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Secondary transporters for nickel and cobalt ions: Theme and variations

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Abstract

Nickel/cobalt transporters (NiCoTs), a family of secondary metal transporters in prokaryotes and fungi, are characterized by an eight-transmembrane-domain (TMD) architecture and mediate high-affinity uptake of cobalt and/or nickel ions into the cells. One of the strongly conserved regions within the NiCoTs is the signature sequence RHA(V/F)DADHI within TMD II. This stretch of amino acid residues plays an important role in the affinity, velocity and specificity of metal transport. Some relatives of the NiCoTs, named HupE, UreJ and UreH, contain a similar signature sequence and are encoded within or adjacent to [NiFe] hydrogenase or urease operons, or elsewhere in the genome of many prokaryotes. HupE and UreH from *Rhodopseudomonas palustris* CGA009 and UreJ from *Cupriavidus necator* H16 were shown to mediate Ni^{2+} transport upon heterologous production in *E. coli*. Other variants of NiCoTs are found in many marine cyanobacteria and in plants. The cyanobacterial proteins are encoded by a segment adjacent to the genes for [Ni] superoxide dismutase and a corresponding putative maturation peptidase. The plant proteins contain N-terminal sequences resembling bipartite transit peptides of thylakoid lumenal and thylakoid integral membrane precursor proteins; expression of a YFP-fusion protein in transfected leaf cells is consistent with targeting of this protein to the plastid, but the function of the plant gene product has yet to be demonstrated.

Introduction

Nickel is an essential component of at least nine metalloenzymes involved in energy and nitrogen metabolism, in detoxification processes and in pathogenesis (Mulrooney & Hausinger 2003). This list includes urease, [NiFe] hydrogenase and [Ni] superoxide dismutase. Cobalt is primarily found in the corrin ring of coenzyme B_{12}, but is also contained in some non-corrin cobalt-dependent enzymes such as [Co] nitrile hydratase (Kobayashi & Shimizu 1999). Synthesis of nickel and cobalt enzymes is dependent on high-affinity uptake of the metal ions from natural environments where they are available only in trace amounts. In metal-polluted environments, however, metal homeostasis is an important issue since metal overload will eventually cause oxidative stress leading to enzyme inactivation, lipid peroxidation and damage of nucleic acids.

Nickel and cobalt uptake in microorganisms is mediated by secondary transporters and by ATP-binding cassette (ABC) systems (see Eitinger & Mandrand-Berthelot 2000; Eitinger 2001; Mulrooney & Hausinger 2003 for reviews). The secondary systems are classified as nickel/cobalt transporters (NiCoTs; TC 2.A.52), a family of prokaryotic and fungal membrane proteins with an eight-transmembrane-domain structure. The best-investigated ABC-type nickel permease is the NikABCDE system of *E. coli*, composed of a periplasmic binding protein (NikA), two integral

membrane proteins (NikBC) and two ABC proteins (NikDE). Certain ABC systems, consisting of three (CbiMQO) or four (CbiMNQO) components and encoded within prokaryotic coenzyme B_{12} biosynthesis gene clusters, are thought to be implicated in cobalt uptake (Roth *et al*. 1993). Since cognate extracellular binding proteins, prerequisites for substrate uptake by prokaryotic ABC permeases, have not been identified, the mechanism of cobalt uptake by CbiM(N)QO remains elusive. Homologs of *cbiMQO* were identified adjacent to urease genes in *Streptococcus salivarius* (Chen & Burne 2003) and *Actinobacillus pleuropneumoniae* (Bossé *et al*. 2001), and shown to be important for urease activity in cells grown under nickel limitation. In contrast to *S. salivarius*, *cbiMQO* is preceded by *cbiKL* in *A. pleuropneumoniae cbiK* and *cbiL* encode membrane proteins with a periplasmic domain. These data imply that CbiMQO homologs may be involved in transport of either one or both metals.

This short survey summarizes data on secondary nickel and cobalt transporters. It describes distribution, topology and function of the well-investigated NiCoTs and gives an initial insight into the role of UreH and HupE/UreJ proteins in metal metabolism. In addition, potential functions of related membrane proteins in marine cyanobacteria and in plants are discussed.

