Helliwell et al. Supplementary Materials

RUNNING HEADER: ALGAE AND VITAMIN B_{12}

Corresponding author:

Alison G. Smith

Department of Plant Sciences,

University of Cambridge,

Downing Street, Cambridge,

CB2 3EA, UK.

Email: as25@cam.ac.uk, tel.: +44 1223 333952

RESEARCH AREA: BIOCHEMISTRY AND METABOLISM

Table S1. Identification of proteins from 2-D DIGE

For each spot, the five Mascot hits with the highest score and at least two unique peptides of rank 1 are listed. For the number and identity of unique peptides, only peptides of rank 1 are considered. Coverage indicates the fraction of the predicted protein that is covered by the identified peptides. Oxidised Met residues are underlined.

Spot no.	Protein ID	Total score	Coverage	No. of unique peptides	Unique peptides
448	154307 (METE)	1505	32%	24	VASAYTR, HLSGLQR, EGLPLKR, LGAGVVDGR, GGAALDMSK, VEEIVSVAR, GAALGAAAGTSR, FAMESYFK, NMVEAAAQAR, FKHLSGLQR, AGWVQSYGSR, GWPETIAALR, SFVDSGILSGR, VQSDAWALQK, ALLDTGAVSSDR, AAQAMQLGLALR, QKVEEIVSVAR, MDADVLTIENSR, GDSGEAELLAVAHK, SDNAMMAALAAAGYGR, TPEAEPHLPPALAAR, AVPILIGPVTFVSLSR, LAASPAAAAAAAGHGAVVQLQR, AELQLAGGAAVAPVAGGVEAAGK
	144070	104		2	
	348055	88		2	
	345759	80		2	
	133859	70		2	
455	154307 (METE)	1273	30%	20	VASAYTR, HLSGLQR, EGLPLKR, LGAGVVDGR, GGAALDMSK, GAALGAAAGTSR, NMVEAAAQAR, AGWVQSYGSR, SFVDSGILSGR, VQSDAWALQK, ALLDTGAVSSDR, AAQAMQLGLALR, QKVEEIVSVAR, MDADVLTIENSR, SVWKDDGTAVALLR, TPEAEPHLPPALAAR, SDNAMMAALAAAGYGR, LAASPAAAAAAAGHGAVVQLQR, AELQLAGGAAVAPVAGGVEAAGK, DIGPGVYDVHSPVVPSVEFIK
	158948	64		2	
	60432	62		2	
	144131	53		2	
	406827	50		2	
456	424119	130	2.0%	3	
	406498	95		2	
	133859	94		2	

	122088	72		2	
	206241	67		2	
976	129593 (SAHH)	326		7	IVLGLIR, VAALHLPK, ATDVMIAGK, DSAAVFAWK, HSLPDGIMR, VMGVSEETTTGVK, DGTLPNPDSTDNAEFK
	400024	51	14%	2	
	189186	50		2	
	340070	49		2	
	286417	46		2	
995	196354 (SHMT2)	588	23%	9	IGTPAMTSR, YSEGQPGAR, YVATPEFR, GLEGNPAIADIR, YADIVTTTTHK, QYSEQVVHNCR, NAVVGDLSAMNPGGVR, ISATSIFFESLPYK, LAVADPEVFALIEDEK
	60432	130		3	
	133859	105		2	
	172839	74		2	
	423338	67		3	

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

The values are the ratio of expression in *C. reinhardtii* cells grown without B_{12} compared to 1000 ng/L vitamin B_{12} for those spots altered by at least two-fold ($P \leq 0.01$) (shown on Figure S5).

Spot no.	Appearance (no.of spot maps)	Identity[1]	Expression ratio	*q*-value[2]	*P*-value (t-test)
432	16	METE?	3.63	0.058	$4.4 \cdot 10^{-4}$
438	14	METE?	4.84	0.036	$1.2 \cdot 10^{-4}$
448	16	METE	8.11	0.012	$2.1 \cdot 10^{-5}$
455	16	METE	6.15	0.036	$1.5 \cdot 10^{-4}$
456	16	METE?	4.45	0.101	$1.7 \cdot 10^{-3}$
457	16	METE	2.66	0.072	$6.3 \cdot 10^{-4}$
976	16	SAHH	2.43	0.036	$1.5 \cdot 10^{-4}$
995	16	SHMT2	2.70	0.073	$7.4 \cdot 10^{-4}$
1002	16	SHMT2	2.12	0.072	$6.7 \cdot 10^{-4}$
1006	16	SHMT2	2.16	0.099	$1.4 \cdot 10^{-3}$
1010	14	unknown	2.53	0.089	$9.7 \cdot 10^{-4}$

[1] Identity of those indicated with ? inferred from equivalent mobility of spots assigned by MS/MS.

