tZOG	tZROG	tZ7G	tZ9G	ΣtZ-types
CK in medium (pmol 100 ml^{-1})	Kn	T0	0.03 ± 0.01	0.24 ± 0.04	ND	0.28 ± 0.04	ND	ND	ND	ND	ND	ND	ND	ND
		T1	5.40 ± 0.98	22.83 ± 2.54	ND	28.23 ± 2.16	0.40 ± 0.27	0.11 ± 0.01	ND	ND	ND	ND	ND	0.58 ± 0.35
		T2	7.40 ± 1.79	66.79 ± 3.92	1.11 ± 0.62	77.01 ± 7.11	ND	0.26 ± 0.07	ND	1.20 ± 0.35	ND	ND	ND	1.46 ± 0.40
	Cc	T0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		T1	1.12 ± 0.30	0.66 ± 0.13	0.36 ± 0.10	2.10 ± 0.49	ND	0.02 ± 0.01	ND	ND	ND	ND	ND	0.02 ± 0.01
		T2	0.96 ± 0.21	1.66 ± 0.30	0.45 ± 0.06	3.04 ± 0.44	ND	0.09 ± 0.03	ND	ND	ND	ND	ND	0.15 ± 0.03
	Mr	T0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		T1	0.55 ± 0.01	1.08 ± 0.51	0.45 ± 0.23	2.16 ± 0.56	1.56 ± 0.29	0.03 ± 0.01	ND	ND	ND	ND	ND	1.58 ± 0.30
		T2	1.14 ± 0.34	4.29 ± 1.01	1.18 ± 0.22	6.50 ± 1.20	1.91 ± 0.43	0.10 ± 0.04	ND	ND	ND	ND	ND	2.01 ± 0.47
	Sp	T0	0.46 ± 0.10	0.05 ± 0.02	0.07 ± 0.02	0.57 ± 0.07	ND	ND	ND	ND	ND	ND	ND	ND
		T1	3111.36 ± 1043.22	24.85 ± 4.88	0.42 ± 0.03	3136.82 ± 1047.85	4.31 ± 1.50	0.08 ± 0.03	ND	ND	ND	ND	ND	4.39 ± 1.52
		T2	6597.68 ± 1573.04	36.17 ± 7.38	ND	6635.32 ± 1564.73	38.03 ± 11.49	0.22 ± 0.08	ND	ND	ND	ND	ND	38.26 ± 11.52
	Mv	T0	0.44 ± 0.13	0.65 ± 0.15	0.15 ± 0.02	1.15 ± 0.24	0.06 ± 0.02	ND	ND	ND	ND	0.01 ± 0.01	ND	0.07 ± 0.02
		T1	123.38 ± 12.31	203.10 ± 11.91	16.57 ± 0.84	342.72 ± 18.39	3.91 ± 0.11	0.21 ± 0.02	ND	ND	ND	ND	ND	4.12 ± 0.09
		T2	445.22 ± 73.00	352.33 ± 37.16	22.70 ± 4.72	832.26 ± 104.11	11.09 ± 0.19	0.34 ± 0.01	ND	ND	ND	ND	ND	11.43 ± 0.21

Continued Tab. S1

	Species	Samples	cZ	cZR	cZRMP	cZOG	cZROG	cZ7G	cZ9G	ΣcZ-types	DHZ	DHZR	DHZRMP	DHZOG	DHZROG	DHZ7G	DHZ9G	ΣDHZ-types	Total Cytokinins
CK in cells (pmol g^{-1} DW)	Kn	T0	6.62 ± 0.71	0.87 ± 0.25	ND	ND	ND	ND	ND	7.49 ± 0.46	ND	ND	ND	ND	ND	ND	ND	ND	18.82 ± 0.62
		T1	20.99 ± 6.07	9.94 ± 2.97	10.63 ± 2.43	ND	ND	ND	ND	41.56 ± 11.46	ND	0.12 ± 0.02	ND	ND	ND	ND	ND	0.12 ± 0.02	62.97 ± 16.57
		T2	2.82 ± 1.26	2.55 ± 0.48	ND	ND	ND	ND	ND	5.13 ± 2.01	ND	ND	ND	ND	ND	0.13 ± 0.02	ND	0.13 ± 0.02	18.66 ± 2.62
	Cc	T0	14.87 ± 4.23	24.89 ± 6.45	907.15 ± 62.96	ND	ND	ND	ND	954.12 ± 59.38	ND	0.36 ± 0.07	ND	ND	ND	6.80 ± 1.90	0.50 ± 0.14	7.65 ± 1.99	1012.48 ± 54.28
		T1	13.81 ± 4.32	1.75 ± 0.22	76.12 ± 4.67	ND	ND	ND	ND	91.77 ± 6.27	ND	0.10 ± 0.02	ND	ND	ND	0.94 ± 0.25	0.04 ± 0.01	1.04 ± 0.24	103.33 ± 6.67
		T2	7.49 ± 1.28	12.10 ± 3.21	219.06 ± 66.77	ND	ND	ND	ND	238.66 ± 70.88	ND	0.20 ± 0.06	ND	ND	0.55 ± 0.09	0.12 ± 0.03	ND	0.89 ± 0.14	263.16 ± 74.59
	Mr	T0	7.74 ± 2.09	2.81 ± 0.82	37.79 ± 10.96	ND	ND	ND	ND	48.33 ± 12.19	ND	ND	ND	ND	ND	ND	ND	ND	62.02 ± 11.34
		T1	16.66 ± 1.15	1.23 ± 0.36	41.05 ± 3.62	ND	0.06 ± 0.01	ND	ND	52.51 ± 11.32	ND	0.07 ± 0.01	ND	ND	ND	ND	ND	0.18 ± 0.04	66.03 ± 12.19
		T2	1.67 ± 0.25	1.67 ± 0.39	30.26 ± 5.54	ND	ND	ND	ND	33.59 ± 5.48	ND	0.05 ± 0.02	ND	ND	ND	0.02 ± 0.01	ND	0.05 ± 0.01	40.37 ± 5.53
	Sp	T0	21.87 ± 4.64	14.42 ± 2.73	48.80 ± 10.09	ND	ND	ND	ND	85.08 ± 13.89	ND	0.18 ± 0.05	ND	ND	ND	ND	0.10 ± 0.03	0.25 ± 0.09	165.39 ± 18.86
		T1	150.86 ± 43.02	14.80 ± 1.33	280.04 ± 71.65	ND	ND	ND	ND	445.70 ± 114.23	ND	0.11 ± 0.03	ND	ND	ND	ND	ND	0.11 ± 0.03	1848.63 ± 222.34
		T2	113.86 ± 24.56	7.08 ± 1.82	171.46 ± 48.81	ND	ND	ND	0.14 ± 0.05	292.53 ± 56.03	0.24 ± 0.08	0.03 ± 0.01	ND	ND	ND	ND	ND	0.27 ± 0.09	1694.49 ± 413.72
	Mv	T0	25.68 ± 4.11	1.49 ± 0.17	26.11 ± 3.21	ND	ND	ND	0.35 ± 0.08	53.63 ± 5.04	ND	0.05 ± 0.02	ND	ND	ND	ND	ND	0.05 ± 0.02	93.03 ± 9.25
		T1	63.84 ± 12.09	8.69 ± 1.00	81.61 ± 17.96	ND	ND	ND	ND	15.14 ± 22.21	ND	0.14 ± 0.03	ND	ND	ND	ND	ND	0.14 ± 0.03	389.93 ± 30.38
		T2	4971.08 ± 1036.82	156.91 ± 50.43	94.05 ± 23.15	ND	0.27 ± 0.10	ND	0.47 ± 0.05	5222.53 ± 1088.16	ND	3.53 ± 1.06	ND	ND	ND	ND	ND	3.53 ± 1.06	7167.88 ± 1514.33
	Species	Samples	cZ	cZR	cZRMP	cZOG	cZROG	cZ7G	cZ9G	ΣcZ-types	DHZ	DHZR	DHZRMP	DHZOG	DHZROG	DHZ7G	DHZ9G	ΣDHZ-types	Total Cytokinins
CK in medium (pmol 100 ml^{-1})	Kn	T0	0.15 ± 0.04	0.11 ± 0.03	1.04 ± 0.28	ND	ND	ND	ND	1.31 ± 0.27	ND	ND	ND	ND	ND	ND	ND	ND	1.58 ± 0.26
		T1	32.69 ± 8.10	17.74 ± 4.71	ND	ND	ND	ND	ND	50.42 ± 6.41	ND	0.36 ± 0.01	ND	ND	ND	ND	ND	0.36 ± 0.01	79.42 ± 6.74
		T2	232.25 ± 70.84	33.78 ± 3.63	8.54 ± 0.71	ND	ND	ND	0.25 ± 0.08	274.82 ± 74.63	ND	1.31 ± 0.32	ND	ND	ND	ND	ND	1.31 ± 0.32	354.59 ± 81.53
	Cc	T0	ND	0.03 ± 0.01	0.50 ± 0.10	ND	ND	ND	ND	0.53 ± 0.11	ND	ND	ND	ND	ND	ND	ND	ND	0.54 ± 0.11
		T1	7.73 ± 2.61	1.13 ± 0.20	12.86 ± 3.81	ND	0.02 ± 0.01	ND	0.02 ± 0.00	21.73 ± 5.70	ND	0.03 ± 0.01	ND	ND	ND	ND	ND	0.03 ± 0.01	23.91 ± 5.95
		T2	16.44 ± 2.65	4.84 ± 1.64	20.40 ± 4.41	ND	0.12 ± 0.01	ND	0.03 ± 0.00	41.84 ± 7.71	ND	0.11 ± 0.04	ND	ND	ND	ND	ND	0.11 ± 0.04	45.14 ± 7.92
	Mr	T0	ND	0.02 ± 0.00	0.41 ± 0.03	ND	ND	ND	ND	0.43 ± 0.03	ND	ND	ND	ND	ND	ND	ND	ND	0.28 ± 0.17
		T1	4.31 ± 1.03	2.31 ± 0.69	ND	ND	0.18 ± 0.06	ND	0.03 ± 0.01	6.82 ± 1.74	ND	0.12 ± 0.02	ND	ND	ND	ND	ND	0.12 ± 0.02	10.67 ± 2.52
		T2	16.85 ± 4.47	8.66 ± 2.88	37.51 ± 7.75	ND	0.90 ± 0.21	ND	0.10 ± 0.02	64.03 ± 14.35	ND	0.56 ± 0.15	ND	ND	ND	ND	ND	0.56 ± 0.15	72.47 ± 16.00
	Sp	T0	0.19 ± 0.03	0.05 ± 0.01	0.87 ± 0.17	ND	ND	ND	ND	1.12 ± 0.13	ND	ND	ND	ND	ND	ND	ND	ND	1.68 ± 0.10
		T1	1728.31 ± 498.05	10.54 ± 1.65	ND	ND	0.06 ± 0.01	ND	0.18 ± 0.03	1739.09 ± 499.63	0.57 ± 0.15	0.18 ± 0.05	ND	ND	ND	ND	ND	0.75 ± 0.19	4881.05 ± 1413.77
		T2	11821.53 ± 1410.45	26.37 ± 1.59	ND	ND	0.33 ± 0.11	ND	3.00 ± 0.74	11851.23 ± 1411.74	0.92 ± 0.15	0.54 ± 0.09	ND	ND	ND	ND	ND	1.46 ± 0.17	18526.27 ± 838.95
	Mv	T0	0.56 ± 0.15	0.17 ± 0.03	1.17 ± 0.28	ND	ND	ND	0.01 ± 0.00	1.92 ± 0.21	ND	ND	ND	ND	ND	ND	ND	ND	3.11 ± 0.36
		T1	1307.65 ± 191.04	53.52 ± 7.47	37.47 ± 8.68	ND	0.01 ± 0.00	ND	0.13 ± 0.02	1398.79 ± 203.67	0.06 ± 0.00	1.50 ± 0.29	ND	ND	ND	ND	ND	1.56 ± 0.29	1747.18 ± 221.06
		T2	2784.86 ± 133.99	97.34 ± 5.18	51.30 ± 5.43	ND	ND	ND	0.34 ± 0.13	2933.84 ± 138.92	ND	3.11 ± 0.25	ND	ND	ND	ND	ND	3.11 ± 0.25	3780.64 ± 235.74

Tab. S2: Genes related to cytokinin biosynthesis, degradation, transport and signaling in the investigated organisms and their corresponding reciprocal BLASTP e-values. Cells with grey background represent no blast hit. Abbreviations: IPT, isopentenyltransferase; LOG, riboside 5'-monophosphate phosphoribohydrolase; ENT, equilibrative nucleoside transporter; PUP, purine permease; CKX, CK oxidase/dehydrogenase; CHK, CHASE domian containing histidine kinase; HPT, His containing phosphotransfer; RRA, type-A response regulator; RRB, type-B response regulator; At, *Arabidopsis thaliana*; Pp, *Physcomitrella patens*; Mp, *Marchantia polymorpha*; Sm, *Spirogloea muscicola*; Me, *Mesotaenium endlicherianum*; Sp, *Spirogyra pratensis*; Cr, *Chara braunii*; Co, *Coleochaete orbicularis*; Kn, *Klebsormidium nitens*; Mv, *Mesostigma viride*; Nos, *Nostoc* sp. PCC7120.