Nickel/cobalt transporter (NiCoT) family

NiCoTs are widespread among bacteria and found in thermoacidophilic archaea and certain fungi including *Schizosaccharomyces pombe* and *Neurospora crassa* (Eitinger *et al*. 2000; Hebbeln & Eitinger 2004). They work efficiently when their substrates are present in the low nanomolar range. A topological model for this type of transporter based on experimental analyses and hydropathy profile alignments (reviewed by Eitinger & Mandrand-Berthelot 2000; Eitinger 2001) is shown in Figure 1. Metal accumulation assays upon heterologous production of various NiCoTs in *E. coli* have identified subtypes with different ion preferences ranging from strict selectivity for nickel through unbiased transport of both ions to a strong preference for cobalt. Interestingly, substrate preference correlates in many cases with the genomic localization of NiCoT genes adjacent to clusters for nickel or cobalt enzymes or enzymes involved in coenzyme B_{12} biosynthesis (Degen *et al*. 1999; Degen & Eitinger 2002; Rodionov *et al*. 2003; Hebbeln & Eitinger 2004). Site-directed mutagenesis has been applied to certain NiCoTs to identify domains and residues that are responsible for the exceptionally high affinity and the differences in selectivity. HoxN, the NiCoT of *Cupriavidus necator* H16 (formerly named *Wautersia eutropha*, *Ralstonia eutropha* and *Alcaligenes eutrophus*), is a selective nickel permease, whereas NhlF from *Rhodococcus rhodochrous* J1 transports both ions but prefers cobalt. The studies showed that the conserved signature in TMD II (RHA(V/F)DADHI) contains residues essential for function. Replacement of the first His residue reduces the affinities of HoxN for Ni^{2+} and of NhlF for Co^{2+} and Ni^{2+}. NhlF is completely inactivated upon replacement of the second His residue and likewise, exchange of this His residue or the preceding Asp residue completely inactivates HoxN. The variable position occupied by a Val (as in HoxN) or Phe (as in NhlF) residue seems to affect transport velocity. Ni^{2+} transport by HoxN is significantly enhanced by a Val-to-Phe mutation and this variant displays low but significant Co^{2+}-transport activity, suggesting that an increase in velocity results in decreased specificity. The converse Phe-to-Val replacement diminishes NhlF activity significantly (Eitinger *et al*. 1997; Degen & Eitinger 2002). In summary, these results suggest that the conserved signature sequence in TMD II is a critical part of the selectivity filter of nickel/cobalt permeases. Our current efforts aim at a deeper insight into the structure of NiCoTs and their metal-binding sites by applying crystallographic techniques.

Prokaryotic UreH

Initial evidence for the existence of alternative secondary nickel transporters in prokaryotes was provided in 1994 when Maeda and colleagues characterized the urease operon of the thermophilic *Bacillus* sp. strain TB-90. This urease operon contains *ureH* as a downstream gene. Its deduced primary structure shares similarity with NiCoT sequences and contains the signature sequence in a predicted transmembrane region. Urease activity upon heterologous expression of the *ure* genes in

