

Unraveling Vitamin B₁₂-Responsive Gene Regulation in Algae^{1[W]}

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Photosynthetic microalgae play a vital role in primary productivity and biogeochemical cycling in both marine and freshwater systems across the globe. However, the growth of these cosmopolitan organisms depends on the bioavailability of nutrients such as vitamins. Approximately one-half of all microalgal species requires vitamin B₁₂ as a growth supplement. The major determinant of algal B₁₂ requirements is defined by the isoform of methionine synthase possessed by an alga, such that the presence of the B₁₂-independent methionine synthase (*METE*) enables growth without this vitamin. Moreover, the widespread but phylogenetically unrelated distribution of B₁₂ auxotrophy across the algal lineages suggests that the *METE* gene has been lost multiple times in evolution. Given that *METE* expression is repressed by the presence of B₁₂, prolonged repression by a reliable source of the vitamin could lead to the accumulation of mutations and eventually gene loss. Here, we probe *METE* gene regulation by B₁₂ and methionine/folate cycle metabolites in both marine and freshwater microalgal species. In addition, we identify a B₁₂-responsive element of *Chlamydomonas reinhardtii* *METE* using a reporter gene approach. We show that complete repression of the reporter occurs via a region spanning −574 to −90 bp upstream of the *METE* start codon. A proteomics study reveals that two other genes (*S-Adenosylhomocysteine hydrolase* and *Serine hydroxymethyltransferase2*) involved in the methionine-folate cycle are also repressed by B₁₂ in *C. reinhardtii*. The strong repressible nature and high sensitivity of the B₁₂-responsive element has promising biotechnological applications as a cost-effective regulatory gene expression tool.

Vitamins are essential nutritional components of the human and animal diet and can lead to an array of deficiency diseases if lacking (Martin et al., 2011). However, it is not just animals that require an external source of vitamins for growth, and in fact, a widespread and complex distribution of vitamin requirements exists across the tree of life (Helliwell et al., 2013). These compounds are vital as cofactors for enzymes of central metabolism, but evidence suggests that there may be a selective advantage to outsource the production of these compounds when an external supply is available through the diet or biotic interactions (Croft et al., 2005; Giovannoni, 2012; Helliwell et al., 2013). Microalgae are diverse photosynthetic eukaryotes in which vitamin auxotrophy is prevalent,

with 22%, approximately 5%, and more than 50% of surveyed species requiring thiamine (vitamin B₁), biotin (B₇), and cobalamin (B₁₂), respectively (Croft et al., 2005, 2006). Improved methodology for the quantification of thiamine and cobalamin in environmental samples revealed a heterogeneous distribution of these vitamins in the oceans, with generally very low levels free in solution in the photic zone (Sañudo-Wilhelmy et al., 2012). It has also been shown, through fertilization experiments, that vitamins are limiting for algal growth in several marine environments (Gobler et al., 2007). Therefore, it is intriguing that many oligotrophic species such as *Ostreococcus tauri* are nonetheless vitamin auxotrophs (Worden et al., 2009; Helliwell et al., 2011) and so must be able to obtain a ready source of these organic micronutrients from their surroundings. In the case of cobalamin, the biosynthesis of this complex tetrapyrrole is restricted to prokaryotes, and specific biotic interactions between algae and bacteria have been shown to take place for B₁₂ acquisition (Croft et al., 2005; Kazamia et al., 2012).

Many genes encoding enzymes of vitamin metabolism are down-regulated by an external supply of these organic micronutrients, giving rise to the suggestion that such feedback inhibition loops can drive gene loss, leading to vitamin auxotrophy (Helliwell et al., 2013). In the case of cobalamin, the loss of the B₁₂-independent methionine synthase gene (*METE*) throughout the algal lineages

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means that species must then depend on the activity of a second isoform of this enzyme (METE), which requires B₁₂ as a cofactor (Croft et al., 2005; Helliwell et al., 2011). Auxotrophy for vitamin B₁₂ shows no phylogenetic relationship, and even strains of the same species have different B₁₂ requirements (Tang et al., 2010). In algae that have both *METE* and *METH*, such as *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*, it has been shown that *METE* transcript levels are repressed by the presence of B₁₂ (Croft et al., 2005; Helliwell et al., 2011). Prolonged repression of the gene by a reliable source of vitamin B₁₂, therefore, could lead to the accumulation of mutations and, eventually, gene loss. Support for this model comes from the discovery of *METE* pseudogenes in the B₁₂-dependent green algae *Volvox carteri* and *Gonium pectorale* (Helliwell et al., 2011).

Gene regulation by B₁₂ has been widely studied in bacteria. In *Escherichia coli* and *Salmonella typhimurium*, a transacting protein, MetR, positively regulates *metE* (Cai et al., 1989; Maxon et al., 1989). In contrast, in many other bacteria, B₁₂-mediated regulation of gene expression occurs via ligand-responsive genetic control elements, known as riboswitches. These sequences are found in the 5' untranslated region (UTR) of regulated mRNAs and bind the ligand directly. Changes in the secondary structure of the mRNA result in altered gene expression, for example by masking the ribosome-binding site (Winkler et al., 2002). B₁₂-responsive riboswitches are widespread within the prokaryote lineages and have been identified in genes involved in B₁₂ transport and biosynthesis (Nahvi et al., 2004) as well as the *metE* gene of *Mycobacterium tuberculosis* (Warner et al., 2007). In mammalian cell lines, where *METH* is translationally up-regulated by B₁₂ supplementation, another mechanism has been reported. There is a 70-bp region in the 5' UTR just upstream of the start codon, which acts as an internal ribosome entry site, or IRES (Oltean and Banerjee, 2003), the secondary structure of which is influenced by B₁₂. However, electrophoretic mobility shift assays revealed that translational activation in the presence of B₁₂ also requires an auxiliary protein, indicating that it is unlikely to be via a riboswitch (Oltean and Banerjee, 2005).

Recently, a combined proteomic and transcriptomic study identified several B₁₂-responsive genes in addition to *METE* in the diatoms *P. tricornutum* (B₁₂ independent) and *Thalassiosira pseudonana* (B₁₂ dependent; Bertrand et al., 2012). These genes include *CBA1*, which has been suggested to encode a novel cobalamin transporter. However, the means by which B₁₂-mediated gene regulation occurs in algae remains unknown. Certainly, no B₁₂-specific riboswitches have been identified in eukaryotes to date, although thiamine pyrophosphate riboswitches have been characterized in thiamine biosynthesis genes, including in *C. reinhardtii* (Croft et al., 2007). Recently, the thiamine pyrophosphate riboswitch in the *THI4* gene was incorporated into constructs upstream of the nucleus-encoded chloroplast protein2 (*NAC2*) gene encoding a regulator of chloroplast translation. Introduction of the plasmid into *C. reinhardtii nac2* mutants resulted in transformants in which complementation by

the *NAC2* transgene was repressed by thiamine (Ramundo et al., 2013). This construct also included a 1.2-kb region upstream of the *METE* gene, and the authors observed some reduction in *NAC2* complementation by exogenous vitamin B₁₂. However, to understand how B₁₂ regulates *METE* gene expression, a more in-depth study is required. Here, we investigate the effect of B₁₂ on gene expression in different species of algae and define the responsive element of the *C. reinhardtii* *METE* gene, providing valuable information on the likely ecological and environmental context necessary for the gene to be lost. As a result, one can begin to address the question of why B₁₂ auxotrophy is so prominent within the many algal lineages.