Species		Biosynthesis		Degradation	Transport		Signaling			
		IPT	LOG	CKX	ENT	PUP	CHK	HPT	RRB	RRA
At		AtIPT1 AT1G68460.1; AtIPT2 AT2G27760.1; AtIPT3 AT3G63110.1; AtIPT4 AT4G24650.1; AtIPT5 AT5G19040.1; AtIPT6 AT1G25410.1; AtIPT7 AT3G23630.1; AtIPT8 AT3G19160.1; AtIPT9 AT5G20040.1	AtLOG1 AT2G28305.1; AtLOG2 AT2G35990.1; AtLOG3 AT2G37210.1; AtLOG4 AT3G53450.1; AtLOG5 AT4G35190.1; AtLOG6 AT5G03270.1; AtLOG7 AT5G06300.1; AtLOG8 AT5G11950.1; AtLOG9 AT5G26140.1	AtCKX1 AT2G41510.1; AtCKX2 AT2G19500.1; AtCKX3 AT5G56970.1; AtCKX4 AT4G29740.2; AtCKX5 AT1G75450.1; AtCKX6 AT3G63440.1; AtCKX7 AT5G21482.1	AtENT1 AT1G70330.1; AtENT3 AT4G05120.1; AtENT4 AT4G05130.1; AtENT6 AT4G05110.1; AtENT7 AT1G61630.1; AT3G09990.1; AT4G05140.1; AtENT8 AT1G02630.1	AtPUP1 AT1G28230.1; AtPUP2 AT2G33750.2; AtPUP14 AT1G19770.1	AHK2 AT5G35750.1; AHK3 AT1G27320.1; AHK4 AT2G01830.1	AtAHP1 AT3G21510.1; AtAHP2 AT3G29350.1; AtAHP3 AT5G39340.1; AtAHP4 AT3G16360.1; AtAHP5 AT1G03430.1	AtARR1 AT3G16857.1; AtARR2 AT4G16110.1; AtARR10 AT4G31920.1; AtARR11 AT1G67710.1; AtARR12 AT2G25180.1; AtARR13 AT2G27070.1; AtARR14 AT2G01760.1; AtARR18 AT5G58080.1; AtARR19 AT1G49190.1; AtARR20 AT3G62670.1; AtARR21 AT5G07210.1	AtARR3 AT1G59940.1; AtARR4 AT1G10470.1; AtARR5 AT3G48100.1; AtARR6 AT5G62920.1; AtARR7 AT1G19050.1; AtARR8 AT2G41310.1; AtARR9 AT3G57040.1; AtARR15 AT1G74890.1; AtARR16 AT2G40670.1; AtARR17 AT3G56380.1;
Pp		PpIPT1 Pp1s96_115V6.1; PpIPT2.1 Pp1s137_3V6.1; PpIPT2.2 Pp1s137_19V6.1; PpIPT3 Pp1s280_8V6.1; PpIPT4 Pp1s64_135V6.1; PpIPT5 Pp1s14_391V6.1; PpIPT6 Pp1s341_1V6.1	Pp3c13_19580V3.1; Pp3c13_19630V3.1; Pp3c4_15150V3.1; Pp3c12_8430V3.1; Pp3c3_20740V3.1; Pp3c27_3060V3.1; Pp3c26_2730V3.1; Pp3c6_28650V3.1; Pp3c6_28670V3.1	Pp3c24_13960V3.1; Pp3c8_18480V3.1; Pp3c20_2380V3.1; Pp3c23_17360V3.1; Pp3c8_18580V3.1; Pp3c23_17550V3.1	Pp3c20_22540V3.1; Pp3c10_22780V3.1; Pp3c2_6580V3.1; Pp3c25_15120V3.1		Pp3c25_8540V3.1; Pp3c16_7610V3.1; Pp3c6_7030V3.1; Pp3c4_1360V3.1; Pp3c9_21840V3.1; Pp3c22_15240V3.1; 7Pp3c18_4650V3.1; Pp3c2_20110V3.1; Pp3c1_20050V3.1; Pp3c21_18040V3.1; Pp3c18_4620V3.1	Pp3c2_13560V3.1; Pp3c17_11700V3.1	PpRRB1 Pp3c23_7400V3.1; PpRRB2 Pp3c8_21000V3.1; PpRRB3 Pp3c24_12830V3.1; PpRRB4 Pp3c11_21510V3.1; PpRRB5 Pp3c7_7640V3.1	PpRRA1 Pp3c8_11160V3.1; PpRRA2 Pp3c20_13600V3.1; PpRRA3 Pp3c23_6560V3.1; PpRRA4 Pp3c23_6230V3.1; PpRRA5 Pp3c20_19070V3.1; PpRRA6 Pp3c19_17660V3.1; PpRRA7 Pp3c22_9010V3.1
Mp	**Candidate counterparts**	Mapoly0033s0012.1; Mapoly0012s0089.1	Mapoly0103s0059.1	Mapoly0124s0014.1; Mapoly0093s0012.1	Mapoly0029s0065.1; Mapoly0031s0146.1; Mapoly0130s0004.1; Mapoly0081s0014.1; Mapoly0011s0177.1; Mapoly0161s0013.1;	Mapoly0050s0085.1	Mapoly0104s0036.1; Mapoly0075s0066.1	Mapoly0091s0072.1	Mapoly0101s0006.1	Mapoly0022s0150.1
	Best hit and e-values	AtIPT9, 2.9E-128; AtIPT9, 2.6E-109	AtLOG3, 3.6E-98	AtCKX6, 6.2E-158; AtCKX1, 6.9E-141	AtENT1, 5.9E-120; AtENT1, 7.8E-103; AtENT1, 3.8E-80; AtENT1, 4.3E-47; AtENT3, 2.1E-167; AtENT3, 6.1E-119	AtPUP5, 4.1E-83	AtAHK3, 3.6E-119; AtAHK3, 1.2E-130	AtAHP1, 3E-45	AtARR1, 5.3E-96;	AtARR16, 9E-52
Sm	**Candidate counterparts**		SM000540S18194		SM000008S22163; SM000035S13075; SM000005S17296		SM000040S14749; SM000172S03053; SM000010S04334; SM000123S25870;	SM000241S08503	SM000050S17052; SM000082S22839	

	Best hit and e-value		AtLOG3, 5E-47		AtENT1, 2E-84; AtENT1, 6.6E-88; AtENT1, 4.4E-19		AtAHK3, 1.7E-91; AtAHK4, 6.6E-104; AtAHK3, 2.1E-94; AtAHK3, 3.1E-141	AtAHP1, 2.5E-31	AtARR2, 3.9E-103; AtARR1, 7.2E-85	
Me	**Candidate counterparts**	ME000044S07449	ME000122S00046		ME000013S00810; ME000057S08473; ME000176S02017;		ME000006S09235; ME000264S04335; ME001156S00139	ME000280S04671	ME000025S04432; ME000320S05406	
	Best hit and e-value	AtIPT9, 1.9E-40	AtLOG1, 6.8E-62		AtENT1, 2.7E-66; AtENT1, 6.7E-43; AtENT1, 1.3E-10		AtAHK4, 3.3E-61; AtAHK4, 4.9E-69; AtAHK4, 1.8E-47	AtAHP1, 4.2E-31	AtARR1, 1.1E-57; AtARR2, 2.5E-93	
Sp	**Candidate counterparts**	Sp29120	Sp599		Sp5182		Sp1900; Sp9495	Sp9618	Sp24873	Sp24942
	Best hit and e-value	AtIPT9, 2.3E-55	AtLOG3, 1E-71		AtENT1, 1.3E-63;		AtAHK3, 1.8E-62; AtAHK3, 4E-88;	AtAHP1, 2.1E-34	AtARR1, 8.9E-94	AtARR6, 3.3E-41
Co	**Candidate counterparts**	Co53722	Co26846		Co52568		Co41325; Co48765; Co26630	Co28567	Co10024; Co12850	
	Best hit and e-value	AtIPT9, 2.5E-106	AtLOG3, 2.2E-83		AtENT1, 1E-40		AtAHK4, 1.7E-139; AtAHK4, 1.6E-123; AtAHK4, 3.3E-77	AtAHP1, 2E-40	AtARR1, 3.6E-88; AtARR2, 2.5E-56	
Cb	**Candidate counterparts**	CHBRA188g00440	CHBRA342g00230		CHBRA11438g00010 CHBRA48g00730		CHBRA645g00050; CHBRA19g00270	CHBRA650g00040		
	Best hit and e-value	AtIPT9, 3.3E-89	AtLOG8, 1.9E-40		AtENT1, 4.3E-29; AtENT1, 1.7E-33		AtAHK4, 4.9E-68; AtAHK3, 1.1E-39	AtAHP1, 2.2E-13		
Kn	**Candidate counterparts**	kfl00008_0210	kfl00647_0030		kfl00001_0140; kfl00001_0130		kfl00331_0060; kfl00880_0020; kfl00564_0090; kfl00095_0250; kfl00542_0050; kfl00035_0070; kfl00015_0240	kfl00529_0020	kfl00133_0040	kfl00066_0050
	Best hit and e-value	AtIPT9, 1E-64	AtLOG4, 7E-79		AtENT3, 5.3E-43; AtENT1, 1.5E-40		AtAHK3, 6.7E-56; AtAHK4, 4.8E-80; AtAHK4, 8.8E-63; AtAHK3, 5.1E-82; AtAHK3, 2.3E-62; AtAHK4, 2.9E-56; AtAHK4, 4.6E-67	AtAHP5, 1E-37	AtARR2, 4.8E-92	AtARR16, 3.3E-41
Mv	**Candidate counterparts**	Mvcomp38114_c4_seq1	Mv34412_c0_seq1		Mvcomp35720_c1_seq2; Mvcomp37527_c1_seq1		Mvcomp38356_c2_seq1	Mvcomp17921_c1_seq1	Mvcomp38151_c10_seq1	
	Best hit and e-value	AtIPT9, 2.5E-39	AT1G50575.1, 1.2E-46		AtENT1, 3.9E-25; AtENT1, 1.9E-6		AtAHK2, 7.9E-25	AtAHP1, 1.3E-25	AtARR2, 1.5E-76	
Um	**Candidate counterparts**	UM007_0238.1	UM060_0095.1		UM012_0154.1			UM004_0159.1	UM011_0122.1	
	Best hit and e-value	AtIPT9, 4.5E-33	AtLOG7, 3.8E-66		AtENT1, 0.12			AtAHP5, 6.1E-20	AtARR2, 2.9E-70	
Cr	**Candidate counterparts**	Cre17.g717350.t1.1	Cre07.g340900.t1.2		Cre02.g082900.t1.1 Cre16.g690319.t1.1			Cre01.g040450.t1.2	Cre05.g243000.t1.1	
	Best hit and e-value	AtIPT9, 1.7E-18	AtLOG7, 1.3E-65		AtENT1, 2.6E-12; AtENT1, 3.5E-8			AtAHP5, 1.2E-30	AtARR14, 9.3E-62	
Nos	**Candidate counterparts**	alr5266; all0220	alr0053	all0324			alr3761; all2875			
	Best hit and e-value	AtIPT9, 1.4E-33; AtIPT4, 3.5E-8	AtLOG3, 7E-4	AtCKX1 AT2G41510.1, 5.8E-36			AtAHK3, 1.2E-68; AtAHK2, 2E-68			

Tab. S3: Primers employed for cloning of the SpCHK genes and characterization of the SpCHKs expression moss lines. The restriction sites are marked with fonts in bold.

Primer	**Sequence (5' to 3')**	**Tm (°C)**
SpCHK1-MluI-F	CTG**ACGCGT**ACAACGAAGCGATTCTGAT	60.3
SpCHK1-SgfI-R	TAG**GCGATCGC**GTTAGAAACGACTTGGAACG	59.6
SpCHK2-F-I	TCATTCTTGAAACTTGAACGGGT	63.8
SpCHK2-R-I	AACACACAGCATAACCCAGC	63.8
SpCHK2-MluI-F	TCTG**ACGCGT**TGTGATGCCAGATCCAGATG	62.7
SpCHK2-BsrGI-R	ACAG**TGTACA**GCTAACCCTTTCCCAAACATT	62.0
SpCHK1-BamHI-F	AGCTT**GGATCC**GATTATGTTCAATCAGAGCCTACTGA	63.5
SpCHK1-SpeI-R	TGTTC**ACTAGT**CGAACCCCTGTTCGAATCG	63.5
SpCHK2- BsrGI -F	GCAT**ATGTAC**AGATTATGCCAGATCCAGATGTATTT	65.3
SpCHK2-SpeI-R	TGTTC**ACTAGT**ACCCTTTCCCAAACATTTCTG	62.0
AtAHK4-BamHI-F	AGCTT**GGATCC**GATTATGAGAAGAGATTTTGTGTATAATAA	62.3
AtAHK4-SpeI-R	TGTTC**ACTAGT**CGACGAAGGTGAGATAGGA	61.0
SpCHK1-P-F	AAATGCAGACGCATCAAAGA	61.6
SpCHK1-P-R	TGACGACATCCTGCAGAGAT	61.6
SpCHK2-P-F	TTTCCCACAACGAACCATCC	63.6
SpCHK2-P-R	TCTTCTTCTCCAATGCCTCCT	63.6

Tab. S4: Algal and moss strains employed in this study

Species	Strain Nr.	Medium	Description
Cosmarium crenatum var. *boltianum*	MZCH561	C	Collected and determined by Kusel, E. in Russian Federation in 1995. Purified by Zhou, H.
Micrasterias radians var. *evoluta*	MZCH672	C	Collected by Krienitz, L. from the Lake Ol Bolossat, Kenia and determined by M. Engels and Skaloud et al. (2011). Purified by Wilkens, A.
Spirogyra pratensis	MZCH10213	WHM	Purchased from culture collection of algae at in University of Texas at Austin (UTEX). The original number was UTEX928. Purified by Zhou, H.
Klebsormidium nitens (Kützing) Lokhorst	MZCH10199	C	Purchased from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES, Japan). The original number was NIES-2285.
Mesostigma viride	NIES-296	C	Purchased from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES, Japan).
Physcomitrella patens (Hedw.) B.S.G.	WTL6	KNOP	The 6th generation spore of strain 16/14. Originally collected in Gransden Wood, Huntingdonshire (GB) by H. L. K Whitehouse in 1968. (Rensing et al., 2008).
PpΔchk1,2	#172	KNOP	The double mutant of PpCHK1 and PpCHK2 (von Schwartzenberg et al., 2016)
PpΔchk1,2,3		KNOP	The triple mutant of PpCHK1, PpCHK2 and PpCHK3. (von Schwartzenberg et al., 2016)

Tab. S5: Sequences sources of the species used in bioinformatics analysis

Species	**Taxonomy**	**Data type**	**Source or reference**
Arabidopsis thaliana	Dicot	Genome	JGI https://phytozome.jgi.doe.gov/pz/portal.html
Physcomitrella patens	Bryophyta	Genome	JGI https://phytozome.jgi.doe.gov/pz/portal.html
Marchantia polymorpha	Liverwort	Genome	JGI https://phytozome.jgi.doe.gov/pz/portal.html
Spirogloea muscicola	Charophyta	Genome	(Cheng *et al.*, 2019)
Mesotaenium endlicherianum	Charophyta	Genome	(Cheng *et al.*, 2019)
Spirogyra pratensis	Charophyta	Transcriptome	(Cooper and Delwiche, 2016)
Coleochaete orbicularis	Charophyta	Transcriptome	(Cooper and Delwiche, 2016)
Chara braunii	Charophyta	Genome	ORCAE https://bioinformatics.psb.ugent.be/orcae/
Klebsormidium nitens	Charophyta	Genome	(Hori et al., 2014)
Mesostigma viride	Charophyta	Transcriptome	(Cooper and Delwiche, 2016)
Ulva mutabilis	Chlorophyta	Genome	ORCAE https://bioinformatics.psb.ugent.be/orcae/
Chlamydomonas reinhardtii	Chlorophyta	Genome	JGI https://phytozome.jgi.doe.gov/pz/portal.html
Nostoc sp. PCC 7120	Cyanobacteria	Genome	KEGG https://www.genome.jp/kegg-bin/show_organism?org=ana

4.2 Zygnematophyceae: from living Algae collections to the establishment of future models

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Contribution of Zhou H.

Participation in planning the structure of the paper; Writing the chapter “Zygnematophyceae-past and future models” of the manuscript; Data compilation for the tables and supplementary tables; Visualization; Experiments for the pictures of life cycle and transformation of Spirogyra in Fig. 2; Revision of the entire manuscript and preceding for publication.

