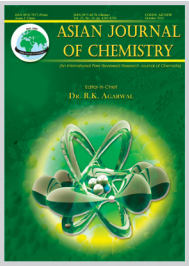
Asian Journal of Chemistry; Vol. 24, No. 6 (2012), 2394-2400

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in



Fusion of MxIRT1 Vesicles and Plasma Membrane is a Key Regulation Step of High Affinity Iron Transport in Response to Iron Supplement in Transgenic Yeast

LI-YONG WANG, BING FENG, PENG LI, GUANG YANG and LI-PING YIN*

College of Life Science, Capital Normal University, Beijing Xisanhua Beilu No. 105, Beijing, 100048, P.R. China

*Corresponding author: Fax: +86 10 68981191; Tel: +86 10 68901692; E-mail: yinlp@mail.cnu.edu.cn

(*Received*: 23 March 2011; *Accepted*: 4 January 2012) AJC-10912

In previous study, it was found that heterologous expression of MxIRT1, one of iron-regulated transporters, sorted from endoplasmic reticulum (ER) to plasma membrane (PM) *via* vesicles trafficking in transgenic *Saccharomyces cerevisiae*. The sorting process was affected by surrounding iron existence. To clarify the detailed molecular mechanism in cells underlying vesicles trafficking responses to iron supplement, gene expression patterns and genetic networks in MxIRT1-transformed DEY1453 (fet3fet4) mutant strain were examined using global-scale microarrays and computational gene expressional analysis tools. The results showed that MxIRT1 transport may be involved in oxygen-dependent reactions, stress signal and membrane vesicles transport *etc*. Based on microarray data, the analyses focused on iron high affinity pathway including vesicle trafficking and membrane fusion. It was remarkably revealed that the expression of MxIRT1 made iron transport system work and most of MxIRT1-vesicles were transmitted toward plasma membrane and docked inside of plasma membrane but without fusion in the absence of iron. However, as supplying iron to the environment, the fusion of MxIRT1-vesicle with plasma membrane was promoted, which was just consistent with results from laser scanning confocal microscope observation. Therefore, it is concluded that MxIRT1 might function in place of FET3/FTR1 complex in yeast, and then induce the iron high affinity pathway from ER to plasma membrane *via* vesicles trafficking in lack of iron. Furthermore, it is newly discovered that the fusion between MxIRT1-vesicles and plasma membrane might play a key step response to iron.

Key Words: MxIRT1, Iron uptake, Microarray, Fusion, Green fluorescent protein.

INTRODUCTION

The genome of *S. cerevisiae* is divided up into 16 chromosomes ranging in size between 250 kb and 2500 kb. The complete genome sequence now defines about 6000 open reading frames (ORFs). A protein-encoding gene is found every 2 kb in the yeast genome, with nearly 70 % of the total sequence being covered[1]. Besides the protein-encoding genes, the yeast genome contains some 120 ribosomal RNA genes in a large tandem array on chromosome XII, 40 genes encoding small nuclear RNAs (sRNAs), 274 tRNA genes (belonging to 42 families), which are scattered throughout the genome, and some 50 copies of the yeast retrotransposons.

Iron deficiency is one of the most common human nutritional disorders in the world today. Indeed, iron is an essential nutrient for virtually all organisms because it plays a critical role in important biochemical processes such as respiration and photosynthesis. Although the fourth abundant in the soil micronutrients, iron is often available in limited amounts because the oxidized form Fe(III), is extremely insoluble at neutral or basic pH. This fact is of particular importance to agriculture because approximately one-third of the world's soils are classified as iron deficiency.

Up to now plants have evolved two effective iron acquisition systems known as strategy I and strategy II to cope with iron deficiency conditions for growth and development. Grasses, also called strategy II plants, respond to iron insufficiency by releasing Fe(III)-binding compounds called phytosiderophores (PS) into the surrounding soil that bind iron and then take up the Fe(III)-PS complex through a high-affinity transporter of Fe(III)-PS, such as Yellow Stripel 1[2]. Most other iron-efficient plants use strategy I and respond to iron deprivation by inducing the activity of membrane-bound Fe(III) chelate reductases that reduce Fe(III) to the more soluble Fe(II) form.

There is striking similarity between iron uptake in strategy I plants and the mechanism of iron uptake in *Saccharomyces cerevisiae*. In *S. cerevisiae*, Fe(III) reductases in the plasma membrane[3-5] reduce extracellular Fe(III) to Fe(II) and then the Fe(II) product is taken up by either of two uptake systems. With low affinity for substrate, one system requires the Fe(II) transporter encoded by the FET4 gene[3], the other has high

affinity for Fe(II), induced in iron deficiency. The high affinity system is encoded by FET3 and FTR1 genes[3].

The transition metals iron and copper are required for respiration. The first evidence of metabolic remodeling occurs when these essential transition metals become limiting. The high affinity elemental iron transport system is comprised of two cell surface proteins, FET3 protein a multicopper oxidase and FTR1 protein a transmembrane iron permease. FET3 protein, accepts copper from CCC2 and then assembles with FTR1 to complex, which transports ferrous iron across the plasma membrane. Most environmental iron is found as ferric iron, which is deoxidized by Fe^{3+}-chelate reductase. The substrate for FET3/FTR1 protein complex is ferrous iron.

