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学 位 論 文 題 目 CHARACTERIZATION OF Na⁺/H⁺ ANTIPORTERS
IN THE CYANOBACTERIUM *SYNECHOCYSTIS* sp. PCC 6803

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The cyanobacterium *Synechocystis* sp. PCC 6803 has five genes for putative Na⁺/H⁺ antiporters (designated *nhaS1*, *nhaS2*, *nhaS3*, *nhaS4*, and *nhaS5*). In this thesis, their products were studied by means of functional expression in *Escherichia coli*, targeted mutagenesis, and phylogenetic analyses.

Na⁺/H⁺ antiporters are membrane proteins that exchange Na⁺ for H⁺, the two most important ions in cell bioenergetics. Na⁺/H⁺ antiporters were discovered by Peter Mitchell and his colleagues in the late 60's and, since then, it has been found that they are widely distributed throughout the biological kingdoms. Chapter 1 describes previous studies of Na⁺/H⁺ antiporters, which will provide an overview of how these universal devices are used by various organisms. It should be stressed that no Na⁺/H⁺ antiporters had been identified in cyanobacteria, despite of accumulating evidence that Na⁺/H⁺ antiporters play important roles in the maintenance of intracellular ion homeostasis during acclimation to changes in environmental conditions.

Chapter 2 describes characterization of *Synechocystis* Na⁺/H⁺ antiporters by functional expression of the *nhaS* genes in *Escherichia coli*. The author successfully induced the expression of *nhaS1*, *nhaS3*, and *nhaS4* under control of an Na⁺-dependent promoter in *E. coli* TO114, a strain that is deficient in Na⁺/H⁺-antiport activity. Inverted membrane vesicles prepared from *nhaS1*/TO114 and *nhaS3*/TO114 cells exhibited Na⁺(Li⁺)/H⁺-antiport activity. Kinetic analysis of the antiport activity revealed that the *nhaS1* gene encodes a low-affinity Na⁺/H⁺ antiporter with a K_m value of 7.7 mM for Na⁺ ions and a K_m value of 2.5 mM for Li⁺ ions, while the *nhaS3* gene encodes a high-affinity Na⁺/H⁺ antiporter with a K_m value of 0.7 mM for Na⁺ ions and a K_m value of 0.01 mM for Li⁺ ions. Transformation of *E. coli* TO114 with the *nhaS1* and *nhaS3* genes increased tolerance to high concentrations of Na⁺ and Li⁺ ions and depletion of K⁺ ions. This is the first demonstration of functional characterization of Na⁺/H⁺ antiporters from cyanobacteria. Inverted membrane vesicles prepared from *nhaS4*/TO114 cells did not have Na⁺/H⁺-antiport activity, and the cells themselves were as sensitive as the original TO114 cells to Na⁺ and Li⁺ ions. However, the *nhaS4*/TO114 cells were tolerant to depletion of K⁺ ions. This result suggested that the *nhaS4* gene might encode a membrane protein that transports Na⁺ and/or K⁺ ions.

Evaluation of effects of targeted inactivation of the *nhaS* genes on growth phenotypes of *Synechocystis* cells is described in Chapter 3. She created single and double mutants of *Synechocystis* in which individual *nhaS* genes were interrupted by insertion of an antibiotic-resistance gene cartridge. The disruption of the *nhaS1*, *nhaS2*, *nhaS4*, and *nhaS5* genes in all copies of the chromosomal DNA was verified by PCR. She failed to disrupt the *nhaS3* gene; homozygous null mutants were not recovered after the mutagenesis. The single mutants that she obtained did not show any phenotypic changes in terms of the sensitivity to growth inhibition by NaCl. *nhaS1 nhaS2* cells grew slower than wild-type cells both in BG11 medium (the standard medium), that contained 18 mM Na⁺, and in a high-salt medium, prepared by adding NaCl, to 0.5 M, to the BG11 medium. The growth retardation of *nhaS1 nhaS2* cells appeared to be greater in the presence of 0.5 M NaCl than in its absence. In contrast, *nhaS4 nhaS5* cells grew as well as

wild-type cells regardless of the presence or absence of 0.5 M NaCl. These results suggested that (i) the function of the *nhaS1* and *nhaS2* genes' products might be complementary, (ii) the *nhaS3* gene is essential for viability of *Synechocystis* cells, and (iii) products of the *nhaS4* and *nhaS5* genes may contribute little to high-salt stress tolerance.