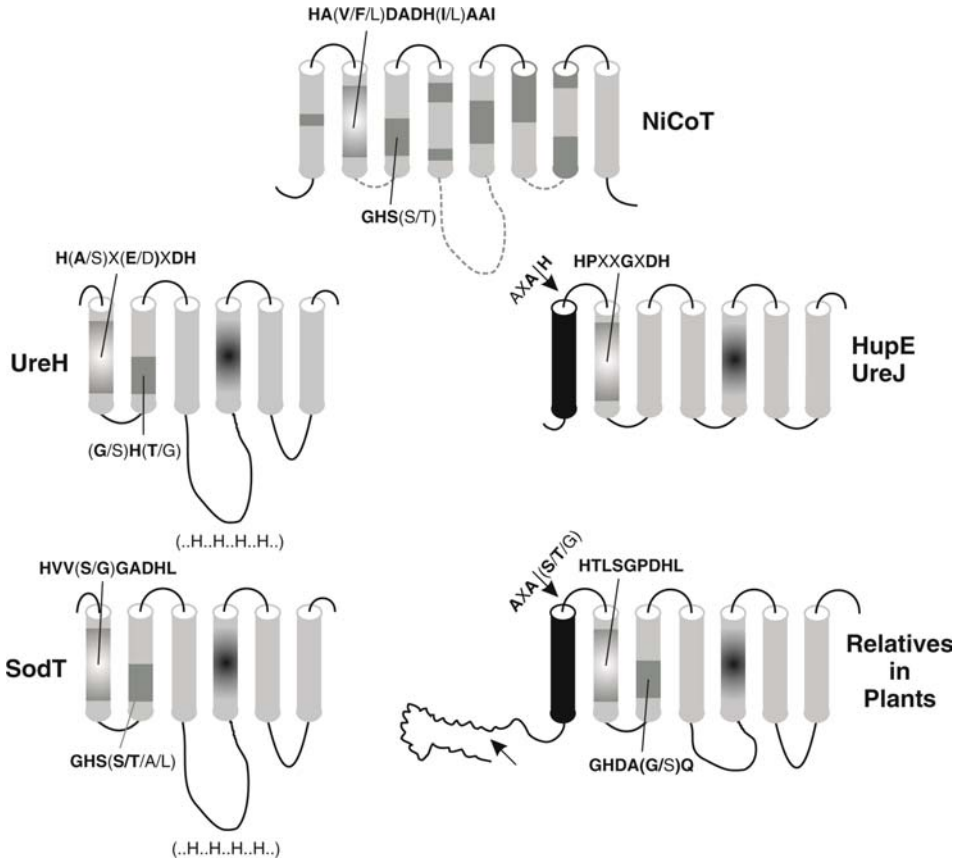

Figure 1. Topology of nickel/cobalt transporters (NiCoT) and some relatives in prokaryotes (UreH, HupE/UreJ, SodT) and in plants. Conserved segments within the NiCoT family, including the cytoplasmic loop between transmembrane domains (TMDs) IV and V, are highlighted. Dashed lines indicate sequence conservation in cytoplasmic loops. The motif in TMD II with the core sequence HX_4DH is considered to be a signature sequence for NiCoTs. Very similar signatures in UreH, HupE/UreJ, SodT and in the plant relatives are shown. Another His-containing motif is conserved in TMD III of the NiCoT sequences, and in TMD II of the UreH, SodT and plant sequences (bold letters indicate strong conservation). The black cylinders in the HupE/UreJ and in the plant transporter models indicate a predicted cleavable leader peptide and the hydrophobic segment of a bipartite thylakoid transit peptide, respectively. Diagonal arrows pointing downwards indicate putative cleavage sites by bacterial signal peptidase I in HupE/UreJ proteins and by lumenal thylakoid processing peptidase in the plant transporters. The diagonal upward-pointing arrow indicates a putative processing site in the stroma. The shading of TMD IV of (i) UreH and the plant transporters and (ii) HupE/UreJ and SodT indicates a signature with one His residue or two His residues, respectively. '..H..H..H..H..' illustrates His motifs with up to 14 His residues in the cytoplasmic loops connecting TMDs III and IV in UreH and SodT proteins.

E. coli was dependent on *ureH* when the recombinants were grown under nickel limitation, and thus, the authors assigned to UreH a potential role in nickel uptake. Relatives of *ureH* are contained in urease operons in *Geobacillus kaustophilus*, *G. stearothermophilus* and *Microbulbifer degradans*. A *ureH* gene-Orf 173, annotated as "hypothetical protein" (Fuhrmann *et al.* 2003) - is located on megaplasmid pHCG3 of *Oligotropha carboxidovorans* immediately downstream of hypB in a [NiFe] hydrogenase gene cluster. In other *ureH*-containing organisms (e.g. *Rhodopseudomonas palustris*), genomic linkage to metal metabolism is not obvious. Alignment of UreH sequences and comparison with NiCoTs resulted in the topological model illustrated in Figure 1. UreH proteins are predicted to contain six TMDs with the amino- and carboxyl-termini oriented to the exterior of the cells. If this prediction is correct, questions of how these proteins are properly inserted into the membrane need to be addressed, since none of the UreHs contains an N-terminal signal peptide. To clarify the role of UreH in metal metabolism we have cloned and expressed in *E. coli* the homolog

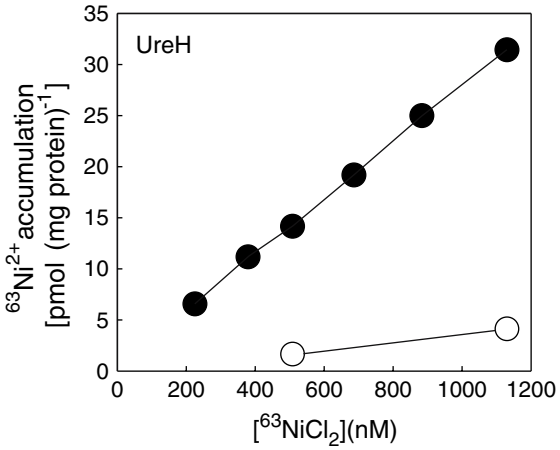

Figure 2. $^{63}Ni^{2+}$ accumulation of *E. coli* XL1-Blue expressing *ureH* from *R. palustris* CGA009 (●) or containing an empty vector (○). *ureH* was amplified by PCR and inserted into a derivative of plasmid pCH675AF (Degen & Eitinger 2002; Hebbeln & Eitinger 2004). Cells were grown in Luria-Bertani broth containing $^{63}NiCl_2$ as indicated. Radioactivity in washed cells was quantitated by liquid scintillation counting.