MxIRT1 is highly homologous to other iron-regulated transporters belonging to the ZIP family and is induced by iron deficiency. In previous study, DEY1453 (fet3fet4) mutant is used to identify MxIRT1, Fe(II) transporter, which is sensitive to iron deficiency. Li *et al*.[6] isolated MxIRT1 from a library of roots of *Malus xiaojinensis* Cheng et Jiang under iron-deficient conditions, which was tested that *Malus xiaojinensis* Cheng et Jiang was an iron-efficient species in the genus Malus[7,8]. Moreover, it is found that molecular mechanism of MxIRT1 *via* membrane vesicles trafficking is involved in response to iron deprivation.

In our study, Affymetrix Yeast_2 Genechip is used, which contains approximately 10928 probe sets for 5,841 of the 5,845 genes present in yeast genome to study the growth of yeast under iron stress. The Affymetrix Microarray Suite Software (MAS version 5.0) was applied for analyzing the reliability of signals for each probe set[9]. In this study, microarray analysis was made for revealing through, which kind of pathway the heterologous MxIRT1 sorted with membrane vesicle trafficking and which process regulated the vesicles transport in response to iron ion signal in yeast cells.

EXPERIMENTAL

Yeast culture: The yeast strains used in this study were *Saccharomyces cerevisiae* strain DEY1453 (MATa/MATa ade2/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::HIS3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2), fet3fet4 double mutant, which was transformed with pDR196-MxIRT1 using the high efficient Li-acetate transformation method (Yeast transformation System, Colontech, USA). Yeast cells were grown in 1 % yeast extract, 2 % peptone supplemented with 2 % glucose (YPD)[6]. After pre-culture to 1 OD600, the cells were transferred to uptake medium (YPAD). Two treatments were used within 3 h: 20*f*ÊM BPDS for iron lack treatment (-Fe for short, *i.e*. there was just a few iron ions in the surrounding for BPDS was a chelator), 30 *f*ÊM Fe for iron supplement treatment (+Fe for short). To reduce experimental variation, we compared two transformants that are pDR196 empty vector and pDR196-MxIRT1 transform into DEY1453 mutant respectively..

RNA extraction and purification: Total RNA was extracted from cell using hot phenol method. The protocol was as follows: 1.5 mL cells were spun down for 1 min in eppendorf tube at 3000 rpm, and then supernatant was poured off. Cell pellet was resuspended in 0.4 mL of TE buffer (plus 50 µL 10 % SDS). 0.4 mL H_2O-saturated phenol (pH < 7.0) was added. The mixture was shaken with vortex for 30s and incubated at 65 ºC for 0.5 h. Following ice bath for 5 min, mixture was spun for 10 min, 4 ºC at > 10000 rpm. Removed aqueous layer into new tube, equal volume of chloroform was added, spinning for 10 min, at > 10000 rpm. Remove aqueous layer into new tube, add 1/10 vol. (50 µL) 3M sodium acetate and 2-2.5 vol. (1-1.5 ml) 100 % cold ethanol and spin at 10000 rpm for 10 min. Wash pellet with 70 % cold ethanol. Air dry, resuspended in RNAs-free H_2O.

To characterize the quality of the RNA sample, it was analyzed on spectrophotometer and then electrophoresis with a 1.2 % agarose, 2 % formaldehyde gel (120 mL gel = 1.44 g agarose, 6.96 mL formaldehyde, 12 mL 10xMOPS, 100 mL DEPC treated water; MOPS and formaldehyde. Samples contained 10 µg of total RNA in 20 µL RNA running buffer consisting of 50 % deionised formamide, 7 % formaldehyde, 0.5 mg/mL ethidium bromide, 1 mM EDTA, 20 % glycerol, 1xMOPS buffer, 0.25 % bromophenol blue.

Microarray analysis: The RNA samples were further purified using RNeasy mini plant kit (Qiagen) for microarray analysis. The RNA yield and quality were determined by spectrophotometry at 260 and 280 nm. The RNA integrity was checked on 1.5 % (w/v) agarose/formaldehyde gel. For microarray analysis, total RNAs were processed for use on Affymetrix yeast GeneChip arrays according to the manufacturer's protocol. In brief, 15 µg of total RNAs were used in a reverse transcription reaction to generate first-strand cDNAs, using the SuperScript III (invitrogen) with random primers. After second-strand synthesis, biotin-labeled target complementary RNAs (cRNAs) were prepared using the BioArray high-yield RNA transcript labeling kit in the presence of buotinlated UTP and CTP. After purification and fragmentation, 15 µg of cRNAs were used in a 300 µL hybridization mixture containing added hybridization controls. A total of 200 µL of the mixture was hybridized on arrays for 16 h at 45 ºC. Standard posthybridation wash and double-stain protocol were applied on an Affymetrix GeneChip fluidics station 450. Arrays were scanned on an Affymetrix GeneChip scanner 2500. The microarray results were analyzed by some open software and public database such as cluster, KEGG.

RESULTS AND DISCUSSION

Total RNAs integrity and purity: The high quality RNA was extracted from yeast. Based on data from spectrophotometer, the quantity of total RNA reached more than 120 µg every sample and an average A260/A280 ratio was between 1.9 and 2.0, which indicated that the integrity and purity was acceptable and RNA was available. 18S rRNA and 28S rRNA were seen distinctly on the gel of RNA formaldehyde denaturing gel electrophoresis (Fig. 1a), which indicated that RNA didn't degrade before cells captured. Purified RNA electrophoresis map and complementary RNA electrophoresis map were shown in Fig.1b and 1c respectively, which indicated that isolated total RNA was available for use.

