

Antagonistic roles of abscisic acid and cytokinin during response to nitrogen depletion in oleaginous microalga *Nannochloropsis oceanica* expand the evolutionary breadth of phytohormone function

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SUMMARY

The origin of phytohormones is poorly understood, and their physiological roles in microalgae remain elusive. Genome comparison of photosynthetic autotrophic eukaryotes has revealed that the biosynthetic pathways of abscisic acid (ABA) and cytokinins (CKs) emerged in unicellular algae. While ABA and CK degradation mechanisms emerged broadly in algal lineages, complete vascular plant-type conjugation pathways emerged prior to the rise of Streptophyta. In microalgae, a complete set of proteins from the canonical ABA and CK sensing and signaling pathways is not essential, but individual components are present, suggesting stepwise recruitment of phytohormone signaling components. In the oleaginous eustigmatophyte *Nannochloropsis oceanica* IMET1, UHPLC-MS/MS detected a wide array of plant hormones, despite a phytohormone profile that is very distinct from that of flowering plants. Time-series transcriptional analysis during nitrogen depletion revealed activation of the ABA biosynthetic pathway and antagonistic transcription of CK biosynthetic genes. Correspondingly, the ABA level increases while the dominant bioactive CK forms decrease. Moreover, exogenous CKs stimulate cell-cycle progression while exogenous ABA acts as both an algal growth repressor and a positive regulator in response to stresses. The presence of such functional flowering plant-like phytohormone signaling systems in *Nannochloropsis* sp. suggests a much earlier origin of phytohormone biosynthesis and degradation than previously believed, and supports the presence in microalgae of as yet unknown conjugation and sensing/signaling systems that may be exploited for microalgal feedstock development.

Keywords: *Nannochloropsis oceanica*, antagonistic synergy, abscisic acid, cytokinin, phytohormone evolution, stress response, cell-cycle progression.

INTRODUCTION

The term ‘microalgae’ refers to a phylogenetically broad and heterogeneous group of unicellular photosynthetic organisms, including autotrophs, heterotrophs (e.g. apicomplexans and dinoflagellates) and mixotrophs (e.g. chlorarachniophytes and *Chromera velia*). They are generally grouped into at least two major phylogenetic lineages:

Stramenopiles (diatoms, brown algae and eustigmatophytes) and Archaeplastida (or Plantae, which include green algae, red algae and glaucophytes, with green algae sharing a common ancestor with modern land plants) (Baldauf, 2008; Archibald, 2009). Some microalgae are considered to be promising biofuel feedstock based on their

ability to grow rapidly and synthesize large amounts of neutral storage lipids (e.g. triacylglycerols) under a variety of conditions; however, few natural strains harbor all the traits required for scalable biofuel production simultaneously. Identification of the molecules and mechanisms underlying these traits should facilitate development of microalgal feedstock (Wijffels and Barbosa, 2010; Georgianna and Mayfield, 2012).

In flowering plants, growth, development and stress responses are regulated by phytohormones, such as those derived from isoprenoids, which primarily include abscisic acid (ABA), brassinosteroids (BRs), cytokinins (CKs) and gibberellins (GAs). These hormones regulate crucial and economically relevant processes such as seed development (Riefler *et al.*, 2006; Bartrina *et al.*, 2011), dormancy (Lee *et al.*, 2010), germination (Linkies *et al.*, 2009), vegetative growth (Linkies *et al.*, 2009) and stress responses (Jeon *et al.*, 2010; Großkinsky *et al.*, 2011). At present, it is generally believed that phytohormones and the associated regulatory mechanisms emerged in an ancient organism prior to the split of seedless plants and seed plants (Rensing *et al.*, 2008). Recent evidence suggests that CK-dependent mechanisms govern development of the bryophyte *Physcomitrella patens*, one of the 'basal' land plants (Richter *et al.*, 2012). ABA signaling also occurs in *P. patens*, as demonstrated by the observation that an ABA-hypersensitive phenotype is produced by disrupting expression of the enzyme encoded by *ABA-insensitive 1-1* (*ABI1-1*) (Komatsu *et al.*, 2009). Orthologs of the GA receptor *GID1* have been identified in *P. patens* and the lycophyte *Selaginella moellendorffii*, which represent those land plants closest to the green algae (Rensing *et al.*, 2008). Expression of the lycophyte gene in GA receptor mutants of rice (*Oryza sativa*) compensated for their inactive native receptors but expression of the bryophyte gene did not (Yasumura *et al.*, 2007), suggesting that there are substantial gaps in our understanding of phytohormone evolution. Furthermore, it is not known whether phytohormones are present in other lower plants, or what functions they may fulfill in those species.

Although phytohormones have been shown to be present and active in several macroalgae (Tarakhovskaya *et al.*, 2007), work with algae, in particular unicellular microalgae, lags far behind work with land plants (Bradley, 1991; Kiseleva *et al.*, 2012). While treatment with exogenous phytohormones was shown to stimulate the growth and stress tolerance of unicellular algae (Kobayashi *et al.*, 1997; Bajguz and Asami, 2004; Bajguz, 2011), limited data on the effects of their endogenous hormone(s) are available. A recent study showed the presence of homologs of some enzymes for auxin, GA, ABA and CK biosynthesis in microalgae (mainly green microalgae) (Kiseleva *et al.*, 2012). However, it remains unclear whether microalgae produce or respond to known plant hormones, and, more specifi-

cally, what the similarities and differences are in the phytohormone profiles, turnover mechanisms, signaling circuitry and function between microalgae and land plants.

To determine whether phytohormone-like metabolic and regulatory mechanisms are present and functional in algae, we performed a genome-wide reconstruction of the metabolic and signaling pathways of selected phytohormones in plants. As it is impractical to cover the entire aquatic photosynthetic world, we focused on ABA and CKs in photosynthetic eukaryotic microalgae. Our results show that the ABA and CK biosynthetic and catabolic pathways arose in various microalgal species, and the higher plant-type conjugation mechanisms emerged prior to the emergence of green algae. The associated ABA and CK signaling mechanisms emerged in microalgae, but the complete set of proteins of canonical sensing/signaling systems did not emerge until the rise of ancient land plants. Moreover, not yet identified ABA and CK conjugation and signaling mechanisms appear to exist in microalgae. The endogenous ABA and CK profile of the oleaginous microalga *Nannochloropsis oceanica* IMET1 revealed the presence of multiple hormones known to be active in flowering plants. Furthermore, temporal profiling of the *N. oceanica* transcriptome and phytohormones during the cellular response to nitrogen depletion revealed an antagonistic synergy between ABA and CK biosynthetic pathways, suggesting functional hormone signaling systems. Algal cell physiological changes caused by an altered CK or ABA status further showed that CKs stimulate growth while ABA acts as both a repressor of growth and a positive regulator in stress responses. These findings suggest antagonistic roles of ABA and CKs during responses to nitrogen depletion in unicellular microalgae, and expand the evolutionary breadth of phytohormone function.

RESULTS

Phytohormone biosynthesis and degradation is conserved in oxygenic photosynthetic eukaryotes

Selecting the genomes for phylogenetic reconstruction of phytohormone biosynthesis pathways. To probe the phylogenetic distribution of phytohormone metabolism in algae, we included all the major algal lineages (Table S1 and Figure S1): (i) green algae, including the picophytoplankton *Micromonas* sp. RCC299 (Worden *et al.*, 2009), the free-living small-genome alga *Ostreococcus tauri* (Palenik *et al.*, 2007), the virus-harboring alga *Chlorella variabilis* NC64A (Blanc *et al.*, 2010), and the laboratory model alga *Chlamydomonas reinhardtii* (Merchant *et al.*, 2007); (ii) red algae, including a primitive alga with simple cellular architecture: *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004); (iii) diatoms, including the marine pennate diatom *Phaeodactylum tricornutum* (Bowler *et al.*, 2008) and the centric diatom *Thalassiosira pseudonana* (Armbrust *et al.*,

2004); (iv) brown algae, including the marine alga *Ectocarpus siliculosus* (Cock et al., 2010); (v) eustigmatophyte, represented by *N. oceanica*. Groups (i) and (ii) are from the Archaeplastida (or Plantae), while groups (iii)–(v) are algae from Stramenopiles.

To test the role of phytohormones in the evolution of multicellularity and differentiation, the multicellular green algae *Volvox carteri* and the multicellular brown alga *E. siliculosus* (Cock et al., 2010) were also included in this analysis. *C. reinhardtii* and *V. carteri* represent sister groups of the land plant lineage. To test the potential link between phytohormones and adaptation to different ecological niches, we also included *Coccomyxa subellipsoidea* C-169 (a polar green microalga) (Blanc et al., 2012), *Fragilariopsis cylindrus* (a typical cold-water diatom) and *C. merolae* (which lives in hot acidic water). To probe the potential roles of phytohormones in energy conversion and storage, two oleaginous algae, *N. oceanica* (Wang et al., 2014) and *Chlorella pyrenoidosa* (a starch-rich facultative autotrophic green microalga), were included in this analysis. Finally, the bryophyte *P. patens* (Rensing et al., 2008) and the lycophyte *S. moellendorffii* (Banks et al., 2011) were selected to represent phylogenetically basal land plants, while the monocot *Zea mays* (Schnable et al., 2009) and the dicot *Arabidopsis thaliana* were included as representatives of flowering plants.

In higher plants, ABA is derived from isoprenoids synthesized in the plastids via the 1-deoxyxylulose 5-phosphate (MEP) pathway. Conversely, CKs are synthesized from isoprenoids that are in turn produced via the mevalonate pathway, which operates in the cytoplasm. Most algae exclusively express enzymes of the MEP pathway, and use that pathway for isoprenoid biosynthesis, while marine diatoms (*P. tricornutum*, *T. pseudonana* and *F. cylindrus*) and brown alga (*E. siliculosus*) appear to use both pathways (Lu et al., 2014). Regardless of the origins of the isopentenyl diphosphate consumed, the ABA and CK biosynthetic pathways are strongly conserved in evolutionary terms, and occur in species ranging from unicellular algae to flowering plants (Figure 2). Thus the emergence of these phytohormone synthetic pathways presumably precedes that of land plants.

