Exploitation of genomic sequences in a systematic analysis to access how cyanobacteria sense environmental stress

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Abstract

The perception and subsequent transduction of environmental signals are primary events in the acclimation of living organisms to changes in their environment. Many of the molecular sensors and transducers of environmental stress cannot be identified by traditional and conventional methods. Therefore, the genomic information has been exploited in a systematic approach to this problem, performing systematic mutagenesis of potential sensors and transducers, namely, histidine kinases and response regulators, respectively, in combination with DNA microarray analysis, to examine the genome-wide expression of genes in the unicellular cyanobacterium Synechocystis sp. PCC 6803. Using targeted mutagenesis, 44 out of the 47 histidine kinases and 42 out of the 45 response regulators of this organism have successfully been inactivated. The resultant mutant libraries were screened by genome-wide DNA microarray analysis and by slot-blot hybridization analysis under various stress and non-stress conditions. Histidine kinases have been identified that perceive and transduce signals of low-temperature, hyperosmotic, and salt stress, as well as manganese deficiency.

Key words: Hyperosmotic stress, histidine kinase, low-temperature stress, Mn deficiency, response regulator, salt stress, sensor of environmental stress, signal transduction, Synechocystis sp. PCC 6803, two-component system.

Introduction

Environmental stress depresses various physiological activities. Under sudden and severe stress, organisms often fail to survive. When environmental conditions change gradually, organisms can acclimate to the new conditions by sensing the changes and expressing a large number of previously unexpressed genes, with the resultant synthesis of large numbers of proteins and metabolites that are involved in protection against environmental stress. This review focuses on the primary events in environmental stress-inducible gene expression and describes an analysis of the sensors of various types of environmental stress and transduction of the respective signals in a cyanobacterium (see also reviews by Los and Murata, 2002, 2004; and by Mikami and Murata, 2003).

Regulation of gene expression in response to changes in environmental conditions

When an environmental stress exceeds a certain threshold level, the activities of some enzymes are inhibited. In particular, enzymes that are involved in the control of gene expression seem to be extremely sensitive to environmental stress. Moreover, the translation machinery appears to be especially sensitive to environmental stress. For example, in photosynthetic organisms, oxidative stress, such as H₂O₂ stress (Nishiyama et al., 2001) and stress due to singlet oxygen (Nishiyama et al., 2004), as well as salt stress (Allakhverdiev et al., 2002), specifically inhibits the translation of transcripts of the psbA genes that encode the precursor to the D1 protein that is an essential component of photosystem II. In addition, the evidence
suggests that the process that is affected by oxidative stress, in this case, is the elongation, during translation, of the precursor to the D1 protein (Nishiyama et al., 2001, 2004).

When environmental stress is relatively mild, organisms express sets of genes that are specific to individual types of stress, as well as some genes whose expression is induced, in common, by a variety of stresses. As a result, specific sets of proteins are synthesized and these proteins, in turn, may synthesize a number of specific stress-related metabolites. The proteins and metabolites that are synthesized de novo are important for the intra- and extracellular reactions that function to acclimate the organism to its new environment. The first step in the acclimation process is the perception of environmental stress and transduction of the resultant signal. Organisms and/or individual cells appear to be equipped with sensors and signal transducers that perceive and transduce signals due to changes in the environment. The sensors and signal transducers appear likely, moreover, to be specific to individual types of environmental stress.

Two-component systems: potential sensors and signal transducers

The existence of two-component systems has been well established in *Escherichia coli* and *Bacillus subtilis* (Stock et al., 2000; Aguilar et al., 2001). Each two-component system consists of a histidine kinase (Hik) and a cognate response regulator (Rre). In *E. coli* and *B. subtilis*, the genes for the two components for a single system are, in many cases, located close to one another on the chromosome. The Hik perceives a change in the environment via its sensor domain and then a conserved histidine residue within the histidine kinase domain is autophosphorylated, with ATP as the donor of the phosphate group (Stock et al., 2000). The phosphate group is transferred from the Hik to a conserved aspartate residue in the receiver domain of the cognate Rre. Upon phosphorylation, the Rre changes its conformation, and this change allows the binding of the Rre to the promoter regions of genes that are located further downstream in the acclimation pathway (Koretke et al., 2000).

Two-component systems have been found in prokaryotes (including cyanobacteria), fungi, yeasts, plants, and lower animals (Koretke et al., 2000), but not in higher animals. In eukaryotes and, in particular, in higher plants and higher animals, Ser/Thr protein kinases are supposed to be the major sensors and transducers of environmental signals (Widmann et al., 1999).

The unicellular cyanobacteria have several features that make them particularly suitable for studies of stress responses at the molecular level. The general features of the plasma and thylakoid membranes of cyanobacterial cells are similar to those of the chloroplasts of higher plants in terms of lipid composition and the assembly of membranes. Therefore, cyanobacteria might be expected to serve as powerful model systems for studies of the molecular mechanisms of the responses and acclimation to stress (Murata and Wada, 1995; Glatz et al., 1999).

Some strains of the cyanobacteria, such as *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*), *Synechococcus* sp. PCC 7942, and *Synechococcus* sp. PCC 7002, are naturally competent and, thus, foreign DNA is incorporated into cells and is integrated into the genome by homologous recombination at high frequency (Williams, 1988; Haselkorn, 1991). For other strains, such as filamentous *Anabaena* sp., other methods of transformation have been developed that are based on the use of broad-host-range plasmids and bacterial conjugation (Elhai and Volk, 1988). As a result, cyanobacteria are widely used by researchers for the production of mutants with disrupted genes of interest (for a review see Vermaas, 1998).

