DNAマイクロアレイを用いた
ラン藻 *Synechocystis* sp. PCC 6803 における
塩ストレス及び高浸透圧ストレス誘導性遺伝子
の網羅的解析

総合研究大学院大学

生命科学研究科 分子生物機構論専攻

兼崎　友

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要旨

本研究は、ラン藻における塩ストレス及び高浸透圧ストレス応答の機構を分子レベルで解明するために行われた。

第1章では、本研究の意義及びストレス応答に関する研究の背景をまとめた。植物のストレス応答機構を解明することの重要性と、そのためのモデル系として、ゲノム解析が終了したラン藻 *Synechocystis* sp. PCC 6803 が有利な材料であることについて述べた。また、*Synechocystis* 用の DNA マイクロアレイを用いた遺伝子発現解析と、ラン藻においてこれまでに明らかにされている塩ストレス耐性の機構をまとめた。

第2章では、塩ストレス及び高浸透圧ストレスが、ラン藻に対して生理レベルでどのような効果を及ぼすかについて解析した結果をまとめた。細胞の生育においては、*Synechocystis* は塩ストレスよりも高浸透圧ストレスに対してより感受性を示した。また、0.5M NaCl もしくは 0.5 M sorbitol が引き起こす高浸透圧ストレスによる脱水効果を比較するため、スピンプローブを用いた EPR 法により細胞質体積の変化を解析した。その結果、0.5M sorbitol は、長時間にわたって細胞質体積を大きく減少させるが、0.5M NaCl による効果は相対的に弱く、しかも一時的なものであることが明らかになった。これらの結果、塩ストレスが細胞に及ぼす主要な効果はイオンによるストレスであり、高浸透圧ストレスとは生理レベルで異なっていることが明らかになった。

第3章では、さらに遺伝子発現のレベルでの塩ストレスと高浸透圧ストレスの比較を行うため、DNA マイクロアレイを用いた網羅的な遺伝子発現解析を行った。その結果、塩ストレス及び高浸透圧ストレスにより、全遺伝子中の約 1 割の遺伝子が、発現の誘導もしくは抑制を受けることが明らかになった。

そのうちの約半数は、コードするタンパク質の機能が同定されていない遺伝子であった。これらは、塩ストレス及び高浸透圧ストレスに対する耐性及び適応の機構が、これまで考えられてきたよりも複雑であることを示唆するものであった。また、DNA マイクロアレイにより得られた発現の誘導比は、従来の Northern blotting 法と比べてもそれほど大きな差が無いことも明らかになった。さらに、ストレスにより発現の変化が見られた遺伝子を誘導比に基づいて分類した結果、塩ストレスもしくは高浸透圧ストレスに特異的な発現の変化を示す遺伝子群が存在することが明らかになった。それらの多くは、細胞内の特定の機能や化合物の代謝に関わる一連のタンパク質をコードした遺伝子群であることが明らかになった。塩ストレス特異的に発現が誘導される遺伝子は、リボゾームタンパク質などの一群であった。高浸透圧ストレス特異的に発現が誘導される遺伝子は、リポタンパク質などの一群であった。さらに、塩ストレスと高浸透圧ストレスの両方で発現が誘導される遺伝子は、熱ショックタンパク質やシグマ 70 因子などの一群であった。同様に、ストレスに特異的な発現の抑制を受ける遺伝子も、同じ機能に関わる遺伝子間で共通の制御を受けている傾向があることが明らかになった。塩ストレス特異的に発現が抑制される遺伝子は、脂質不飽和化酵素などの一群であった。また、塩ストレスと高浸透圧ストレスの両方で発現が抑制される遺伝子は、光化学系Ⅰやフィコビリゾームを構成するタンパク質などの一群であった。これらの結果は、細胞における塩ストレスと高浸透圧ストレスの作用点の違い、及びシグナル伝達経路の違いを示唆するものであった。一方で、両方のストレスに共通した遺伝子発現制御の機構が存在することも示唆された。

第 4 章では、総合考察として、DNA マイクロアレイによる解析の利点及び問題点に関して論じた。また、本研究の今後の展望について考察した。

目次

第１章

研究背景

環境応答及び適応の機構に関する研究の意義

生物は地球上の様々な環境に棲息している。そして、刻々と変化する苛酷な自然環境の中で様々な生存戦略を駆使して生きている。生命の歴史とは、環境応答と適応の歴史と言っても過言ではなく、そのメカニズムを理解することは、生物と環境との関わりや生物進化の歴史を考える上で非常に重要なことである。

一方、近年、地球規模での温暖化が問題になっている。温暖化によって起こる被害については諸説様々であるが、一般的に気候変動の振幅が大きくなり、局地的な豪雨や暴風雨、熱波、寒波などの異常気象が増えると言われている。その結果、生態系や農作物に大きな被害が及ぶと考えられるが、その被害規模は、世界的な森林面積の減少や増え続ける地球人口などを考慮すれば、年を追うごとに甚大なものになると思われる。また、開発による地球規模での水資源及び土壌汚染も深刻化してきている。生活排水による湖沼の富栄養化や工業排水による重金属汚染など、生態系のバランスを崩しかねない問題が数多く指摘されている。こうした問題は、地球生態系と人間社会の共存を考える上で、避けて通ることのできない道であり、生物学だけでなくあらゆる分野の研究においても考慮すべき課題である。こうした課題に取り組む上で、環境ストレス耐性植物の作出による耕地面積の拡大や、バイオレミディエーションによる環境の再生といった研究は、今後ますます重要になると考えられる。その発展のためには、生物のストレス応答及び環境適応のメカニズムを分子レベルにまで掘り下げて理解することが不可欠であり、膨大な基礎研究に基づく情報の蓄積を行っていかねばならない。

植物のストレス応答及び環境適応

生物が環境の変化に対してどのように応答するかを調べる上で、植物は非常に有用なサンプルである。なぜなら、植物は移動することができないため、温度や乾燥といった環境ストレスから逃げることができないからである。植物にとって環境ストレスに対する防御機構を保持することは生存戦略として重要な意味を持つ。植物が自然界から受ける環境ストレスは、塩、乾燥、高温、低温、凍結、紫外線、酸化、栄養欠乏、及び物理刺激など多種多様であり、それぞれのストレス応答と耐性の機構に関しては、これまでにも多くの研究が行われてきた。近年は特に、分子レベルでのメカニズムの解明に注目が集まっている。一般に、植物細胞は外部環境の変化に伴い様々な遺伝子やタンパク質の発現を誘導することが、ディファレンシャルディスプレイ法や二次元電気泳動法などにより明らかにされている（Strizhov *et al.*, 1997 ; Banzet *et al.*, 1998 ; Majoul *et al.*, 2000 ; Dunaeva and Adamska, 2001)。これらの手法により同定された環境ストレスへの耐性に関わる遺伝子、及びタンパク質に関する知見の一部は、生産性や耐病性を高めた植物の作出などに応用され (Cushman and Bohnert, 2000)、今やそうした遺伝子組換え植物が市場に投入される時代に入った。既に、トウモロコシ、大豆、ジャガイモ、ワタ及びナタネなどの植物で、遺伝子組換え植物の市場投入が始まっている（厚生労働省安全性審査済み遺伝子組換え植物リスト (http://www.mhlw.go.jp/topics/idenshi/ list.html)。しかし、これまでにストレス応答や適応の機構に関わっているタンパク質とそれをコードする遺伝子の発現制御のメカニズムについて明らかにされているのは、ゲノムにコードされている数千、数万の遺伝子のうち、ほんの一握りに過ぎない。ストレス応答及び環境適応のために、いったいどれだけの数の遺伝子及びそこにコードされるタ

ンパク質が、関わっているのか。それらの遺伝子の発現はどのように制御されているのか。こうした点を明らかにするために、さらなる研究が必要とされている。こうした研究を進める上で、ゲノム解析の終了したモデル生物は非常に有利な材料となる。中でも、ラン藻 *Synechocystis* sp. PCC 6803 は、ゲノムレベルでの遺伝子発現の解析が可能な貴重な材料であり、進化的に見ても高等植物と同じ酸素発生型の光合成を行う性質から、葉緑体の起源と考えられており、モデル系としてよく用いられている。

研究材料としてのラン藻

地球に原始生命が誕生してから 35 億年程度の時間が経ったと考えられている。その中でも、ラン藻 (シアノバクテリア) の一群は最も古い生物の一つであり、地球上のあらゆる陸域・水域の生態系に存在している。その細胞は大腸菌と同じく 2 枚の生体膜（外膜と細胞質膜）に囲まれており、内部には複雑なチラコイド膜が形成されている (図 1-1)。ラン藻の色は主に、クロロフィル、カロチノイド、色素タンパクであるフィコビリンなどにより決定される。特に、フィコビリンは、青色のフィコシアニンと赤色のフィコエリトリンという二つの色素を含んでいるが、通常はフィコシアニンの方がはるかに多い。その結果、多くのラン藻は青緑色を呈している。ラン藻属の中でよく知られているものとしては、湖沼のピコ植物プランクトン（細胞サイズが 2 μm 以下の植物プランクトン）の代表ともいえる *Synechococcus* 及び *Synechocystis* や、水面にアオコ (水の華) を発生させる *Microcystis* 及び *Anabaena* 等がある。湖沼におけるラン藻類の大発生は、毒性化学物質を生成するといった水質問題の原因となることが多いため、有害成分の同定や異常増殖の防止などを目的とした、

多くの研究が行われている。また、ラン藻は南極の湖や 80℃以上にもなる高温高圧の熱水鉱泉といった、極端な環境条件にも生息する生物として知られている。驚くべきことに、南極の湖沼で見つかった、*Oscillatoriales*、*Nodularia* 及び *Nostoc* や、高温泉で見つかった好熱性 *Synechococcus* などのラン藻は、これまでに知られた通常温度で生息するラン藻と同属別系統の種であった (Hitzfeld *et al.*, 2000; Miller *et al.*, 2000)。これらのラン藻属は多くの系統を持ち、その分類の一部はリボソーム RNA などの塩基配列による分子系統を反映していないという問題も残っているが、こうした事実はラン藻類のそれぞれの属が特殊な環境条件に適応する能力を持っていること、また、これらのラン藻属のゲノムには極限環境で生きていくための様々なタンパク質をコードした遺伝子情報が存在することを示している。このように、ラン藻はストレス応答や環境適応の分子機構の解明という点においても興味深い材料であるため、多くの研究に用いられている。これら約 1500 種にも及ぶラン藻類の中で、最も分子生物学的手法を用いた解析が進んでいるのが、単細胞性のラン藻、*Synechocystis* sp. PCC 6803 である (図 1-1)。*Synechocystis* sp. PCC 6803 (以下 *Synechocystis*) は、1996 年に Kaneko らにより、全生物中 4 番目に全ゲノム配列が解読された生物で、その全遺伝子数は 3168 個であることが知られている。また、*Synechocystis* は細胞外に存在する DNA 断片を取り込み、自らの遺伝子中の相同領域と組換えを起こす性質を有しており、この性質を利用して容易に遺伝子破壊株を作製することができる。そのため、分子生物学的手法を用いた遺伝子の機能解析にも有用な生物である。さらに、ポストゲノム解析として注目される、トランスクリプトーム及びプロテオームといった手法が既に確立されており、現在最も研究が進んだモデル生物の一つである。中でも、近年開発された DNA マイクロアレイ法による解析は、モデル生物としての *Synechocystis* の価値を飛躍的に高めた。

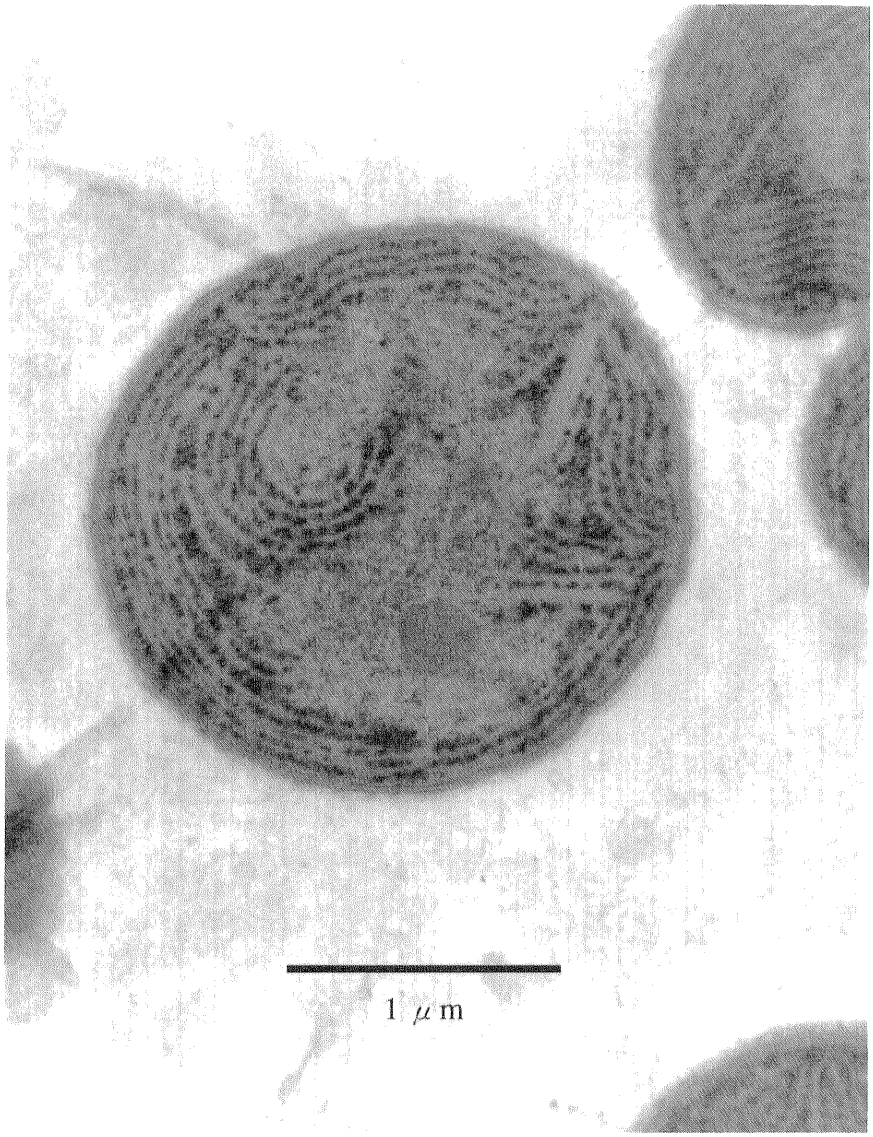


図1-1　ラン藻 *Synechocystis* sp. PCC 6803
淡水性のラン藻で、1.2 M 程度の NaCl 濃度まで生育可能。ゲノムプロジェクトにより、1996年に全ゲノム配列が解析された。

ラン藻における DNA マイクロアレイ法

DNA マイクロアレイ法とは、ガラス基板上に数千の遺伝子断片をスポットした“アレイ”に対し、生育条件の異なる細胞から抽出された全 RNA もしくは mRNA から逆転写反応により合成した２種類の cDNA を競争的にハイブリダイズさせることで、両細胞間で発現量が変化した遺伝子を同定することを可能にした技術である (DeRisi *et al.*, 1997; DeRisi and Iyer, 1999)。*Synechocystis* 用の DNA マイクロアレイ、CyanoCHIP (TaKaRa, 京都) は、全遺伝子の 97% (3079 / 3168 遺伝子) をカバーしており、遺伝子発現の網羅的な解析を可能にした強力な研究ツールである (図 1-2)。本アレイ上にスポットされた遺伝子断片は、PCR により増幅された各 open reading frame の終始コドン側から 1000 bp の double strand DNA である。ただし、全長が 1000 bp に満たない open reading frame に関しては、タンパク質コード領域の全長配列を増幅したものを用いている。本アレイを用いることで、ラン藻において様々なストレスによって発現量が変化する遺伝子をゲノムレベルで解析することが可能になった。真核細胞と異なり、ラン藻のような原核生物では、mRNA は転写後にすぐに翻訳されるため、こうした遺伝子発現の解析は、様々なタンパク質の発現量と相関があると考えられる。これまでに、強光シフト及び低温シフトにより発現の誘導、もしくは抑制を受ける遺伝子についての報告があり、既知の遺伝子だけでなく、多くの機能未同定遺伝子の発現が変化することが分かってきた (Hihara *et al.*, 2001; Suzuki *et al.*, 2001)。さらに、塩、浸透圧、高温、栄養欠乏、及び機械刺激などのストレスによって、どのような遺伝子の発現が制御されているかが解明されれば、ラン藻におけるストレスと遺伝子発現の関係、及び個々のストレスの本質を理解することが可能になると予想される。

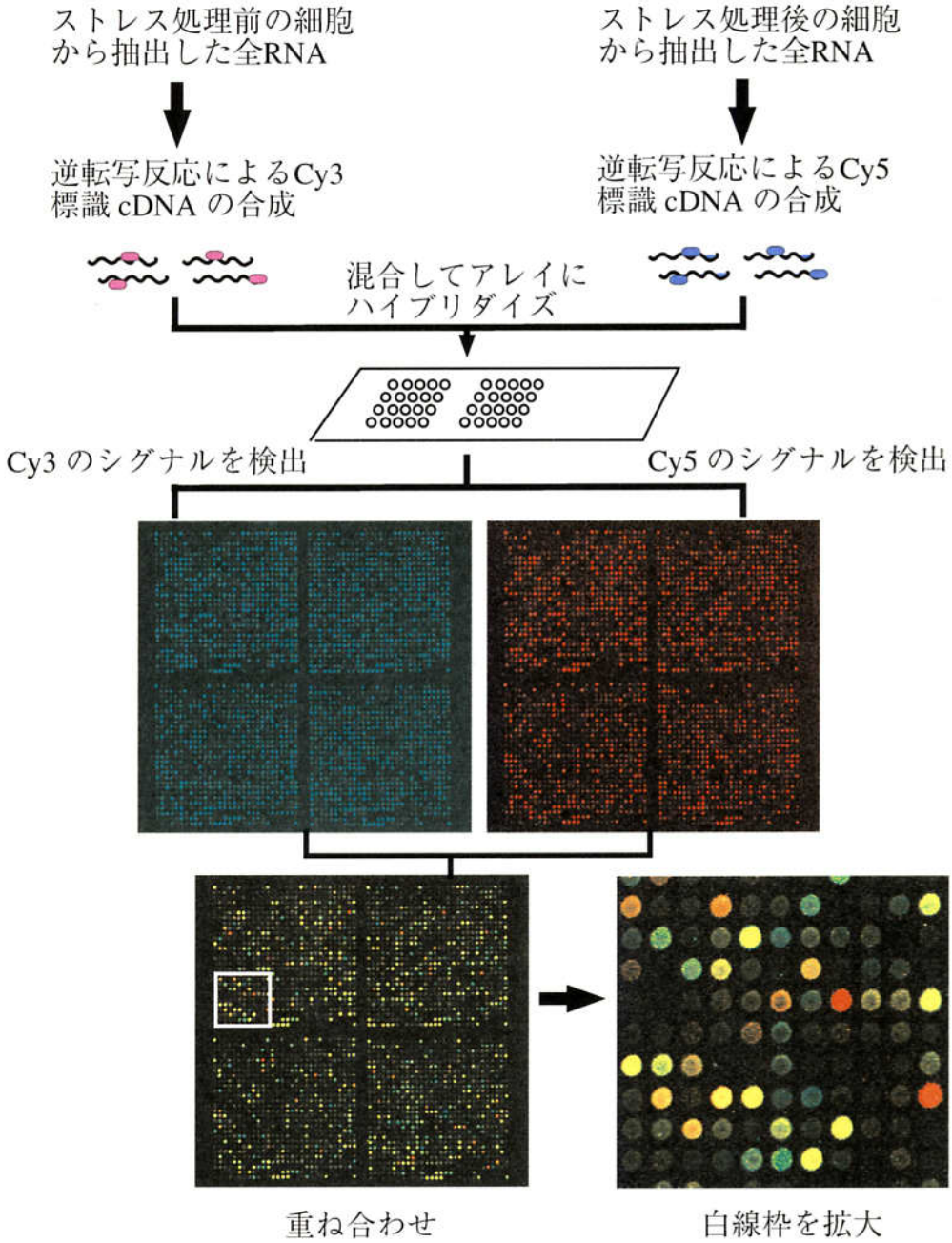


図1-2　DNA マイクロアレイによる遺伝子発現解析例

レーザー照射により得られた画像を重ね合わせ、それぞれのスポットにおけるシグナル強度の変化をストレスの前後で比較する。赤い点は、処理により発現が誘導された遺伝子。緑の点は、処理により発現が抑制された遺伝子。黄色い点は処理により発現の変化が見られなかった遺伝子。

塩ストレス及び高浸透圧ストレスと耐性の分子機構

NaCl に代表される塩ストレスや脱水を伴う高浸透圧ストレスは、植物やラン藻の生育を左右する重大なストレスの一つである。一般に、NaCl ストレスは、細胞に対して浸透圧とイオンによる二つの効果を及ぼす。土壌中もしくは水中の NaCl 濃度の上昇は、水ポテンシャルの低下をまねき、植物の水分吸収を阻害する。また、細胞中に Na^+ イオンが流入することで、タンパク質の変性や酵素活性の低下、及び膜輸送系の阻害が起こると考えられている。そのため、植物細胞は塩ストレスにさらされると、細胞質から過剰な Na^+ イオンの排出を行う。さらに適合溶質と呼ばれる低分子有機化合物を蓄積することで膨圧の低下を防いだりして、水分の保持や浸透圧の調節を行っている (Hayashi *et al.*, 1999)。

Synechocystis においても、Na^+ イオンの排出に関わる Na^+/H^+ アンチポーターをコードした遺伝子ホモログの多重破壊株では、塩ストレス条件下での生育が阻害されることから、同様の機構が保存されていると考えられる (Inaba *et al.*, 2001)。また、*Synechocystis* では、塩ストレスによって糖アルコールの一種であるグルコシルグリセロールが合成、蓄積される (Mikkat *et al.*, 1996)。また、グルコシルグリセロールの合成に関わるタンパク質、GgpS 及び StpA をコードする *ggpS* 遺伝子及び *stpA* 遺伝子は、塩ストレスにより発現が誘導され、また、これらの遺伝子の破壊株は、塩ストレス条件下での生育が阻害される (Kay *et al.*, 1998; Hagemann *et al.*, 1997)。こうした知見から、グルコシルグリセロールが塩耐性に関わっていると考えられる。他にも、グリコプロテアーゼ (Gcp) や脂質不飽和化酵素 (Des) 等をコードする遺伝子の破壊株で塩ストレスにより光合成による酸素発生活性が低下した表現型が得られることが知られている (Zuther

et al., 1998; Allakhverdiev *et al.*, 1999)。

このように、幾つかの遺伝子が、*Synechocystis* の塩耐性に関わっていることは明らかになってきているが、ゲノムレベルで見た場合、どれだけの数の遺伝子が塩ストレス応答、及び塩耐性の機構に関わっているかは、未だ不明である。また、塩ストレスはイオンストレスと高浸透圧ストレスの二つの効果を持つとされるが、こうした遺伝子の発現の誘導が塩ストレスに特異的なのか、それとも高浸透圧ストレスに特異的なのかといった詳細な解析は未だ為されていない。こうした課題を解決する上で、DNA マイクロアレイによるゲノムレベルでの遺伝子発現解析は非常に有用な手段であり、個々のストレスの本質を理解する上で極めて重要な情報を得られるものと考えられる。

第２章

ラン藻 *Synechocystis* sp. PCC 6803 における塩ストレス及び高浸透圧ストレスによる生育阻害及び細胞質体積の変化

2.1 序論

ラン藻は、砂漠や高塩濃度の湖沼にも生存しており (Garcia-Pichel and Pringault, 2001; Brock, 1976)、植物の塩ストレスや高浸透圧ストレスに対する応答機構を分子レベルでの研究を行う上で有用なモデル生物である。一般に、高濃度の NaCl による塩ストレスや、細胞内に浸透しない sorbitol のような物質による高浸透圧ストレスは、水チャンネルを介した細胞質からの水分子の漏出を引き起こすと考えられている (Allakhverdiev *et al.*, 2000a,b)。しかし、ラン藻の場合、ナトリウムイオン (Na^+) が短時間で細胞質に取り込まれるため (Reed *et al.*, 1986)、塩ストレスの初期過程における細胞への効果は、イオンストレスによる影響と高浸透圧ストレスによる影響とを分けて考える必要がある。こうした違いは、生育や遺伝子発現など、様々なレベルで起きていると考えられるが、これまでに詳細な解析は行われてこなかった。そこで、まず我々は塩ストレスと高浸透圧ストレスがラン藻の生育と細胞質体積にどのような影響を与えるかについて検討を行った。その結果、ラン藻の生育は高浸透圧ストレスに対し、より感受性であることが明らかになった。また、ラン藻の細胞質体積は、0.5 M sorbitol による高浸透圧ストレスにより短時間で初期レベルの 30% 程度まで減少し、以後 2 時間はほぼ同じレベルを保つが、0.5 M NaCl による塩ストレスによる処理では、一旦初期レベルの 70% まで減少するが、処理後 45 分で元のレベルにまで回復することが明らかになった。

2.2 材料及び実験方法

実験に用いた細胞と生育条件

本実験で用いた、ラン藻 *Synechocystis* sp. PCC 6803 (Glucose 耐性株) は Dr. J. D. K. Williams (Du Pont de Nemours & Company, Inc., Wilmington, DE) より供与していただいた。細胞は、50 ml の BG-11 液体培地 (Stanier *et al.*, 1971) 中で、34 ℃、光強度 70 μ E/m^{2}s 条件下で培養し、OD_{730}＝0.2-0.3 の対数増殖期に達した段階で実験に用いた (Wada and Murata, 1989)。細胞への塩処理、及び高浸透圧処理は、予め調製した 5 M NaCl 溶液、もしくは 5 M sorbitol 溶液から適量分取し液体培地に加えることで目的濃度にした。

生育曲線の測定

細胞が OD_{730}＝0.2 の対数増殖期に達した段階で、塩もしくは高浸透圧処理を行い、最終濃度 0.5 M とした。以後 12 時間おきに培養液の OD_{730} を測定し、処理を行わなかった細胞との生育を比較した。OD_{730} の測定は、Model 200-20 分光光度計 (日立計測器サービス, 東京) を用いて行った。

細胞質体積の測定

細胞質体積の測定はスピンプローブを用いた EPR 法により行った (Blumward *et al.*, 1983, 1984; Allakhverdiev *et al.*, 2000a)。細胞は 30 ℃、8000 x *g* で 10 分間遠心して集菌し、400 μ g Chl/ml になるように 1.0 mM

2,2,6,6-tetramethyl-4-oxopiperidinooxy free radical (TEMPO)、20 mM $K_3[Fe(CN)_6]$ 及び 75 mM Na_2Mn-EDTA 混液に懸濁した。40 μl の懸濁液を直径 0.02 cm のガラスキャピラリーチューブに封入し室温で測定を行った。ブランクとしては、1.0 mM TEMPO のみを封入した 40 μl キャピラリーを用いた。測定は、model ESP 300E (Bruker, Karlsruhe, Germany) により、暗所で以下の条件下で行った。Field modulation 100kHz; microwave frequency 11.72GHz; modulation amplitude 0.4 mT; microwave power 10 mW; time constant 80 ms; scan rate 0.4 Gs^{-1}.