RESULTS

Defining the Regulation of *METE* by B₁₂

To understand the regulation of *METE* in *C. reinhardtii*, the time scale and concentration dependence of B₁₂ repression was investigated using reverse transcription (RT)-quantitative real-time PCR (qPCR). The addition of vitamin B₁₂ resulted in a significant reduction of *METE* transcript level within 2 h, with an estimated half-life of about 45 min (Fig. 1A). By varying the concentration of B₁₂, as little as 50 ng L⁻¹ could reduce the transcript level to about 30% of that seen in the absence of B₁₂ (Fig. 1B, solid line), although it should be pointed out that even with 1,000 ng L⁻¹ (a concentration that supports the growth of algal B₁₂ auxotrophs; Bertrand et al., 2012; Kazamia et al., 2012), some transcript can still be detected. The same trend was observed for the marine diatom *P. tricornutum* (Fig. 1B, dashed line). To establish if the transcript levels correlated with the *METE* protein, western-blot analysis was carried out using polyclonal antibodies against *C. reinhardtii* *METE* (Schneider et al., 2008). A band of the expected size (approximately 86.5 kD) was detected in protein extracts prepared from *C. reinhardtii* grown without B₁₂ (Fig. 1C). After the addition of vitamin B₁₂ (1,000 ng L⁻¹), the *METE* protein took over 24 h to drop to an undetectable level, with a half-life of approximately 18 h (Fig. 1C), in other words much slower than the transcript (Fig. 1A). Furthermore, over 200 ng L⁻¹ vitamin B₁₂ was required for the *METE* protein to reach an undetectable level (Fig. 1D). Similar lack of correlation between the responses of transcript and protein levels has been observed for environmental samples (Bertrand et al., 2013).

Characterization of the Vitamin B₁₂-Responsive Element of *METE* in *C. reinhardtii*

To identify the B₁₂ regulatory region of *C. reinhardtii* *METE*, we used a reporter gene approach. Regions upstream of the *METE* translational start but downstream of the preceding gene (Fig. 2A) were joined to the dual reporter gene *sh.BLE-GFP* encoding the *Streptoalloteichus hindustanus* zeocin-binding protein (Sh.Ble) fused to GFP

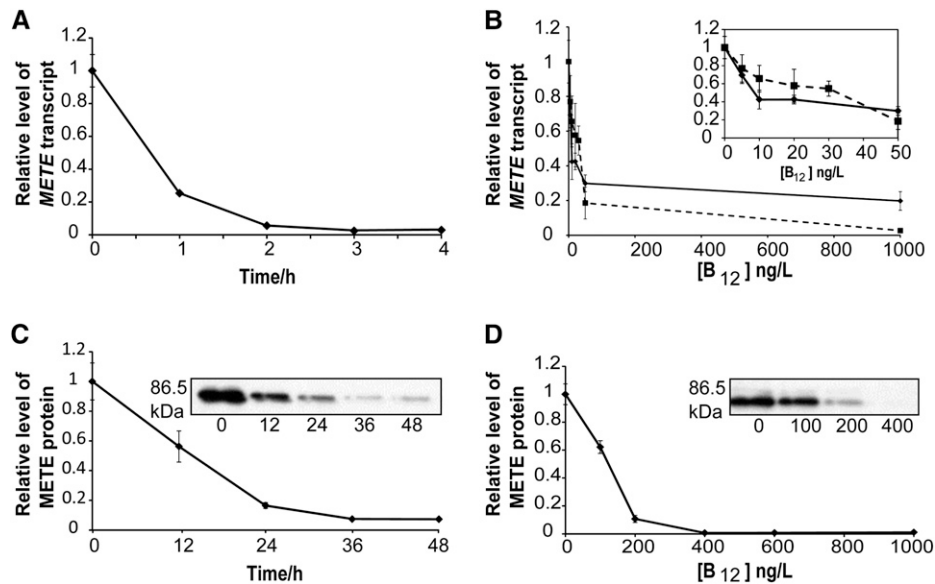


Figure 1. The role of vitamin B₁₂ on *METE* expression. A, RT-qPCR analysis of *METE* expression in *C. reinhardtii* over time after the addition of vitamin B₁₂ (1,000 ng L⁻¹) normalized to expression at time 0 (i.e. prior to B₁₂ addition). Transcript expression was quantified and normalized using *RACK1* as a housekeeping gene (Mus et al., 2007; Dubini et al., 2009). Values are means and error bars denote the range ($n = 2$). B, *METE* transcript abundance after the addition of different concentrations of vitamin B₁₂ for *C. reinhardtii* (as described above; solid line) and the marine diatom *P. tricornutum* (as normalized to *Histone H4*, *30S Ribosomal protein S1*, and *TATA box-binding protein* as housekeeping genes [Siaut et al., 2007]; dashed line). Values are means and error bars denote the range ($n = 2$). C, *METE* protein abundance after the addition of vitamin B₁₂ (1,000 ng L⁻¹); as normalized to the quantity of loaded protein calculated from a Coomassie Brilliant Blue-stained gel. Values are means and SE ($n = 3$). D, *METE* protein abundance after the addition of different concentrations of vitamin B₁₂. Values are means and SE ($n = 3$).

(Supplemental Fig. S1). Plasmid pMS4-1 contains the *METE* upstream region from -574 to the start codon, whereas pMS4-2 contains the same region but with the -89 to +1 region replaced with the 5' UTR of the *C. reinhardtii* *Ribulose Bisphosphate Carboxylase/Oxygenase Small Subunit2* (*RBCS2*) (a control gene that is not repressed by B₁₂). The -89 position was chosen on the basis of EST data from the genome database showing that the longest transcripts extend to this position. This suggests that -89 marks the start of the *METE* 5' UTR, with the promoter element upstream. Plasmid pMS4-3 has the converse arrangement, with the *HSP70A/RBCS2* promoter element fused to the 89-bp *METE* 5' UTR. Finally, pRBCS2 contains both the *HSP70A/RBCS2* promoter and the *RBCS2* 5' UTR, with no *METE* sequence, serving as a negative control in terms of B₁₂-responsiveness. The plasmids were introduced into *C. reinhardtii* cell wall-less mutant15 (cw15), and transformants were selected on the basis of zeocin resistance. Approximately the same number of transformants were obtained for each construct, confirming that the *METE* -574 to -90 upstream sequence was able to drive expression of the reporter gene.