The complete nucleotide sequence of the genome of *Synechocystis* (Kaneko et al., 1996) was determined in 1996, and the annotated sequence information is now available via the internet. Moreover, random mutagenesis with transposons (Bhya et al., 2001) or with antibiotic-resistance cassettes can be achieved in cyanobacteria (Suzuki et al., 2000), allowing the random disruption of genes and determination of their respective functions under certain stress conditions. The availability of the complete genome sequence also allows the sites of mutations to be located with ease and genes of interest to be identified. Another powerful technique, which is also based on the availability of the complete sequence of the genome, is the construction of ‘knockout’ libraries of specific sets of genes. This review describes the successful application of this technique, which has allowed identification of almost all the sensors and transducers of environmental signals in *Synechocystis* (Suzuki et al., 2000; Paithoonrangsarid et al., 2004). In addition, DNA microarray analysis of the genome-wide expression of genes has made it possible to examine both the expression of individual genes under specific stress conditions and the effects of specific mutations on gene expression.

*Synechocystis* includes 44 putative genes for Hiks on its chromosome (Kaneko et al., 1996; Mizuno et al., 1996) and three putative genes for Hiks on its plasmids (Kaneko et al., 2003), all of which are candidates for sensors of environmental signals. Indeed, recent studies have demonstrated that Hiks in *Synechocystis* are involved in the perception and transduction of signals due to low-temperature stress (Suzuki et al., 2000, 2001), phosphate deprivation (Hirani et al., 2001; Suzuki et al., 2004), high osmolarity (Mikami et al., 2002; Paithoonrangsarid et al., 2004), heavy metal ions (López-Maury et al., 2002; Ogawa et al., 2002; Yamaguchi et al., 2002), and high concentrations of NaCl (Marin et al., 2003; Shoumskaya et al., 2005).
Genome-based analysis for the identification of potential sensors of environmental stress

In *E. coli* and *B. subtilis*, most of the genes for Hiks and Rres are located close to one another on the chromosome and their products from individual two-component systems. Moreover, in many cases, these genes are located in the vicinity of functionally related genes. However, genes for Hiks and Rres in *Synechocystis* are distributed rather randomly on the chromosome. Among the 44 genes for Hiks on the *Synechocystis* chromosome, 14 are located in the vicinity of genes for potentially cognate Rres, whereas genes for the other 30 Hiks are located separately from any genes for Rres. Thus, it is difficult to predict the pairs of Hiks and Rres that function as individual two-component systems, and it is necessary to introduce new methods to investigate the ways in which individual Hiks and Rres are involved in specific signal-transduction pathways. Systematic mutagenesis of genes was performed for all the involved in specific signal-transduction pathways. Systematic mutagenesis of genes was performed for all the potential sensors of environmental stress and the transducers of such signals and the genome-wide expression of genes were examined using DNA microarrays.

Information about cyanobacterial genomes and the genome-wide expression of genes

The entire nucleotide sequence of the *Synechocystis* genome was determined by Kaneko *et al.* (1996). The genome was the first cyanobacterial genome to be sequenced but now the genome sequences of a number of cyanobacterial strains are available, such as *Anabaena* sp. PCC 7120 (or Nostoc sp. PCC 7120; Kaneko *et al.*, 2001), *Thermosynechococcus elongatus* BP-1 (Nakamura *et al.*, 2002), *Gloeobacter violaceus* PCC 7421 (Nakamura *et al.*, 2003), *Synechococcus* sp. WH8102 (Palenik *et al.*, 2003), *Synechococcus* sp. PCC 6301 (M Sugita, unpublished data; data are available from GenBank under accession no. AP008231), *Prochlorococcus marinus* SS120 (Dufresne *et al.*, 2003), *P. marinus* MED4 and *P. marinus* MIT9313 (Rocap *et al.*, 2003), and *Nostoc punctiforme* (http://genome.jgi-psf.org/finished_microbes/nospu/nospu.home.html). The complete genome sequences of *Anabaena variabilis* ATCC29413, *Trichodesmium erythraeum* IMS101, and *Synechococcus* sp. PCC 7942 should be available in the near future, and progress is being made on the genomes of *Synechococcus* sp. PCC 7002 and *Crocosphaera watsonii* strain WH8501.

Genome sequences provide vast amounts of basic information, which can be exploited for the genome-wide study of gene expression. In 1999, Takara Bio Co. (Ohtu, Japan) initiated the production of a genome-wide cDNA microarray for the analysis of gene expression in *Synechocystis*. Their cDNA microarray covers 3079 of the 3165 (97%) genes on the chromosome of *Synechocystis* (99 genes for transposases are excluded from this calculation). Recently, Kaneko *et al.* (2003) reported the entire sequences of all four of the plasmids (pSYSM, pSYSX, pSYSA, and pSYSG) harboured by *Synechocystis*. There are 132, 110, 106, and 49 putative genes, respectively, on these plasmids, namely, 397 genes in all. However, these genes are not included in the cDNA microarray from Takara Bio Co.