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REVIEW PAPER

Zygnematophyceae: from living algae collections to the establishment of future models

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Abstract

The class of conjugating green algae, Zygnematophyceae (Conjugatophyceae), is extremely rich in species and has attracted the interest of phycologists for a long time. It is now widely accepted that this class of charophyte algae holds a key position in the phylogenetic tree of streptophytes, where they represent the closest relatives to all land plants (embryophytes). It is increasingly evident that robust model plants that can be easily cultivated and genetically transformed are necessary to better understand the process of terrestrialization and the related molecular, cellular, and physiological adaptations. Living algae collections play an important role, not only for phylogenomic-based taxonomy but also for screening for suitable model organisms. For this review, we screened six major public algae collections for Zygnematophyceae strains and established a cumulative list comprising 738 different taxa (including species, subspecies, varieties, and forms). From the described biodiversity with 8883 registered taxa (AlgaeBase) the cultured Zygnematophyceae taxa worldwide cover only ~8.3%. We review the past research on this clade of algae and discuss it from the perspective of establishing a model organism. We present data on the life cycle of the genera *Micrasterias* and *Spirogyra*, representing the orders Desmidiales and Zygnematales, and outline the current status of genetic transformation of Zygnematophyceae algae and future research perspectives.

Keywords: Closteriaceae, conjugating green algae, Desmidiaceae, Gonatozygaceae, Mesotaeniaceae, model organism, Peniaceae, Zygnematophyceae.

Introduction

The class Zygnematophyceae

Streptophytic algae of the class Zygnematophyceae, also called conjugating green algae or Conjugatophyceae (Guiry, 2013), are characterized by their special type of sexual reproduction, in which zygote formation occurs as a result of conjugation without the presence of flagellate gametes. The monophyletic class Zygnematophyceae comprises more than 4100 described species (Guiry and Guiry, 2019) and includes unicellular as well as filamentous forms. These species occur in almost all freshwater habitats worldwide, including extreme habitats such as acidic and ultra-oligotrophic bodies of water (e.g. peat bogs and fens). For a long time, their taxonomy was mainly based on morphological traits and grouped the Zygnematophyceae into two orders (Desmidiales and Zygnematales) and six families (Closteriaceae, Mesotaeniaceae, Desmidiaceae, Peniaceae, Gonatozygaceae, and Zygnemataceae). Recently, phylogenomic work on Zygnematophyceae including the taxon *Spirogloea muscicola* (de Bary) Melkonian comb. nov. has revealed the necessity of establishing the new subclass of

Spirogloeophycidae (Cheng *et al.*, 2019). It is noteworthy that new Zygnematophyceae species are regularly found and described (e.g. Coesel and van Geest, 2014).

Zygnematophyceae, and especially the family Desmidiaceae, have attracted the attention of researchers because of their unique cell morphology (Drawert and Mix, 1961; Meindl, 1993; Lütz-Meindl, 2016), and they seem ideal organisms for cytomorphogenetic studies. It is noteworthy, for example, that the Desmid *Micrasterias thomasiana* was chosen for the first visualization of the cellulose synthase complex in the plasma membrane (Giddings *et al.*, 1980).

The class Zygnematophyceae is attracting increasing interest from a growing number of evolutionary scientists since it has been shown to represent the closest relatives to the land plants (Wodniok *et al.*, 2011; Wickett *et al.*, 2014; Zhong *et al.*, 2015; Leebens-Mack *et al.*, 2019). It is now widely accepted that the ancestors of extant Zygnematophyceae were the group of organisms that were able to initiate the conquest of land in the middle Cambrian to early Ordovician, ~500 to 470 million years ago (Morris *et al.*, 2018; Puttick *et al.*, 2018), thus giving rise to the embryophytes, which have strongly diversified and shaped the natural history of the earth as a result of their enormous biomass and the increase of atmospheric oxygen content they have brought about. Zygnematophyceae and charophyte algae in general have been described as "evolutionary giants" (Domozych *et al.*, 2016) and it is evident that comparative genomics and the inference of ancestral plant traits is attracting more and more attention (Delaux *et al.*, 2012, 2015; Ju *et al.*, 2015; Rensing, 2017).

Phylogenic studies assume a single origin of land plants, which was initialized by the ancestors of Zygnematophyceae, thus opening the embryophytic line (Kenrick and Crane, 1997; Puttick *et al.*, 2018). It is an interesting question why the ancestors of Zygnematophyceae and not of other classes of algae were able to adapt to land and to evolve higly diverse and complex embryophytes, although survival in aerial habitats has also been achieved by many species of other algal groups, for example, from the Klebsormidiophyceae and Chlorophyceae. A detailed analysis of the genomes of *Mesotaenium endlicherianum* and *Spirogloea muscicola* reported by Cheng *et al.* (2019) suggested that horizontal gene transfer from soil bacteria to the ancestors of Zygnematophyceae occurred, giving rise to the *GRAS* and *PYL/PYR/RCAR* genes, which are restricted to the Zygnematophyceae and embryophytes. It is speculated that the common ancestor of Zygnematophyceae and embryophytes already lived in a subaerial or terrestrial environment (see also Harholt *et al.*, 2016). In order to further determine the ancestral traits of Zygnematophyceae on a molecular, cell biological, and physiological level, it is necessary to establish and analyse a large number of well-defined unialgal reference cultures. Sequence-based studies will then allow the depiction of a robust phylogeny within this class, enabling reliable conclusions to be drawn on basal ancestral and symplesiomorphic properties.

Taxonomy of Zygnematophyceae

While considerable phylogenomic work has been able to unequivocally determine the sister group of land plants, the molecular phylogeny within the class Zygnematophyceae can so far be regarded as only preliminary. As the current classification of the Zygnematophyceae with 55–60 different genera (most of which were described more than 100 years ago) is based purely on morphological features, which can be highly variable, it is not surprising that the traditional species arrangement disagrees with DNA sequence-based clustering for many genera (Gontcharov, 2008). A polyphyletic nature of the genera *Cosmarium*, *Euastrum*, *Staurodesmus*, and *Actinotaenium*, and *Netrium*, *Cylindrocystis*, and *Mesotaenium*, was shown by Gontcharov and Melkonian (2008, 2010). For the filamentous Desmidiaceae, Hall *et al.* (2008*b*) revealed the polyphyletic nature of *Desmidium* and *Spondylosium*. It is evident that taxa such as *Spirotaenia*, for which the exact taxonomic position needs a revision (Gontcharov and Melkonian, 2004), have been misplaced, as morphological characteristics alone can be misleading. *Spirotaenia minuta* has been shown be an early divergent streptophyte alga, as it clusters together with *Mesostigma* and *Chlorokybus* in phylogenomic analyses (Cheng *et al.*, 2019; Leebens-Mack *et al.*, 2019). A detailed phylogenomic analysis of the taxon *Spirotaenia muscicola* [CACC 0214, renamed to *Spirogloea muscicola* (de Bary) Melkonian] even led to the establishment of a new subclass (Spirogloeophycidae), new order (Spirogloeales), new family (Spirogloeaceae) and new genus (*Spirogloea*). *Spirogloea muscicola* was located at a basal position, close to the branching point between Zygnematophyceae and embryophytes (Cheng *et al.*, 2019).

Taken together, these works clearly demonstrate the necessity of comprehensive taxonomic and phylogenomic revisions within the class Zygnematophyceae using a large number of taxon samples and a large set of genes. It is evident that providing the greatest possible number of taxon samples is an essential prerequisite—and a challenging task.

The phylogenetic trees constructed on the basis of three combined nucleotide sequences—*SSU rDNA*, *rbcL*, and *cp LSU rDNA* by Gontcharov and Melkonian (2010), *rbcL*, *psaA*, and *coxIII* by Hall *et al.* (2008*a*), and *atpB*, *psbC*, and *rbcL* by Stancheva *et al.* (2014)—show many similarities despite the fact that different genes and taxa samples were used. Generally, the phylogenetic trees reflect the classical clades of Zygnematales and Desmidiales and, with a few exceptions, display the families in accordance with the classical taxonomy. All three trees (Hall *et al.*, 2008*a*; Gontcharov and Melkonian, 2010; Stancheva *et al.*, 2014) support the idea that most of the species belonging to the Desmidiales, and especially those of the family Desmidiaceae, represent a highly derived group. While most taxa of the Desmidiales are unicellular life forms, filamentous taxa also occur. The trait of filamentous growth in Desmidiales appears in two well-separated clades (Gontcharov and Melkonian, 2011). Given the fact that earlier as well as later divergent Desmidiales are unicellular, it can be assumed that the filamentous growth within Desmidiales is a secondary rather than a plesiomorphic characteristic. Analyses of a large set of plant transcriptomes allowed the inference of a high number of whole-genome duplications, especially in the order Desmidiales (Leebens-Mack *et al.*, 2019). This molecular feature is likely to be one of the drivers leading to the extreme richness of forms and taxa.

Regarding the topology of Zygnematales clades in the phylogenetic trees, it seems that filamentous as well as unicellular growth could have arisen *de novo* as homoplasic traits. In all three trees the filamentous *Zygnemopsis* clusters in the same branch together with the unicellular *Cylindrocystis*. Furthermore, the filamentous *Mougeotia* taxa appear to be derived from and are surrounded by unicellular taxa, again suggesting that filamentous growth is possibly an apomorphic characteristic here.

Interestingly, all three trees suggest that the filamentous *Spirogyra* taxa are early divergent Zygnemataceae. However, so far it seems impossible to consider filamentous growth as an ancestral trait, as certain *Mesotaenium* taxa were shown by Gontcharov and Melkonian (2010) to be earlier divergent than *Spirogyra*. Based on the available phylogenetic trees it seems impossible to connect either filamentous or unicellular growth with ancestral traits within Zygnematales. From today's perspective a representative Zygnematales model could have either form of growth, again underscoring that an unequivocal fixing of the topology of the Zygnematophyceae tree is essential. With a substantial number of Zygnematophyceae genomes being sequenced in the 10KP (10,000 Plants) genome sequencing project (Cheng *et al.*, 2018), we can assume that the subsequent phylogenomics will solve this issue in the near future.

Representation of Zygnematophyceae in public culture collections

It is evident that the aim to revise the taxonomy of class Zygnematophyceae is closely related to the understanding of the evolution of conjugating algae and to the selection of representative model organisms. A robust phylogenetic tree will enable the inference of ancestral traits and will be the basis for the choice of models representative of certain orders or families. The ongoing phylogenomic research certainly needs the sustainable hosting and availability of a maximum number of Zygnematophyceae taxa in public culture collections as resources for *ex-situ* conserved genetic material. In view of the large number (4120) of described Zygnematophyceae species (Guiry and Guiry, 2019), and the total number of taxa, including all infraspecific ranks, currently being as high as 8883 (M.D. Guiry, personal communication), this can best be achieved by combining the strains contained within several culture collections.

Analysis of the representation of taxa in six of the major freshwater living algae collections resulted in a total number of 2527 Zygnematophyceae strains, of which a few are cultured in more than one collection. The cumulative taxa list, however, represents only 738 different taxa (including infraspecific ranks) cultured in the six different collections (see Supplementary Table S1 at *JXB* online). As the analysis of culture collections did not include smaller collections or any from which the Zygnematophyceae strains and taxa could not be extracted from the website of the collection, the number of cultivated taxa representatives must be regarded as slightly underestimated. A worldwide list of algae collections, including their URLs, can be retrieved from the website of the Microalgae and Zygnematophyceae Collection Hamburg (MZCH; https://www.biologie.uni-hamburg.de/en/einrichtungen/wissenschaftliche-sammlungen/algensammlung.html) as well as from other collections and from the website of the World Federation of Culture Collections (WFCC; http://www.wfcc.info/index.php/collections/).

As the different culture collections have different foci, and only one collection (MZCH) focuses on Zygnematophyceae, it is evident that the number of strains and different taxa in different collections is highly variable. The number of strains ranged from 113 to 886 and the number of taxa from 68 to 347 in the six selected collections (Table 1). It is noteworthy that there is a low level of redundancy of taxa between the collections as, of the 738 different taxa, 499 are cultured in only one culture collection.

Generally, our preliminary meta-analysis clearly indicates a large gap between the huge biodiversity of Zygnematophyceae, with 8883 current taxa (4120 species, 13 subspecies, 3555 varieties, and 1195 forms; M.D. Guiry, personal communication; Guiry and Guiry, 2019) and their coverage by public culture collections, with only ~738 different current taxa (including infraspecific taxa). Taken together, the relative overall representation of Zygnematophyceae taxa in public culture collections is only ~8.3%, with representation being slightly higher for the Desmidiales taxa (8.9%) than for the Zygnematales taxa (5.3%) (see Table 2 and Supplementary Table S2). With regard to the global overall Zygnematophyceae biodiversity, it must

Table 1. Numbers of Zygnematophyceae strains and taxa in selected public culture collections (as at December 2019)

Name of collection	Acronym	Zygnematophyceae strains	Zygnematophyceae taxa	Taxa occurring only in this collection
Central Collection of Algae Cultures	CCAC	622	275	145
Coimbra Collection of Algae	ACOI	886	295	133
Microalgae and Zygnematophyceae Collection Hamburg	MZCH (SVCK)	621	347	155
Microbial Culture Collection at the NIES	NIES	157	68	34
SAG – Culture Collection of Algae	SAG	128	103	8
UTEX Culture Collection of Algae	UTEX	113	73	24

Collections with more than 100 strains and the possibility of extracting the Zygnematophyceae taxa from the collection's website are listed. A certain number of strains and taxa are cultured in more than one culture collection. A cumulative list of the cultured Zygnematophyceae taxa can be found in Supplementary Table S1. Reference publications for the living collections are: CCAC, Surek and Melkonian (2004); ACOI, Santos and Santos (2004); MZCH, von Schwartzenberg *et al.* (2013); NIES, Kasai (2009); SAG, Friedl and Lorenz (2012); UTEX, Nobles *et al.* (2016).

be assumed that, especially in the tropics, a large number of undescribed taxa exist (Coesel and van Geest, 2014). Thus, it is evident that further sampling and establishment of reference cultures is necessary at a large scale in order to allow the development of a robust phylogeny from which ancestral traits and ideal model organisms representative of the different Zygnematophyceae groups can be inferred.

Zygnematophyceae past and future models

In the past decade, evolutionary studies revealing the Zygnematophyceae to be a sister clade of land plants initiated increasing attention of researchers regarding not only phylogenetic analyses but also molecular mechanisms and unique cell biological features, including physiological functions related to the transition from water to land. Studies of molecular mechanisms and of cell biology require appropriate model organisms that enable detailed experimental and genetic manipulation (Leonelli and Ankeny, 2013).

Requirements of Zygnematophyceae model organisms

First, the model organisms should be easy to culture and maintain under laboratory conditions. Most of the Zygnematophyceae accessions can be cultured at low cost, as only water, macro- and micronutrients, vitamins, and light are required. For some strains, the addition of complex biological nutrients, such as soil or plant extracts, facilitates culture and fast algal growth.

Second, the life cycle of the candidate organisms has to be known and should be controllable *in vitro* in order to have experimental access to all developmental stages and cell types.

