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Homologous mutations in two diverse sulphate transporters have similar effects

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Abstract Mutations in the human sulphate transporter gene, *DTDST*, have been implicated in several diseases. Analysis of affected patients has linked disease symptoms to faulty sulphate transporter activity. We have reproduced two of these mutations in SHST1, a homologous member of the family isolated from the tropical legume, *Stylosanthes hamata*. Both mutations significantly reduce sulphate transport activity of SHST1. These results indicate that conserved residues between distinct members of the family may share essential roles in structure or function. The results also suggest that putative helix 9 may be important for stability and/or trafficking of SHST1 to the plasma membrane.

Key words: Sulfate transporter; Site-directed mutagenesis; Diastrophic dysplasia; Transmembrane helix

1. Introduction

High affinity sulphate transporters are members of an expanding family of anion transporters [1] and have been isolated from many different organisms, including plants, fungi and mammals. Although most of the characterised members have been shown to transport sulphate (for example, those from plants [2], yeast [3] and rat [4]), homologues with other specificities have recently been found. Three members of this family have been isolated from humans and are of considerable interest for two reasons. Firstly, all three have been implicated in different diseases and there are now a number of mutations described from affected patients. Secondly, some of the human members transport anions other than sulphate and show different modes of energisation. For example, pendrin is a chloride/iodide transporter and mutations in this gene cause a form of congenital deafness [5]. Similarly, DRA appears to be a chloride/bicarbonate exchanger and has been implicated in congenital chloride diarrhoea [6,7].

Mutations in the human *DTDST* gene, which encodes a sulphate transporter, cause a series of chondrodysplasias of differing severity [8,9]. Diastrophic dysplasia was the first characterised [8], and appears to be due to extremely low expression of the *DTDST* gene [10]. Patients live a normal life span but show severe skeletal deformities which are thought to result from undersulphation of proteoglycans [8]. Following isolation of the *DTDST* gene, patients with related but more severe chondrodysplasias such as atelosteogenesis type II and achondrogenesis type 1B were also examined for mutations in the same gene. Several such mutations have now been identified. Biochemical analysis of chondrocytes and fibroblasts from patients indicated that there is very little sulphate transport activity in most mutants so far analysed [9–12]. In their most severe forms, chondrodysplasias are fatal and many of the mutations responsible for the lethal variants are frameshifts or altered splice sites, consistent with a complete absence of sulphate transport due to the DTDST protein. However, there are also some point mutations that show the severe phenotype. Naturally occurring point mutations with deleterious effects can sometimes give useful information for protein structure and function studies. We were interested to test whether homologous mutations would have similar effects in another member of the sulphate transporter family. Our aim in the present work was to characterise the effects of some of these mutations by using expression of a homologous sulphate transporter from the tropical legume *Stylosanthes hamata* in yeast as a model system. Expression in yeast is an attractive system for structure–function analysis since yeast is relatively simple to grow and use for transport studies.

S. hamata possesses three homologous sulphate transporters, two of which have a K_m for sulphate of around 10 μM and the third with a 10-fold lower affinity [2]. Functional expression of these transporters in yeast was used to isolate their cDNAs and to characterise the biochemical properties of the transporters [2]. A comparison of the protein sequences of DTDST and SHST1, one of the high affinity sulphate transporters from *S. hamata*, indicates that several of the disease-causing mutations occur in conserved amino acids. Although both proteins transport sulphate, they are likely to be energised differently. DTDST is thought to be a sulphate/chloride antiporter [8] whereas SHST1 is a proton-coupled symporter [2]. We aimed to gain information on structure and function of this family of transporters by reproducing some of the disease-causing mutations in SHST1 and analysing the properties of the mutant transporters.