We then tested the responses of several independent transformants for each construct in the absence and presence of B₁₂ (1,000 ng L⁻¹). As expected, growth in zeocin of the lines transformed with the control plasmid pRBCS2 was unaffected by B₁₂ (Fig. 2B). In contrast, the majority of transformants containing pMS4-1 and pMS4-2

were unable to grow in medium containing both zeocin and B₁₂ (Fig. 2, C and D). This indicates that the upstream region of *METE* from -574 to -90 is sufficient for repression by B₁₂. For those transformants that were not responsive, a likely explanation comes from the fact that nuclear transformation in *C. reinhardtii* occurs via random insertion (through nonhomologous end joining; Tam and Lefebvre, 1993), which can result in rearrangements/partial deletion of the transforming DNA. It is possible, therefore, that the *METE* sequence has been lost in some of the recovered transformants, and sh.BLE expression is due to integration downstream of actively expressed endogenous genes. For 15 out of 16 transformants containing pMS4-3 (which has the *METE* 5' UTR only), there was no response to B₁₂ (Fig. 2E), and although in one case there was some growth reduction in zeocin, it was only partial. To assess whether the presence of the -89 to +1 region enhances sensitivity to vitamin B₁₂, we performed a B₁₂ dosage experiment using representative transformants for pMS4-1 and pMS4-2. In both cases, the response was identical, with a complete suppression of growth induced with 100 ng L⁻¹ B₁₂ (Supplemental Fig. S2). Two further constructs were also analyzed in this study: pMS4-4 (-574 to -376) and pMS4-5 (-375 to +1). Lines transformed with these plasmids showed no or little responsiveness to vitamin B₁₂ (Fig. 2, G and F, respectively), indicating that the B₁₂-responsive element has most likely been disrupted in these constructs.

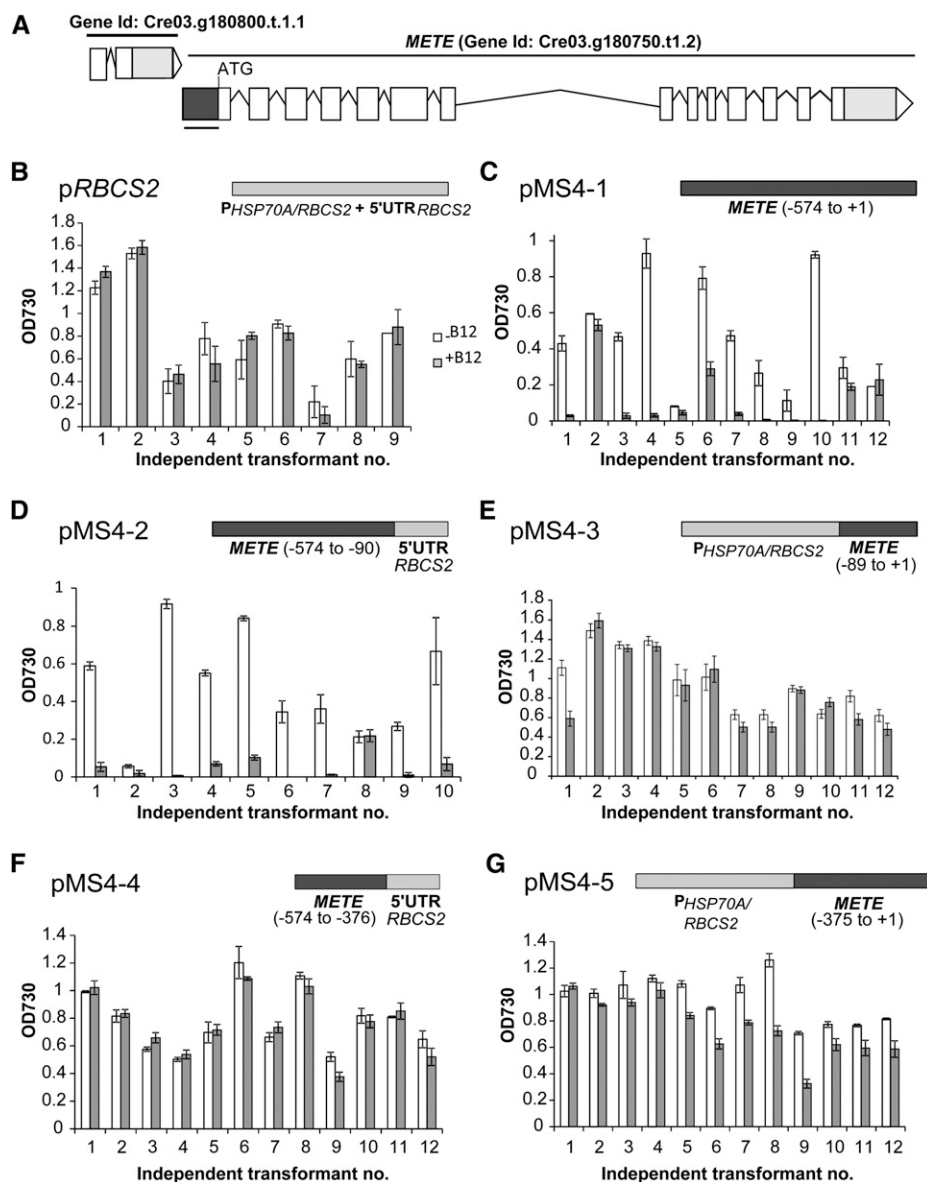


Figure 2. Probing the vitamin B₁₂ regulatory element of *METE* using a reporter gene approach. A, Genome browser view of the *METE* gene model, downstream of the preceding gene (gene identifier Cre03.g180800.t1.1). B to G, Effects of vitamin B₁₂ (1,000 ng L⁻¹) on growth (optical density at 730 nm [OD₇₃₀]) at 96 h in the presence of zeocin (10 μg mL⁻¹) for independent transformant lines containing reporter constructs: pRBCS2 (B), pMS4-1 (C), pMS4-2 (D), pMS4-3 (E), pMS4-4 (F), and pMS4-5 (G). White columns, Without B₁₂ treatment; gray columns, with B₁₂. Error bars denote SE (*n* = 3).