Third, in contrast to seed plants, culture under axenic conditions is essential for Zygnematophyceae model organisms (as well as for other microalgae), in order to avoid the potential influence of other microorganisms during the experiment (Keshtacher-Liebso *et al.*, 1995). Studies investigating the interactions between microbiota and algal physiology represent a growing research field, and for this purpose standard culture without the presence of other microorganisms should be possible as a control.

Fourth, genome-scale sequence information and transcriptome datasets of the candidate model organism are crucial for gaining insights into evolutionary questions and cellular phenomena (Joyce and Palsson, 2006) and also for functional gene analyses. Fragmented sequence information of some Zygnematophyceae species is already available, but it is not sufficient for detailed comparative and functional genomic studies (Rensing, 2017). Ploidy and genome size are important parameters for simplifying the whole-genome sequencing of candidate model organisms. To date, no high-coverage and annotated genome sequence for any Zygnematophyceae alga is publicly available, however, it can be assumed that these resources will be established in the near future.

Fifth, and last but not least, the availability of molecular tools that enable genetic transformation and gene manipulation is a prerequisite to carry out reverse genetic studies and to access in detail the molecular and cell biological characteristics of the model organisms (Leonelli and Ankeny, 2013).

During the past decades, several Zygnematophyceae algae have been used and proposed as model organisms for studying different biological processes (Table 3). Out of the six Zygnematophyceae families, four are covered by the work mentioned in Table 3. For the families Gonatozygaceae and Mesotaeniaceae, only fragmented work is available in the literature.

Model organisms for Desmidiales

Algae of the order Desmidiales are characterized by their symmetrical cell architecture. Unicellular members of the Desmidiales from the genera *Micrasterias*, *Penium*, and *Closterium* represent marvellous systems for studying cell physiology, development, and sexual reproduction.

For more than 50 years, the genus *Micrasterias* has been considered important for studying cell wall biochemistry, cell division, and morphogenesis because of its extremely differentiated and ornamented cell morphology (Ueda and Yoshioka, 1976; Giddings *et al.*, 1980; Meindl, 1993; Lütz-Meindl, 2016)). After cytokinesis, the new daughter semicells undergo a series of cytomorphogenetic changes when establishing their parental pattern (Lacalli, 1975; Vannerum *et al.*, 2011). The morphological variation, cell wall development, and function of dictyosomes, and the role of the cytoskeleton as well as physiological responses that are involved in the process of *Micrasterias* cell pattern formation are reviewed in Lütz-Meindl (2016). However, experimental platforms allowing elucidation of the molecular basis of these processes are still lacking. *Micrasterias denticulata,* which has a large cell size, has been described in terms of its vegetative growth; *in vitro* sexual reproduction has not been reported yet. It was the first Desmidiaceae alga to be transiently transformed via particle bombardment with constructs containing heterologous promoters. Transformation was applied for investigation of the subcellular localization of expansin (Vannerum *et al.*, 2010, 2011).

Life cycle of homothallic Micrasterias radians *var.* evoluta *under laboratory conditions*

In the MZCH culture collection it was observed (S. Körner and K. von Schwartzenberg, unpublished results) that *M. radians*

Table 2. Taxa described and covered by six major living algae collections (see Table 1) and percentage of taxa coverage

Order	Species described	Taxa described	Strains in collections	Taxa in collections	Coverage of taxa by collections (%)
Desmidiales	2919	7452	2112	661	8.9
Zygnematales	1201	1431	414	76	5.3
Spirogloeales	1	1	1	1	–

Table 3. Overview of properties of potential Zygnematophyceae model organisms based on selected published and unpublished work

Order	Genus (Family)	Research focus	Available genomic resources	Life cycle *in vitro*	Transient transformation	Stable transformation	Reference
Desmidiales	*Micrasterias denticulata* (Desmidiaceae)	Cell morphogenesis, cell wall deposition, cytoskeleton	n.r.	n.r.	Particle bombardment	n.r.	Vannerum *et al.* (2010)
	Micrasterias radians var. *evoluta* (Desmidiaceae) MZCH 518		n.r.	In this review	Protoplasts (PEG), particle bombardment	n.r.	Zhou and von Schwartzenberg (unpublished work)
	Penium margaritaceum (Peniaceae)		n.r.	Closed	n.r.	Agrobacterium	Sørensen *et al.* (2014)
	Closterium peracerosum–strigosum–littorale complex (Closteriaceae)	Sexual reproduction	Genome in preparation	Closed	Particle bombardment	Particle bombardment	Abe et al. (2008, 2011); Kanda *et al.* (2017)
Zygnematales	*Mougeotia scalaris* (Zygnemataceae)	Cell division	n.r.	n.r.	Particle bombardment	n.r.	Regensdorff *et al.* (2018)
	Spirogyra pratensis (Zygnemataceae) MZCH 10213	Signaling	Partial transcriptome	In this review	Protoplasts (PEG), particle bombardment	n.r.	Ju *et al.* (2015); Zhou and von Schwartzenberg (unpublished work)

n.r., Not reported; PEG, polyethylene glycol.

var. *evoluta* (MZCH 518) performed conjugation, zygospore formation, and germination under *in vitro* conditions (Fig. 1). As the strain originated from a clonal culture, it is considered homothallic.

Under our laboratory conditions using liquid C medium or modified Woods Hole medium (WHM), *M. radians* var. *evoluta* undergoes in most cases only asexual growth via cell division. Mitosis occurs every 2–3 days and is followed by the regenerative growth of the young semicells, during which the cell shape is re-established. Following the mitotic division of the nucleus, the two semicells separate at the isthmus region and form new daughter cells by bulging of the septum, which originates from the inward centripetal growth of the cell wall girdle. Each of the new semicells successively builds its lobes, including the polar and two lateral lobes (Fig. 1B), followed by the first and second doubling of the lateral lobes (Fig. 1C, D), and finally develops a morphology identical to that of the old semicells. Both semicells contain a single chloroplast that copies the shape of the outer cell wall (Carter, 1919).

Sexual reproduction in *M. radians* var. *evoluta* was reliably found only in the non-axenic line. Diploid zygospores (Fig. 1E) are formed by conjugation between vegetative cells that have undergone transformation into gametangia (Lenzenweger, 1968; Coesel and Teixeira, 1974; Blackburn and Tyler, 1980, 1981). The mature spores (Fig. 1F) are dark brown and spherical in appearance, have a thick three-layered cell wall, and are covered with spines. Zygnematophyceae zygospores have been reported to be extremely stress tolerant and to survive unfavourable abiotic stress scenarios such as desiccation, exposure to antibiotics, temperature variation, and high radiation (Holzinger and Pichrtová, 2016). Meiosis takes place within the zygospore and only two meiotic products are maintained, being typical for Desmidiaceae (Rieth, 1961) (Fig. 1F). Germination of dormant spores occurs after rewetting them with fresh medium for a few days. With swelling, the mesospore is ruptured, releasing a germination vesicle, which is still covered by the thin hyaline endospore wall. The remaining empty zygospore consists of the exospore (the outer spiny wall) and the brown-colored middle wall layer (Fig. 1G). Thereafter, the two meiotic products develop into *Cosmarium*-like germlings within the germination vesicle (Fig. 1G). The two germlings are then released from the vesicle (Fig. 1H) and undergo a first vegetative cell division to generate prevegetative cells (Fig. 1I), which consist of one *Cosmarium*-like and one *Micrasterias*-like semicell. Further vegetative division leads to the formation of normal star-shaped *M. radians* var. *evoluta* cells (A. Wilkens, H. Zhou, S. Bock, V. Schwekendiek, S. Körner, E. Woelken, K. von Schwartzenberg, unpublished results). The first attempts to transiently transform *M. radians* by polyethylene glycol (PEG)-mediated protoplast formation (Fig. 1J–L) have been successful. However, the protoplasts were unable to regenerate into vegetative cells. From the family Peniaceae, *Penium margaritaceum* has been described as a valuable model organism for elucidating the fundamental principles of cell wall development, cell expansion, and secretion dynamics (Domozych *et al.*, 2014, 2016). The fast growth of the simple cylindrical large cells, which possess only a primary

cell wall, makes *Penium* an excellent experimental organism for studying the effects of many chemical agents on cell wall development (Domozych *et al.*, 2011). The conjugation process was documented (Tsuchikane *et al.*, 2011) and *Agrobacterium tumefaciens*-mediated transformation was established based on this growth stage. RNA interference enabled the study of cell wall modification and formation, pectin methylesterase, and cellulose synthase (Sørensen *et al.*, 2014). Surprisingly, no follow-up study on transgenic *P. margaritaceum* has been published since 2014.

The genus *Closterium* is probably the best-established model species among the unicellular Zygnematophyceae for studying sexual reproduction processes (Sekimoto *et al.*, 2014; Sekimoto, 2017; Tsuchikane and Sekimoto, 2019). *Closterium peracerosum–strigosum–littorale* complex was the first Zygnematophyceae alga to be successfully transiently transformed via biolistic bombardment using endogenous promoters (Abe *et al.*, 2008). Stable nuclear gene transformation by random insertion into the genome was established by using the same technique (Abe *et al.*, 2011). Further studies targeting genes of interest by techniques such as knockdown of expression by antisense RNA and CRISPR/Cas9-based knockout of receptor-like protein kinase (RLK) in the *C. peracerosum–strigosum–littorale* complex were first (and so far exclusively) reported by the working group of Hiroyuki Sekimoto (Hirano *et al.*, 2015; Kanda *et al.*, 2017).

Model organisms for Zygnematales

As outlined above, the order Zygnematales is considered to be early divergent among the Zygnematophyceae, and therefore holds an important position for studies of the origin of land plant traits including growth- and development-related regulatory networks (Delwiche and Cooper, 2015).

As a member of the family Zygnemataceae, the genus *Mougeotia* has attracted attention, especially with respect to the movement of its flat-shaped chloroplast, which is controlled by gradients of phytochrome (Pfr) (e.g. Wagner and Klein,1981). The growth and cell division of *Mougeotia* have also been analysed, showing some land-plant-like features, and might further provide insight into the evolution of the mitotic and cytokinetic apparatus of streptophytes (Pickett-Heaps and Wetherbee, 1987; Brown and Lemmon, 1993; Buschmann and Zachgo, 2016). Transient genetic transformation of *Mougeotia scalaris* was achieved by utilizing vectors with a homologous promoter (tubulinA) by means of biolistic transformation (Regensdorff *et al.*, 2018).

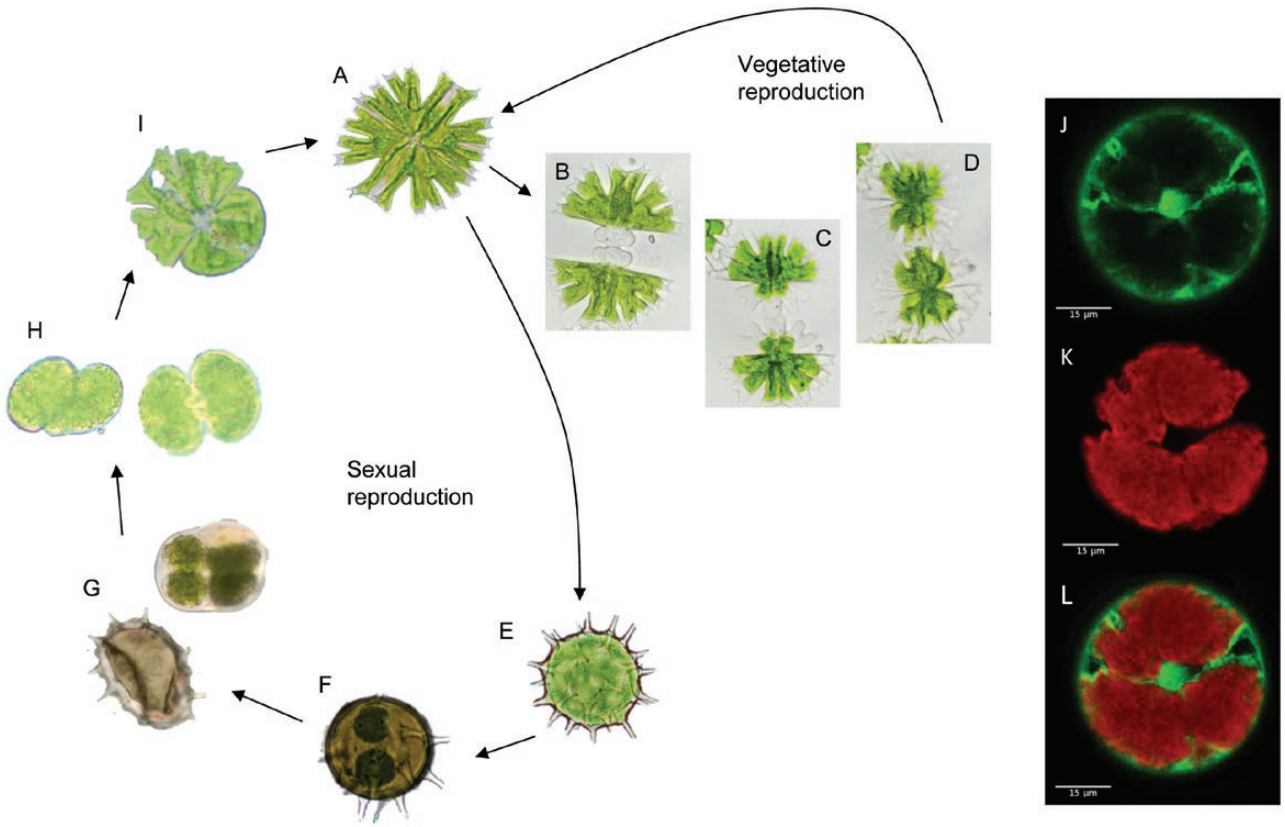

Fig. 1. Life cycle of *Micrasterias radians* var. *evoluta* (MZCH 518). Vegetative reproduction (1n) is illustrated in (A–D). Conjugation of two gametes deriving from vegetative cells leads to the formation of a zygospore (2n) (E), which becomes brown during its maturing process (F). Meiosis takes place within the zygospore, creating a germination vesicle, which is released from the zygospore (G). Two germlings (1n) (H) are released from the germination vesicle and become pre-vegetative cells (I) and finally vegetative cells (A) through cell division. (J–L) Fluorescence microscopy images of transformed protoplasts using green fluorescent protein (GFP) expression constructs. (J) GFP signals; (K) intrinsic fluorescence of chlorophyll; (L) Merged image of J and K. (A–D, J–L) Images by courtesy of Alwine Wilkens.

Ethylene production and an ethylene-induced cell elongation response were demonstrated in *Spirogyra pratensis*, which is an unbranched filamentous alga also belonging to the family Zygnemataceae (Ju *et al.*, 2015). Transcriptomic analysis of *S. pratensis* revealed the regulatory role of ethylene in cell wall metabolism, photosynthesis, and stress responses (Van de Poel *et al.*, 2016). Genetic transformation has not been reported for this species yet, but transient gene expression protocols are currently being developed by the authors of this review. Preliminary results showed the successful regeneration of transformed protoplasts after PEG-mediated transfection, as well as vegetative cell transformation via particle bombardment (Fig. 2F–I). So far there has been no report on the generation of stable transformants for a species from the Zygnematales.