ABA biosynthesis pathways. ABA may be produced via two biosynthetic routes: a 'direct' pathway from farnesyl pyrophosphate (the fungal route) (Siewers et al., 2006) or an 'indirect' pathway via cleavage of a carotenoid precursor (the higher plant route) (Nambara and Marion-Poll, 2005). With the exception of the P450 monooxygenase gene *BcABA3*, all of the genes involved in the fungal 'direct' pathway (Siewers et al., 2004, 2006) were apparently present in each of the investigated species. *BcABA3* has been identified as an indispensable component of the 'direct' pathway (Siewers et al., 2006). In addition, the putative P450 mono-

oxygenase genes *BcABA1* and *BcABA2* from *N. oceanica* responded to nitrogen deprivation in opposing ways (Data S2), which is not consistent with the co-regulatory mechanism observed for the *BcABA1–4* gene cluster during ABA accumulation in the gray mold *Botrytis cinerea* (Siewers et al., 2006). Although other enzymes in *N. oceanica* may perform the *BcABA3* function, and mechanisms regulating *BcABA1* and *BcABA2* transcription may be different, these results suggest that the 'direct' ABA pathway, at least the canonical one, is probably absent in algae.

The gene encoding the first enzyme of the 'indirect' pathway, zeaxanthin epoxidase (ZEP), was identified in all of the studied species except *C. merolae*. The enzyme that catalyzes the next step in this pathway, ABA-deficient protein 4 (ABA4), has not been identified in *C. merolae*, *P. tricornutum*, *T. pseudonana*, *F. cylindrus* or *C. subellipsoidea* C-169 (Figure 2). The downstream genes of the pathway were apparently present in each of the species studied here except *O. tauri* and *C. merolae* (Figure 2). Most of the algal genomes examined in this work therefore have a complete set of genes encoding enzymes of the 'indirect' pathway, which suggests that this pathway emerged in algae.

CK biosynthesis pathways. In Arabidopsis, the first and committed step in the CK biosynthesis pathway is catalyzed by isopentenyltransferases (IPTs), which form two functional classes: prokaryote-type tRNA IPTs (AtIPT2 and 9) and ATP/ADP IPTs (AtIPT1, 3–8). The putative algal IPTs harbor the conserved domains found in Arabidopsis and exhibit higher similarity to tRNA IPTs than to ATP/ADP IPTs (Table S3 and Figure S2a). Thus the ATP/ADP IPTs appear to have emerged after the split between Chlorophyta and land plants (Frébort et al., 2011).

The phylogeny of the adenosine kinases (AKs) that convert CKs into the corresponding nucleotides revealed a high level of sequence conservation among the photosynthetic eukaryotes, ranging from unicellular algae to flowering plants (Figure S3). Moreover, the putative algal AKs harbor a pentose moiety binding motif (indicated by asterisks in Figure S3) that are also found in the equivalent enzyme from the moss *P. patens*, which is known to be functional (Vo et al., 1998). Although no algal AKs have been experimentally validated, these findings reveal that functional AKs are potentially broadly present in algae.

Cytokinin riboside 5'-monophosphate phosphoribohydrolase (LOG) catalyzes the final step in synthesis of bioactive CKs (Kurakawa et al., 2007; Kuroha et al., 2009). Putative LOG genes are present in all of the investigated species, albeit at a relatively low gene dose in algae (Figure 2 and Table S4). LOG-like proteins have diverged into four clades (Figure S2b). Interestingly, while most land plants harbor multiple copies of the LOG genes in each genome, all Stramenopile algae plus several green algae (e.g. *C. pyrenoidosa*, *C. subellipsoidea* C-169, *C. reinhardtii*

and *V. carteri*) harbor only a single LOG (Figure S2b and Table S4).

The cellular levels of phytohormones are controlled by the precise balance between biosynthesis and turnover. Turnover is achieved via two mechanisms: conjugation and degradation (Figure 1 and Doc S1). The observed distribution of seed plant-type glucosyl transferases suggests that the conjugations of ABA and CKs are probably peculiar to the green plants (Figure 2). The degradation pathways for ABA (CYP707A1–A4) appear to emerge across a deep algal phylogeny, while the occurrence of cytokinin oxidase/dehydrogenase, which is responsible for complete

cleavage of the side chain of base and nucleoside forms of isoprenoid CKs, is only present sporadically (Figure 2). Therefore, the higher plant-type hormone degradation mechanisms arose in various microalgae, but the higher plant-type conjugation mechanisms did not emerge until the rise of green algae.

Phytohormone signaling: distinct distribution profiles of ligand receptors and the corresponding signaling components in algae

The presence of ligand receptors and relevant signaling components is often taken as evidence for the existence of

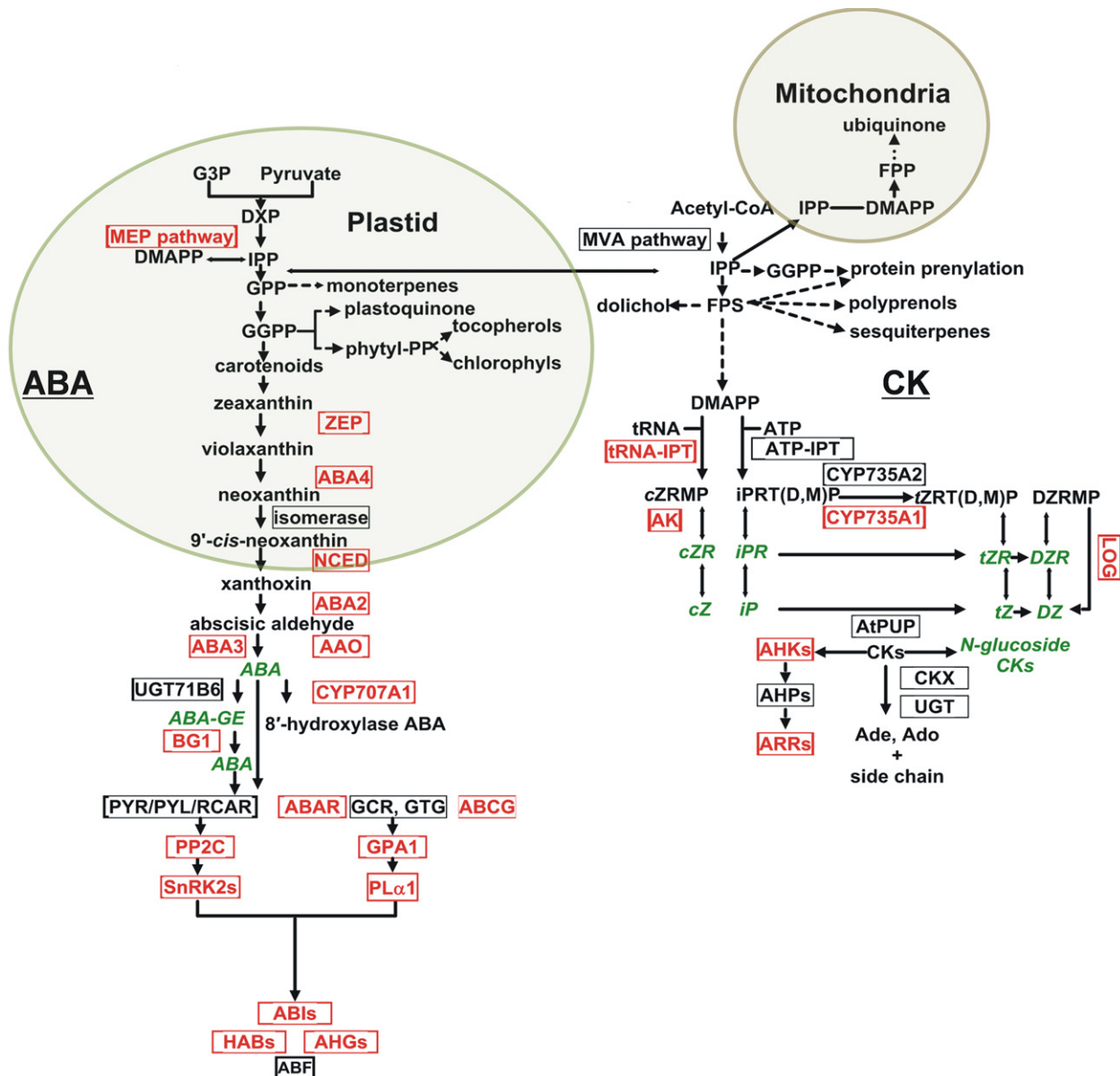
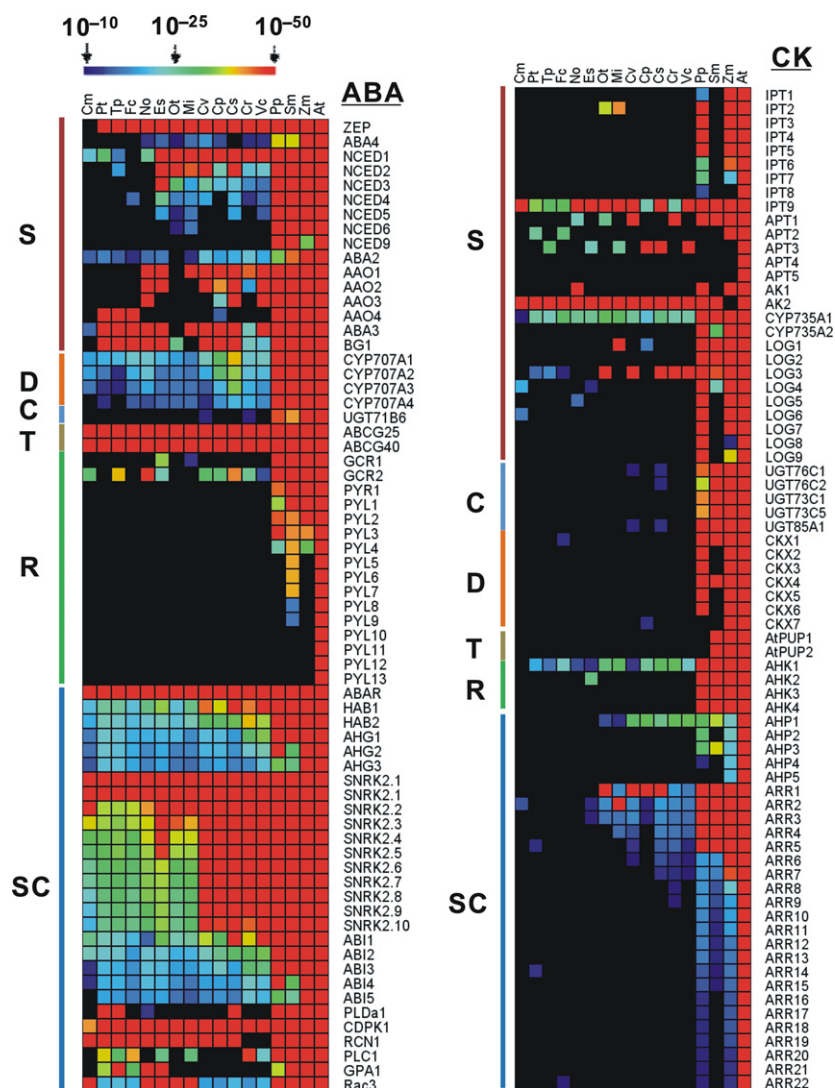


Figure 1. Comparison of ABA and CK metabolic and signaling pathways between *Arabidopsis* and *N. oceanica*.

