Identification of a gene, ccr-1 (sll1242), required for chill-light tolerance and growth at 15 °C in Synechocystis sp. PCC 6803

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Synechocystis sp. PCC 6803 exposed to chill (5 °C)-light (100 μmol photons m⁻² s⁻¹) stress loses its ability to reinitiate growth. From a random insertion mutant library of Synechocystis sp. PCC 6803, a sll1242 mutant showing increased sensitivity to chill plus light was isolated. Mutant reconstruction and complementation with the wild-type gene confirmed the role of sll1242 in maintaining chill-light tolerance. At 15 °C, the autotrophic and mixotrophic growth of the mutant were both inhibited, paralleled by decreased photosynthetic activity. The expression of sll1242 was upregulated in Synechocystis sp. PCC 6803 after transfer from 30 to 15 °C at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹. sll1242, named ccr (cyanobacterial cold resistance gene)-1, may be required for cold acclimation of cyanobacteria in light.

INTRODUCTION

Cyanobacteria are a group of oxygenic photosynthetic prokaryotes distributed in a vast array of habitats, including open seas, inland lakes, freshwater ponds of polar regions, hot springs and certain terrestrial environments (Castenholz, 2001). In temperate and subtropical zones, they are adapted to fluctuations of temperature over four seasons, typically from 4 to >30 °C. Synechocystis sp. PCC 6803 is a unicellular mesophilic cyanobacterium whose optimal temperature for autotrophic growth is ~30 °C, and preferred temperature between 25 and 40 °C (Tasaka et al., 1996). It is one of the research models for the biology of cyanobacteria, including cold acclimation.

The cold tolerance of cyanobacteria is one of the key factors that limit their global distribution. Genes involved in cold acclimation in cyanobacteria may also be applied to the genetic modification of higher plants. It has been established that the fluidity of the biomembrane plays an important role in the cold acclimation of cyanobacteria and that it is affected by the degree of unsaturation of membrane lipids (Tasaka et al., 1996; Szalontai et al., 2000; Inaba et al., 2003). Four types of acyl-lipid desaturases found in cyanobacteria introduce double bonds at the Δ6, Δ9, Δ12 and ω3 positions of C18 fatty acids, and they are encoded by desD, desC (desC1 and desC2), desA and desB, respectively (Murata & Wada, 1995; Chintalapati et al., 2006). When the temperature is lowered, transcripts of desA, desB and desC accumulate in Synechococcus sp. PCC 7002, transcripts of desA, desB and desD accumulate in Synechocystis sp. PCC 6803, and the level of unsaturation of membrane glycerolipids is increased (Sakamoto et al., 1997; Kis et al., 1998). Consequently, the fluidity of the membrane is maintained at low temperature (Murata & Wada, 1995; Sakamoto & Murata, 2002). Such a low temperature-induced expression requires light (Kis et al., 1998). In different species, a combination of cold and light stresses may exert different effects on the cell activities of mutants with reduced membrane fluidity. In Synechocystis sp. PCC 6803, the decrease of membrane fluidity may depress the recovery of the photosystem II (PSII) protein complex from photoinhibition damage, reducing photosynthesis activity (Gombos et al., 1992; Tasaka et al., 1996). In Synechococcus sp. PCC 7002, a high light-tolerant species, however, no such effect has been found (Sakamoto & Bryant, 2002).

Low temperature also affects the expression of other genes. Heat-shock proteins ClpB, ClpP1, HtpG and GroEL are required for cold tolerance and/or are upregulated at low temperature (Porankiewicz & Clarke, 1997; Porankiewicz et al., 1998; Hossain & Nakamoto, 2002). Transcripts and/or proteins encoded by rbp (RNA-binding protein) (Sato, 1995), crhC (RNA helicase) (Chamot et al., 1999; Chamot & Owttrim, 2000) and btpA (required for stabilization of photosystem I, PSI) (Zak & Pakrasi, 2000) accumulate at low temperature in cyanobacteria. With the application of DNA microarray analysis, many more genes have been shown to be up- or down-regulated in the cold (Suzuki et al., 2001).