2. Materials and methods

2.1. Molecular biology

Standard procedures were used for bacterial plasmid isolation and transformation into *Escherichia coli*. SHST1 in the yeast expression vector pYES3 [3] was used for site-directed mutagenesis, using the Quikchange method from Stratagene. Mutagenic oligonucleotides were 25–30 bases long and included the codon changes shown in Table 1. The entire *shst1* cDNA was sequenced following mutagenesis to confirm that no PCR-derived errors had arisen.

2.2. Yeast growth and transformation

Saccharomyces cerevisiae strain YSD1, which has a deletion in the native sulphate transporter gene [3], was used for expression of

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SHST1 and the mutants derived from it. Sulphate-free growth medium was prepared as previously described [3] and supplemented with either 38.26 mg/l homocysteine thiolactone or 100 μM sulphate. For the growth experiments, yeast cultures were grown at 28°C, with shaking. Optical density was measured using a Klett–Summerson calorimeter. Yeast transformation was by a LiCl/PEG method [13].

2.3. Sulphate uptake assays

Sulphate uptake assays were based on those described by Smith et al. [2,3]. Yeast cells were grown to mid-log phase in sulphate-free medium minus uracil and supplemented with homocysteine thiolactone and 2% galactose. Cells were then harvested by centrifugation at $5000 \times g$ for 15 min, washed and resuspended in sulphur-free growth medium, and incubated at 28°C for 30 min prior to the uptake measurements. Cells (50 μl) were then incubated with [^{35}S]sodium sulphate for the times indicated and the reaction stopped by rapid centrifugation through a silicon oil layer into 5 μl 40% perchloric acid. The [^{35}S]sulphate concentration was 50 μM unless otherwise indicated. Radioactivity in the pellet was determined by liquid scintillation counting.

2.4. Western blotting of plasma membrane fractions

Yeast cells for plasma membrane preparation were grown to mid-log phase in sulphate-free medium minus uracil and supplemented with homocysteine thiolactone and 2% galactose. Cells were lysed and plasma membranes isolated on a sucrose density gradient following the procedure of Katzmann et al. [14]. The level of protein in cell lysates from each mutant was determined using the BCA™ Protein Assay Reagent (Pierce) and the concentration adjusted so that a similar level of protein was loaded onto each sucrose gradient. Plasma membrane fractions were harvested and protein was precipitated with trichloroacetic acid as described by Katzmann et al. [14]. Protein was then resuspended in sample buffer (6.25 mM Tris–HCl pH 6.8, 5% SDS, 6 M urea, 500 mM dithiothreitol, 10% glycerol and 0.002% bromophenol blue) and heated at 70°C for 25 min prior to loading onto a gel. Proteins were resolved by SDS–PAGE and blotted onto nitrocellulose using a semidry transfer protocol [15]. Rabbits were used to raise polyclonal antisera against a 24 kDa C-terminal fragment of SHST1 following standard procedures [16]. Immunoreactive SHST1 was detected with a horseradish peroxidase-conjugated goat anti-rabbit IgG (ICN) and enhanced chemiluminescence (Pierce, SuperSignal™ Substrate).

3. Results

3.1. Sequence alignment and construction of mutants

An alignment of SHST1 with DTDST is shown in Fig. 1. The primary sequence shows only 24% identity. More extensive sequence analysis has identified several motifs that are conserved throughout the entire family [1]. Despite the low level of sequence homology, the hydropathy plots are clearly similar and are consistent with the presence of 12 transmembrane helices (not shown). This suggests that the overall structure and perhaps also the sulphate transport pathway are conserved, despite differences in energisation. Several mutations responsible for chondrodysplasias affect conserved residues and two such mutations were chosen to study: N425D in putative helix 9 and L483P in putative helix 11 (Fig. 1). Both mutations are non-conservative changes, introducing a negative charge or a helix-breaking residue into putative transmembrane regions. Both are also implicated in the most severe disease, achondrogenesis type 1B, which is thought to result from a complete loss of sulphate transport [10,11]. N425 is in putative transmembrane helix 9 which forms part of a conserved region that has been identified as a signature sequence of this transporter family (see Fig. 1B) [1]. An asparagine residue is present at this position in sulphate transporters from all species so far examined with the exception of *Caenorhabditis elegans* [1]. The only completely invariant residue in this region is E418 [1]. This residue is predicted to be located at the N-terminal end of putative helix 9. Because of its high conservation, this residue is potentially important for sulphate transporter function.