Pathways are indicated by black arrows. Omitted steps are indicated by dotted arrows. Enzymes are labeled in boxes. Enzymes labeled in red have orthologs in *N. oceanica*. Italic green text indicates compounds that are present in *N. oceanica*. See Doc S1 and Table S2 for details.



an active hormone regulatory pathway (Hauser *et al.*, 2011). However, for both ABA and CK, in contrast to the extensive phylogenetic conservation of biosynthesis genes, the distribution patterns of canonical receptors and signaling components are quite distinct: despite the absence of ligand-binding receptors for both ABA and CKs, orthologs of ABA signaling components are ubiquitous, but not those of CKs (Figure 2).

Orthologs of the downstream phosphatases from the ABA signaling pathway [e.g. HYPERSENSITIVE TO ABA 29 (HAB), ABA-HYPERSENSITIVE GERMINATION (AHG), Sucrose Non-Fermenting Kinase 2 proteins (SNRK2s)] that interact directly with the ABA receptor PYR/PYL/RCAR (PYRABACTIN RESISTANT/PYRABACTIN RESISTANT-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR) (Nishimura *et al.*, 2009) were identified in all species examined (Figure 2). However, PYR/PYL/RCAR proteins have not been identified in algal lineages. To date, PYR/PYL/RCAR proteins from plants lower than

liverworts (Marchantiophyta) have not been reported to bind to ABA or have any specific functions *in vivo* (Hauser *et al.*, 2011). On the other hand, although ABA plays a fundamental role in regulating multiple responses to diverse stimuli in human cells, no PYR/PYL/RCAR homologs or cytosolic receptors for ABA have yet been described (Tossi *et al.*, 2012). Thus it is likely that the canonical ABA sensing/signaling system is essential only in land plants. However, the ABA-hypersensitive phenotype observed in an ABI1-defective strain of *P. patens* (Komatsu *et al.*, 2009) suggests that ABA regulates metabolic activity via certain mechanisms in mosses. We therefore propose that, in lower species, although ABA is likely to be a bioactive regulator, proteins in the sensing/signaling pathways (e.g. PYR/PYL/RCAR) differ significantly from their counterparts in vascular plants. The individual components of the PYR/PYL/RCAR-PROTEIN PHOSPHATASE 2C-SNRK2 signaling system appear to be recruited in a stepwise manner during evolution.

For CKs, orthologs of *Arabidopsis* histidine kinase 1 [AHK1; lacking the CK binding cyclases/histidine kinase-associated sensory extracellular (CHASE) domain] are common in the sampled algal genomes (Figure 2 and Table S5). Orthologs of *Arabidopsis* histidine kinases AHK4 (or CK response 1), AHK2 and AHK3, which contain the CHASE domain (Anantharaman and Aravind 2001), have only been found in the cyanobacteria *Synechocystis* sp. PCC 6803 (slr1759) and the brown alga *E. siliculosus* (Esi0034_0049) (Table S5). On the other hand, three varieties of *Arabidopsis* response regulators (ARRs) have been described (Kim *et al.*, 2006). Types A and C contain solely the RR domain, but only type A has been experimentally identified as a negative regulator of CK responses (To *et al.*, 2004). Type B ARRs contain an Myb domain in addition to the RR domain. In algae, orthologs of type A (or type C) ARRs are ubiquitous (e.g. g1318 in *N. oceanica*), but putative type B ARRs are found only in green algae. The emergence of type B ARRs therefore pre-dated the emergence of the Chlorophyta (Riaño-Pachón *et al.*, 2008). Therefore, components of the CK signaling system appear to have emerged in microalgae, but a full set of proteins of the canonical signaling pathway appears not to have emerged until the rise of green plants.

The phytohormone profile of *N. oceanica* resembles those of higher plants but has different dominant species and biosynthetic intermediates

Our genome-based metabolic reconstruction supports the existence of ABA and CK biosynthesis in microalgae; however, several important genes in these hormone biosynthesis and signaling pathways were not identified in the studied algae. Moreover, several of the relevant biosynthetic genes are P450 enzymes, whose substrates and products may only be reliably identified by experiments. We therefore selected *N. oceanica*, an industrial oleaginous microalga, as a model for hormone profiling. This species is an emerging research model of algal biofuels, due to its capabilities for robust growth and high oil productivity in large-scale cultivation (Radakovits *et al.*, 2012; Vieler *et al.*, 2012; Wang *et al.*, 2012, 2014; Wei *et al.*, 2013; Li *et al.*, 2014; Corteggiani Carpinelli *et al.*, 2014; Lu *et al.*, 2014).

The profile of the phytohormone metabolites of *N. oceanica* was determined using ultra-high performance liquid chromatography-electrospray tandem mass spectrometry method (UHPLC-MS/MS). Consistent with genome-based predictions, *N. oceanica* cells contain significant quantities of ABA at a concentration of approximately 10 pmol g⁻¹ dry weight (DW) (range from 6.02 to 16.48 pmol g⁻¹ DW, i.e. 1.59 to 4.36 ng g⁻¹ DW), which is similar to that of some liverworts (11–700 pmol g⁻¹ DW; Dietz and Hartung, 1998) but significantly lower than that of aquatic plants (56–3293 pmol g⁻¹ DW; Schiller *et al.*

1997). Despite the absence of known high-plant UDP-glucosyl transferases (UTGs), a significant amount of ABA-β-D-glucopyranosyl ester (ABA-GE) was detected (45.71 ± 5.10 ng g⁻¹ DW). The key step of the 'indirect' ABA biosynthesis pathway, xanthoxin biosynthesis, is catalyzed by a family of carotenoid cleavage dioxygenases (CCDs). In higher plants, either 9-*cis*-neoxanthin or 9-*cis*-violaxanthin is used as the substrate for xanthoxin production. However, the substrate specificities of the algal CCDs cannot be directly inferred from their phylogenetic relationships with analogous proteins from other species (Figure S4). 9-*cis*-neoxanthin has been detected in green algae, but not in Eustigmatophyta (e.g. *Nannochloropsis oculata* CCAP849/1), diatoms (*Phaeodactylum tricornutum*) or red algae (*Cyanidium caldarium*) (Takaichi and Mirauro, 1998), which suggests that 9-*cis*-violaxanthin may be the substrate of the CCDs in these non-green algae. Alternatively, otherwise undetermined CCD substrates may be important for algal ABA synthesis, particularly given the substrate promiscuity of the characterized CCDs (Auldrige *et al.*, 2006).

Both isoprenoid and aromatic CKs were present in *N. oceanica*, with the former at a higher level (Figure 3a,b), which is typical in higher plants (Sakakibara, 2006). For *N. oceanica*, the total amount of *cis* isomers was equivalent to that of *trans* ones (Figure 3a). In addition, *N. oceanica* produced a substantial amount of dihydrozeatin (DHZ), although DHZ metabolites have not previously been identified in algae (Stirk *et al.*, 2003) (Figure 3a). As for the CK species, although isopentenyladenine (iP) was a principle component in non-vascular plants such as mosses (i.e. *P. patens*; von Schwartzberg *et al.*, 2007) or certain algae (Stirk *et al.*, 2009), it was present at a low level in *N. oceanica* (Figure 3a).

The aromatic CK benzyladenine (BA) was not detected in *N. oceanica*, but low levels of topolins (BA hydroxylated analogs) are present (0.65%). This is very different from the CK profile in green microalgae, which produce much higher levels of BA and topolins (Ördög *et al.*, 2004) (Figure 4a). The two topolin isomers (*ortho* and *meta*) were present (Figure 3b), but at levels two orders of magnitude lower than their isoprenoid counterparts (Figure 3b).

Various glucoside CK metabolites (reserves of bioactive CKs in higher plants; Sakakibara, 2006) were also detected in *N. oceanica* (Figure 3a,b). *Cis*-zeatin-*O*-glucoside (19.28%) and *trans*-zeatin-*O*-glucoside (11.4%) account for a large proportion of total CKs (Figure 3a). *O*-glucosides may be reversibly cleaved to the corresponding bioactive free base or riboside (Sakakibara, 2006). These *O*-glucosides may function as active CK sources in *N. oceanica* if their biological functions in this species are similar to those observed in higher plants. On the other hand, in contrast to previous findings in macroalgae (Stirk *et al.*, 2003), *N*⁶-glucosides were also found in *N. oceanica* (Figure 3a,b). This observation contrasts with the absence of known CK