DNA microarrays are powerful tools for the study of the genome-wide expression of genes in response to various kinds of environmental stress. They have been used to examine gene expression in response to low-temperature stress (Suzuki *et al.*, 2001), high-temperature stress (Inaba *et al.*, 2003; Suzuki *et al.*, 2005), hyperosmotic stress (Mikami *et al.*, 2002; Pathoonrangsarid *et al.*, 2004; Shapiguzov *et al.*, 2005), salt stress (Kanesaki *et al.*, 2002; Marin *et al.*, 2003, 2004; Shoumskaya *et al.*, 2005), strong-light stress (Hihara *et al.*, 2001; Huang *et al.*, 2002; Tu *et al.*, 2004), UV light stress (Huang *et al.*, 2002), H$_2$O$_2$ stress (Singh *et al.*, 2004), and ion-deficiency and ion-excess stress (Yamaguchi *et al.*, 2002; Singh *et al.*, 2003; Suzuki *et al.*, 2004). Each type of stress enhances and represses the expression of several hundred genes!

DNA microarray analysis revealed the differential expression of genes upon exposure of *Synechocystis* cells to salt stress and hyperosmotic stress (Kanesaki *et al.*, 2002; Shoumkskaya *et al.*, 2005). The effects of salt stress and hyperosmotic stress have been confused by some researchers, who were unable to distinguish between them. Figure 1 shows that, in *Synechocystis*, these two kinds of stress enhance, in common, the expression of genes for heat-shock proteins.

![Fig. 1. Some salt stress-inducible genes and hyperosmotic stress-inducible genes are the same and some are different. The diagram includes genes that are induced during incubation for 20 min in the presence of 0.5 M NaCl (salt stress) and of 0.5 M sorbitol (hyperosmotic stress). Abbreviation: GG, glucosylglycerol. With permission from the authors, this figure was adapted from (i) Shoumkskaya *et al.* (2005) and (ii) Kanesaki *et al.* (2002). Copyright (2002), with permission from Elsevier.](image-url)
proteins (hspA, dnaK2, groEL2, and clpB1), for the synthesis of glycerol (ggpS and gldP), for a sigma factor (sigD), and for a high-light-inducible protein (hilA). However, salt stress, but not hyperosmotic stress, enhances the expression of genes for ribosomal proteins (rpl2, rpl5, rpl4, and rpl23) and for proteases (ctpA, prc, and fisH). By contrast, hyperosmotic stress, but not salt stress, enhances the expression of genes for the synthesis of lipids and lipoproteins (fabG and rlpA) and for other functions (repA). These findings clearly demonstrate that Synechocystis cells recognize salt stress and hyperosmotic stress as different signals.

Mutant libraries
Synechocystis harboured 3661 putative genes of which 47 are genes for Hiks and 45 are genes for Rres (http://www.kazusa.or.jp/cyanobase/Synechocystis/index.html). There are 44 genes for Hiks on the chromosome, and two genes and one gene on plasmids pSYSX and pSYSM, respectively. The 44 genes for Hiks on the chromosome were named hik1 through hik44. Three putative Hiks, namely, Hik11, Hik17, and Hik37, might be inactive as histidine kinases because the conserved histidine residue in the Hik domain is absent from these Hiks. Hik32 might also be inactive as a histidine kinase because the hik32 (sll1473) gene is part of a larger gene, namely, sll1473-sll1475, which is interrupted by a transposon (sll1474) in the strain that was used for genome sequencing and systematic mutagenesis (Okamoto et al., 1999). The gene for the Hik encoded by pSYSM is designated hik45, and the genes for Hiks encoded by pSYSX are designated hik46 and hik47. There are 42 genes for Rres located on the chromosome, and two and one on plasmids pSYSX and pSYSM, respectively. The 42 genes for Rres on the chromosome were designated rre41 through rre42. The rre43 gene is located on pSYSM, and rre44 and rre45 are on pSYSX.

Each of the putative hik genes in Synechocystis was inactivated by insertion of a spectinomycin-resistance gene cassette to create a gene-knockout library (Suzuki et al., 2000; Cyanomutants, http://www.kazusa.or.jp/cyanobase/Synechocystis/mutants/). This library has proved to be a powerful tool for the identification of sensors of various stimuli and the corresponding signal transducers in Synechocystis (Suzuki et al., 2000, 2004; Yamaguchi et al., 2002; Marin et al., 2003; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005). The genome-wide expression of genes in wild-type cells and in all the lines of hik mutant cells was examined with the DNA microarray from Takara Bio Co. in an attempt to identify various Hiks that might be involved in the regulation of expression of stress-inducible genes.

Positive and negative regulation of gene expression
There are two types of regulation of gene expression, namely, positive regulation and negative regulation. In positive regulation (Fig. 2A, B), a Hik is inactive under non-stress conditions and, as a result, the corresponding Rre is active. Genes that encode this type of two-component system are silent (Fig. 2A) or expressed (Fig. 2B). When cells are exposed to the appropriate environmental stress, the Hik is activated (by phosphorylation in response to the stress) and then the signal is transferred to the Rre. The activated Rre enhances the expression of genes that are silent under non-stress conditions (Fig. 2A) or represses the expression of genes that are expressed under non-stress conditions (Fig. 2B). Most types of stress-inducible gene expression in Synechocystis are associated with this type of regulation.

In the negative regulation of stress-inducible gene expression (Fig. 2C, D), the Hik is active under non-stress conditions and, as a result, the corresponding Rre is active. The expression of the genes controlled by this type of two-component system is either enhanced (Fig. 2C) or repressed (Fig. 2D). When the appropriate environmental stress is applied, the Hik becomes inactive and the Rre also becomes inactive. Genes that are expressed or repressed under non-stress conditions become silent (Fig. 2C) or are released from repression (Fig. 2D), respectively. As a result, the levels of expression of genes decrease in the former case (Fig. 2C) and appear to increase in the latter case (Fig. 2D).