Table 1
Mutations introduced into SHST1 and the resulting mutant strains

Amino acid change	Codon change	Yeast strain
E387Q	G(1219)AA→CAG	YSD24
N395D	A(1243)AC→GAC	YSD23
N395A	A(1243)AC→GCT	YSD19
L453P	C(1417)TT→CCA	YSD22

The equivalent residues in SHST1 are N395, L453 and E387. Site-directed mutagenesis was used to introduce the four mutations shown in Table 1. The resulting plasmids carried the mutations: N395D, N395A, L453P and E387Q. The N395A mutant was constructed in order to compare the effects of introducing a charged or a non-polar residue into this position. The mutant plasmids were then transformed into the sulphate uptake-deficient yeast strain YSD1 [3] to give strains YSD23(N395D), YSD19(N395A), YSD22(L453P) and YSD-24 (E387Q).

3.2. The effects of mutations in SHST1

The mutant plasmids were tested for their ability to complement the yeast strain YSD1 on plates containing 100 μM sulphate with galactose as the carbon source. The N395D mutation completely abolished complementation while the mutations L453P, N395A and E387Q did allow complementation of YSD1 (Table 2). The growth rates in liquid medium with 100 μM sulphate were also examined (Fig. 2). YSD22(L453P) grew at a rate comparable to the wild type, YSD7(SHST1), although the final cell density was slightly less. YSD24(E387) took longer to enter exponential phase than YSD7(SHST1) but then grew at a similar rate. YSD19-(N395A) grew quite slowly in liquid medium.

Sulphate uptake by each of the mutant strains was determined and the results are shown in Table 2. YSD23(N395D) did not have any measurable sulphate uptake activity, consistent with its failure to grow on low sulphate medium. YSD24(E387Q), YSD19(N395A) and YSD22(L453P) had low but significant levels of sulphate transport. Although YSD19-(N395A) and YSD24(E387Q) retained only about 15% of the wild type level of activity, this was sufficient for complementation of YSD1. The concentration dependence of uptake was determined for the three mutants that did retain some function. The wild type SHST1 had a K_m of 9 μM, which agrees well with the previously published value of 10 μM [2]. Each of the three mutations, E387Q, N395A and L453P, resulted in a slightly lower affinity for sulphate (Table 2). Therefore the mutations at these positions affect the level of transport activity much more than the affinity of the transporter for sulphate.

3.3. Determination of the level of expression of SHST1 mutant proteins in the plasma membrane

The low level of sulphate transport activity observed in all mutants could result from the loss of function of SHST1 or from failure of SHST1 to be correctly trafficked to the plasma membrane. To distinguish between these two alternatives, plasma membranes from each of the mutant strains were iso-

