Construction of cDNA library from iron-deficiency induced maize roots and screening and identification of iron-stress gene *Fdr3*

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Abstract To isolate Fe-deficient related (*Fdr*) genes, an expression cDNA library of 4.5×10^5 pfu/μg has been constructed from maize roots in iron-stress. 6 clones have been screened from the cDNA library by differential hybridization screening. It is proved that an *Fdr3* cDNA clone expressed stronger under iron-deficient condition than under iron-sufficient one by Northern blot and Western blot.

Keywords: **maize, iron-deficient cDNA library, differential hybridization screening, iron-deficient related (*fdr*) clone.**

Iron (Fe) is one of the essential microelements for plant growth. However, available Fe element is often limited in calcific soils in China and other countries over the world. Two different strategies of root response to Fe deficiency have been identified in various plants[1]. Strategy I occurs in all plants except grasses, in which plasma membrane-bound reductase activity is induced with enhanced net excretion of protons. In Strategy II [2], grasses respond to Fe-deficiency stress by enhancing the release of phytosiderophores (PS) which form chelate with Fe^{3+}. Uptake of Fe^{3+}-PS is mediated by a specific transporter in the plasma membrane of root cells of grasses. According to the amount of PS from high to low, the important crops are ordered by barley > rye > wheat > oat > maize > Chinese sorghum≫ rice[3,4].

Maize was classified as Strategy II due to PS (2′-deoxymugineic acid DMA)[5] which was secreted from roots in Fe-deficient. Von Wiren et al.[6] confirmed that iron inefficiency in the maize mutant *ys1*(*Zea mays* L. cv *Yellow-Stripe*) is caused by a defect in the uptake system for Fe(III)-PS. To characterize this defect further, Von Wiren designed an uptake experiment with double-labeled ^{59}Fe-[^{14}C]-DMA by the Fe-efficient maize cultivator Alice and the Fe-inefficient mutant *ys1*. The

results suggested that Fe-DMA was taken up by the roots as the intact chelate in both cultivators; moreover, they indicated the existence of a high- and a low-affinity uptake systems mediating Fe-PS transport across the root plasma membrane in maize. Apparently the mutation responsible for Fe-inefficiency in *ys1* affected the high-affinity uptake and led to the decrease in activity and/or number of Fe-PS transporters[7]. However, Baguaes et al.[8] proved that NADH-Fe (III)-EDTA reductase was induced under Fe-deficiency and led maize roots to uptake more iron. Fe(III) reductase activity on roots surface was enhanced and pH value decreased in maize (WF9) rhizosphere but Fe-PS has not been tested in this Fe-efficient cultivator. During intercroping between maize and peanut in calcific soil, the peanut did not show chlorosis as usual and the fact that the products of peanut had increased, indicated that the secreted material under Fe-deficiency in maize could be available for the iron uptake in dicotyledonous plants[9]. Its mechanism has not been understood yet. Thus it is important to study the Fe-stress molecular mechanism of maize in order to develop a new strategy to control plants with high-iron efficiency.

1 Materials and methods

(i) Plant material. Seeds of Yedan No.12 sensitive to Fe deficiency were surface-sterilized in 0.5%—1% (*v*/*v*) NaOCl, and germinated at 27℃ for 36 h. Seedlings with 1-cm roots were transferred to the liquid aerated nutrient solution (Hoagland) without supplying Fe (–Fe treatment) at 25℃ for 11—24 d. While Fe-deficiency symptoms occurred, the root tips including root hair region around 2 cm in length were obtained and stored at –80℃ for use. At the same time, the control plants supplied with iron (1.4 mol/L +Fe treatment) were cultured under normal conditions.

(ii) Construction of cDNA library from Fe-stressed maize roots. A λZAP express cDNA library from Fe-deficient maize roots was constructed according to the protocol of ZAP Express™ cDNA Synthesis Kit and ZAP Express™ cDNA Gigapack Gold II Cloing Kit (Strategene, 1996). Above all, the reverse transcriptase (MMLV-RT)-mediated synthesis of the first-strand cDNAs was primed with the linker-primer containing

Xho I site: 5′GAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTT3′
"GAGA"sequence *Xho* I Poly (dT)

The "GAGA" sequence can protect the *Xho* I restriction enzyme recognition site and the 18-base poly(dT) sequence. Moreover, 5-methyl dCTP instead of dCTP avoided digestion from restriction enzymes. The second-strand cDNAs were synthesized by RNaseH and polymerase I at 16℃ for 25 h, and then the uneven termini of the double-stranded cDNAs were nibbled or filled in with cloned DNA polymerase. *Eco*R I adapters were ligated to the blunt ends. Size fractionation was spun on a Sephacryl ®S-500 column, followed by *Xho* I digestion, then cDNAs with *Xho* I and *Eco*R I cohensived ends were recombined into *Xho* I and *Eco*R I sites of λZAP vectors. Having been packaged *in vitro*, recombinant phage was transducted into XL1-Blue MRF′ host strain. The cDNA library has been titered and the ratio of recombinants has been detected.