Microarray analysis of gene expression in yeast after iron stress: Two independent microarray analyses were performed to obtain an overall picture of gene regulation. To

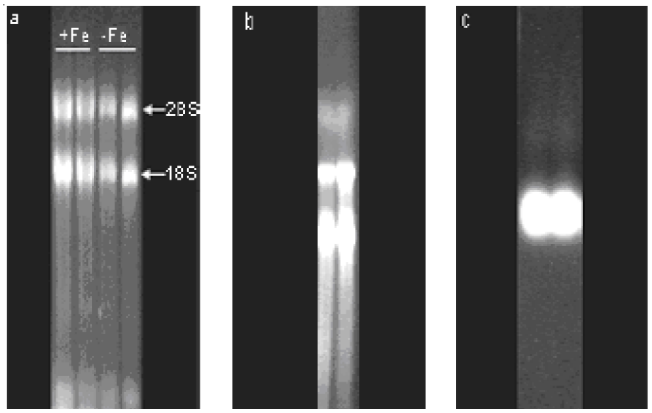


Fig. 1. Analysis of the products of RNA fractionised by electrophoresis in formaldehyde denaturing gel and results were visualized by autoradiography. +Fe represents cells in supplementary iron solution and -Fe in deficient iron solution. a: Total RNA electrophoresis map b: Purified RNA electrophoresis map c: Complementary RNA electrophoresis map

reduce experimental variation, RNA were derived from two independent cell culture. Then total RNAs were prepared from the replicates of the pooled samples.

In this study, about 53 % of 5,744 probe sets was present in microarray analyses, about 46 % was absent and less 1 % was marginal. Changes in RNA levels in response to iron stress treatment (*i.e.* induced or repressed) were assessed using Affymetrix GeneChip Operating Software (GCOS) as described. Different expression genes were identified by the following criterion: the average of fold changes in expression level between the two microarray analyses was greater than 2.0 or less than -2.0.

Using the *p*-value ($p < 0.05$) criteria and removing genes from the variation experiment, a total of 895 differentially expressed genes were identified, of these, 392 were up-regulated while 503 were down-regulated in -Fe compared with controls (30 μM +Fe).