METE Is Not Repressed in Species That Lack METH

Repression of *METE* by vitamin B₁₂ has been shown previously in *C. reinhardtii* and the marine diatom *P. tricornutum* (Helliwell et al., 2011; Bertrand et al., 2012), both of which also encode *METH*. However, there are examples of species that have *METE* only, such as the freshwater green alga *Coccomyxa subellipsoidea* and the thermophilic red alga *Cyanidioschyzon merolae* (Helliwell et al., 2011). In these species, we hypothesized that *METE* would not respond to B₁₂ in the same way as in *C. reinhardtii*, because repression by vitamin B₁₂ would leave the cells devoid of Met synthase activity altogether. Comparison of the *METE* amino acid sequence of *C. subellipsoidea*, *C. merolae*, and *P. tricornutum* with the *C. reinhardtii* *METE* antibody-binding site reveals sequence conservation between species (Fig. 3A). Therefore,

we tested the *C. reinhardtii* *METE* antibody (Schneider et al., 2008) on protein samples extracted from these species. The predicted molecular mass of *METE* from *C. subellipsoidea* is 82 kD, and a band slightly smaller than that of *C. reinhardtii* *METE* (predicted size of 86.5 kD) was detected for samples extracted from *C. subellipsoidea* (Fig. 3B). Given that *METE* was present in both the sample extracted from cells grown with 10 μg L⁻¹ B₁₂ and those grown without (Fig. 3B) and at a similar level (Fig. 3C), we conclude that *METE* is not repressed by B₁₂ in *C. subellipsoidea*. No bands were detectable on western blots of protein extracts from *C. merolae* or *P. tricornutum*, indicating that these proteins are not recognized by the antibody, probably on account of decreased conservation in the region of the antibody-binding site (Fig. 3A). Using an RT-PCR approach, however, we did detect a *METE*

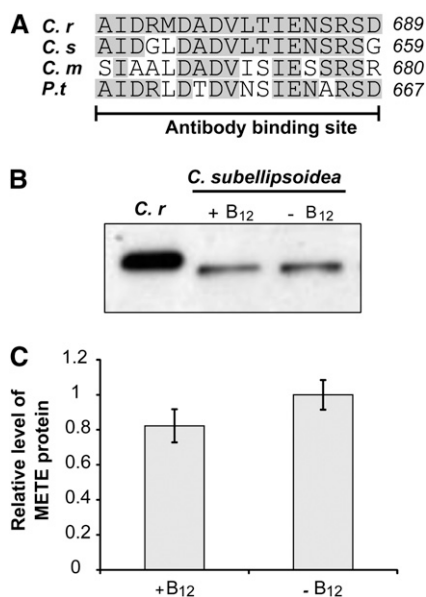


Figure 3. METE is expressed in the presence of vitamin B₁₂ in *C. subellipsoidea*, which lacks *METH*. A, Comparison of the METE amino acid sequences of *C. subellipsoidea* (*C.s*), *C. merolae* (*C.m*), and *P. tricornutum* (*P.t*) with the *C. reinhardtii* (*C.r*) METE antibody-binding site (Schneider et al., 2008). Shared residues are shaded gray. B, METE abundance in *C. subellipsoidea* in the presence (10 $\mu\text{g L}^{-1}$) and absence of vitamin B₁₂ using western blotting with the *C. reinhardtii* METE antibody (Schneider et al., 2008). C, Quantification of METE levels in *C. subellipsoidea* in the presence (10 $\mu\text{g L}^{-1}$) and absence of B₁₂. Values are means and SE ($n = 3$).

transcript with complementary DNA (cDNA) prepared from *C. merolae*, and this was present whether the cells were grown with B₁₂ even at 10 $\mu\text{g L}^{-1}$ (Supplemental Figs. S3 and S4). Thus, in two different algal groups, chlorophytes (*C. subellipsoidea*) and rhodophytes (*C. merolae*), *METE* repression by B₁₂ does not occur when *METH* is absent.

Exploring the Roles of Other Factors Involved in METE Regulation in *C. reinhardtii*

Next, we investigated if other factors regulate *C. reinhardtii* *METE*. Met synthase catalyzes the synthesis of Met from homocysteine and methyl-tetrahydrofolate through the transfer of a methyl group from methyl-tetrahydrofolate (which in turn generates tetrahydrofolate; Fig. 4). This is an essential reaction in C₁ metabolism, and in animals, which have only *METH*, lack of B₁₂ as cofactor causes changes in the balance of intracellular folate derivatives. This ultimately leads to a phenomenon known as folate trapping (Scott and Weir, 1994). In *E. coli*, which has both *METE* and *METH*, Met has a negative regulatory effect on *METE* expression (Milner et al., 1969; Weissbach and Brot, 1991), whereas homocysteine has a positive regulatory role in *E. coli*, *S. typhimurium*, and *Ralstonia solanacearum* (Urbanowski and Stauffer, 1989a, 1989b; Plener et al., 2012). Croft et al. (2005) showed that, in the absence of B₁₂, Met and folate

can support the growth of the B₁₂-dependent green alga *Lobomonas rostrata* when supplemented at concentrations of 10 mM and 1 μM , respectively. To determine if these metabolites have an effect on *METE* protein abundance in *C. reinhardtii*, western-blot analysis was carried out on samples extracted from cultures supplemented with different concentrations of Met and folate. No changes were observed with either Met or folate (Fig. 5A). To assess the effect of homocysteine, we included this metabolite at 100 $\mu\text{g mL}^{-1}$ (0.74 mM) to *C. reinhardtii* cultures grown under three different B₁₂ treatments: no B₁₂, 100 ng L⁻¹ B₁₂, and 1,000 ng L⁻¹ B₁₂. Again, no effect on *METE* protein content was observed by western-blot analysis (Fig. 5B). Therefore, based on these data, we conclude that, unlike in *E. coli*, homocysteine, Met, and folate have no role in the regulation of *METE* in *C. reinhardtii*.

Proteomics Analysis of the Effect of Vitamin B₁₂ on Algal Metabolism

Having defined more clearly the effect of B₁₂ on *METE* expression and investigated the role of other factors, we took a proteomics approach to examine the effect of B₁₂ on *C. reinhardtii* metabolism more generally. *C. reinhardtii* was grown in either the absence or presence of B₁₂, at 1,000 ng L⁻¹ to ensure complete repression of *METE*. Proteins were extracted from cultures grown to exponential phase ($n = 4$) and analyzed by two-dimensional difference gel electrophoresis (2D-DIGE; Unlü et al., 1997; Lilley and Friedman, 2004). The eight individual samples were each labeled with Cy5, and then mixed with a Cy3-labeled standard made of equal amounts of each sample. The Cy3/Cy5 mix was separated by two-dimensional gel electrophoresis, followed by silver staining. A total of 1,190 spots were found to be present in at least 12 of the 16 spot maps, and these were analyzed by comparison of the Cy3 and Cy5 intensities. Of these, just 11 spots (0.9%) demonstrated differential abundance of at least 2-fold. Strikingly, all are from proteins that are down-regulated in the presence of vitamin B₁₂ ($P \leq 0.01$; Supplemental Fig. S5; Supplemental Tables S1 and S2).