from *R. palustris* CGA009. The results of metal accumulation assays with the recombinant, shown in Figure 2, clearly indicate that UreH of *R. palustris* functions as a nickel permease. In additional experiments performed in the presence of non-labelled divalent metal ions as potential inhibitors of nickel transport, only cobalt had a detectable but minor effect (data not shown), suggesting that Ni^{2+} is the preferred substrate for *R. palustris* UreH.

Bacterial HupE and UreJ

HupE/UreJ proteins are widespread among bacteria and encoded within certain [NiFe] hydrogenase and urease gene clusters, as originally reported by Hidalgo *et al.* (1992) and Baginsky *et al.* (2004) for hydrogenase operons and by McMillan *et al.* (1998) for the urease operon of *Bordetella bronchiseptica*. *hupE* genes linked to hydrogenase genes are found in *Aquifex aeolicus*, *Azorhizobium caulinodans*, *Cupriavidus metalliduran*s, *Methylobium petroleophilum* (formerly *Rubrivivax gelatinosus*), *Methylococcus capsulatus*, *Rhizobium leguminosarum*, *Rhodobacter sphaeroides*, *R. palustris*, and *Thiobacillus denitrificans*. Linkage of *ureJ* to urease genes was detected in many organisms including *Acinetobacter* sp., *Bordetella bronchiseptica*, *B. parapertussis*, *B. pertussis*, *C. metallidurans*, *C. necator* H16, *Deinococcus radiodurans*, *Mesorhizobium loti*, *Mesorhizobium* sp. BNC1, *Methylobacillus flagellatus*, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, *P. syringae*, and *Ralstonia solanacearum*. Based on the genomic localization of these genes, a potential function of the proteins in nickel uptake has been proposed (McMillan *et al.* 1998; Baginsky *et al.* 2004). A function in cobalt transport has been ascribed to the HupE/UreJ homologs in cyanobacteria. In these organisms, the transporter genes are not linked to nickel enzymes. Rather, they are under control of a coenzyme B_{12}-dependent riboswitch element and thus are linked to cobalt metabolism (Rodionov *et al.* 2003). The topological model for HupE/UreJ shown in Figure 1 indicates an N-terminal signal peptide with a conserved cleavage site which was detected by SIGNALP 3.0 (Bendtsen *et al.* 2004) in a great number of HupE/UreJ proteins. Similar results have recently been reported for certain HupE proteins encoded in hydrogenase gene clusters (Baginsky *et al.* 2004). Mature HupE/UreJ proteins are predicted to fold into six TMDs. TMD I contains the signature sequence with two His residues. The distance between these His residues is six amino acid residues compared to five as found in NiCoTs and UreHs.

We have experimentally analyzed HupE from *R. palustris* CGA009 and UreJ from *C. necator* H16, which are encoded in [NiFe] hydrogenase and urease operons, respectively. The two genes were amplified by PCR and inserted into an expression plasmid. As illustrated in Figure 3, expression of both *hupE* and *ureJ* conferred nickel transport activity on *E. coli* cells. As in the case of UreH (see above), among a series of divalent cations tested only cobalt had a minor inhibitory effect on HupE- and UreJ-mediated nickel transport (data not shown). Deletion mutants of *R. palustris* and *C. necator* lacking *hupE* and *ureJ* were constructed. Even when surplus nickel was present in the growth medium, the HupE-negative mutant of *R. palustris* was strongly impaired in hydrogenase activity but had normal urease activity. The *ureJ* mutation in *C. necator* greatly diminished urease activity under both nickel limitation and nickel excess (data not shown). These results lead us to speculate that HupE- and UreJ-mediated nickel transport across the membrane may be specifically