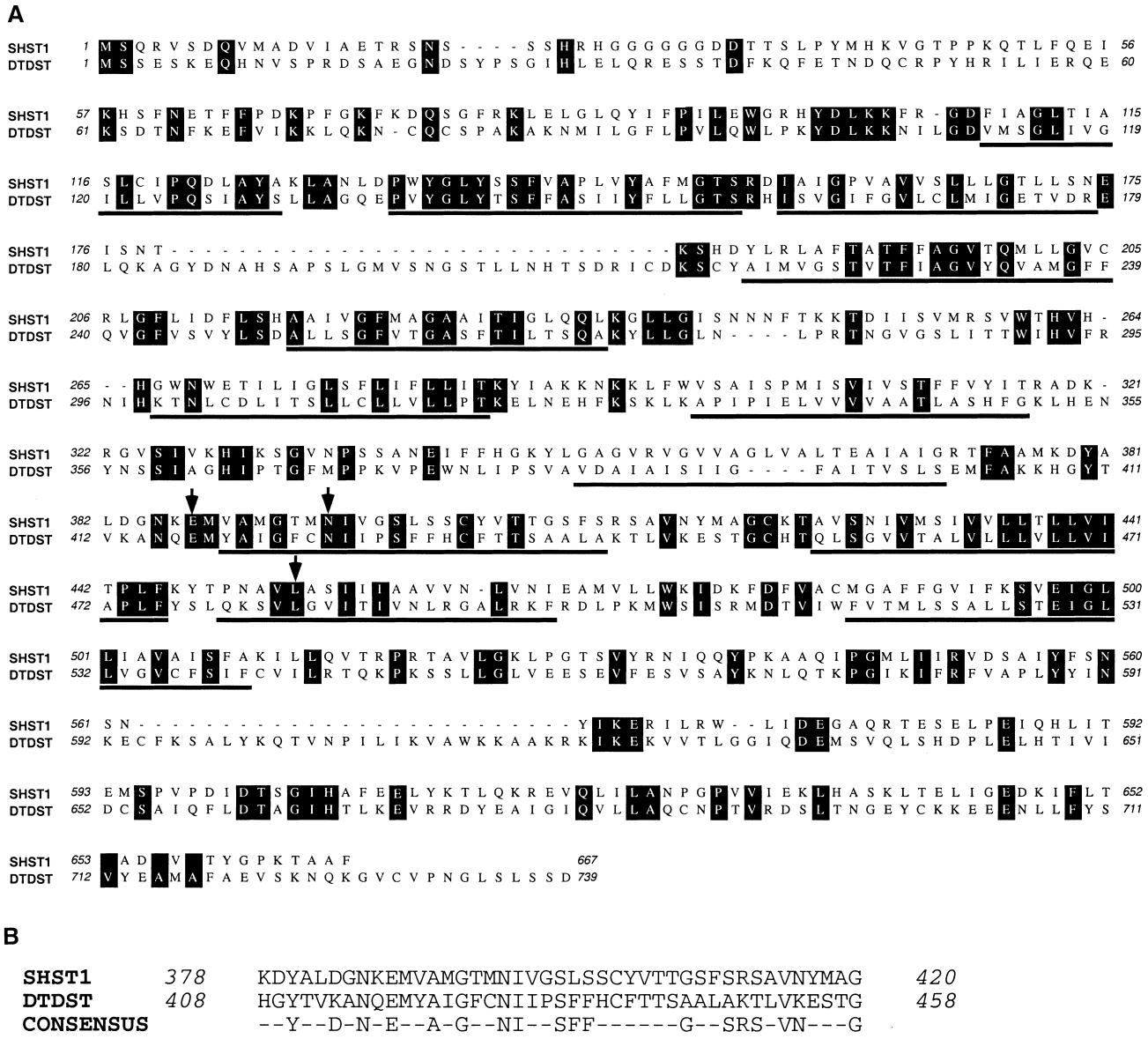

Fig. 1. A: Alignment of SHST1 and DTDST. Alignment was performed using ClustalW. Identical residues are shaded and those mutated in this study are indicated by arrows. Putative transmembrane helices are indicated by bars below the sequence. B: The conserved motif around putative helix 9 identified by Saier et al. [1].

lated and used for Western blotting as described in Section 2. Fig. 3 shows the results of this experiment. SHST1 is predicted to have a molecular weight of 73 kDa but was consistently observed to run at 140 kDa, although a number of different sample preparation conditions were tested. SHST1 appears not to fully denature under the running conditions and therefore may run as a dimer or run aberrantly as a result of its hydrophobicity. Secondary transporters are usually thought to function as monomers, although this has not been investigated for SHST1. None of the mutations completely abolished plasma membrane expression of SHST1 but only the L453P mutant had a similar level of expression to the wild type. SHST1 with the L453P mutation ran even higher than the wild type protein and the band was quite smeared, presumably because of conformational differences. Expression of the E387Q mutant was very low and, of the two changes made at position 395, the N395D mutant had a lower level of SHST1 protein in the plasma membrane than the N395A mutant.