Life cycle of Spirogyra pratensis *under laboratory conditions*

Spirogyra pratensis exhibits fast vegetative growth in both C and WHM media under standard culture conditions. Cell division takes place through a special pattern of cytokinesis integrating cleavage and cell plate formation, which is regarded as an important phylogenetic indicator demonstrating the algal ancestry of the phragmoplast/cell plate cytokinesis of land plants (Fowke and Pickett-Heaps, 1969*a*, *b*).

Sexual reproduction of *S. pratensis* (MZCH 10213), including conjugation process and zygospore formation, is possible on solid C medium (Fig. 2A–E). Both lateral and scalariform conjugation can be observed, with the latter occurring with a much higher frequency. In the process of lateral conjugation, bulges emerge on both sides of the transverse septum of two adjacent cells in the same filament and grow to a conjugation tube. In the process of scalariform conjugation (Fig. 2B), a protuberance appears first in one filament and then in the adjacent position on the other filament. The subsequent growth of these protuberances separates the two conjugating filaments, and forms the conjugation bridge. The two conjugating cells behave as donor and recipient gametangia. The protoplast of the mobile gamete migrates via the conjugation tube to the recipient gametangium, and syngamic fusion results in the formation of an elliptical-shaped diploid zygospore, which is covered by a thick cell wall (Fig. 2C, D). After a period of dormancy, germination was occasionally observed when zygospores were supplied with fresh medium and incubated under light (see also Agrawal and Chaudhary, 1994). In this process, the diploid nucleus undergoes meiosis to produce four haploid nuclei, of which only one survives (Fowke and Pickett-Heaps, 1971). The zygospore with the one remaining haploid nucleus gradually enlarges and ruptures to release a germ tube (Fig. 2E). Finally, a new vegetative filament is formed through repeated cell divisions of the germ tube.

Outlook

Research in Zygnematophyceae and other charophyte algae will continue to increase, as can be deduced from recent initiatives such as MAdLand (Molucular Adaptation to Land) and the 10 KP genome sequencing project (Cheng *et al.*, 2018), where Zygnematophyceae are contributing to a deepened understanding of early plant evolution, especially the conquest of terrestrial habitats. With regard to the availability of representative research organisms with the potential to serve as model algae, we have to take into account the relatively low coverage of the natural biodiversity in public living collections. Thus, the establishment of new isolates and reference cultures might lead to new and alternative models in future.

With respect to the high degree of diversity and the occurrence of reductive evolution, it appears appropriate to develop more than just one model organism for the class

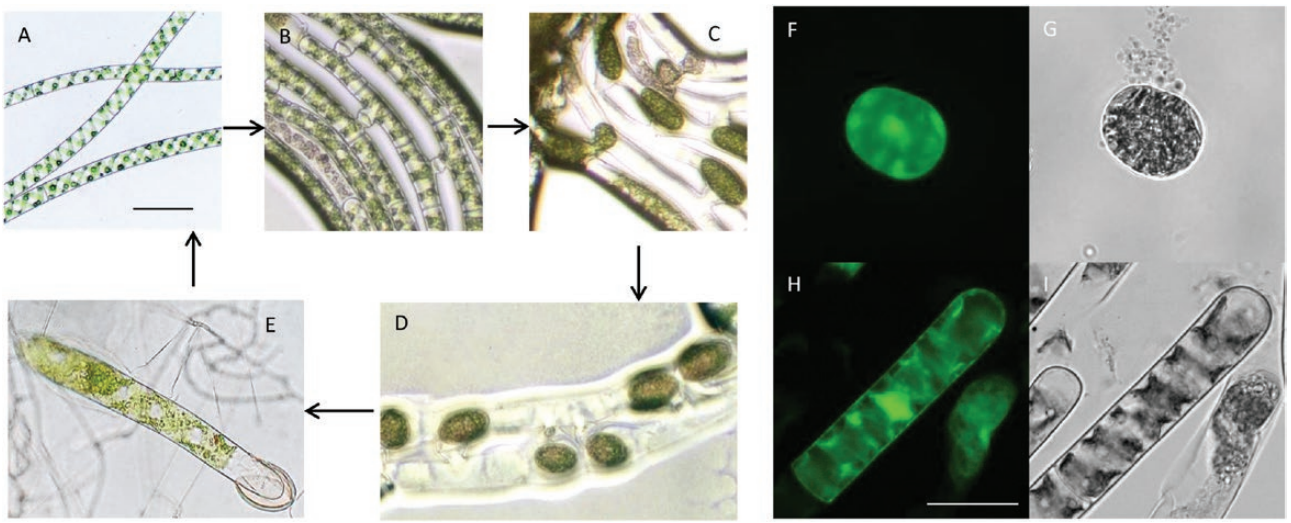

Fig. 2. (A–E) Life cycle of *Spirogyra pratensis* (MZCH 10190). (A) Vegetative filaments. (B) Scalariform conjugation was induced on the agar medium plate. (C) Migration of the protoplast from one gamete to its conjugation partner via the conjugation tube. Syngamic fusion resulted in the formation of diploid zygospores (image courtesy of Janet Schwab). (E) Germination of zygospores occurred under favorable conditions after a period of dormancy (image courtesy of Thaís Ferreira de Oliveira). (F–J) Fluorescence microscopy images of transformed *S. pratensis* using green fluorescent protein (GFP) expression constructs. (F) GFP channel and (G) bright-field image of a regenerating protoplast after polyethylene glycol-mediated transformation; (H) GFP channel and (I) bright-field image of a biolistically transformed vegetative cell. Scale bar=50 μm.

Zygnematophyceae. While—although to different extents—considerable research has been published on Closteriaceae, Peniaceae, Desmidiaceae, and Zygnemataceae and has covered the question of suitability as a model organism, the existing reports on Gonatozygaceae and Mesotaeniaceae seem limited in this respect. Due to the basal position in phylogenetic trees of the family Mesotaeniaceae in particular (Hall *et al.* 2008*a*; Gontcharov and Melkonian, 2010; Stancheva *et al.* 2014, Cheng *et al.* 2019), further work to identify potential model organisms for this family also seems necessary.

Almost any field of molecular plant research is important to be addressed for the class Zygnematophyceae; the ancestral roots of adaptation to stress, the capacity of acclimation, signaling and phytohormones, regulation of the life cycle, and microbiome interaction are some examples of subjects for which research projects are starting to emerge. It is evident that besides enlarging the pool of available accessions and reference cultures, the further development of molecular resources such as high-quality genomes/transcriptomes and the availability of transgenic approaches allowing reverse genetic studies are important prerequisites for the emerging research on Zygnematophyceae.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Distribution of taxa and number of strains in six major living algae collections.

Table S2. Strain and taxa numbers for Zygnematophyceae families as represented in six major living algae collections (see Table 1).

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4.3 Expanding the molecular toolbox for Zygnamatophyceae — transient genetic transformation of the desmid *Micrasterias radians* var. *evoluta*

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Contributions of Zhou H.

Lab work: PEG-mediated transformation of protoplasts (Fig. 2 and Table 1); Particle bombardment transformation of vegetative cells (Fig. 3 and Table 2); Intracellular labelling the actin filaments (Fig. 4).

Manuscript: Participation in designing the project; Writing the manuscript and working on the review and editing.

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Understanding and using algae

Expanding the molecular toolbox for Zygnematophyceae – transient genetic transformation of the desmid *Micrasterias radians* var. *evoluta*

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ABSTRACT

In the past, various species of the unicellular algal genus *Micrasterias* (Zygnematophyceae) have been used for research in the fields of cell biology and physiology. Relatively large cell size, highly differentiated cell shape and a remarkable evolutionary position make *Micrasterias* interesting, especially for cytomorphogenetic studies. However, within this genus a model organism enabling molecular research has not yet been established. *Micrasterias radians* var. *evoluta* (W.B.Turner) Krieger allows easy culturing under axenic conditions and performs its whole life cycle *in vitro* thus fulfilling two important prerequisites for a model organism. In this work resources allowing transient expression of transgenes were developed. First, axenic lines were obtained by the treatment of zygospores with a cocktail of antibiotics followed by germination and regeneration. In order to allow transgene expression we isolated the 5′ -flanking region of the *M. radians* var. *evoluta Actin1* gene (*MrACT1*) and fused it to the green fluorescent protein (GFP). A higher promoter activity compared with various heterologous promoters regarding GFP expression was observed. Transient transgene expression was achieved by polyethylene glycol (PEG)-mediated protoplast transformation, yielding a rate of 3.9% transformed cells per surviving protoplasts. Transgene expression was also achieved by particle bombardment of vegetative cells. Employing the established protocol, a Lifeact-GFP fusion protein for labelling F-actin was expressed, allowing visualization of the actin cytoskeleton in *M. radians* var. *evoluta*.

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Introduction

Since it has been confirmed that algae of the class Zygnematophyceae represent the group most closely related to land plants, these organisms have attracted increasing attention for research in cell biology, developmental biology, physiology and phylogenomics. A deeper understanding of Zygnematophyceae will help in elucidating evolutionary mechanisms in the water-to-land transition (Delwiche & Cooper, 2015; Domozych *et al.*, 2016). Desmidiaceae represents the most species-rich family among the Zygnematophyceae, comprising unicellular as well as filamentous forms. In phylogenetic trees Desmidiaceae appear as a derived clade (e.g. Gontcharov & Melkonian, 2011).

As typical members of the unicellular Desmidiaceae, algae of the genus *Micrasterias*, which possess an extremely complex and ornamented cellular architecture, have previously been considered as models for studying cell biology and physiology (Meindl, 1993; Lutz-Meindl, 2016). Vegetative cells of *Micrasterias* consist of two bilaterally symmetrical semi-cells that are characterized by a central polar lobe and flanking lateral lobes that vary in numbers across species (Škaloud *et al.*, 2011). During the process of asexual reproduction, the semi-cells are separated by a septum formed soon after mitotic division of the nucleus. Mitosis is followed by a series of cytomorphogenetic processes in which the daughter semi-cells establish the morphology of the parental semi-cells (Kiermayer, 1968; Lacalli, 1975; Vannerum *et al.*, 2011; Zhou & von Schwartzenberg, 2020). This peculiar growth pattern of *Micrasterias* made it attractive for research on revealing cell shaping and regulation in the process of cytomorphogenesis (Lutz-Meindl, 2016).

Algae from the genus *Micrasterias*, like many Desmidiaceae, predominantly inhabit peatlands at different altitudes and climate zones. This genus has been shown to be adapted to a variety of abiotic stress factors such as UV radiation, drought, varying temperatures, as well as pollutants such as heavy metals (Meindl & Lütz, 1996; Affenzeller *et al.*, 2009; Volland *et al.*, 2014). Understanding the adaptive mechanisms of these freshwater algae that enable them to cope with different stress factors will be helpful for studies of evolutionary processes related to the land plant transition. In this context the establishment of new and robust models becomes more and more important (Rensing, 2017), as can be inferred from work on comparative genomics/

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transcriptomics of charophytes (e.g. Hori *et al.*, 2014; Nishiyama *et al.*, 2018; Cheng *et al.*, 2019; Jiao *et al.*, 2020). For the Desmidiaceae, the most species-rich taxonomy group in Charophyta, the lack of suitable models is a limiting factor for this kind of evolutionary research.

In the past, it has been difficult to identify potential model organism candidates that exhibit fast growth under axenic conditions and can be easily genetically manipulated (Zhou & von Schwartzenberg, 2020). Despite considerable DNA sequence resources, no high coverage transcriptome and genome dataset has been reported for *Micrasterias*, only fragmented sequences are accessible for several species. Transgene expression was first achieved for *Micrasterias denticulata* using the construct pSA405A containing a heterologous promoter from *Closterium* (Closteriaceae), transferred via particle bombardment, but this yielded relatively low transformation rates (Vannerum *et al.*, 2010, 2011, 2012). Increased transformation efficiencies can be obtained when using homologous promoters as reported for *Closterium peracerosum-strigosum-littorale* complex (*C. psl* complex) (Abe *et al.*, 2008) and *Mougeotia scalaris* (Regensdorff *et al.*, 2018). Stable transformation of Zygnematophyceae algae was so far reported for *C. psl* complex (Abe *et al.*, 2011; Hirano *et al.*, 2015; Kanda *et al.*, 2017) and *Penium margaritaceum* (Sørensen *et al.*, 2014). However, versatile genetic transformation protocols which are easily applicable and enable efficient subcellular localization, as well as genomic manipulation, are still lacking for Desmidiaceae.

In this paper we describe a procedure where an axenic culture of *Micrasterias radians* var. *evoluta* (MZCH 672), preselected from the Microalgae and Zygnematophyceae Collection Hamburg (MZCH), was established. With this axenic strain, we established a highly efficient transient genetic transformation system via different approaches (PEG mediated protoplast transfection and particle bombardment) utilizing the green fluorescent protein (GFP) as a reporter. The endogenous *Actin1* promoter (p*MrACT1*) was isolated and enabled higher expression efficiency compared with heterologous promoters. We demonstrate that the established protocol is useful for *in vivo* gene expression and subcellular localization studies. As a proof of concept, we expressed the fluorescent tag Lifeact (Riedl *et al.*, 2008; Regensdorff *et al.*, 2018) for labelling the cellular actin network. The proposed resources established for *M. radians* var. *evoluta* make this organism a candidate model facilitating molecular and cell biological research for Desmidiaceae.

Material and methods

Algal cultures

The algal strain employed in this study was *Micrasterias radians* var. *evoluta* (W.B.Turner) Krieger, which was selected from the Microalgae and Zygnematophyceae Collection Hamburg (MZCH, strain No. 518) (von Schwartzenberg *et al.*, 2013). The alga was originally collected in 2001 by L. Krienitz from Lake Ol Bolossat, Kenya; the variety '*evoluta*' was determined by Škaloud *et al.* (2011). An axenic derivative of strain MZCH 518 is listed under the number 672 in MZCH. Cultures were maintained in 100 ml Erlenmeyer flasks in the modified C-medium (Ichimura, 1971; Supplementary table 1) under the standard growth condition (23°C, 30 µmol photons m^{-2} s^{-1} with 16 h light: 8 h dark cycle). The working cultures were cultivated in 500 ml Schott flasks with aeration (sterile air ~500 ml min^{-1}) under standard conditions except that the light intensity was increased to 80 µmol photons m^{-2} s^{-1}. The culture medium was refreshed weekly to ensure rapid growth. For the purposes of cultivation on solid C-medium, 1.5% plant agar (Duchefa, Haarlem, the Netherlands) was supplemented.

Purification of Micrasterias radians *var.* evoluta

For the purification of *M. radians* var. *evoluta*, mature and dry zygospores were suspended in C-medium and treated with serial dilution of two antibiotic mixtures modified from Droop (1967). Mix I contained 10 mg ml^{-1} ampicillin, 1.6 mg ml^{-1} streptomycin, 1.6 mg ml^{-1} cycloheximide (actidione) and 0.8 mg ml^{-1} chloramphenicol while Mix II contained 16 mg ml^{-1} ampicillin, 4 mg ml^{-1} streptomycin and 0.016 mg ml^{-1} chloramphenicol. The treated zygospores were incubated under standard growth conditions (23°C, 30 µmol photons m^{-2} s^{-1} with 16 h light: 8 h dark cycle) until germination and growing up to culture suspensions. The absence of microbial contaminants was tested by cultivation of the culture aliquots on LB-agar for bacteria and PDA-agar for fungi.