Random insertion mutagenesis is an alternative method to transcriptomic analysis to find genes involved in cold acclimation. In the study of the chill-light sensitivity of a desD mutant of Synechocystis sp. PCC 6803, we found a clear

Abbreviations: ARG, ability to reinitiate growth; Em, erythromycin; Km, kanamycin.
difference between this mutant and the wild-type strain. Based on this finding, we attempted to screen chill-light-sensitive mutants from a mutant library of Synechocystis sp. PCC 6803 and to identify novel genes involved in cold acclimation. In this paper, we report that ccr-1 (sll1242), whose homologues are widely distributed in mesophilic cyanobacteria, is required for growth at low temperature.

METHODS

Strains, culture conditions and transformation. Synechocystis sp. PCC 6803 was from J. Zhao, Peking University, and was cultured in BG11 with or without glucose (5 mM) in 250 to 500 ml flasks at 30 °C under continuous illumination at 30 μmol photons m⁻² s⁻¹. Mutants were grown in the medium with kanamycin (Km, 20 μg ml⁻¹) or erythromycin (Em, 5 μg ml⁻¹), or both.

Transformation of Synechocystis sp. PCC 6803 and mutants was performed according to Williams (1988). The complete segregation of a mutant was confirmed by PCR.

Evaluation of the sensitivity of a strain to cold or chill-light stress. The sensitivity of a strain to 15 °C was calculated as the percentage of its autotrophic growth rate at this temperature compared with that of the wild-type. Strains were inoculated in 500 ml flasks in triplicate with initial OD730 0.2, and grown at 30, 20 or 15 °C on a shaker (120 r.p.m.) at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹. The turbidity was measured every 24 h, and growth rates in the exponential phase were calculated. The tolerance of a strain to 5 °C was calculated as the percentage of its ability to reinitiate growth (ARG) at this temperature compared with that of the wild-type. Cells grown at 30 °C were diluted with BG11 to OD730 0.05 in 100 ml flasks, and 5 ml aliquots were taken from the flasks and transferred into test tubes in triplicate, and exposed to chill stress (5 ± 1 °C) with or without light (100 μmol photons m⁻² s⁻¹) for 5 days. Sterile 1 M glucose solution (25 μl) was added to each test tube after exposure to the chill stress, and the test tubes were warmed to 30 °C to allow the treated cells to grow at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹ for 3.5 days. Samples not exposed to the chill stress were used as the control. The ARG was calculated as OD730 (treated)/OD730 (control) × 100%, in which OD730 is the turbidity after photomixotrophic growth at 30 °C for 3.5 days. Data represent the mean ± SD of values from three tests.

To find the correlation between the ARG and log (c.f.u. ml⁻¹), we also spread the cells on BG11 + glucose plates after the chill stress, employing different 10-fold dilutions to determine the ability of cells to form colonies under photomixotrophic conditions.

Screening of mutants of increased sensitivity to chill-light stress and localization of the antibiotic-resistance marker. A random insertion mutant library of Synechocystis sp. PCC 6803 was generated as previously reported (Kong et al., 2003), with modifications. The genomic DNA library constructed with pUC19 was partially digested with Alul and HaeIII, instead of Sau3AI, until most of the plasmid DNA was cut once. DNA fragments larger than 6 kb were eluted from an electrophoresis agarose gel. A Km' marker was excised from pHBI68 (R. Kong & X. Xu, unpublished results) with KpnI, blunted with T4 DNA polymerase, ligated with the partially digested library DNA fragments, and electroporated into Escherichia coli DH10B, resulting in a random insertion library in E. coli. The Km' marker was derived from C.K2 of pRL446 (Elhai & Wolk, 1988), called C.K2d in this report for convenience. The total plasmid DNA of the secondary library was then pooled together and used to transform Synechocystis sp. PCC 6803. Transformants were maintained in BG11 + glucose with Km in 96-well microtitre plates.

The library was replicated, producing two additional copies; one copy was maintained at 30 °C at a photosynthetic photon flux density of 20 μmol photons m⁻² s⁻¹, and the other was incubated at 5 °C and 100 μmol photons m⁻² s⁻¹ for 5 days. Mutants exposed to chill-light stress were allowed to grow at 30 °C for 4 days. Those unable to grow were regarded as candidates for increased chill-light sensitivity and subjected to further screening under the same conditions. Candidate mutants were confirmed by evaluating tolerance to chill-light stress as described above, and those with an ARG < 20% of that of the wild-type were considered to have significantly increased chill-light sensitivity.