Changes in phenotype due to mutations of Hiks and Rres, which reflect the effects of these Hiks and Rres on gene expression, differ between positive regulation and negative regulation. A knockout mutation of either the Hik or the Rre in a two-component system for negative regulation has a marked effect on gene expression. The expression of genes under a negatively regulating two-component system is either enhanced or repressed under non-stress conditions (as described in the following section). Therefore, a specific signal-transduction pathway with a specific Hik and a specific Rre can be identified with relative ease in cases of negative regulation. By contrast, knockout mutation of a Hik and an Rre in a two-component system that operates via positive regulation does not have a significant effect on gene expression under non-stress conditions. In this type of two-component system, the identification of the Hik and the Rre in a specific signal-transduction pathway requires the screening of knockout libraries of hik and rre genes under individual types of stress.

Negative regulators of gene expression
The effects of mutation of each hik gene on gene expression in mutant Synechocystis cells that had been grown under normal conditions were analysed, using the DNA microarray specific for Synechocystis. No significant alterations in gene expression were evident in most of the mutants. However, three of the mutants, Δhik27, Δhik34, and Δhik20, each with a mutation in the indicated histidine kinase, were unique because, in these mutants, the expression...
of some genes was enhanced and that of some other genes was repressed. The results indicated that Hik27, Hik34, and Hik20 might be involved in signal transduction and the negative regulation of gene expression. As described below, Hik27 is a sensor of Mn²⁺ ion deficiency (Yamaguchi et al., 2002) and Hik34 is involved in the regulation of expression of heat-shock genes (Suzuki et al., 2005). Hik20 seems to be involved in sensing of K⁺ ions (N Murata, I Suzuki, unpublished results).

The His27-Rre16 two-component system negatively regulates the expression of genes for the Mn transporter

Using the DNA microarray, gene expression was compared in Δhik27 mutant cells with that in wild-type cells during growth under normal conditions, namely, in BG-11 medium that contained 9 μM Mn²⁺ ions (Yamaguchi et al., 2002). Marked changes, with induction factors higher than 10 due to mutation of the hik27 gene (slr0640), were recognized only in the expression of three genes, namely, mntC, mntA, and mntB (Fig. 3A), which constitute the mntCAB operon that encodes subunits of the ABC-type Mn²⁺ transporter (Bartsevich and Pakrasi, 1995, 1996). The results in Fig. 3A suggest that, under normal growth conditions, Hik27 might transduce a signal that represses the expression of the mntCAB operon and, moreover, that disappearance of this signal, due to inactivation of Hik27, might allow the mntCAB operon to be expressed. This scenario corresponds to the scheme presented in Fig. 2D.

Hik27 is a typical histidine kinase with 441 amino acid residues. It has a relative molecular mass of 48 kDa and includes a phosphorylatable histidine residue in the histidine kinase domain in the carboxy-terminal region and two membrane-spanning domains in the amino-terminal region (Yamaguchi et al., 2002; http://www.kazusa.or.jp/cyano/Synechocystis/cgi-bin/ranso-orfsearch.cgi?type=orf&name=slr0640). It is likely that the region between the two membrane-spanning domains is located in the periplasmic space and perceives the extracellular concentration of Mn²⁺ ions.

The effects were examined of various mutations on gene expression in mutant cells which had been grown in BG-11 medium that contained 9 μM Mn²⁺ ions. The mutation in Δrre16 cells enhanced the expression of only three genes, namely, mntC, mntA, and mntB. This phenomenon was
very similar to the change in gene expression detected in the \( \Delta hik27 \) mutant. The results in Fig. 3B suggest that Rre16 in its active form represses the expression of the \( mntCAB \) operon and that the inactivation of Rre16 in \( \Delta rre16 \) mutant cells eliminated the repressive effect of Rre16, allowing expression of the \( mntCAB \) operon.

The response regulator Rre16 consists of 234 amino acid residues and has a relative molecular mass of 26 kDa (http://www.kazusa.or.jp/cyano/Synechocystis/cgi-bin/ ranso-orfsearch.cgi?type=orf&name=slr1837). It contains a receiver domain that includes a phosphorylatable aspartate residue and a putative DNA-binding domain in the carboxy-terminal region. This structure resembles that of OmpR from \( E. coli \), which is a regulator of responses to hyperosmotic stress (Martinez-Hackert and Stock, 1997).

These findings suggest that Hik27 and Rre16 constitute a two-component system, acting as a sensor and a signal transducer of Mn\(^{2+}\) ion deficiency. Ogawa et al. (2002) identified this two-component system independently by a traditional method.

**Hik34 negatively regulates the expression of heat-shock genes**

Mutation of Hik34 altered the genome-wide expression of genes under normal conditions, namely, at a growth temperature of 34 °C (Suzuki et al., 2005). In \( \Delta hik34 \) cells, the level of the transcript of the heat-shock gene \( htpG \) was approximately 3-fold higher than that in wild-type cells. Levels of transcripts of other heat-shock genes, such as \( groES \) and \( groEL1 \), were also elevated. These results suggest that Hik34 might act as a negative regulator of the expression of these genes under normal growth conditions. The mutation in \( hik34 \) also enhanced the expression of some genes that are related to photosynthesis.

Since mutation of the \( hik34 \) gene enhanced the expression of some heat-shock genes under normal growth conditions, the library of mutated Hiks was screened for survival at a high temperature, such as 48 °C. All types of cell examined were tolerant to the heat stress at 48 °C for 2 h. However, when cells were exposed to 48 °C for 3 h, only \( \Delta hik34 \) cells survived and other mutant and wild-type cells died. These results clearly indicated that the enhanced expression of heat-shock genes, as a consequence of the mutation of Hik34, under normal growth conditions contributed to the acquisition of thermotolerance by \( \Delta hik34 \) cells (Suzuki et al., 2005).