Isolation of Actin1 *promoter from* Micrasterias radians *var.* evoluta *and cloning to GFP expression vector*

Due to the lack of sequence data for *M. radians* var. *evoluta*, degenerate primers (Act-Fwd 01, Act-Fwd 02, Act-Rv 01 and Act-Rv 02) were designed to clasp the coding region of *Actin1* gene (*MrACT1*) based on an alignment of homologues derived from eight plant species (*M. denticulata* (DQ987611.1), *M. rotata* (HG805276.1), *Penium margaritaceum* (Seq. Nb.: 8436), *Mougeotia scalaris* (Seq. Nb.: 17221), *Spirogyra pratensis* (Seq. Nb.: 24,003), *Klebsormidium nitens* (Seq. Nb.: 21459), *Mesostigma viride* (Seq. Nb.: 64,879) (Delwiche & Cooper, 2015) and *Arabidopsis thaliana* (U42007.1). Total genomic DNA was isolated from *M. radians* var. *evoluta* by the cetyltrimethyl ammonium bromide (CTAB) method (Murray & Thompson, 1980) and was used as a template for the following PCRs. PCR was performed with the DreamTaq™ polymerase kit

(Thermo Fisher Scientific, Bremen, Germany). The gene-specific TAILB primers were nested reverse primers designed according to the clasped coding sequence of *MrACT1* gene and used for isolation of the promoter regions via Thermal Asymmetric Interlaced (TAIL) PCR following the protocol described by Liu & Whittier (1995) and Liu *et al.* (1995). High-efficiency TAIL (Hi-TAIL) PCR was performed according to Liu & Chen (2007) with the gene-specific TAILC primers, which were nested reverse primers binding to the promoter region isolated via TAIL-PCR. The forward arbitrary primers AD1-4 and LAD1-4 used for both TAIL and Hi-TAIL were the same as the ones used by Liu *et al.* (1995) and Liu & Chen (2007). The PCR products were checked via agarose gels and afterwards sequenced by commercial Sanger sequencing. Rapid amplification of 5′ cDNA ends (5′-RACE) was performed with a ligation dependent method according to Dallmeier & Neyts (2013). The total RNA was isolated according to Oñate-Sánchez & Vicente-Carbajosa (2008). The cDNA used for RACE-PCR was synthesized by using the RevertAid Reverse Transcriptase (Thermo Scientific) with the specific 5′-phosphorylated primer cDNAsy. Ligation of cDNA was performed with the T4 RNA Ligase (Thermo Scientific). The circularized cDNA was amplified by inverted PCR with the specific forward (RACEFW2) and reverse (TAILB1) primers.

The GFP expression vector pSA405A (Abe *et al.*, 2008) served as backbone for the construction of the transformation vector, in which the endogenous *MrACT1* promoter was used to drive cGFP expression. The 1158 bp *MrACT1* promoter sequence was amplified from the genomic DNA with the Q5 high-fidelity DNA polymerase (New England Biolabs, Frankfurt/Main, Germany) using the primer pair FwProm and RvProm, which contains the restriction enzyme *Eco*RI and *Spe*I recognition sites in the 5′ end respectively. After restriction digestion, the PCR product was inserted into the corresponding sites of pSA405A. The sequence information of the primers used for all PCRs is listed in Supplementary table 2. The *MrACT1* promoter sequence and the partial coding sequence were submitted to GenBank (MN844879).

Protoplast isolation

Protoplast isolation and transfection of *M. radians* var. *evoluta* with the plasmid pAW001 via polyethylene glycol (PEG) was performed using a modified method published for *Physcomitrella patens* by Schaefer *et al.* (1990). Protoplasts were generated from *M. radians* var. *evoluta* by enzymatic digestion with combination of 1% driselase (Sigma-Aldrich, Munich, Germany) and 1% cellulase (Sigma-Aldrich, Munich, Germany) in the digestion buffer (pH 5.6) containing 0.25 M mannitol, 10 mM $CaCl_2$, 3 mM $MgSO_4$ and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES). After overnight incubation, undigested cells and cell debris in the supernatant were carefully removed by gentle pipetting. The isolated protoplasts were transferred to a sterile round bottom tube (13 ml) and sedimented by centrifugation (100 *g*, 5 min). The protoplasts were re-suspended in digestion buffer and the washing step was repeated twice. The density of the protoplasts was determined microscopically by a Neubauer haemocytometer.

PEG mediated protoplast transfection of Micrasterias radians *var.* evoluta

The isolated protoplasts were suspended in 3M medium (0.25 M mannitol, 15 mM $MgCl_2$, 0.1% MES, pH 5.6) and adjusted to a density of 1.0×10^6 protoplasts ml^{-1}. Transfection of *M. radians* var. *evoluta* was performed by mixing 200 µl of protoplast suspension with 20 µg plasmid DNA (~1 µg $µl^{-1}$) and 200 µl PEG solution (40% PEG 4000 and 0.1 M $Ca(NO_3)_2$ in 3M medium with 2-fold dilution). The mixture was subsequently incubated at room temperature for different durations followed by heat shock as indicated in the Results section. Afterwards the assay mixture was stepwise diluted by addition of 3M medium to a final volume of 7 ml. For cultivation of the treated protoplasts, the regeneration (REG) buffer (pH 5.8) was prepared from the C-medium supplemented with 0.1% $CaCl_2$, 2.5% glucose and 2.5% mannitol. GFP signal was microscopically observed after protoplast incubation for 2 days (1 day in dark and 1 day with 30 µmol photons m^{-2} s^{-1} irradiation under standard growth condition) using a Zeiss Axio Imager A2 (Carl Zeiss Microscopy, Jena, Germany).

Transformation of Micrasterias radians *var.* evoluta *by particle bombardment*

Micrasterias radians var. *evoluta* grown in liquid C-medium under standard conditions for 5–7 days was harvested by centrifugation (200 *g*, 5 min) and washed with fresh C-medium. Approximately 5×10^4 cells were transferred to the centre of 90 mm diameter Petri dishes containing C-medium with 1.5% agar and spread in an area with a diameter of ~5 cm. The samples were dried until no obvious liquid was visible and subsequently cultivated for 6 days under standard conditions (23°C, 30 µmol photons m^{-2} s^{-1} with 16 h light: 8 h dark cycle). The preparation of plasmid DNA coated gold particles was followed according to the protocol described by Regensdorff *et al.* (2018). The bombardment of the *M. radians* var. *evoluta* was performed using the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad Laboratories, Feldkirchen, Germany) according to the manufacturer's instructions. The following parameters were applied: the pressure

capacity of rupture discs was 1100, 1350 or 1550 psi. The distance from the stopping screen to cell targets was 6.0 cm or 9.0 cm. The size of gold particles used in this study was 0.6 µm or 1.0 µm (Bio-Rad). After bombardment, the plates were incubated at 23°C in dim light (15 µmol photons m^{-2} s^{-1}). After culturing for 3 days, cells were observed for GFP expression with a fluorescence microscope (Zeiss Axio Imager A2, Carl Zeiss Microscopy, Jena, Germany) and/or laser scanning confocal microscope (LSCM, Leica Microsystem, Wetzlar, Germany) equipped with a VisiScope live cell imaging system (Visitron Systems GmbH, Puchheim, Germany).

Results

Preparation of axenic cultures Micrasterias radians *var.* evoluta

?Axenization of *M. radians* var. *evoluta* was achieved by treatment of zygospores with dilution series of two different antibiotics mixtures: Mix I containing ampicillin, streptomycin, actidione and chloramphenicol and Mix II containing ampicillin, streptomycin and chloramphenicol. The zygospores treated with dilution series of Mix II yielded either exclusively dead cells or surviving algae cells with microorganisms still being present. However, an axenic culture was obtained in the treated samples with 1:4 dilution of Mix I, which inactivated the bacteria and fungi but did not affect the viability of zygospores. The axenic status of the obtained strain was confirmed by growth tests on LB and PDA agar plates. The new strain, entered into the MZCH collection under the accession No. 672, was used in all further experiments.

Isolation of the Micrasterias radians *var.* evoluta Actin1 *promoter and construction of GFP expression vector*

With the aim to highly express genes of interest in *M. radians* var. *evoluta*, the endogenous *Actin1* gene (*MrACT1*) was chosen for promoter isolation. Since no sequence information was available for this *Micrasterias* species, the *MrACT1* promoter was isolated in three steps as follows. Firstly, a 740 bp genomic fragment of the *MrACT1* coding region was amplified with degenerate primers (Act-Fwd 02 and Act-Rv 02). Relying on the sequence of the coding region, the Thermal Asymmetric Interlaced (TAIL) PCR was performed to extend the 5'-flanking region by ~550 bp. With the High-efficiency TAIL (Hi-TAIL) PCR, another ~1500 bp were obtained. In total, 1838 bp of genomic sequence upstream and 937 bp downstream from the putative start codon of *MrACT1* were identified (Fig. 1a; accession no. MN844879). The sequence obtained via the ligation based 5′ RACE-PCR was about 300 bp. It was aligned with the identified genomic sequence of *MrACT1* gene (Supplementary fig. 1) and the result revealed that the transcriptional start is located 175 bp upstream of the start codon (Fig. 1a and Supplementary fig. 1). The sequence also contained a 32 bp sequence from the coding region due to the methodology. By comparison of the gDNA and cDNA sequences of the coding region of *Actin* genes from different organisms (Supplementary figs 1 and 2), one intron was identified in the 5′ UTR (nucleotides −121 to −18) and a further three were localized in the known coding sequence (nucleotides 59 to 195, 363 to 452 and 681 to 770). The putative TATA-Box is positioned 29 bp upstream from the transcriptional start and 204 bp upstream from the translational start. A 1158 bp fragment including the start codon was amplified from the *MrACT1* promoter region and was cloned to the *Eco*RI and *Spe*I sites of the vector pSA405A (Abe *et al.*, 2008) to generate the plasmid pAW001 (Fig. 1b), in which the original p*CpCAB* promoter was thereby substituted by the promoter p*MrACT1* transcriptionally fused to cGFP.

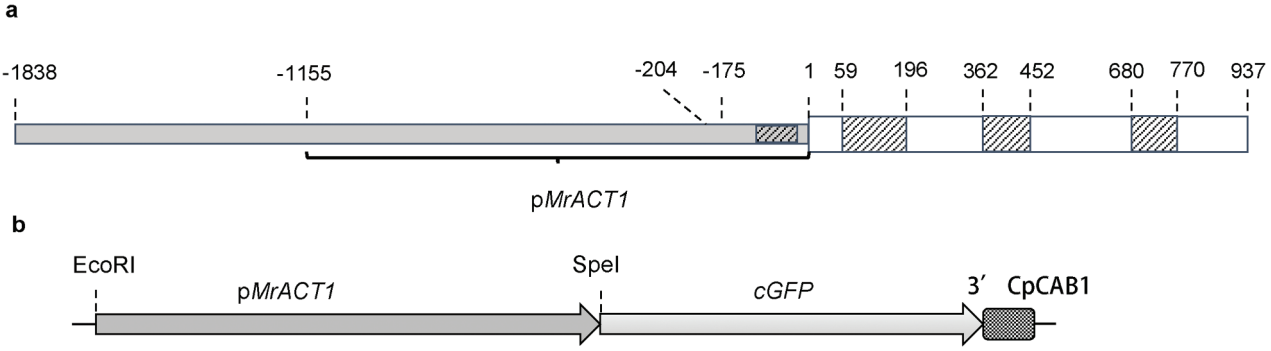

Fig 1. Schematic diagram of the partial 5′ region of *Micrasterias radians* var. *evoluta Actin1* gene plus partial coding region as determined after TAIL PCR and 5′RACE (a) and the structure of the p*MrACT1*:cGFP cassette in the plasmid pAW001 (b). The A-residue in the start codon was defined as position 1. At 204 bp upstream the presumed TATA-box is located. Open boxes with white background represent the coding sequence, dashed boxes represent introns and boxes with grey background represent the 5′ flanking region.

Efficient transient protoplast transformation via PEG mediated transfection using heterologous and endogenous promoters

The supercoiled plasmid pAW001 containing the endogenous p*MrACT1* promoter was delivered to the isolated protoplasts of *M. radians* var. *evoluta* via PEG mediated transfection. GFP expression could be detected in protoplasts after 2 days of incubation. As shown in Fig. 2a–c, the GFP protein was targeted to the nuclei and cytoplasm. This indicated that the p*MrACT1* promoter was functional and can be used for transgene expression in this alga. The transformation efficiency was strongly affected by the incubation conditions during the PEG-mediated DNA delivery (Fig. 2d). When the protoplasts were mixed with 20 µg plasmid DNA and incubated at room temperature (RT) for an extended period of 40 min, the transformation efficiency was increased to 1.53%. An incubation period of 50 min leads to a decrease in transformation efficiency due to reduction of cell viability. Heat shock at 42°C following the addition of PEG solution obtained a higher yield of transiently transformed protoplasts. A threefold increase of the transformation efficiency was found using a 5 min heat shock rather than 1 min. Therefore, a 5 min heat shock followed by 25 min of incubation at room temperature was established as a standard protocol for further PEG-mediated transfection of *M. radians* var. *evoluta* protoplasts. The survival rate ranged between 78.2% and 85%. Further cultivation of the protoplasts showed that they survived in the regeneration medium for 2 weeks even though the number of cells showing GFP signal decreased. The protoplasts were found to be unable to regenerate the normal *M. radians* var. *evoluta* morphology and did not undergo cell division.