4. Discussion

We have characterised four mutations of SHST1, all of which significantly affect sulphate uptake activity. Two of the mutations were designed to mimic changes in the *DTDST* gene which have been shown to cause achondrogenesis type 1B [9,11,12]. It has been suggested that this phenotype results from a complete loss of DTDST function [9]. Both mutations are non-conservative changes so severe effects were to be expected. Surprisingly, the two mutations had very different effects in SHST1. While YSD22(L453P) did retain some sulphate transport activity, the N395D mutation completely abolished function.

L453 is predicted to occur within putative transmembrane

Table 2
Properties of SHST1 mutants

Mutant strain	Complementation[a]	K_m (μM)[b]	V^b_{max} (nmol/min/mg protein)
YSD7(SHST1)	+++	9 ± 2	3.1 ± 0.3
YSD6 (pYES)	−	–	< 0.2
YSD24(E387Q)	+++	13 ± 4	0.5 ± 0.1
YSD23 (N395D)	−	–	< 0.2
YSD19(N395A)	++	14 ± 5	0.4 ± 0.2
YSD22(L453P)	+++	14 ± 2	0.9 ± 0.2

The values shown are the mean ± S.D. from at least three independent experiments.
[a]Complementation of YSD1 by each mutant plasmid as indicated by growth on minimal galactose plates supplemented with 100 μM sulphate. +++, wild type growth; ++, moderate growth; +, poor growth; −, no growth.
[b]Cells were incubated for 2 min in the presence of a range of concentrations of [^{35}S]sulphate. The Michaelis–Menten equation was fitted to the data.

helix 11 (Fig. 1) although there is at present no information on the topology of any member of this anion transporter family. Leucine is a hydrophobic residue which is a good helix former. It was replaced by proline which is less hydrophobic and a helix breaker. The L453P mutation substantially reduced the level of sulphate uptake activity of SHST1 but had only a minor effect on the affinity for sulphate. The L453P mutant protein was targeted to the plasma membrane as well as the wild type SHST1 which suggests that no gross structural defect had occurred as a result of this mutation. Disruption to the helicity of this region within the membrane, which may alter the positions of crucial amino acid residues relative to each other, could account for the reduction in sulphate transport activity. Information on the topology of SHST1 is required to confirm this interpretation.

The homologous mutation in the *DTDST* gene, L483P, appears to completely abolish sulphate transport in chondrocytes and fibroblasts of patients [10,11]. This has been determined from uptake assays and is suggested by the fact that patients homozygous for L483P have achondrogenesis type 1B, the most severe chondrodysplasia. It is, therefore, somewhat surprising that the equivalent mutation in SHST1 retained about one third of the wild type activity. This could be a result of high expression levels from the GAL promoter in the yeast expression system. As mentioned above, we observed that two mutations were able to complement YSD1 although their sulphate uptake activity was only about 15% of the wild type. This suggests that the expression level is very high. The lower expression level of DTDST in chondrocytes may be insufficient to give measurable sulphate transport activity. Alternatively, the different phenotypes may reflect functional differences between DTDST and SHST1 since their primary sequence is only 24% identical and the two transporters are energised differently.