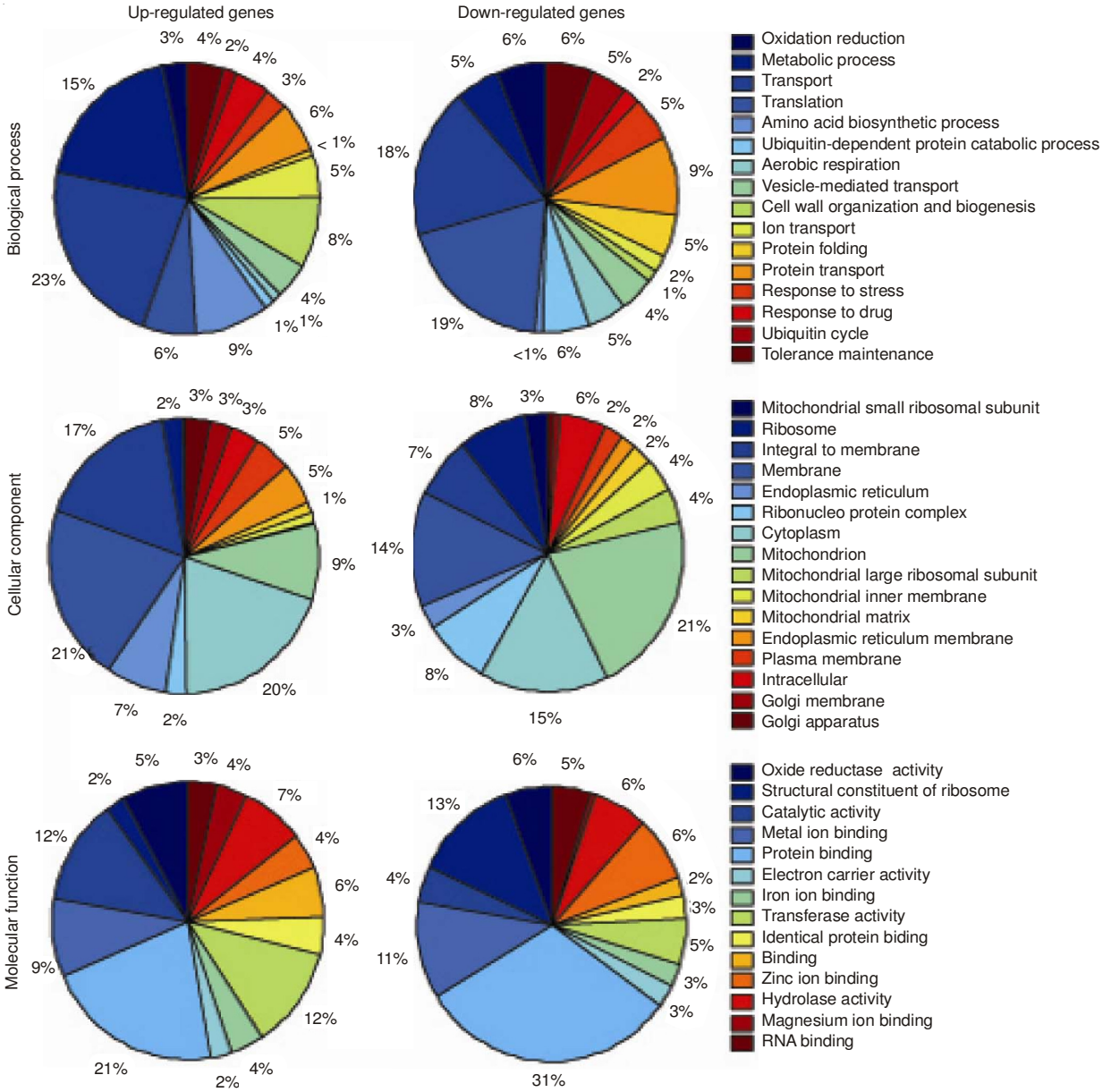


Fig. 2. Pie charts showing the percentage of up-regulated and down-regulated genes in each of the functional categories identified by microarray analysis. Gene ontology classification of 392 up-regulated and 503 down-regulated genes in transgenic yeast of iron deficiency *vs.* iron supplement.

TABLE-1
UP- REGULATED GENES INVOLVED IN IRON AND COPPER HIGH AFFINITY TRANSPORT SYSTEM IN IRON DEFICIENCY

Gene	-Fe/+Fe fold change	Description
FTR1	5.36	High affinity iron permease involved in the transport of iron across the plasma membrane; forms complex with FET3 protein; expression is regulated by iron.
CTR1	3.7	High-affinity copper transporter of the plasma membrane, mediates nearly all copper uptake under low copper conditions; transcriptionally induced at low copper levels and degraded at high copper levels.
ATX1	2.25	Golgi membrane protein involved in manganese homeostasis; overproduction suppresses the sod1 (copper, zinc superoxide dismutase) null mutation.
CCC2	2.61	Cu(+2)-transporting P-type ATPase, required for export of copper from the cytosol into an extracytosolic compartment; has similarity to human proteins involved in Menkes and Wilsons diseases.
FRE1	10.55	Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels.
FRE2	4.82	Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low iron levels but not by low copper levels.
FRE3	4.05	Ferric reductase, reduces siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels.
FRE8	15.9	Protein with sequence similarity to iron/copper reductases, involved in iron homeostasis; deletion mutant has iron deficiency/accumulation growth defects; expression increased in the absence of copper-responsive transcription factor MAC1 protein.

TABLE-2
HIGHLY EXPRESSED GENES INVOLVED IN VESICLE TRAFFICKING IN CELLS IN IRON DEFICIENCY

Vesicle trafficking	-Fe/+Fe fold change	Description
SEC24	5.58	Subunit of the coatomer complex (COPII), in complex with Sec23p; involved in ER-to-Golgi transport.
SEC31	2.43	Essential phosphoprote in component (p150) of the COPII coat of secretary pathway vesicles, in complex with Sec13p; required for ER-derived transport vesicle formation.
COP1	2.19	α-Subunit of COPI vesicle coatomer complex, which surrounds transport vesicles in the early secretory pathway.
RET2	2.43	δ-Subunit of the coatomer complex (COPI), which coats Golgi-derived transport vesicles; involved in retrograde Golgi-to- ER transport.
SEC27	2.28	Essential beta'-coat protein of the COPI coatomer, involved in retrograde Golgi-to-ER and CGN-TGN transport; contains WD40 domains that mediate cargo selective interactions; 45% sequence identity to mammalian beta'-COP.
SEC21	2.14	γ-Subunit of coatomer, a heptameric protein complex that together with Arf1p forms the COPI coat; involved in ER to Golgi transport of selective cargo.
CHC1	6.28	Clathrin heavy chain, subunit of the major coat protein involved in intracellular protein transport and endocytosis; three heavy chains form the clathrin triskelion structural component; the light chain (CLC1) is thought to regulate function.
SNC1	-2.04	Vesicle membrane receptor protein (v-SNARE) involved in the fusion between Golgi-derived secretory vesicles with the plasma membrane; proposed to be involved in endocytosis; member of the synaptobrevin/VAMP family of R-type v-SNARE proteins.
SNC2	-4.55	Vesicle membrane receptor protein (v-SNARE) involved in the fusion between Golgi-derived secretory vesicles with the plasma membrane; member of the synaptobrevin/VAMP family of R-type v-SNARE proteins.
VPS27	-4.38	Endosomal protein that forms a complex with Hse1p; required for recycling Golgi proteins, forming luminal membranes and sorting ubiquitinated proteins destined for degradation; has Ubiquitin Interaction Motifs which bind ubiquitin (Ubi4p).
VPS36	-3.16	Component of the ESCRT-II complex; contains the GLUE (GRAM Like Ubiquitin binding in EAP45) domain which is involved in interactions with ESCRT-I and ubiquitin-dependent sorting of proteins into the endosome.

Functional category assignment: The differentially expressed genes were annotated using database from Affymetrix website, clustered by cluster 3.0, treeview and some software which was commercially available. In -Fe *vs.* +Fe, 20 % of the 392 up-regulated genes were involved in biological processes, 45 % were in cellular components, and the remaining 35 % comprised molecular function (Fig. 2). Annotation of the 503 down-regulated genes revealed that the majority of genes were involved in biological process (23 %) and cellular component (48 %) with the remainder of genes involved in molecular function (29 %, Fig. 2).