[2] Gillet S, Decottignies P, Chardonnet S, Le Maréchal P (2006) Cadmium response and redoxin targets in *Chlamydomonas reinhardtii*: a proteomic approach. *Photosyn Res* **89**: 201-211.

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

Protein	Protein identifier	Subunit molecular mass (kDa)	pI	Expression ratio		Evidence for multiple isoforms
				2D-DIGE	RT-qPCR	
METE	154307	86.8	5.94	5.9 [1)]	7.0	√
METH	(76715) [3)]				no significant difference	
SAHH	129593	52.7	5.39	2.4	(1.7) [2)]	
SHMT1	194461	54.8 [3)]	8.65 [3)]			
SHMT2	196354	51.9	6.25	2.5 [1)]	2.2	√
SHMT3	196400	49.0 [3)]	8.14 [3)]			

[1)] Average values, assuming that METE migrated to six adjacent spots, and SHMT2 to three adjacent spots (see Figure S5)

[2)] The statistical *P*-value exceeds 0.05

[3)] The values are given for the mature protein, after removal of putative mitochondrial target peptide predicted by TargetP

Table S4. **Strains, origin, and growth conditions for each alga grown during this study**

Species	Origin	Type of Growth Media	Reference	Conditions
Chlamydomonas reinhardtii strain 12[1], a wild-type strain derived from strain 137c	*Chlamydomonas* Culture Collection	TAP	2	25 °C, 140 rpm under continuous light (100-150 μE m^{-2} s^{-1})
C. reinhardtii cell-wall-deficient-strain (cw15, mt^-)				
Phaeodactylum tricornutum (CCAP 1052/1B)	Culture Collection of Algae and Protozoa (CCAP)	f/2	3,4	15°C, 16:8 hours light:dark cycle
Coccomyxa subellipsoidea (C-169)	A gift from Dr James Van Etten (Department of Plant Pathology, University of Nebraska-Lincoln).	Bold's Basal Media (BBM)	5,6	25 °C, 140 rpm under continuous light (100-150 μE m^{-2} s^{-1})
Cyanidioschyzon merolae (M10)	A gift from Kan Tanaka (Tokyo Institute of Technology, Japan)	Double Strength Modified Allen (MA2)	7	37 °C , with shaking (~110 revs/ minute)

[1]Croft MT, Lawrence AD, Raux-Deery E., Warren, MJ, Smith AG. (2005) Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. *Nature* **438**:90-93.

[2]Rochaix J-D, Mayfield S, Goldschmidt-Clermont M, Erickson J (1988) Molecular Biology of *Chlamydomonas*. *Plant Molecular Biology - a Practical Approach*, ed Shaw CH (IRL Press, Oxford), pp 253-275.

[3]Guillard RRL, and Ryther JH (1962) Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea Cleve. (Can. J. Microbiol), pp 229-239.

[4]Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. *Smith W.L. and Chanley M.H (Eds.) Culture of Marine Invertebrate Animals.*, (Plenum Press, New York, USA), pp 26-60.

[5]Bold HC (1949) The morphology of *Chlamydomonas chlamydogama*, sp. nov. Bulletin of the Torrey Botanical Club. **76**:101-108.

[6]Bischoff HW, Bold, H.C. (1963) Phycological Studies. IV. Some soil algae from Enchanted Rock and related algal species. (University of Texas Publication) **6318**: 1–95.

[7]Minoda A, Sakagami R, Yagisawa F, Kuroiwa T, Tanaka K (2004) Improvement of culture conditions and evidence for nuclear transformation by homologous recombination in a red alga, *Cyanidioschyzon merolae* 10D. *Plant and Cell Physiology* **45**:667-671.