In contrast, the N395D mutation did completely abolish sulphate transport activity and in this respect behaved similarly to its *DTDST* counterpart which is also responsible for achondrogenesis type 1B. A severe effect is also to be expected with the introduction of a charged residue into a putative transmembrane helix (helix 9). Expression of the protein in the plasma membrane was severely reduced but not completely abolished, suggesting that the N395D mutation affects both targeting and function of SHST1. Asparagine is a polar residue so we were interested in whether its polarity was important for function of SHST1, and potentially for all members of the family given its high level of conservation. To investigate this, we introduced a non-polar amino acid, alanine, into position 395. The N395A mutation had less severe effects than the N395D mutation but still resulted in significant impairment of transporter function. If N395 did have a direct role in transport due to its hydrogen bonding capacity we would expect the N395A change to result in a complete loss of function since alanine is unable to form hydrogen

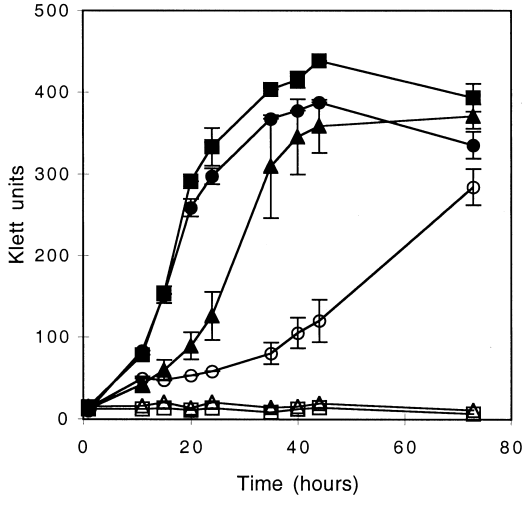

Fig. 2. Growth curves of mutant and control strains on minimal galactose medium with 100 μM sulphate as the sole source of sulphur. Each curve is the mean of three experiments, with standard error of the mean indicated. Error bars are shown only if they are larger than the symbol. YSD7(SHST1), ■; YSD6(pYES), △; YSD24(E387Q), ▲; YSD19(N395A), ○; YSD23(N395D), □; YSD22(L453P), ●.

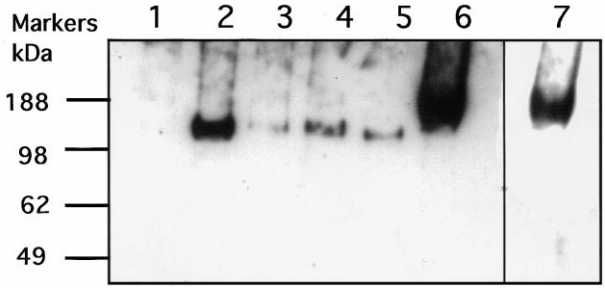

Fig. 3. Western blot of plasma membrane protein from mutant yeast strains. Cells were grown and plasma membranes prepared as described in Section 2. Plasma membrane proteins were separated by SDS–PAGE, transferred to nitrocellulose and probed with polyclonal antisera to SHST1. Lane 1, YSD6(pYES); lane 2, YSD7(SHST1); lane 3, YSD24(E387Q); lane 4, YSD19(N395A); lane 5, YSD23(N395D); lane 6, YSD22(L453P). Lane 7 is the same as lane 6 but is a shorter exposure of the blot to X-ray film.

bonds. The retention of some activity rules out a direct mechanistic role for N395. Both sulphate transport and the level of expression in the plasma membrane were very low for this mutant. The major effect of this mutation is therefore on stability and/or trafficking of the transporter. However, it is possible that activity of the SHST1 in the plasma membrane was also affected by the mutation. N395 is likely to be involved in interactions with other residues which contribute to the stability and/or folding of the transporter. Helix 9 is somewhat atypical in that it contains a number of other polar residues as well as three glycine residues. This region may therefore be quite flexible, and also has the potential for hydrogen bonding. It has recently been shown that glycine residues within a transmembrane helix of the lactose permease from *E. coli* are essential for function [17].