From these 11 hits, there were three groups of three adjacent spots that have the same molecular masses (Supplemental Fig. S5), implying that they are different isoforms of the same protein. One spot from each of these groups was excised from the gels and identified by tandem mass spectrometry (MS/MS; details of spot identification are given in Supplemental Table S1). The two larger groups of three spots were identified as *METE*. When corrected for the different abundance of each putative isoform, this protein is down-regulated 5.9-fold overall. A possible modification of *METE*, which might affect mobility leading to multiple spots, is phosphorylation, since this has been predicted previously for *C. reinhardtii* (Kurvari et al., 1995; Schneider et al., 2008), and *METE* from tobacco (*Nicotiana tabacum*) appeared as seven isoforms on a two-dimensional gel (Moscatelli et al., 2005). The third group of adjacent spots,

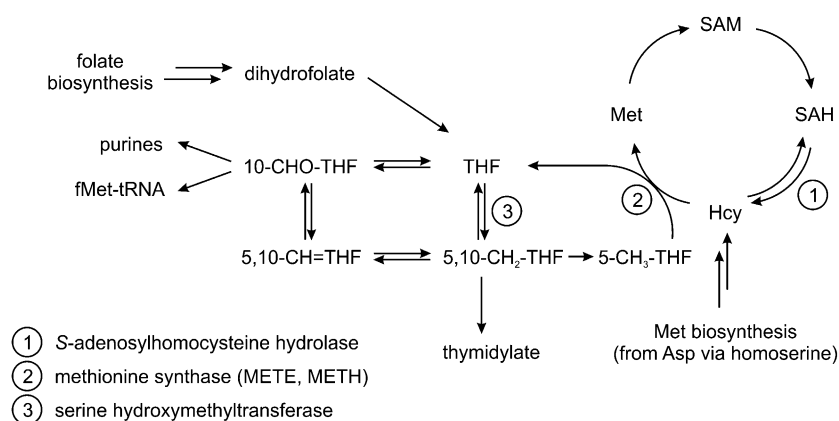


Figure 4. Simplified scheme of C₁ metabolism. 10-CHO-THF, 10-Formyl-tetrahydrofolate; 5,10-CH⁺-THF, 5,10-methenyl-tetrahydrofolate; 5,10-CH₂-THF, 5,10-methylene-tetrahydrofolate; 5-CH₃-THF, 5-methyl-tetrahydrofolate; Hcy, homocysteine; SAH, S-adenosylhomocysteine.

down-regulated an average of 2.5-fold, was serine hydroxymethyltransferase2 (SHMT2), but MS/MS data did not give any information on the nature of these isoforms. Of the two individual spots, one, down-regulated 2.43-fold, was S-adenosylhomocysteine hydrolase (SAHH), while the last (marked with an asterisk in Supplemental Fig. S5) could not be identified. Interestingly, all three identified proteins are neighbors in the metabolic network of C₁ compounds (Fig. 4): SAHH is part of the activated methyl cycle and catalyzes the hydrolysis of S-adenosylhomocysteine to yield homocysteine, the substrate of Met synthase, while SHMT catalyzes the reversible transfer of a C₁ group from Ser to tetrahydrofolate. RT-qPCR showed that expression ratios for *METE*, *SAHH*, and *SHMT2* are similar at the transcript level compared with the protein level (Fig. 6; Supplemental Table S3), thus validating the 2D-DIGE approach. Similarly, B₁₂ was confirmed to not affect transcript abundance of *METH*, as evidenced previously (Croft et al., 2005).

Having identified two further genes that respond to vitamin B₁₂ in *C. reinhardtii*, we used the bioinformatics tool MEME (Multiple EM for Motif Elicitation; Bailey and Elkan, 1994) to search for common sequence motifs shared between the 1-kb upstream regions of the *SAHH* and *SHMT2* genes and the *METE* (-574 to -90) regulatory element. The *METH* upstream region was used as a negative control sequence (Bailey and Elkan, 1994). This approach identified one common sequence motif, CTGCGCTG (E-value of 1.6E + 005), present in the *SAHH*, *SHMT2*, and *METE* sequences. However, since the reporter construct pMS4-4 (-574 to -376) contains this motif, and regulation by B₁₂ in this construct is impaired, this motif alone is not sufficient for B₁₂-mediated transcriptional regulation. Understanding exactly how these genes are regulated in the presence of B₁₂, and whether the mode by which this occurs is conserved, remains an exciting prospect.

DISCUSSION

Our study of vitamin B₁₂-mediated regulation of gene expression in algae has provided important insights into the mechanism of this process and its biological

consequences. We have defined in detail the response of the *C. reinhardtii* *METE* gene to vitamin B₁₂ and shown that it is not sensitive to other C₁ metabolites (folate, Met, or DL-homocysteine), and we also found two other targets of B₁₂ repression, SAHH and SHMT, which are both involved in the C₁ cycle. In addition, we have successfully isolated a B₁₂-responsive element located upstream of the transcription start of *METE*, which is sufficient to control the expression of a reporter construct in

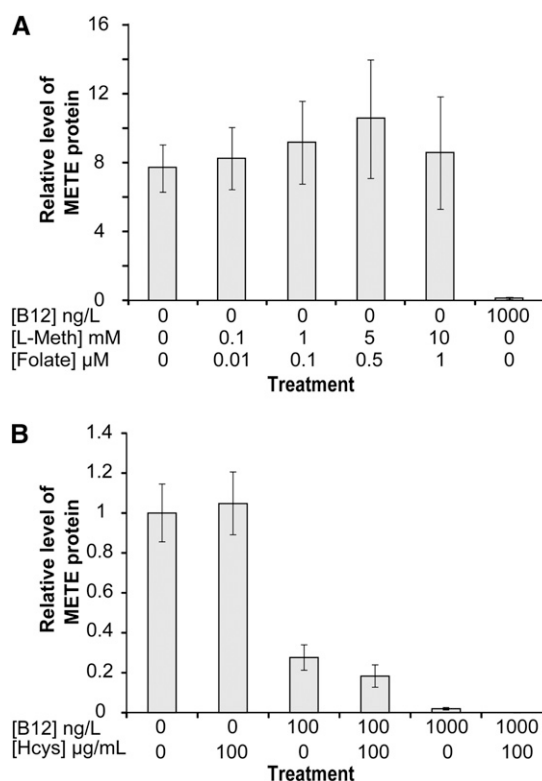


Figure 5. Effects of C₁ metabolites on METE protein abundance. A, METE protein in response to different concentrations of L-Met (L-Meth) and folate (48 h). B, METE protein in response to DL-homocysteine (Hcys) in different B₁₂ treatments (48 h). Values are means and SE (n = 3).