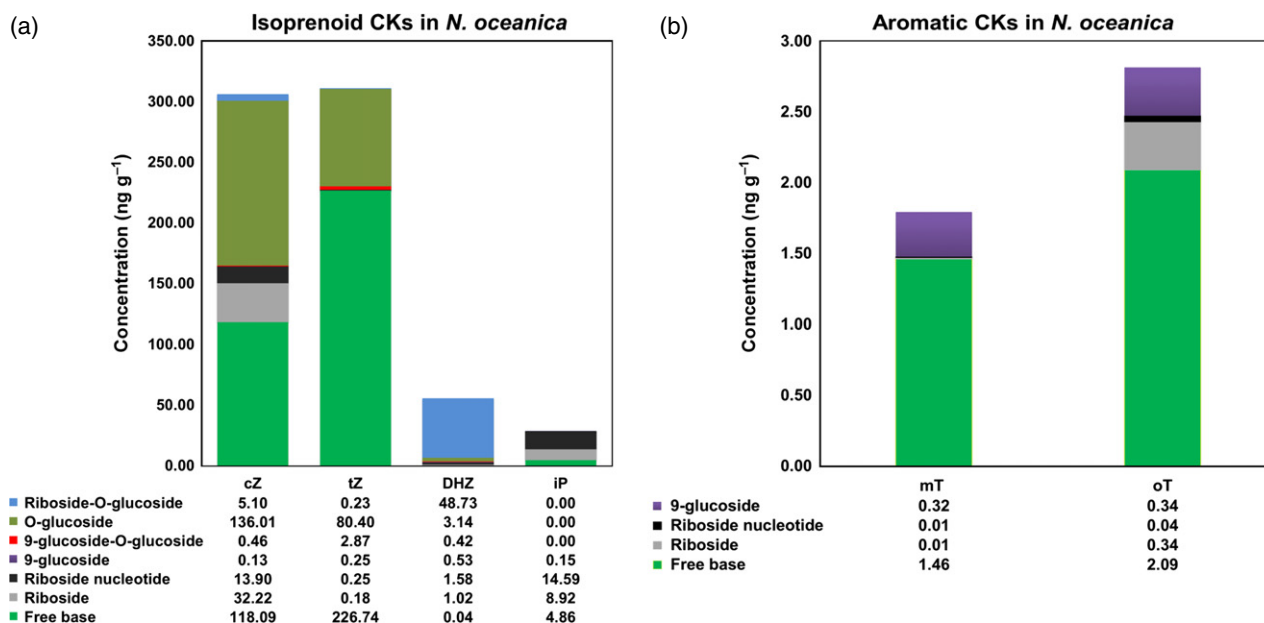


Figure 3. Composition and concentration of endogenous CKs in *N. oceanica*.

(a) Isoprenoid CKs.

(b) Aromatic CKs.

Abbreviations: cZ, *cis*-zeatin types; tZ, *trans*-zeatin types; DHZ, dihydrozeatin types; iP, isopentenyladenine types; mT, *meta*-topolin types; oT, *ortho*-topolin types. Note that 'nucleotides' include nucleoside monophosphates, nucleoside diphosphates and nucleoside triphosphates, and that iP metabolites cannot form O-glucosides as their molecules do not contain a hydroxyl group that can be glucosylated.

glucosyltransferases in *N. oceanica* (Figure 2), suggesting the presence of as yet unidentified glycosylation pathways. If validated, this finding adds another layer of complexity and diversity to the mechanisms of hormonal homeostasis in *N. oceanica* and algae in general.

The most prominent features of the CK profile for *N. oceanica* include the observations that: (i) no BA metabolites were detected, and topolin conjugates (approximately 0.65%) were at a much lower level than in the Chlorophyta (19–66%) (Figure 4a and Table S6), (ii) the ratio of isoprenoid (99.34%) to aromatic CKs (0.65%) is >150, much higher than that in Chlorophyta (< 3.5-fold; Stirk *et al.*, 2013) (Figure 4b and Table S6), and (iii) DHZ is present in *N. oceanica* (as for bryophytes and Arabidopsis; Novák *et al.*, 2008; Frébort *et al.*, 2011) (Figure 4c), but absent in green algae (Stirk *et al.*, 2013) (Figure 4a). On the other hand, the CK profile of *N. oceanica* also differs from that of bryophyte and higher plants. For example, the level of *cis*-zeatin (cZ) is 10-fold higher than that of iP in *N. oceanica*; however, a reverse trend was observed in Arabidopsis (0.35-fold; Novák *et al.*, 2008) (Figure 4c). Moreover, despite being the dominant types in *P. patens* (von Schwardenberg *et al.*, 2007), iP-type CKs are present only at a low level in *N. oceanica* (Figure 4c). Additionally, although cZ is over twice as abundant as *trans*-zeatin (tZ) in *P. patens*, they are present at equivalent levels in *N. oceanica* (Figure 4c).

Phytohormone synthesis is actively involved in nitrogen-depletion induced stress responses in *N. oceanica*

Little was known about the functional roles of phytohormones in microalgae, particularly at the molecular level. In oleaginous microalgae such as *Nannochloropsis* sp., triacylglycerols (i.e. the microalgal 'oil') are the main carbon and energy reserve under nitrogen deprivation or other stress conditions (Radakovits *et al.*, 2012; Vieler *et al.*, 2012; Li *et al.*, 2014; Wang *et al.*, 2014). Therefore, to assess the physiological relevance of phytohormones in microalgae, we monitored the transcriptional activities of ABA and CK metabolism pathways during nitrogen depletion stress. A large-scale, highly reproducible transcriptome dataset from *N. oceanica* was generated via mRNA sequencing (mRNA-Seq) at six time points (3, 4, 6, 12, 24 and 48 h) under both N-replete (N+) and N-depleted (N−) conditions respectively (each with three biological replicates) (Li *et al.*, 2014). The transcriptional dynamics were further validated by quantitative PCR analysis of selected genes over the six time points (Doc S2). This time-series dataset of 36 transcriptomes demonstrated the diversity and dynamics of phytohormone-related transcripts during stress responses in *N. oceanica* (Data S2).

In higher plants, ABA signaling contributed to the response to abiotic stress (Raghavendra *et al.*, 2010). In *N. oceanica*, nitrogen deprivation increased the abundance

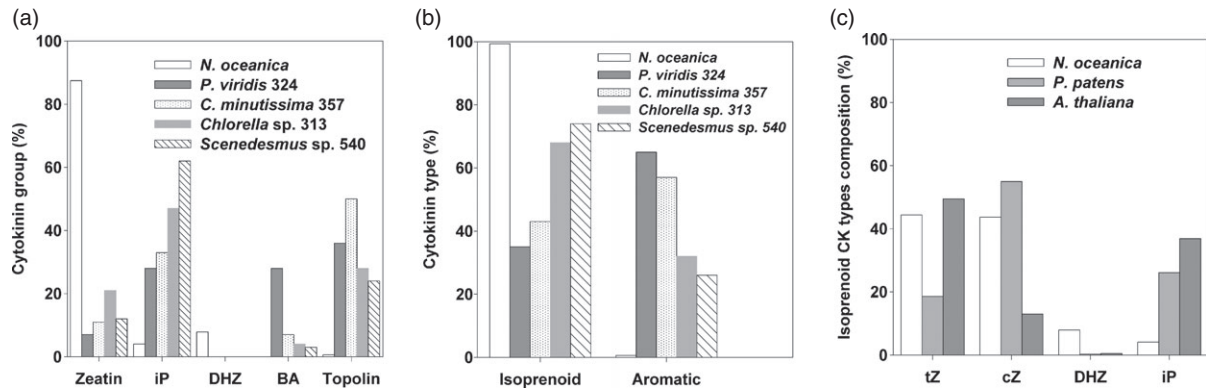


Figure 4. Comparison of the CK profile among *N. oceanica*, *P. patens*, Arabidopsis, and green algae.

(a) Comparison of the CK types between *N. oceanica* and green microalgae.

(b) Comparison of the CK groups between *N. oceanica* and green microalgae. The numbers in parentheses specify algal strain numbers.

(c) Comparisons of the isoprenoid CK types among *N. oceanica*, *P. patens* and Arabidopsis.

of 9-*cis*-epoxycarotenoid dioxygenase (NCED) and MoCo sulfurase (ABA3) transcripts (Figure 5a,b and Data S3; see Table S7 for conserved functional domains). Transcripts of ABCG40 (the ABA transporter) and ABI1 (which is implicated in the early events of ABA signaling) were also slightly up-regulated (Figure 5a). Meanwhile, the transcript level of CYP707A1 (involved in the main ABA catabolic pathway; Figure 1) was moderately repressed (Figure 5a, b). The rates of both synthesis and catabolism of ABA are thus regulated as part of the response to nitrogen deprivation. This strongly suggests that ABA-based processes are important at least under N-depleted conditions in *N. oceanica*.

In vascular plants, CKs regulate biotic (Jeon *et al.*, 2010; Großkinsky *et al.*, 2011) and abiotic stress (Ha *et al.*, 2012) responses, both of which involve extensive interplay between CK and ABA. Stresses such as drought and high salinity reduce the biosynthesis of CKs (Ha *et al.*, 2012). In *N. oceanica*, nitrogen deprivation suppressed the transcript level of CK synthesis genes such as *IPT*, *AK* and *APT* (encoding adenine phosphoribosyl transferase; Schnorr *et al.*, 1996), but induced that of CK receptor *ARR* gene g1318 (Figure 5a,c and Data S3; see Table S7 for conserved functional domains). In Arabidopsis, type A RRs function as negative regulators of ABA signaling (To *et al.*, 2004). *ARR* encoded by g1318 resembles the type A RR genes from Arabidopsis in that it only contains the RR domain. Thus it is probably a type A RR. These results suggest that CKs exert an opposite effect on ABA under N-depleted conditions in *N. oceanica*. The distinctive dynamics of transcription of the ABA and CK biosynthetic genes are consistent with the present notion that CKs function as antagonists of ABA in various growth and physiological processes in higher plants, including environmental stress responses (Ha *et al.*, 2012). However, much remains to be elucidated regarding the role of ABA or CKs in the microalgal stress response, such as whether

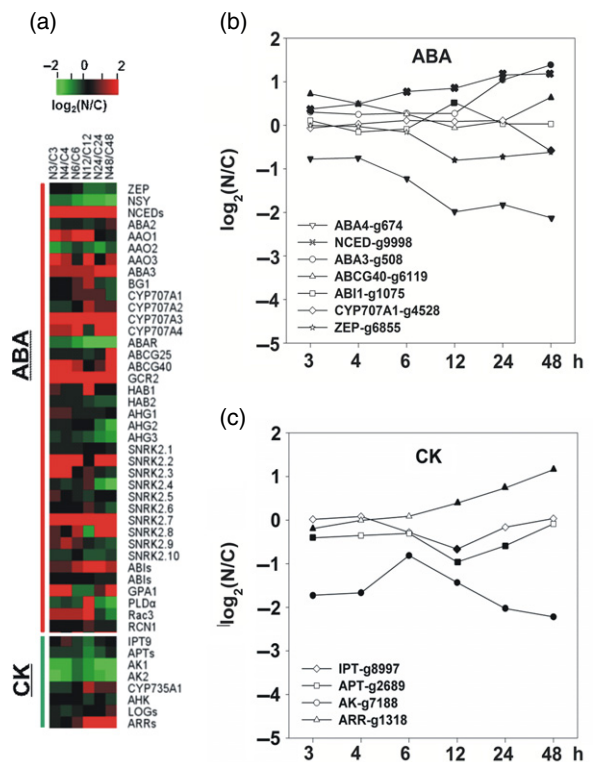


Figure 5. Transcriptional dynamics of ABA and CK metabolism and signaling genes in *N. oceanica* during nitrogen depletion.