Despite the conservation of E387, it is apparently not necessary for sulphate transport. Nevertheless, YSD24(E387Q) had substantially reduced sulphate uptake activity and very low expression of the mutant transporter in the plasma membrane. This mutation, like N395A, appears to primarily affect normal trafficking of SHST1 to the plasma membrane although a direct effect on sulphate uptake activity as well cannot be excluded. E387 is located at the N-terminal end of putative transmembrane helix 9. The similar phenotype observed for the E387Q and N395A mutants suggests that interactions of polar residues in this helix are important for folding and/or stability of SHST1.

In conclusion, we have shown that two mutations of conserved amino acids which affect the human sulphate/chloride exchanger encoded by the *DTDST* gene also affect the function of the plant sulphate/proton symporter, SHST1. Further, we have found that mutations in putative helix 9 primarily affect stability and/or trafficking of SHST1. We are continuing to use site-directed mutagenesis to elucidate the role of this helix in the mechanism of sulphate transport.

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References

[1] Saier Jr., M.H., Eng, B.H., Fard, S., Garg, J., Haggerty, D.A., Hutchinson, W.J., Jack, D.L., Lai, E.C., Liu, H.J., Nusinew, D.P., Omar, A.M., Pao, S.S., Paulsen, I.T., Quan, J.A., Sliwinski, M., Tseng, T.T., Wachi, S. and Young, G.B. (1999) Biochim. Biophys. Acta 1422, 1–56.
[2] Smith, F.W., Ealing, P.M., Hawkesford, M.J. and Clarkson, D.T. (1995) Proc. Natl. Acad. Sci. USA 92, 9373–9377.
[3] Smith, F.W., Hawkesford, M.J., Prosser, I.M. and Clarkson, D.T. (1995) Mol. Gen. Genet. 247, 709–715.
[4] Bissig, M., Hagenbuch, B., Stieger, B., Koller, T. and Meier, P.J. (1994) J. Biol. Chem. 269, 3017–3021.
[5] Scott, D.A., Wang, R., Kreman, T.M., Sheffield, V.C. and Karnishki, L.P. (1999) Nature Genet. 21, 440–443.
[6] Moseley, R.H., Hoglund, P., Wu, G.D., Silberg, D.G., Haila, S., de la Chapelle, A., Holmberg, C. and Kere, J. (1999) Am. J. Physiol. 276, G185–192.
[7] Melvin, J.E., Park, K., Richardson, L., Schultheis, P.J. and Shull, G.E. (1999) J. Biol. Chem. 274, 22855–22861.
[8] Hastbacka, J., de la Chapelle, A., Mahtani, M.M., Clines, G., Reeve-Daly, M.P., Daly, M., Hamilton, B.A., Kusumi, K., Trivedi, B. and Weaver, A. (1994) Cell 78, 1073–1087.
[9] Hastbacka, J., Superti-Furga, A., Wilcox, W.R., Rimoin, D.L., Cohn, D.H. and Lander, E.S. (1996) Am. J. Med. Genet. 58, 255–262.
[10] Hastbacka, J., Kerrebrock, A., Mokkala, K., Clines, G., Lovett, M., Kaitila, I., de la Chapelle, A. and Lander, E.S. (1999) Eur. J. Hum. Genet. 7, 664–670.
[11] Rossi, A., Bonaventure, J., Delezoide, A.L., Cetta, G. and Superti-Furga, A. (1996) J. Biol. Chem. 271, 18456–18464.
[12] Superti-Furga, A., Hastbacka, J., Wilcox, W.R., Cohn, D.H., van der Harten, H.J., Rossi, A., Blau, N., Rimoin, D.L., Steinmann, B., Lander, E.S. and Gitzelmann, R. (1996) Nature Genet. 12, 100–102.
[13] Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. (1995) Yeast 11, 355–360.
[14] Katzmann, D.J., Epping, E.A. and Moye-Rowley, W.S. (1999) Mol. Cell. Biol. 19, 2998–3009.
[15] Towbin, H.T., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4554.
[16] Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold spring Harbor, NY.
[17] Kaback, H.R. (1997) Proc. Natl. Acad. Sci. USA 94, 5539–5543.