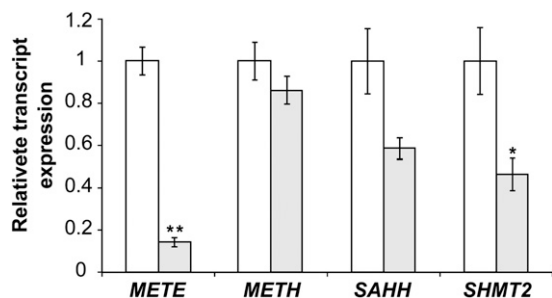


Figure 6. Investigating other B₁₂-responsive genes in *C. reinhardtii*. Transcript abundance is shown when *C. reinhardtii* is grown in the presence or absence of vitamin B₁₂ (1,000 ng L⁻¹). Transcripts were quantified using RT-qPCR with *RACK1* as the reference gene (Mus et al., 2007; Dubini et al., 2009). For each gene, the result was normalized by expression in the absence of B₁₂. **P* ≤ 0.05, ***P* ≤ 0.001 compared with expression in the absence of B₁₂ (two-tailed Student's *t* test). Values are means and se (*n* = 3).

transformed lines of *C. reinhardtii*, with the same responsiveness to exogenous B₁₂ as the endogenous *METE* gene. Since the *METE* 5' UTR does not confer this responsiveness, it is unlikely that *METE* is regulated by a B₁₂ riboswitch as it is in bacteria (Nahvi et al., 2004; Warner et al., 2007) or by an IRES as in mammals (Oltean and Banerjee, 2003, 2005).

The tight repression of *METE* exhibited in *C. reinhardtii* is also observed in the unrelated diatom *P. tricornutum*, which also possesses *METH* (Croft et al., 2005; Helliwell et al., 2011; Bertrand et al., 2012). This implies that there is a strong cellular advantage to having the gene switched off when possible (i.e. in the presence of B₁₂). In *E. coli*, MetH has a more than 100-fold higher turnover number than MetE (González et al., 1992), and in the absence of the vitamin, MetE is the second most abundant protein (Karlin et al., 2001), presumably to allow efficient C₁ metabolism. Intriguingly, a recent study of thermal tolerance in *C. reinhardtii* found that nonlethal heat shock treatment resulted in the repression of *METE* gene transcription (Xie et al., 2013), and cells could only survive if supplied with B₁₂, presumably to allow *METH* to function. Reduction in *METH* levels using artificial microRNA (amiRNA) suppressed thermal tolerance. This suggests that having both isoforms of Met synthase provides a degree of environmental flexibility. On the other hand, if there is a reliable and abundant external source of B₁₂, and these conditions prevail over a long period of time, the gene may be lost entirely, which is the derived state of many algal species spanning multiple diverse phyla (Croft et al., 2005; Helliwell et al., 2011). However, natural levels of B₁₂ are reportedly less than 10 ng L⁻¹ (10 pM) in freshwater (Kurata, 1986) and perhaps up to 70 ng L⁻¹ in seawater (Carlucci, 1970; Gobler et al., 2007), with more recent measurements indicating a lower range in the photic zone of the Southern Ocean and North Atlantic Ocean (Panzeca et al., 2009; Sañudo-Wilhelmy et al., 2012). The extracellular concentration of B₁₂ necessary to repress the *METE* transcript (Fig. 1B) far exceeds the amount of free

cobalamin estimated to be present in characterized freshwater and certain marine ecosystems. How, then, has B₁₂ auxotrophy become so common in the algal lineages? There are several examples where algae obtain vitamin B₁₂ via a symbiotic relationship with bacteria (Croft et al., 2005; Wagner-Döbler et al., 2010; Kazamia et al., 2012). A plausible explanation, therefore, is that provision of B₁₂ by associated bacteria could lead to *METE* loss, which, in turn, would bind the organisms further together. Indeed, this mechanism may have played a role in shaping the evolution of vitamin auxotrophy more generally (Helliwell et al., 2013).

In this regard, it is also relevant that neither Met, nor homocysteine influences *METE* expression in *C. reinhardtii*, which is in contrast to several bacterial species studied (Urbanowski and Stauffer, 1989a, 1989b; Weissbach and Brot, 1991; Plener et al., 2012). Since, in the absence of B₁₂, the growth of the B₁₂-dependent alga *L. rostrata*, a close relative of *C. reinhardtii*, can be supplemented by Met and folate (at concentrations of 10 mM and 1 μM, respectively), it seems plausible that these compounds do enter the *C. reinhardtii* cell. Therefore, our data suggest that exogenous levels of vitamin B₁₂ are likely to be the primary factor governing the loss of *METE*. In contrast to *C. reinhardtii* and *P. tricornutum*, in species that lack *METH* (i.e. *C. subellipsoidea* and *C. merolae*; Fig. 3), vitamin B₁₂ does not affect the expression of *METE*. This suggests that there must have been selection for an alteration in how *METE* is regulated, either prior to or shortly after the loss of *METH* in these species. It is noteworthy that *C. merolae*, a thermophilic red alga, has *METE* but not *METH*, since in *C. reinhardtii*, the latter enzyme has a higher thermotolerance compared with *METE* (Xie et al., 2013). In the hot sulfurous springs in which *C. merolae* lives, temperatures can exceed 45°C, with pH being as low as 1.5 (Matsuzaki et al., 2004). It is quite likely, therefore, that B₁₂ degrades under such conditions and that *C. merolae* does not encounter vitamin B₁₂ in its environment at all, which could explain its loss of *METH* in this species. Presumably, selection on *METE* to enhance thermotolerance must also have occurred. As an aside to this, the fact that *METE* is not repressed by B₁₂ in all algae must also be considered when utilizing *METE* as a biomarker for evaluating the B₁₂ status of microbial communities in the open field as has been proposed (Bertrand et al., 2013).

Besides probing *METE* regulation, we also identified further genes that are responsive to vitamin B₁₂ in *C. reinhardtii*, using a 2D-DIGE proteomics approach. The most pronounced regulation was found for *METE*, which was 7-fold more abundant in the absence of B₁₂ (Supplemental Table S3). Two other proteins were identified as down-regulated by B₁₂, SAHH and SHMT2, which catalyze adjacent reactions in C₁ metabolism (Fig. 4). The SHMT protein was also observed to increase in the marine diatom *P. tricornutum* in response to B₁₂ depletion (Bertrand et al., 2012), but to our knowledge this is the first report of SAHH being responsive to vitamin B₁₂. Raised levels of these two enzymes are likely to increase the flux of metabolites

toward Met synthase (Fig. 4; Supplemental Fig. S5). Therefore, the probable purpose of this regulation pattern is to ensure sufficient Met and S-adenosylmethionine levels in the cytosol when the less efficient METE protein has to operate. The strikingly few changes in global gene expression we detected illustrate how *C. reinhardtii* subtly adjusts its metabolism to switch from a nonutilizing to a B₁₂-utilizing organism.