Inserted genes were determined by inverse PCR, sequencing and similarity searches in the Kazusa cyanobacterial genome database (www.kazusa.or.jp/cyano/cyano.html).

PCR and RT-PCR. PCRs for the purpose of molecular cloning were conducted using Taq/II (1:1) DNA polymerases (Fermentas UAB). dA was added to the ends of PCR products using Taq DNA polymerase before cloning into pMD18-T (Takara), a T vector.

To generate a DNA fragment containing sll1242: C.K2d, PCR was performed with the genomic DNA of the sll1242: C.K2d mutant as the template, using primers sll1242-1 (caagaggtgtatgtgcctca) and sll1242-2 (ggcgttggaaaatgtcggga).

Inverse PCR was performed with genomic DNA restricted and self-ligated. One microgram of genomic DNA from the mutants was digested with Sau3AI, self-ligated in a 50 μl reaction system at 16 °C for 24 h, precipitated with ethanol, and redissolved in 10 μl distilled water. Two microplots of the self-ligated DNA was used in PCR using primers C.K2-16 (acttgcacagcataccgtcgt) and C.K2-2 (cctcttccgaccatcaacag) or C.K2-8 (cctcttccgacactcaaga)/C.K2-15 (ttgagacacaacagcgt).

RT-PCR was conducted as previously described (Ning & Xu, 2004). The relative concentration of cDNA templates was evaluated after serial twofold dilutions by PCRs using primers 16SrRNA-1 (ctctgtgctcgattgca) and 16SrRNA-2 (gactgccccttcagtc) for the cDNA of 16S rRNA. Primers used for sll1242 were sll1242rt1 (aagttgctccagtggg) and sll1242rt2 (ccgctgccaccaactctg). Two independent experiments were performed, which showed consistent results.

Construction of plasmids

Molecular manipulations were performed by standard protocols. Molecular-tool enzymes were used according to the instructions provided by the manufacturers. Sticky ends of DNA fragments were blunted using T4 DNA polymerase (Promega). PCR products were purified by recovery from agarose gels using a DNA gel extraction kit (Jingmei) and cloned using pMD18-T. Restriction enzymes and T4 DNA ligase were purchased from Takara.

Construction of the plasmid for targeted insertion in desD. A DNA fragment containing desD generated by PCR using primers sll0262-1 (cactcttttcagcattgacctgctg) and sll0262-2 (ttgtgaccctgccct) was cloned into pMD18-T and inserted at the BsuI site of desD by C.K2 excised from pRL446 with PvuI, resulting in pHB451.

Construction of the plasmid for complementation of the desD::C.K2 mutant. The DNA fragment containing desD was generated by PCR using primers desD-1 (actctgtacctctgacct) and desD-2 (gcctgcttgctgccc) cloned into the Smal site of pUC19, and confirmed by sequencing, resulting in pHB654. C.CE2, a Cm' and Em' cassette, was excised from pRL598 (Elhai & Wolk, 1988).
Construction of the plasmid for complementation of the \textit{sl}l2142::C.K2d mutant. The DNA fragment containing \textit{sl}l2142 was generated by PCR using primers Gp206-3 (caacagaacttgcccgaaga) and Gp206-6 (agccatagctatgcaaga), cloned into pMD18-T, and confirmed by sequencing, resulting in pHB916. C.CE2 excised from pRL598 with SalI was inserted into the \textit{SalI} site of pHB916. The \textit{sl}l2142::C.CE2 fragment was then excised from pHB937 with SphI and KpnI, and ligated with T4 DNA polymerase, and ligated with the blunted \textit{sl}l2142–C.CE2 fragment. The resulting plasmid pHB945 was used to transform the \textit{sl}l2142::C.K2d mutant.