Table S5. Primers and conditions used for RT-PCR

Gene	Primer name	Sequence (5'→3')
P. tricornutum[1]		
METE	METE-F1	ACGACGCGTATGTTTTCCAT
	METE-R1	TGGTAGTTGCTCGTGAT
H4	HISTONE H4-F1	AGGTCCTTCGCGACAATATC
	HISTONE H4-R1	ACGGAATCACGAATGACGTT
RPS	RPS-F1	CGAAGTCAACCAGGAAACCAA
	RPS-R1	GTGCAAGAGACCGGACATACC
TBP	TBP-F1	ACCGGAGTCAAGAGCACACAC
	TBP-R1	CGGAATGCGCGTATACCAGT
C. reinhardtii[2]		
METE	46-METE-F	GCCGCCCTGCGCAACATGGT
	47-METE-R	CAGTCCACGCAGCGGCTGGTT
METH	40-METH-F	TGATCCGTGCCTTCCCCATTG
	41-METH-R	GTCGTCGCCCGCAGCCTTGT
SAHH	6-SAH1NOR-F	CCAAGAAGGTGATGGGCGTGTCT
	7-SAH1NOR-R	GCGGTAGTGGGCGGGCTTGTA
SHMT2	8-SHMT2NOR-F	CGCGCTGCTGATGGTGGACAT
	9-SHMT2NOR-R	TCGGCGGCAGGCACGGTGAA
RACK1	RACK1-for	GCCACACCGAGTGGGTGTCGTGCG
	RACK1-rev	CCTTGCCGCCCGAGGCGCACAGCG
C. merolae[3]		
METE	METE-F1	AAACCAGCACTTTGCGATCT
	METE_R1	GATTTTCCAGAATGCCTCCA
EF1a	EF1a_F	GGCTTTGTA4TGCGGAGACAG
	EF1a _R	CCGCCTCTTTCTTGTTGACC

[1] For *P. tricornutum,* reaction mixtures contained 4 ng cDNA, 100 nM of each primer, and 3.5 mM $MgCl_2$ .Cycling conditions on a Qiagen Rotor-Gene Q instrument were: 95°C for 10 min, followed by cycles of 95°C for 5 s and 65°C for 15 s, up to a total of 40 cycles.

[2]For *C. reinhardtii,* reaction mixtures contained 400 nM of each primer, 2 mM $MgCl_2$ and 5% DMSO. To amplify *METE*, 6.4 mM $(NH_4)_2SO_4$ and 27 mM Tris, pH 8.8, was further included in the reaction mixture (*RACK1* could be amplified in both buffer conditions). Amplification conditions on a DNA Engine PTC-200 thermal cycler equipped with a Chromo4 detector (Bio-Rad) were as follows: 1 cycle of 95 °C for 2 min; 40 cycles of 95 °C for 20 s, 65 °C for 30 s, 72 °C for 30 s, 83 °C for 5 s after which fluorescence was read; followed by 72 °C for 7 min). RACK1 (=CBLP) primers are from Erikson et al., 2009.

[3]For *C. merolae,* reaction mixtures contained 125 nM of each primer, 2 mM $MgCl_2$, and 5% DMSO. Amplification conditions on a TC-5_{12} gradient instrument (Techne) were: 94 °C for 5 minutes, cycles of (94 °C for 45 s, 63 °C 45 s, 72 °C for 2 min), and 72 °C for 10 min. Cycle number was optimized (Figure S4) to 35 and 23 cycles for *METE* and *EF1a* respectively.

Table S6 PCR primers employed for the amplification of DNA fragments for reporter construct generation. The Chloramphenicol (Chl) resistance gene was isolated from plasmid pBAD24 (Guzman et al., 1995), the origin of replication was isolated from pUC18, HSP70A/RBCS2 fusion and METE promoters were isolated from plasmid pRBCS2 and pMETE-Ble.GFP, respectively.