2.3 実験結果

2.3.1 塩ストレス及び高浸透圧ストレスによる生育阻害効果

ラン藻の生育における、塩ストレス及び高浸透圧ストレスの効果を図 2-1 に示した。対数増殖期に入った細胞 ($O.D._{730} = 0.2$) の培養液に 0.5 M NaCl による塩ストレス及び 0.5 M sorbitol による高浸透圧ストレスをかけることで、細胞の生育速度はそれぞれ、55%及び 40%に減少した。NaCl は水溶液中では解離するため、0.5 M NaCl は、1 osmol の浸透圧を生じるはずである。しかし、生育阻害効果は、sorbitol の方が大きかった。これらより、*Synechocystis* は塩ストレスよりも高浸透圧ストレスに対してより強い感受性を示すことが明らかになった。

2.3.2 塩ストレス及び高浸透圧ストレスの細胞質体積への影響

細胞質体積の変化はそれぞれのストレスによって引き起こされる高浸透圧効果による細胞質からの水分漏出の程度を反映すると考えられる。そのため、細胞質体積の変化を追跡することで、高浸透圧効果のみを比較することができる。本実験では、スピンプローブを用いた EPR 法によって塩ストレス及び高浸透圧ストレスを受けた細胞の細胞質体積の経時変化を観測した。結果を図 2-2 及び 2-3 に示す。実験に使用したスピンプローブである TEMPO ラジカルは安定な水溶性ラジカルであり、細胞質内にも浸透できる (図 2-2A) (Blumwald *et al.*, 1983)。予め TEMPO ラジカルを混合した細胞溶液に対し、細胞質内に浸透しない消光剤 (Mn(II)-EDTA) を加えることで、細胞外に存在するラジカルのスペク

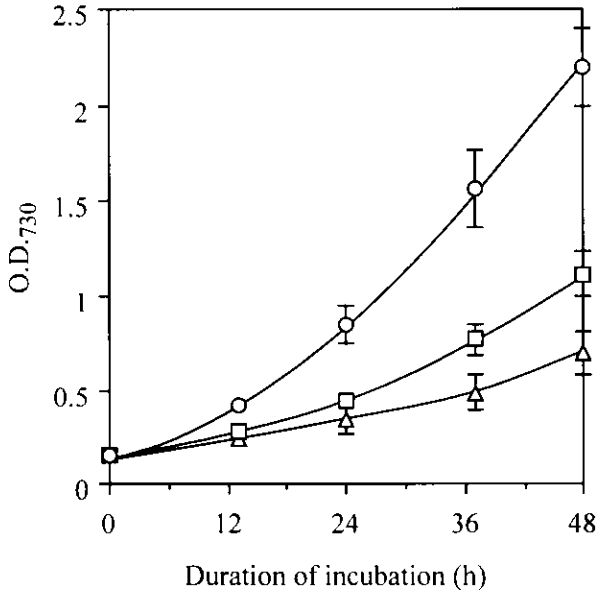


図2-1　0.5 M NaCl 及び 0.5 M sorbitol による *Synechocystis* の生育阻害効果

○, BG-11 培地、□, BG-11 培地 + 0.5 M NaCl、△, BG-11 培地 + 0.5 M sorbitol
細胞濃度が、OD_{730} = 0.2 になった段階 (図中の 0 時間) で、塩処理及び高浸透圧処理を行った。図中の各点及び標準誤差は、3 回の独立した実験を平均して算出した。

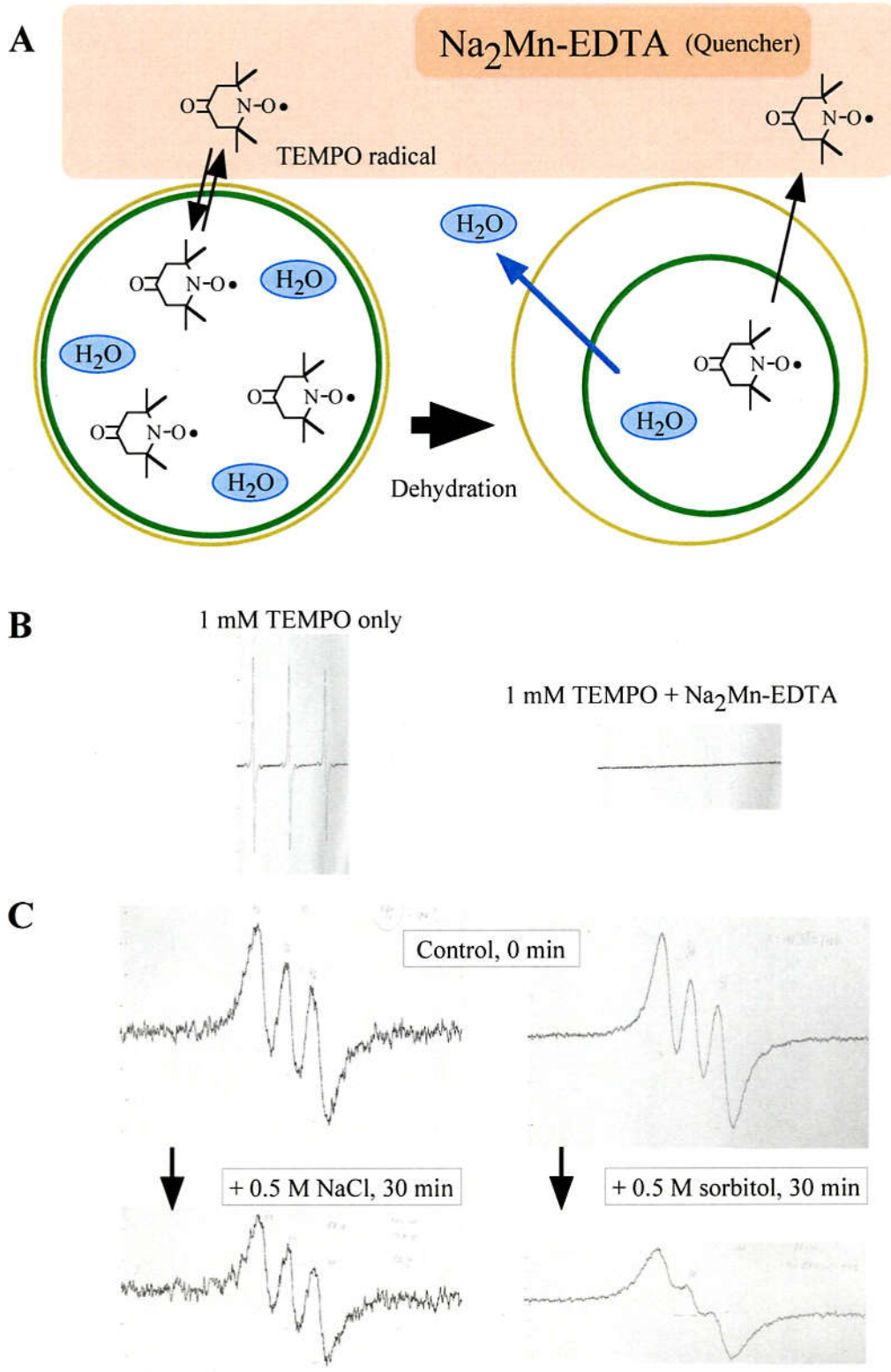


図 2-2　スピンプローブ法による細胞質体積の測定

A, TEMPO ラジカルによるスピンプローブ法の概念図。B, 参照実験。TEMPOラジカルのEPRスペクトル (左) と消光剤存在下でブロードになったスペクトル (右)。C, 細胞質内のラジカル由来のスペクトル及び塩ストレス及び高浸透圧ストレスをかけて 30 分後のEPRスペクトル。

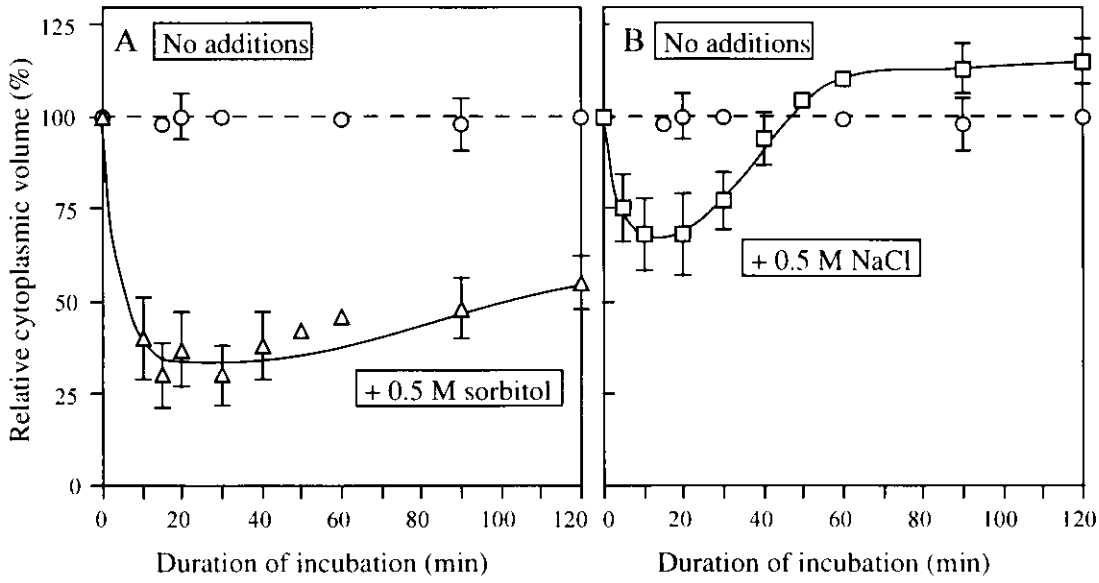


図2-3　細胞質体積に及ぼす 0.5 M NaCl 及び 0.5 M sorbitol の効果

○, BG-11 培地、△, BG-11 培地 + 0.5 M sorbitol、□, BG-11 培地 + 0.5 M NaCl。スピンプローブにより得られる EPR スペクトルから、細胞質体積の変化を追跡した。図中の各点及び標準誤差は、3 回の独立した実験を平均して算出した。

トルがブロードになりピークの検出が困難になる。これにより細胞質内のラジカルに由来するシグナルだけを観測することが可能になる。この状態で塩ストレスもしくは高浸透圧ストレスをかけると、水分の漏出と共に細胞外に放出されたラジカルのピークがブロードになるため、観測される EPR スペクトルの強度が減少する (図 2-2C)。逆に、細胞が水分を再吸収し、再び TEMPO ラジカルが細胞内に取り込まれるとシグナル強度は増大する。これにより EPR スペクトルの強度比から相対的な細胞質体積の変化を追跡することが可能である。

細胞を 0.5 M sorbitol 存在下で処理した場合、細胞質の体積は、高浸透圧処理後 10 分で初期レベルの 30% 程度まで減少し、以後 2 時間はほぼ同じレベルを保つ (図 2-3)。一方、細胞を 0.5 M NaCl 存在下で処理した場合、細胞質の体積は、塩処理後 10 分で初期レベルの 70% まで減少するが、処理後 45 分で元のレベルにまで回復することが明らかになった。こうした細胞質体積の減少は、水チャンネルを介した細胞質からの水分子の漏出によることが知られている (Allakhverdiev *et al.*, 2000a,b)。また、細胞質体積の回復は Na^+/H^+ アンチポーターによる Na^+ の排出 (Inaba *et al.*, 2001)、及び *Synechocystis* の適合溶質として知られるグルコシルグリセロールの蓄積による膨圧の増加の結果、水分子の再吸収により起こると考えられている (Hagemann *et al.*, 1996)。我々は以前、ラン藻 *Synechococcus* sp. PCC 7942 において 0.5 M NaCl 処理及び 1 M sorbitol 処理により、30 分以内に細胞質の体積がそれぞれ 85% 及び 45% 減少することを報告しているが (Allakhverdiev *et al.*, 2000a,b)、今回の結果でも塩ストレス及び浸透圧ストレスの効果は、それらと同様の傾向を示した。NaCl による塩ストレスでは、ナトリウムイオンが細胞質膜を透過して細胞内外のイオン濃度差が減少するため、sorbitol による高浸透圧ストレスの場合に見られるような脱水効果は小さかった。

2.4 考察

2.4.1 塩ストレス及び高浸透圧ストレスが細胞に及ぼす影響

塩ストレスと高浸透圧ストレスが細胞に与える影響は、生理的なレベルで異なっていることが、図 2-1、2-3 の結果より明らかになった。NaCl は水溶液中では解離するため、0.5 M NaCl であれば、1 osmol の浸透圧を生じるはずであるが、生育阻害効果は、0.5 M sorbitol の方が大きかった。また、細胞質体積の減少に関しても、0.5 M NaCl よりも 0.5 M sorbitol による効果の方が強かった。これらの結果は、*Synechocystis* の細胞内に、ナトリウムイオンが取り込こまれることで、相対的な浸透圧差が緩和されていることによると考えられる。これまでに、同属他種のラン藻 *Synechocystis* PCC 6714 などで細胞外に存在する高濃度の塩 (NaCl 等) が数分で細胞質に取り込まれることが知られており、おそらく *Synechocystis* sp. PCC 6803 でも同様の機構が存在すると考えられる（Reed *et al.*, 1986; Nitschmann and Packer, 1992)。このことは、細胞が塩ストレスにより受ける高浸透圧効果は非常に小さく、モル濃度に比例しないことを示唆している。また、図 2-3 で示された結果から、NaCl によるラン藻の生育阻害は主にイオンストレスによるものと考えられる。ナトリウムイオンが細胞質膜を透過し、細胞質に高濃度に蓄積することがイオンストレスの主な特徴であり、膜を介した浸透圧の誘導は主要な効果ではないことが明らかになった。こうした結果から、塩ストレスと浸透圧ストレスは、別々の刺激として細胞に検知されている可能性が示された。

しかし、塩ストレスと高浸透圧ストレスに共通した効果も完全に無視できるほど小さくは無い。例えば、sorbitol のような細胞質膜を透過しない物質が

培養液中に高濃度で存在した場合、細胞は主に高浸透圧による脱水ストレスを受ける (Allakhverdiev *et al.*, 2000a)。しかし、この場合も、脱水効果によって細胞質体積が減少した分だけ、細胞質に存在するイオン濃度が上昇するため、若干のイオンストレスが引き起こされると考えられる。図 2-3 において、高浸透圧ストレス処理後 10 分後の細胞では、細胞質体積が初期レベルの 30% にまで収縮しているが、この状態の細胞質内のイオン濃度は 3 倍上昇していると考えられる。こうした塩ストレスと高浸透圧ストレスの共通の効果は、DNA マイクロアレイなどの遺伝子発現の解析においても考慮する必要がある。

第３章

ラン藻 *Synechocystis* sp. PCC 6803 における塩ストレス及び高浸透圧ストレスによる遺伝子発現の変化

3.1 序論

ラン藻などのバクテリアや、酵母、藻類などは、多くのストレス誘導性遺伝子の発現を誘導することで、多様な環境ストレスに適応している (Los and Murata, 1999; Hagemann *et al.*, 1997)。これまでにラン藻 *Synechocystis* sp. PCC 6803 では、NaCl による塩ストレスによって、グルコシルグリセロールリン酸合成酵素遺伝子 (*ggpS*)、RNA ヘリカーゼ遺伝子 (*crh*)、GroEL 遺伝子 (*cpn60*)、クロロフィル結合タンパク質遺伝子 (*isiA*)、フェレドキシン：$NADP^+$ 還元酵素遺伝子 (*petH*) などの発現が誘導されることが知られている (Engelbrecht *et al.*, 1999; Vinnemeier and Hagemann, 1999; van Thor *et al.*, 2000)。しかし、これらの遺伝子の発現を誘導するのが、NaCl のイオンによる効果なのか、それとも浸透圧による効果なのか、といった検討はこれまで行われてこなかった。また、塩ストレスと高浸透圧ストレスという用語は、時として混同されて用いられることがあり、高濃度の塩にさらされた細胞組織で発現が誘導された遺伝子が Osmo-inducible gene として定義されるケースもある (van Wuytswinkel *et al.*, 2000; Figge *et al.*, 2001)。しかし、これまでに得られた知見から考えると、塩ストレスと高浸透圧ストレスは、異なるシグナルとして細胞に検知されている可能性が高い。本稿第２章の結果からも明らかなように、塩ストレスと高浸透圧ストレスが細胞質体積に及ぼす効果は大きく異なっている。高浸透圧ストレスによる細胞質体積の減少はなかなか回復しないが、塩ストレスによるそれは一過的であった。こうした効果の違いから、ラン藻は塩ストレスと高浸透圧ストレスに対して異なる応答をしていると考えられる。同様に、これらのストレスにより発現が誘導される遺伝子群も塩ストレスと高浸透圧ストレスとでは異なっている可能性が考えられる。そこで、本実験では塩ストレスと高浸透圧ストレ

スによりどれだけの遺伝子の発現が制御されるのかをゲノムスケールで調べるために、DNA マイクロアレイを用いた解析を行い、両者の違いを明らかにすることを試みた。その結果、塩ストレスと高浸透圧ストレスが異なる遺伝子群の発現制御に関わっていることを明らかにした。一方で、熱ショックタンパク質など、二つのストレスに共通の発現制御が見られる遺伝子の一群が存在することも明らかにした。さらに、多くの機能未同定タンパク質をコードした遺伝子群の発現が誘導もしくは抑制されていることを明らかにした。

3.2 材料及び実験方法

実験に用いた細胞と生育条件

本実験で用いた、ラン藻 *Synechocystis* sp. PCC 6803 (Glucose 耐性株) は Dr. J. D. K. Williams (Du Pont de Nemours & Company, Inc., Wilmington, DE) より供与していただいた。細胞は、50 ml の BG-11 液体培地 (Stanier *et al.*, 1971) 中で、34 ℃、光強度 70 μ E/m^{2}s 条件下で培養し、OD_{730}＝0.2-0.3 の対数増殖期に達した段階で実験に用いた (Wada and Murata, 1989)。細胞への塩処理、及び高浸透圧処理は、予め調製した 5 M NaCl 溶液、もしくは 5 M sorbitol 溶液から適量分取し液体培地に加えることで目的濃度にした。Mannitol による処理の際には 2 M 溶液より行った。

DNA マイクロアレイ法による遺伝子の発現解析

塩処理もしくは高浸透圧処理を行った細胞は、30 分間の処理後、直ちに 50 ml の phenol-ethanol 混液 (1 : 10, w/v) で殺菌処理後、集菌し、total RNA の抽出を行った (Los *et al.*, 1997)。RNA は、DnaseI (Nippon Gene, 東京)で処理し、混在する DNA を除いた。DNA マイクロアレイ (CyanoCHIP; TaKaRa, 京都) 用の cDNA は、20 μ g の total RNA から AMV reverse transcriptase (Amersham Pharmasia Biotech, Buckinghamshire, UK) で逆転写して合成した。cDNA の標識には Cy3-dUTP 及び Cy5-dUTP を用いた。ハイブリダイゼーションは 65 ℃で 16 時間行った。その後、2x SSC (1 x SSC; 150 mM NaCl + 15 mM sodium citrate) 溶液で 60 ℃、10 分間、0.2 x SSC + 0.1% SDS 溶液で 60℃、10 分間、さらに 0.2 x

SSC 溶液で室温、10 分間の洗浄を行った。最後に、純水で 2 分間洗浄し、ガラスに付着した水滴をエアースプレイで取り除き、GMS 418 Array Scanner (Affimetrix, Woburn, MA) でシグナルの読み取りを行った。シグナルは、ImaGene ver. 4.0 program (BioDiscovery, Los Angels, CA) により定量した。

Northern blotting 解析

細胞からの全 RNA の抽出、及び Northern blotting 解析は Los らの手法にしたがった (Los *et al.*, 1997)。プローブ作成のために用いた PCR 用プライマーの配列を以下に示した (配列は左より 5′-3′。F: 順方向, R: 逆方向)。

sll1799-F : GGCATGACCCAGATATTTGAT

sll1799-R : GATGATCAGCAAGTTCCGCTC

slr0967-F : TTCAACAACTGCTGGAAACC

slr0967-R : ATCGTCGCCGCCGCCAATACCAATGC

sll0330-F : CGCCGAAGAACTAGCCAAACAAGGTC

sll0330-R : GATGACTCTGCCAACCCACCAAAATT

sll1862-F : AGGAAATTACTTGCTGTCGTTAGATGG

sll1862-R : TACAGGAGGAGAAACAGGATAAGCAT

sll1514-F : TTCTTTACAATCCCCTGC

sll1514-R : TCATCTAGGGTCAGGAG

これらのプライマーを用いて PCR で増幅したそれぞれの Open reading frame の一部は T-vector (Invitrogen, Carlsbad, CA) でサブクローニングした。その後、制限酵素 (*EcoRI* / *SalI*) で切り出し、精製したフラグメント 100 ng を、アルカリホスファターゼでラベルし (AlkPhos Direct kit; Amersham Pharmasia Biotech,

Buckinghamshire, UK) プローブとして使用した。ハイブリダイゼーション後、洗浄したメンブレンは、基質となる CDP-star 溶液に浸し、アルカリホスファターゼとの反応により生じる化学発光を、Luminescence image analyzer (LAS-1000; Fuji-Photo Film, 東京, 日本)で検出し、シグナルの定量を行った。

3.3 実験結果

3.3.1 DNA マイクロアレイによる遺伝子発現解析

塩ストレスと高浸透圧ストレスの生理的な効果が異なるならば、これらのストレスにより発現の制御を受ける遺伝子も異なっている可能性が考えられる。そこで、ラン藻の細胞が塩ストレス及び高浸透圧ストレスによりどのように遺伝子発現の調節を行っているかをゲノムレベルで調べるために、DNA マイクロアレイ法による解析を行った。実験には、塩もしくは高浸透圧による処理後 30 分の細胞を用いた。本条件では NaCl 処理した細胞は主にイオンストレスを受け、また sorbitol 処理では高浸透圧ストレスによる細胞質体積の減少が最大となる (図 2-3)。また、筆者らが *Synechocystis* において塩ストレス及び高浸透圧ストレスにより発現が誘導されることを見出した、低分子量熱ショックタンパク質遺伝子 (*hspA*) の発現の時間変化を Northern 解析により調べた結果 (図 3-1)、細胞に対して 0.5 M NaCl もしくは 0.5 M sorbitol によるストレスを与えた場合、*hspA* 遺伝子の発現量は 30 分でほぼ最大に達することが明らかになった。

対照実験として、ストレスをかける前の細胞から抽出した全 RNA から、Cy3 及び Cy5 で別々に標識した cDNA を合成し、アレイにハイブリダイズさせた結果を図 3-2A に示した。それぞれの遺伝子に対応する個々のスポットのシグナル強度は、全 mRNA のシグナル強度の総和に対する割合で算出する Global standarization 法に基づいて補正した。また、各遺伝子の発現の変化は、ストレスをかける前の全 mRNA 量に対する割合とストレスをかけた後の割合の比を取って比較した。一般に、塩濃度の上昇などにより、転写活性の低下や mRNA の安定性の変化などが起こると考えられるが、本補正法を用いることで、

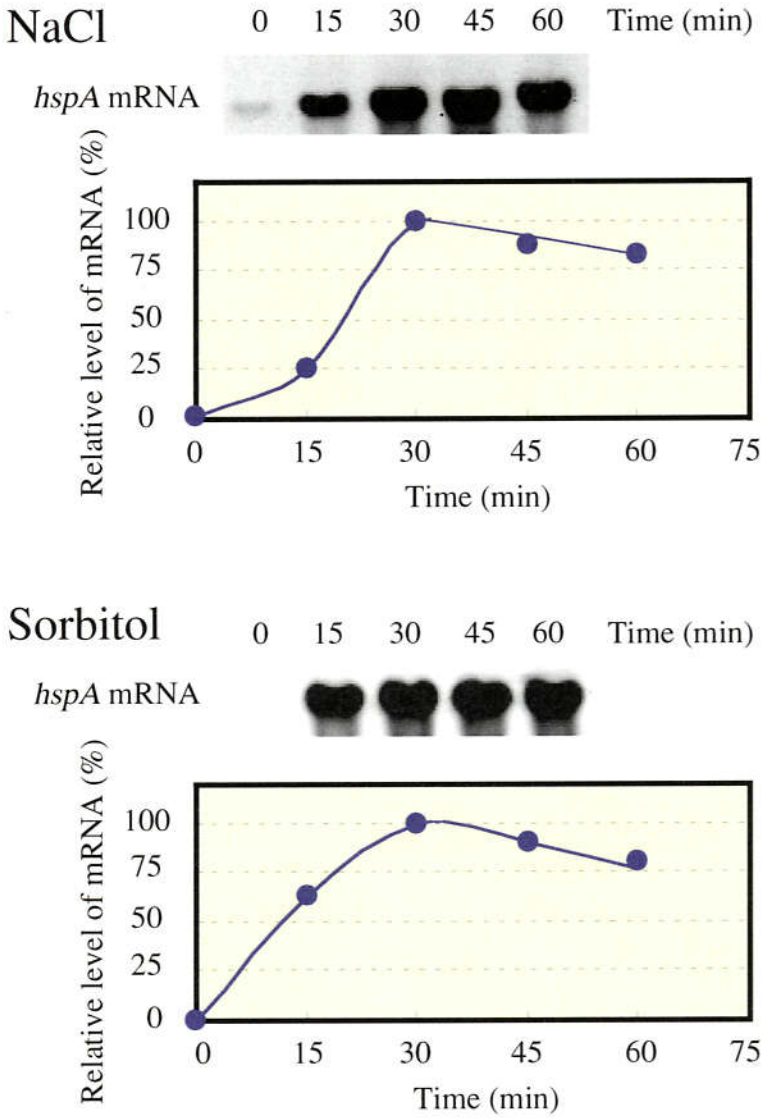


図3-1　*hspA* 遺伝子の発現の時間変化

対数増殖期の細胞 (OD_{730} = 0.2) を 0.5 M NaCl もしくは 0.5 M sorbitol で処理し、15分ごとに集菌し、全RNAを抽出した。

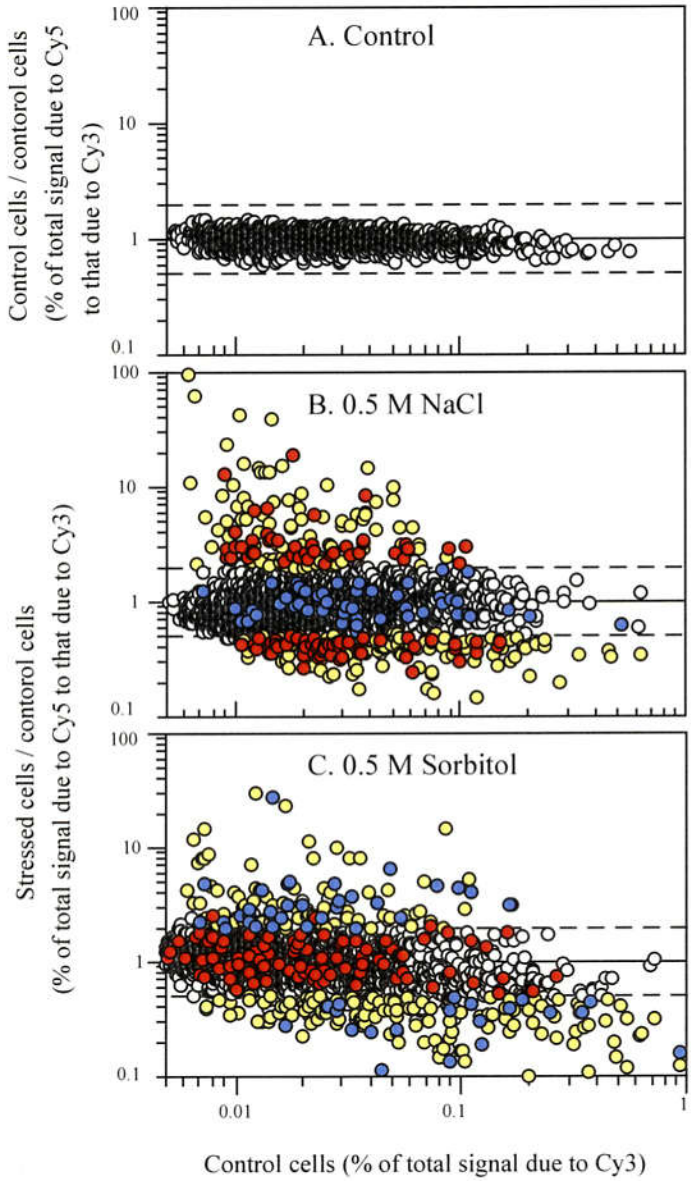


図3-2　DNAマイクロアレイによる塩ストレス及び高浸透圧ストレス誘導性遺伝子の解析

A, 内部標準実験。ストレス処理を行っていない細胞から抽出した同一の全 RNA より、逆転写反応により Cy3 及び Cy5 で標識された cDNA を合成し、アレイに対しハイブリダイズさせた結果。B, 0.5 M NaCl で 30 分処理した細胞より抽出した全 RNA を用いた結果。C, 0.5 M sorbitol で 30 分処理した細胞より抽出した全 RNA を用いた結果。図中の実線は、Cy5/Cy3 比 = 1 (発現の変化無し)を示し、破線は、Cy5/Cy3 比 ＞ 2 もしくは、Cy5/Cy3 比 ＜ 0.5 (遺伝子の発現が2倍以上増減している) を示す。図中の赤、青、及び黄の各点は、それぞれ塩ストレス特異的、高浸透圧ストレス特異的、及び両方のストレスによって発現が誘導もしくは抑制される遺伝子を示す。6 回の独立した実験を行い、同様のパターンを得た。

他の遺伝子に比べ、ストレス下で全 mRNA 量に対する割合が相対的に上昇、もしくは低下する遺伝子を見出すことが容易になる。同一の RNA を別々の色素で標識した場合、その比は理論的には 1：1 になるが、図 3-2A に示したように、全ての点は参照として図示した 2 倍及び 0.5 倍の破線の内側に現れた。この対照実験から、全 mRNA 量に対する割合が 2 倍以上増加した、もしくは 2 倍以上減少した遺伝子は、発現が有意に変化した遺伝子であるといえる。よって、本実験ではこの値を閾値として設定し、以後の解析を行った。

図 3-2B は、0.5 M NaCl 条件で 30 分処理した細胞での個々の遺伝子発現パターンの変化を示している。塩ストレスにより、147 個の遺伝子の発現が誘導された。また、228 個の遺伝子の発現が抑制された。一方、多くの遺伝子の発現は 2 倍以内の増減の範囲であり、塩ストレスによっては変化しないことが明らかになった。*Synechocystis* において、塩ストレスは遺伝子発現を抑制する効果があるという報告があるが (Hagemann *et al.*, 1994)、塩処理前の状態で相対的に強く発現している遺伝子の多くが塩処理により発現が抑制される傾向が見られたことは、こうした結果を裏付けるものであった。

図 3-2C は、0.5 M sorbitol 条件で 30 分処理した細胞での個々の遺伝子発現パターンの変化を示している。高浸透圧ストレスにより、113 個の遺伝子の発現が誘導された。また、161 個の遺伝子の発現が抑制された。また、高浸透圧ストレスの場合も、多くの遺伝子の発現は 2 倍以内の増減の範囲であった。我々は、0.5 M mannitol で処理した細胞を用いて同様の実験を行ったが、発現変化のパターンはほぼ同じであり (図 3-3)、また、sorbitol により強く発現が誘導される遺伝子は、mannitol によっても同様の発現誘導が確認された。これは、sorbitol による遺伝子発現の変化が高浸透圧ストレスによるものであり、sorbitol に特異的な変化ではないことを示している。