(a) Overview of the transcriptional profile. Red and green indicate genes that are up- and down-regulated, respectively.

(b, c) Transcriptional dynamics of ABA-related genes (b) and CK-related genes (c). Solid symbols indicate genes whose transcriptional levels differ significantly between N-replete (N⁺, control) and N-depleted (N⁻) cultures. The conserved domains of transcriptionally regulated ABA- and CK-related proteins in *N. oceanica* compared with Arabidopsis counterparts are provided in Table S7 (see detailed analysis in Data S2 and S3).

ABA and CK metabolites show an antagonistic response to nitrogen depletion, and what the potential functions of ABA and CK are during nitrogen depletion.

To answer these questions, *N. oceanica* cells under both N+ and N− conditions were harvested at 6, 12, 24, 48 and 72 h for investigation of temporal changes in intracellular ABA and CK profiles using UHPLC-MS/MS. The ABA level was remarkably increased following onset of nitrogen depletion ($P \leq 0.001$) and remained at elevated levels in algal cells during nitrogen depletion (Figure 6a). Similarly, the ABA level was found to be elevated in the green microalga *Dunaliella* sp. under nitrogen starvation (Tominaga *et al.*, 1993). This was consistent with the increased transcriptional abundance of *NCED* and *ABA3*, which suggested they encode committed enzymes in ABA biosynthesis in *N. oceanica* (Figure 1). In contrast, the genes encoding ZEP and ABA4, which are involved in production of neoxanthin, a component of light-harvesting complex II (Figure 1), were transcriptionally depressed under N− conditions (Figure 5a,b). This implies that, in *N. oceanica*, neoxanthin synthesis may not be the limiting process in ABA synthesis, and that its primary role appears to be in photosynthesis (Normanly *et al.*, 2004). This is

further supported by the observation that transcripts encoding ZEP do not increase under osmotic stress, and conversion of xanthoxin to ABA is independent of stress conditions in higher plants (Normanly *et al.*, 2004).

CKs are classified, based on physiological functions, into active forms, transport forms and storage forms. The free bases (*iP* and *tZ*) are active forms (Takei *et al.*, 2001), while isopentenyladenine riboside, the *cis*- and *trans*-zeatin ribosides (*cZR* and *tZR*) and dihydrozeatin riboside (DHZR) are transport forms. The *cZ*-type CKs act as transient storage forms, which are transported to certain locations and converted to the *trans* isomers (Sakakibara, 2010). Conjugation of CKs with glucose leads to the formation of *O*- and/or *N*-glucosides, both of which are storage forms and lack CK activity (Sakakibara, 2006). The bioactivity of CK nucleotides remains to be determined, as they are usually inactive in enzyme assays but bind to certain CK receptors (von Schwanzenberg *et al.*, 2007).

The intracellular contents of 33 major CK species over a 72 h period, and the total content of six CK classes, revealed

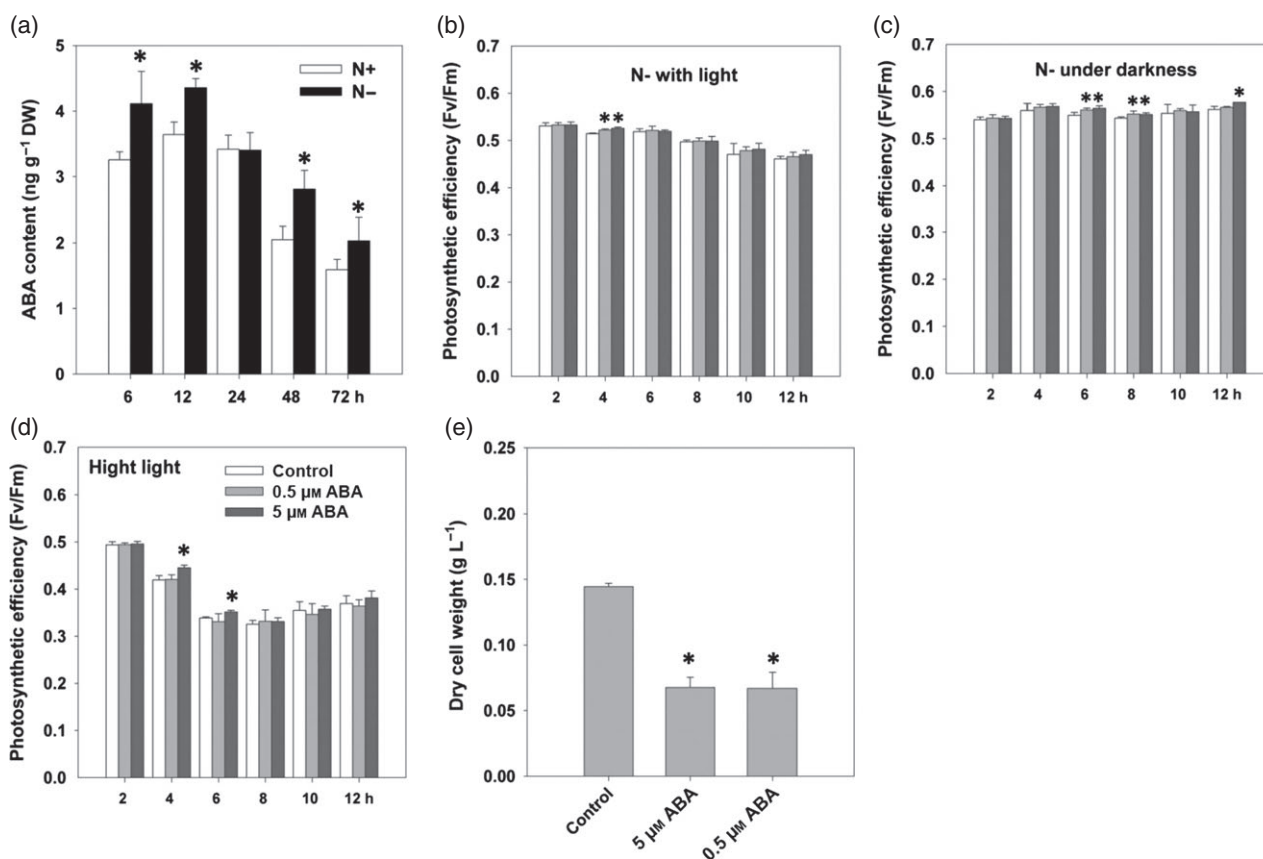


Figure 6. Effects of ABA on *N. oceanica* cells under nitrogen depletion.

(a) Dynamics of ABA levels under N− conditions as a function of time.

(b) Effect of ABA under N− conditions at 50 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ light.

(c) Effect of ABA under N− conditions in darkness.

(d) Effect of ABA on *N. oceanica* under high light stress (150 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$).

(e) Dry weight of *N. oceanica* cells in the presence or absence of ABA (control) for 5 days under optimal growth conditions.

Values are means and SD of four biological replicates. Asterisks indicate statistically significant differences compared with the control conditions ($P < 0.05$).

distinct patterns of dynamics in relative abundance (Figure 7a). Total CK (excluding the glycoside conjugates) decreased after 12 h of nitrogen depletion ($P \leq 0.001$) and remained at a decreased level in the subsequent period, despite a transient increase at 6 h (Figure 7b). Simultaneously, levels of isoprenoid CKs (excluding their glycoside conjugates) exhibited a trend similar to that of total CK (Figure 7b). As for isoprenoid CK types, the total amount of *tZ*-, *cZ*- and *iP*-type CKs (excluding glycoside conjugates) decreased under N- conditions at 72 h ($P \leq 0.05$; Figure 7c).

The free bases *tZ* and *iP* underwent rapid decreases after 12 or 24 h, respectively, upon nitrogen depletion ($P \leq 0.001$, Figures 7a and S5). In addition, the free base *cZ*, which generally has lower or no activity in plants, decreased after 12 h of nitrogen depletion (Figures 7a and S5). On the other hand, although DHZ increased under N- conditions, it accumulated at a very low level relative to other free bases (Figures 7a and S5). Considering its weak activity in a bud-induction bioassay (von Schwartzenberg *et al.*, 2007), DHZ is unlikely to be an

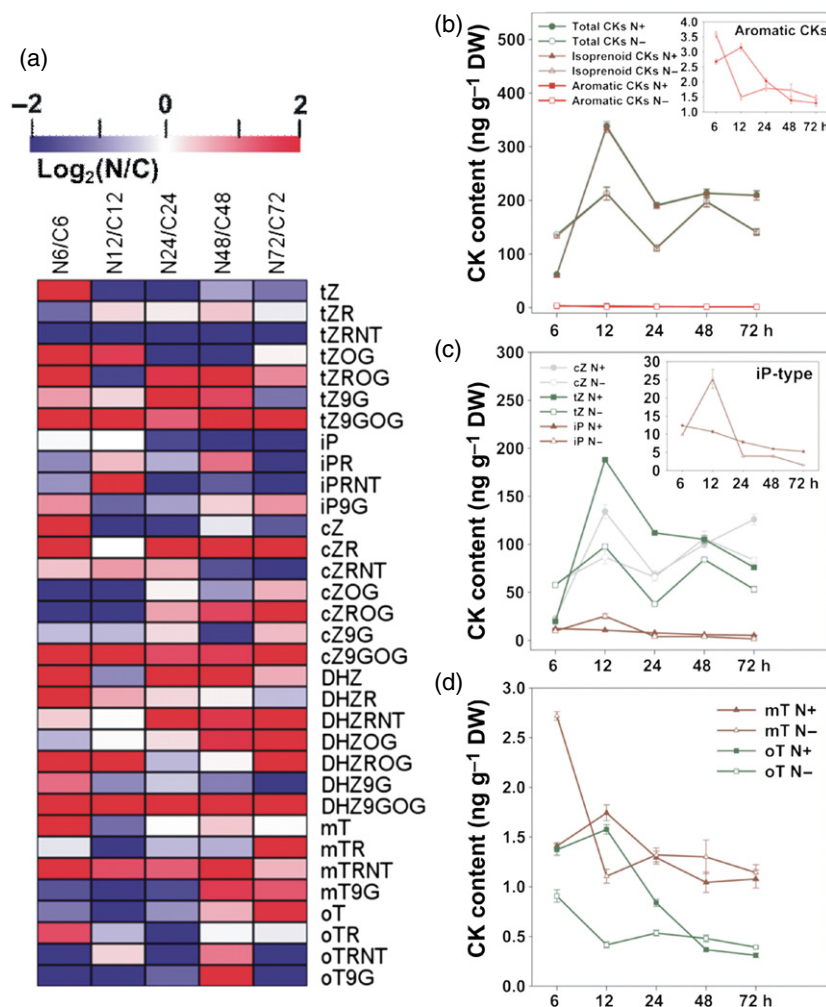


Figure 7. Dynamics of the cellular content of CKs in *N. oceanica* as a function of time under N+ and N- conditions.