Since Hik34 appeared to be a negative regulator of heat shock-responsive genes, it is postulated that its overexpression should result in repressed expression of these genes. Therefore, Hik34 was overexpressed and the effects of elevated levels of Hik34 on gene expression were examined using the DNA microarray (Suzuki et al., 2005). The genome-wide expression of genes in Hik34-overexpressing cells was compared with that in control cells. Overexpression of the \( hik34 \) gene in \( Synechocystis \) cells clearly depressed the expression of several heat-shock genes, including the \( htpG \), \( hspa \), \( groESL1 \), \( dnaK2 \), and \( groEL2 \) genes. These results supported the hypothesis that Hik34 negatively regulates the expression of heat-shock genes. Inactivation of the \( hik34 \) gene and overexpression of the \( hik34 \) gene had opposite effects on the expression of heat-shock genes at the normal growth temperature. This observation is consistent with the hypothesis that Hik34 plays an important role in the regulation of the expression of heat-shock genes.

Hik34 consists of 464 amino acid residues and it includes a conserved signature motif of histidine kinases, H1, which includes a phosphorylatable histidine residue at position 238 from the amino terminus (http://www.kazusa.or.jp/cyano/Synechocystis/cgi-bin/orfinfo.cgi?title=Chr&name=slr1285&iden=1). However, Hik34 lacks other signature motifs of Hiks, such as the N, G1 (DXGXG), F, and G2 (GXGXG) motifs, which are normally located downstream of the H1 motif and probably play important roles in the binding of ATP, which serves as the phosphate donor in the autophosphorylation of Hiks (Mizuno et al., 1996). Despite the absence of the signature motifs in Hik34, a recombinant Hik34 protein was autophosphorylatable in vitro in the presence of ATP as the phosphate donor (Suzuki et al., 2005). Thus, Hik34 is an unusual Hik and appears to be
unique among the Hiks encoded by the chromosome and plasmids of Synechocystis. Moreover, to date, no homologues of Hik34 have been found in any organisms other than cyanobacteria. Modification of the ATP-binding motif in the Hik domain of Hik34 might be related to the way in which this kinase functions.

**Positive regulators**

Mutation of all the other genes (42 genes) apart from hik27, hik34, and hik20 did not induce any significant changes in gene expression, as determined with the genome-wide DNA microarray. Therefore, it is likely that the Hiks encoded by these 42 genes regulate gene expression in a positive manner (Fig. 2A, B). In such cases, it is necessary to screen the library of hik mutants under specific kinds of stress, such as high-salt stress (Marin et al., 2003; Shoumskaya et al., 2005) and hyperosmotic stress (Paithoonrangsarid et al., 2004).

**Hik33 perceives low-temperature stress via the rigidification of membrane lipids**

During the screening of the library of mutant hik genes, Hik33 had originally been identified in Synechocystis as a regulator of the low-temperature-inducible expression of a reporter gene. This reporter gene included the promoter of the desB gene for the o3 desaturase of Synechocystis and the luxAB gene for a bacterial luciferase (Suzuki et al., 2000). Subsequent analysis of the genome-wide pattern of gene expression with the DNA microarray demonstrated that Hik33 regulates the expression of most of the genes that are induced at low temperature (Suzuki et al., 2001). Figure 4A shows that the inducibility by low temperature of 21 of the 36 low-temperature-inducible genes with induction factors higher than 4.0 was under the control of Hik33. By contrast, the level of expression of the other low temperature-inducible genes was not significantly altered by mutation of Hik33.

The amino acid sequence of the Hik33 sensory kinase contains several conserved regions, namely, a type-P linker, a leucine zipper, and PAS domain (Los and Murata, 1999, 2002, 2004; Mikami et al., 2003). The type-P linker consists of two helical regions in tandem that are assumed to transduce stress signals via intramolecular structural changes that occur as a result of interactions between the two helical regions and lead to intermolecular dimerization of membrane proteins (Williams and Stewart, 1999; Aravind et al., 2003). In Hik33, low-temperature stress might promote a conformational change in the type-P linker, with the subsequent activation of Hik33 as a result of dimerization of the protein (Los and Murata, 2000, 2004). Thus, low-temperature stress might trigger the dimerization of Hik33.

There are two transmembrane domains in the aminoterminal region of Hik33 (Mikami et al., 2003; Los and Murata, 2004). It has been suggested that changes in membrane fluidity might be involved in the sensing of temperature (Murata and Los, 1997; Los and Murata, 2000; Vigh et al., 1998). It is likely that the transmembrane domains of Hik33 are able to recognize a change in membrane fluidity at low temperatures (Los and Murata, 1999, 2000, 2004). It was demonstrated previously, as evidence for this model, that the expression in Synechocystis of the desA gene, a low-temperature-inducible gene that encodes the Δ12 fatty-acid desaturase, is induced by the rigidification of the plasma membrane under isothermal conditions that results from the Pd-catalysed hydrogenation of membrane lipids (Vigh et al., 1993).