The majority of the up-regulated genes seem to be related to five major biological changes induced by iron limitation in yeast, metabolic process (19 %), transport (24 %), amino acid biosynthetic process (9 %; Fig. 2), membrane (22 %) and membrane integrity (17 %) in cellular component, protein binding, metal ion binding in molecular function, which implied that transport of MxIRT1 may be connected with these genes.

The three largest functional categories (except for unclassified protein) of the down-regulated genes are translation (20 %) and transport (18 %) (Fig. 2). The down-regulated genes

belonging to mitochondrion category mainly include those involved in Krebs cycle and carbohydrate metabolism.

The genes involved in MxIRT1 Transport pathway in iron deficiency: Under iron limitation, DEY1453 (fet3fet4) cells could not survive. However, MxIRT1 transformed DEY1453 would grow up and therefore it was speculated that MxIRT1 transport might cooperate with some genes about iron transport and make cells grown. As shown in Table-1, it was observed that the expression of genes about copper transport system was up-regulated, such as CCC2, ATX1, and CTR1. Based on data from microarray, copper transport definitely occurred under iron limitation. For copper transport was necessary for high affinity iron transport system[10], it might play a role in MxIRT1 transport pathway (Fig. 3). The over expression of genes (FRE family) associated with ferric reductase also indicated that MxIRT1 might benefit for ferrous iron transport.

FRE1-3 which expression were induced by low copper and iron levels was ferric reductase and cupric reductase, reduced siderophore-bound iron and oxidized copper prior to 6 uptake by transporters. FRE8 was protein with sequence similarity to iron/copper reductases, involved in iron homeostasis; deletion mutant had iron deficiency/accumulation growth defects; expression increased in the absence of copper-responsive transcription factor MAC1 protein.

The genes of MxIRT1-vesicles trafficking in yeast in the absence of iron: MxIRT1 was predicted to be an integral membrane protein with a metal-binding domain[6]. After MxIRT1 was transformed into yeast mutant, MxIRT1 transport possible pathways to the plasma membrane was depicted in Fig. 2 based on data from microarray. A set of transcript about membrane vesicles trafficking changed significantly, listed on Table-2. COP1, RET2, SEC27, SEC21, SEC31, SEC24 were involved in the coatomers of COPI and COPII. From endoplasmic reticulum (ER) to Golgi SEC31 protein and SEC24 protein was essential component of the COPII coat of secretory pathway vesicles. COP1, RET2, SEC27, SEC21 were up-regulated which indicated COPI vesicle coatomer complex might participate in MxIRT1 protein transport. From Golgi to endosome, CHC1 defined a clathrin heavy chain of was up-regulated, more than 6 times fold, which implicated that clathrin vesicle was increasingly formed. As shown in Fig. 2, SNC1 and SNC2 genes about SNARE in yeast were almost down-regulated, especially, SNC2, about -4.55 fold. This might implied that after clathrin embraced MxIRT1, most vesicles had not immediately fused with plasma membrane under iron deprivation, which also was identified by observation from confocal microscope (Fig.3a-c). In Fig.3a, MxIRT1 occurred in plasma induced by iron limitation. As surrounding iron concentration increasing, MxIRT1 was delivered to plasma membrane (Fig. 3b). Although high affinity iron-transport system was known to take place induced by iron limitation, there was increased transcription of genes that encoded vesicle delivery which suggested that high affinity transport system might be related to vesicle trafficking.

Comparison of RNA extraction methods for microarrays: Total RNA extracted from yeast has generally been considered suitable for studying gene expression patterns using modern molecular techniques, such as quantitative RT-PCR or microarrays. To the best of our knowledge, there are three