Several heterologous promoters were tested for activity with the established protocol for transient expression in protoplasts of *M. radians* var. *evoluta*. The used plasmids contained a GFP reporter driven by various promoters from different organisms such as *ubiquitin* promoter from *Z. mays* (p*ZmUBI*) (Christensen & Quail, 1996), *Actin1* promoter from *O. sativa* (p*OsACT1*) (Zeidler *et al.*, 1999), p*CpCAB*

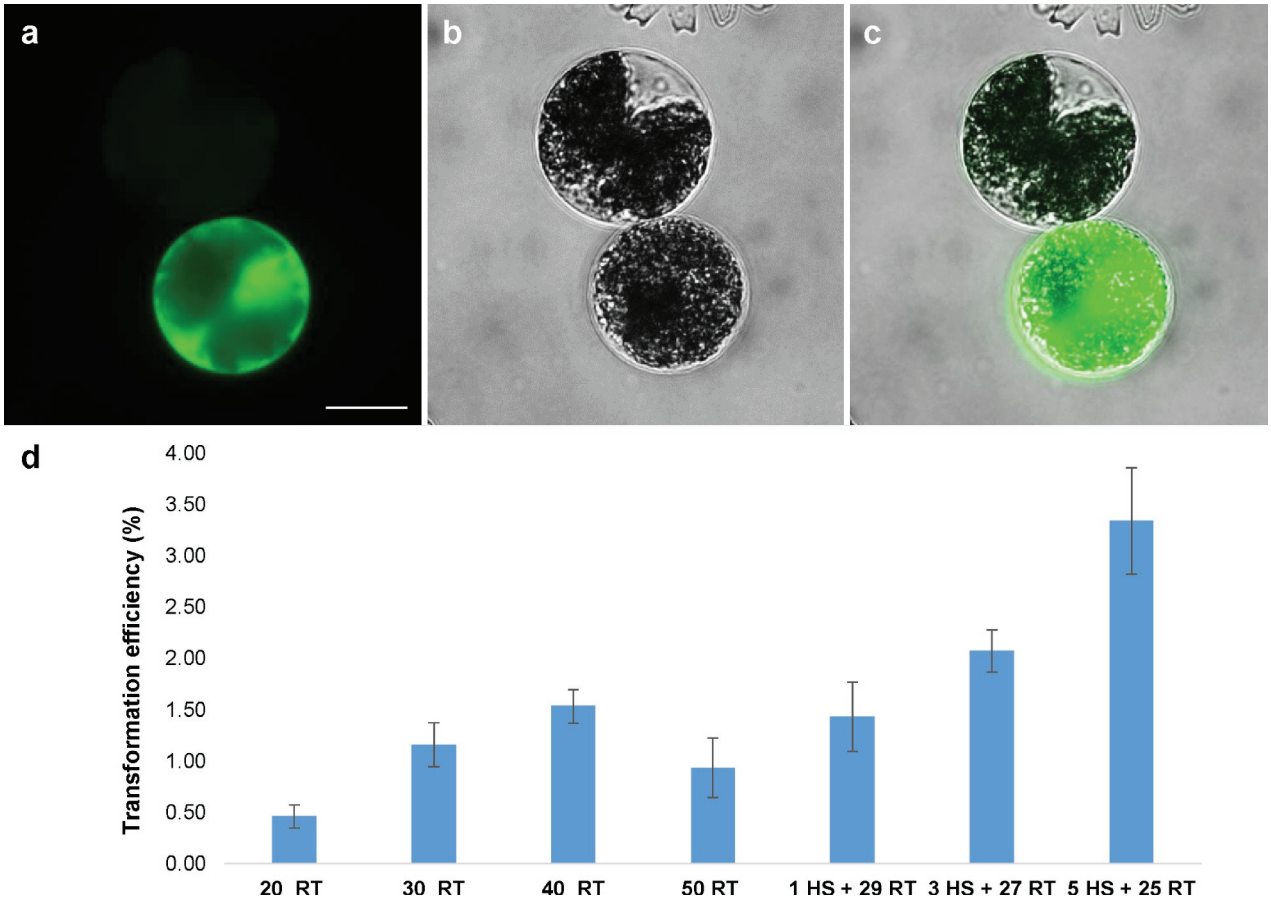

Fig 2. Transient GFP expression in protoplasts of *Micrasterias radians* var. *evoluta* (a–c) and the effect of different transfection conditions on the transformation efficiency (d). Fluorescent microscopy pictures show GFP fluorescence (a), bright field (b) and merged images (c) of the protoplasts (Zeiss Axio Imager A2). Protoplasts were transformed with the plasmid pAW001 containing endogenous promoter p*MrACT1*. The DNA amount was 20 µg for each transfection. The volume of protoplast suspension for transfection assay was 200 µl. The survival rates (no. of surviving cells related to the initial no. of protoplasts) were: 85.0% for the 20 RT; 84.0% for 30 RT; 82.5% for 40 RT; 78.2% for 50 RT; 83.9% for 1 HS + 29 RT; 82.6% for 3 HS + 27 RT; 81.6% for 5 HS + 25 RT. Scale bar, 20 µm. The numbers below x-axis (d) designate the duration of the transfection incubations in minutes, RT – room temperature; HS – 42°C heat shock. The transformation efficiency shows mean values with SD from three replicates.

promoter from *Closterium* (Abe *et al.*, 2008) and *a-tubulin1* gene promoter from *M. scalaris* (p*MsTUA1*) (Regensdorff *et al.*, 2018). As shown in Table 1, the promoters p*OsACT1* and p*ZmUBI* gave no GFP signal. Cells showing GFP fluorescence were found in the transformation assays with the plasmids pSA405A carrying p*CpCAB* and pMD containing p*MsTUA1*. However, the expression efficiency of the promoter p*CpCAB* was much lower than that of the promoter p*MsTUA1* and the p*MrACT1*. The activities displayed by these promoters were in the following descending order: p*MrACT1* > p*MsTUA1* > p*CpCAB*.

Transformation of Micrasterias radians *var.* evoluta *vegetative cells via particle bombardment*

The plasmids pAW001, pMD and pSA405A bearing promoters found active in *M. radians* var. *evoluta* were coated to gold particles and successfully delivered to vegetative cells by particle bombardment. The GFP signal was detected after 3 days of cultivation under dim light in cells bombarded with the plasmids pMD and pAW001 (Fig. 3), while no signal was found in the transformation with plasmid pSA405A. As in the comparative assays performed with the PEG mediated protoplast transfection, the promoter activity of p*MrACT1* was higher than that of the other two promoters.

Attempts were made to further determine the optimum conditions for the bombardment of *M. radians* var. *evoluta* by variation of parameters such as helium pressure, bombardment distance and gold particle size (Table 2) using the plasmid pAW001. Comparing the three pressures used, it was found that 1100 psi was sufficient to attain a relatively high number of cells expressing GFP and further increase of pressure (1350 and 1550 psi) did not improve the numbers but caused instead more cell damage. Using a pressure of 1100 psi, a higher number of GFP expressing cells were observed when the sample was placed at the shorter distance of 6 cm from the stopping screen. Utilization of both the 0.6 µm and 1.0 µm gold particles resulted in similar numbers of GFP expressing cells. The bombarded samples were observed *in situ* every second day. The number of cells showing GFP fluorescence started decreasing after 5 days, which was mainly due to a decrease of cell vitality. When the transformants were transferred to liquid culture, a few of them recovered and still displayed cell division. However, some cells kept a high level of GFP expression for a long period (3 weeks) but were unable to divide.

Table 1. GFP expression efficiencies using promoters from different genes and species in *Micrasterias radians* var. *evoluta* in per cent of surviving protoplasts. The efficiency shows mean values with SD from 3 replicates.

Plasmid	Promoter	Gene	Species	Efficiency (%)
pBAS	p*OsACT1*	Actin1	*O. sativa*	0.00 ± 0.00
pLNU	p*ZmUBI*	Ubiquitin	*Z. mays*	0.00 ± 0.00
pSA405A	p*CpCAB*	Chlorophyll *a* binding protein	*C. psl complex*	0.03 ± 0.01
pMD	p*MsTUA1*	Tubulin1	*M. scalaris*	0.66 ± 0.23
pAW001	p*MrACT1*	Actin1	*M. radians*	3.88 ± 0.34

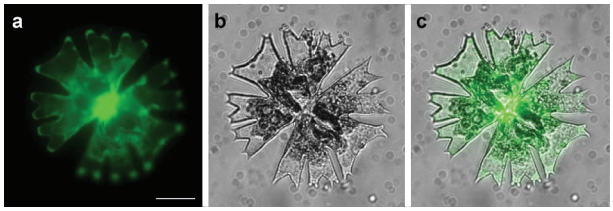

Fig 3. GFP expression in vegetative cell of *Micrasterias radians* var. *evoluta* cultured for 3 days after particle bombardment with plasmid pAW001. Pictures showing (a) GFP fluorescence (b) cell in bright field, and (c) merged images, taken with the Zeiss Axio Imager A2. Scale bar: 20 µm.

Table 2. Numbers of GFP expressing cells of *Micrasterias radians* var. *evoluta* by particle bombardment with different DNA delivery parameters. Number of transformants was calculated as mean values (± SD, n = 3).

Plasmid	Gold particle (µm)	DNA amount per shot (µg)	He pressure (psi)	Distance (cm)	No. of transformants (cells/plate)
pMD	0.6	6	1100	6	5.0 ± 2.0
pAW001	0.6	6	1100	6	18.3 ± 5.7
pAW001	0.6	6	1350	6	13.7 ± 5.1
pAW001	0.6	6	1550	6	11.0 ± 4.6
pAW001	0.6	6	1100	9	1.3 ± 0.6
pAW001	0.6	2	1100	6	3.7 ± 1.5
pAW001	1.0	2	1100	6	2.3 ± 1.5

Lifeact tagged GFP was expressed for labelling actin filaments

As a proof of concept, the construct pMD-Lifeact which contains a chimeric gene containing the *M. scalaris a-tubulin1* gene (*MsTUA1*) promoter, driving the expression of the Lifeact actin-binding sequence translationally fused to the cGFP reporter sequence (Regensdorff *et al.*, 2018), was introduced into *M. radians* var. *evoluta* vegetative cells. Particle bombardment was carried out under the optimum condition determined before. The transformed cells were observed under a confocal laser scanning microscope (CLSM) for actin structures after 3 days of cultivation on the original agar plate. It was confirmed that the Lifeact-cGFP was able to be efficiently expressed and to visualize F-actin cables in *M. radians* var. *evoluta* cells (Fig. 4). The Lifeact fluorescence labelled dense filament bundles which seemed to be distributed throughout the cytosol including the tips of the lobes and the isthmus area and branch into a fine meshwork (Fig. 4a). In the lobe area, some of the long bundles were aligned along the longitudinal axis (Fig. 4d).

Discussion

Owing to its important phylogenetic position the green algal lineage and unique cell properties, the genus *Micrasterias*, within the Zygnematophyceae, represents an attractive model for studying aspects of cell biology and physiology (Meindl, 1993; Domozych *et al.*, 2016; Lutz-Meindl, 2016). *Micrasterias radians* var. *evoluta* is a candidate model organism for experimental research due to its growth properties and closed life cycle under laboratory conditions (Zhou & von Schwartzenberg, 2020). Under our standard growth conditions, the alga showed a growth rate of ~0.062 mg dry weight per day and ml. Although this biomass production is considerably lower than that of the microalga *Chlorella* sp., which can gain about 10 times more dry weight per day (Watanabe & Saiki, 1997), it is largely sufficient for biological experiments.

For avoidance of microbial impacts in physiological and molecular experiments with this algal species, a standard axenic culture had to be established. Thus, a cocktail of antibiotics, which was described for purification of diatoms, flagellates and multicellular chlorophytes (Droop, 1967), was utilized to remove microbial contaminants associated with the cell surface of *M. radians* var. *evoluta*. A treatment of the culture with antibiotic mixtures failed since vegetative algal cells were susceptible to the antibiotics. However, an axenic culture was obtained by germination of zygospores previously treated with broad spectrum antibiotics ampicillin and chloramphenicol which are effective against prokaryotes, and actidione which is effective against fungi (Schneider-Poetsch *et al.*, 2010). The strategy used to obtain an axenic culture of *M. radians* var. *evoluta* might be applicable also for other Zygnematophycean species, provided that zygospores are available and able to germinate under laboratory conditions. However, the composition of the antibiotic cocktail might have to be adapted with respect by the nature of the original algal–bacterial/fungi community.

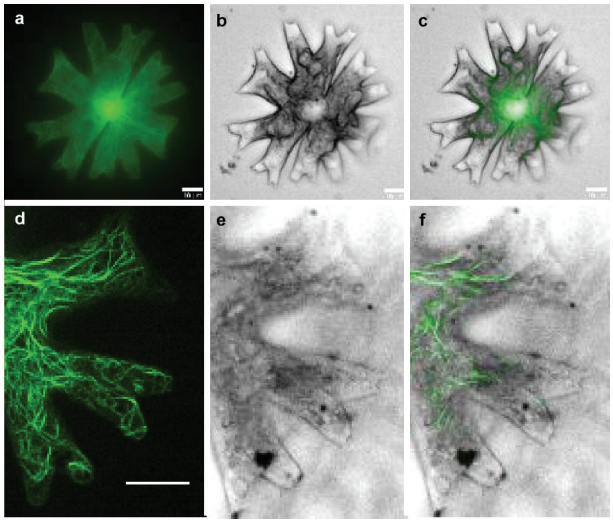

Fig 4. Intracellular distribution of actin filaments in a vegetative cell of *Micrasterias radians* var. *evoluta*, visualized with Lifeact-cGFP. GFP fluorescence, cell in bright field and merged images of the F-actin in entire cell (a–c) and lobes (d–e) were taken with a confocal laser scanning microscope (CLSM) equipped with a VisiScope live cell imaging system. Scale bar: 10 µm.

With the standard axenic culture of *M. radians* var. *evoluta*, we developed protocols for genetic transformation of both, protoplasts and vegetative cells that could be further used for different experimental purposes. A suitable promoter enabling efficient gene expression in transgenic cells is one of the major requirements for the establishment of a robust transformation system. It was reported that several heterologous promoters were able to drive GFP (and cGFP) expression in *M. denticulata* (Vannerum *et al.*, 2010). However, it seems difficult to exploit this protocol as a routine in molecular studies of *Micrasterias* due to the low expression efficiency that might result from the incompatibility of the heterologous promoters. Therefore, we isolated 1838 bp sequence upstream from the start codon of *MrACT1* gene by two rounds of thermal asymmetric interlaced (TAIL)-PCR. 1158 bp sequence including one intron and the ATG was demonstrated to possess high promoter activity in driving the cGFP expression via PEG mediated protoplasts transformation of *M. radians* var. *evoluta*. The promoter and the transformation protocol could be used in future research involving cell-based experiments for gene functional studies, subcellular localization and *in vivo* protein-protein interactions (Sheen, 2001). For PEG-mediated transfection of *M. radians* var. *evoluta* protoplasts we determined that heat shock during the co-incubation of the protoplasts with DNA plays an important role in promoting transfection efficiency, which was also reported for *Physcomitrella patens* (Schaefer, 1994) and *Elaeis guineensis* (Masani *et al.*, 2014).

Using the efficient transient expression system, different heterologous promoters were tested for their activities in gene expression in *M. radians* var. *evoluta*. The promotor activity was deduced from the relative number of protoplasts displaying visible GFP fluorescence. Promoters suitable for GFP expression usually caused GFP fluorescence of different levels among the transformed protoplasts, presumably due to the different physiological states of the cells.