Our findings present a significant contribution to the understanding of B₁₂-specific gene regulation and metabolism in algae. Additionally, this work has unexpected applications for algal biotechnology. Inducible gene expression systems are highly desirable for the controlled expression of genes of interest. However, existing platforms are generally regulated by cues that are toxic to the cell and/or physiologically intrusive, such as the upstream elements of the *Nitrate Reductase*, *Cytochrome C6*, and *Carbonic Anhydrase1* genes (Schroda, 2006), which are sensitive to nitrate limitation, toxic metals (including copper, cobalt, and nickel), and CO₂, respectively. Given the minimal effect of vitamin B₁₂ on global protein expression in *C. reinhardtii*, the novel B₁₂ regulatory system we have identified is likely to have many fewer indirect metabolic effects. Moreover, the micronutrient quantities (as low as 200 ng L⁻¹) sufficient for the complete repression of transgene expression enables cost-effective scale up. For example, just 0.2 g of vitamin B₁₂ would be sufficient to regulate gene expression in 10⁶ L of culture. The combined assets of high sensitivity and metabolic specificity make this an apt gene expression tool for industrial purposes.

MATERIALS AND METHODS

Strains and Growth Conditions

Escherichia coli DH5 α was grown in lysogeny broth medium and used in all cloning steps. Details of source and growth conditions for *Chlamydomonas reinhardtii* strains 12 and cw15 (cw15, mt⁻), *Coccomyxa subellipsoidea* C-169, *Phaeodactylum tricornutum* CCAP 1052/1B, and *Cyanidioschyzon merolae* M10 are provided in Supplemental Table S4.

RNA Extraction and RT-qPCR

Total RNA was extracted as described by Helliwell et al. (2011) and subsequently treated with the Ambion Turbo DNase-Free Kit to remove genomic DNA. RNA was reverse transcribed into cDNA with SuperScript II (Invitrogen). Details of the primers and specific amplification conditions used for RT-qPCR are given in Supplemental Table S5. For each gene, primers were designed for different exons so that genomic DNA contamination could be assessed. For *P. tricornutum*, RT-qPCR was carried using Qiagen SYBR Green MasterMix using 4 ng of cDNA and 0.1 μ M of each primer per reaction. Unique single products were verified by melt-curve analysis. No amplification was detected in no reverse transcriptase control samples, confirming the absence of genomic DNA. Cycle threshold values of two technical replicates were calculated using Rotor-Gene Q Software version 2.02, and primer efficiency was calculated with LinRegPCR (Ramakers et al., 2003). The expression of each gene was calculated by raising the efficiency of each primer pair to the negative cycle threshold value and then normalizing this by dividing by the mean expression of reference genes (*Histone H4*, *30S Ribosomal protein S1*, and *TATA box-binding protein*). Representative RT-qPCR samples were additionally checked by agarose gel electrophoresis. Similar protocols were followed for *C. reinhardtii*, except that the SYBR Green JumpStart Taq ReadyMix kit (Sigma

no. S5193) was used, with 3.3 ng of cDNA from 3.3 ng of RNA and 400 nM of each primer. The expression of each gene was calculated by raising the efficiency of the different primer pairs to the negative cycle threshold value and then normalizing this to *Receptor for Activated C Protein Kinase1* as the reference gene. For *C. merolae*, PCR was used to amplify sequences from cDNA using Taq DNA polymerase (Bioline). Reaction mixtures contained 125 nM of each primer, 2 mM MgCl₂, and 5% (v/v) dimethyl sulfoxide. Amplification conditions on a TC-5₁₂ gradient instrument (Techne) were as follows: 94°C for 5 min, cycles of 94°C for 45 s, 63°C for 45 s, and 72°C for 2 min, and 72°C for 10 min. Cycle number was optimized (Supplemental Figure S4), and 35 and 23 cycles were chosen for *METE* and *Elongation factor1a*, respectively.

Western Blotting

Cells from 50 mL of midexponential phase culture were harvested by centrifugation (600g, 5 min), resuspended in 150 μ L of extraction buffer (97.1 mM potassium phosphate buffer, pH 7.5, 5 mM dithiothreitol [DTT], and 2 mM EDTA, pH 8), and lysed using sonication (Biorupter; Diagenode). Protein concentration was quantified using a Bradford assay (Bradford, 1976), and 4 μ g of protein was separated by SDS-PAGE on 12% (w/v) gels with a 5% (w/v) stacking gel, blotted onto a nitrocellulose membrane (Protran BA 83; Whatman), and labeled with METE antibodies (Schneider et al., 2008). Band intensity (integrated density) was quantified using ImageJ (Schneider et al., 2012), and relative METE levels were calculated (normalized to the quantity of loaded protein calculated from a Coomassie Brilliant Blue-stained gel).

Plasmid Construction

Plasmids were generated using Gibson assembly (Gibson et al., 2009). PCR was carried out using the iProof DNA polymerase (Bio-Rad) plus 3% (v/v) dimethyl sulfoxide with the primers listed in Supplemental Table S6. Transformation into *E. coli*, isolation of positive clones, and sequence confirmation were according to standard protocols.

Genetic Manipulation of *C. reinhardtii* and Reporter Gene Growth Assays

Transformation of a cell wall-less strain of *C. reinhardtii* (cw15, mt⁻) was carried out by electroporation via a procedure modified from Shimogawara et al. (1998). Briefly, 2-d-old cultures (1 \times 10⁶ cells mL⁻¹ to 5 \times 10⁶ cells mL⁻¹) were harvested via centrifugation (at 1,500g) and resuspended in Tris-acetate phosphate (TAP) medium + 60 mM Suc. Concentrated cells (300 μ L) were combined with 3 μ g of linear plasmid DNA per transformation in a chilled 0.4-mm-gap cuvette. Electroporation was performed with a Bio-Rad Gene Pulser Xcell set to exponential decay protocol, 800-V field strength, 25 μ F, and no shunt resistance. A typical pulse time of 9.8 to 15 ms was obtained. Cells were subsequently transferred to 10 mL of TAP + 60 mM Suc and incubated at 20°C in low light for 16 to 18 h. For selection, cells were spread on TAP 1.5% (w/v) agar plates containing 10 μ g mL⁻¹ zeocin. Colonies appeared after 5 d and were further processed after 7 d.

To assess the effect of vitamin B₁₂ on the various upstream sequences controlling the *BLE* reporter gene, growth assays were used to test resistance to zeocin in the presence of B₁₂. For each transformed line of *C. reinhardtii*, three replicate precultures were set up on 24-well plates on nonselective TAP medium (without zeocin) either with B₁₂ (1,000 ng L⁻¹) or without. After 72 h, 15 μ L of these precultures was inoculated into 50 mL of the appropriate medium for the plus or minus B₁₂ condition, this time with 10 μ g mL⁻¹ zeocin. Growth was then monitored over time by measuring the optical density at 730 nm.