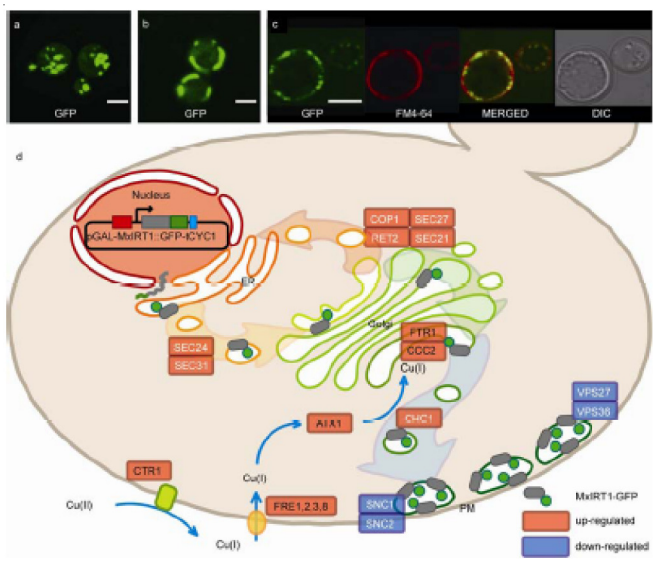


Fig. 3. A hypothetic sketch of MxIRT1-vecicle trafficking pathway in transformed yeast cells under iron deficient. a to c: MxIRT1-GFP-vesicle dynamic localization in transgenic yeast with different iron treatment by laser scanning confocal microscope. (a) Fe deficient treatment (20 μM BPDS-chelater) for 72 h, MxIRT1-GFP-vesicles occurred largely in the cytoplasm. (b) Added Fe treatment (30 μM Fe^{2+}) for 24 h, MxIRT1-GFP-vesicles move forward to the PM and get together by the PM. (c) MxIRT1-GFP-vesicles is getting to fuse to PM when 30μM iron added for 12 h. MxIRT1-GFP-vesicles (green) were partly colocalized (MERGED) with PM stained by FM4-64 (red). DIC, differential interference contrast. Bar = 4 μm. (d) A hypothetic sketch of MxIRT1-GFP trafficking pathway under iron deficient .Expression of pGAL-MxIRT1::GFP-tCYC1 gene was induced by galactose. Aft1/Aft2, namely transcriptional activators responded to iron deficient stress and promoted related-genes expression, such as the genes of coatmer proteins. After MxIRT1 synthesis in the endoplasmic reticulum, the COPII vesicles (SEC24 and SEC31 encode for the COPII vesicles' coatomer; Sec24p is also a receptor protein) are formed on the surface of the rough endoplasmic reticulum and reach the *cis*-Golgi, wrapping around the MxIRT1 protein. After that, clathrin vesicles are formed in the trans-Golgi membrane (CHC1 encodes clathrin heavy chain; CLC1 encodes clathrin light chain) and are docked at the plasma membrane nearby. When there was lack of iron in environment, the fusion-related genes were down regulated, for example, SNC1 and SNC2. However, as iron is added, the MxIRT1 vesicles were to fuse with the PM and MxIRT1, namely Fe^{2+}-transporters were released into the PM and then iron ions would be transported. The fusion between MxIRT1-vesicles and the PM might play an important role in the response of transgenic yeast to iron supplements.

Note: MxIRT1-GFP fused proteins are shown in gray and green symbol, Fe deficiency up-regulated genes are boxed with red and down-regulated genes with blue. MxIRT1-GFP fused proteins are shown in gray and green.

methods about RNA extraction from yeast including hot phenol method, trizol reagent and RNA isolation kits. In general, for total RNA, as long as A260/A280 ratio was between 1.9 and 2.0, 18S rRNA and 28S rRNA were shown distinctly on the gel, RNA would be considered usable. By comparing commercially available RNA isolation kits, RNA extracted from yeast with hot phenol method is often of proper integrity and quality for microarray experiments based on A260/A280 ratio and gel electrophoresis. Trizol reagents were ready-to-use monophasic solutions of phenol and guanidine isothiocyanate suitable for isolating total RNA DNA and proteins. However, breaking and dissolving yeast cell wall were often troublesome, there-

fore, integrity and purity of total RNA with Trizol reagents was not as good as other methods.

Up-regulated genes involved in high affinity iron transport system in iron deficiency: MxIRT1 was the gene encoding an Fe(II) transporter. A *fet3 fet4* double mutant, although viable, was extremely sensitive to iron limitation for high and low affinity transport systems were damaged. In this report, we used DEY1453 (fet3fet4) mutant strain to identify how MxIRT1 target to plasma membrane and played a role in response to iron supply.

For *Saccharomyces cerevisiae*, FET3 protein and FTR1 protein comprised the cell-surface high-affinity iron uptake system, which imported iron when it was present in low concentrations. FET3 protein was a multicopper oxidase and FTR1 protein was a transmembrane permease. FET3 protein oxidizes ferrous iron to ferric iron, which was then transported across the plasma membrane by FTR1 protein[11]. It was that FET3 protein must binds with Cu(I) for functions. Without neither FET3 protein or FTR1 protein, the another would be retained in the ER and didn't work. In microarray analysis, expression of FTR1 was highly up-regulated under iron-deficient condition, which implied that MxIRT1 protein might replace FET3 protein and assembly with FTR1 at Golgi and then transport toward the plasma membrane. On the basis of confocal microscope results, the presence of many MxIRT1-vesicles were near the plasma membrane, which also indicated MxIRT1 might function as a number of high affinity transport system and was trafficked to plasma membrane through vesicle.

Because FET3 protein must binds with Cu(I) for functions, the pathway of copper ion entering cell belongs to iron high affinity transport system. Copper ions entered *S. cerevisiae* as Cu(I) through a combination of copper high affinity and low affinity transporter[12]. The high affinity uptake system consisted of two transporters, CTR1 and CTR3, as well as the FRE1 metalloreductase. Low affinity copper uptake occurred through Fet4 and Smf1 transporter. Once in the cells, Cu(I) ions were transported to sites of utilization by two known soluble Cu(I)-binding metallochaperones, ATX1 and CCC1[13]. The ATX1 metallochaperone shuttled Cu(I) to the CCC2 P-type ATPase transporter localized in post-Golgi. The ATPases translocated Cu(I) ions into the lumen of secretory vesicles for incorporation into secretory copper metalloenzymes such as the FET3 and ceruloplasmin Cu oxidases in yeast[14].

In iron deficiency, copper cotransport functioned in wild type yeast. The copper cotransport mainly provided copper ion for FET3 protein. Microarray analysis also proved that copper cotransport still occurred in DEY1453 (fet3fet4) mutant transformed by MxIRT1, expression genes about copper transport, for instance, CTR1, ATX1, CCC2, were up-regulated, which indicated that copper high affinity uptake system initiated for coping with iron high affinity transport system.