DNA fragment	Primer	Primer sequence (5'-3')
Chl resistance		
Chl - RPS29	RPS29-BAD F	AATGCCCGAATGTTGGGTATCTAGCTCACACGGCAGTTTGTAAGGTGCTGAGGCGTTGCTAAATCAGTAAGTTGGCAGCATCAC
	BAD-PUC R	CCTTAAACGCCTGGTGCTACGCAGATACCAAATACTGTCCTTCTAGTG
Origin of replication		
ORI-RBCS2	BAD-PUC F	CCTTAAACGCCTGGTGCTACGCAGATACCAAATACTGTCCTTCTAGTG
	pUC-HSP70A R	CAAGCCTCAGCGAGCTCCCCGCCGTCGTACCGAGCTCGAATTCGTAATC
ORI-METE	BAD-PUC F	CCTTAAACGCCTGGTGCTACGCAGATACCAAATACTGTCCTTCTAGTG
	pUC-METE R	GTTGTAGGCTCTGGTCCTGACCTACGTACCGAGCTCGAATTCGTAATC
Promoter & 5'UTR		
HSP70A/RBCS2 promoter	pUC-HSP70A F	TGACCATGATTACGAATTCGAGCTCGGTACGACGGCGGGGAGCTCGCTGAGGCTTGACAT
-RBCS2 UTR	HSP70A-Ble R	GGTCAGCTTGGCCATTTAAGATGTTGAGTGACTTCTCTTGT
METE promoter	pUC-pMETE F	TGACCATGATTACGAATTCGAGCTCGGTACGTAGGTCAGGACCAGAGCCTACAAC
- METE UTR	METE UTR R	GTTGACAGATGTTGATTCCCAAGGAC
METE promoter	pUC-pMETE F	TGACCATGATTACGAATTCGAGCTCGGTACGTAGGTCAGGACCAGAGCCTACAAC
- RBCS2 UTR	pMETE-RBCS2ble R	CAGCTTGGCCATTTTAAGATGTTGAGTGACATGTCACTTAAATAATCGGCCTG
HSP70A/RBCS2 promoter	pUC-HSP70A F	TGACCATGATTACGAATTCGAGCTCGGTACGACGGCGGGGAGCTCGCTGAGGCTTGACAT
- METE UTR	pRBCS2-METE R	CCTTGTGCAGTCAGACAGAATTGCAAAACAACAATCGGATGCTCATGCATGGTACCAATTCTCTTGTAAAAAAGTAAAGAACATAGGCC
Reporter & 3'UTR		
RBCS2 UTR - Ble.GFP	RBCS2-Ble F	GTCACTCAACATCTTAAAATGGCCAAGCTGACCAGCGCCGTT

	GFP-RPS29 R	CAACGCCTCAGCACCTTACAAACTGCCGTGTGAGCTAGATACCCAACATTCGGGCATTTTACTTGTACAGCTCGTCCATGCCGT
METE UTR-Ble.GFP	METE-Ble F	TTGTTGTTTTGCAATTCTGTCTGACTGCACAAGGAACCCGTCCTTGGGAATCAACATCTGTCAACATGGCCAAGCTGACCAGCGCCGTT
	GFP-RPS29 R	CAACGCCTCAGCACCTTACAAACTGCCGTGTGAGCTAGATACCCAACATTCGGGCATTTTACTTGTACAGCTCGTCCATGCCGT