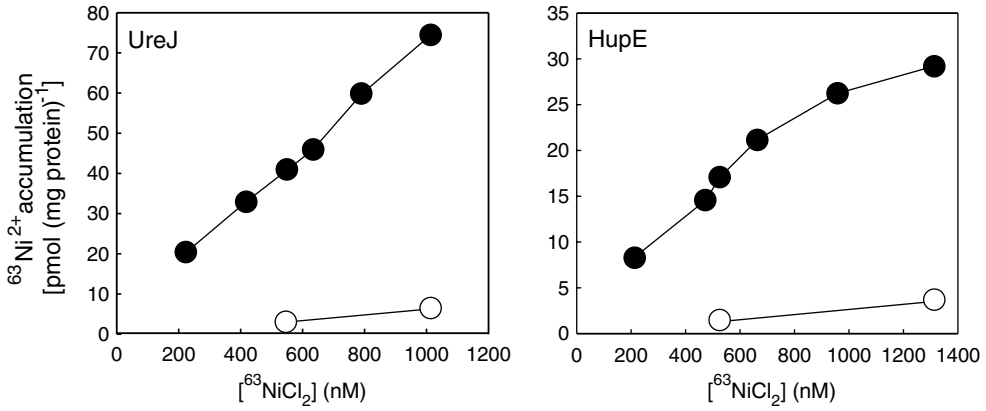

Figure 3. $^{63}Ni^{2+}$ accumulation of *E. coli* XL1-Blue expressing *hupE* from *R. palustris* CGA009 or *ureJ* from *C. necator* H16 (●) or containing an empty vector (○). The two genes were amplified by PCR and inserted into a derivative of plasmid pCH675AF (Degen & Eitinger 2002; Hebbeln & Eitinger 2004). Cells were grown in Luria-Bertani broth containing $^{63}NiCl_2$ as indicated. Radioactivity in washed cells was quantitated by liquid scintillation counting.

linked to intracellular nickel incorporation into the corresponding metalloenzymes.

Variants in marine cyanobacteria and plastids

Membrane proteins (named SodT) with a predicted function in nickel transport are encoded in the genomes of many marine cyanobacteria. In *Prochlorococcus marinus* strains MED4, MIT9313 (Rocap *et al.* 2003) and SS120 (Dufresne *et al.* 2003), in *Synechococcus* strain WH8102 (Palenik *et al.* 2003), and in several uncultured organisms analyzed by environmental shotgun sequencing (Venter *et al.* 2004), SodT is encoded by a segment adjacent to *sodN* and *sodX*, coding for [Ni] superoxide dismutase (SOD) and a putative maturation peptidase, respectively, as depicted in Figure 4. Ni-dependent SOD activity has recently been detected in *E. coli* cells expressing *sodN* and *sodX* from *P. marinus* MIT9313 (Eitinger 2004). SodT proteins are clearly related to UreH (Figure 1). They have a predicted six-TMD structure and contain a number of His residues in a hydrophilic loop between TMDs III and IV. Although genomic localization and similarity to UreH and other nickel transporters argue for a role in nickel uptake, experimental evidence for this function is still missing. We attempted to produce and analyze epitope-tagged SodT proteins from *P. marinus* MED4, MIT9313 and SS120 in *E. coli*. Unfortunately, only trace amounts of the proteins were detectable and the patterns observed in immunoblots indicated degradation. Consistent with this result, expression of *sodT* did not enhance nickel uptake of *E. coli* cells (J. Suhr & T. Eitinger, unpublished result).

Close relatives of SodT are encoded in plants, including the model plant *Arabidopsis thaliana* and, based on genomic sequences and expressed sequence tags collected in the TIGR gene indices database, many crop plants (e.g. barley, cotton, grape, lettuce, potato, rice, soybean, tomato and wheat). Computer analyses with the programs TARGETP 1.01 (Emanuelsson *et al.* 2000) and CHLOROP 1.1 (Emanuelsson *et al.* 1999) predicted for most of these proteins that they are localized in plastids. As illustrated in Figure 1, they have an N-terminal bipartite transit peptide reminiscent of thylakoid lumenal and thylakoid integral membrane precursor proteins (Schleiff & Klösgen 2001; Tissier *et al.* 2002; Gómez *et al.* 2003). The *A. thaliana* genome contains two loci (At2g16800 and At4g35080) encoding SodT-related proteins. The cellular location of the protein encoded by At2g16800 was analyzed experimentally and the results are shown in Figure 5. Translational fusions with a modified yellow fluorescent protein at the C-terminus clearly localize to the chloroplast in transfected leaf cells, although the membrane localization of the protein within the organelle has not so far been investigated. Thus far, attempts to produce these proteins or N-terminally truncated variants in *E. coli* or