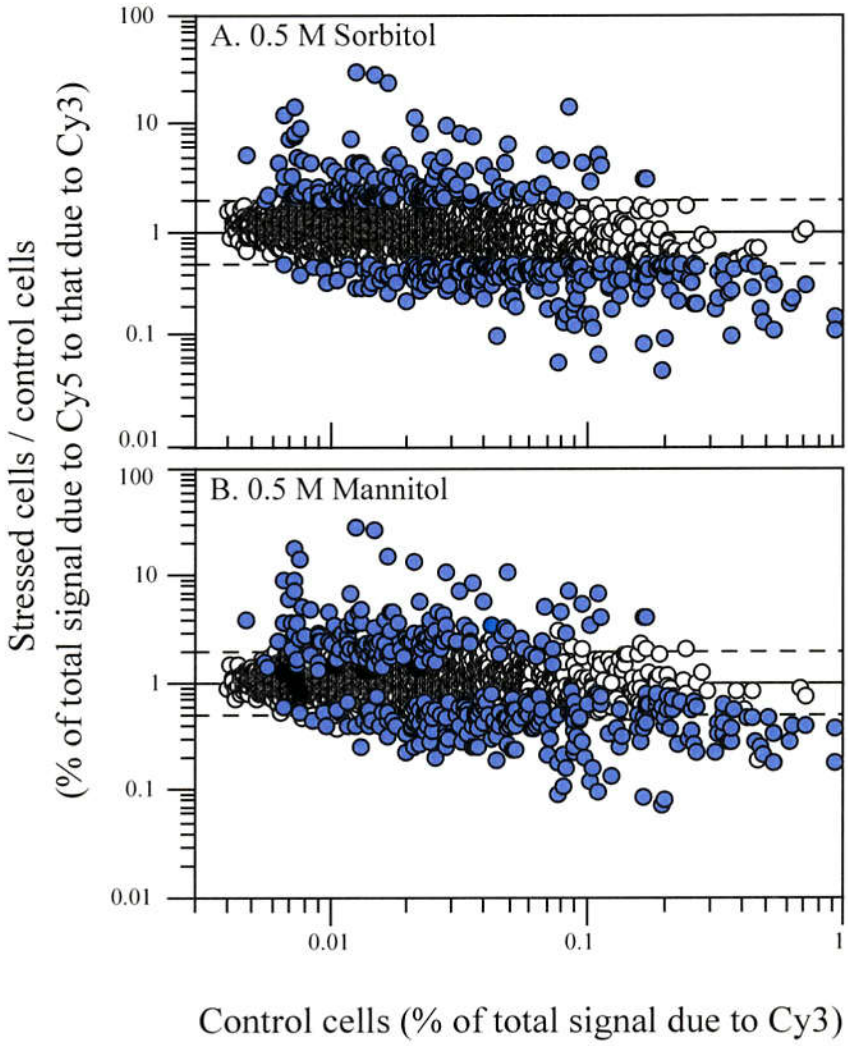


図3-3　sorbitol 及び mannitol による高浸透圧ストレスと遺伝子発現のパターンの変化

図中の青点は、sorbitol によって発現が誘導もしくは抑制された遺伝子。

DNA マイクロアレイにより得られた結果は、かずさ DNA 研究所が公表している *Synechocystis* sp. PCC 6803 のゲノムデータベース (Cyanobase; http://www.kazusa.or.jp/cyano/) の情報に基づいて解析した。各遺伝子がコードするアミノ酸配列の相同性に基づいた命名、及び機能は、Cyanobase に従った。解析の結果、塩ストレス特異的、もしくは高浸透圧ストレス特異的な発現制御を受ける遺伝子の一群が存在することが明らかになった。また、一群の遺伝子は、塩ストレス及び高浸透圧ストレスの両方によって発現の誘導もしくは抑制が行われていることも明らかになった (図 3-2B, 3-2C)。この発見をもとに、塩ストレス及び高浸透圧ストレスにより、遺伝子の発現が 3 倍以上増えた遺伝子を表 1 にまとめた。さらに、塩ストレス及び高浸透圧ストレスにより特異的に発現が変化する遺伝子を選別し、3 つのグループに分類した。それぞれに、塩ストレスにより特異的に発現が誘導される遺伝子群、高浸透圧ストレスにより特異的に発現が誘導される遺伝子群、及び両方のストレスで発現が誘導される遺伝子群をまとめた。表 2 には、塩ストレス及び高浸透圧ストレスにより、遺伝子の発現が 3 倍以上抑制された遺伝子をまとめた。これらに関しても、同様にストレスに対する発現の特異性から、遺伝子のグループ分けを行った。

3.3.2 Northern 解析による結果の追試

DNA マイクロアレイ解析の結果、ストレス特異的な発現の変化を示す遺伝子が多数存在することが明らかになった。しかし、DNA マイクロアレイ解析における問題点として、プローブのクロスハイブリダイゼーションや、残存した標識色素のアレイに対する非特異的な結合などが考えられる。そこで、マイクロアレイによる結果の確認のため、Northern blotting 法により表 1 で分類さ

表1　塩ストレスもしくは高浸透圧ストレスによって発現が誘導される遺伝子

ORF number	Gene	Product	0.5 M NaCl	0.5 M sorbitol
			誘導比 ± 標準誤差	
塩ストレスによって発現が強く誘導される遺伝子（倍）				
slr1704		Protein of unknown function	17.4 ± 1.2	2.3 ± 0.6
slr0967		Protein of unknown function	16.0 ± 3.6	2.5 ± 0.6
sll1621		Membrane protein	8.3 ± 1.1	1.5 ± 0.3
sll1515		Protein of unknown function	7.6 ± 1.1	0.8 ± 0.2
slr1501		Protein of unknown function	7.0 ± 1.2	1.4 ± 0.5
sll1864		Chloride channel protein	6.1 ± 1.9	1.1 ± 0.2
slr1932		Protein of unknown function	5.4 ± 0.6	1.2 ± 0.1
sll1022		Protein of unknown function	4.7 ± 0.4	1.6 ± 0.6
ssr2194		Protein of unknown function	4.7 ± 1.4	1.4 ± 0.4
slr1738		Protein of unknown function	4.4 ± 0.2	1.9 ± 0.4
slr0095		O-Methyltransferase	4.2 ± 0.1	1.4 ± 0.2
sll1086		Protein of unknown function	4.0 ± 0.6	1.6 ± 0.2
slr0529		Protein of unknown function	3.9 ± 0.7	1.3 ± 0.2
sll1167	*pbp*	Penicillin-binding protein	3.8 ± 0.2	1.3 ± 0.5
slr1259		Protein of unknown function	3.7 ± 0.7	1.1 ± 0.2
sll0905	*maf*	maf protein	3.6 ± 0.7	1.3 ± 0.4
sll1236		Protein of unknown function	3.2 ± 0.6	1.1 ± 0.4
slr1916		Esterase	3.2 ± 0.5	1.5 ± 0.4
sll1594	*rbcR*	Rubisco operon regulator	3.2 ± 0.4	1.2 ± 0.2
slr1971		Protein of unknown function	3.1 ± 0.7	1.7 ± 0.5
sll1723		Protein of unknown function	3.1 ± 0.6	1.6 ± 0.4
Slr1604	*ftsH*	Cell division protein	3.1 ± 0.0	1.6 ± 0.3
sll1491		Protein of unknown function	3.0 ± 0.4	1.4 ± 0.3
slr1751	*prc*	Carboxy-terminal protease	3.0 ± 0.2	1.4 ± 0.2
sll0188		Protein of unknown function	3.0 ± 0.2	1.7 ± 0.5
slr1608	*gdhB*	Glucose dehydrogenase-B	3.0 ± 0.4	1.4 ± 0.3
sll0938		Aspartate transaminase	3.0 ± 0.6	1.3 ± 0.4
sll1799	α *rpl3*	50S ribosomal protein L3	3.0 ± 0.2	0.8 ± 0.2
高浸透圧ストレスによって発現が強く誘導される遺伝子				
sll0330	*fabG*	3-ketoacyl-ACP reductase	1.2 ± 0.2	23.9 ± 6.5
slr0423	*rlpA*	Rare lipoprotein A	1.2 ± 0.1	6.2 ± 0.4
slr1748		Protein of unknown function	1.7 ± 0.4	4.6 ± 0.6
slr1119		Protein of unknown function	1.3 ± 0.2	4.2 ± 1.1
slr0381		Lactoylglutathione lyase	0.9 ± 0.1	3.9 ± 0.9
sll0430	*htpG*	Heat-shock protein Hsp90	1.9 ± 0.1	3.8 ± 0.7
ssr1256		Protein of unknown function	0.7 ± 0.1	3.7 ± 0.3
ssl3177	*repA*	Rare lipoprotein A	1.6 ± 0.2	3.6 ± 0.5

sll0185			Protein of unknown function	1.3 ± 0.4	3.3 ± 0.5
sll0293			Protein of unknown function	0.9 ± 0.1	3.1 ± 0.6
slr0753		*p*	P protein	0.6 ± 0.1	3.0 ± 1.0
塩ストレスと高浸透圧ストレスの両方で発現が強く誘導される遺伝子					
sll1862	ε		Protein of unknown function	93.8 ± 7.5	17.2 ± 5.3
sll1514		*hspA*	Heat-shock protein Hsp17	56.2 ± 10.7	26.5 ± 4.5
sll1863	ε		Protein of unknown function	52.7 ± 10.1	8.3 ± 1.8
sll0528			Protein of unknown function	40.0 ± 6.5	20.0 ± 6.0
slr1544			Protein of unknown function	20.3 ± 3.3	7.8 ± 0.9
slr1516		*sodB*	Superoxide dismutase	16.5 ± 1.6	4.9 ± 0.7
sll0939			Protein of unknown function	15.3 ± 3.2	3.5 ± 0.9
sll0846			Protein of unknown function	14.9 ± 1.5	10.1 ± 1.4
slr1674	ε		Protein of unknown function	14.2 ± 0.8	10.6 ± 1.2
ssr2595			High light-inducible protein	13.4 ± 1.6	7.0 ± 1.4
sll1106			Protein of unknown function	12.6 ± 2.8	3.4 ± 0.8
sll1085	ε	*glpD*	Glycerol-3-P dehydrogenase	11.8 ± 2.1	3.2 ± 0.5
sll0306		*rpoD*	RNA polymerase sigma factor	11.5 ± 0.3	7.2 ± 2.3
sll0170		*dnaK*	DnaK protein	11.5 ± 2.8	4.1 ± 0.9
sll1566	ε	*ggpS*	Glucosylglycerol-P synthase	10.7 ± 1.5	3.7 ± 0.5
slr1675	ε	*hypA*	Hydrogenase-related protein	10.1 ± 1.5	7.8 ± 2.1
slr1963			Protein of unknown function	10.0 ± 1.4	9.1 ± 2.4
slr0093		*dnaJ*	DnaJ protein	9.6 ± 1.6	3.2 ± 0.8
sll1884			Protein of unknown function	9.4 ± 1.1	3.9 ± 0.3
slr1687	γ		Protein of unknown function	9.4 ± 2.2	3.2 ± 0.9
slr1641		*clpB*	ClpB protein	8.4 ± 1.2	3.5 ± 1.2
sll0788	β		Protein of unknown function	8.3 ± 1.3	7.0 ± 0.6
sll1483			Protein of unknown function	7.8 ± 1.3	22.9 ± 0.6
slr1603			Protein of unknown function	7.2 ± 0.8	3.9 ± 1.1
slr0959			Protein of unknown function	6.6 ± 1.2	4.2 ± 1.1
ssl1633			CAB/ELIP/HLIP superfamily	6.5 ± 0.9	5.1 ± 0.5
sll0416		*groEL2*	60-kDa chaperonin	6.2 ± 0.4	3.2 ± 1.1
ssl3044	γ		Hydrogenase component	5.7 ± 1.5	3.5 ± 1.2
ssr2016			Protein of unknown function	5.5 ± 0.9	4.6 ± 0.5
ssl2542		*hliA*	HLIP	5.0 ± 0.8	4.8 ± 1.8
slr1204		*htrA*	Serine protease	4.8 ± 0.3	4.6 ± 0.7
sll0789	β	*copR*	Response regulator	4.4 ± 0.9	3.9 ± 0.9
ssl2971			Protein of unknown function	4.3 ± 0.7	4.0 ± 0.2
slr0581			Protein of unknown function	3.9 ± 0.7	3.3 ± 0.3

対数増殖期の細胞（OD_{730} = 0.2）を 0.5 M NaCl 及び 0.5 M sorbitol で 30 分処理し、全 RNA を抽出した。表中の数値は、細胞中の全 mRNA 量に対するそれぞれの遺伝子の発現割合を処理前と処理後で比を取った値、及び標準誤差を

示す。数値は、独立に行われた6回の実験の平均値。 Open reading frame の命名は、 Kaneko *et al.* (1995, 1996)らの報告に基づく。全遺伝子のリストは、以下のサイトに公開している。 http//synecho.genome.ad.jp: 8081/

表2　塩ストレスもしくは高浸透圧ストレスによって発現が抑制される遺伝子

ORF number	Gene	Product	0.5 M NaCl	0.5 M sorbitol
			Extent of repression	
塩ストレスによって発現が強く抑制される遺伝子　(倍)				
slr0294		Protein of unknown function	3.7 ± 0.9	1.3 ± 0.2
sll1453	*nrtD*	Nitrate transport protein	3.5 ± 0.4	1.3 ± 0.6
ssr0536		Protein of unknown function	3.4 ± 0.5	1.7 ± 0.2
slr1658		Protein of unknown function	3.3 ± 0.5	1.3 ± 0.2
sll1693		Protein of unknown function	3.2 ± 0.6	1.7 ± 0.5
slr0213	*guaA*	GMP synthetase	3.1 ± 0.8	1.7 ± 0.2
ssl2084	*acp*	Acyl carrier protein	3.1 ± 0.5	1.4 ± 0.6
slr0591		Protein of unknown function	3.0 ± 0.8	0.9 ± 0.2
sll0262	*desD*	Delta-6 desaturase	3.0 ± 0.3	1.3 ± 0.3
ssl2874		Protein of unknown function	3.0 ± 0.7	1.2 ± 0.5
高浸透圧ストレスによって発現が強く抑制される遺伝子				
slr1634		Protein of unknown function	1.5 ± 0.7	7.2 ± 1.0
sll0381	ζ	Protein of unknown function	0.5 ± 0.1	5.9 ± 1.1
sll0382	ζ	Protein of unknown function	0.5 ± 0.1	3.7 ± 0.6
slr1272		Protein of unknown function	1.4 ± 0.1	3.7 ± 0.7
sll1626	*lexA*	SOS regulatory protein	1.2 ± 0.5	3.4 ± 1.1
sll0383	ζ *cbiM*	CbiM protein	0.5 ± 0.0	3.0 ± 0.9
塩ストレスと高浸透圧ストレスの両方で発現が強く誘導される遺伝子				
sll1783	η	Protein of unknown function	8.9 ± 2.0	6.8 ± 0.2
slr1854	η	Protein of unknown function	8.9 ± 1.6	10.3 ± 2.0
sll1785	η	Protein of unknown function	8.0 ± 2.1	5.4 ± 0.5
slr1852	η	Protein of unknown function	7.7 ± 1.3	5.5 ± 1.0
sll1784	η	Protein of unknown function	7.2 ± 1.6	5.0 ± 0.5
slr0737	*psaD*	Photosystem I subunit II	5.8 ± 0.9	3.6 ± 0.1
slr1855	η	Protein of unknown function	5.5 ± 1.2	5.0 ± 0.7
slr2051	*cpcG*	Phycobilisome rod-core linker	5.5 ± 1.1	4.3 ± 0.3
slr1277	*gspD*	Secretion pathway protein D	4.9 ± 0.7	3.7 ± 0.4
sll0819	*psaF*	Photosystem I subunit III	4.5 ± 1.3	4.5 ± 0.8
sll1091		Bacteriochlorophyll synthase	3.9 ± 0.3	3.0 ± 0.5
slr0335	*apcE*	Phycobilisome LCM linker	3.8 ± 0.6	4.3 ± 0.1
slr0653	*rpoDI*	RNA polymerase sigma factor	3.8 ± 1.2	4.0 ± 0.5
slr1459	*apcF*	Phycobilisome core component	3.8 ± 0.5	3.6 ± 1.0
sll1305	η	Protein of unknown function	3.7 ± 0.8	3.0 ± 0.6
slr2067	ι *apcA*	Allophycocyanin a chain	3.6 ± 0.7	4.1 ± 0.1
slr1986	ι *apcB*	Allophycocyanin b chain	3.6 ± 0.5	3.3 ± 0.4
sll0427	*psbO*	Mn-stabilizing polypeptide	3.5 ± 0.8	3.5 ± 0.3
sll1580	θ *cpcC*	Phycocyanin-associated linker	3.4 ± 0.8	8.2 ± 0.4

sll1577	θ	*cpcB*	Phycocyanin b subunit	3.4 ± 1.1	7.2 ± 0.8
sll1712			DNA-binding protein HU	3.4 ± 1.0	3.7 ± 1.1
slr1859			Protein of unknown function	3.3 ± 0.4	3.0 ± 0.6
sll0661		*ycf35*	Protein of unknown function	3.3 ± 0.8	3.6 ± 0.8
slr1276			Protein of unknown function	3.2 ± 0.1	3.4 ± 0.6
slr0011		*rbcX*	Protein of unknown function	3.1 ± 0.8	3.5 ± 1.0
slr1856	η		Protein of unknown function	3.0 ± 0.3	4.2 ± 0.6

詳細は表１に記載。

れた幾つかの遺伝子の発現を塩ストレス及び高浸透圧ストレス条件下で比較した (図 3-4 及び表 3)。塩ストレス特異的な発現誘導を示す遺伝子群に含まれる *sll1799* (*rpl3*) 及び *slr0967*、高浸透圧特異的な発現誘導を示す遺伝子群に含まれる *sll0330* (*fabG*)、両方のストレスにより発現が誘導される遺伝子群に含まれる *sll1862* 及び *sll1514* (*hspA*) 遺伝子の発現は、それぞれアレイと同様の傾向を示した (図 3-4)。さらに Northern 法で得られたシグナルを 16S rRNA 量で補正し、通常条件での発現量を 1 とした場合の誘導比を、DNA マイクロアレイの結果と並べた (表 3)。その結果、DNA マイクロアレイで得られる遺伝子発現の誘導比は、Northern 法で得られる値の約 1/2 程度であることが明らかになった。DNA マイクロアレイ解析でのシグナル補正は Global standarization 法によるため、直接の比較はできないが、Northern 法とほぼ同程度の検出感度で得られることが明らかになった。

3.3.3 塩ストレス特異的に発現が誘導される遺伝子群

塩ストレスにより特異的に発現が誘導された遺伝子のうち、3 倍以上発現が誘導されたのはリボゾームタンパク質 (*rpl3*)、タンパク質の修飾や分解にかかわる酵素 (*prc* 及び *ftsH*)、塩化物イオンチャンネル (*sll1864*)、転写調節因子 (*rbcR*)、細胞膜の機能に関わるタンパク質 (*sll1621*、*pbp* 及び *maf*)、解糖系の酵素 (*gdhB*)、及び多くの機能未同定タンパク質 (*slr1704*、*slr0967*、*sll1515*、*slr1501*、*slr1932* 及び *sll1022*) といった遺伝子であった。

Synechocystis のゲノム DNA 上では、リボゾームタンパク質 *rpl2*, *rpl3*, *rpl4* 及び *rpl23* は一つのオペロンを構成している (図 3-5A)。塩ストレスによってこれらの遺伝子の発現量はそれぞれ 2.0 倍、3.0 倍、2.3 倍、及び 2.7 倍増加

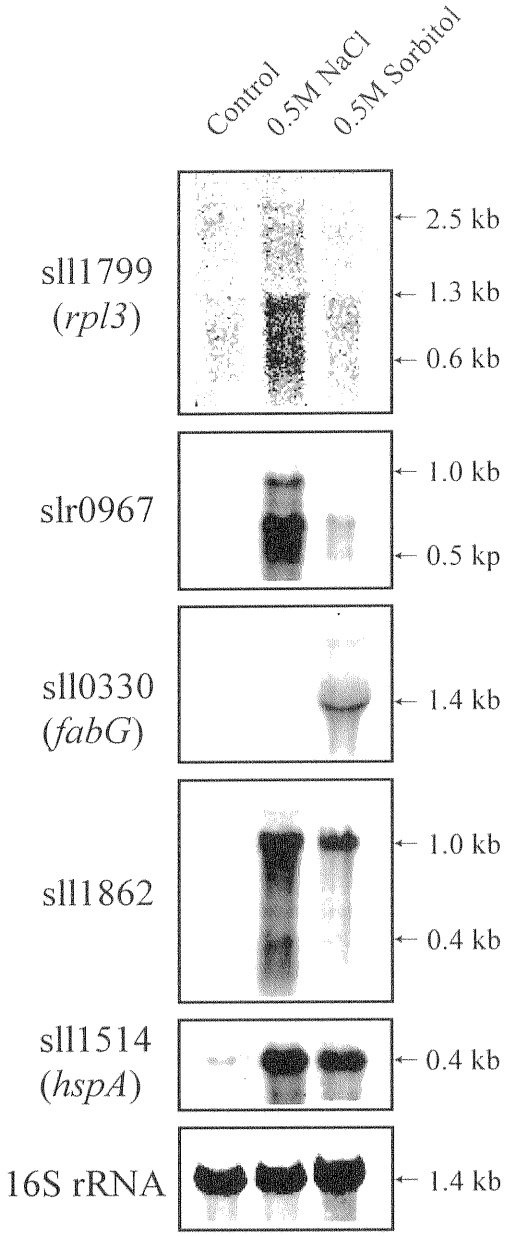


図3-4　Northern blotting 法による塩ストレス及び高浸透圧ストレス誘導性遺伝子の発現解析

0.5M NaCl もしくは 0.5 M sorbitol で 30 分処理した細胞から抽出した全 RNA を用いて解析を行った。実験には 5 μg の全 RNA を用い、1.4% formaldehyde 変性ゲル (1%) で泳動を行った。2回の独立の実験により同様の結果を得た。

表3　**Northern 法と DNA マイクロアレイ法の比較**

Northern 法によって得られた各 orf に対応する mRNA のバンド (図 3-4) は定量後、16S rRNA 量で補正した。通常状態での発現量を 1 として誘導比を算出し、DNA マイクロアレイにより得られた結果と比較した。

ORF	Northern		マイクロアレイ	
	NaCl	sorbitol	NaCl	sorbitol
sll1799	**2.8**	1.1	**3.0**	0.8
slr0967	**33.5**	4.1	**16.0**	2.5
sll0330	1.8	**53.1**	1.2	**23.9**
sll1862	**135.8**	**37.9**	**93.8**	**17.2**
sll1514	**129.9**	**71.9**	**56.2**	**26.5**

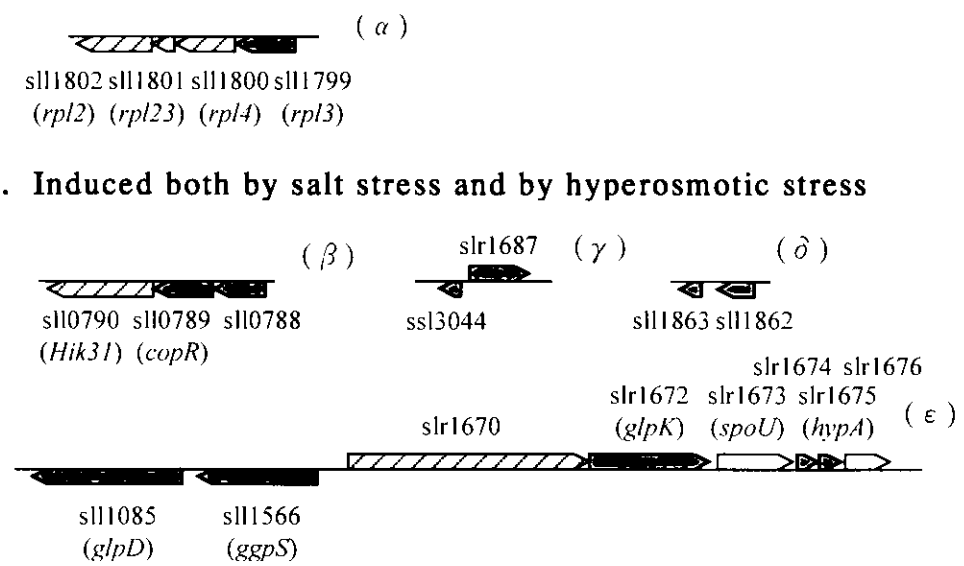


図3-5　塩ストレス及び高浸透圧ストレスにより発現の制御を受ける遺伝子クラスター

A, 塩ストレス特異的に発現が誘導される遺伝子クラスター。B, 塩ストレス及び高浸透圧ストレスにより発現が誘導されるクラスター。C, 高浸透圧ストレス特異的に発現の抑制が見られる遺伝子クラスター。D, 塩ストレス及び高浸透圧ストレスにより発現の抑制が見られる遺伝子クラスター。それぞれの矢印の向きは遺伝子の転写方向を示し、矢印の長さは Open reading frame の長さに対応する。黒い矢印は発現が 3 倍以上誘導、もしくは抑制された遺伝子 (表 1 及び 2 参照) を示す。斜線のついた矢印は発現の変化が2倍以上3倍以下であった遺伝子を示す。白い矢印は塩ストレス及び高浸透圧ストレスにより発現に変化が見られなかった遺伝子を示す。

した。また、Northern 解析の結果、これらの遺伝子は同じ転写単位を構成している可能性が高いことが明らかになった (図 3-4)。既に明らかにされている、リボゾームラージサブユニットの結晶立体構造と比較すると、*rpl2*、*rpl3*、及び *rpl4* 遺伝子がコードする L2、L3、及び L4 タンパク質は、ペプチジルトランスファー活性部位の近傍に存在することが明らかになった (Ban *et al.*, 2000; Nissen *et al.*, 2000)。

ATP 依存性金属プロテアーゼ、FtsH はチラコイド膜に存在し、失活した D1 タンパク質の分解に関わっていることが、シロイヌナズナで報告されている (Lindahl *et al.*, 1996, 2000)。*Synechocystis* のゲノムには *ftsH* のホモログは 4 つ存在しており、それらは、*sll1463*、*slr0228*、*slr1390* 及び *slr1604* の各 ORF にコードされている (Kaneko *et al.*, 1995, 1996)。このうち、*slr1390* 及び *slr1604* 遺伝子の発現は、塩ストレスにより 2.1 倍、及び 3.1 倍増加したが、他の ORF の発現に変化は見られなかった。

カルボキシル末端プロテアーゼ、CtpA は、光化学系 II の反応中心に存在する D1 タンパク質の修飾に関わる酵素で、D1 タンパク質前駆体の C 末端の 16 残基を切断し、成熟した D1 タンパク質を生成する反応を触媒する (Shestakov *et al.*, 1994; Inagaki N *et al.*, 2001)。CtpA をコードする遺伝子、*ctpA*、及びそのホモログである *prc* 遺伝子の発現は塩ストレスにより、それぞれ 2.7 倍及び 3.0 倍誘導されたが、高浸透圧ストレスによっては誘導されなかった。

ラン藻は、バクテリアから動物細胞まで広く保存されている塩化物イオンチャンネル (CLC ファミリー) (Thiemann *et al.*, 1992) の遺伝子ホモログを一つだけ持つが、その発現も塩ストレス特異的に誘導された。一方、ナトリウムイオンの取り込み、及び排出に関わる、sodium-coupled permease 及び Na^+/H^+ アンチポーターをコードする遺伝子、また、ナトリウムの排出過程で取り込まれ

ることが知られているカリウムイオンのトランスポーター遺伝子などの発現は塩ストレスによっても誘導されなかった。

3.3.4 高浸透圧ストレス特異的に発現が誘導される遺伝子群

高浸透圧ストレスにより特異的に発現が誘導された遺伝子のうち、3 倍以上発現が誘導されたのは、3-ケトアシル-ACP 還元酵素 (*fabG*)、レアリポタンパク質 (*rlpA* 及び *repA*)、熱ショックタンパク質 Hsp90 (*htpG*)、ラクトグルタチオン分解酵素 (*slr0381*) 及び機能未同定タンパク質 (*slr1748*、*slr1119*、*sll0185* 及び *sll0293*) 遺伝子であった。これらの遺伝子の発現は塩ストレスによっては誘導されなかったことから、塩ストレスと高浸透圧ストレスによって発現が制御される遺伝子群は明らかに異なっていることが明らかになった。*Synechocystis* において、塩ストレスでは誘導されず、高浸透圧ストレスのみによって発現が誘導される遺伝子が報告されるのは、これが初めてである。

3-ケトアシル-ACP 還元酵素、FabG は、3-ケトアシル-ACP を還元して 3-ヒドロキシアシル-ACP を生成する反応を触媒する、脂肪酸合成過程における炭素鎖伸長反応に必要な酵素である。*Synechocystis* のゲノムには、*sll0330*、*slr1994*、及び *slr0886* の 3 種類の FabG のホモログをコードする遺伝子が存在するが、高浸透圧ストレスで発現が誘導されるのは、*sll0330* 遺伝子のみであった。またこれらのどの遺伝子の発現も、塩ストレスによっては誘導されなかった。一般に、短鎖脂質の炭素鎖伸長反応に関わる 3-ケトアシル-ACP 還元酵素の C 末端には特異的なモチーフが存在するが、*sll0330* 遺伝子がコードするタンパク質の予想アミノ酸配列の C 末端にはそれらは見当たらなかった。