Supplementary Figures

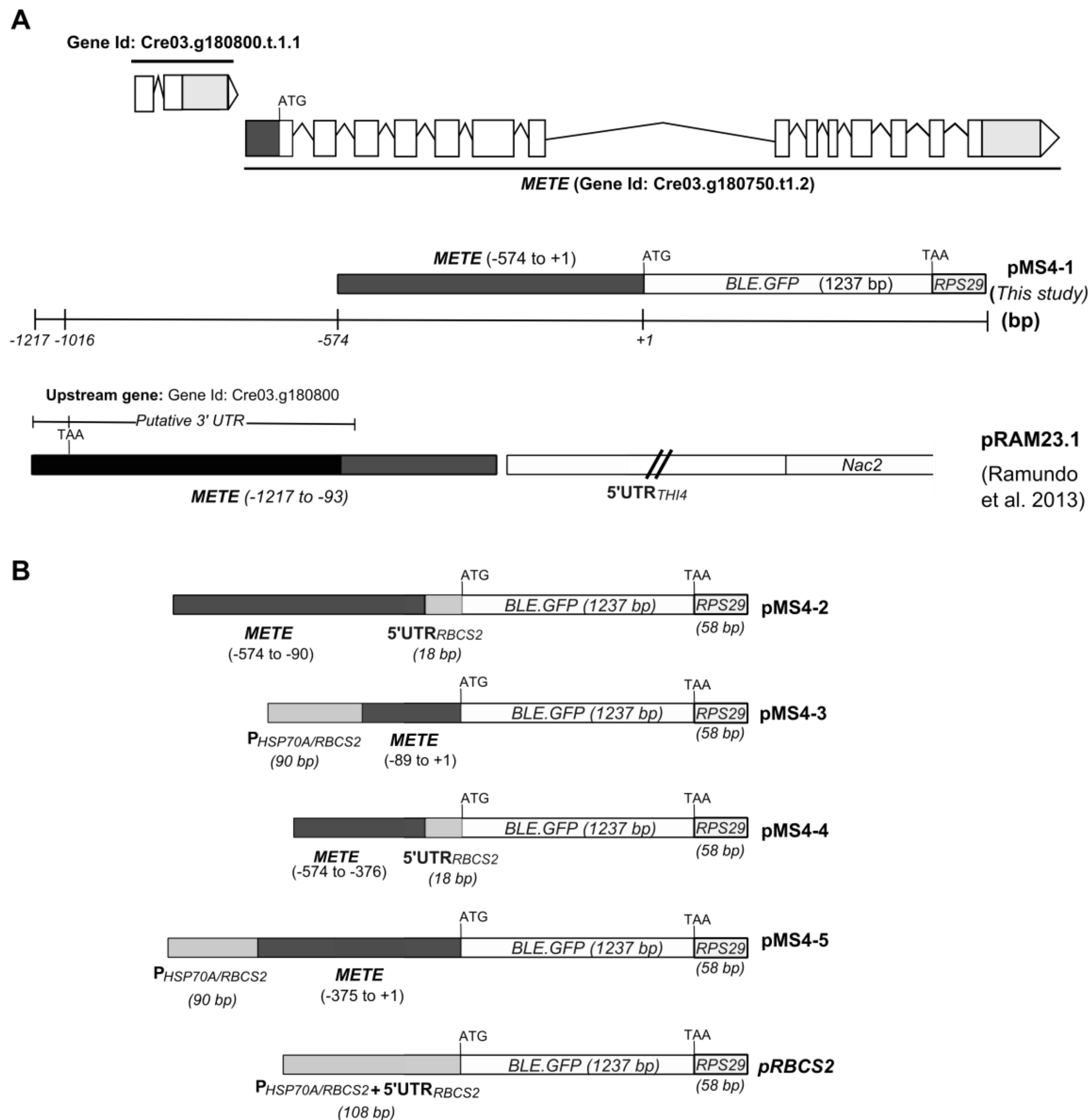

Figure S1. Schematic diagram of reporter constructs used in this study. A. Plasmid pMS4-1 incorporates -574 to +1bp upstream of the *C. reinhardtii METE* gene as the promoter and 5'UTR for the reporter gene *sh.Ble.GFP.* Shown underneath for comparison is the pRAM23.1 construct used by Ramundo et al. (2013), which showed a slight response to B_{12}. This contains a region upstream of *METE,* but it contains part of the coding sequence of the upstream gene (gene Id: Cre03.g180800 (v3)), and extends to -93 of *METE* **B.** All other constructs used in this study.