Yeasts exhibit three primary mechanisms of iron accumulation: 1) siderophore-mediated; 2) ferrous iron transporter-mediated; 3) ferroxidase, permease complex-mediated[15-17]. All three mechanisms are metalloreductase-dependent with the Fe^{3+}/Fe^{2+} redox reaction catalyzed by this enzyme activity required either at the end of the accumulation process (siderophore-mediated uptake) or at the beginning (the transporter and permease pathways). Therefore, under iron deprivation, oxidization reduction related genes were up-regulated. In fact, FRE1-3 that functioned as copper and ferric reductase was over-expressed and the expression had all more than 4 times increase, based on microarray analysis.

Up-regulated gene of MxIRT1-vesicle trafficking pathway in yeast in iron deficiency: Functional expression of MxIRT1 in a yeast mutant (fet3fet4) defective for iron uptake suggests that MxIRT1 protein, have the activity of iron acquisition. The function of iron uptake associated to MxIRT1 protein mentioned above suggested MxIRT1 should be a plasma integral membrane protein. Vesicles would carry MxIRT1 from ER to the Golgi complex. Once they reached the *trans* Golgi network (TGN), MxIRT1 proteins were ready to be sorted and targeted for delivery to the plasma membrane by clathrin-coated vesicles. So sorting processes of MxIRT1 was suggested that iron starvation promote protein trafficking *via* membrane vesicles transporting[6,18].

Over the past years, we had observed that MxIRT1 protein was expressed and delivered to plasma membrane nearby but only accumulated into bigger patch and seemed no fusion initiated in lack of iron for 24-72 h (no galactose induced) with a laser scanning confocal microscope. However, it should be noted that some MxIRT1 might fuse with plasma membrane for they had to absorb a few iron ions in the surrounding to survive the cells, which might be consistent with low expression of SNC1 and SNC2, which worked as vesicle membrane receptor protein under iron deprivation. For the fuse amounted to a small number due to little iron concentration in the solution, it speculated that there was no observation about the fuse. As environmental iron increasing, most of MxIRT1 protein vesicles started to be fused to plasma membrane (Fig. 3c), which implied that the fusion of MxIRT1-vesicles and plasma membrane might be in response to iron signal. Based on research about iron transport in yeast and microarray data, a hypothetical model about MxIRT1 transport pathway was proposed (Fig. 3d).

Early electron microscopy studies of cells led to a vesicle transport hypothesis for protein trafficking[19]. Vesicular transport intermediates bud from a donor organelle and then fuse with an acceptor organelle. Budding intermediates were initially identified by their electron-dense coats and were found on the plasma membrane and intracellular organelles[20]. Three major classes of these coated vesicles had now been purified: COPI-(coatomer) and COPII-coated vesicles (where COP stands for coat protein complex) and clathrin-coated vesicles[21]. COPI coat components traffic primarily from the Golgi to the endoplasmic reticulum (ER) and between Golgi cisternae. The COPII coat complex is a key determinant in the fidelity of sorting and transport of newly synthetized biosynthetic cargo out of the endoplasmic reticulum and on towards the Golgi from where it can be directed to the plasma membrane or endosome or out of the cell by clathrin-coated vesicles[22]. Clathrin-coated vesicles are named after the protein that self-polymerizes into a lattice around these vesicles as they bud from the plasma membrane, *trans*-Golgi network (TGN) , endosomes and the plasma membrane.

In iron deficiency, genes about vesicle coat in COPII and COPI such as SEC24, SEC31, SEC21, RET2, *etc*., were all up-regulated. SEC24 and SEC31 were subunit of the coatomer

complex (COPII). SEC23/24 and SEC13/31 could self-assemble to form COPII cage-like particles. In complex with SEC23 and SEC31 involved in endoplasmic reticulum-to-Golgi transport. The subunit SEC24 in COPII has the site of recognizing cargo signal namely Asp-X-Glu, which was found in MxIRT1 amino acid sequence. SEC21 was gamma subunit of coatomer, a heptameric protein complex that together with ARF1 protein forms the COPI coat. Expression changes of these genes indicated that MxIRT1 protein might transport *via* COPII and COPI coat complex. CHC1, Clathrin heavy chain, subunit of the major coat protein involved in intracellular protein transport and endocytosis and exocytosis. Each clathrin molecule consists of three heavy chains, which the inner portion of each heave chain is linked to a smaller light chain, and three light chains, joined together at the center to form a three-legged triskelion The CHC1 of among them were up-regulated, which indicated that MxIRT1 protein might bedelivered out of TGN in the form of clathrin-coated vesicles.

Down-regulated genes involved in vesicle budding and fusion in iron deficiency: Vesicle budding and docking and fusion were major sorting procession in the exocytosis route that deliver proteins and lipids to multiple destinations in the cell, including the cell surface, Golgi complex, vacuole, *etc*. VPS27 was coding endosomal protein that forms a complex with Hse1p, required for recycling Golgi proteins, forming inter caveolae membranes. VPS36 was component of the ESCRT-II complex (endosomal sorting complexes required for transport, ESCRT), contains the GLUE (GRAM Like Ubiquitin binding in EAP45) domain, which is involved in interactions with ESCRT-I and ubiquitin-dependent sorting of proteins into the endosome or plasma membrane. It was emphasized that there was a ubiquition binding site in MxIRT1 amino acid sequence[6,23]. Down regulation of these genes indicate that vesicle budding nearby plasma membrane might be arrested under iron limitation.