Construction of the plasmid containing \textit{sl}l2142::luxAB. The \textit{luxAB-\Omega} fragment excised from pRL58 (Black & Wolk, 1993) with Smal was ligated with ApaI-cut and T4 DNA polymerase-blunted pHB916. The correct orientation was identified by restriction analysis with \textit{HindIII}, resulting in pHB2119.

Luciferase activity. The activity of bacterial luciferase was assayed according to Elhai & Wolk (1990). \textit{Synechocystis} sp. PCC 6803 \textit{sl}l2142::luxAB-\Omega was mixotrophically cultured in 250 ml flasks at 30°C at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹ to OD₅₅₀ ~ 1.0, and transferred to 15°C at 30 or 3 μmol photons m⁻² s⁻¹. Samples were taken from the culture at the indicated time intervals and assayed for luciferase activity. Data represent the mean ± SD of measurements from triplicate samples.

Fatty acid composition. Total lipids of cells grown in BG11+glucose in 500 ml flasks at 15°C for 6 days or 30°C for 4 days (OD₇₃₀ ~ 1.5) were collected by centrifugation (6000 g), extracted in petroleum ether/diethyl ether (1:1, v/v) for 3 h, and transesterified in 1 ml 0.4 M KOH/methanol. The fatty acid methyl esters were analysed by GC (HP5890E, Hewlett Packard), employing a glass column 1 ml 0.4 M KOH/methanol. The fatty acid methyl esters were analysed by GC (HP5890E, Hewlett Packard), employing a glass column 1 ml 0.4 M KOH/methanol. The fatty acid methyl esters were analysed by GC (1.8 m × 2 mm) packed with 5% (w/v) diethylene glycol succinate (DEGS). Fatty acids were identified by comparison with the retention times of standards. Quantification was based on known amounts of internal standards (17:0 fatty acid) added to the sample before injection. The relative content of fatty acids (mol%) was expressed with respect to total fatty acids, and data represent the average or mean of values from two or three independent experiments.

Photosynthetic activity. Cells grown autotrophically in 250 ml flasks at 30°C to OD₇₃₀ 0.8–1.0 were collected by centrifugation (6000 g), extracted in petroleum ether/diethyl ether (1:1, v/v) for 3 h, and transesterified in 1 ml 0.4 M KOH/methanol. The fatty acid methyl esters were analysed by GC (HP5890E, Hewlett Packard), employing a glass column 1 ml 0.4 M KOH/methanol. The fatty acid methyl esters were analysed by GC (1.8 m × 2 mm) packed with 5% (w/v) diethylene glycol succinate (DEGS). Fatty acids were identified by comparison with the retention times of standards. Quantification was based on known amounts of internal standards (17:0 fatty acid) added to the sample before injection. The relative content of fatty acids (mol%) was expressed with respect to total fatty acids, and data represent the average or mean of values from two or three independent experiments.

RESULTS AND DISCUSSION

Selection of mutants with increased sensitivity to chill-light stress

\textit{Synechocystis} sp. PCC 6803 kept at 5°C without light could survive for >2 months (data not shown). However, if exposed to light of 100 μmol photons m⁻² s⁻¹, cells completely lost viability, as seen from the c.f.u. ml⁻¹ (Fig. 1) and ARG results (data not shown), at 5°C within 10 days. To set up the conditions to screen for mutants of increased chill-light sensitivity, we first tested a \textit{desD}::C.K2 mutant (Fig. 2A) of \textit{Synechocystis} sp. PCC 6803. After exposure to the chill-light stress for 5 days, the ARG of the \textit{desD}::C.K2 mutant decreased to 5.6 ± 3.0% of that of the wild-type strain (Table 1). In this study, the percentage ARG relative to the wild-type was used to evaluate the tolerance of a mutant to chill-light stress. Complementation of the mutant by integration of \textit{desD} (Fig. 2A) into a platform (Williams, 1988) in the genome restored the ARG to 113.6 ± 52.0%. The increased sensitivity of the \textit{desD} mutant to the chill-light stress probably reflects the role of 18:3 fatty
acids in chill-light tolerance. On the other hand, these results showed the efficacy of chill plus light in identifying a candidate gene required for cold acclimation.