Advantage has recently been taken of a genetically modified strain of Synechocystis in which the membrane lipids have been rigidified by manipulating the extent of unsaturation of the constituent fatty acids (Inaba et al., 2003). In earlier studies, a series of mutants was produced in which the extent of unsaturation of fatty acids is modified in a step-wise manner (Tasaka et al., 1996). In one of these mutants, the desA and desD genes, which encode the Δ12 and Δ6 fatty-acid desaturases, respectively, are both inactive as a result of targeted mutagenesis. Cells of the desA^-/desD^- double mutant synthesize only a saturated C16 fatty acid and a mono-unsaturated C18 fatty acid, regardless of growth temperature, whereas wild-type cells synthesize di-unsaturated and tri-unsaturated C18 fatty acids in addition to the mono-unsaturated C18 fatty acid (Tasaka et al., 1996). FTIR spectrometry revealed that the double mutation of the desA and desD genes rigidified the plasma membrane of Synechocystis at physiological temperatures (Szalontai et al., 2000).

The DNA microarray was used to examine the effects of the above-described membrane rigidification on the expression of genes in response to low temperature in Synechocystis (Inaba et al., 2003). The changes in gene expression in wild-type and desA^-/desD^- cells were monitored after they had been grown at 34 °C and then incubated at 22 °C for 30 min. In wild-type cells, low-temperature stress enhanced more than 2-fold the levels of expression of 168 genes. In desA^-/desD^- cells, in addition to the enhancement of the levels of expression of the same set of 168 low-temperature-inducible genes, the expression of 96 additional genes appeared to be enhanced. The results indicated that the rigidification of membrane lipids had enhanced the response of gene expression to low temperature in Synechocystis. By contrast, under isothermal conditions, the double mutation had no significant effect on gene expression.

The low-temperature-inducible genes could be divided into three groups according to the effects of the double mutation (Inaba et al., 2003). The first group consisted of genes that were not induced by low temperature in wild-type cells but were strongly induced by low temperature in desA^-/desD^- cells. The second group consisted of genes
Fig. 4. Hypothetical schemes showing the two-component systems that are involved in the transduction of low-temperature stress, salt stress, and hyperosmotic stress, as well as the genes that are under the control of the individual two-component systems. Primary signals are represented by open arrows. Hiks are indicated as ellipses, Rres are indicated as hexagons, and selectively regulated genes are shown in boxes. Uncharacterized mechanisms are represented by question marks. Genes with induction factors higher than 3.0 are included in these schemes. (A) whose low-temperature inducibility was moderately enhanced by the double mutation. The third group consisted of genes whose inducibility by low temperature was unaffected by the double mutation. These results suggest that the expression in response to low temperature of the genes in these three groups might be regulated by different mechanisms with respect to membrane rigidity. The induction of the expression of the genes in the first group might require a higher degree of rigidification of membrane lipids than the low-temperature responses of genes in the second and third groups. The rigidification of membrane lipids did not enhance the low-temperature-inducibility of genes in the third group, perhaps because the rigidity of membranes in wild-type cells is sufficient at low temperatures for the maximum induction of expression of these genes.

To examine whether Hik33 might regulate the low-temperature-responsive gene expression that depends on membrane rigidity, the DNA microarray was used to examine gene expression in desA/desD/hik33 cells, in which the hik33 gene had been mutated in addition to mutation of the desA and desD genes. Mutation of Hik33 abolished or significantly reduced the inducibility by low temperature of 10 out of the 17 genes in the second group and of 7 out of the 25 genes in the third group. By contrast, mutation of Hik33 had no significant effect on the low-temperature inducibility of genes in the first group. These results indicate that Hik33 regulates the expression of many genes in the second and third groups. These results also suggest that the activity of Hik33 in the sensing of low temperature depends on membrane rigidity and that there are at least two other low-temperature sensors, one of which depends on membrane rigidification, while the other functions independently of membrane rigidity (Inaba et al., 2003).

It has been postulated that the fluidity of membrane lipids might be involved in the perception of heat stress (Vigh et al., 1998). To examine this hypothesis in Synechocystis, the effects were investigated of membrane rigidification, caused by double mutation of the desA and desD genes, on the regulation of gene expression upon exposure of cells to heat shock. DNA microarray analysis revealed that heat shock at 42 °C for 10 min induced the expression of a large number of genes in wild-type Synechocystis. These heat-inducible genes were induced to a similar extent in desA/desD cells. Thus, it is likely that, in Synechocystis at least, membrane rigidity is not involved in the regulation of gene expression in response

Low-temperature stress; adapted originally from Suzuki et al. (2001) and Mikami K et al. (2002). Copyright Blackwell Publishing and reproduced with permission, with inclusion of more recent results. (B) Salt stress; adapted, with permission, from Shoumskaya et al. (2005). (C) Hyperosmotic stress; adapted from Paithoonrangsarid et al. (2004) and Shoumskaya et al. (2005).
to heat stress and, therefore, in the perception of such stress (Inaba et al., 2003).

**Two-component systems involved in the acclimation to salt stress**

The response to salt stress has been investigated in detail in model micro-organisms, such as E. coli and B. subtilis, and in photoautotrophic cyanobacteria. *Synechocystis* is able to acclimate to concentrations of NaCl as high as 1.2 M by synthesis de novo or by uptake of the compatible solute glucosylglycerol (Reed and Stewart, 1985; Hagemann et al., 1997), with the additional involvement of ion exchangers, such as Na\(^+\)/H\(^+\) antiporters (Inaba et al., 2001). Recently, all of the salt-regulated genes in *Synechocystis* were identified using the DNA microarray from Takara Bio Co. (Kanesaki et al., 2002). Homologues of salt-inducible genes in *Synechocystis* are also regulated by salt stress in higher plants (Bohnet et al., 2001), an observation that suggests that this cyanobacterium might serve as a good model system for investigations of the responses of plants to salt stress.

