1 <center>**ELECTRONIC SUPPLEMENTARY INFORMATION (SI)**</center>

2

3 **TITLE**

4 Linking gene expression to productivity to unravel long- and short-term responses of

5 seagrasses exposed to CO_2 in volcanic vents

6

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12 **APPENDIX 1**

13 **Supplementary information on Results.** Selection, stability and ranking of reference

14 genes in *Cymodocea nodosa*. Results of two-way ANOVA test on productivity of *C. nodosa*.

15

16 **Table S1**. Selection of reference genes in *Cymodocea nodosa* based on the BestKeeper

17 approach [1]. Lowest standard deviation (SD) is underlined. *n*, number of plant samples

18 in the tested conditions; GM, geometric mean of C_T-value; AM, arithmetic mean of

19 C_T-value; Min and Max, the extreme values of C_T; SD, standard deviation of C_T;

20 CV, coefficient of variance as percentage.

	18S	**elF4A**	**GAPDH**	**UBI**
n	12	12	12	12
GM [CT]	22.53	27.83	26.05	27.31
AM [CT]	22.54	27.83	26.06	27.31
min [CT]	21.43	26.91	25.12	26.44
max [CT]	23.38	28.72	27.26	28.23
SD [± CT]	0.50	<u>0.35</u>	0.55	0.46
CV [% CT]	2.23	1.28	2.12	1.67

21

22 **Table S2.** Expression stability of candidate RGs as calculated by geNorm [2] in *Cymodocea*

23 *nodosa*. Best candidate genes are underlined.

Gene name	Average expression stability (*M*)
eIF4A	<u>0.60</u>
GAPDH	<u>0.60</u>
18S	0.71
UBI	0.83

24

25

26 **Table S3.** Expression stability of candidate RGs as calculated by NormFinder [3] in *Cymodocea*

27 *nodosa*. Best candidate genes are underlined.

Gene name	Stability value	Standard error
eIF4A	<u>0.07</u>	0.10
18S	0.25	0.07
GAPDH	0.25	0.07
UBI	0.31	0.08

28

29

30 **Table S4.** Ranking of the reference gene candidates by BestKeeper, geNorm, and

31 NormFinder. The three best RGs selected for RT-qPCR assays are highlighted in

32 bold. S.D.: standard deviation; Stab. Val.: stability value; *M*: average expression

33 stability.

Bestkeeper	NormFinder	geNorm
(S.D.)	(Stab. Val.)	(*M*)
eIF4A (0.35)	**eIF4A** (0.07)	**eIF4A** (0.60)
UBI (0.46)	**18S** (0.25)	**GAPDH** (0.60)
18S (0.50)	GAPDH (0.25)	18S (0.71)
GAPDH (0.55)	UBI (0.31)	UBI (0.83)

34

35

36   **Table S5.** Results of ANOSIM multivariate gene expression analysis. *In situ* incubations

37   indicate gene expression of CO_2 plants incubated in CO_2 water with respect to

38   Reference plants incubated in Reference water. Transplant incubations (Reference

39   plants) indicate gene expression of Reference plants incubated in CO_2 water with

40   respect to Reference plants incubated in Reference water. Transplant incubations

41   (CO_2 plants) indicate gene expression of CO_2 plants incubated in Reference water

42   with respect to CO_2 plants incubated in CO_2 water.

Global Test			
	Sample statistic (Global R)	Significance level	Average dissimilarity
In situ incubations	0.37	10%	10.39
Transplant incubations (Reference plants)	-0.074	70%	8.08
Transplant incubations (CO_2 plants)	0.37	20%	6.51

43

44

45 **Table S6.** Relative expression report for RT-qPCR experiments obtained with REST

46 2009 [4]. Expression values (with Standard error, Std. Error) and P(H1) (Probability of

47 alternate hypothesis that difference between sample and control groups is due only to

48 chance) are reported for each gene. (a) *In situ* incubations indicate relative gene

49 expression of CO_2 plants incubated in CO_2 water with respect to Reference plants

50 incubated in Reference water. (b) Transplant incubations (Reference plants) indicate

51 relative gene expression of Reference plants incubated in CO_2 water with respect to

52 Reference plants incubated in Reference water. (c) Transplant incubations (CO_2

53 plants) indicate relative gene expression of CO_2 plants incubated in Reference water

54 with respect to CO_2 plants incubated in CO_2 water. Underlined values indicate P(H1)

55 < 0.1.