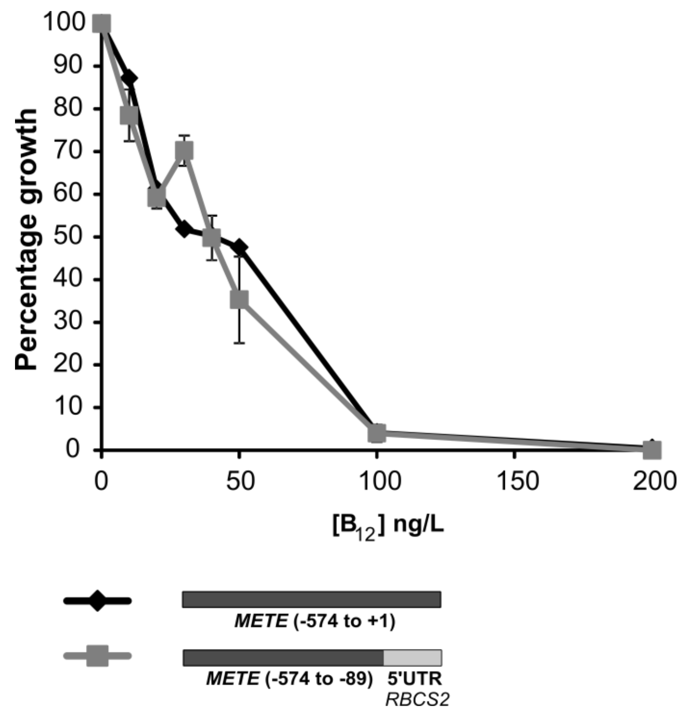

Figure S2. B_{12} dose response of pMS 4-1 (transformant no. 4, Fig. 2C) and pMS 4-2 (transformant no. 3, Fig. 2D) to different concentrations of vitamin B_{12}. Growth was calculated as a percentage of maximal growth i.e. at t=120 h in the $-B_{12}$ treatment. Error bars denote standard error (n=3).

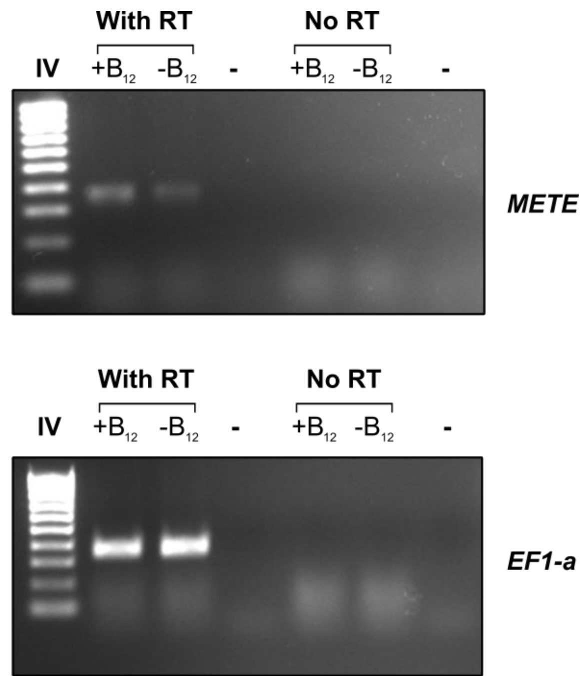

Figure S3. *METE* expression is not repressed by B_{12} in the thermophilic red alga *C. merolae*. RT-PCR analysis of transcripts for *METE* and *EF1-A* following growth + B_{12} or - B_{12}. The following cycle number for each primer set was used throughout: *METE* (35 cycles) and *EF1-A* (23 cycles), see Figure S4.

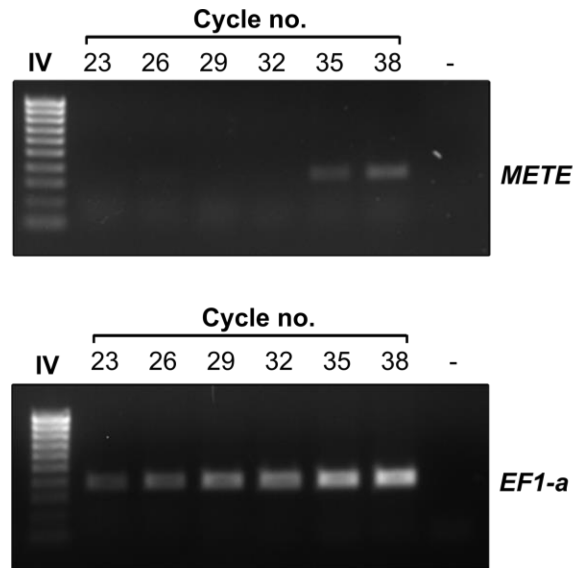

Figure S4. RT-PCR cycle number optimization for *METE* and *EF1-A* expression in *C. merolae.* RT-PCR analysis was performed on cDNA made from RNA harvested from cultures of *C. merolae* grown without B_{12}.