The molecular mechanisms involved in the regulation of the salt-induced expression of genes are unknown for the most part. In heterotrophic bacteria, such as *E. coli* and *B. subtilis*, expression of a number of salt-inducible genes is regulated by the activities of the sigma factors RpoS and SigB, respectively (Hecker et al., 1996). By contrast, in the photoautotrophic micro-organism *Synechocystis*, SigF seems to be involved in such regulation (Huckauf et al., 2000). However, none of these sigma factors perceives salt signals directly, and the salt sensor(s) that are located upstream of these sigma factors in the salt signal-transducing pathway remain to be characterized.

The DNA microarray was used to investigate the impact of mutations in each Hik on changes in gene expression upon exposure of cells to salt stress (Marin et al., 2003). The comprehensive screening revealed that the inducibility of gene expression by elevated levels of NaCl was significantly affected in ΔHik16 (slr1805), ΔHik33 (sll0698), ΔHik34 (slr1285), and ΔHik41 (sll1229) mutant cells. In each of these mutants, several genes were no longer induced by salt or their inducibility by salt was markedly reduced.

By screening an Rre knockout library by RNA slot-blot hybridization and with the genome-wide DNA microarray, three Hik-Rre systems were identified, namely, Hik33-Rre31, Hik10-Rre3, and Hik16-Hik41-Rre17, as well as another system that included Rre1, that appeared to be involved in the perception of salt stress and transduction of the signal (Shoumksaya et al., 2005). In addition, further extensive screening of the Hik and Rre mutant libraries by DNA microarray analysis and slot-blot hybridization analysis revealed the existence of another two-component system, namely, Hik10-Rre3. Figure 4B shows a hypothetical model of the salt signal-transducing systems that involve these Hiks and Rres; this model includes the salt-inducible genes that are controlled by the individual Hik-Rre systems.

The Hik33-Rre31 two-component system regulates the inducibility by high concentrations of NaCl of the expression of ten genes. Inactivation of either Hik33 or Rre31 resulted in the elimination of or a marked reduction in the salt stress-inducible expression of seven genes. These findings indicate that Hik33 and Rre31 are tightly coupled in the signal transduction pathway. The Hik10-Rre3 two-component system regulates the salt stress-inducible expression of only one gene, *htrA*, which encodes a serine protease.

The Hik16-Hik41-Rre17 system regulates the hyperosmotic stress-inducible expression of three genes, namely, *sll0939, slr0967*, and *sll0938*. Inactivation of Hik16, of Hik41 or of Rre17 eliminated the expression of these genes, suggesting that Hik16, Hik41, and Rre17 are all essential for the perception of hyperosmotic stress and for transduction of the signal that controls the expression of these genes. It seems likely that Hik41 acts downstream of Hik16 since Hik41 is a hybrid-type histidine kinase that contains both a signal-receiver domain and a histidine kinase domain, whereas Hik16 is a typical Hik with a histidine kinase domain and potential sensory domain that, hypothetically, spans the membrane seven times. It is also possible that Hik16 and Hik41 might perceive salt stress as a complex.

The Hik34-Rre1 two-component system regulates the expression of 26 salt stress-inducible genes. The inducible expression of these genes was abolished or significantly reduced by mutation of either Hik34 or Rre1, suggesting the tight coupling of these two components.

The expression of the *sigB, dnaJ*, and *riml* genes, as well as that of five other salt stress-inducible genes, was controlled by Rre1 but not by Hik34. Screening this laboratory’s library of mutant Hiks by RNA slot-blot hybridization with a probe derived from the *sigB* gene did not allow a Hik to be identified that might act upstream of Rre1 in the signal transduction pathway that regulates the expression of this gene. These results suggest that such a Hik might be encoded by a gene that is incompletely replaced by the corresponding mutated gene in the line of mutant cells. Evidence was found for incomplete replacement of the respective genes for Hik in ΔHik2, ΔHik11, and ΔHik26 cells. Experiments with a yeast two-hybrid system revealed that Hik2 could associate very tightly with Rre1. Therefore, it seems very likely that Hik2 and Rre1 might constitute a two-component system that regulates the expression of all eight genes in response to salt stress.

These studies with the DNA microarray revealed that expression of 26 of the 64 salt stress-inducible genes was not controlled by any of the five Hiks and four Rres discussed above (Fig. 4B). Screening this Rre knockout library by slot-blot hybridization with probes derived from
genes in this group of 14 genes, such as \textit{ndhR} and \textit{sll1862}, did not allow candidates for Rres that might act as signal transducers to be identified. However, the Rre knockout library included three mutant lines, ΔRre23, ΔRre25, and ΔRre26, in which the complete replacement of the \textit{rre} gene had not been achieved (http://www.kazusa.or.jp/cyanobase/Synechocystis/mutants/). Thus, possible candidates for signal transducers include Rre23, Rre25, and Rre26. It is, however, more likely that the signals, due to salt stress, that induce the expression of these genes are perceived by unknown mechanisms that are separate from the typical Hik-Rre two-component systems. Such signals might act directly to regulate either the transcription or the stability of the transcripts of these inducible genes.

\textbf{Two-component systems involved in signalling of hyperosmotic stress}

Earlier work from this laboratory demonstrated that perception of hyperosmotic stress involves a histidine kinase, Hik33, and other unknown components (Suzuki \textit{et al}., 2001; Mikami \textit{et al}., 2002), and that the sets of inducible genes differed between salt stress and osmotic stress (Fig. 1; Kanesaki \textit{et al}., 2002). However, it is postulated that both types of stress might share common sensors and response regulators. With this possibility in mind, the effects of inactivation in Hik33, Hik34, Hik16, Hik41, and Hik10 on the induction of gene expression by hyperosmotic stress were examined.