HtpG タンパク質は、バクテリア、酵母から高等生物の器官にまで広く

保存される、Hsp90 ファミリーの一員である (Buchner, 1999)。*Synechococcus* sp. PCC 7942 では、*htpG* 遺伝子の発現は熱ショックにより誘導され、高温耐性に必須なタンパク質であることが既に報告されている (Tanaka *et al.*, 1999)。*slr0381* 遺伝子は、ラクトイルグルタチオンリアーゼのホモログをコードしており、細胞内の酸化還元状態の制御に関わる化合物であるグルタチオンの代謝に関わっている可能性が考えられる (Cabiscol *et al.*, 2000)。しかし、これらの遺伝子の発現が、高浸透圧条件下でのみ誘導されることの生物学的な意味は不明である。

3.3.5 塩ストレス及び高浸透圧ストレスの両方で発現が誘導される遺伝子群

塩ストレス及び高浸透圧ストレスの両方により発現の誘導が見られた遺伝子のうち、両方のストレスにより 3 倍以上発現が誘導された遺伝子は、グルコシルグリセロール合成系の遺伝子 (*glpD* 及び *ggpS*)、熱ショックタンパク質 (*hspA*、*dnaK*、*dnaJ*、*htrA*、*groEL2*、及び *clpB*)、スーパーオキシドジスムターゼ (*sodB*)、ヒドロゲナーゼ関連の遺伝子(*hypA*)、強光誘導性遺伝子 (*hliA*、*ssr2595*、及び *ssl1633*)、シグマ 70 因子 (*rpoD*) 及びレスポンスレギュレーター (*copR*)、及び機能未同定の遺伝子群であった (表 1)。

グルコシルグリセロールの合成に関わる *glpD* 及び *ggpS* 遺伝子は、それぞれ、グリセロールリン酸化酵素及びグルコシルグリセロールリン酸合成酵素をコードしている (Hagemann *et al.*, 1996)。また、これらの遺伝子は *Synechocystis* のゲノム上に隣り合って並んでいる。図 3-5B に、表 1 の遺伝子のうち、クラスターを構成するものを図示した。図中のギリシャ文字は、表中の文字に対応する。

熱ショックタンパク質遺伝子の発現は、枯草菌において熱ショックのみ

ならず、塩ストレス、エタノール添加、及び栄養欠乏により誘導されることが知られている (Hecker *et al.*, 1996)。*Synechocystis* においては、熱ショックにより熱ショックタンパク質遺伝子の発現が誘導されることは既に報告されているが (Lehel *et al.*, 1992; Lee *et al.*, 1998)、それ以外のストレスによりこれらの遺伝子の発現誘導を確かめられたのは、今回が初めてである。HtrA (DegP) は、セリンプロテアーゼの一種で、チラコイド膜に局在し、強光失活した D1 タンパク質などの膜局在性のタンパク質を分解する (Itzhaki *et al.*, 1998)。HspA は、熱変性したタンパク質を安定化し、DnaK/DnaJ/GrpE 及び GroEL/GroES シャペロンネットワークによるタンパク質のリフォールディング行い易くする (Török *et al.*, 2001)。

3.3.6 塩ストレス特異的に発現が抑制される遺伝子群

塩ストレスにより特異的に発現が抑制された遺伝子のうち、3 倍以上発現が抑制されたのは、脂質不飽和化酵素 (*desD*)、アシル基運搬酵素 (*acp*)、GMP 合成酵素 (*guaA*)、硝酸トランスポーター (*nrtD*)、及び機能未同定の遺伝子群 (*slr0294*、*ssr0536*、*slr1658*、*sll1693*、*ssl0213*、*slr0591*、及び *ssl2874*) であった (表 2)。

Synechocystis のゲノム中には、4 種類の脂質不飽和化酵素の遺伝子 (*desA*、*desB*、*desC*、及び *desD*) が存在しており、それぞれがアシル脂質の炭素鎖上の異なる部位に二重結合を導入するタンパク質をコードしている (Murata and Wada, 1995)。これらのタンパク質は、生体膜の構造と機能を調節する、重要な働きを持っている (Los and Murata, 1998)。我々は、これらの脂質不飽和化酵素のうち *desB*、*desC*、及び *desD* 遺伝子の発現が塩ストレスにより、それぞれ

2.0 倍、2.7 倍、及び 3.0 倍抑制されることを見出した (*desB* 及び *desC* 遺伝子は表 2 にはリストされていない)。これらの結果は、膜脂質の不飽和度と塩ストレス適応機構に関係があることを示唆していると考えられる。

3.3.7 高浸透圧ストレス特異的に発現が抑制される遺伝子群

高浸透圧ストレスにより特異的に発現が抑制された遺伝子のうち、3 倍以上発現が抑制されたのは、コバルトトランスポーター (*cbiM*)、SOS 応答転写抑制因子 (*lexA*) 及び機能未同定遺伝子群 (*slr1634*、*sll0381*、*sll0382*、及び *slr1272*)であった。

これらのうち、*sll0381*、*sll0382*、及び *cbiM* 遺伝子は、一つの遺伝子クラスターに含まれており、共通の発現制御を受けている可能性が高い (図 3-5C)。コバルトイオンはコバラミンの合成に必要であるが、高浸透圧との関係は解明されていない。

SOS 応答は、細胞において DNA の損傷が起きた場合に観察される応答機構で、大腸菌で多くの知見が得られている (Shinagawa, 1996)。転写抑制因子である *lexA* 遺伝子の発現抑制は、高浸透圧ストレスにより DNA の損傷が起きたことによるものと考えられる。

3.3.8 塩ストレス及び高浸透圧ストレスの両方で発現が抑制される遺伝子群

塩ストレス及び高浸透圧ストレスの両方により発現が抑制された遺伝子のうち、両方のストレスにより 3 倍以上発現が誘導された遺伝子は、光化学系 I (*psaD* 及び *psaF*)、フィコビリゾーム構成タンパク質 (*cpcB*、*cpcC*、*cpcG*、

apcA、*apcB*、*apcE* 及び *apcF*)、シグマ 70 因子 (*rpoDI*)、運搬タンパク質 (*gspD*) 及び機能未同定タンパク質遺伝子群であった。

光化学系 I、及びフィコビリゾームを構成するタンパク質の多くは、ゲノム上で遺伝子クラスターを形成している (図 3-5D)。これらのクラスターに含まれる遺伝子は全て、塩ストレス及び高浸透圧ストレスの両方により発現が 2 倍以上抑制された。

また、物質輸送経路に関わるタンパク質の遺伝子 *gspD* の発現も両方のストレスによって抑制された。GspD タンパク質は、外膜に局在することが、*Pseudomonas aeruginosa* で報告されている (Bleves *et al.*, 1999)。物質輸送経路に関わるタンパク質の遺伝子としては、*Synechocystis* は 2 つの *gspD* 遺伝子、及び 2 つの *hofG* 遺伝子をコードしているが、これら全てが両方のストレスにより発現が 2 倍以上抑制された。

3.4 考察

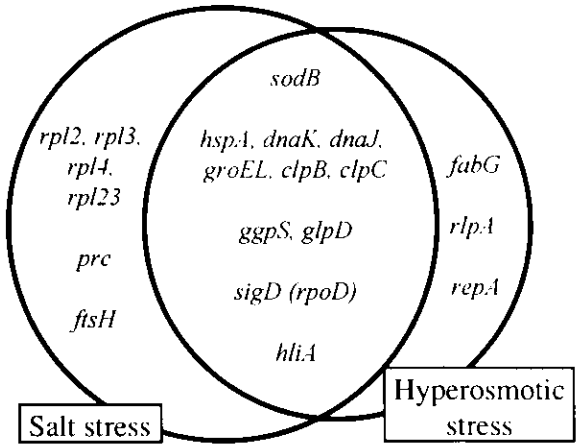
3.4.1 ストレス特異的な発現の変化を示す機能既知遺伝子の分類

DNA マイクロアレイにより明らかになった、数多くのストレス特異的な発現の誘導もしくは抑制を示す遺伝子群のうち、推測されるアミノ酸配列が機能既知のタンパク質とホモロジーを示すものを、機能別に分類し、図 3-6 に示した。その結果、リボゾームのサブユニットや、脂質不飽和化酵素など、特定の機能もしくは物質代謝に関わる一群の遺伝子の発現は、ストレス特異的に誘導されることが明らかになった。また、両方のストレスによって発現が誘導される遺伝子も、熱ショックタンパク質やグルコシルグリセロール合成経路の酵素など、特定の機能及び代謝に関わるものが多いことが明らかになった。一般に、細胞における塩ストレスと高浸透圧ストレスの効果は、転写、翻訳、タンパク質の活性など多岐に及ぶと考えられるが、少なくとも図 3-6 で示された遺伝子発現の傾向から、これらのストレスの作用点は明らかに異なっていることが示唆された。

3.4.2 塩ストレス特異的な発現の誘導もしくは抑制を受ける遺伝子群

塩ストレス特異的に発現が誘導された遺伝子は、主に塩ストレスによって引き起こされるイオンストレスによって発現の調節を受けていると考えられる。例えば、高い塩濃度はリボゾームの構造の不安定化を引き起こすとされるため、細胞は L2、L3、及び L4 タンパク質の *de novo* 合成を行うことでリボゾームの活性を維持している可能性が考えられる。また、光化学系 II の反応中

A. Stress-inducible genes



B. Stress-repressible genes

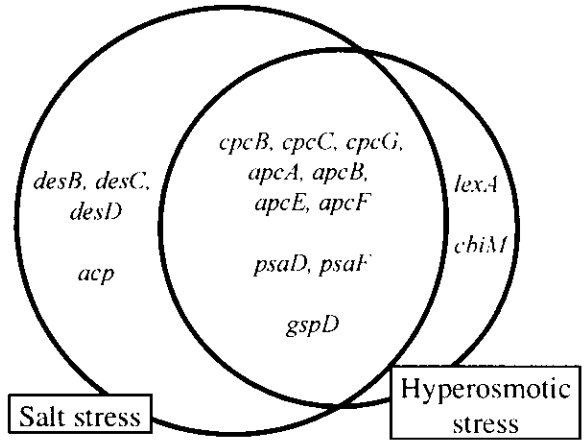


図3-6 塩ストレス及び高浸透圧ストレスにより発現の制御を受ける遺伝子の分類

A, 塩ストレス及び高浸透圧ストレスにより発現が誘導される遺伝子の場合。
B, 塩ストレス及び高浸透圧ストレスにより発現が抑制される遺伝子の場合。
それぞれの円の大きさは、それぞれのストレスにより発現が変化した遺伝子数に比例する。円の重なり部分は、両方のストレスによって発現が誘導もしくは抑制された遺伝子を示す。

心に存在するD1 タンパク質の分解に関わるプロテアーゼ (FtsH)、及び *de novo* 合成された D1 タンパク質前駆体の修飾に関わるカルボキシル末端プロテアーゼ (CtpA, Prc) の遺伝子の発現が塩ストレス特異的に誘導されることは、イオンストレスによりD1タンパク質の代謝が阻害を受ける可能性を示唆している。一般に、D1 タンパク質は、強光存在下では容易に失活する性質を持っており (Aro *et al.*, 1993)、植物細胞は損傷したD1タンパク質を光化学系 II の反応中心からはがしてストロマのプロテアーゼで分解し、新たに合成された D1 タンパクを反応中心に結合させることで光合成活性を維持している。この D1 タンパク質の素早い代謝は、光化学系 II の活性の調節に非常に重要な役割を果たしている。これらの結果は、塩ストレスにより細胞内での D1 タンパク質の代謝が、転写レベル、翻訳レベルのみならず、失活後の分解やタンパク質前駆体の修飾のレベルにおいても妨げられている可能性を示唆している。FtsH、CtpA 及び Prc 等のタンパク質をコードする遺伝子の発現誘導は、おそらくこうしたストレスによる代謝阻害を補償するためと考えられる。

また、塩化物イオントランスポーターホモログの遺伝子が塩ストレス特異的に発現の誘導を受けたことは、細胞質内への塩化物イオンの浸透が起きている可能性及びその濃度の調整が細胞質体積の回復や塩耐性に重要であることを示唆している。逆に、ナトリウムイオン量の調節や塩耐性に関わると考えられている sodium-coupled permease、Na^+/H^+ アンチポーター (Inaba *et al.*, 2001)、及びカリウムイオンチャンネルなどの遺伝子の発現は塩ストレスによって誘導されなかった。これは、これらのタンパク質が既に十分な量で存在している可能性、及び遺伝子発現以外のレベルでタンパク質の活性を制御されている可能性を示唆している。

3.4.3 高浸透圧ストレス特異的な発現の誘導もしくは抑制を受ける遺伝子群

一般に、高浸透圧ストレスによる細胞からの水分の漏出は、細胞質体積を減少させ、原形質分離やペリプラズムの構造や状態の変化を引き起こす (Wilkinson and Northcote, 1980)。また細胞質体積が減少することで細胞質膜の脂質や膜タンパク質は凝縮されると考えられている (Wilkinson and Northcote, 1980)。こうしたペリプラズム、もしくは細胞質膜の状態の変化は、細胞が高浸透圧ストレスを感じて遺伝子発現の調節を行う上での最初のシグナルとなる可能性が考えられる。

実際、高浸透圧ストレス特異的に誘導された遺伝子がコードするタンパク質の中には、その機能が細胞壁やペプチドグリカンの合成との関連が示唆されるものが見出された。例えば、高浸透圧ストレスにより最も強く発現の誘導の見られた遺伝子である、*fabG* (*sll0330*) 遺伝子がコードするタンパク質と高い相同性を持つ *Zea mays* L. の GL8 タンパク質は、表皮ワックスを構成する化合物に含まれる超長鎖脂肪酸の合成に関わっていることが明らかになっている (Xu *et al.*, 1997)。こうした表皮ワックスは、疎水バリアとしての働きを持つとされており、推測ではあるが、*sll0330* 遺伝子がコードしているタンパク質は、高浸透圧耐性に関わる何らかの化合物、もしくは細胞壁を構成するリポポリサッカライドなどの特殊な脂肪酸化合物の合成に関わっている可能性が考えられる。

また、高浸透圧ストレスによってのみ、その発現が誘導されたレアリポタンパク質、*rlpA*、及び *repA* 遺伝子も細胞質膜やペプチドグリカンの合成と関わっている可能性がある。大腸菌では、新たに合成されたレアリポタンパク質は、一旦、ペリプラズムに輸送された後、脂肪酸を付加され細胞質膜に局在することが知られている (Takase *et al.*, 1987)。さらに *rlpA* 遺伝子は、大腸菌のゲ

ノム上でペプチドグリカン合成系の酵素、*mrdAB* と共にオペロンを形成している。高浸透圧により細胞質体積が減少した場合、原形質分離が起こりペプチドグリカン層は大きくダメージを受けると予想されるが、細胞はこうしたタンパク質を新たに合成し、細胞壁の再構築を行うのかもしれない。

3.4.4 塩ストレス及び高浸透圧ストレスの両方で発現が誘導もしくは抑制される遺伝子群

高浸透圧ストレスにより、細胞質体積が減少すると、細胞質内の構成物やイオンの濃度が増加するため、細胞には若干の塩ストレスもかかっている可能性が考えられる。両方のストレスによって発現の誘導が観測された遺伝子の一群のうち、塩ストレスによってより強い誘導が見られるものが多かったことは、こうした塩濃度の上昇による結果である可能性も考えられる。例えば、グルコシルグリセロールの合成に関わる、*glpD* 及び *ggpS* 遺伝子は、高浸透圧条件よりも塩ストレス条件下でより強く発現しており、このことはグルコシルグリセロールが、塩ストレスに特異的な適合溶質である可能性を示している。

一方、熱ショックタンパク質遺伝子の発現は、塩ストレスや高浸透圧ストレスが多くのタンパク質の安定性に深く関わっていることを示している。塩ストレスや高浸透圧ストレス条件下では、細胞は失活したタンパク質が細胞内に蓄積させないために、シャペロンやプロテアーゼを合成していると考えられる。さらに、Hagemann *et al.* (1994) らは、run-on assay により、684 mM の NaCl 存在下では *Synechocystis* の遺伝子の発現は、抑制されることを報告している。これらのシャペロンは、ストレスによって低下した、遺伝子発現に関わる何らかのタンパク質の活性を補償するために発現が誘導されるのかもしれない。

塩ストレス及び高浸透圧ストレス下で、転写レベルでの遺伝子発現の調節もしくは転写の阻害が起きている可能性を示す別の要素は、これら両方のストレスにより、二つの *rpoD* 遺伝子 (*sll0306* 及び *sll2012*; Goto-Seki *et al.* (1999) らによるラン藻のシグマ 70 因子の分類に従うと、それぞれ *sigB* 及び *sigD* に対応する) の発現が誘導され (表 1)、逆に *rpoD1* 遺伝子 (*sigA*) の発現が抑制されたことである (表 2) (*sll2012* は表 1 にはリストされていない)。*Synechocystis* は、5 つのシグマ 70 因子のホモログを持っている。発現の抑制が見られた *rpoDI* 遺伝子は、生育に必須なシグマ因子であるが、*sll0306* 及び *sll2012* を含む他の 4 つのホモログは破壊株においても通常条件での生育に影響が無いことが知られている (Goto-Seki *et al.*, 1999)。こうしたシグマ因子は、転写の阻害を補償するために発現するものと考えられる。大腸菌や枯草菌では、熱ショックタンパク質のストレス依存的な発現は、特異的なシグマ因子によって制御されている。しかし、*Synechocystis* においては、熱ショックタンパク質遺伝子の塩ストレス及び高浸透圧ストレス下における発現が、シグマ因子によってどのような制御を受けているかは未だ解明されていない。しかし、*sll2012* 及び *sll0306* 遺伝子は、枯草菌の *sigB* 遺伝子と高い配列相同性を示すため、同様の制御を受けている可能性も考えられる。さらに、両方のストレスにより、*rpoDI* 遺伝子の発現が抑制され、逆に 2 つのシグマ 70 遺伝子 (*slr0306* 及び *sll2012*) の発現が誘導されたことは、細胞がストレスに応じて細胞内のシグマ因子数の調節を行ったり、シグマ依存的な遺伝子の発現調節を行ったりしている可能性を示唆している。

一方、塩ストレス及び高浸透圧ストレスにより発現が抑制された遺伝子には、光化学系 I、及びフィコビリゾームを構成するタンパク質をコードする遺伝子が多く含まれたことは、光合成活性の調節が遺伝子発現のレベルで行われ

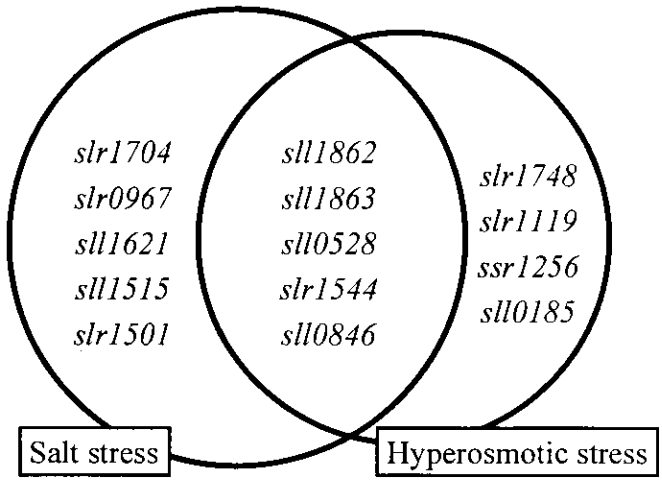
ていることを示している。我々は、塩ストレス及び高浸透圧ストレスにより、光化学系 I の電子伝達活性が阻害されることを既に報告しているが (Allakhverdiev *et al.*, 2000a,b)、これらの遺伝子の発現が抑制されることが光合成活性の調節に重要であると考えられる。また、これらのタンパク質の多くはチラコイド膜に局在しており、塩ストレス及び高浸透圧ストレスにより膜の構造や膜タンパク質の活性が阻害される可能性が示唆される。また、物質輸送経路に関わるタンパク質をコードする遺伝子群の発現も両方のストレスによって抑制されたことは、塩ストレス及び高浸透圧ストレスのターゲットが、外膜や細胞質膜、及び膜タンパク質の活性など、幅広い範囲に及ぶ可能性を支持している。

3.4.5 ストレス特異的な発現の変化を示す機能未同定遺伝子の分類

DNA マイクロアレイによる解析の結果、新たに得られた知見として、塩ストレスもしくは高浸透圧ストレスによって発現の誘導もしくは抑制が見られた遺伝子のうちの約半数はコードしているタンパク質の機能が未同定であることがあげられる。この結果は、塩ストレスや高浸透圧ストレスに対する適応の分子機構のうち、未だ明らかにされていないものが数多く存在する可能性を示唆している。図 3-7 には、機能未同定遺伝子についてストレス依存的な発現パターンによる分類の結果をまとめた。

塩ストレス特異的に発現が誘導された遺伝子のうち、*slr0967* 遺伝子は、AXLXX pentapeptide repeats を持つ、ラン藻から高等植物まで広く保存された遺伝子ファミリーの一つであった。しかしこれらの pentapeptide repeats の機能は未だ明らかにされていない。*Synechocystis* は、このモチーフを持つタンパク質

A. Stress-inducible genes



B. Stress-repressible genes

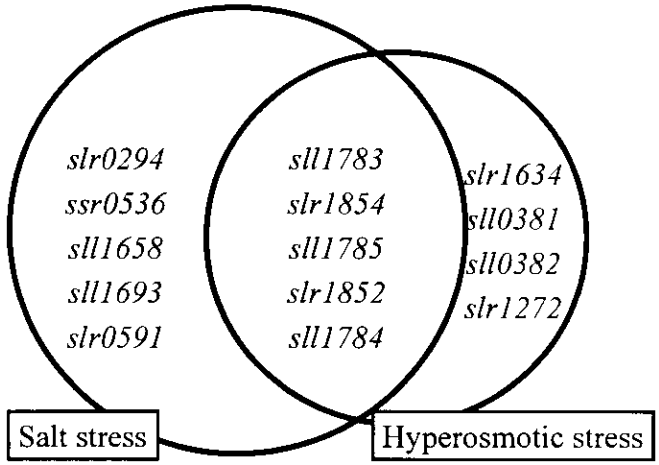


図3-7　塩ストレス及び高浸透圧ストレスにより発現の制御を受ける機能未知遺伝子群の分類

A, 塩ストレス及び高浸透圧ストレスにより発現が誘導される遺伝子の場合。
B, 塩ストレス及び高浸透圧ストレスにより発現が抑制される遺伝子の場合。
それぞれの円の大きさは、それぞれのストレスにより発現が変化した遺伝子数に比例する。円の重なり部分は、両方のストレスによって発現が誘導もしくは抑制された遺伝子を示す。

の遺伝子のホモログを16個コードしているが、それらのうち *slr0967* 遺伝子の発現のみが塩ストレスによって誘導されることが明らかになった。図 3-7 で示したように、特定の代謝機能に関わる一群のタンパク質をコードした遺伝子の発現はストレス特異的な傾向を示すことから、本実験で見つかった機能未同定タンパク質の機能も、図 3-6 で分類されたタンパク質の機能と関連している可能性も考えられる。

塩ストレスと高浸透圧ストレスの両方のストレスで、発現の抑制を受けた遺伝子のうち、2 つの機能未同定遺伝子のクラスターが見つかった (図 3-5Dη)。これらの遺伝子クラスターはゲノム上で逆方向に並んでおり、転写調節に関わる領域を共有している可能性がある。これらの遺伝子クラスターは、塩ストレス及び高浸透圧ストレスのみならず、低温ストレスによっても発現が抑制される (Suzuki *et al.*, 2001)。この結果は、これらの遺伝子が、多様なストレス適応の機構に関わっている可能性を示している。また、ストレス特異的な発現を示す遺伝子群は、その上流配列に発現の制御に関わる共通のモチーフが存在する可能性も示唆されたが、これまでのところ明確な配列は見つかっていない。

3.4.6 塩ストレス及び高浸透圧ストレスとシグナル伝達

図 3-6 及び 3-7 から明らかなように、*Synechocystis* においてストレスに適応するために発現の誘導もしくは抑制が行われる遺伝子の種類と機能は、塩ストレスと高浸透圧ストレスの間で異なっている。これは、塩ストレスと高浸透圧ストレスに適応するためのメカニズムが明らかに異なっているということを示している。これらの結果から、*Synechocystis* は塩ストレスと高浸透圧ストレスを別個のストレスとして検知している可能性が高い。また、このことか

ら、ストレスに特異的なシグナル伝達の経路が存在する可能性が考えられる。一般に、バクテリアのシグナル伝達にはヒスチジンキナーゼと呼ばれるシグナル伝達系のタンパク質が関わっていることが多く、*Synechocystis* でも既に、低温を検知するヒスチジンキナーゼが同定されている (Suzuki *et al.*, 2000)。塩ストレス及び浸透圧ストレスの検知とシグナル伝達に関与するヒスチジンキナーゼ、及びこれらのストレスに適応するための遺伝子の発現制御に関わるタンパク質の同定が今後の課題である。

第４章

総合考察

本研究は、ラン藻の塩ストレス及び高浸透圧ストレス応答の機構を分子レベルで解明することを目標に行われた。その結果、これらのストレスがラン藻の生育、細胞質体積及び遺伝子発現に対して異なる効果を及ぼすことを明らかにした。

本研究で用いられた DNA マイクロアレイという手法は、これまで個別に解析されていた遺伝子の発現を、ゲノムレベルで解析することを可能にした技術である。ほぼ全遺伝子を網羅する CyanoCHIP の最大の利点は、塩ストレス及び高浸透圧ストレスにより発現パターンが変化する遺伝子は、ここで示されたもの以外には存在しないという情報が得られることにある。逆を言えば、細胞の中には塩ストレス及び高浸透圧ストレスによって発現に変化が見られない遺伝子のほうが圧倒的に多いということである。本研究により明らかになった、ストレスにより発現が誘導、もしくは抑制される遺伝子数の全遺伝子数に対する割合は、塩ストレスで 11.8%、高浸透圧ストレスで 8.6% と、共に一割前後でしかない。こうした知見は、これらのストレスによって発現の誘導もしくは抑制を受ける遺伝子がコードするタンパク質の機能が、塩耐性もしくは塩ストレス適応において非常に重要な役割を果たすであろうことを示唆している。また、これらの遺伝子の約半数が、機能未同定のタンパク質をコードしていたという結果は、こうした遺伝子の機能解析がストレスの本質を理解する上で極めて重要であることを示した。現在、今回明らかになった機能未同定遺伝子群の個別破壊株の作製を計画中であり、これらの遺伝子がこれまでに明らかにされている塩耐性や高浸透圧耐性の機構とどのように関わっているのかを検討していきたい。

また、DNA マイクロアレイのような網羅的解析法の利点として、異なるストレス間での遺伝子発現のパターンを比較することで、個々のストレスに固

有の性質、及び特異的なシグナル伝達経路を明らかにすることが可能性である。例えば、塩、高浸透圧のみならず、酸化、可視光、紫外線、高温、低温、栄養欠乏など、様々な条件での遺伝子発現のパターンを比較することで、それぞれのストレスに特異的に制御される遺伝子群が明らかになると予想される。こうした遺伝子の発現を制御するシスエレメントの解析や、二成分制御系などのシグナル伝達に関わるタンパク質をコードした遺伝子の破壊株を用いた解析により、最終的には、*Synechocystis* の全てのシグナル伝達回路を描くことが可能になると思われる。こうした情報の蓄積は、生物進化や物質生産分野での効果的な応用への第一歩となると考えられる。

一方、DNA マイクロアレイにより得られた結果の解析には、幾つかの問題を考慮する必要がある。例えば、プローブの非特異的なハイブリダイズにより見かけ上、発現が変化しているように見える遺伝子が存在する可能性があることや、mRNA の発現量とタンパク質の合成量は必ずしも一致しないこと、また、本実験では参照実験により、有意な mRNA の発現量の変化を 2 倍として定義したが、実際どれだけの変化が生物学的に意味を持つかは明らかではないことなどである。こうした問題を解決し、発現が変化した遺伝子の機能とストレス耐性の関わりを調べるためには、タンパク質レベルでの発現解析との比較が必要であり、プロテオーム解析などと合わせて検討する必要である。また、タンパク質自身の活性が、ストレスによりどのように変化するかといった情報も重要であり、実際、GgpS タンパク質では、塩濃度の上昇により酵素活性自体が上がることが知られている (Hagemann *et al.*, 1996)。こうした、翻訳後の酵素活性の調節と塩耐性の機構との関係は、まだ未解明な部分が多く、今後の大きな課題であると考えられる。

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付録

総合研究大学院大学 生命科学研究科　　分子生物機構論　専攻

ふりがな
氏　名　　兼崎（かねさき）　友（ゆう）　　（学籍番号：　983202　）

博士論文題目

DNA マイクロアレイを用いたラン藻 *Synechocystis* sp. PCC 6803 における塩ストレス及び高浸透圧ストレス誘導性遺伝子の網羅的解析

(1) 主要論文

Kanesaki, Y., Suzuki, I., Allakhverdiev S. I., Mikami, K., and Murata, N. (2002) Salt stress and hyperosmotic stress regulate the expression of different sets of genes in *Synechocystis* sp. PCC 6803. *Biochem. Biophys. Res. Commun.* **290**, 339-348.