2D-DIGE

Proteins were extracted from frozen cell pellets of *C. reinhardtii* cw15 by a procedure modified from Gillet et al. (2006). Cells from 50 mL of culture, grown either with B₁₂ (1 μ g L⁻¹) or with no addition, were resuspended in 500 μ L of 50 mM Tris-Cl, pH 8.0, and 0.07% (w/v) DTT containing protease inhibitor cocktail (Roche no. 11697498001) and lysed by three cycles of freezing in liquid nitrogen and thawing at room temperature. After the removal of debris via centrifugation (45 min, 15,000g, 4°C), nucleic acids were removed by the addition of 2% (w/v) streptomycin sulfate and centrifugation for 45 min at 60,000g, 4°C. Proteins were precipitated with 10% (w/v) TCA and 80% (v/v) acetone at -20°C for 24 h and centrifuged (15 min, 17,000g, 4°C), washed once with acetone and once with 80% (v/v) acetone, dried in a SpeedVac centrifuge, and dissolved in 200 μ L of 10 mM

Tris-Cl, pH 8.0, 5 mM magnesium acetate, 8 M urea, and 2% (w/v) amidosulfobetaine-14. Protein concentration was determined with the DC Protein Assay kit (Bio-Rad) using bovine serum albumin as a standard.

For each of eight samples (four samples per condition), 50 μ g of protein was labeled with 18 μ M Cy5 dye (GE Healthcare no. RPK0275) and a standard, made of equal amounts of each sample, was labeled with Cy3 (no. RPK0273), according to the manufacturer's instructions (for 30 min at 4°C). After quenching with 0.8 mM Lys for 10 min, individual Cy5-labeled samples were combined with the same amount of Cy3-labeled standard, and 1 volume of 4% (w/v) CHAPS, 8 M urea, 2% (w/v) DTT, and 2% (w/v) IPG Buffer pH 4-7 (GE Healthcare no. 17-6000-86) was added and the solution was incubated for 15 min at 4°C. Another 8 volumes of 4% (w/v) CHAPS, 8 M urea, 0.2% (w/v) DTT, and 1% (v/v) IPG Buffer/pharmalytes was added before isoelectric focusing was performed using a 13-cm Immobiline DryStrip pH 4-7 (GE Healthcare no. 17-6001-13). Proteins were separated on an Ettan IPGphor isoelectric focusing unit for 10 h at 20 V, 1 h at 500 V, 1 h at 1,000 V, and 5 h at 8,000 V and subsequently in the second dimension by SDS-PAGE using 12% (v/v) polyacrylamide. Cy3 and Cy5 spot maps were generated by scanning the gel with a Typhoon 9400 imager (GE Healthcare). Eight two-dimensional gels were run in total, loaded with equal amounts of sample and standard. Matching, quantification, and evaluation of spots were carried out using the software package DeCyder 5.02 (GE Healthcare). To calculate overall expression ratios for the proteins with several putative isoforms, the ratios of the individual isoforms were weighted by the average intensity of the Cy3-labeled standard spot.

For spot identification, gels were silver stained according to Carpentier et al. (2005). Excised gel pieces were destained with 30 mM $K_3Fe(CN)_6/100$ mM $Na_2S_2O_3$, washed with water, and dried under vacuum. Gel pieces were incubated with 10 mM DTT in 50 mM NH_4HCO_3 for 1 h at 56°C, then with 5 mM iodoacetamide in 50 mM NH_4HCO_3 at room temperature in the dark for 45 min, before they were washed with 50 mM NH_4HCO_3 in 50% (v/v) CH_3CN and dried under vacuum. Protein was digested with 12.5 ng μL^{-1} trypsin in 50 mM NH_4HCO_3 at 37°C overnight, and peptides were extracted with 50% (v/v) CH_3CN in 5% (v/v) formic acid.

Liquid chromatography-MS/MS experiments were performed using the NanoLC-1D Plus (Eksigent Technologies) HPLC system and an LTQ Orbitrap mass spectrometer (ThermoFisher). Separation of peptides was performed by reverse-phase chromatography at a flow rate of 300 nL min^{-1} and an LC-Packings (Dionex) PepMap 100 column (C18; 75 μ m i.d. \times 150 mm, 3- μ m particle size). Peptides were loaded onto a precolumn (Dionex Acclaim PepMap 100 C18; 5- μ m particle size, 100 A, 300 μ m i.d. \times 5 mm) from the autosampler with 0.1% (v/v) formic acid for 5 min at a flow rate of 10 μ L min^{-1} . After this period, the 10-port valve was switched to allow elution of peptides from the precolumn onto the analytical column. Solvent A was water + 0.1% (v/v) formic acid and solvent B was acetonitrile + 0.1% (w/v) formic acid. The gradient employed was 5% to 50% (v/v) B in 40 min. The liquid chromatography eluent was sprayed into the mass spectrometer by means of a New Objective nano-spray source. All mass-to-charge ratio values of eluting ions were measured in the Orbitrap mass analyzer, set at a resolution of 7,500. Peptide ions with charge states of 2+ and 3+ were then isolated and fragmented in the LTQ linear ion trap by collision-induced dissociation, and MS/MS spectra were acquired. Postrun, the data were processed using Bioworks Browser (version 3.3.1 SP1; ThermoFisher) and submitted to the Mascot Daemon 2.2.0 search algorithm (Matrix Science; Perkins et al., 1999) to search against the proteins predicted from assembly version 4.0 of the *C. reinhardtii* genome sequence (Merchant et al., 2007), using a fixed modification of carbamidomethylation and a variable modification of oxidation. A protein was considered identified if the highest Mascot score was above the background composed of the remaining hits (Supplemental Table S1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Schematic diagram of the reporter constructs used in this study.

Supplemental Figure S2. B_{12} dose response of pMS4-1 and pMS4-2 to different concentrations of vitamin B_{12} .

Supplemental Figure S3. RT-PCR reveals that *METE* expression is not repressed by B_{12} in the thermophilic red alga *C. merolae*.

Supplemental Figure S4. RT-PCR cycle number optimization for *METE* and *Elongation factor1a* expression in *C. merolae*.

Supplemental Figure S5. Investigating B_{12} -responsive genes in *C. reinhardtii* by 2D-DIGE.

Supplemental Table S1. Identification of proteins from 2D-DIGE.

Supplemental Table S2. Expression ratio of identified spots from 2D-DIGE.

Supplemental Table S3. Characteristics of proteins involved in C_1 metabolism in *C. reinhardtii* down-regulated by B_{12} .

Supplemental Table S4. Strains, origins, and growth conditions for each alga grown during this study.

Supplemental Table S5. Primers and conditions used for RT-PCR.

Supplemental Table S6. PCR primers employed for the amplification of DNA fragments for reporter construct generation.

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