(a) In situ incubations			
Gene	Expression	Std. Error	P(H1)
psaJ	-1.2235	0.5444	0.351
psaC	-1.5637	0.3760	0.268
psbA	-3.1933	0.2377	0.000 ***
psbD	-1.1583	0.4907	0.330
LHCA1	-6.1860	0.1217	0.000 ***
FD	-2.1205	0.3018	0.058
rbcL	-1.7125	0.2872	0.037 *
ATPA	-1.0020	0.5949	0.461
PEPC	-4.6031	0.2156	0.058
SUS	-1.7949	0.2028	0.000 ***
BCA	-4.3563	0.1641	0.000 ***
SOD	-2.6559	0.2845	0.000 ***
CAT	-2.1268	0.2985	0.107
APX7	-1.7292	0.2444	0.000 ***
APX6	-2.0806	0.3129	0.000 ***
LBP	-1.4587	0.3902	0.000 ***
GSH-S	-2.2658	0.2362	0.000 ***
GR	-3.1383	0.2030	0.037 *
MTP	-2.7702	0.1626	0.000 ***
MT	-3.4187	0.2834	0.000 ***

56

57

(b) Transplant incubations (Reference plants)			
Gene	**Expression**	**Std. Error**	**P(H1)**
psaJ	-1.6625	0.6732	0.602
psaC	-2.0596	0.3616	0.212
psbA	-1.5462	0.2615	0.192
psbD	-1.6521	0.9551	0.704
LHCA1	1.0088	0.6195	0.969
FD	1.2004	1.1975	0.793
rbcL	-1.5319	0.4360	0.358
ATPA	-1.5760	0.6649	0.528
PEPC	-2.3135	0.1910	0.000 ***
SUS	-1.1906	0.7741	0.778
BCA	-1.2465	0.3835	0.819
SOD	1.2143	0.5284	0.713
CAT	-1.1480	0.4479	1.000
APX7	1.1068	0.6279	0.704
APX6	-1.4624	0.3958	0.389
LBP	-1.2174	0.9836	0.894
GSH-S	-1.8769	0.2877	0.212
GR	-1.7806	0.2851	0.000 ***
MTP	-1.0744	0.9455	0.894
MT	-1.0346	0.3725	0.894

58

(c) Transplant incubations (CO_2 plants)			
Gene	**Expression**	**Std. Error**	**P(H1)**
psaJ	1.5063	0.5530	0.000 ***
psaC	-1.7183	0.2825	0.127
psbA	2.9783	2.2956	0.096
psbD	2.0434	0.9608	0.190
LHCA1	4.4506	2.5997	0.074
FD	1.6250	1.6696	0.529
rbcL	1.3850	0.6230	0.000 ***
ATPA	1.1772	0.7049	0.704
PEPC	2.1562	2.1473	0.497
SUS	2.2300	0.8250	0.095
BCA	2.6931	1.9187	0.096
SOD	2.4607	1.7860	0.101
CAT	1.9327	1.0148	0.000 ***
APX7	1.7995	0.6850	0.095
APX6	1.5200	0.7488	0.000 ***
LBP	1.6729	0.7234	0.601
GSH-S	2.1214	0.9188	0.000 ***
GR	3.7192	2.3486	0.096
MTP	3.6992	1.5363	0.075
MT	5.8535	5.7004	0.101

59

60   **Table S7.** Results of two-way ANOVA test (fixed crossed factors) to assess the effects of the

61   "origin" of the plants (plants from CO_2 or reference site) and the "water" used in the incubations

62   (water from CO_2 or reference site) of *Cymodocea nodosa* plants in Vulcano.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Origin	1	0.0796	0.0796	1.484	0.23457	
Water	1	0.6161	0.6161	11.481	0.00233	**
Origin x Water	1	0.1448	0.1448	2.698	0.113	
Residuals	25	1.3415	0.0537			

63

64

65   **Table S8.** Results of Principal Components Analysis (PCA) conducted considering the

66   combined contributions of all GOIs and NPP. Percent variation explained by the first three axes

67   and individual contribution of NPP and targeted genes are indicated.

PC	Eigenvalue	%variance
1	9.2212	66.465
2	3.5521	25.603
3	1.1004	7.9316

	PC 1	PC 2	PC 3
NPP	0.1737	0.0222	0.3656
psaJ	0.0461	0.0163	-0.0246
psaC	0.0110	0.0999	0.5127
psbA	0.2576	0.0840	0.0199
psbD	0.0188	0.0812	-0.2113
LHCA1	0.2701	0.4156	-0.1125
FD	0.0487	0.2814	0.1668
rbcL	0.0476	0.1879	0.0989
ATPA	-0.0107	0.0423	0.0005
PEPC	0.3636	0.0326	0.2246
SUS	0.0558	0.2356	-0.1749
BCA	0.1960	0.3233	0.1975
SOD	0.3551	-0.1987	0.0293
CAT	0.2353	0.0265	-0.3159
APX7	0.0029	0.3011	-0.0525
APX6	0.1043	0.1223	0.2124
LBP	-0.0125	0.2373	0.0102
GSH-S	0.2933	-0.1526	-0.0730
GR	0.4466	-0.3454	0.1849
MTP	0.0836	0.4169	-0.1732
MT	0.4055	-0.0834	-0.4116

68

69 **Table S9.** Spearman's rank correlation between Net plant productivity (NPP) and

70 mean gene expression from the incubations of *Cymodocea nodosa* plants in Vulcano.