Systematic mutagenesis of almost all the \textit{hik} and \textit{rre} genes in \textit{Synechocystis}, in combination with the genome-wide analysis of gene expression at the transcriptional level, allowed four Hik-Rre systems to be identified, namely Hik33-Rre 31, Hik34-Rre1, Hik16-Hik41-Rre17, and Hik10-Rre3, and another potential two-component system, namely Hik2-Rre1, for the perception of hyperosmotic stress and transduction of the signal. The expression of most of the hyperosmotic stress-inducible genes was controlled by these Hik-Rre systems. Figure 4C shows the signal transduction pathways that operate when cells are exposed to hyperosmotic stress, as well as the hyperosmotic stress-inducible genes whose expression is controlled by the individual Hik-Rre systems.

The Hik33-Rre31 two-component system regulates the inducible expression of 11 genes. The Hik10-Rre3 two-component system regulates the hyperosmotic stress-inducible expression of only the \textit{htrA} gene, as in the case of the salt inducibility of gene expression. The Hik16-Hik41-Rre17 system regulates the hyperosmotic stress-inducible expression of two genes, namely, \textit{sll0939} and \textit{sbr0967}. The Hik34-Rre1 two-component system regulates 19 hyperosmotic stress-inducible genes. However, all the inducible genes are not the same as the salt stress-inducible genes that are under the control of the Hik34-Rre1 system. Hyperosmotic stress-inducible expression was abolished or significantly reduced by mutation of either Hik34 or Rre1, suggesting the tight coupling of these two components in the perception and transduction of hyperosmotic signals.

The expression of the \textit{sigB} gene and four other hyperosmotic stress-inducible genes was controlled by Rre1, but not by Hik34. Screening of the library of mutant Hiks by RNA slot-blot hybridization with a probe derived from the \textit{sigB} gene did not allow a Hik to be identified that might act upstream of Rre1 in the signal transduction pathway that regulates the expression of this gene. It seems most likely that Hik2 and Rre1 might constitute a two-component system that regulates the expression of \textit{sigB} and four other genes in response to hyperosmotic stress, a situation similar to that described in the earlier discussion of salt stress.

The studies with the DNA microarray specific for \textit{Synechocystis} revealed that expression of 14 of the 48 hyperosmotic stress-inducible genes was not controlled by any of the five Hiks and four Rres discussed above (Fig. 4C). Screening of the Rre knockout library by slot-blot hybridization, as performed in the analysis of the salt-signalling pathway, did not allow candidates for Rres that might act as signal transducers to be identified. It is likely that the signals due to hyperosmotic stress that induce the expression of these genes are perceived by unknown mechanisms that are distinct from Hik-Rre two-component systems.

\textbf{Identical Hik-Rre systems perceive salt stress and hyperosmotic stress but regulate different sets of genes in response to each type of stress}

It was found that the Hik-Rre systems for the perception and transduction of the salt-stress signal and the hyperosmotic stress-signal were identical. The induction factors of the salt stress-inducible and hyperosmotic stress-inducible genes that are controlled by each two-component system were compared and it was found that these genes responded to the two types of stress to different respective extents. In addition, the Hik33-Rre31 system regulated the expression of genes, namely, \textit{fabcG} and \textit{gloA}, that were specifically induced by hyperosmotic stress, whereas the Hik34-Rre1 system regulated the expression of seven and four genes that were specifically induced by salt stress and by hyperosmotic stress, respectively. These observations suggest that the perception of salt stress and hyperosmotic stress by the Hik-Rre systems is complex and that salt stress and hyperosmotic stress are perceived as distinct signals by the individual Hik-Rre systems. This hypothetical scenario strongly suggests the presence of as-yet-unidentified components that provide the sensory systems with their respective activities to induce responses specific to salt stress and hyperosmotic stress.

\textbf{Conclusion}

In this review, it has been demonstrated that genome-based systematic analysis is a powerful technique with which it is
possible to identify, with relative ease, the Hiks and Rres that are involved in the perception and transduction of stress signals. Without this approach, it would have been very difficult to show that Hik33 regulates the expression of most of the low-temperature-inducible genes in *Synechocystis*, that membrane rigidification is intimately involved in the sensing of low temperature, and that five two-component systems are involved in the perception of salt stress and hyperosmotic stress and the transduction of the respective signals. However, although such analysis has allowed some details of these signal-transduction pathways to be defined, it now has to be determined how the same two-component system can perceive and transduce more than one kind of environmental signal (Los and Murata, 2002, 2004; Mikami *et al.*, 2003). For example, Hik33 is involved in the sensing of low temperature, salt stress, and hyperosmotic stress; Hik34 is involved in the sensing of salt stress, hyperosmotic stress, and possibly heat stress. These findings cannot be explained by the current model of two-component systems, in which a Hik perceives a specific stress and regulates the expression of a particular set of genes via the phosphorylation-dependent activation (or inactivation) of its cognate Rre. To answer this question, it will be necessary to study more details of the mechanisms of signal perception and transduction. It is probable that there exist as-yet-unidentified components that are important in determining the specificity of the responses to individual types of stress. It is also possible that the sensors of environmental signals are highly organized protein complexes, in which Hiks, Rres and various unidentified components are somehow associated. To identify these components, new techniques which, most probably, will also exploit the information encoded in the genomes of cyanobacteria and other organisms, will have to be developed and introduced.

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