The p*OsACT1* and p*ZmUBI* promoters are both capable of driving strong gene expression in land plants such as rice and *Physcomitrella* (McElroy *et al.*, 1990; von Schwartzenberg *et al.*, 2007; Lindner *et al.*, 2014). However, transformation of *M. radians* var. *evoluta* using these promoters yielded no GFP expressing cells. The promoter p*CpCAB1* (Abe *et al.*, 2008) from *C. psl* complex representing an organism from the same

order of Desmidiales is active in *M. radians* var. *evoluta* protoplasts, while the activity was 100-fold lower than that of the endogenous promoter p*MrACT1*. The plasmid pMD generated in the laboratory of H. Buschmann (Osnabrück University, Germany) was found to have high capability for driving gene expression in *M. radians* var. *evoluta*. Apparently the *Mougeotia scalaris MsTUA1* promoter, which showed activity in tobacco leaves as well (Regensdorff *et al.*, 2018), can be used in a broad range of organisms. The high expression efficiency achieved by using the p*MsTUA1*, together with the homologous p*MrACT1* are helpful for transgene expression including resistance genes for antibiotic selection in future. Further studies will reveal whether p*MrACT1* shows activity also in distantly related algae or seed plants. Despite the relatively high efficiency of 3.88% of transformed protoplasts (related to the number of surviving cells) a crucial limitation of the PEG-mediated transfection is that so far no Desmidiaceae protoplast was successfully regenerated into intact vegetative cells. Presumably factors like the loss of cell polarity in protoplasts make it impossible for the cells to regenerate and undergo cell division. In this respect Desmidiales seem to differ from Zygnematales algae such as *Zygnema* and *Spirogyra* where protoplast regeneration has been reported (Ohiwa, 1977; Zhou & von Schwartzenberg, 2020).

Since protoplast transformation did not allow regeneration and cell division, we applied biolistic transformation using *M. radians* var. *evoluta* vegetative cells. It was confirmed that the transformation efficiency of the plasmid pAW001 was higher than that of constructs containing heterologous promoters. For standardization of the biolistic transformation protocol, the influence of a few factors on the transformation efficiency and the recovery of the transformants were investigated by using the parameters employed by Vannerum *et al.* (2010) as a starting point. Factors like lower acceleration pressures coupled to a short shooting distance were found to improve the transformation rate and recovery of transformants. This might be due to the reduction of stress imposed during the bombardment process enabling cells to survive and continue growth at higher rates.

Using the established transformation platform, the F-actin in *M. radians* var. *evoluta* was labelled by expression of the Lifeact tagged cGFP protein. The distribution pattern of the filamentous network was similar to the results published by Meindl *et al.* (1994) for *M. denticulata* in which actin bundles were visualized by fluorescently labelled phalloidin. Lifeact allows an efficient and highly specific labelling of F-actin in the cell so that the filaments in the complete cytosol including the lobe tips could be clearly visualized with low background (Fig. 4a and d). Furthermore, unlike the restriction for labelling of fixed samples by usage of phalloidin, the major advantages of *in vivo* expression of actin-binding tags are that it confers a relatively low toxicity and facilitates live-cell imaging analysis (Riedl *et al.*, 2008; Melak *et al.*, 2017). Tagging actin with GFP derivatives might help to study actin dynamics and its role in *Micrasterias* growth and morphogenesis in future. Aside from the actin protein, the subcellular localization of other gene products related to the morphological phenotypes could also be achieved by employing this transient expression system using the GFP fusion protein.

In conclusion, we propose the *M. radians* var. *evoluta* as a potential model organism for future molecular studies on Desmidiaceae, as this alga exhibits the presented features such as easy culturing under axenic conditions, the completion of the life cycle and the possibility of transient genetic transformation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary material

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at 10.1080/09670262.2020.1768298

Supplementary table 1: The composition of C-medium used for *Micrasterias radians* var. *evoluta* cultivation.

Supplementary table 2: Primers used for amplification of partial coding sequence and 5′ flanking region of *Actin1* gene from *Micrasterias radians* var. *evoluta*.

Supplementary fig. 1: Alignment of partial of *Micrasterias radians* var. *evoluta Actin1* genomic DNA (gDNA) sequence and nucleotide sequence obtained by the ligation based 5′ RACE-PCR (5′ RACE).

Supplementary fig. 2: Alignment of the partial coding sequences from *Actin* genes of various organisms and partial *MrACT1* gDNA sequence.

Author contributions

H. Zhou: design of the project, genetic transformation of *M. radians* var. *evoluta* protoplasts and vegetative cells,

writing of manuscript, reviewing and developing the manuscript for submission. A. Wilkens: experiments for purification of the axenic culture, isolation and characterization of the endogenous promoter, genetic transformation of *M. radians* var. *evoluta* protoplasts and vegetative cells, reviewing and developing the manuscript for submission. D. Hanelt: reviewing and developing the manuscript for submission. K. von Schwartzenberg: design of the project, reviewing and developing the manuscript for submission.

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5. Appendix

5.1 Publication with co-authorship:

de Vries J, de Vries S, Curtis BA, **Zhou H**, Penny S, Feussner K, et al. 2020. Heat stress response in the closest algal relatives of land plants reveals conserved stress signaling circuits. The Plant Journal, doi: 10.1111/tpj.14782

5.2 Conference contributions:

Posters

Zhou H., Bartels S., Salameh N., Kopečný D., Hanelt D., v. Schwartzenberg K. (2018): Searching for functions of cytokinins in the streptophyte alga *Klebsormidium nitens*, Auxins and Cytokinins in Plant Development (ACPD) international symposium 2018, Prague, Czech Republic.

Zhou H., Hanelt D., v. Schwartzenberg K. (2018): Towards establishment of a genetic transformation system in charophyte green algae –Zygenematophyceae, Conference of the Phycology Section DBG, Berchtesgaden, Germany.

Talks

Zhou H., Hanelt D., v. Schwartzenberg K. (2019): Development of molecular toolkits in Charophyte green algae, International Conference for Young Marine Researchers, Hamburg, Germany.

5.3 Vector cards

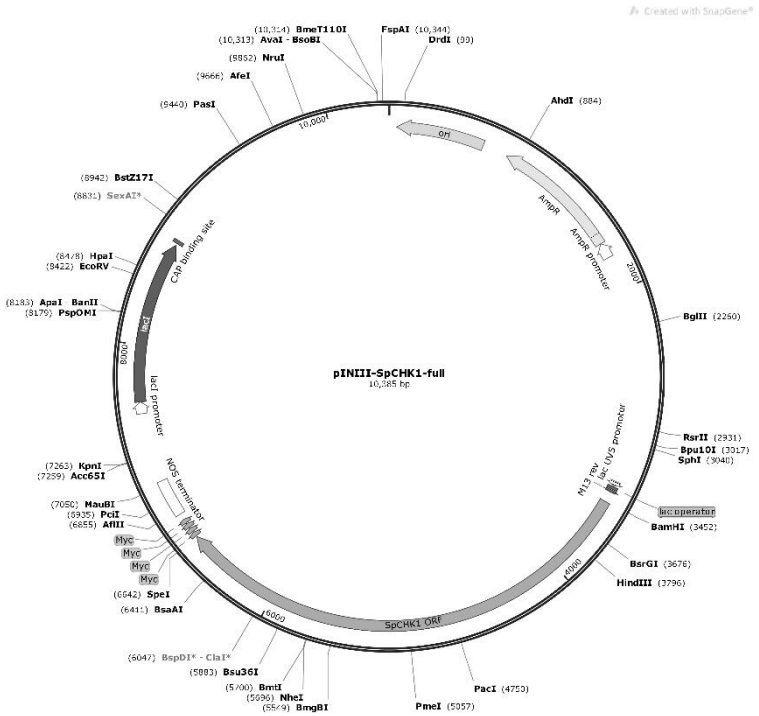

Figure S1. Vector card of pINIII-SpCHK1 for cytokinin binding assay, derived from pINIII-4xMyc (kindly provided by David Zalabák, Palacký University, CZ).

>pINIII-SpCHK1 (10385 bp)

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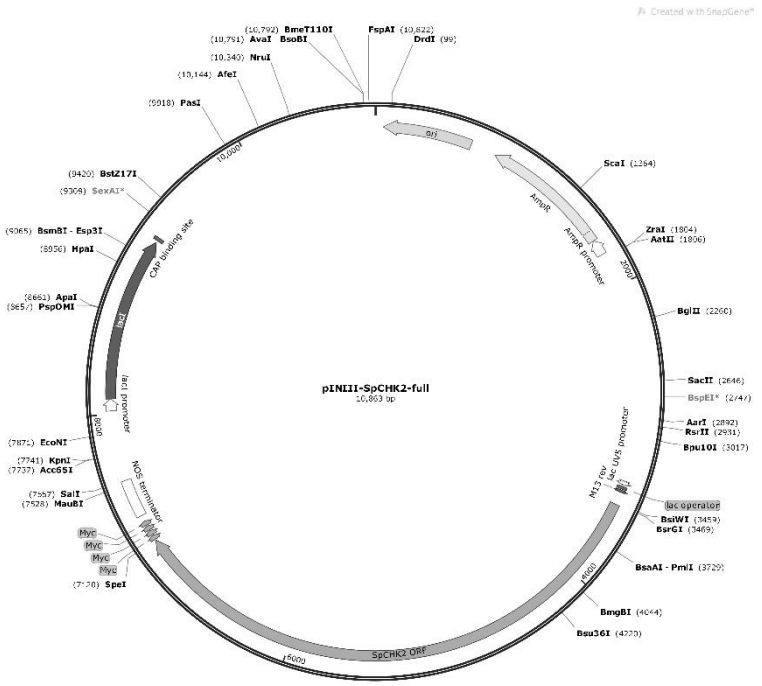

Figure S2. Vector card of pINIII-SpCHK2 for cytokinin binding assay, derived from pINIII-4xMyc (kindly provided by David Zalabák, Palacký University, CZ).

>pINIII-SpCHK2 (10863 bp)

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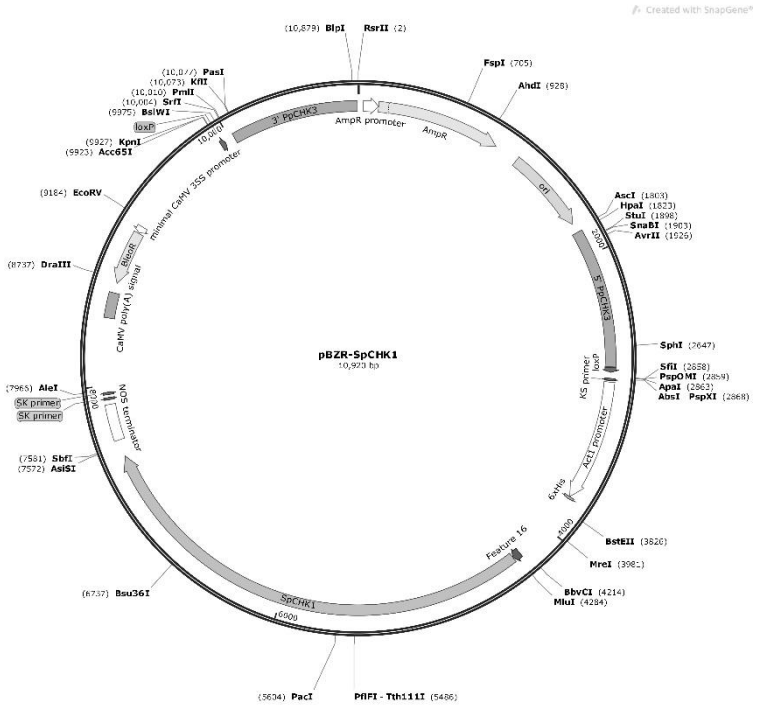

Figure S3. Vector card of pBZR-SpCHK1 for knock-in expression of SpCHK1 in *PpΔchk1,2* double mutant, derived from pBZR-PpCHK3-ko (von Schwartzenberg *et al.,* 2016).

>pBZR-SpCHK1 (10920 bp)

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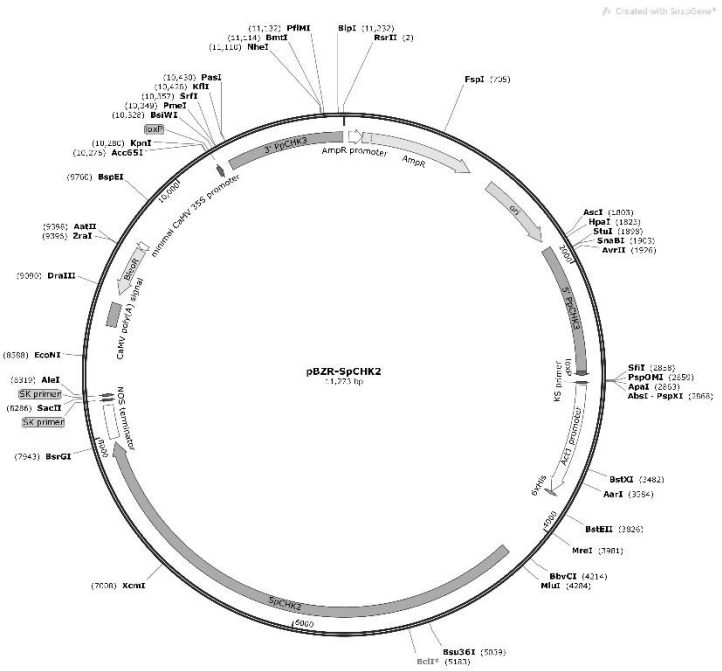

Figure S4. Vector card of pBZR-SpCHK2 for knock-in expression of SpCHK2 in *PpΔchk1,2* double mutant, derived from pBZR-PpCHK3-ko (von Schwartzenberg *et al.,* 2016).

>pBZR-SpCHK2 (11273 bp)

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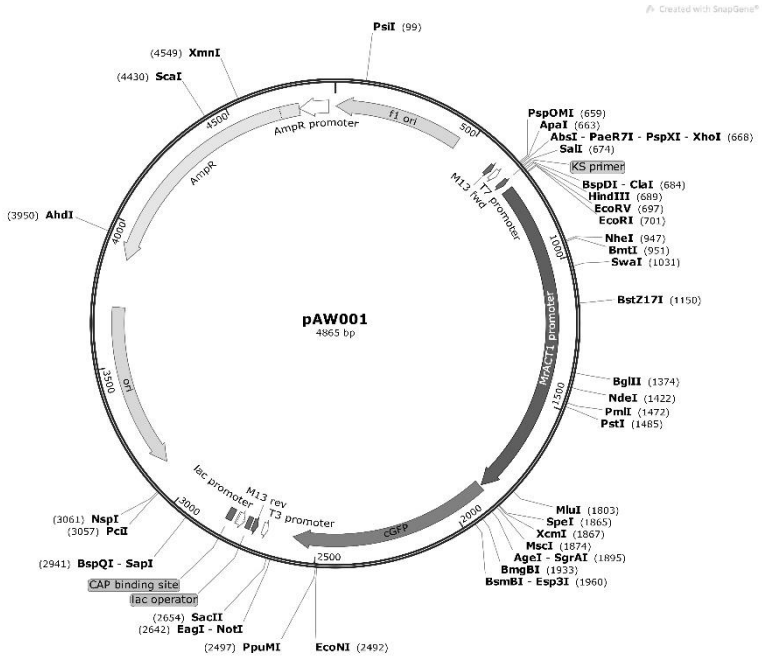

Figure S5. Vector card of pAW001 for transient transformation of *Micrasterias radians* var. *evoluta,* derived from pSA405A (Abe *et al.,* 2008).

>pAW001 (4865 bp)

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7. Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, den

Unterschrift