	Spearman coefficient (ρ)	p-value	
NPP	1	0	
psaJ	0.49	0.0064	**
psaC	0.3	0.1099	
psbA	0.49	0.0064	**
psbD	-0.06	0.738	
LHCA1	0.49	0.0064	**
FD	0.29	0.132	
rbcL	0.29	0.132	
ATPA	-0.15	0.4266	
PEPC	0.59	0.0007	***
SUS	-0.27	0.1525	
BCA	0.29	0.132	
SOD	0.59	0.0007	***
CAT	0.29	0.1236	
APX7	-0.07	0.7143	
APX6	0.29	0.132	
LBP	-0.07	0.7143	
GSH-S	0.39	0.0356	*
GR	0.59	0.0007	***
MTP	-0.07	0.7143	
MT	0.29	0.1236	

71

72   **APPENDIX 2**

73   **Supplementary information on Material and Methods**. Gene expression protocol

74   and information on Reference genes (RGs) and Genes of interest (GOIs).

75

76   *RNA extraction and cDNA preparation*

77   RNA was extracted from *C. nodosa* leaves with the Aurum™ Total RNA Mini Kit

78   (BIO-RAD) following manufacturer's instructions and retro-transcribed in cDNA

79   with the iScript™ cDNA synthesis kit (BIO-RAD), according to standard protocol.

80   RNA quantity and purity were assured by Nano-Drop (ND-1000 UV-Vis

81   spectrophotometer; NanoDrop Technologies) and by gel electrophoresis. The 260/280

82   nm and 260/230 nm ratios were always about 2.0, while gel electrophoresis showed

83   intact RNA, with sharp ribosomal bands. Total RNA (500 ng) was retro-transcribed in

84   cDNA with the iScript™ cDNA synthesis kit (BIO-RAD), according to standard

85   protocol.

86

87   *Gene selection and RT-qPCR*

88   A Reverse Transcription -quantitative Polymerase Chain Reaction (RT-qPCR)

89   analysis was performed in order to investigate differences in expression levels of

90   genes belonging to pathways potentially affected by changes in CO_2 availability

91   (photosynthesis, carbon utilization, free-radical detoxification and oxidative-stress

92   response, and metal detoxification) (Supplementary Table S10).

93   Given the lack of genomic (or transcriptomic) resources available for this species,

94   specific primers for putative reference genes (RGs) and genes of interest (GOIs) were

95   designed considering the alignment of conserved regions in other monocotyledons

96   such as *Posidonia oceanica*, *Zostera marina*, *Oryza sativa*, *Zea mays*. Sequences were

97   identified using the search function in the seagrass EST database Dr. Zompo

98   (http://drzompo.uni-muenster.de) or the generic online database GenBank

99   (http://www.ncbi.nlm.nih.gov/genbank).

100 Alignments were performed with ClustalW embedded in BioEdit v. 7.0.5.3 [5]. Primers

101 were designed with the primer analysis software Primer3 v. 0.4.0 [6,7] and Gene Runner

102 v. 3.05 (Hasting Software, Inc.). Primers' selection conditions included primer length

103 (18-23 bp), T_m (60°C), GC content (≥50%) and product size (130 to 230 bp). Primers

104 for *SUS* and *rbcL* were designed from the known *C. nodosa* sequences available in the

105 GenBank database (Acc. no. AM292651.2 and U80688.1, respectively). In some

106 cases primer pairs established in previous works for closely related species were used

107 (see references in Table S10).

108 To determine the specificity of the amplification, designed primer pairs were first

109 tested in PCR, according to the reaction conditions detailed in [8]. Amplified PCR

110 products were then analyzed by 1.5% agarose gel electrophoresis in TBE buffer. The

111 resulting bands were excised from the gel and extracted with the GenElute[TM] Gel

112 Extraction Kit (SIGMA). Sequence reactions were obtained with the BigDye

113 Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA),

114 purified in automation using the Agencourt CleanSEQ Dye terminator removal Kit

115 (Agencourt Bioscience Corporation, 500 Cummins Center, Suite 2450, Beverly MA

116 01915 - USA) and a robotic station Biomek FX (Beckman Coulter, Fullerton, CA).