(iii) Differential hybridization screening. For differential screening of the iron-stressed cDNA library, Fe-sufficient(+) and Fe-deficient(–) cDNA probes were synthesized from (–) and (+) mRNA template by RT-PCR of random primers. After repeated clone screening using *in situ* hybridization of bacteriophage plagues, the 6 plaques were selected from the cDNA library, and then were converted to the pBK-CMV phagmid by *in vivo* rapid excision. The 6 clones were sequenced and homogeneously analyzed by TaKaRa Biotech (Japan).

(iv) Molecular hybridization. *Fdr3* clone was digested with *Eco*R I /*Xho* I. Then *Fdr3* fragment was recovered by Glassmilk either as probe of Northern blotting or as antigen of Western immunoblot analysis.

Total RNA from Fe-deficient maize roots was isolated using the acid guanidinium thiocyanatephenol-chloroform method and then electrophoresized through agarose gel with 30 μg total RNA. Northern hybridization was analyzed by probe with labeled ^{32}P by nick translation after transferring total RNA to nitrocellulose filters.

Solution protein of maize roots was extracted and SDS-polyacrylamide gel (PAGE) was run by the Laemnil system with 30 μg protein adding to each lane. Different protein bands were shown by silver staining. Meanwhile another one was transferred onto the PVDF-membrane and blocked with *E. coli* protein and 5% nonfat milk for Western blotting.

Fdr3 cDNA was reconstructed into pGEX vector. Transformer was induced with IPTG. Fusion protein was extracted and antiserum was made through raising in mice. The first antibody was added to the blocked membrane at 37℃ for 30 min. After washing the second antibody for anti-mouse IgG-HRP (horseradish peroxidase) was added at 37℃ for 30 min. The color was developed with DAB.

2 Results and discussion

(i) Construction of cDNA library from Fe-stressed maize roots. 2.95 mg total RNA were extracted from 5 g maize roots that were induced for 11—14 d in iron-deficiency. The RNA production rate was 0.59%. 20 μg poly(A)+RNA was purified from 2 mg total RNA by Oligo poly(dT) cellulose column. 1.5 μg cDNAs around 500 bp—10 kb in length were obtained from 10 μg mRNA. Reversed transcription rate was about 15% of mRNA. Recombination rates of cDNA library were 70%—76% by Blue-White screening (This index is very important. Please don't ignore it). The final λZAP express cDNA library of 4.5×10^5 pfu/μL (plaque forming unit/μL) for iron-deficiency induced maize roots was constructed and the titer of the amplified cDNA library was 1.03×10^{11} pfu/mL.

(ii) Differential hybridization screening of the cDNA library. 6 clones were selected from 1.25×10^5 pfu recombinants by differential hybridization screening (fig. 1). Among them, expression of the cDNA clones was enhanced in Fe-deficient. Specific-induced clones have not been found so far.

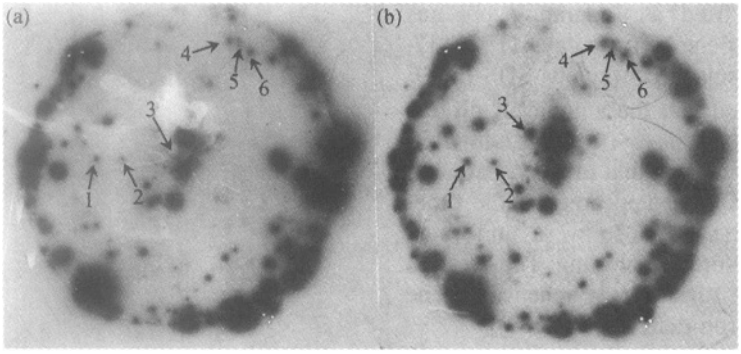

Fig. 1. Autoradiography of differential hybridization screening. (a) (+) cDNA probes; (b) (−)cDNA probes. Arrows show that the 6 clones in Fe-deficiency enhanced their expression.

It is more difficult to pick out the amount-different clones than to pick out the specific clones from cDNA library by differential screening. So more stringent conditions were employed during screening, such as determining exactly the amounts of the probe and the time of transferring NC-film, controlling strictly the conditions of prehybridization and hybridization and autoradiography, etc.