(2) 参考論文

Suzuki, I., Kanesaki, Y., Mikami, K., Kanehisa, M., and Murata, N. (2001) Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. *Mol. Microbiol.* **40**, 235-244.

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Salt Stress and Hyperosmotic Stress Regulate the Expression of Different Sets of Genes in *Synechocystis* sp. PCC 6803

Yu Kanesaki,*,† Iwane Suzuki,*,† Suleyman I. Allakhverdiev,*
Koji Mikami,* and Norio Murata*,†,[1]

Department of Regulation Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan; and †Department of Molecular Biomechanics, School of Life Science, Graduate University for Advanced Studies, Okazaki 444-8585, Japan

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Acclimation of microorganisms to environmental stress is closely related to the expression of various genes. We report here that salt stress and hyperosmotic stress have different effects on the cytoplasmic volume and gene expression in *Synechocystis* sp. PCC 6803. DNA microarray analysis indicated that salt stress strongly induced the genes for some ribosomal proteins. Hyperosmotic stress strongly induced the genes for 3-ketoacyl-acyl carrier protein reductase and rare lipoprotein A. Genes whose expression was induced both by salt stress and by hyperosmotic stress included those for heat-shock proteins and the enzymes for the synthesis of glucosylglycerol. We also found that each kind of stress induced a number of genes for proteins of unknown function. Our findings suggest that *Synechocystis* recognizes salt stress and hyperosmotic stress as different stimuli, although mechanisms common to the responses to each form of stress might also contribute to gene expression.

Key Words: **DNA microarray; cyanobacteria; salt stress; hyperosmotic stress.**

Microorganisms including cyanobacteria acclimate to various kinds of environmental stress by regulating the expression of numerous stress-inducible genes (1–3). For example, when the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is exposed to salt stress, expression of the following genes is induced: the *ggpS* gene for glucosylglycerolphosphate synthase (4); the *crh* gene for RNA helicase (3); the *isiA* gene for iron-stress-inducible protein A (5); the *isiB* gene for flavodoxin (5) and the *petH* gene for ferredoxin:$NADP^+$ reductase (6).

[1] To whom correspondence and reprint requests should be addressed at National Institute for Basic Biology, Okazaki 444-8585, Japan. Fax: (+81) 564 54 4866. E-mail: murata@nibb.ac.jp.

The terms salt stress and hyperosmotic stress have often been used in a confusing manner, such that genes induced upon exposure of organisms to high concentrations of NaCl have sometimes been defined as osmo-stressed genes (7, 8). However, the accumulated evidence suggests that the two kinds of stress are perceived as different signals. Incubation of the cyanobacterium *Synechococcus* sp. PCC 7942 in medium supplemented with 1 M sorbitol decreases the cytoplasmic volume to 45% of the original value (9), whereas incubation in 0.5 M NaCl only decreases the volume to 85% of the original value (10). These findings suggest that cyanobacterial cells might respond to salt stress and hyperosmotic stress in different ways. We wondered whether these two kinds of stress might induce the same set or different sets of genes. To examine this issue, we used a DNA microarray to investigate gene expression in *Synechocystis* in response to salt stress and to hyperosmotic stress.

DNA microarrays allow monitoring of changes in levels of transcripts of almost all genes in specific organisms (11, 12). Such arrays have been used to examine gene expression in response to various kinds of stress in *Saccharomyces cerevisiae* (13) and in *Synechocystis* (14, 15).

In the present study, we obtained clear evidence that salt stress and hyperosmotic stress regulate different sets of genes, although expression of some genes was induced in common by both kinds of stress. Furthermore, we found that expression of a number of genes for proteins of unknown function was induced or repressed by salt stress and hyperosmotic stress.

MATERIALS AND METHODS

Strain and culture conditions. A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was kindly provided by Dr. J. G. K. Williams (Du Pont de Nemours & Co., Inc., Wilmington, DE). Cells were grown at 34°C in 50 ml of BG-11 medium (16) buffered with 20 mM Hepes–

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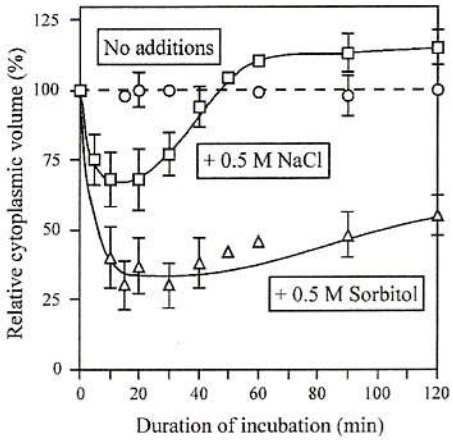

FIG. 1. Effects of NaCl and sorbitol on the volume of *Synechocystis* cells. Circles, control (no additions); squares, 0.5 M NaCl was added at time 0; triangles, 0.5 M sorbitol was added at time 0. The volume of cells was monitored by EPR spectrometry (see text). Each point represents the mean ± SE of results of six independent experiments.

NaOH (pH 7.5) under continuous illumination from incandescent lamps, as described previously (17). Cells at the exponential growth phase (OD_{730} = 0.2) were incubated with 0.5 M NaCl, sorbitol, or mannitol, after addition of an appropriate volume of a 5 M solution of NaCl or sorbitol or a 2 M solution of mannitol to the culture. Cells were incubated under the standard growth conditions for 30 min unless otherwise noted.

Measurement of cytoplasmic volume. The cytoplasmic volume was determined by electron paramagnetic resonance (EPR) spectrometry as described previously (9, 18). Cells were harvested by centrifugation at 30°C at 8000*g* for 10 min and were resuspended at 400 μg Chl ml^{-1} in a solution of 1.0 mM 2,2,6,6-tetramethyl-4-oxopiperidinooxy free radical (TEMPO; a spin probe), 20 mM $K_3[Fe(CN)_6]_3$ and 75 mM Na_2Mn–EDTA. TEMPO that was oxidized by $K_3[Fe(CN)_6]_3$ penetrated plasma membranes rapidly and reached an equilibrium in all phases of the suspension of cells. The addition of the paramagnetic quencher Na_2Mn–EDTA, which cannot penetrate the plasma membrane, broadened all the EPR signals except those that originated within the space bounded by the plasma membrane. The internal volume of cells could be calculated from the difference between the EPR spectrum obtained from the treated cells and that from control cells. Cells were enclosed in a sealed glass capillary tube (i.d., 0.02 cm) in a final volume of 40 μl and EPR spectra were recorded at room temperature in an EPR spectrometer (Model ESP 300E; Bruker, Karlsruhe, Germany). The EPR signal from the 40-μl capillary filled with 1.0 mM TEMPO alone was measured as a blank control. Measurements were made in darkness under the following conditions: 100 kHz field modulation at a microwave frequency of 11.72 GHz; a modulation amplitude of 0.4 mT; microwave power of 10 mW; a time constant of 80 ms; and a scan rate of 0.4 Gs^{-1}.

Profiles of transcriptional activity. Cells that had been exposed to salt stress or hyperosmotic stress were killed instantaneously by the addition of 50 ml of a mixture of phenol and ethanol (1:10, w/v) to 50 ml of the cell suspension and then total RNA was extracted as described previously (19). The RNA was treated with DNase I (Nippon Gene, Tokyo, Japan) to remove contaminating DNA (19).

A *Synechocystis* DNA microarray (CyanoCHIP) was obtained from TaKaRa Co. Ltd. (Kyoto, Japan). This microarray covered 3079 of the 3168 open reading frames of *Synechocystis* (20). We used Cy3 dye-labeled and Cy5 dye-labeled cDNA, synthesized by reverse transcription of 20 μg of total RNA, for hybridizations, which were allowed to proceed at 65°C for 16 h. After the incubation, the microarrays were rinsed with 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate) at room temperature. They were washed with 2× SSC at 60°C for 10 min and 0.2× SSC 0.1% SDS at 60°C for 10 min and then rinsed with distilled water at room temperature for 2 min. Moisture was removed with an air spray prior to analysis with the array scanner (GMS418; Affimetrix, Woburn, MA). Each signal was quantified with the ImaGene ver. 4.0 program (BioDiscovery, Los Angeles, CA). The signal from each gene on the microarray was normalized by reference to the total intensity of signals from all genes with

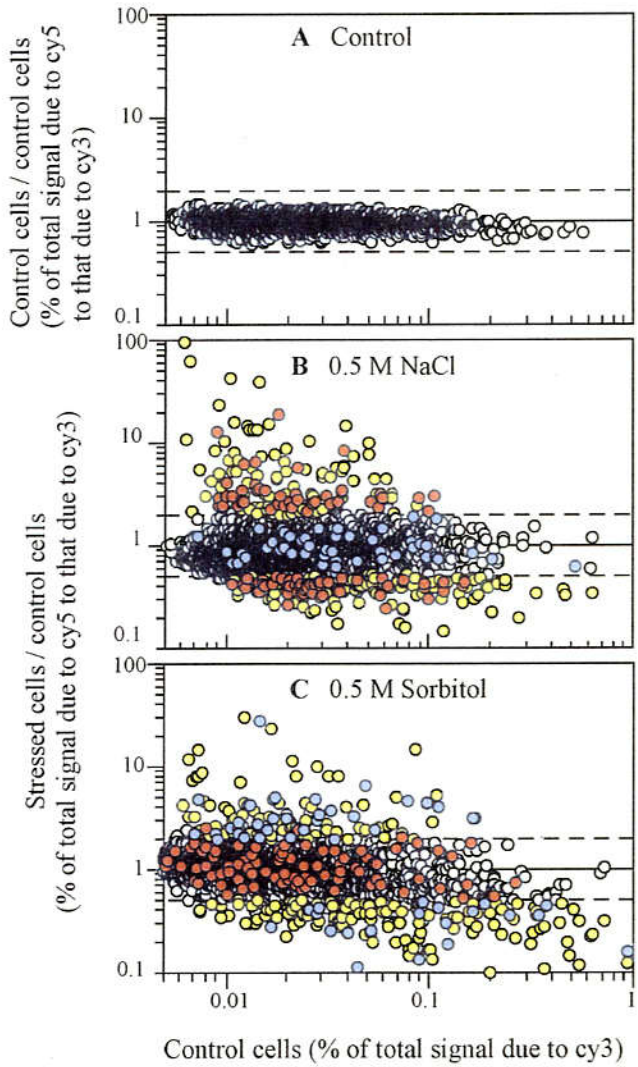

FIG. 2. DNA microarray analysis of salt stress-induced and hyperosmotic stress-induced gene expression. (A) Internal-control experiment (control experiment). RNA extracted from unstressed cells was used to synthesize both Cy3-labeled cDNA and Cy5-labeled cDNA. Dashed lines correspond to reference lines, which indicate the limit of experimental deviations. (B) RNA, extracted from cells that had been exposed to 0.5 M NaCl for 30 min, was compared with RNA that from unstressed cells. (C) RNA, extracted from cells that had been exposed to 0.5 M sorbitol for 30 min, was compared with RNA from unstressed cells. Red circles correspond to genes whose expression was regulated only by NaCl stress. Blue circles correspond to genes whose expression was regulated only by sorbitol. Yellow circles correspond to genes whose expression was affected both by NaCl and by sorbitol. The assay was repeated six times in independent experiments and essentially the same results were obtained in every case.

the exception of genes for rRNAs. Then we calculated changes in the level of the transcript of each gene relative to the total level of mRNA.

RESULTS AND DISCUSSION

Effects of NaCl and Sorbitol on Cytoplasmic Volume

Salt stress and hyperosmotic stress depressed the growth of *Synechocystis* cells. Addition to the standard culture medium of 0.5 M NaCl or 0.5 M sorbitol decreased the growth rate to about 50% of the original level (data not shown). In our subsequent analysis, we used 0.5 M NaCl and 0.5 M sorbitol to induce salt stress and hyperosmotic stress, respectively.

We examined the effects of NaCl and sorbitol on the cytoplasmic volume of *Synechocystis* cells by monitoring the EPR spectrum of TEMPO, as described under Materials and Methods (Fig. 1). When cells were incubated with 0.5 M sorbitol, the cytoplasmic volume decreased to 30% of the original level within 10 min and then remained at this level subsequently. When cells were incubated with 0.5 M NaCl, the cytoplasmic volume decreased to 70% of the original level within 10 min but returned to the original level within 45 min of the start of exposure to salt stress. These observations were consistent with our previous finding that incubation of *Synechococcus* sp. PCC 7942 with 1 M sorbitol and with 0.5 M NaCl decreased the cytoplasmic volume to 45 and 85% of original level, respectively, within 30 min (9, 10). The previous reports that Na^+ ions penetrate the plasma membrane might explain why NaCl is less effective than sorbitol in exerting hyperosmotic pressure (21, 22).

The results in Fig. 1 suggested that the inhibitory effect of 0.5 M NaCl on the cell growth might be due mainly to ionic stress. Therefore, we postulated that an increase in the concentration of Na^+ ions in the cytoplasm, due to the penetration of ions through the cell membrane, might be the main feature of salt stress, with osmotic pressure across the plasma membrane being a minor feature of such stress. By contrast, osmotic pressure due to sorbitol results from the fact that the plasma membrane is impermeable to this compound (9). However, sorbitol might also have an ionic effect, albeit a minor one, because a decrease in cytoplasmic volume to 30% of the original value would lead to a three-fold increase in the concentrations of salts in the cytosol. Thus, our findings suggest that the stress due to NaCl and the stress due to sorbitol might have different effects on cell physiology and might be recognized as different stimuli by cyanobacterial cells.

Analysis of Gene Expression with a DNA Microarray

To study the responses of *Synechocystis* cells to salt stress and hyperosmotic stress in terms of gene expression, we used a DNA microarray. First, we performed an internal-control experiment in which Cy3- and Cy5-labeled cDNAs were synthesized from total RNA that had been extracted from unstressed cells. Figure 2A shows that all the data points appeared between the two reference lines, which corresponded to ratios of expression of 2.0 and 0.5. This control experiment suggested that data points that appeared above the upper reference line or below the lower reference line could be regarded as representing those genes whose expression was induced or repressed, respectively.

Figure 2B shows the effects of the incubation of *Synechocystis* cells with 0.5 M NaCl for 30 min on the expression of individual genes. Upon exposure to the salt stress, 147 genes appeared above the upper reference line and 228 genes appeared below the lower reference line. However, most of the genes appeared between the two reference lines, indicating that the expression of most genes was unaffected by salt stress. Figure 2C shows the effects of incubating *Synechocystis* cells with 0.5 M sorbitol on the expression of individual genes. Upon exposure to the hyperosmotic stress, 113 genes appeared above the upper reference line and 161 genes appeared below the lower reference line. We also examined the effect of 0.5 M mannitol on *Synechocystis* cells; the profile of gene expression was essentially the same as that obtained with 0.5 M sorbitol. This observation indicated that the changes in gene expression due to the sorbitol were caused by hyperosmotic stress and not specifically by sorbitol. We also examined the effects of extended incubation for 2 h with 0.5 M NaCl or 0.5 M sorbitol on the pattern of gene expression. We obtained essentially the same results as those obtained after a 30-min incubation (data not shown).

The genes, whose expression was highly enhanced or was strongly repressed to an extent that corresponded to a ratio greater than 3:1, are listed in Tables 1 and 2. We found that half of genes, whose expression was enhanced or was repressed by each kind of stress, encode proteins of unknown functions. It suggests that a number of proteins of unknown functions may be related to the known or unknown mechanisms for the acclimation to these kinds of stress. We also found that salt stress and hyperosmotic stress regulate the expression of different sets of genes (see red spots or blue spots in Figs. 2B and 2C). It should be noted here, however, that salt stress tends to depress gene expression in general (23). Eventually, gene expression was reduced to 75 and 90% of the control level in response to 0.5 M NaCl and 0.5 M sorbitol, respectively. The results in Figs. 2B and 2C reveal the changes in the contribution of the expression of individual genes relative to the total level of gene expression, which was estimated from signals that reflected the extent of hybridization.

TABLE 1

Genes Whose Expression Was Enhanced by Salt and/or Hyperosmotic Stress

ORF number	Gene	Product	Extent of enhancement	
			0.5 M NaCl	0.5 M sorbitol
		Genes whose expression was strongly enhanced by salt stress (fold)		
slr1704		Protein of unknown function	17.4 ± 1.2	2.3 ± 0.6
slr0967		Protein of unknown function	16.0 ± 3.6	2.5 ± 0.6
sll1621		Membrane protein	8.3 ± 1.1	1.5 ± 0.3
sll1515		Protein of unknown function	7.6 ± 1.1	0.8 ± 0.2
slr1501		Protein of unknown function	7.0 ± 1.2	1.4 ± 0.5
sll1864		Chloride channel protein	6.1 ± 1.9	1.1 ± 0.2
slr1932		Protein of unknown function	5.4 ± 0.6	1.2 ± 0.1
sll1022		Protein of unknown function	4.7 ± 0.4	1.6 ± 0.6
ssr2194		Protein of unknown function	4.7 ± 1.4	1.4 ± 0.4
slr1738		Protein of unknown function	4.4 ± 0.2	1.9 ± 0.4
slr0095		*O*-Methyltransferase	4.2 ± 0.1	1.4 ± 0.2
sll1086		Protein of unknown function	4.0 ± 0.6	1.6 ± 0.2
slr0529		Protein of unknown function	3.9 ± 0.7	1.3 ± 0.2
sll1167	*pbp*	Penicillin-binding protein	3.8 ± 0.2	1.3 ± 0.5
slr1259		Protein of unknown function	3.7 ± 0.7	1.1 ± 0.2
sll0905	*maf*	maf protein	3.6 ± 0.7	1.3 ± 0.4
sll1236		Protein of unknown function	3.2 ± 0.6	1.1 ± 0.4
slr1916		Esterase	3.2 ± 0.5	1.5 ± 0.4
sll1594	*rbcR*	Rubisco operon regulator	3.2 ± 0.4	1.2 ± 0.2
slr1971		Protein of unknown function	3.1 ± 0.7	1.7 ± 0.5
sll1723		Protein of unknown function	3.1 ± 0.6	1.6 ± 0.4
Slr1604	*ftsH*	Cell division protein	3.1 ± 0.0	1.6 ± 0.3
sll1491		Protein of unknown function	3.0 ± 0.4	1.4 ± 0.3
slr1751	*prc*	Carboxy-terminal protease	3.0 ± 0.2	1.4 ± 0.2
sll0188		Protein of unknown function	3.0 ± 0.2	1.7 ± 0.5
slr1608	*gdhB*	Glucose dehydrogenase-B	3.0 ± 0.4	1.4 ± 0.3
sll0938		Aspartate transaminase	3.0 ± 0.6	1.3 ± 0.4
sll1799	*α rp13*	50S ribosomal protein L3	3.0 ± 0.2	0.8 ± 0.2
		Genes whose expression was strongly enhanced by hyperosmotic stress		
sll0330	*fabG*	3-Ketoacyl-ACP reductase	1.2 ± 0.2	23.9 ± 6.5
slr0423	*rlpA*	Rare lipoprotein A	1.2 ± 0.1	6.2 ± 0.4
slr1748		Protein of unknown function	1.7 ± 0.4	4.6 ± 0.6
slr1119		Protein of unknown function	1.3 ± 0.2	4.2 ± 1.1
slr0381		Protein of unknown function	0.9 ± 0.1	3.9 ± 0.9
sll0430	*htpG*	Heat-shock protein Hsp90	1.9 ± 0.1	3.8 ± 0.7
ssr1256		Protein of unknown function	0.7 ± 0.1	3.7 ± 0.3
ssl3177	*repA*	Rare lipoprotein A	1.6 ± 0.2	3.6 ± 0.5
sll0185		Protein of unknown function	1.3 ± 0.4	3.3 ± 0.5
sll0293		Protein of unknown function	0.9 ± 0.1	3.1 ± 0.6
slr0753	*p*	P protein	0.6 ± 0.1	3.0 ± 1.0
		Genes whose expression was strongly enhanced by salt and hyperosmotic stress		
sll1862	*ϵ*	Protein of unknown function	93.8 ± 7.5	17.2 ± 5.3
sll1514	*hspA*	Heat-shock protein Hsp17	56.2 ± 10.7	26.5 ± 4.5
sll1863	*ϵ*	Protein of unknown function	52.7 ± 10.1	8.3 ± 1.8
sll0528		Protein of unknown function	40.0 ± 6.5	20.0 ± 6.0
slr1544		Protein of unknown function	20.3 ± 3.3	7.8 ± 0.9
slr1516	*sodB*	Superoxide dismutase	16.5 ± 1.6	4.9 ± 0.7
sll0939		Protein of unknown function	15.3 ± 3.2	3.5 ± 0.9
sll0846		Protein of unknown function	14.9 ± 1.5	10.1 ± 1.4
slr1674	*ϵ*	Protein of unknown function	14.2 ± 0.8	10.6 ± 1.2
ssr2595		High light-inducible protein	13.4 ± 1.6	7.0 ± 1.4
sll1106		Protein of unknown function	12.6 ± 2.8	3.4 ± 0.8
sll1085	*ϵ glpD*	Glycerol-3-P dehydrogenase	11.8 ± 2.1	3.2 ± 0.5
sll0306	*rpoD*	RNA polymerase sigma factor	11.5 ± 0.3	7.2 ± 2.3
sll0170	*dnaK*	DnaK protein	11.5 ± 2.8	4.1 ± 0.9

TABLE 1—*Continued*

ORF number	Gene	Product	Extent of enhancement	
			0.5 M NaCl	0.5 M sorbitol
sll1566	ϵ *ggpS*	Glucosylglycerol-P synthase	10.7 ± 1.5	3.7 ± 0.5
slr1675	ϵ *hypA*	Hydrogenase-related protein	10.1 ± 1.5	7.8 ± 2.1
slr1963		Protein of unknown function	10.0 ± 1.4	9.1 ± 2.4
slr0093	*dnaJ*	DnaJ protein	9.6 ± 1.6	3.2 ± 0.8
sll1884		Protein of unknown function	9.4 ± 1.1	3.9 ± 0.3
slr1687	γ	Protein of unknown function	9.4 ± 2.2	3.2 ± 0.9
slr1641	*clpB*	ClpB protein	8.4 ± 1.2	3.5 ± 1.2
sll0788	β	Protein of unknown function	8.3 ± 1.3	7.0 ± 0.6
sll1483		Protein of unknown function	7.8 ± 1.3	22.9 ± 0.6
slr1603		Protein of unknown function	7.2 ± 0.8	3.9 ± 1.1
slr0959		Protein of unknown function	6.6 ± 1.2	4.2 ± 1.1
ssl1633		CAB/ELIP/HLIP superfamily	6.5 ± 0.9	5.1 ± 0.5
sll0416	*groEL2*	60-kDa chaperonin	6.2 ± 0.4	3.2 ± 1.1
ssl3044	γ	Hydrogenase component	5.7 ± 1.5	3.5 ± 1.2
ssr2016		Protein of unknown function	5.5 ± 0.9	4.6 ± 0.5
ssl2542	*hliA*	HLIP	5.0 ± 0.8	4.8 ± 1.8
slr1204	*htrA*	Serine protease	4.8 ± 0.3	4.6 ± 0.7
sll0789	β *copR*	Response regulator	4.4 ± 0.9	3.9 ± 0.9
ssl2971		Protein of unknown function	4.3 ± 0.7	4.0 ± 0.2
slr0581		Protein of unknown function	3.9 ± 0.7	3.3 ± 0.3

Note. Cells grown at 34°C (to OD_{730} = 0.2) were incubated with 0.5 M NaCl or 0.5 M sorbitol for 30 min. Each value indicates the ratio of the level of expression in stressed cells to that in control cells. Values shown are means ± SE of results of three independent experiments. The numbering of ORFs corresponds to that of Kaneko *et al.* (20, 29). Genes marked by the same Greek letter are located in tandem on the *Synechocystis* genome.

Genes Whose Expression Was Strongly Enhanced by Salt Stress

Figure 3 shows that salt stress due to 0.5 M NaCl strongly induced the expression of genes for proteins involved in translation (*rpl3*), the modification and degradation of proteins (*prc* and *ftsH*). The expression of these genes was not induced by osmotic stress due to 0.5 M sorbitol.

In *Synechocystis,* genes for ribosomal proteins, namely, *rpl2, rpl3, rpl4,* and *rpl23* are located in a putative ribosomal-protein operon (20). Salt stress enhanced the expression of these genes by factors of 2.0, 3.0, 2.3, and 2.7, respectively. The crystallographic structure of the large subunit of ribosomes has revealed that the ribosomal proteins, L2, L3 and L4, encoded by *rpl2, rpl3,* and *rpl4,* respectively, are located near the peptidyltransferase center (24, 25). These observations suggest that salt stress might destabilize ribosomes and that the synthesis *de novo* of L2, L3, and L4 might be necessary for maintenance of the activities of ribosomes.

The D1 protein at the photochemical reaction center of the photosystem II complex is rapidly damaged under strong light (26). The rapid degradation of photodamaged D1 and regeneration of D1 by synthesis *de novo* are important if photosynthetic organisms are to maintain the activity of photosystem II. We found that the proteins that are involved in the degradation of photodamaged D1 are also induced by salt stress. Lindahl *et al.* demonstrated that an ATP-dependent metalloprotease, FtsH, located in the thylakoid membrane in *Arabidopsis thaliana,* degrades photodamaged D1 (27, 28). *Synechocystis* has four open reading frames (ORFs) that encode homologs of FtsH, namely, *sll1463, slr0228, slr1390,* and *slr1604* (20, 29). We found that the expression of *slr1390* and *slr1604* was enhanced 2.1- and 3.1-fold by salt stress, respectively, whereas levels of expression of the other proteins were basically unchanged.

The CtpA protein catalyzes the cleavage of the carboxy-terminal region of the precursor to the D1 protein to generate the mature D1 protein (30). The expression of the *ctpA* gene and that of a homologous gene, *prc,* were also enhanced 2.7- and 3.0-fold by salt stress but were unaffected by hyperosmotic stress. These results suggest that salt stress might inhibit the turnover of D1 not only at the transcriptional and translational levels, but also at the levels of degradation of photodamaged D1 and the processing of the precursor to D1. The enhanced expression of the *ftsH, prc* and *ctpA* genes might compensate for the inhibition via the synthesis of FtsH, Prc, and CtpA.

Genes Whose Expression Was Strongly Enhanced by Hyperosmotic Stress

Hyperosmotic stress strongly induced the expression of the *fabG* gene for 3-ketoacyl-ACP reductase, the *rlpA* and *repA* genes for rare lipoproteins. Salt stress

TABLE 2

Genes Whose Expression Was Repressed by Salt and/or Hyperosmotic Stress

ORF number	Gene	Product	Extent of repression	
			0.5 M NaCl	0.5 M sorbitol
		Genes whose expression was strongly repressed by salt stress (fold)		
slr0294		Protein of unknown function	3.7 ± 0.9	1.3 ± 0.2
sll1453	*nrtD*	Nitrate transport protein	3.5 ± 0.4	1.3 ± 0.6
ssr0536		Protein of unknown function	3.4 ± 0.5	1.7 ± 0.2
slr1658		Protein of unknown function	3.3 ± 0.5	1.3 ± 0.2
sll1693		Protein of unknown function	3.2 ± 0.6	1.7 ± 0.5
slr0213	*guaA*	GMP synthetase	3.1 ± 0.8	1.7 ± 0.2
ssl2084	*acp*	Acyl carrier protein	3.1 ± 0.5	1.4 ± 0.6
slr0591		Protein of unknown function	3.0 ± 0.8	0.9 ± 0.2
sll0262	*desD*	Delta-6 desaturase	3.0 ± 0.3	1.3 ± 0.3
ssl2874		Protein of unknown function	3.0 ± 0.7	1.2 ± 0.5
		Genes whose expression was strongly repressed by hyperosmotic stress		
slr1634		Protein of unknown function	1.5 ± 0.7	7.2 ± 1.0
sll0381	ζ	Protein of unknown function	0.5 ± 0.1	5.9 ± 1.1
sll0382	ζ	Protein of unknown function	0.5 ± 0.1	3.7 ± 0.6
slr1272		Protein of unknown function	1.4 ± 0.1	3.7 ± 0.7
sll1626	*lexA*	SOS regulatory protein	1.2 ± 0.5	3.4 ± 1.1
sll0383	ζ *cbiM*	CbiM protein	0.5 ± 0.0	3.0 ± 0.9
		Genes whose expression was strongly repressed by salt and hyperosmotic stress		
sll1783	η	Protein of unknown function	8.9 ± 2.0	6.8 ± 0.2
slr1854	η	Protein of unknown function	8.9 ± 1.6	10.3 ± 2.0
sll1785	η	Protein of unknown function	8.0 ± 2.1	5.4 ± 0.5
slr1852	η	Protein of unknown function	7.7 ± 1.3	5.5 ± 1.0
sll1784	η	Protein of unknown function	7.2 ± 1.6	5.0 ± 0.5
slr0737	*psaD*	Photosystem I subunit II	5.8 ± 0.9	3.6 ± 0.1
slr1855	η	Protein of unknown function	5.5 ± 1.2	5.0 ± 0.7
slr2051	*cpcG*	Phycobilisome rod-core linker	5.5 ± 1.1	4.3 ± 0.3
slr1277	*gspD*	Secretion pathway protein D	4.9 ± 0.7	3.7 ± 0.4
sll0819	*psaF*	Photosystem I subunit III	4.5 ± 1.3	4.5 ± 0.8
sll1091		Bacteriochlorophyll synthase	3.9 ± 0.3	3.0 ± 0.5
slr0335	*apcE*	Phycobilisome LCM linker	3.8 ± 0.6	4.3 ± 0.1
slr0653	*rpoDI*	RNA polymerase sigma factor	3.8 ± 1.2	4.0 ± 0.5
slr1459	*apcF*	Phycobilisome core component	3.8 ± 0.5	3.6 ± 1.0
sll1305	η	Protein of unknown function	3.7 ± 0.8	3.0 ± 0.6
slr2067	ι *apcA*	Allophycocyanin a chain	3.6 ± 0.7	4.1 ± 0.1
slr1986	ι *apcB*	Allophycocyanin b chain	3.6 ± 0.5	3.3 ± 0.4
sll0427	*psbO*	Mn-stabilizing polypeptide	3.5 ± 0.8	3.5 ± 0.3
sll1580	θ *cpcC*	Phycocyanin-associated linker	3.4 ± 0.8	8.2 ± 0.4
sll1577	θ *cpcB*	Phycocyanin b subunit	3.4 ± 1.1	7.2 ± 0.8
sll1712		DNA-binding protein HU	3.4 ± 1.0	3.7 ± 1.1
slr1859		Protein of unknown function	3.3 ± 0.4	3.0 ± 0.6
sll0661	*ycf35*	Protein of unknown function	3.3 ± 0.8	3.6 ± 0.8
slr1276		Protein of unknown function	3.2 ± 0.1	3.4 ± 0.6
slr0011	*rbcX*	Protein of unknown function	3.1 ± 0.8	3.5 ± 1.0
slr1856	η	Protein of unknown function	3.0 ± 0.3	4.2 ± 0.6

Note. See Table 1 for details.

did not induce the expression of any of these genes, the result suggests again that salt stress and hyperosmotic stress affect the expression of different sets of genes.