From microarray data, under iron starvation, some genes about SNAREs were down regulated such as SNC1 and SNC2 which functioned as vesicle membrane receptor protein (v-SNARE) involved in the fusion between Golgi-derived secretory vesicles with the plasma membrane. Member of the v-SNARE proteins requires the participation of two kind of SNAREs on transport vesicles membrane (v-SNARE) and target membranes (t-SNARE) for fusion[24]. SNC1 and SNC2 were yeast-unique v-SNARE involved in the fusion of Golgi-vesicles with plasma membrane[25]. Their down-expression indicated there was no fusion to plasma membrane, which also implied that SNC1 and SNC2 were fusion regulatory factors responding to iron existence in solution. Under iron-sufficient condition, vesicles with MxIRT1 protein started to fuse with plasma membrane and transport MxIRT1 protein into plasma membrane, consistent with results from our previous microscope. Here now, we further proved that MxIRT1 protein transport toward plasma membrane from endoplasmic reticulum *via* vesicle trafficking and docking inside plasma membrane in iron deficient. It was indicated that the fusion of MxIRT1-vesicle with plasma membrane might work as a key regulating step, while SNARE responded to surrounding iron existence.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 30770178); and Technical Development Key Project of Beijing Education Community (No. KZ200710028013). We are indebted to Prof. Nicolaus von Wire´n (Institute for Plant Nutrition, University of Hohenheim, Germany) for providing the yeast strains DEY1453. We could like to acknowledge Fang Zhang for assistance in the microscope observation. We acknowledge Su-Lan Bai for skillful microarray experiment and Minxin Co.Ltd. in Shanghai for microarray analysis.

REFERENCES

1. B. Dujon, *Trends Genet.*, **12**, 263 (1996).
2. C. Curie, Z. Panaviene, C. Loulergue, S.L. Dellaporta, J.F. Briat and E.L. Walker, *Nature*, **409**, 346 (2001).
3. D.R. Dix, J.T. Bridgham, M.A. Broderius, C.A. Byersdorfer and D. Eide, *J. Biol. Chem.*, **269**, 26092 (1994).
4. D. Eide, S. Davis-Kaplan, I. Jordan, D. Sipe and J. Kaplan, *J. Biol. Chem.*, **267**, 20774 (1992).
5. E. Lesuisse and P. Labbe, *J. Gen. Microbiol.*, **135**, 257 (1989).
6. P. Li, J.L. Qi, L. Wang, Q.N. Huang, Z.H. Han and L.P. Yin, *Plant Sci.*, **171**, 52 (2006).
7. Z.H. Han, T. Shen, R.F. Korcak and V.C. Baligar, *J. Plant. Nutr.*, **17**, 579 (1994).
8. Z.H. Han, Q. Wang and T. Shen, *J. Plant. Nutr.*, **17**, 1257 (1994).
9. R. Wang, O. Mamoru, X. Xing and N.M. Crawford, *Plant Physiol*., **132**, 556 (2003).
10. R. Stearman, D.S. Yuan, Y. Yamaguchi-Iwai, R.D. Klausner and A. Dancis, *Science,* **271**, 1552 (1996).
11. J. Kaplan, D. McVey Ward, R.J. Crisp and C.C. Philpott, *J. Biochim. Biophys.*, **1763**, 646 (2006)..
12. S. Puig and D.J. Thiele, *Curr. Opin. Chem. Biol.*, **6**, 171 (2002).
13. S. Puig and D.J. Thiele, *Chem. Biol.*, **6**, 171(2001).
14. A. Paul, F.P. Cobine and D.R. Winge, *Biochim. Biophys. Acta*, **1763**, 759 (2006).
15. A. Van Ho, D.M. Ward and J. Kaplan, *Ann. Rev. Microbiol.*, **56**, 237 (2002).
16. D.J. Kosman, *Mol. Microbio.*, **147**, 1185 (2003).
17. D.H. Howard, *FEMS Immunol. Med. Microbiol.,* **40**, 95 (2004).
18. L.P. Yin, *Chin. Sci. Bull.*, **45**, 1127 (2000).
19. L.P. Yin, T. Sun, W. Li, Q.N. Huang and X.L. Liu, *Prog. Nat. Sci.*, **14**, 522 (2004).
20. T.F. Roth and K.R. Porter, *J. Cell. Biol.*, **20**, 313 (1964).
21. H.T. McMahon and I.G. Mills, *Curr. Opin. Cell Biol.*, **16**, 379 (2004).
22. R.A. Crowther and B.M. Pearse, *J. Cell. Biol.*, **91**, 790 (1981).
23. L. Kerkeb, I. Mukherjee, I. Chatterjee, B. Lahner, D.E. Salt and E.L. Connolly, *Plant Physiol. Prev.*, **107**, 13282 (2008).
24. S.E. Verrier, M. Willmann, D. Wenzel, U. Winter, G.F. von Mollard and H.-D. Soling, *Eur. J. Cell Biol.*, **87**, 863 (2008).
25. J. Samaj, F. Baluska and D. Menzel, in eds.: M.H. Sato, R.L. Ohniwa and T. Uemura, Plant Endocytosis. SNAREs in Plant Endocytosis and the Post-Golgi Traffic, Springer-Verlag Berlin Heidelberg, pp. 197–215 (2005).
26. G. Yang, F. Ma, Y. Wang, C.G. Feng, P, Li, Y. Xu, W.Z. Zhao and L.P. Yin, *Chinese Sci.*, **55**, 3298 (2010).