Based on a previously reported procedure (Kong et al., 2003), but taking measures to avoid insertion by multiple DNA fragments, we constructed a random insertion mutant library of Synechocystis sp. PCC 6803. From about 6000 Km-resistant colonies, we identified 14 mutants of increased chill-light sensitivity whose ARG under chill-light conditions was < 20% of that of the wild-type. As shown by inverse PCR and sequencing, six interrupted genes were identified in these mutants, including sll0158 (gltB), sll0726 (pgm), sll2142, slr0193 and slr0688.

**sll2142 is required for chill-light tolerance**

The sensitivity of the selected mutants to chill-light stress could be caused by chill or light, or both. We tested these mutants at 5 °C exposed to different light intensities. In the dark, all mutants showed high ARG values; exposed to light of 100 μmol photons m⁻² s⁻¹, all of them showed significantly increased sensitivity (ARG < 10% of that of the wild-type) (Table 1). However, at 15 μmol photons m⁻² s⁻¹, the mutant sll2142::C.K2d showed an ARG higher than that of desD::C.K2, but significantly lower than that of the other two mutants, sll0158::C.K2d and sll0726::C.K2d (Table 1). Therefore, sll2142::C.K2d, like desD::C.K2, was more sensitive to low light at 5 °C than the other two mutants. At 30 °C, these two mutants grew as the wild-type, even at 100 μmol photons m⁻² s⁻¹ (data not shown). Apparently, it was the combination of chill and light that impaired mutants sll2142::C.K2d and desD::C.K2, with the light playing a crucial role.

The C.K2d Km-resistance fragment was inserted at the AluI site 65 bp from the 5’ end of sll2142 (Fig. 2B). To confirm the role of sll2142 in chill-light tolerance, we generated a PCR product containing sll2142::C.K2d and transformed Synechocystis sp. PCC 6803 with the DNA fragment to reconstruct the sll2142 mutant. The phenotype of the reconstructed mutant was identical to that of the original (data not shown). In addition, complementation of the mutant with sll2142 restored its tolerance to chill-light stress (Fig. 2B, Table 1).

**sll2142 is required for autotrophic growth at 15 °C**

At a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹, Synechocystis sp. PCC 6803 grows autotrophically at 15 °C with 0.47 ± 0.03 doublings per day. Under the same conditions, the sll2142::C.K2d mutant grew at 0.16 ± 0.02 doublings per day, 34.5% that of the wild-type. When complemented by wild-type sll2142, the percentage increased to 85.5%. At 30 °C, the mutant (0.92 ± 0.05 doublings per day) was similar to the wild-type (1.03 ± 0.08 doublings per day). As described elsewhere (Sakamoto & Bryant, 1998), Synechocystis sp. PCC 6803 cells aggregate at 15 °C. We examined the wild-type and mutant cells under the microscope and found that both formed aggregates of comparable size (data not shown).

We compared the photosynthesis activity of the sll2142 mutant with that of the wild-type after incubation at 15 °C at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹. Before the treatment, photosynthetic activities were about the same in the mutant and wild-type. After incubation at 15 °C for over 24 h, the mutant gradually lost photosynthetic activity, while the wild-type showed no reduction (Fig. 3). On the third day at 15 °C, the oxygen evolution rate of the mutant was 24.2% that of the wild-type [160.6 ± 21.0 μmol O₂ (mg chlorophyll)⁻¹ h⁻¹]. This shows that sll2142, at least, is required to maintain the photosynthesis activity at such a low temperature in Synechocystis sp. PCC6803. Due to the effect of sll2142 on chill-light tolerance and autotrophic growth at 15 °C, we named this gene ccr (cyanobacterial cold resistance gene)-1.