The efflux of water from cells due to hyperosmotic stress reduces the volume of the cytoplasmic space and such reduction can lead to plasmolysis and changes the state of the periplasmic space (31). A decrease in the cytoplasmic space might cause close packing of membrane lipids and membrane proteins in the plasma membrane (31). Such changes in the periplasm and/or the plasma membrane might be the first signal of hyperosmotic stress that is perceived by the cell and that

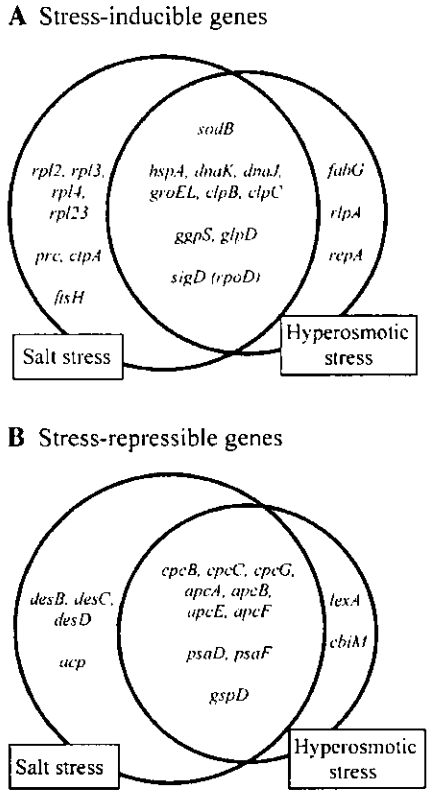

FIG. 3. Categorization of salt stress-regulated and hyperosmotic stress-regulated genes by reference to the specific stress that affected gene expression. Each of the two overlapping circles in A and B includes genes whose expression was altered by salt stress or hyperosmotic stress. The overlapping regions of the circles include those genes whose expression was affected by both kinds of stress. The area of each circle and of each overlapping region reflects the number of genes whose expression was affected by each kind of stress. (A) Stress-inducible genes. (B) Stress-repressible genes. Genes annotated by Cyanobase (http://www.kazusa.or.jp/cyano/) are indicated (20).

results in changes in the levels of expression of certain genes.

The FabG protein, 3-ketoacyl-ACP reductase, which catalyzes the production of 3-hydroxyacyl-ACP (ACP, acyl carrier protein) from 3-ketoacyl-ACP, is required for the elongation of carbon chains in the synthesis of fatty acids. *Synechocystis* has three *fabG* genes: *sll0330, slr1994,* and *slr0886.* However, of these three, only sll0330 was induced by hyperosmotic stress, suggesting that synthesis *de novo* of lipids might be related to the acclimation of the cyanobacterium to hyperosmotic stress. If the cytoplasm shrinks and membranes become closely packed under hyperosmotic stress, synthesis *de novo* of membrane lipids might assist in restoration of the original volume of the cytoplasm.

The RlpA protein, rare lipoprotein A, in *Escherichia coli* is exported to the periplasm where it is fatty-acylated to allow anchorage in the plasma membrane (32). It is previously demonstrated that the gene for this protein is located in a cluster of *mrdAB* genes for peptidoglycan synthesis in *E. coli* (33). *Synechocystis* has two genes for homologs of rare lipoprotein A (*rlpA* and *repA*) and both were specifically induced by hyperosmotic stress. Shrinkage of the cytoplasm by plasmolysis might damage the peptidoglycan layer. Thus, it is possible that the cell might strengthen or reconstruct the cell wall under hyperosmotic stress.

Genes Whose Expression Was Enhanced by Both Kinds of Stress

Both salt stress and hyperosmotic stress induced the expression of genes for heat-shock proteins (*hspA, dnaK, dnaJ, htrA, groEL2,* and *clpB*), superoxide dismutase (*sodB*), proteins for the synthesis of glucosylglycerol (*glpD* and *ggpS*), and sigma 70 factors (two *rpoD* genes). The efflux of water from cells due to hyperosmotic stress reduces the volume of the cytoplasmic space and, thus, increases the concentration of ions in the cytosol. Therefore, hyperosmotic stress might be expected to have effects similar to those of salt stress. Such effects might explain why a large number of genes were induced both by salt stress and by hyperosmotic stress.

The *glpD* and *ggpS* genes for proteins involved in the synthesis of glucosylglycerol are located in tandem on the chromosome of *Synechocystis* (20). Hagemann *et al.* demonstrated that GgpS is a key enzyme of the synthesis of glucosylglycerol, which is synthesized as a major compatible solute upon exposure of *Synechocystis* to salt stress (34).

The expression of genes for heat-shock proteins (*hspA, dnaK, dnaJ, htrA, groEL2,* and *clpB*) was induced both by salt stress and by hyperosmotic stress. It is demonstrated that, in *Bacillus subtilis,* the expression of these genes is induced not only by heat shock but also by salt stress, by ethanol and by starvation (1). In *Synechocystis,* heat shock induces the expression of these heat-shock genes (35, 36). In the present study, we found that a group of heat-shock proteins was also induced both by salt stress and by hyperosmotic stress in *Synechocystis.* The HtrA protein (DegP), a serine protease that is localized on the thylakoid membrane, degrades membrane-bound proteins that include photodamaged D1 (37). Török *et al.* demonstrated that HspA stabilizes proteins that have been heat-denatured *in vitro* for subsequent refolding by the DnaK/DnaJ/GrpE and GroEL/ES chaperone network (38). These observations suggest that the effect of both salt stress and hyperosmotic stress might be related to the stability of proteins. Cells might avoid the accumulation of denatured proteins by synthesizing chaperones and proteases under salt stress and hyperosmotic stress. Furthermore, It is demonstrated that both transcription and translation in *Synechocystis* cells might be inhibited when cells are incubated in medium supplemented with 684 mM NaCl (23). Thus, chaperones

might protect the transcriptional and translational apparatus under salt stress, as well as under hyperosmotic stress.

Both salt stress and hyperosmotic stress induced the expression of the two *rpoD* genes (*sll2012* and *sll0306*) that encode sigma 70 factors. These findings suggest that transcription might be one of the targets of salt stress and hyperosmotic stress. The induction of these genes might represent the strategy whereby *Synechocystis* compensates for the salt stress-induced depression of transcription. In *E. coli* and *B. subtilis,* the stress-dependent expression of genes for heat-shock proteins is regulated by specific sigma factors, namely, SigH and SigB, respectively (39). However, it is unclear which sigma factor regulates the expression of genes for heat-shock proteins in *Synechocystis.* The induction both by salt stress and by hyperosmotic stress of two *rpoD* genes (*sll2012* and *sll0306*), which are homologous to genes for SigB of *B. subtilis,* suggests that these sigma factors might be involved in regulation of the expression of heat-shock proteins.

Genes Whose Expression Was Strongly Repressed by Salt Stress

Figure 3B shows the genes whose levels of expression were reduced by more than 3.0-fold upon incubation of cells with 0.5 M NaCl or 0.5 M sorbitol for 30 min. Salt stress specifically depressed the expression of the genes for a desaturase (*desD*) and acyl carrier protein (*acp*).

The genome of *Synechocystis* includes the genes for four acyl-lipid desaturases (*desA, desB, desC,* and *desD*), which introduce double bonds into fatty-acyl chains (40). These enzymes play an important role in the maintenance of the structure and function of biological membranes (2). We found that expression of *desB, desC,* and *desD* genes was specifically reduced by salt stress, with reductions of 2.0-, 2.7-, and 3.0-fold, respectively.

Genes Whose Expression Was Strongly Repressed by Hyperosmotic Stress

Hyperosmotic stress specifically depressed the expression of the *cbiM* gene, the *lexA* gene. The *cbiM* (*sll0383*) genes are included in the gene cluster that encodes components of ABC-type cobalt transporter. Cobalt ions are necessary for the biosynthesis of cobalamin. The *lexA* gene encodes a transcriptional repressor of SOS function. The SOS response has been well characterized in *E. coli,* in which the reduce expression of the *lexA* gene under severe hyperosmotic stress results in damage to DNA (41).

Genes Whose Expression Was Repressed by Both Kinds of Stress

Both salt stress and hyperosmotic stress depressed the expression of genes for components of photosystem I (*psaD* and *psaF*), components of phycobilisomes (*cpcB, cpcC, cpcG, apcA, apcB, apcE,* and *apcF*), a sigma factor (*rpoDI*), a general secretion protein (*gspD*) and proteins of unknown function.

Most of the genes for components of photosystem I and phycobilisomes are located in tandem on the *Synechocystis* chromosome (20). We demonstrated previously that salt stress and hyperosmotic stress have inhibitory effects on the electron-transfer activity of photosystem I (9, 10). Repression of the expression of genes for photosystem I and phycobilisomes might be important for maintenance of a certain level of photosynthetic activity.

Synechocystis has five homologs of sigma-70 factors. The *rpoD1* gene (*slr0653*) encodes an indispensable sigma-70 factor, whereas the other *rpoD* genes encode sigma-70 factors that are not essential for growth under normal conditions (42). Both salt stress and hyperosmotic stress induced the enhanced expression of sigma-70 factors, slr0306 and sll2012, which are not essential for normal growth but might be involved in regulation of the expression of stress-inducible genes in *Synechocystis.*

Expression of the *gspD* gene for a protein in the general secretion pathway was also depressed by both salt stress and hyperosmotic stress. The GspD protein is located in the outer membrane in *Pseudomonas aeruginosa* (43). The repressed expression of the *gspD* gene suggests again that the outer membrane and plasma membrane are the targets of salt stress and hyperosmotic stress.

The regulation by salt stress and hyperosmotic stress of the expression of different sets of genes suggests that different mechanisms are operative in *Synechocystis* for acclimation to salt stress and hyperosmotic stress and, moreover, that *Synechocystis* recognizes salt stress and hyperosmotic stress as different kinds of stress. However, the components of the signaling pathways for salt stress and hyperosmotic stress remain to be identified.

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Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*

Iwane Suzuki,[1] Yu Kanesaki,[1] Koji Mikami,[1] Minoru Kanehisa[2] and Norio Murata[1]*
[1]*Department of Regulation Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan.*
[2]*Institute for Chemical Research, Kyoto University, Uji 611-0011, Japan.*

Summary

A histidine kinase, Hik33, appears to sense decreases in temperature and to regulate the expression of certain cold-inducible genes in the cyanobacterium *Synechocystis* sp. PCC6803. To examine the role of Hik33 in the regulation of gene expression, we analysed a ΔHik33 mutant using the DNA microarray technique. In wild-type cells, genes that were strongly induced at low temperature encoded proteins that were predominantly subunits of the transcriptional and translational machinery. Most cold-repressible genes encoded components of the photosynthetic machinery. Mutation of the *hik33* gene suppressed the expression of some of these cold-regulated genes, which could be divided into three groups according to the effect of the mutation of *hik33*. In the first group, regulation of gene expression by low temperature was totally abolished; in the second group, the extent of such regulation was reduced by half; and, in the third group, such regulation was totally unaffected. These results suggest that expression of the genes in the first group is regulated solely by Hik33, expression of genes in the third group is regulated by an as yet unidentified cold sensor, and expression of genes in the second group is regulated by both these cold sensors.

Introduction

Some organisms acclimate to low temperatures by regulating the expression of certain genes. For example, enhanced expression of genes for acyl-lipid desaturases at low temperatures is one of the essential events for acclimation to low temperature, increasing the extent of *cis*-unsaturation of the fatty acids in membrane lipids and, thus, maintaining the fluidity of membranes at an appropriate level (Murata and Los, 1997). Inactivation of the *desA* and *desD* genes for Δ9 and Δ12 desaturases reduces the capacity for acclimation to low temperature of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*; Tasaka *et al.*, 1996). Desaturation of fatty acids in membrane lipids at low temperatures has been well characterized in various other poikilothermic organisms, such as several strains of cyanobacteria (Sato and Murata, 1980; Murata and Wada, 1995; Sakamoto *et al.*, 1997), *Bacillus subtilis* (Aguilar *et al.*, 1999), *Tetrahymena* (Thompson and Nozawa, 1977), *Acanthamoeba* (Avery *et al.*, 1995), *Caenorhabditis elegans* (Tanaka *et al.*, 1996) and carp (Cossins, 1994).

We found recently that a membrane-associated histidine kinase, Hik33, and a soluble histidine kinase, Hik19, appear to be involved in the perception and transduction of the low-temperature signal that regulates the low temperature-dependent induction of the genes for ω3 and Δ6 acyl-lipid desaturase and for RNA helicase in *Synechocystis* (Suzuki *et al.*, 2000). We suggested that a change in membrane fluidity might be important for the perception of temperature that results in induction of the synthesis of the desaturases (Vigh *et al.*, 1993). Therefore, we concluded that Hik33, which is located in membranes, is a low-temperature sensor that regulates the expression of some low temperature-inducible genes in *Synechocystis* (Suzuki *et al.*, 2000).

Various cold-inducible genes have been identified in cyanobacteria (Los and Murata, 1999), for example genes for fatty acid desaturases (Murata and Wada, 1995), RNA-binding proteins (Sato, 1995), ribosomal protein S21 (Sato *et al.*, 1997), ribosomal protein L9, cytochrome C_M (Malakhov *et al.*, 1999) and two RNA helicases (Chamot *et al.*, 1999). Thus, acclimation to low-temperature stress includes the co-ordinated regulation of the expression of multiple genes. The roles of proteins encoded by low temperature-inducible genes in the acclimation to low temperature are not fully understood. It is also not yet clear how all the genes on the chromosome are regulated during acclimation to low temperature.

As described below, we used a recently developed technique, known as DNA microarray analysis (Watson *et al.*, 1998; DeRisi and Iyer, 1999), to monitor simultaneous changes in levels of transcripts of almost all the genes in *Synechocystis*. We found that low temperature induced the expression of genes for some components of the translational and transcriptional machinery and

Accepted 29 January, 2001. *For correspondence. E-mail murata@nibb.ac.jp; Tel. (+81) 564 55 7600; Fax (+81) 564 54 4866.

repressed the expression of genes for components of photosystem I and phycobilisomes. Mutation of Hik33 suppressed the induction by low temperature of the expression of certain genes for components of the transcriptional and translational machinery and also decreased the extent of suppression of the expression of some cold-repressible genes, such as genes for proteins in the photosynthetic apparatus.

Results

Modulation of gene expression by inactivation of the hik33 *gene*

From our earlier work (Suzuki *et al.*, 2000), the *hik33* gene appeared to be involved in the cold-induced expression of certain genes. In this study, we examined whether Hik33 might affect the expression of certain genes under isothermal conditions using a DNA microarray. Figure 1A shows the effects of the inactivation of Hik33 on the expression of individual genes when wild-type and mutant cells were grown at the same temperature (34°C). The inactivation of Hik33 enhanced the expression of one gene and repressed the expression of several others. However, most data points appeared between two lines, which corresponded to ratios of the expression between the two cultures being 2:1 and 1:2, respectively (Fig. 1A), indicating that the respective levels of expression of each gene were similar in the two types of cell.

In order to verify our results, we prepared Cy3- and Cy5-labelled cDNAs from a sample of RNA that had been extracted from wild-type cells grown at 34°C and allowed the cDNAs to hybridize to the DNA microarray (Fig. 1B). All data points appeared between the two reference lines, which corresponded to ratios of the expression being 2.0 and 0.5. Even for genes with signals as low as 0.01% of the total signal, the ratios of levels were between 2:1 and 1:2. These results suggested that data points that appeared above the line 2:1 or below the line 1:2 in Fig. 1A corresponded to genes whose expression had been significantly enhanced or repressed by the inactivation of Hik33.

The genes whose expression was affected by the inactivation of Hik33 are listed in Table 1. The inactivation of Hik33 quadrupled the levels of expression of the *hlyA* gene, which encodes a homologue of haemolysin (Bhaya *et al.*, 1999). It also repressed the expression of genes designated sll1407, sll1404 (*exbB*), sll1406 (*fhuA*), sll1408, slr1484 and slr1485.

To verify the results in Fig. 1A and Table 1, we examined the levels of expression of the *hlyA* gene and sll1404 by Northern blotting analysis (Fig. 2). The *hlyA* gene was more strongly expressed in ΔHik33 cells than in wild-type cells. Incubation of ΔHik33 cells at 22°C for 20 min slightly depressed the expression of this gene. In wild-type cells, the downward shift in temperature to 22°C induced the expression of *hlyA*, although the level of expression at 22°C was much lower than in ΔHik33 cells at both 34°C and 22°C (Fig. 2A).

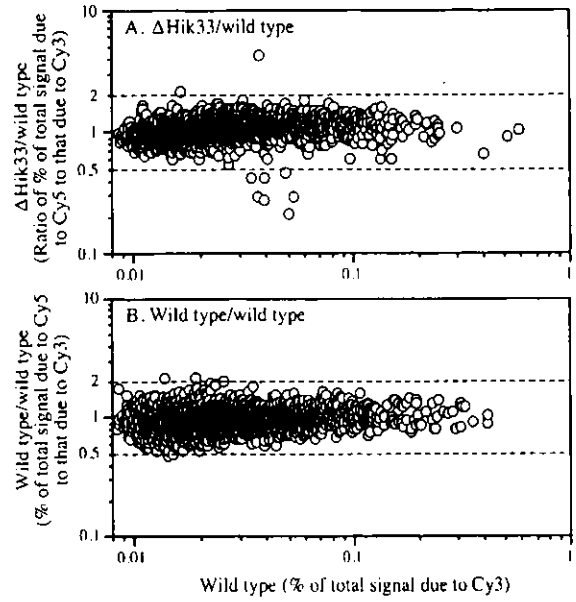

Fig. 1. The expression of genes as revealed by DNA microarray analysis.
A. Wild-type cells, grown at 34°C for 16 h, were compared with ΔHik33 cells grown under the same conditions. After growth at 34°C for 16 h, cells were harvested, and total RNA was extracted. The RNA from wild-type and ΔHik33 cells was used to synthesize Cy3-labelled and Cy5-labelled cDNAs respectively.
B. Control experiment with wild-type cells grown at 34°C. RNA from wild-type cells was used to synthesize Cy3- and Cy5-labelled cDNAs, which were allowed to hybridize to the DNA microarray. Dashed lines correspond to reference lines that indicate the limit of experimental deviations.

The sll1404 transcripts appeared as a smear. Their lengths ranged from 0.8 kb to 5.6 kb, and they were abundant in wild-type cells grown at 34°C. Incubation of wild-type cells at 22°C for 20 min markedly depressed the levels of these transcripts. Two bands of RNAs of apparently 2.1 kb and 1.4 kb were considered to be artifacts because of the dense bands of rRNA of 1.4 kb and 2.5 kb. The largest RNA, which was 5.6 kb long, was most probably the transcript of the entire gene cluster that consists of sll1404, sll1405, sll1406, sll1407 and sll1408 (see *Discussion*), and its appearance suggests that these genes were co-transcribed as a single operon. The expression of this operon was repressed by low temperature in wild-type cells. Inactivation of Hik33 almost completely eliminated the transcription of the operon irrespective of the growth temperature of the cells. The results obtained from the DNA microarray were qualitatively consistent with the results of Northern blotting analysis.

Table 1. Genes whose expression was strongly enhanced or strongly suppressed by inactivation of Hik33.

ORF no.	Gene	Product	34°C cells	22°C cells
Enhanced expression			ΔHik33/WT	ΔHik33/WT
sll1951	*hlyA*	Haemolysin	4.29 ± 1.39	2.15 ± 0.70
Suppressed expression			WT/ΔHik33	WT/ΔHik33
sll1407		Methyl transferase	6.73 ± 1.27	3.41 ± 2.22
sll1404	*exbB*	Biopolymer transport	5.10 ± 0.53	1.82 ± 0.67
sll1406	*fhuA*	Ferrichrome-iron transport	4.20 ± 0.32	3.28 ± 1.72
sll1408		Transcriptional regulator	4.15 ± 1.48	3.00 ± 1.72
slr1485		Protein of unknown function	2.80 ± 0.93	1.02 ± 0.50
slr1484		Protein of unknown function	2.49 ± 0.17	1.78 ± 0.48

Wild-type and ΔHik33 cells were grown at 34°C for 16 h (34°C cells) and then incubated at 22°C for 20 min (22°C cells). Each value indicates the ratio of levels of expression in wild-type cells (WT) and ΔHik33 cells (ΔHik33). Genes that gave ratios greater than 2.0 are listed. The values shown are the means ± SE of results of four experiments. The numbering of ORFs corresponds to that of Kaneko *et al.* (1995; 1996). The complete list can be accessed at http://www.genome.ad.jp/kegg/expression

Cold-induced genes in wild-type and ΔHik33 cells

Figure 3 shows the effects of the inactivation of Hik33 on the cold-inducible expression of genes. Most of the data points lie between the two reference lines in the case of both wild-type and ΔHik33 cells, indicating that the expression of the majority of genes was not affected by low temperature in either type of cell. Spots that do not fall between the reference lines correspond to those genes for which the downward shift in temperature altered the level of expression (Fig. 3). The extent of deviations from the reference lines was much smaller for ΔHik33 cells than for wild-type cells (Fig. 3), suggesting that the ability of various genes to respond to low temperature was reduced by the inactivation of Hik33.

Table 2 lists the genes whose levels of expression were more than doubled in wild-type cells during incubation at 22°C for 20 min. These genes encode proteins involved in transcription (*crh*, *rpoD*, *nusG* and *rpoA*), translation (*fus*, *rbp1*, *rpl23*, *rpl3*, *rpl4*, *rps12 and rpl20*), dehydrogenation of NADH (*ndhD2 and ndhD6*), synthesis of cofactors (*folE and cbiM*) and synthesis of peptide glycan (*murA*), as well as proteins of unknown function (slr0082, slr1544, *ycf39*, sll1911, slr0551, sll0668, slr1974 and slr0955).

Inactivation of Hik33 eliminated the cold inducibility of the *ndhD2*, *rpoD*, slr1544, *folE* and *murA* genes. Furthermore, inactivation of Hik33 reduced the levels of expression of the slr0082, *crh*, *fus*, *ycf39*, *ndhD6* and sll1911 genes. In contrast, cold induction of the *rbp1*, *rpl3*, *rpl23*, *rpl4*, *rps12*, *rpl20*, *nusG* and *rpoA* genes was unaltered by the mutation.

The cold inducibility of *desA*, *desB* and *desD* genes for acyl-lipid desaturases in *Synechocystis* has been well established (Los *et al.*, 1997), and the activity of the *desB* promoter was used to identify the cold sensor, Hik33 (Suzuki *et al.*, 2000). However, the cold inducibility of these genes did not appear to be significant in the DNA microarray assay. Inactivation of the *hik33* gene reduced the levels of induction of the *desB and desD* genes

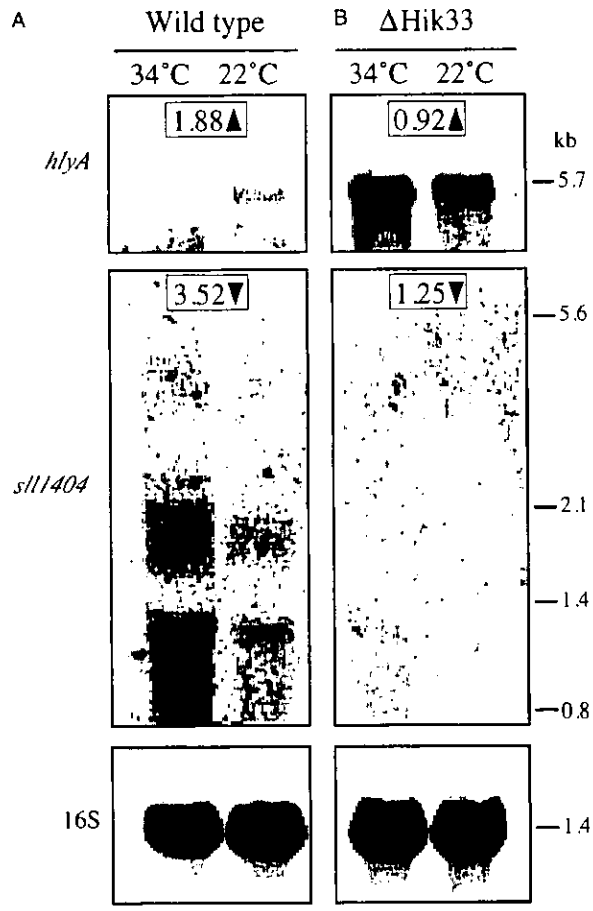

Fig. 2. Northern blotting analysis of the temperature-dependent expression of genes that were affected by inactivation of Hik33. Total RNA was extracted from wild-type cells (A) and ΔHik33 cells (B) that had either been grown at 34°C for 16 h or had been grown at 34°C for 16 h and then incubated at 22°C for 20 min. Aliquots (20 μg) of the extracted RNA were fractionated on 1.2% agarose gels that contained 1.4 M formaldehyde. Numbers in squares indicate ratios for the induction (▲) and repression (▼) by low temperature of individual genes that were determined by DNA microarray analysis.

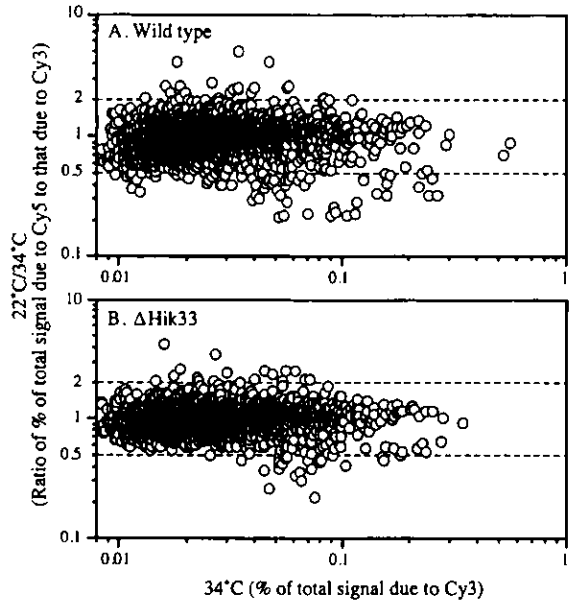

Fig. 3. Regulation of gene expression by low temperature in wild-type and ΔHik33 cells.
A. Wild-type cells, grown at 34°C for 16 h (34°C cells), were compared with wild-type cells that had been grown at 34°C for 16 h and then incubated at 22°C for 20 min (22°C cells).
B. ΔHik33 cells, grown at 34°C for 16 h, were compared with ΔHik33 cells that had been grown at 34°C for 16 h and then incubated at 22°C for 20 min. RNA extracted from 34°C cells was used to synthesize Cy3-labelled cDNAs, and RNA extracted from 22°C cells was used to synthesize Cy5-labelled cDNAs. The cDNAs were mixed and allowed to hybridize to the DNA microarray. Dashed lines correspond to reference lines that indicate the limit of experimental deviations.

from 1.82 ± 0.23 and 1.90 ± 0.43 to 1.45 ± 0.11 and 1.67 ± 0.43 respectively (data not shown).

We also examined the effect of extended incubation at 22°C (namely 60 min instead of 20 min) on the pattern of gene expression. Essentially the same results as for the incubation for 20 min were obtained on the cold inducibility and effects of inactivation of Hik33 (data not shown).

To confirm the effects of the inactivation of Hik33 on the cold induction of genes listed in Table 2, we examined the cold induction of the various genes by Northern blotting analysis (Fig. 4). Induction of the slr0082 gene in ΔHik33 cells by low temperature was much less efficient than in wild-type cells (Fig. 4). The low temperature-dependent induction of the *rpoD*, *ndhD2* and sll1544 genes was almost eliminated by mutation of the *hik33* gene. However, expression of the *rbp1* and *rpl3* genes was unaffected by the mutation. Thus, the results of DNA microarray analysis were qualitatively consistent with those obtained by Northern blotting. It appears that genes whose expression is enhanced by low temperature can be divided into three groups according to the effect of the inactivation of Hik33. In the first group, the regulation of gene expression by low temperature was totally eliminated; in the second group, the extent of such regulation was reduced and, in the third group, regulation was unaffected.