(a) Dynamics of the levels of the 33 CK species. The fold change of the CK content was calculated as $\log_2[cc(N-)/cc(N+)]$, where *cc* is the content of CK species. (b) Dynamics of the levels of total CKs, isoprenoid CKs and aromatic CKs. Isoprenoid CKs account for a large proportion of the total CKs (99.34%), while aromatic CKs contribute only a trivial amount (0.65%). In addition, total CKs and isoprenoid CKs exhibit identical trends during nitrogen depletion.

(c, d) Dynamics of the total amount of major isoprenoid CK types (c) and major aromatic CK types (d). The amount of indicated CK types represents the sum of this type of CKs excluding the glycoside conjugates.

Time refers to the duration (in hours) since onset of N+ or N- conditions. Values represent means \pm SD ($n = 3$). Abbreviations: *tZ*, *trans*-zeatin; *tZR*, *trans*-zeatin riboside; *tZRNT*, *tZR* nucleotide; *tZOG*, *trans*-zeatin-*O*-glucoside; *tZROG*, *trans*-zeatin riboside-*O*-glucoside; *tZ9G*, *trans*-zeatin-9-glucoside; *tZ9GOG*, *trans*-zeatin-9-glucoside-*O*-glucoside; *cZ*, *cis*-zeatin; *cZR*, *cis*-zeatin riboside; *cZRNT*, *cZR* nucleotide; *cZOG*, *cis*-zeatin-*O*-glucoside; *cZROG*, *cis*-zeatin riboside-*O*-glucoside; *cZ9G*, *cis*-zeatin-9-glucoside; *cZ9GOG*, *cis*-zeatin-9-glucoside-*O*-glucoside; *iP*, *isopentenyladenine*; *iPR*, *isopentenyladenine* riboside; *iPRNT*, *iPR* nucleotide; *iP9G*, *isopentenyladenine*-9-glucoside; *DHZ*, dihydrozeatin; *DHZR*, dihydrozeatin riboside; *DHZRNT*, dihydrozeatin riboside nucleotide; *DHZOG*, dihydrozeatin-*O*-glucoside; *DHZROG*, dihydrozeatin riboside-*O*-glucoside; *DHZ9G*, dihydrozeatin-9-glucoside; *DHZ9GOG*, dihydrozeatin-9-glucoside-*O*-glucoside; *mT*, *meta*-topolin; *mTR*, *meta*-topolin riboside; *mTRNT*, *mTR* nucleotide; *mT9G*, *meta*-topolin-9-glucoside; *oT*, *ortho*-topolin; *oTR*, *ortho*-topolin riboside; *oTRNT*, *oTR* nucleotide; *oT9G*, *ortho*-topolin-9-glucoside.

active contributor in the *N. oceanica* response to nitrogen depletion.

Among the translocated forms, *tZR* and *DHZR* were found at very low and largely stable basal levels, while *cZR* showed a fourfold increase over 24 h and remained at an elevated level under N[−] conditions (Figures 7a and S5). Meanwhile, *tZR* nucleotide and *iPR* nucleotide (sum of mono-, di-, and triphosphates) showed a gradual decrease after onset of nitrogen depletion with the exception of *iPR* nucleotide at 12 h (Figures 7a and S5). The level of *cZRNT* slightly increased in the first 24 h, but showed a decreased level at 72 h (Figures 7a and S5). The level of dihydrozeatin riboside nucleotide increased under N[−] conditions after 24 h, but its basal level is quite low (Figures 7a and S5).

The total amount of aromatic CKs transiently decreased (2.1-fold) after 12 h of nitrogen depletion, and then remained largely stable relative to the control (Figure 7b). The *ortho*-topolin-type CKs underwent a progressive decrease during the first 24 h of exposure to N[−] conditions. The total amount of *meta*-topolin-type CKs transiently increased (1.93-fold) during 6 h nitrogen depletion, but decreased to 60% of the control at 12 h. The dynamics of aromatic CK species showed more complex changes than isoprenoid CKs during nitrogen depletion (Figures 7a and S6). The free topolin bases, especially *meta*-topolin, are generally considered as the most active forms of this compound. Under N[−] conditions, *meta*-topolin increased at 6 h (twofold), then decreased by 60% at 12 h, and eventually returned to the control level. In contrast, *ortho*-topolin decreased rapidly to 25% of the control at 12 h, but showed a moderately elevated accumulation at 72 h (1.25-fold, Figures 7a and S6). Despite fluctuations and wide discrepancy in the levels of various CK species, the levels of dominant bioactive CK species (i.e. free bases *tZ* and *iP*) decreased upon nitrogen depletion. Therefore, ABA and CKs are actively involved in the cellular response to nitrogen depletion, where they act antagonistically at both transcriptional and metabolite levels.

Physiological roles of ABA or CKs in *N. oceanica*: ABA alleviates stress damage and CKs promote growth

To assess their potential functions in *N. oceanica*, the levels of ABA and CK were perturbed by supplementing ABA and 6-benzyladenine (BA, an artificial CK) to algal cultures at concentrations of 0.5 and 5 μM . For ABA-treated and untreated cultures, algal cells at the linear growth phase were inoculated into nitrogen-free medium, and the cellular responses (in terms of the maximum quantum yield of photosystem II, F_v/F_m) were tracked for 12 h. Under 50 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$, cells treated with either 0.5 or 5 μM ABA showed a higher F_v/F_m (which implies elevated stress tolerance) than the ABA-untreated control ($P \leq 0.05$; Figure 6b). Under darkness, the photosynthetic efficiency

was also significantly higher in ABA-treated cells than the control ($P \leq 0.05$; Figure 6c).

To assess whether ABA plays a role in the response to environmental stresses other than nitrogen depletion, algal cells in the linear growth phase were inoculated into fresh N⁺ medium containing 0.5 or 5 μM ABA under various light intensities. Under 150 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$, the F_v/F_m gradually decreased with time, but the stress tolerance of cells treated with 5 μM ABA slightly improved compared to the ABA-free control ($P \leq 0.05$; Figure 6d). Under a higher light intensity of 300 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$, the F_v/F_m values decreased more rapidly (from 0.60 to 0.35 within 12 h) than under 150 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$, and both 0.5 and 5 μM ABA-treated cells showed elevated stress tolerance (relative to the ABA-free control), but the discrepancy in F_v/F_m values between ABA-treated and untreated cells under 300 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ was not widened, when compared with that under 150 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ (Figure S7).

It is noteworthy that, in higher plants, the effects of manipulating the endogenous hormone pattern by supplementing hormones are only moderate, and sometimes are pleiotropic (George *et al.*, 2008), which may be due to the sophisticated and efficient modulation of hormone levels by the cells to minimize exogenous disturbances (Hartig and Beck, 2005). In addition, internal protective reactions may be present in *N. oceanica*, as indicated by the elevated F_v/F_m of the control cells (without ABA treatment) under a light intensity of 300 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ (the F_v/F_m value increased from 0.35 at 12 h to 0.45 at 24 h; Figure S7). Thus the discrepancy of maximum quantum yield of photosystem II between ABA-treated and ABA-untreated cells should be more dramatic considering these internal protective effects. Moreover, a growth-retarding effect was observed for ABA, as algal biomass cultured under optimal conditions decreased by 53.2 and 53.6%, respectively, relative to the control, after 5 and 0.5 μM ABA treatment for 5 days ($P \leq 0.005$; Figure 6e). Taken together, ABA may be a stress hormone in *N. oceanica*, similar to its role in plants (Nambara and Marion-Poll, 2005) and the green algae *C. reinhardtii* (Yoshida *et al.*, 2003, 2004) and *Haematococcus pluvialis* (Kobayashi *et al.*, 1997).

The life-cycle progression was also investigated in *N. oceanica* cells with perturbed CK levels. Algal cells were firstly synchronized by the standard method of alternating light/dark periods (12/12 h) followed by 24 h darkness (Umen and Goodenough, 2001). At the end of the dark period, the vast majority of cells show almost complete synchronization (Umen and Goodenough, 2001; Bišová and Zachleder, 2014). Synchronized cells were used to inoculate experimental cultures with or without BA under continuous light. Cellular DNA content was monitored using flow cytometry. The cell cycle comprises four phases: Gap 1 (G1, *n* copies of DNA), DNA synthesis (S, *n*–2*n*

copies of DNA), Gap 2 (G2, $2n$ copies of DNA) and mitosis (M, $2n$ copies of DNA). The DNA content of cells during the G2 and M phases cannot be distinguished by flow cytometry, and thus this is referred to as the G₂M phase (Figure 8a) (De Veylder *et al.*, 2007). Prior to the transfer to light, only approximately 5.6% of the cells were in the G₂M phase indicating that a large proportion of the cell had completed mitosis. With the onset of light, the G1 peak decreased in both BA-treated cultures and the control, suggesting progression of the cell-cycle from G1 into S. Notably, the G1 peak decreased faster in BA-treated cultures than controls (Figure 8b). In contrast, the G₂M peak of cells in both the control and BA-treated populations increased, the latter more dramatically (Figure 8c). Thus, BA stimulated cell-cycle progression from G1 to S phase and S to G₂M phase. These results are supported by microscopic observations. Whereas cell division hardly occurred in control cultures in the first 4 h under light, cell division was observed in the algal population treated with BA (Figure 8d). Moreover, total algal biomass increased by 72% in

N+ medium after 5 μ M BA treatment for 5 days relative to the control ($P \leq 0.001$; Figure 8e). Therefore, CKs are involved in the control of *N. oceanica* cell-cycle progression.