117 Products were analyzed on an Automated Capillary Electrophoresis Sequencer 3730

118 DNA Analyzer (Applied Biosystems). The identity of each sequence was confirmed

119 using the blastn function. All obtained *C. nodosa* sequences are deposited in GenBank

120 under the accession numbers shown in Table S10.

121   We set primers for 8 RGs and 20 GOIs, but 4 RGs (*L31*, *UBC13*, *EF1A* and *TBP*)

122   were discarded due to double or no amplifications [8]

123   RT-qPCR was performed in MicroAmp Optical 384-Well reaction plate (Applied

124   Biosystems) with Optical Adhesive Covers (Applied Biosystems) on a Viia7 Real

125   Time PCR System (Applied Biosystems), using Sybr Green as fluorescence detection

126   chemistry. The PCR consisted of 5 μL Fast SYBR® Green Master Mix (Applied

127   Biosystems), 1 μL 1:50-diluted cDNA template, and 0.7 pmol μL^{-1} of each primer, in

128   a total reaction volume of 10 μL. Thermal profile was as follows: 20 sec at 95°C, 40

129   cycles of 1 sec at 95°C and 20 sec at 60°C. For determining the specificity of the

130   reaction, the melting curve of each product from 60 to 95°C was also assessed. All

131   RT-qPCR reactions were carried out in triplicate and each assay included three no-

132   template negative controls (NTC) for each primer pair [9]. The technical variation

133   among the triplicates was checked and individual outliers were excluded when the SD

134   was more than 0.3.

135   PCR efficiencies for all primer pairs have been calculated from the slopes of standard

136   curves of the threshold cycle (C_T) *vs*. cDNA concentration, with the equation $E = 10^{-1/slope} - 1$.

137    $^{1/slope}$-1. Primer's sequences, percent efficiencies (*E*) and regression coefficients (R^2)

138   of RGs and GOIs are reported in Table S10. All $E \geq 84\%$ and all R^2 were ≥ 0.97. To

139   normalize target gene-expression data, three different algorithms BestKeeper [1],

140   geNorm [2] and NormFinder [3] were utilized to identify the best RGs in our

141   experimental conditions, among the four candidate RGs selected for amplification: the

142   Eukaryotic initiation factor 4A (eIF4A), the Glyceraldehyde 3-phosphate

143   dehydrogenase (GAPDH), the 18S ribosomal RNA and the Ubiquitin (UBI). All RGs

144   belong to different functional classes in order to reduce the possibility that they might

145   be co-regulated. For the relative quantification of RT-qPCR data, we used REST-

146   MCS© (Relative Expression Software Tool) [4]. The mathematical model used is based

147   on the correction for exact PCR efficiencies and the mean crossing point deviation

148   between sample group(s) and control group(s), and it allows the use of multiple

149   reference genes for the normalization of target gene expression levels [10].

150

151   **TABLES**

152

153 **Table S10**. List of reference genes (RGs) and genes of interest (GOIs) in *Cymodocea nodosa* assessed using RT-qPCR. Gene symbol and

154 protein name, GenBank accession number, primer sequence, reference, amplicon size (S, base pair), percent of efficiency (*E*), correlation

155 coefficient (R^2) and Swiss-Prot best hit with corresponding *E*-value are given.

Gene symbol	Protein name	GenBank	Primer Sequences 5' ->3'	Reference	S	*%E*	R^2	Swiss-Prot best hit	E-Value
Reference genes (RGs)									
18S	Ribosomal RNA 18S	KT200607	F:AACGAGACCTCAGCCTGCTA R:AAGATTACCCAAGCCTGTCG	8	200	100	0.97	-	-
eIF4A	Eukaryotic initiation factor 4A	KT200591	F:TTCTGCAAGGGTCTTGACGT R:TCACACCCAAGTAGTCACCAAG	11	194	100	0.99	sp|Q40466.1|IF413_TOBAC	2e-37
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	KT200590	F:AGGTTCTTCCTGCTTTGAATG R:CTTCCTTGATTGCTGCCTTG	8	139	100	0.99	sp|P34783.1|G3P_ATRNU	5e-34
UBI	Ubiquitin	KT200589	F:CACCCTCGCTGACTACAACA R:TTTCTCAGCCTGACGACCTT	8	195	96	0.99	sp|P31753.2|RS27A_ASPOF	1e-18
Genes of interest (GOIs)									
psaJ	Photosystem I reaction center subunit IX	KT200587	F:GGTTTGGGTCTTTAGCAGGTC R:GAATGGGTGGGAGGAGAAAT	12	155	100	0.97	sp|Q6EW33.1|PSAJ_NYMAL	8e-20
psaC	Photosystem I iron-sulfurcenter	KT200593	F:TCTTGGGATGGGTGTAAAGC R:GGTTGTCTCATTCCATAAATACA		135	100	0.99	sp|P62090.2|PSAC_ARATH	4e-41
psbA	Photosystem II protein D1	KT200596	F:GACTGCAATTTTAGAGAGACGC R:CAGAAGTTGCAGTCAATAAGGTAG	12	137	97	0.99	sp|P27201.3|PSBA_LANPU	5e-71