Cold-repressible genes in wild-type and Δ Hik33 cells

Table 3 lists the cold-repressible genes whose levels of expression were reduced more than twofold after incubation of wild-type cells at 22°C for 20 min. These genes encoded components of photosystem I (*psaD*, *psaF*, *psaJ* and *psaL*), components of phycobilisomes (*apcA*, *apcB*, *apcC*, *apcE*, *apcF*, *cpcA*, *cpcC* and *cpcG*), a protein involved in the photosynthetic transport of electrons (*petA*) and proteins involved in the synthesis of porphyrin compounds (*chlP*, *hemA*, *hemF* and *ho1*). Low temperature also depressed the expression of several genes for proteins of unknown function. In particular, the expression of the sll1783, sll1784, sll1785, slr1852, slr1855, slr1856 and sll1549 genes was strongly depressed by low temperature. Extended incubation of wild-type cells at 22°C for 60 min did not change the pattern of gene expression (data not shown), suggesting that early events of gene expression in *Synechocystis* cells were observed in our experimental system.

The inactivation of Hik33 affected the expression of most of the cold-repressible genes, although the extent of the effect depended on the individual gene. Cold repression of the slr0147, slr0145, slr0144, slr0149, slr0151, *hemF*, *im30*, slr1535, *lrtA* and *AT103* genes was diminished by the mutation of the *hik33* gene. In contrast, the expression of some cold-repressible genes, such as *sufA*, *apcF and cpcG*, was unaffected by the mutation. The extent of the cold-dependent regulation of expression of other cold-repressible genes was reduced somewhat by the mutation. Thus, the cold-repressible genes can also be divided into three groups according to the effect of the elimination of the activity of Hik33. In the first group, the regulation of gene expression by low temperature was totally abolished; in the second group, it was reduced somewhat and, in the third group, regulation was unaffected.

Discussion

Effects of inactivation of Hik33 on gene expression

Expression of the *hlyA* gene for a homologue of haemolysin was enhanced by low temperature and also by mutation of the *hik33* gene for Hik33 (Figs 1 and 2 and Table 1). The expression of two gene clusters, sll1404 to sll1408 and slr1484 plus slr1485, which encode components related to transport (Braun *et al.*, 1994) and some unknown proteins, was depressed by low temperature and by mutation of the *hik33* gene (Figs 1 and 2 and Table 1).

Figure 5A shows the genomic structure of the sll1404–sll1405–sll1406–sll1407–sll1408 operon and the slr1484–slr1485 operon. The two operons are located adjacent to

Table 2. Cold-inducible genes and effects of the inactivation of Hik33 on their cold induction.

ORF no.	Gene	Product	Wild type 22°C/34°C	ΔHik33 22°C/34°C
Genes in which cold inducibility was diminished in ΔHik33				
slr1291	*ndhD2*	NADH dehydrogenase subunit 4	2.80 ± 0.55	0.89 ± 0.14
slr1544		Protein of unknown function	2.65 ± 0.68	0.89 ± 0.25
sll2012	*rpoD*	σ^{70} factor of RNA polymerase	2.53 ± 0.69	0.73 ± 0.14
slr0426	*folE*	GTP cyclohydrolase I	2.31 ± 0.37	0.96 ± 0.16
slr0017	*murA*	Enoylpyruvate transferase	2.03 ± 0.56	1.01 ± 0.14
Genes in which cold inducibility was marginally affected in ΔHik33				
slr0082		Protein of unknown function	5.01 ± 0.27	2.13 ± 0.06
slr0083	*crh*	ATP-dependent RNA helicase	4.74 ± 1.28	2.86 ± 0.80
slr1105	*fus*	Elongation factor EF-G	2.44 ± 0.64	1.34 ± 0.19
slr0399	*ycf39*	Protein of unknown function	2.40 ± 0.87	1.14 ± 0.16
slr2009	*ndhD6*	NADH dehydrogenase subunit 4	2.12 ± 0.18	1.66 ± 0.32
sll1911		Protein of unknown function	2.01 ± 0.21	1.29 ± 0.21
Genes in which cold inducibility was unaffected in ΔHik33				
sll0517	*rbp1*	RNA-binding protein 1	3.98 ± 0.68	4.16 ± 0.81
sll1801	*rpl23*	50S ribosomal protein L23	2.65 ± 0.73	2.32 ± 0.26
sll1799	*rpl3*	50S ribosomal protein L3	2.63 ± 0.70	2.44 ± 0.43
sll1742	*nusG*	Transcription antiterminator	2.59 ± 0.83	3.57 ± 1.11
slr0551		Protein of unknown function	2.42 ± 0.87	2.54 ± 0.43
sll0668		Protein of unknown function	2.41 ± 0.60	2.57 ± 0.60
sll1818	*rpoA*	α-subunit of RNA polymerase	2.28 ± 0.64	2.08 ± 0.24
sll0383	*cbiM*	Cobalamin biosynthesis protein M	2.10 ± 0.22	1.77 ± 0.14
sll1800	*rpl4*	50S ribosomal protein L4	2.08 ± 0.09	2.14 ± 0.08
slr1974		Protein of unknown function	2.04 ± 0.79	2.12 ± 0.35
sll1096	*rps12*	30S ribosomal protein S12	2.02 ± 0.25	2.00 ± 0.24
slr0955		Protein of unknown function	2.02 ± 0.47	2.36 ± 0.51
sll0767	*rpl20*	50S ribosomal protein L20	2.01 ± 0.63	2.05 ± 0.28

Cells were grown at 34°C for 16 h (34°C cells) and then incubated at 22°C for 20 min (22°C cells). Each value indicates the ratio of levels of the mRNA from 22°C cells and 34°C cells. Genes that gave ratios greater than 2.0 are listed. The values shown are the means ± SE of the results of three independent experiments. The numbering of ORFs corresponds to that of Kaneko *et al.* (1995; 1996). The complete list can be accessed at http://www.genome.ad.jp/kegg/expression

one another on opposite strands of DNA, and they appear to share an upstream regulatory region.

Cold-inducible genes in Synechocystis

The DNA microarray analysis demonstrated that expression of a number of genes was enhanced after a downward shift in temperature. The genes encode components of the transcriptional and translational machinery, and they include *crh*, *rpoD*, *fus*, *nusG* and *rpoA*, genes for subunits of NADH dehydrogenase and a number of genes for proteins of unknown function.

We demonstrated previously that low temperature inhibits the repair of photosystem II after light-induced inactivation and that the synthesis of proteins *de novo* is essential for the repair of the photosynthetic machinery (Gombos *et al.*, 1994; Wada *et al.*, 1994; Kanervo *et al.*, 1997; Sippola *et al.*, 1998). Our finding that low temperature induces the expression of various genes for components of the protein-synthesizing machinery demonstrates the presence in cyanobacterial cells of a mechanism that compensates for the depression of protein synthesis at low temperature by the synthesis *de novo* of the proteins that are required for protein synthesis.

The slr1544 gene is located downstream of ssr2595 (*hliB*; Fig. 5B), which is a homologue of the *hliA* gene and induced by strong light in *Synechococcus* (Dolganov *et al.*, 1995). Northern blotting analysis revealed that the hybridizing transcript was ≈700 bases long (Fig. 4). Such a transcript might correspond to the co-transcript of slr1544 (309 bases) and ssr2595 (210 bases).

Cold-repressible genes in Synechocystis

The DNA microarray analysis demonstrated that low temperature strongly repressed the expression of a variety of genes, in particular genes for subunits of the photosystem I complex, such as *psaD*, *psaF*, *psaJ* and *psaL*, and genes for subunits of phycobilisomes, such as *apcE*, *apcB*, *apcA*, *cpcA*, *apcC*, *cpcC*, *apcF* and *cpcG*. This observation may be related to the findings of Constant *et al.* (1997) and Alfonso *et al.* (1999; 2000) that exposure of *Synechocystis* sp. PCC6714 cells to strong light repressed expression of the *psaE* gene for a subunit of photosystem I and the cluster of *cpcB* and *cpcA* genes for subunits of phycobilisomes. As photosystem II activity is decreased at low temperature (Gombos *et al.*, 1994; Wada *et al.*, 1994; Kanervo *et al.*, 1997; Sippola *et al.*, 1998), the repression of expression of the genes for photosystem I and

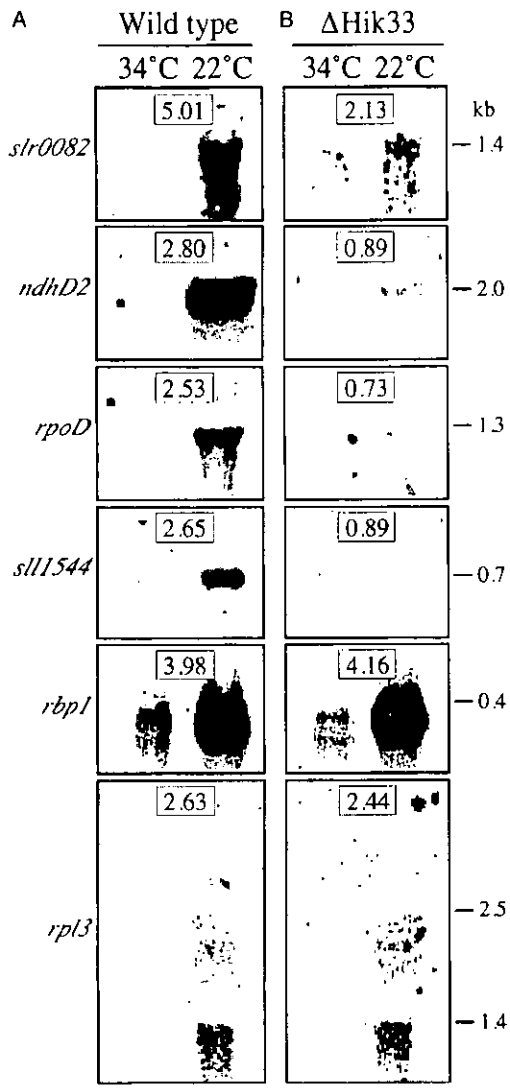

Fig. 4. Temperature-dependent expression of genes that was affected by inactivation of Hik33. Total RNA was extracted from wild-type cells (A) and ΔHik33 cells (B) that had either been grown at 34°C for 16 h or been grown at 34°C for 16 h and then incubated at 22°C for 20 min. Aliquots of 20 μg of RNA were fractionated on 1.2% agarose gels that contained 1.4 M formaldehyde. Numbers in squares indicate ratios for the induction by low temperature of individual genes that were determined by DNA microarray analysis.

phycobilisomes might be important in the maintenance of a balance between the activities of both photosystems.

Low temperature also depressed the expression of the *chlP*, *hemA*, *hemF* and *ho1* genes, which encode proteins involved in the synthesis of porphyrin compounds, such as chlorophyll synthase, glutamyl-tRNA reductase, co-porphyrinogen III oxidase and haem oxygenase respectively. Repression of the expression of genes that are related to biosynthesis of the photosynthetic pigments appears to be co-ordinated with that of certain genes for components of photosystem I and phycobilisomes.

The expression of genes for several proteins of unknown function was also strongly repressed by low temperature in wild-type cells. In particular, the expression of genes in two gene clusters, namely sll1783–sll1784–sll1785–sll1304–sll1305 and slr1852–(slr1853)–slr1854–slr1855–slr1856 (Fig. 5C), was strongly depressed at low temperature. These gene clusters are located on opposite DNA strands and share an upstream regulatory region. The extent of the suppression of expression of these genes after the shift to 22°C was larger than that of other cold-repressible genes (Table 3), suggesting, perhaps, that the suppression of their expression might play a particularly important role in acclimation of *Synechocystis* to low temperature.

The expression of the gene clusters of sll1549–sll1550 (Fig. 5D) and slr0144 to slr0151 (Fig. 5E) was also strongly suppressed at 22°C (Table 3). These gene clusters are located in tandem in the vicinity of some of the genes for photosynthetic components, such as *psaL*, *psaI* and *petF*. The proteins of unknown function, encoded by the other genes in the cluster, might be related to photosynthesis.

Understanding the mechanisms of acclimation of cyanobacterial cells to environmental stresses requires identification of genes whose expression is altered during acclimation. DNA microarray analysis allowed us to identify such genes during acclimation to low temperature. We identified a number of genes for proteins of unknown function in *Synechocystis*, whose expression was induced or repressed by low temperature. Our current efforts to identify the functions of these novel proteins might enhance our understanding of the molecular mechanisms of acclimation to low temperature.

Pathways for transduction of low-temperature signals in Synechocystis

The mutation of the *hik33* gene led to the complete loss of the low temperature-dependent regulation of expression of certain genes, such as *ndhD2*, *rpoD*, slr1544, sll0144, sll0145 and sll0147. The low temperature-inducible regulation of other genes, such as slr0082, *crh*, sll1783 and slr1852, was inhibited to a limited extent by mutation of the *hik33* gene (Tables 2 and 3). In contrast, the low temperature-dependent regulation of genes for ribosomal proteins and of the *sufA* and *apcF* genes was unaffected by mutation of the *hik33* gene (Tables 2 and 3). Therefore, cold-regulated genes can be divided into three groups according to the effects of inactivation of Hik33 on their expression.

The cold regulation of the expression of the genes in the first group is completely under the control of Hik33, whereas that of the genes in the third group is independent of the activity of Hik33. It is very likely that an additional cold sensor exists in *Synechocystis* that initiates another signal transduction pathway for the cold

Table 3. Cold-repressible genes and effects of the inactivation of Hik33 on their cold repression.

ORF no.	Gene	Product	Wild type 34°C/22°C	ΔHik33 34°C/22°C
Genes in which cold repression was diminished in ΔHik33				
slr0147		Protein of unknown function	2.98 ± 0.75	1.08 ± 0.24
slr0145		Protein of unknown function	2.58 ± 0.21	0.98 ± 0.12
slr0144		Protein of unknown function	2.54 ± 0.16	1.25 ± 0.07
sll1185	*hemF*	Coproporphyrinogen III oxidase	2.50 ± 0.94	0.79 ± 0.03
sll0617	*im30*	Chloroplast membrane protein	2.31 ± 0.21	1.09 ± 0.12
slr1535		Protein of unknown function	2.13 ± 0.17	0.90 ± 0.13
sll0947	*lrtA*	Light-repressed protein	2.10 ± 0.32	1.05 ± 0.10
slr0149		Protein of unknown function	2.05 ± 0.25	0.98 ± 0.24
slr0151		Protein of unknown function	2.03 ± 0.28	1.07 ± 0.16
Genes in which cold repression was marginally affected in ΔHik33				
sll1783		Protein of unknown function	4.89 ± 1.86	1.68 ± 0.44
slr1852		Protein of unknown function	4.69 ± 0.98	2.76 ± 0.46
sll1091	*chlP*	Chlorophyll synthase	4.63 ± 0.68	1.47 ± 0.21
sll1785		Protein of unknown function	4.44 ± 1.57	2.06 ± 0.09
sll1549		Protein of unknown function	4.35 ± 1.02	1.77 ± 0.40
slr1855		Protein of unknown function	4.27 ± 1.02	2.35 ± 0.16
sll1784		Protein of unknown function	4.18 ± 1.10	2.04 ± 0.40
slr0737	*psaD*	Subunit II of photosystem I	3.83 ± 0.77	2.25 ± 0.19
slr0335	*apcE*	Phycobilisome core–membrane linker	3.59 ± 0.99	1.82 ± 0.22
slr1986	*apcB*	Allophycocyanin B chain	3.18 ± 0.71	1.74 ± 0.36
sll0819	*psaF*	Subunit III of photosystem I	3.12 ± 0.71	2.27 ± 0.23
slr2067	*apcA*	Allophycocyanin A chain	3.08 ± 0.42	1.83 ± 0.17
slr1808	*hemA*	Glutamyl-transfer RNA reductase	2.91 ± 0.33	1.84 ± 0.15
slr1856		σ-regulatory protein	2.76 ± 0.89	1.17 ± 0.18
sll1578	*cpcA*	Phycocyanin A chain	2.64 ± 0.59	1.76 ± 0.18
sml0008	*psaJ*	Subunit IX of photosystem I	2.64 ± 0.55	1.64 ± 0.48
slr1128		Protein of unknown function	2.52 ± 0.59	1.55 ± 0.27
ssr3383	*apcC*	Phycobilisome LC linker protein	2.47 ± 0.32	1.72 ± 0.38
slr1655	*psaL*	Subunit XI of photosystem I	2.46 ± 0.37	1.57 ± 0.30
slr1854		Protein of unknown function	2.41 ± 0.26	1.92 ± 0.17
sll1184	*ho1*	Haem oxidase	2.39 ± 0.19	1.31 ± 0.18
sll1304		Protein of unknown function	2.33 ± 0.28	1.57 ± 0.07
sll1305		Protein of unknown function	2.33 ± 0.22	1.57 ± 0.07
sll1579	*cpcC*	Phycocyanin linker protein	2.17 ± 0.08	1.92 ± 0.08
sll1550		Protein of unknown function	2.14 ± 0.33	1.59 ± 0.20
slr1752		Protein of unknown function	2.12 ± 0.19	1.34 ± 0.22
sll1214	*AT103*	Phytochrome-regulated bZIP protein	2.09 ± 0.24	1.26 ± 0.16
sll1712		DNA-binding protein HU	2.07 ± 0.19	1.60 ± 0.27
sll0051		Protein of unknown function	2.03 ± 0.26	1.47 ± 0.31
Genes in which cold repression was unaffected in ΔHik33				
slr1295	*sufA*	Iron transport protein	3.02 ± 0.45	2.24 ± 0.50
slr1459	*apcF*	Component of phycobilisome core	2.65 ± 0.91	2.10 ± 0.29
slr2051	*cpcG*	Phycobilisome rod–core linker	2.44 ± 0.65	2.08 ± 0.19
slr1397		Protein of unknown function	2.14 ± 0.68	1.63 ± 0.40
sll1621		Protein of unknown function	2.07 ± 0.45	1.81 ± 0.44
sll1580	*cpcC*	Phycocyanin linker protein	2.06 ± 0.35	1.95 ± 0.27
slr1841		Protein of unknown function	2.03 ± 0.41	2.06 ± 0.24
sll1317	*petA*	Apocytochrome f	2.03 ± 0.50	1.76 ± 0.22

See Table 2 for details. The complete list can be accessed at http://www.genome.ad.jp/kegg/expression

regulation of the expression of genes in the third group. The expression of genes in the second group might be controlled by both Hik33 and the unidentified cold sensor. A search for the unidentified sensor is in progress.

Experimental procedures

Cells and culture conditions

A strain of *Synechocystis* sp. PCC 6803 that is tolerant to glucose (Williams, 1988) was provided by Dr J. G. K. Williams (DuPont de Nemours). The mutant with a defect in histidine kinase 33 (ΔHik33) was obtained as described previously (Suzuki *et al.*, 2000). Wild-type and ΔHik33 cells were grown at 34°C in BG-11 medium (Stanier *et al.*, 1971) buffered with 20 mM HEPES–NaOH (pH 7.5) under continuous illumination from incandescent lamps, as described previously (Wada and Murata, 1989).

DNA microarray analysis of gene expression

A DNA microarray, CyanoCHIP, was obtained from Takara

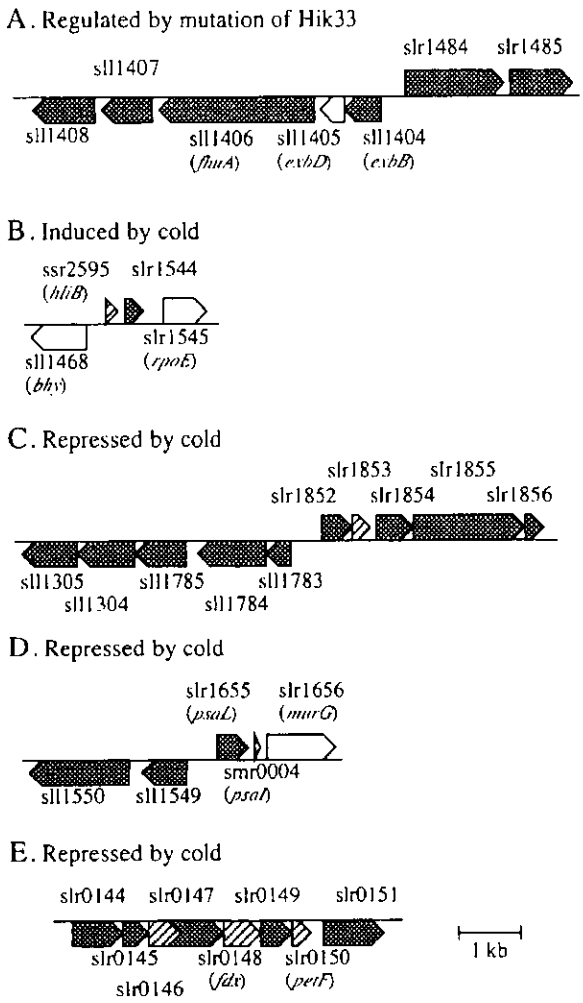

Fig. 5. Gene clusters in which gene expression was affected by inactivation of Hik33 and by a downward shift in temperature. Arrows indicate directions of transcription and lengths of open reading frames. Shaded arrows, genes whose levels of expression changed by more than twofold (listed in Tables 1, 2 or 3); slashed arrows, genes whose levels of expression changed by 1.6- to 2.0-fold; open arrows, genes that were apparently unregulated by inactivation of Hik33 and a downward shift in temperature (< 1.1-fold).
A. Gene clusters *exbB–exbD–fhuA*–sll1407–sll1408 and slr1484–slr1485, whose expression was suppressed by inactivation of Hik33.
B. Gene cluster *hliB* and slr1544 whose expression was enhanced by low temperature.
C. Gene clusters sll1783–sll1784–sll1785–sll1304–sll1305 and slr1852–slr1853–slr1854–slr1855–slr1856, whose expression was suppressed by low temperature.
D. Gene clusters sll1549–sll1550 and *psaL–psaI*, whose expression was suppressed by low temperature.
E. Gene cluster slr0144–slr0145–slr0146–slr0147–*fdx*–slr0149–*petF*-slr0151, whose expression was suppressed by low temperature.

Shuzo. This microarray covers 3079 genes (≈97% of the total genes) and does not cover 89 insertion sequences that are related to transposons in *Synechocystis* (Kaneko *et al.*, 1995; 1996). Fluorescent dye-labelled cDNAs were synthesized from 20 μg of total RNA in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech). Reverse transcription was performed in 40 μl of a mixture that contained 300 pmol of random hexamer, 0.5 mM each of dATP, dGTP and dCTP, 0.2 mM dTTP, 0.1 mM Cy3-dUTP or Cy5-dUTP, 100 units of RNase inhibitor and 50 units of AMV reverse transcriptase XL in 1× reaction buffer attached to the reverse transcriptase. After incubation at 42°C for 1 h, 50 units of the reverse transcriptase were added again, and incubation was continued further at 42°C for 1 h. All the reagents apart from Cy3-dUTP and Cy5-dUTP were purchased from Takara Shuzo. The reaction was stopped by gel filtration on a Centri-Sep spin column (PE Applied Biosystems Japan). The eluate from the column was purified by washing with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1, v/v), and the labelled cDNA was precipitated in ethanol.

Hybridization of the mixture of Cy3- and Cy5-labelled cDNAs with DNA that had been immobilized on the DNA microarray was allowed to proceed at 65°C for 14–18 h in 20 μl of 4× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.2% SDS, 5× Denhardt's solution and 100 ng μl^{-1} denatured salmon sperm DNA. After hybridization, the microarray was rinsed with 2× SSC at room temperature, washed with 2× SSC at 60°C for 10 min, with 0.2× SSC plus 0.1% SDS at 60°C for 10 min and finally with 0.2× SSC at room temperature. After the final rinse, the microarray was centrifuged at 1000 r.p.m. for 2 min to remove residual salts before scanning on an array scanner (GMS 418; Genetic MicroSystems).

The intensity of each signal was determined with scan-analysing software (ImaGene version 2.0; BioDiscovery). The fluorescent signal specific for each gene was normalized by reference to the sum of the fluorescent signals specific for all 3079 genes. The large amount of rRNA in the original preparation of RNA resulted in high background signals from each spot on the microarray. Thus, the relative levels of mRNAs were very low. However, the results were reproducible, suggesting that background levels were similar in independent experiments and that the results were, at least qualitatively, reliable.

Northern blotting analysis

Total RNA was extracted from cells as described previously (Los *et al.*, 1997). The RNA was then incubated at 37°C for 30 min with 100 units of RNase-free DNase I (Nippon Gene) to eliminate contaminating genomic DNA, purified by washing with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1, v/v) and precipitated in ethanol. Northern blotting analysis was performed as described previously (Los *et al.*, 1997). DNA fragments corresponding to the *rpoD*, *ndhD2*, *hlyA*, *sll1544*, *sll1404*, *rbp1*, *rpl3* genes and the gene for 16S rRNA were conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech), and the resultant conjugates were used as probes. After hybridization, blots were soaked in CDP-star solution (Amersham Pharmacia Biotech), and signals from hybridized transcripts were detected with a luminescence image analyser (LAS-1000; Fuji-Photo Film).

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The pathway for perception and transduction of low-temperature signals in *Synechocystis*

Iwane Suzuki[1], Dmitry A.Los[1,2], Yu Kanesaki[1], Koji Mikami[1] and Norio Murata[1,3]

[1]Department of Regulation Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan and [2]Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Street 35, 127276 Moscow, Russia

[3]Corresponding author
e-mail: murata@nibb.ac.jp

Low temperature is an important environmental factor that has effects on all living organisms. Various low-temperature-inducible genes encode products that are essential for acclimation to low temperature, but low-temperature sensors and signal transducers have not been identified. However, systematic disruption of putative genes for histidine kinases and random mutagenesis of almost all the genes in the genome of the cyanobacterium *Synechocystis* sp. PCC 6803 have allowed us to identify two histidine kinases and a response regulator as components of the pathway for perception and transduction of low-temperature signals. Inactivation, by targeted mutagenesis, of the gene for each of the two histidine kinases and inactivation of the gene for the response regulator depressed the transcription of several low-temperature-inducible genes.

Keywords: histidine kinase/low-temperature-inducible gene/response regulator/signal perception and transduction/*Synechocystis*

Introduction

Poikilothermic organisms, such as prokaryotes, plants and fish, sense changes in ambient temperature and acclimate to such changes with greater or lesser efficiency (Kaye and Guy, 1995; Thieringer *et al.*, 1998). Numerous low-temperature-inducible genes have been found in a wide range of organisms, e.g. *csp* genes for cold-shock proteins in *Escherichia coli* (Jones *et al.*, 1987) and *Bacillus subtilis* (Willimsky *et al.*, 1992), *cor* and *cas* genes in plants (Gilmour *et al.*, 1992; Wolfraim *et al.*, 1993), and genes for fatty acid desaturases in cyanobacteria (Wada *et al.*, 1990; Murata and Wada, 1995; Murata and Los, 1997), plants (Gibson *et al.*, 1994) and fish (Tiku *et al.*, 1996). These low-temperature-inducible genes are thought to play an important role in acclimation to low temperature. To date, however, mechanisms for the perception and transduction of low-temperature signals remain to be characterized.

In a previous study (Vigh *et al.*, 1993), we demonstrated that decreases in the degree of unsaturation of fatty acids in the plasma membrane of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) by catalytic hydrogenation *in vivo* enhanced the expression of the *desA* gene for the Δ12 acyl-lipid desaturase, which is otherwise induced primarily by low temperature. Thus, a change in membrane fluidity appears to be important for the perception of temperature that results in induction of the synthesis of the desaturases (Los *et al.*, 1997). However, it remains unclear how a change in membrane fluidity is perceived and how the signal is transduced from the membrane to chromosomes to induce the expression of low-temperature-inducible genes, such as genes for desaturases.

The physical state of the membrane also affects the high-temperature-induced expression of heat-shock genes (Vigh *et al.*, 1998). Modification, by genetic manipulation, of the ratio of unsaturated to saturated fatty acids in *Saccharomyces cerevisiae* has a significant effect on the expression of the heat-shock genes *hsp70* and *hsp82* (Carratu *et al.*, 1996). These findings support the hypothesis that changes in the state of the membrane might also be important in the regulation of the expression of heat-shock genes.

Physical and chemical stimuli that are generated extra- and intracellularly are perceived by a group of proteins that includes histidine kinases. These proteins are localized on the plasma membrane or in the cytosol in various prokaryotes (Appleby *et al.*, 1996), yeast (Maeda *et al.*, 1994) and plants (Chang *et al.*, 1993; Kakimoto, 1996). It seems likely, therefore, that temperature-induced changes in membrane fluidity might be mediated by a membrane-bound histidine kinase. Kaneko *et al.* (1995, 1996) determined the sequence of the genome of *Synechocystis* and identified 43 putative genes for histidine kinases (Mizuno *et al.*, 1996).

In this study we attempted to identify components of the pathway for perception and transduction of low-temperature signals. Among the various low-temperature-inducible genes that had been identified previously, we focused on the *desB* gene for ω3 fatty acid desaturase in *Synechocystis* because the low-temperature-induced expression of this gene has been characterized in detail (Los *et al.*, 1997; Los and Murata, 1998). We monitored the promoter response of the *desB* gene to low temperature using a gene for bacterial luciferase as the reporter. Systematic mutagenesis of genes for histidine kinases and random mutagenesis of almost all the genes in the genome allowed us to identify genes for two histidine kinases and a response regulator as components of the pathway for perception and transduction of low-temperature signals.