To assess a potential link of this finding to microalgal biotechnology, the growth rate of *N. oceanica* was measured under N+ or N– conditions in photobioreactors with or without BA. Cell cultures at $5 \times 10^7 \text{ ml}^{-1}$ were collected and suspended in either N+ or N– medium. BA was applied to cells under N– conditions. The growth ratio was monitored by counting cell number for 144 h. Exogenous BA has a promoting effect on algal growth under N– conditions, as cell number was significantly increased in BA-supplemented cultures relative to the non-treated control ($P \leq 0.05$; Figure 9a). The DWs of cultures treated with 0.5 and 5 μ M BA were 36% and 23% higher than that of the control (cultured under N– conditions without BA) at 144 h, respectively ($P \leq 0.05$; Figure 9b). This evidence collectively suggests that CKs play a role in algal cell-cycle progression and have a stimulating effect on growth, as in

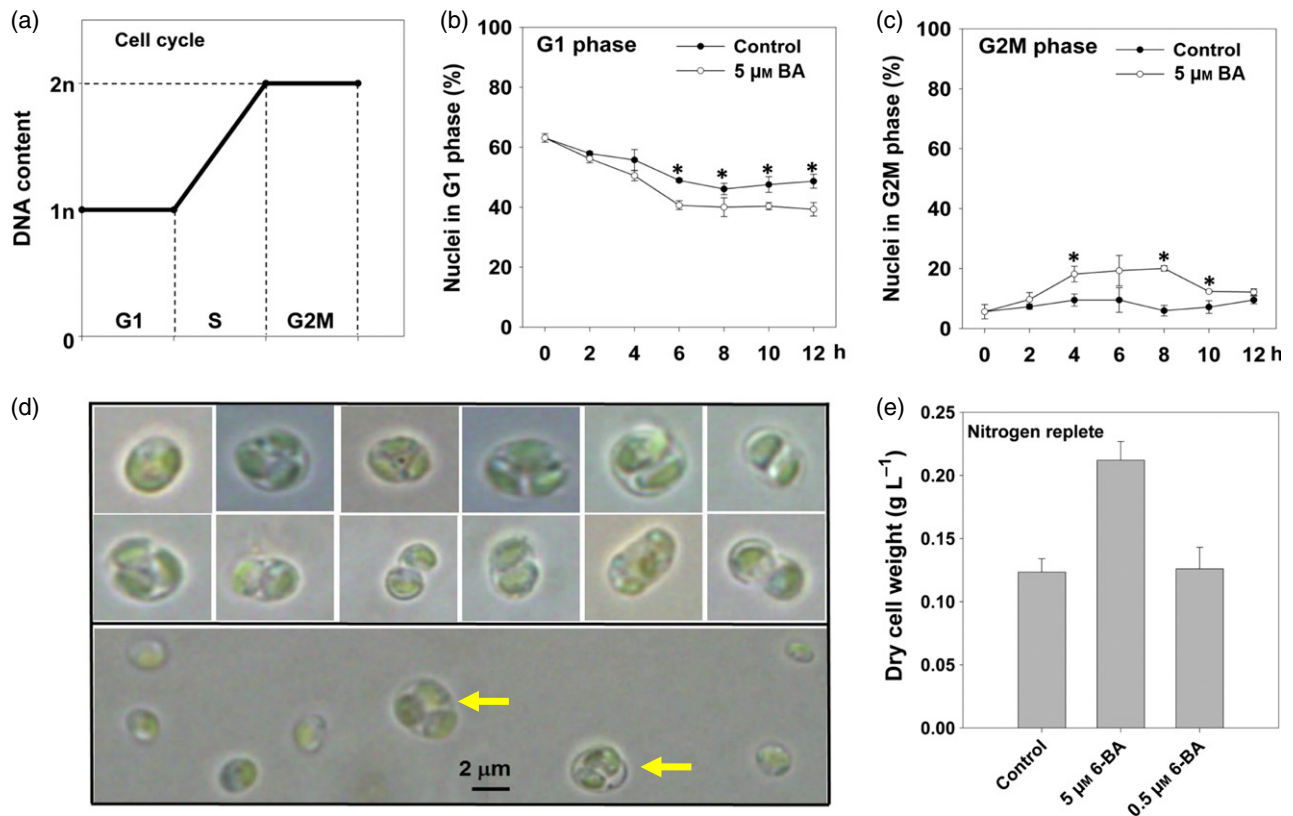


Figure 8. Effects of 6-benzyladenine (BA, an artificial CK) on cell-cycle progression of *N. oceanica*.

(a) Schematic representation of the relationship between DNA content and cell-cycle phase.

(b) Dynamics of cells in G₁ phase in BA-treated and untreated populations.

(c) Dynamics of cells in G₂M phase in BA-treated and untreated populations.

(d) Photomicrographs of cells after BA application. Yellow arrows indicate mother cells with at least two protoplasts.

In (b)–(d), cultures of the synchronous algal cells were divided into two halves, one of which received 5 μ M 6-BA while the other served as control. Cells were harvested every 2 h. The DNA content of 10 000 cells was analyzed in each case by flow cytometry and the percentages of cells attributed to cell-cycle phases G1, S and G₂M were quantified.

(e) Dry weight of *N. oceanica* cells in the presence or absence of BA (control) in N+ medium for 5 days.

green plants (Sakakibara, 2006) including some green microalgae (Tian *et al.*, 2006; Piotrowska and Czerpak, 2009; Stirk *et al.*, 2011; Park *et al.*, 2013; Tate *et al.*, 2013).

DISCUSSION

It is proposed that the phytohormones in present-day flowering plants evolved from pre-existing primary metabolic systems in algae (Kenrick and Crane, 1997). A recent study showed that algae, and green algae in particular, are enriched in hormone-related reactions (Chae *et al.*, 2014). However, the potential roles of phytohormones in algae remain unclear. Here we show that microalgae from both Stramenopile and Archaeplastida lineages possess relatively simple but self-contained ABA and CK metabolic systems that are comparable with those in flowering plants. ABA and CK biosynthetic and catabolic pathways appear to have developed widely in microalgae, but higher plant-type conjugation mechanisms did not emerge until after evolution of the green algae (Figure 10). Moreover, algae and especially non-green algae (diatoms, brown algae, eustigmatophyte algae and red algae) appear to utilize as yet unknown conjugation mechanisms, as supported by the presence of ABA-GE and diverse glucosyl CKs in *N. oceanica* despite its lack of land plant-type glucosyltransferases. In addition, the key enzyme β -glucosidase (BG1), which catalyzes the one-step hydrolysis of ABA-GE (Xu *et al.*, 2012), is present in most of the sampled algae, whereas the ABA glucosyltransferase UGT71B6 (Xu *et al.*, 2012) is found exclusively in green plants (including green algae). The phytohormone profile reveals that *N. oceanica* produces all of the essential, biologically active, forms of ABA and CK at concentrations that are comparable to those found in vascular plants. Moreover, diverse free and conjugated forms of these hormones were detected. Collectively, these results suggest the presence of a quite well-developed and complete ABA and CK metabolism system in *N. oceanica*. However, the microalgal signaling pathways appear to be hormone-specific. The canonical signaling cascade of ABA is quite well conserved in algae, but that of CK is not.

Although receptors for both ABA and CKs are absent in algae, the primitive form of CK receptors (AHK1) is ubiquitous. Stepwise evolution may have played a key role in emergence of hormone sensing/signaling mechanisms in modern plants, which may operate in parallel to those demonstrated for mammal hormone systems (Bridgham *et al.*, 2006).

The transcriptional dynamics of ABA and CK biosynthetic genes in *N. oceanica* during nitrogen deprivation strongly suggest a key role for hormones in microalgal stress responses. Notably, synthesis and degradation pathways for these hormones were transcriptionally active during both nitrogen-replete and nitrogen-depleted conditions. Interestingly, in this alga, CKs appear to function as antagonists of ABA at both the transcriptional and metabolic levels, in the same way as has been proposed for Arabidopsis (Ha *et al.*, 2012). The temporal oscillation of ABA and CKs indicated a sophisticated role for these hormones in orchestrating cellular homeostasis as a regulatory mechanism to cope with environmental stress or other processes. In the case of CK accumulation achieved by exogenously added BA, cell-cycle progression of *N. oceanica* was stimulated and the growth rate was markedly increased regardless of nitrogen availability. Moreover, it was recently reported that endogenous CKs are involved in the responses to light and cell division in green microalgae (Stirk *et al.*, 2013). Thus CKs may act as signaling molecules during cell-cycle progression in *N. oceanica*. On the other hand, ABA enhanced the stress tolerance of this alga, as supported by the mitigation of high light damage and nitrogen starvation damage in ABA-treated cells. These results suggest that these hormones play diverse and active roles in the nitrogen-deprivation response, and that CKs function antagonistically with ABA during this process in *N. oceanica*. At present, it is not clear whether the main targets of the physiological effects of these hormones are hormone-producing cells (autocrine signaling) or other cells (endocrine signaling, which typically requires multi-cellularity).