	Harvesting Complex gene 1		R:AGTTCATCACCATCGCCTTC					B6_ARATH	
FD	Ferredoxin, chloroplastic	KT200600	F:ATGGTGAGCACCCCCTTC R:GGGTGACGAGCTTGACCTT	13	148	89	0.99	sp|Q43517.1|FER1_SOLLC	3e-24
rbcL	RuBisCO large subunit	U80688.1	F:GCTGCCGAATCTTCTACTGG R:CACGTTGGTAACGGAACCTT	13	176	88	0.99	sp|P31196.2|RBL_NEMMU	1e-34
ATPA	ATP synthase subunit alpha	KT200595	F:TATCTGGCGATCTATTCAAT R:AACTCACGTAATCGTTGACC		226	88	0.98	sp|Q02848.1|ATPA_ANTSP	3e-28
PEPC	Phosphoenolpyruvate carboxylase	KT200592	F:AAGTTCCTACGCAGGCTTGA R:TGCCATCATTCTAGCCAACA		174	93	0.99	sp|P51059.1|CAPP2_MAIZE	5e-53
SUS	Sucrose synthase	AM29265 1.2	F:GATCCCAAGTTCAACATTGTCT R:CTCGCCATGGAGAAGATGAT		188	95	0.99	sp|Q41607.1|SUS2_TULGE	3e-27
BCA	Beta carbonic anhydrase	KT200602	F:TACTGGGGTTTCAACCAGGA R:GGCTGTGACCAACGACTAAGA		153	99	0.99	sp|Q94CE3.1|BCA5_ARATH	1e-38
SOD	Copper/zinc superoxide dismutase	KT200606	F:GGAATGTCACAGCTGCAGAA R:CACCCGCATTTCCTGTAGTC		171	94	0.99	sp|O04996.3|SODC_SOLCS	6e-32
CAT	Catalase	KT200585	F:CATCACATGCTGGGTTTCAC R:ACCGATCCTGGACATCTGAC	11	175	84	0.99	sp|P49317.1|CATA3_NICPL	8e-33
APX7	L-ascorbate peroxidase 7, chloroplastic	KT200605	F:AAGAGGGGAGGAGCTAATGG R:GCTGGCAAGCTGAAACAAGT		150	93	0.99	sp|Q7XJ02.1|APX7_ORYSJ	3e-34
APX6	L-ascorbate peroxidase 6	KT200603	F:TAATTGCTGTTGCCGGCTCT R:TCCTTTTTCACGGAAGCAAC		161	99	0.99	sp|Q8GY91.1|APX6_ARATH	6e-24
LBP	Luminal binding protein	KT200583	F:ACCCGAGCTCGGTTTGAAGA R:ATTCTTGTGCTTCCACCAAC	11	137	90	0.99	sp|Q03685.1|BIP5_TOBAC	2e-35
GSH-S	Glutathione synthase	KT200598	F:TAGGTTTCGCCAATTCTTGC R:AAGGGGTGGTTCTCCCAGAT	11	213	100	0.99	sp|P46416.3|GSHB_ARATH	4e-29
GR	Glutathione reductase	KT200599	F:TCCTCCAAGCTTAGTGCTTCA		134	100	0.99	sp|P48642.2|GSH	2e-37

			R:ACACAACCAGACGGTGTCAA					RC_ORYSJ
MTP	Metal tolerance protein	KT200586	F:CTCGTTTCCTGAGGTTCTGC	11	183	91	0.99	sp|Q6Z7K5.1|MTP3_ORYSJ	3e-26
			R:TTTGCTGCTGTCATGGCTAC					
MT	Metallothionein	KT200588	F:CATGTCGACCTGTGACAACTG	11	195	96	0.99	sp|Q40256.1|MT3_MUSAC	2e-22
			R:TATTAATGGCCACAGGTGCAG					

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