Results

Systematic mutation of putative genes for histidine kinases in pdesB::lux cells

In order to monitor the inducibility by low temperature of the *desB* gene, we generated a strain of *Synechocystis*,

designated *pdesB::lux*, in which the promoter region of the *desB* gene was ligated to the coding region of the *luxAB* gene for a bacterial luciferase (Los *et al.*, 1997). Thus, luciferase activity, monitored in terms of luminescence, could be used as an indicator of low-temperature-inducible changes in the activity of the *desB* promoter.

The products of some of the 43 putative genes for histidine kinases in the genome of *Synechocystis* (Kaneko *et al.*, 1995, 1996; Mizuno *et al.*, 1996) might plausibly be expected to function as sensors or transducers of environmental or intracellular stimuli (Appleby *et al.*, 1996; Mizuno *et al.*, 1996). We designated the histidine kinases Hik1–Hik43 and their genes *hik1–hik43*. To investigate the contributions of the various histidine kinases to the induction by low temperature of transcription of the *desB* gene, we attempted to inactivate each of the genes for histidine kinase in *pdesB::lux* cells by inserting a spectinomycin-resistance gene (*Sp*^{r}) cassette (Prentki *et al.*, 1991) into the coding region or by replacing part of the coding region with the cassette, creating a gene-knockout library.

Each cyanobacterial cell contains >10 copies of the chromosome (Mann and Carr, 1974). Therefore, replacement of the wild-type chromosomes by mutated chromosomes required a lengthy period of time under selective pressure due to spectinomycin in the medium. The 43 lines of transformed cells were cultured for 6 months on agar-solidified BG-11 medium supplemented with 20 μg/ml spectinomycin. If the gene that was the target of the mutation were not essential, we would expect all the copies of the wild-type chromosome eventually to disappear. If the gene were essential, some copies of the wild-type chromosome would be expected to remain. Therefore, using the polymerase chain reaction (PCR), we monitored the extent to which wild-type chromosomes had been replaced by mutated chromosomes (data not shown). The wild-type gene was completely absent from all copies of the chromosome in 33 of our mutant strains. It appeared, therefore, that these 33 *hik* genes were not essential for survival under our growth conditions. Eight *hik* genes were not completely removed. However, the number of copies of wild-type chromosomes relative to the total number of copies of the chromosome was quite small in each case (data not shown). It seemed likely that these eight *hik* genes were important for the growth and survival of *Synechocystis* cells under our culture conditions. We failed to obtain any spectinomycin-resistant cells in our attempts to mutate the *hik13* (sll1003; this nomenclature refers to designations of open reading frames by Kaneko *et al.*, 1995, 1996) and *hik15* (sll1353) genes. These two *hik* genes might have been essential for growth under our conditions. Detailed information about these mutants can be found on the web page on which mutants of *Synechocystis* are listed: CyanoMutant, at http://www.kazusa.or.jp/cyano/mutants/.

Inactivation of the hik33 and hik19 genes prevents the induction by low temperature of luciferase activity in pdesB::lux cells

We examined luciferase activity after a decrease in growth temperature in *pdesB::lux* cells and in each line of cells with a mutation in a gene for histidine kinase. Figure 1A shows that the shift in growth temperature

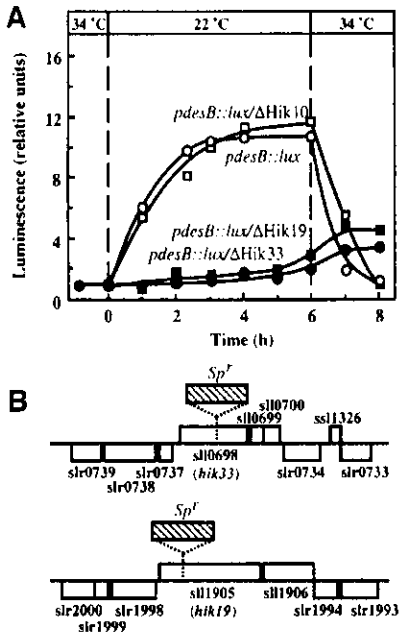

Fig. 1. Temperature-dependent changes in the activity of the *desB* promoter in *pdesB::lux*, *pdesB::lux*/ΔHik10, *pdesB::lux*/ΔHik33 and *pdesB::lux*/ΔHik19 cells, and sites of insertion of the *Sp*^{r} cassette in *pdesB::lux*/ΔHik33 and *pdesB::lux*/ΔHik19 cells. (**A**) Cells were grown on agar-solidified medium at 34°C and then transferred to 22°C. Luciferase activity was measured in terms of the intensity of luminescence, as described in Materials and methods. ○, *pdesB::lux*; □, *pdesB::lux*/ΔHik10; ●, *pdesB::lux*/ΔHik33; ■, *pdesB::lux*/ΔHik19. The results are the averages of results of three independent experiments. (**B**) The *Nco*I sites in the *hik33* (sll0698) and *hik19* (sll1905) genes at which the *Sp*^{r} cassette (hatched rectangle) was inserted are indicated by dashed vertical lines. Open rectangles indicate open reading frames.

from 34 to 22°C increased the luciferase activity ~10-fold in *pdesB::lux* cells. By contrast, in the two mutants *pdesB::lux*/ΔHik33 and *pdesB::lux*/ΔHik19, in which, respectively, the *hik33* gene and the *hik19* gene had been inactivated by the insertion of the *Sp*^{r} cassette, no increase in luciferase activity was observed upon incubation of cells at 22°C (Figure 1A). This result indicated that mutations in the *hik33* and *hik19* genes eliminated the inducibility by low temperature of the *desB* promoter. By contrast, the luciferase activity in other lines with mutant genes for histidine kinase resembled that in *pdesB::lux*; for example, the response of luciferase activity to the shift in temperature in *pdesB::lux*/ΔHik10 cells, in which the *hik10* gene (slr0533) had been inactivated, was the same as that in *pdesB::lux* cells (Figure 1A). The sites of insertion of the *Sp*^{r} cassette in *hik33* and *hik19* genes are shown in Figure 1B. Analysis by PCR revealed, however, that the native genes had not been completely eliminated in these lines, suggesting that the genes were essential under our growth conditions.

Expression of low-temperature-inducible genes in wild-type, ΔHik33 and ΔHik19 cells

We next inactivated, separately, the *hik33* and *hik19* genes in wild-type cells to examine the effects of these genes on the expression of low-temperature-inducible

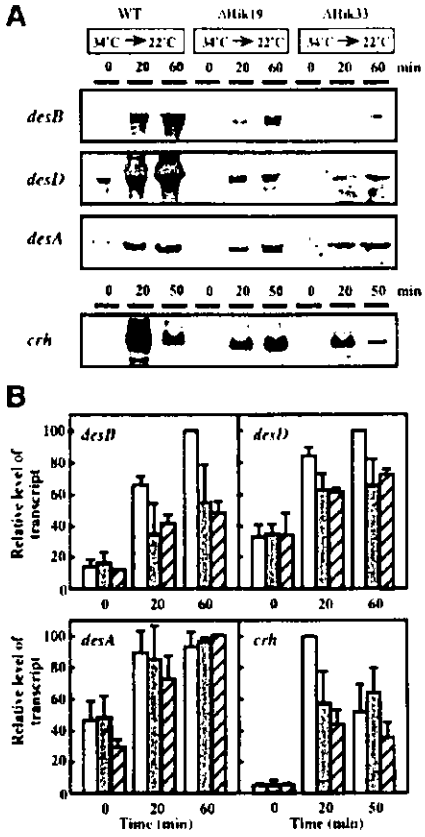

Fig. 2. Induction of *desB*, *desD*, *desA* and *crh* genes in wild-type, ΔHik33 and ΔHik19 cells after a downward shift in temperature. Cells that had been grown at 34°C for 16 h were transferred to 22°C and incubated for the periods of time indicated. Northern blotting analysis of the expression of the *desB*, *desD*, *desA* and *crh* genes was performed as described by Los *et al.* (1997). Total RNA (30 μg for analysis of *des* mRNAs and 5 μg for analysis of *crh* mRNA) was loaded in each lane. (A) Northern blots. (B) Quantification of transcripts. Open bars, wild-type cells; filled bars, ΔHik19 cells; hatched bars, ΔHik33 cells. The results are the averages from three independent experiments with experimental deviations.

genes. There are four genes for fatty acid desaturases in *Synechocystis*. The *desC* gene is expressed constitutively, while the *desA*, *desB* and *desD* genes are induced after a downward shift in temperature (Los *et al.*, 1997). We performed Northern blotting analysis to examine the expression of the *desB*, *desD* and *desA* genes in wild-type, ΔHik19 and ΔHik33 cells before and after a shift in temperature from 34 to 22°C (Figure 2A). As observed previously in wild-type cells (Los *et al.*, 1997), the increase in the level of the *desB* transcript was the most conspicuous. There was also a distinct increase in the level of the *desD* transcript. The level of the *desA* transcript increased least of all among the transcripts of the three genes for desaturases. Prior to exposure of cells to 22°C, the levels of the transcripts of the *desB*, *desD* and *desA* genes in the mutant cells were as low as those in wild-type cells (Figure 2A). However, the extent of induction at 22°C of the *desB* and *desD* genes, but not that of the *desA* gene, appeared to be reduced in both ΔHik19 and ΔHik33 mutant cells.

Figure 2B shows the quantitative changes in the levels of the transcripts. The level of the *desB* transcript in wild-type cells was 8-fold higher after incubation for 60 min at 22°C than before the incubation. However, inactivation of the *hik33* gene significantly depressed the low-temperature-induced increase in the level of the *desB* transcript. The low-temperature-induced increase in the level of the *desD* transcript in ΔHik33 cells was also reduced to two-thirds of that in wild-type cells after incubation for 60 min at 22°C. In addition, inactivation of the *hik19* gene depressed the low-temperature-induced enhancement of expression of the *desB* and *desD* genes (Figure 2B). However, the low-temperature-induced enhancement of the expression of the *desA* gene was unaffected by the inactivation of *hik33* and *hik19* (Figure 2B). These results indicated that inactivation of *hik33* and *hik19* suppressed the low-temperature-induced expression of the *desB* and *desD* genes, but not of the *desA* gene.

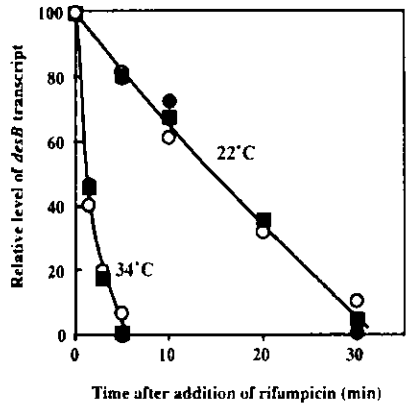

Fig. 3. Dependence on temperature of the stability of *desB* mRNA in wild-type, ΔHik33 and ΔHik19 cells. Cells were grown at 34°C for 16 h and then incubated for 2 h either at 22 or 34°C. An inhibitor of transcription, rifampicin, was added to the cultures at 50 μg/ml at time zero. Aliquots of cultures were withdrawn at the times indicated and levels of *desB* mRNA were determined by Northern blotting analysis. ○, wild-type cells; ●, ΔHik33 cells; ■, ΔHik19 cells. The results are averages from three independent experiments.

The *crh* gene for a homolog of RNA helicase is also a gene that is induced at low temperatures (Chamot *et al.*, 1999). Figure 2 shows that the level of *crh* mRNA increased within 20 min after a shift in temperature from 34 to 22°C and then decreased during further incubation at 22°C. The pattern of expression of *crh* mRNA in wild-type cells differed from that of the mRNAs for desaturases. Nonetheless, the increases in the level of *crh* mRNA in 20 min in ΔHik33 and ΔHik19 cells were smaller than those in wild-type cells.

Degradation of desB mRNA in wild-type, ΔHik33 and ΔHik19 cells

In general, the level of an mRNA is regulated by the rate of transcription of the corresponding gene and the stability of the mRNA itself. These factors also control the accumulation of *desB* mRNA when *Synechocystis* cells are exposed to a low temperature (Los *et al.*, 1997). Therefore, we compared the stability of *desB* mRNA at 34 and 22°C in wild-type, ΔHik33 and ΔHik19 cells in the presence of an inhibitor of transcription, rifampicin. Figure 3 shows that the rate of degradation of *desB* mRNA was the same in wild-type and mutant cells at both high and low

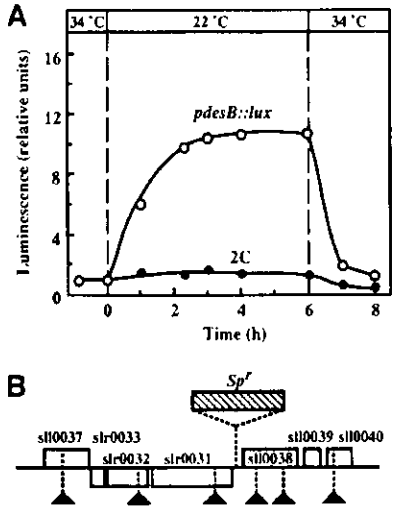

Fig. 4. Changes in the activity of luciferase upon a change in temperature in *pdesB::lux* and 2C cells. (**A**) *pdesB::lux* and 2C cells were grown on agar-solidified medium at 34°C and then transferred to 22°C. Luciferase activity was measured in terms of the intensity of luminescence, as described in Materials and methods. ○, *pdesB::lux*; ●, mutant 2C. The results are the averages of three independent experiments. (**B**) The site of insertion of the Sp^r cassette in the chromosome of mutant 2C cells (hatched rectangle) and the sites of insertion of the Sp^r cassette in wild-type cells (triangles) are indicated. The open reading frame sll0038 corresponds to the response regulator Rer1.

temperatures. The half-life of the *desB* transcript was 15 min at 22°C and 1 min at 34°C. These results indicated that the reduction in the low-temperature-induced accumulation of the *desB* transcript in ΔHik33 and ΔHik19 cells was due to a decrease in the rate of transcription and not to a decrease in the stability of the mRNA, and they suggested, moreover, that both Hik33 and Hik19 might be involved in the low-temperature-induced regulation of transcription of the *desB* gene.

Random mutagenesis and screening of mutants with altered expression of the desB gene

In order to find other components in the pathway for low-temperature signaling, we used random mutagenesis. We introduced the Sp^r cassette randomly into the chromosome of *pdesB::lux* cells by cassette mutagenesis (Hagemann *et al.*, 1996). From among ~20 000 spectinomycin-resistant mutants, we isolated 18 mutants in which the response of luciferase activity to a downward shift in temperature was different from that in parental *pdesB::lux* cells (Figure 4). We postulated that in cells of each of these mutant lines a component of the low-temperature signal-transduction pathway might have been mutated. We determined sequences on both sides of the sites of insertion of the cassette in these mutants. We found that in two of the 18 mutants the *hik19* gene for Hik19 had been inactivated by insertion of the Sp^r cassette (data not shown).

In another of these mutant lines, designated 2C, in which the *desB* promoter was not activated by low temperature (Figure 4A), the Sp^r cassette had been inserted in the upstream region of two operons, as shown in Figure 4B. The results suggested that one of the genes in the vicinity of the site of insertion encoded a component of the low-temperature signal-transduction pathway. In order to identify the gene responsible for the elimination of the transcriptional activity, we inactivated five putative genes separately (sll0037, slr0032, slr0031, sll0038, sll0040) in *pdesB::lux* by inserting an Sp^r cassette, as indicated by triangles in Figure 4B. Inactivation of gene sll0038, but not of any of the other genes, depressed the low-temperature-induced increase in luciferase activity in the same way as observed in 2C cells (data not shown). The amino acid sequence deduced from the nucleotide sequence of this gene indicated that its product was one of the 38 response regulators identified in *Synechocystis* (Mizuno *et al.*, 1996). We designated the gene *rer1* and its product Rer1.

Inactivation of the rer1 gene in wild-type cells inhibited the induction of the desB gene by low temperature

To examine the role of Rer1 in the regulation of expression of low-temperature-inducible genes, we inactivated the *rer1* gene in wild-type cells by inserting the Sp^r cassette at the *Msc*I site. The extent of the low-temperature-dependent induction of the *desB* transcript was reduced to half that in wild-type cells (Figure 5). By contrast, the inducibility by low temperature of the *desD*, *desA* and *crh* genes was unaffected by the mutation (Figure 5). These results indicated that Rer1 might specifically regulate the expression of the *desB* gene, but not that of the other genes examined.

Discussion

Advantages of using Synechocystis for systematic inactivation of histidine kinases

There are 43 putative genes for histidine kinases in the chromosome of *Synechocystis* (Kaneko *et al.*, 1995, 1996; Mizuno *et al.*, 1996). Since histidine kinases have been shown, in many cases, to be sensors or components of signal-transducing systems (Appleby *et al.*, 1996; Mizuno *et al.*, 1996), some of the histidine kinases in *Synechocystis* might also be expected to have similar functions. However, it is impossible to predict the function of each individual histidine kinase on the basis of its primary structure. The functions of ~30 histidine kinases have been identified in *E.coli*, but these enzymes have no obvious counterparts in *Synechocystis* (Mizuno *et al.*, 1996).

Using *Synechocystis*, we were able to identify genes for histidine kinases that appear to be involved in the perception and transduction of low-temperature signals even though these genes are essential for survival and cannot be completely eliminated. Each cell of *Synechocystis* contains ~10 identical copies of the chromosome and, thus, essential genes can be inactivated to some extent but not totally with detectable changes in phenotype. The *hik33* and *hik19* genes were not completely eliminated even under selective pressure due to spectinomycin and, therefore, they can be regarded as essential genes. It is impossible to identify genes that might correspond to *hik33* and *hik19* in experiments with *E.coli* or *B.subtilis* because each cell of these bacteria contains only a single copy of the chromosome.

Characteristics of Hik33

The amino acid sequence deduced from the *hik33* gene (sll0698) indicates that Hik33 contains 663 amino acid

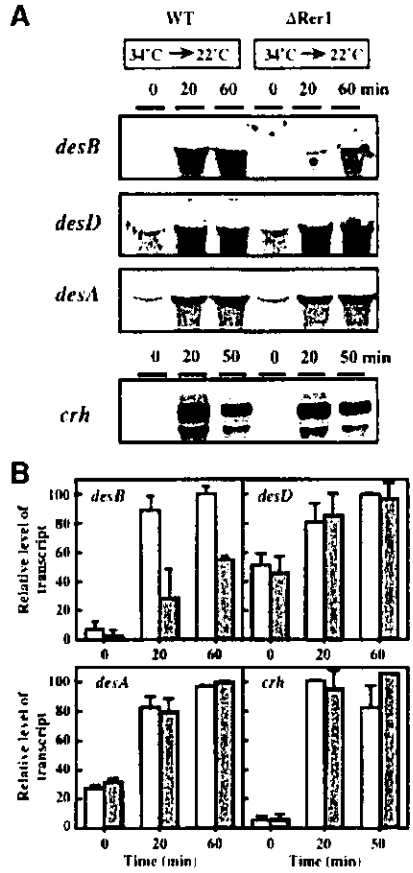

Fig. 5. Induction of *desB*, *desD*, *desA* and *crh* genes in wild-type and ΔRer1 cells after a downward shift in temperature. Cells that had been grown at 34°C for 16 h were transferred to 22°C and incubated for the periods of time indicated. Northern blotting analysis of the expression of *desB*, *desD* and *desA* genes was performed as described by Los *et al.* (1997). Total RNA (30 μg for analysis of *des* mRNAs and 5 μg for analysis of *crh* mRNA) was loaded in each lane. (**A**) Northern blots. (**B**) Quantification of transcripts. Open bars, wild-type cells; filled bars, ΔRer1 cells. The results are the averages of results from three independent experiments with experimental deviations.

residues. The strongly conserved histidine kinase domain is located near the C-terminus. An analysis using computer programs that predict the localization of proteins, such as PSORT (Nakai and Horton, 1999) and HMMTOP (Tusnady and Simon, 1998), indicated that Hik33 has two hydrophobic helices that might, in theory, span the membrane. A putative leucine zipper motif and a putative coiled-coil sequence are located between the second hydrophobic helix and the histidine kinase domain. These motifs are involved in the dimerization of a number of histidine kinases and are important for their activities (Lau *et al.*, 1997; Yaku and Mizuno, 1997; Singh *et al.*, 1998). Thus, we can predict that one or both of these sequences might be involved in the dimerization of Hik33 and the regulation of its activity.

A reduction in the fluidity of the plasma membrane of a cyanobacterium appears to be a primary signal for the low-temperature-induced expression of the genes for desaturases (Vigh *et al.*, 1993; Murata and Los, 1997). The properties of Hik33 appear to be consistent with those of a sensor that can detect a decrease in membrane fluidity.

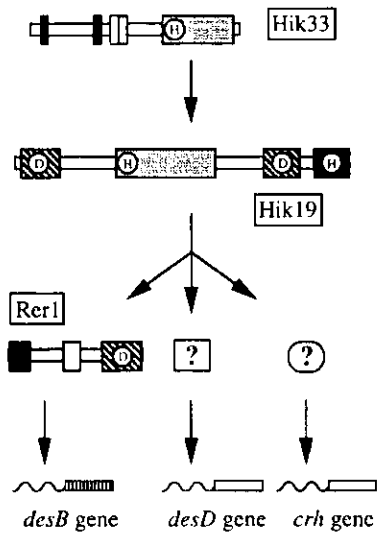

Fig. 6. A hypothetical scheme for the pathway for low-temperature signal transduction in *Synechocystis*. The histidine kinase domains, the receiver domains and the histidine phospho-transfer (Hpt) domain of Hik33, Hik19 and Rer1 are indicated by gray rectangles, hatched rectangles and a filled rectangle, respectively. The histidine and aspartate residues that might be involved in the phospho-relay reaction are indicated by H and D in circles, respectively. Filled rectangles, a gray rectangle and an open rectangle in Hik33 indicate the putative membrane-spanning domains, the coiled-coil domain and leucine-zipper domain, respectively. Closed and open rectangles in Rer1 indicate regions homologous to the HMG box and the Ah receptor nuclear translocator, respectively (see the text for details).

A reduction in the fluidity of the membrane at sites at which Hik33 is located might alter the structure of Hik33, influencing the spatial relationship between monomers of the dimerized protein and thereby altering activity.

We searched for proteins homologous to Hik33 in standard databases. The kinase domain of Hik33, which includes an autophosphorylatable histidine residue and an ATP-binding motif (Park *et al.*, 1998), was very similar to those of histidine kinases from bacteria, yeast, fungi and plants. However, the sequences outside the kinase domain, including the membrane-spanning domains, appeared to be unique. One relatively homologous gene was identified, namely *ycf26*, which was found in the chloroplast genome of the red alga *Porphyra purpurea* (Reith and Munholland, 1995). This gene encodes a homolog of histidine kinase of 656 amino acid residues. About 48% of the residues outside the histidine kinase domain are identical to those in Hik33 (data not shown). The hydropathy profiles of Hik33 and Ycf26 are also very similar (data not shown). However, it is unclear what kinds of signal might be perceived and what genes might be regulated by Ycf26.

Hik33 is also homologous to a Ycf26-like protein found in the chloroplast genome of *Cyanidium caldarium* (DDBJ/EMBL/GenBank accession No. AF022186) and to a protein encoded by the *yycG* gene in *B.subtilis* (Kunst *et al.*, 1997). These proteins also contain putative membrane-spanning domains and coiled-coil motifs. This indicates that a pathway for the perception and transduction of cold signals identified in *Synechocystis* might be a common feature of the responses of many organisms to cold.

Characteristics of Hik19

The amino acid sequence deduced from the *hik19* gene (sll1905) indicates that Hik19 contains 1014 amino acid residues and computer analysis suggests that it might be a soluble protein in the cytosol. A strongly conserved histidine kinase domain is located in a central region of the protein. One signal-receiver domain is localized at the N-terminus and another is near the C-terminal region. Furthermore, a histidine phospho-transfer (Hpt) domain is located at the C-terminus. Thus, Hik19 is a hybrid-type histidine kinase (Mizuno *et al.*, 1996). The receiver domain at the N-terminus might accept a phosphate group from some other histidine kinase or protein that contains phosphorylated histidine and Hik19 might function downstream of the membrane-bound sensor Hik33. Hik19 is more likely than Hik33 to be a transducer of the low-temperature signal.

Characteristics of Rer1

The amino acid sequence deduced from the *rer1* gene indicates that Rer1 contains 402 amino acid residues. Unlike most response regulators that have a signal-receiver domain at the N-terminus, Rer1 has a signal-receiver domain at the C-terminus. However, the N-terminal region is homologous to the DNA-binding domain, known as an HMG box, found in regulators of transcription in vertebrates, such as the Sox6 and Sox5 proteins (Connor *et al.*, 1995). The central part of Rer1 is similar to the transcriptional activation domain of the aryl hydrocarbon (Ah) receptor nuclear translocator (*Arnt*; Li *et al.*, 1994; Figure 6). Although Rer1 does not have a structure typical of response regulators, it is possible that it functions as a DNA-binding regulator of transcription.

Characterization of the ΔHik33, ΔHik19 and ΔRer1 mutants

The extent of the low-temperature-inducible expression of the *desB* transcript in ΔHik33 cells was half that in wild-type cells (Figure 2). The low levels of Hik33 and Hik19 might be responsible for the low level of accumulation of the *desB* transcript in the mutant cells. However, we found that the low-temperature-induced activation of the *desB* promoter in *pdesB::lux*/ΔHik33 or *pdesB::lux*/ΔHik19 cells, monitored in terms of luciferase activity, was almost completely eliminated by the inactivation of the *hik19* genes or the *hik33* genes (Figure 1). The discrepancy between the extent of depression of the accumulation of the transcript and the extent of transcriptional activation might be explained by an increase in the stability of transcripts at low temperature, as shown in Figure 3 and in the previous report (Los *et al.*, 1997). The half-life of the *desB* transcript increased 15-fold after a shift in growth temperature from 34 to 22°C in wild-type cells and in ΔHik33 and ΔHik19 cells. It is unclear how the transcripts of *desB*, *desD* and *desA* genes might be stabilized at low temperature.

Although inactivation of *hik33* and *hik19* reduced the low-temperature-induced accumulation of *desB* and *desD* transcripts (Figure 2), inactivation of the *rer1* gene resulted in a reduction in the level of the *desB* transcript, while levels of *desD*, *desA* and *crh* transcripts were unaffected (Figure 5). These results indicate that Hik33 and Hik19 might be involved in a common mechanism that regulates the expression of *desB*, *desD* and *crh* genes, and that Rer1 might specifically regulate the expression of the *desB* gene (Figure 6). In *E.coli*, a hybrid-type histidine kinase, ArcB, transfers a phosphate group via its Hpt domain to several receivers, such as ArcA, OmpR and CheY (Perraud *et al.*, 1999). Hik19 might also transfer phosphate groups to some, as yet unidentified, response regulators that, perhaps, contain a receiver domain and it might, thus, regulate the expression of *desD*, *crh* and certain other low-temperature-inducible genes.

A hypothetical pathway for perception and transduction of low-temperature signals

Figure 6 shows a hypothetical scheme for the transduction of low-temperature signals. Hik33 may span the plasma membrane twice and forms a dimer, whose structure may be influenced by the physical characteristics of lipids in the plasma membrane, such as their fluidity (or the extent of molecular motion), which is controlled by temperature and the extent of unsaturation of the fatty acids. When the temperature is decreased or the fatty acids are more saturated, the histidine residue in the histidine kinase domain may be phosphorylated. A phosphate group is then transferred to Hik19, and finally to Rer1, which regulates the expression of the *desB* gene. Hik19 and Hik33 are also involved in the regulation of expression of the *crh* and *desD* genes. However, we have not yet identified the response regulators (or transcriptional regulators) of these genes.

In *E.coli*, heat stress induces the expression of several genes, whose products are involved in the folding and degradation of denatured proteins. Some of these genes are regulated by the typical two-component system CpxA–CpxR. CpxA is a histidine kinase, which is bound to the plasma membrane and is autophosphorylated under heat stress. Phosphorylated CpxA transfers a phosphate group to the response regulator CpxR, which activates the transcription of several heat-inducible genes, such as *degP*, which encodes a protease, and *dsbA*, which encodes a disulfide isomerase (Mileykovskaya and Dowhan, 1997). The polypeptide deduced from the *cpxA* gene is different from Hik33 except in its histidine kinase domain. The pathway for low-temperature signal transduction in *Synechocystis* (Figure 6) appears to be more complex than the two-component system for high-temperature signal transduction in *E.coli*.

The expression of several sets of genes in response to low temperature occurs in all poikilothermic organisms examined to date. A pathway for the perception and transduction of low-temperature signals that includes two histidine kinases identified in *Synechocystis* might be a common feature of the responses of many organisms to low temperatures.

Materials and methods

Cells and culture conditions

A strain of *Synechocystis* sp. PCC 6803, which is tolerant to glucose (Williams, 1988), was obtained from Dr Williams at Dupont Co. Ltd. We generated strain *pdesB::lux*, in which the coding region of the *desB* gene was replaced by the *luxAB* gene for bacterial luciferase, as described previously (Los *et al.*, 1997). In this construct, the *luxAB* gene was expressed under the control of the *desB* promoter. Wild-type cells were grown at 34°C in BG-11 medium (Stanier *et al.*, 1971) buffered with

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