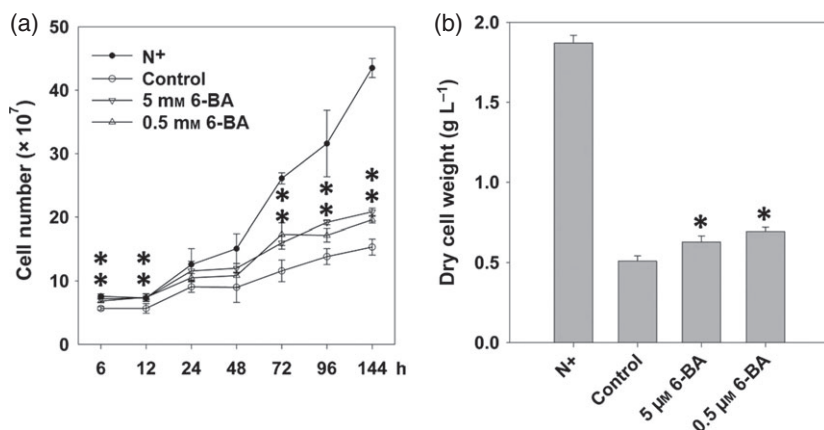


Figure 9. Effect of 6-benzyladenine (BA, an artificial CK) on the growth of *N. oceanica* in photobioreactors.

(a) Growth curve of *N. oceanica* under N⁺ and N⁻ conditions in the presence of BA (0.5 or 5 μM) or its absence (control). (b) Dry weight of *N. oceanica* cells under N⁺ and N⁻ conditions in the presence of BA (0.5 or 5 μM) or its absence (control) for 144 h. Cell numbers were determined at the time points indicated. Values are means ± SD of four independent experiments. Asterisks indicate statistically significant differences between BA-treated cultures and the control ($P < 0.05$).

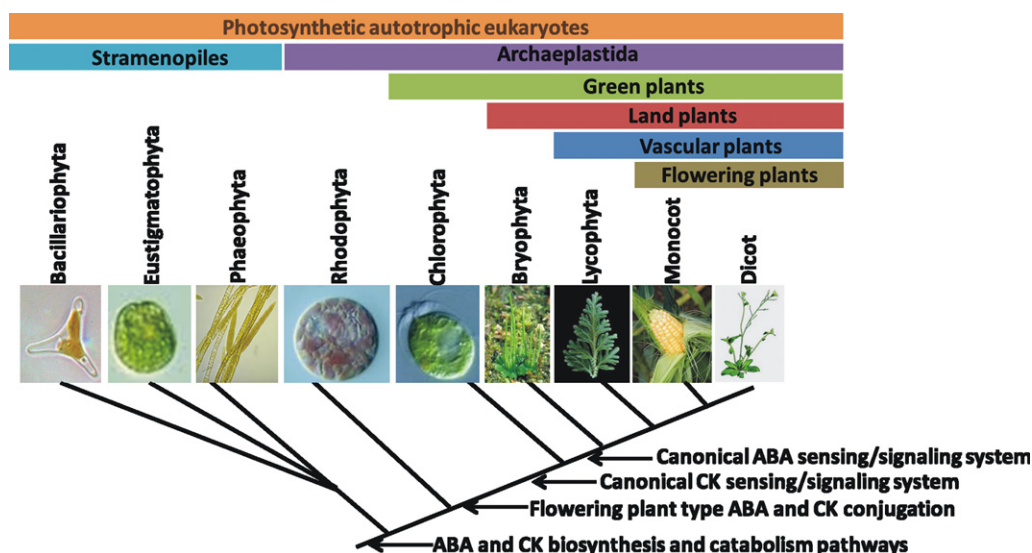


Figure 10. Origin and evolution of ABA and CK metabolism and signaling pathways in oxygenic photosynthetic eukaryotes.

Bacillariophyta: diatoms *P. tricornutum*, *T. pseudonana* and *F. cylindrus*; Eustigmatophyte: *N. oceanica*; Phaeophyte: brown alga *E. siliculosus*; Rhodophyte; red alga *C. merolae*; Chlorophyte: green alga *Micromonas* sp. RCC299, *O. tauri*, *C. variabilis* NC64A, *C. pyrenoidosa*, *C. subellipsoidea* C-169, *C. reinhardtii* and *V. carteri*; Bryophyte: *P. patens*; Lycophyte: *S. moellendorffii*; monocot: *Z. mays*; dicot: *A. thaliana*.

Our findings provide new insights into the forces that have shaped plant evolution, which is characterized by two key transitions: first from unicellular organisms to multicellular life forms, and then from aquatic lifestyles to terrestrial colonization. Phytohormones have been suggested to play vital roles in these transformational events (Rensing *et al.*, 2008; Blanc *et al.*, 2010). Recent findings showed that the canonical auxin and strigolactone systems were at least partially present before land colonization by plants (De Smet *et al.*, 2011; Delaux *et al.*, 2012). By comparing hormone signaling pathways across evolutionarily diverse taxa, we found that the transition from unicellular to multicellular organisms required far fewer new hormone-related genes than the transition from aquatic to terrestrial life. ABA was thought to play a crucial role in conquest of the land by plants (Takezawa *et al.*, 2011). However, our results suggested that primitive functions of ABA emerged in aquatic unicellular species such as *N. oceanica* (although a complete set of land plant sensing/signaling system is still missing). On the other hand, CKs play active roles in present day algal species (e.g. Stramenopiles), but the canonical higher plant-type CK signaling pathway most likely did not arise until after the emergence of ancient green microalgae (Figure 10). Thus primitive properties of ABA and CK may have been present at the unicellular phase of plant evolution. On the other hand, it should be noted that microalgae may possess additional signaling mechanisms that diverge significantly from the canonical model for seed plants (although this requires further investigations). ABA and CKs are found from bacteria to humans (Takezawa *et al.*, 2011; Spíchal, 2012), but the canonical higher-plant

sensing/signaling system has not been identified in most of these organisms (Tossi *et al.*, 2012).

In summary, photosynthetic eukaryotic microalgae produce a wide range of phytohormones using highly conserved biosynthetic enzymes and as yet unknown sensing/signaling systems. In the industrial oleaginous microalga *N. oceanica*, ABA and CKs are involved in the response to nitrogen depletion. Dissection of the mechanism should accelerate rational engineering of microalgal feedstock for enhanced biomass and biofuel productivity.

EXPERIMENTAL PROCEDURES

Data sources and phylogenetic analysis

Genome sequences for the sampled organisms were retrieved from the websites listed in Table S1. Proteins from all genomes were blasted with the Arabidopsis proteins (Table S2). Sequences ultimately selected for Figure 2 are listed in Data S1. Identification of conserved domains, sequence alignment and phylogenetic analysis were performed using Pfam (Finn *et al.*, 2008), ClustalW (Chenna *et al.*, 2003), gBlocks (Talavera and Castresana, 2007), ProtTest (Darriba *et al.*, 2011) and PML 3.0 (Guindon *et al.*, 2010). Details are provided in Doc S2.

Phytohormone analysis

The ABA and CK contents were determined as previously described (Novák *et al.*, 2008; Turečková *et al.*, 2009). Further details are provided in Doc S2.

Dynamics of ABA and CK metabolite and transcript abundance

N. oceanica cells in the linear growth phase were harvested and washed with axenic seawater, and then were inoculated into N+

and N[−] medium. Aliquots of culture were collected at five time points (6, 12, 24, 48 and 72 h, with three biological replicates) for ABA and CK profile determination. Three biological replicates were also collected for mRNA-Seq analysis at six time points (3, 4, 6, 12, 24 and 48 h). The results were validated by quantitative PCR of selected genes. Additional details are provided in Doc S2 and in a previous publication (Li *et al.*, 2014).

Responses of *N. oceanica* to exogenous ABA and CKs

Cells were synchronized by alternating light/dark (12/12 h) cycles with a final extended dark period (Zachleder, 1994; Umen and Goodenough, 2001). Synchronous cells were supplemented with indicated concentration of hormones and cultured under 50 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ light. Cell aliquots were collected for DW determination or cell-cycle analysis by flow cytometry as described previously (Marie *et al.*, 2001) with modifications (Doc S2). Cell division in randomly selected fields was investigated microscopically. Alternatively, cultures at $5 \times 10^7 \text{ ml}^{-1}$ were collected and suspended in N⁺ or N[−] medium. BA was applied to cells under N[−] conditions. DWs and cell counts were determined at the indicated times. Further, cultures at $5 \times 10^7 \text{ ml}^{-1}$ were harvested and re-suspended into N⁺ medium supplemented with 0.5 or 5 μM ABA under high light (150 or 300 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$) or into N[−] medium with 0.5 or 5 μM ABA under darkness or 50 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$. The F_v/F_m value was determined using IMAGING-PAM (Lu *et al.*, 2014). Details are provided in Doc S2.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

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

Figure S1. Phylogenetic relationship of organisms analyzed in this study.

Figure S2. Phylogenetic analysis of isopentenyltransferases (IPTs) and cytokinin riboside 5'-monophosphate phosphoribohydrolases (LOGs).

Figure S3. Alignment of the amino acid sequences of adenosine kinases.

Figure S4. Phylogenetic analysis of the ABA biosynthetic enzymes 9-*cis*-epoxycarotenoid dioxygenases.

Figure S5. Dynamics of the isoprenoid CK species in response to nitrogen deprivation as a function of time.

Figure S6. Dynamics of aromatic CK species in response to nitrogen deprivation as a function of time.

Figure S7. Effects of ABA on *N. oceanica* cells under 300 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ light.

Table S1. Databases used as sequence and annotation sources.

Table S2. List of proteins involved in ABA and CK metabolic and signaling pathways in Arabidopsis.

Table S3. Comparison of sequence identity and conserved domains of isopentenyltransferase proteins between algal species and Arabidopsis.

Table S4. Comparison of sequence identity and conserved domains of cytokinin riboside 5'-monophosphate phosphoribohydrolase proteins between algal species and Arabidopsis.

Table S5. Comparison of sequence identity and conserved domains of ABA and CK signaling proteins between algal species and Arabidopsis.

Table S6. Comparisons of the CK profile of *N. oceanica* with those of green microalgae.

Table S7. Comparison of sequence identity and conserved domains of ABA and CK-related proteins (transcriptionally regulated) between *N. oceanica* and Arabidopsis.

Data S1. List of proteins involved in ABA and CK metabolism and signaling pathways that were used to create the similarity heatmap (Figure 2).

Data S2. Time-course transcriptional dynamics of ABA and CK pathways in *N. oceanica* upon nitrogen depletion.

Data S3. Transcriptional dynamics of the genes related to ABA and CK metabolism and signaling in *N. oceanica* upon nitrogen depletion.

Doc S1. Current model of ABA and CK metabolism in Arabidopsis.

Doc S2. Additional experimental procedures.

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