In the literature on cold acclimation in Synechocystis sp. PCC 6803, a temperature of ~ 20 °C is usually used for cold stress. However, unlike results with a desA desD double mutant (Tasaka et al., 1996), we found much less or no difference between the sll2142 mutant (0.50 ± 0.03 doublings per day) and the wild-type (0.54 ± 0.01 doublings per day) at 20 °C. The requirement for sll2142 for growth at 15 °C, but not

### Table 1. Sensitivity of the sll2142::C.K2d mutant to chill-light stress

Values shown are the ratio (%) of the ARG of the mutant to that of the wild-type. All determinations were carried out at 5 °C. ND, Not determined.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Light intensity (μmol photons m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>desD::C.K2</td>
<td>5.6 ± 3.0</td>
</tr>
<tr>
<td>Complemented desD::C.K2</td>
<td>113.6 ± 52.0</td>
</tr>
<tr>
<td>sll2142::C.K2d</td>
<td>4.4 ± 1.8</td>
</tr>
<tr>
<td>Complemented sll2142::C.K2d</td>
<td>93.2 ± 13.0</td>
</tr>
<tr>
<td>sll0726::C.K2d</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>sll0158::C.K2d</td>
<td>5.5 ± 4.5</td>
</tr>
</tbody>
</table>
Effects of sll1242 on mixotrophic growth at 15 °C and transcriptional analyses

Under mixotrophic conditions, the growth rate of the sll1242 mutant was 59.9 % that of the wild-type (0.68 ± 0.09 doublings per day) at 15 °C, and 91.7 % that of the wild-type (1.65 ± 0.04 doublings per day) at 30 °C. At 15 °C, the photosynthetic activity of the mutant was reduced to 40.7 % that of the wild-type [212.4 ± 37.1 μmol O₂ (mg chlorophyll)⁻¹ h⁻¹] within 3 days, which partially explained the inhibition of the mixotrophic growth of the mutant at this temperature.

We employed a DNA microarray to analyse the effect of sll1242 on gene expression at 15 °C. Many other bacteria (data not shown). Based on the results presented in this paper, we propose that sll1242, and possibly its homologues in other cyanobacteria, is required for chill-light tolerance and growth at low temperature.

Table 2. Fatty acid composition (mol%) of the total glycerolipids from the wild-type and sll1242 mutant strains

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Wild-type</th>
<th>sll1242::C.K2d</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>15 °C</td>
<td>30 °C</td>
</tr>
<tr>
<td>16:0</td>
<td>43.73 ± 3.36</td>
<td>48.67 ± 1.42</td>
</tr>
<tr>
<td>16:1 (9)</td>
<td>7.50 ± 1.21</td>
<td>5.33 ± 1.24</td>
</tr>
<tr>
<td>18:0</td>
<td>1.90 ± 0.04</td>
<td>1.71 ± 0.69</td>
</tr>
<tr>
<td>18:1 (9)</td>
<td>11.42 ± 5.48</td>
<td>12.23 ± 4.53</td>
</tr>
<tr>
<td>18:2 (9,12)</td>
<td>9.67 ± 0.33</td>
<td>15.71 ± 0.93</td>
</tr>
<tr>
<td>18:3 (6,9,12)</td>
<td>10.63 ± 1.50</td>
<td>13.93 ± 3.62</td>
</tr>
<tr>
<td>18:4 (6,9,12,15)</td>
<td>9.46 ± 1.12</td>
<td>1.35 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>5.95 ± 0.69</td>
<td>1.07 ± 0.24</td>
</tr>
</tbody>
</table>
Fig. 4. Induced expression of sll1242 in Synechocystis sp. PCC 6803 under mixotrophic conditions after transfer from 30 to 15°C. (A) RT-PCR detection of the relative mRNA level of sll1242 in cells at 30°C (4 days; lanes 1–6, serial twofold dilutions of cDNA) and 15°C (4 days; lanes 7–12, serial twofold dilutions of cDNA) at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹. (B) Insertion of luxAB-Ω into sll1242 and complementation. (C) Expression of luxAB from the promoter of sll1242. 1, sll1242::luxAB-Ω transferred from 30°C, 30 μmol photons m⁻² s⁻¹, to 15°C, 30 μmol photons m⁻² s⁻¹; 2, sll1242::luxAB-Ω transferred from 30°C, 30 μmol photons m⁻² s⁻¹, to 15°C, 30 μmol photons m⁻² s⁻¹, to 15°C, 30 μmol photons m⁻² s⁻¹, to 30°C, 30 μmol photons m⁻² s⁻¹, to 30°C, 30 μmol photons m⁻² s⁻¹, Chla, chlorophyll a.

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