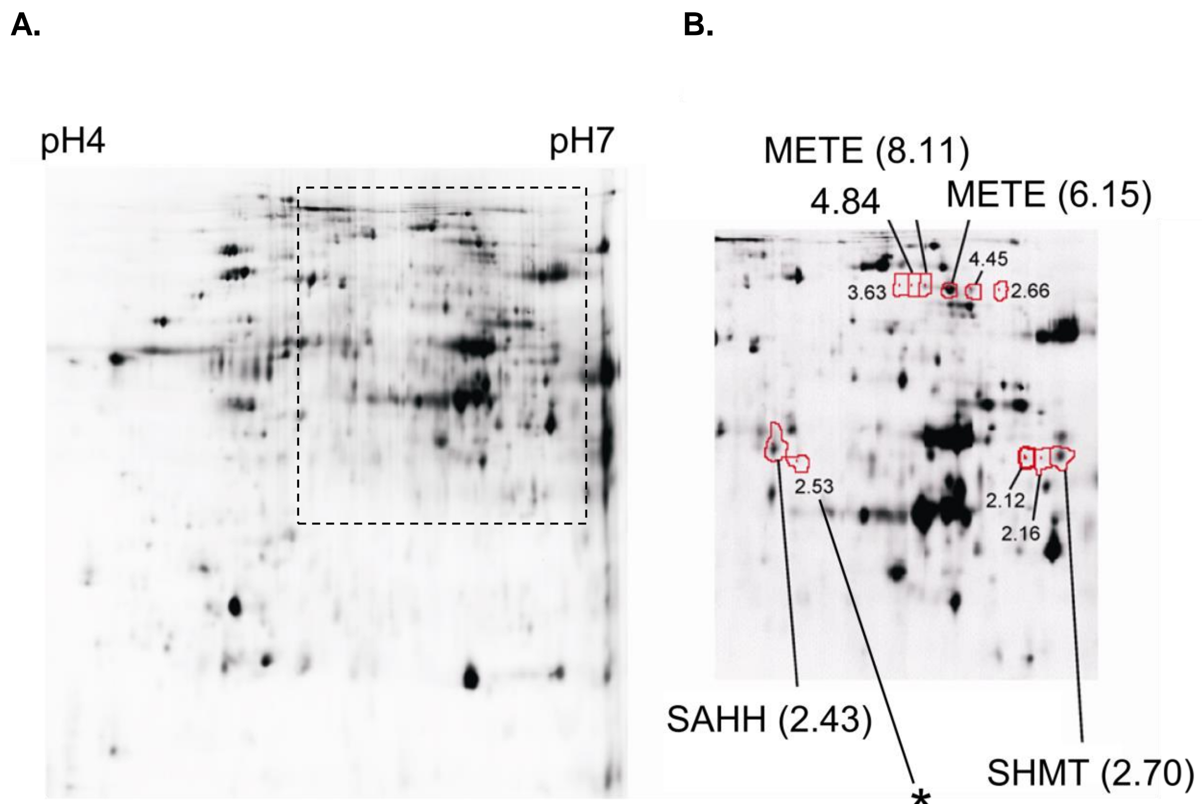

Figure S5. Investigating B_{12}-responsive genes in *C. reinhardtii* by 2D-DIGE Algae were grown in the presence or absence of 1000 ng/L vitamin B_{12}, then extracted protein samples were labelled with Cy5, mixed with a Cy3-labeled standard (made up of equal amounts of each of the samples pooled) and separated by 2D gel electrophoresis, followed by silver staining. **A.** Representative Cy5 spot map for proteins from *C. reinhardtii* grown in the absence of B_{12}. **B.** Expanded region of gel shown by dashed box in panel A. The spots outlined in red are those that were found to be down-regulated by B_{12}, by at least a twofold difference in regulation and $P \leq 0.01$ (two-tailed t-test). The actual values are indicated next to the highlighted spots. To identify the proteins, spots were excised from the gel, digested with trypsin and analysed by MS/MS. Three spots were identified as METE (B12-independent methionine synthase), SAHH (S-adenosylhomocysteine hydrolase) and SHMT2 (serine hydroxymethyltransferase isoform 2). The spot indicated with an asterisk could not be identified.

SUPPLEMENTARY REFERENCES

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Eriksson M, Moseley JL, Tottey S, Del Campo JA, Quinn J, Kim Y, Merchant S. (2004) Genetic dissection of nutritional copper signaling in *Chlamydomonas* distinguishes regulatory and target genes. *Genetics* **168**: 795-807

Ramundo S, Rahire M, Schaad O, Rochaix J-D (2013) Repression of essential chloroplast genes reveals new signaling pathways and regulatory feedback loops in *Chlamydomonas*. *Plant Cell* **25**: 167-186