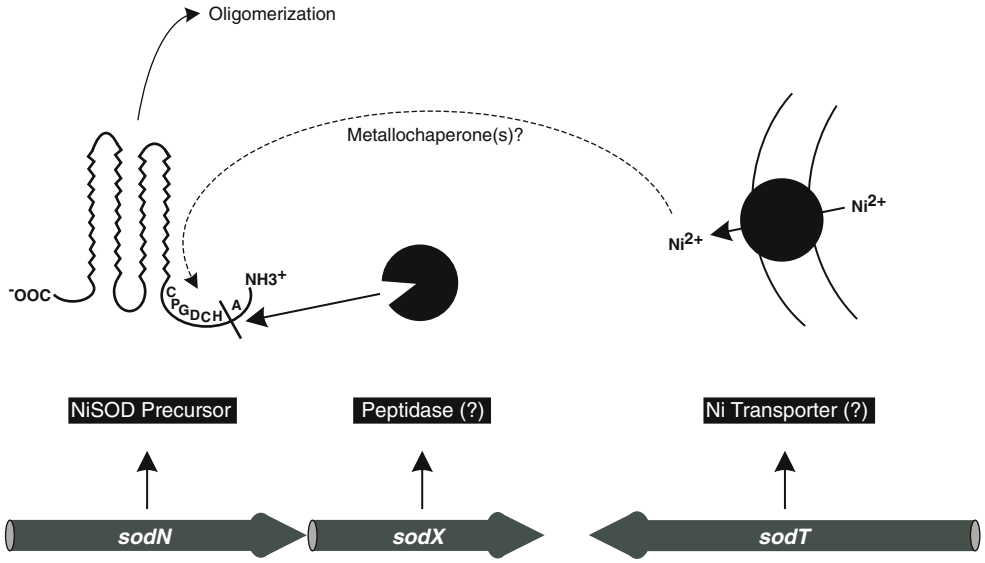

Figure 4. Genetic localization and putative role of SodT and SodX in maturation of [Ni] superoxide dismutase in many marine cyanobacteria. *sodN* encodes the NiSOD precursor which undergoes N-terminal proteolysis, catalyzed by SodX, to release the nickel-binding amino group of His-1. SodT presumably acts as a Ni^{2+} transporter in the cytoplasmic membrane. Intracellular nickel trafficking and incorporation into the NiSOD subunits has not yet been analyzed. By analogy to the hexameric *Streptomyces* enzymes (Barondeau *et al*. 2004; Wuerges *et al*. 2004), it is likely that marine cyanobacterial NiSOD has an oligomeric structure.

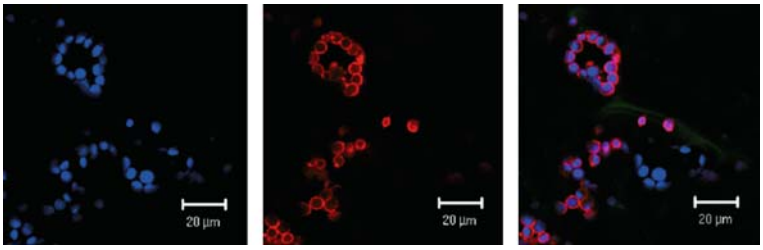

Figure 5. Localization of plant SodT-like protein in plastids. The coding region of *A. thaliana* sequence At2g16800 was cloned into an expression vector upstream of an open reading frame for a modified yellow fluorescent protein (YFP: Nagai *et al*. 2002). This construct was transformed into *Agrobacterium tumefaciens* GV3101 and a cell suspension infiltrated into the intercellular space of leaves of tobacco plants (*Nicotiana tabacum*) as described by Batoko *et al*. (2000). Expression of the fusion protein was examined by confocal microscopy after 3 days. Left-hand panel: chlorophyll fluorescence (blue); middle panel: YFP fluorescence (red); right-hand panel: merged image. YFP fluorescence is restricted to the chloroplasts of individual cells, as not all cells in the leaf were transfected with *A. tumefaciens*.

fission yeast yielded neither stable proteins nor enhanced nickel uptake in the recombinants (J. Suhr & T. Eitinger, unpublished results). At the current time, it is not yet possible to assign a function to these proteins in plastid physiology in plants.

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