Chapter 4 describes phylogenetic analyses of the Na⁺/H⁺ antiporters and putative homologues of the Na⁺/H⁺ antiporters from widely divergent phyla. BLAST search results indicated that NhaS1 and NhaS2 are similar to NhaP of *Pseudomonas aeruginosa* and eukaryotic Na⁺/H⁺ antiporters, while NhaS3, NhaS4, and NhaS5 are similar to NapA of *Enterococcus hirae*. Comparison of deduced amino acid sequences of the NhaS proteins to those of the Na⁺/H⁺ antiporters revealed significant similarities within the putative fifth and sixth transmembrane segments, of the NhaS proteins, and corresponding regions of the Na⁺/H⁺ antiporters. A phylogenetic tree based on the evolutionary distances revealed that 127 Na⁺/H⁺ antiporter homologues from 53 species, including eukaryotes and prokaryotes, cluster with two groups, which are named NhaP/NHE and NapA families. The ubiquitous distribution of the members of the two families throughout the biological kingdoms indicates that the two types of proteins diverged before the divergence of major lineages in prokaryotes. Na⁺/H⁺ antiporter homologues from various cyanobacteria form five distinct groups, namely, NhaS1-5 subfamilies. NhaS3 orthologs exist in all cyanobacteria where the entire genome sequence is available, suggesting that NhaS3 might be of particular importance to cyanobacteria. This is in agreement with the fact that the *nhaS3* gene is essential for the viability of *Synechocystis* (Chapter 3). The NhaS1 and NhaS2 subfamilies belong to the NhaP/NHE family, while the others belong to the NapA family. The findings that NhaS1 and NhaS3 are low-affinity and high-affinity Na⁺/H⁺ antiporters, respectively (Chapter 2), are consistent with the common kinetic properties of each type of Na⁺/H⁺ antiporters. The *Arabidopsis* proteins cluster with six groups, and two of them (SOS1 and AtNHX1) belong to the NhaP/NHE family. SOS1 and NhaS1 form a monophyletic group with a bootstrap value less than 50% and are closely related to NhaS2. These results might indicate the possibility that the *Arabidopsis* genes were acquired from the ancestor of plastids, although the relationship between these proteins are not well resolved.

論文の審査結果の要旨

Na⁺/H⁺ アンチポーターは、植物や微生物の細胞の塩濃度と pH の恒常性を保持する機構において重要な役割を担うと考えられている。ラン藻 *Synechocystis* sp. PCC 6803 (*Synechocystis*) ではゲノムの全塩基配列が 1996 年に決定され、5つの Na⁺/H⁺ アンチポーターホモログの遺伝子が見い出されていた。申請者は植物細胞のモデルとして *Synechocystis* を材料に選び、Na⁺/H⁺ アンチポーターの同定と機能解析を行った。論文は次の4章から構成されている。

第1章: General Introduction さまざまな種類の生物・細胞からの Na⁺/H⁺ アンチポーターのクローニングとその機能、構造、活性・遺伝子発現の調節、細胞内局在、生理学的役割について詳細に記述した上で、本研究の目的を述べている。

第2章: Functional characterization of Na⁺/H⁺ antiporters of *Synechocystis* by expression in *Escherichia coli* *Synechocystis* の各ホモログ遺伝子 (*nhaS1-5*) を大腸菌の Na⁺/H⁺ アンチポーター遺伝子欠損株に導入し、細胞質膜から調製した反転膜小胞の Na⁺/H⁺ アンチポート活性を指標にして相補テストを行った。その結果、*nhaS1* と *nhaS3* がそれぞれ Na⁺ および Li⁺ に対して低親和性、高親和性の Na⁺(Li⁺)/H⁺ アンチポーターをコードしていることを明らかにした。

第3章: Evaluation of effects of targeted mutagenesis of genes for Na⁺/H⁺ antiporter homologues in *Synechocystis* *nhaS1-5* の生理学的役割について知見を得るために、各遺伝子を薬剤耐性遺伝子の挿入によって破壊し、低・高塩濃度条件下での細胞増殖を調べた。*nhaS1*、*nhaS2*、*nhaS4*、*nhaS5* の破壊は、増殖速度に影響を及ぼさなかった。*nhaS3* は *Synechocystis* のゲノムのコピーのおよそ 10% でしか破壊することができず、その *nhaS3* 変異株は低塩濃度培地において野生株よりも顕著に低い増殖速度を示した。*nhaS1* と *nhaS2* の両方を破壊した株は、低塩濃度培地において野生株よりも増殖速度が低く、高塩濃度培地では増殖の阻害がさらに顕著になった。*nhaS4* と *nhaS5* の両方を破壊した株は、野生株と同様に増殖した。以上の結果は、(1) *nhaS3* は *Synechocystis* の生存に必須であること、(2) *nhaS1* と *nhaS2* の産物が *Synechocystis* において相補的に機能し、耐塩性に関与していること、(3) *nhaS4* と *nhaS5* の産物の耐塩性への関与が低いこと、を示唆している。

第4章: Phylogenetic analysis of Na⁺/H⁺ antiporter homologues: from cyanobacteria to higher plants 近年、多くの生物でゲノムプロジェクトが行われ、Na⁺/H⁺ アンチポーターホモログの遺伝子が見い出されている事実を踏まえて、Na⁺/H⁺ アンチポーターおよびホモログの推定アミノ酸配列について系統分類学的解析を行った。その結果、高等植物 (*Arabidopsis thaliana*) には *Synechocystis* の各ホモログと同じファミリーに属する Na⁺/H⁺ アンチポーターホモログが多数存在し、そのうちの1つ (*SOS1*) が *NhaS1* と近い関係にあることが明らかになった。これは、植物が *SOS1* 遺伝子を葉緑体の祖先から獲得した可能性を示唆している。

なお、以上の研究成果のうち第2・3章の内容は J. Bacteriol. 誌に掲載された。

本研究は、光合成生物からはじめて Na^+/H^+ アンチポーターを同定し、その耐塩性における重要な機能を解析したものであり、また、高等植物の Na^+/H^+ アンチポーターホモログの機能を解明する上で有用な情報を提供している。したがって、学位論文として十分な内容を有すると判断し、合格と判定した。

審査委員会は、専門領域と関連領域に関する口述試験を行った。その結果、審査委員は一致して、稲葉昌美の研究内容、専門分野に関する知識と理解が学位取得の条件を十分に満たすものと判定した。

英語に関しては、数遍の英語原著論文を発表しており、博士論文の英語と考えあわせて、博士として十分な能力を備えていると判断した。