(iii) Sequence analysis and Northern blotting of *Fdr3*. According to the nucleotide sequences of *fdr3* gene, the putative sequence of amino acids is shown as follows:

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FDR3   1 MQTTTATTPLADRLRRYTPALASLARALYDARGALCTVRAVPEGPPRTGK
      51 TAQLTVACDQGELHVHVDADAAPEAIALEPEAACRAAVASLYLAGPLAAL
     101 ARHGATRPAVRDVRLAAPSASRAGVLHLEYDHDGAPTGAATGATTGATVA
     151 VSAALAAALAERIGPRGRGVLTPLAALALPTRLRLRTRHATPALLTLRPG
     201 DVLLGWPAAPGPAPHATLQATLLWGAANGHAVHAHARIDSRNVILESSPY
     251 AMNHDPDLSLRAAPDAASSPLDVSDVELPVHIEVVTVNPIGQIAALQPG
     301 ILALPVALADADIRLVAHGQTLAPGELVAIGDQLGHIRRIANADERR
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Fdr3 cDNA is 3.3 kb. Then *Fdr3* subclone is 2.5 kb, including ORF 1068 bp in length.

Homologous analysis of *Fdr3* cDNA was performed extensively by amino acid database of GenBank+PDS+SwissPort+Spupdate+PIR, but no any other homologous genes have been found. So *Fdr3*'s function has not been ensured. But the result of Northern blotting indicated that *Fdr3*-mRNA emerged earlier and more under iron-deficiency (on days 14 and 17) than under iron-sufficiency (only on day 17). Although the amount of inducible *Fdr3*-mRNA on days 14 or 17 under iron-deficiency respectively was less than that on day 17 under iron-sufficiency, the sum amount of *Fdr3*-mRNA in Fe-deficiency was far more than that in Fe-sufficiency (fig. 2).

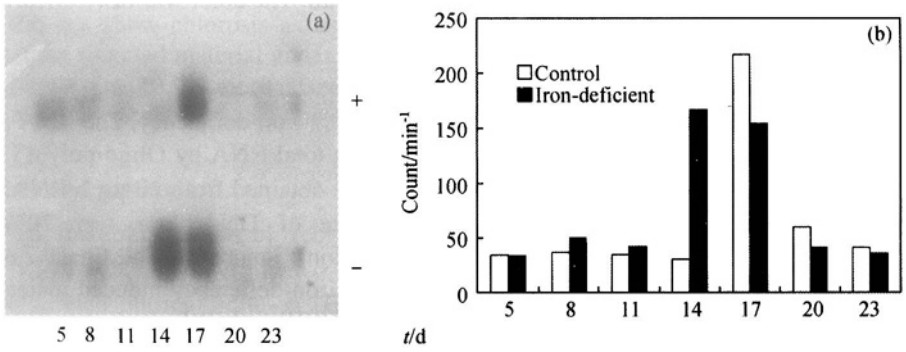

Fig. 2. Autoradiograph of *Fdr3* Northern blot (a) and column figure of liquid scintilation spectrophotometry (b).

(iv) SDS-PAGE and Western blotting. According to the results of Northern blotting, SDS-PAGE was used as separating proteins induced in iron-deficiency from days 11, 14, 17, 20 and 23. The result of continuous SDS-PAGE (7%—15%) showed that the amount of protein band about 40 ku on days 14 and 17 in iron-deficiency was more than that of +Fe treatment. Almost no protein band appeared on the other days (figure omitted). Those were uniformed with the result of Northern blotting. The Western blotting indicated that *Fdr3* enhanced gene expression under iron-deficiency (fig. 3).

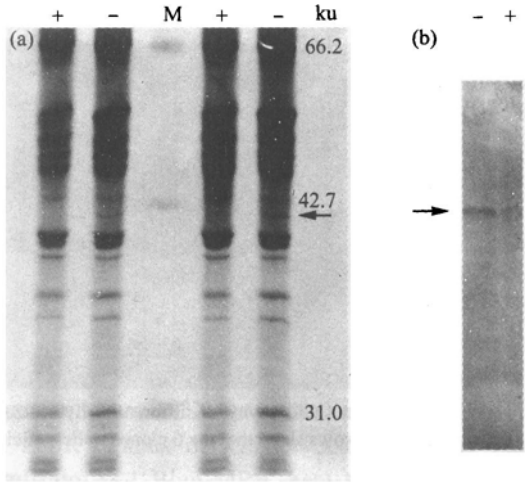

Fig. 3. Western blotting of solution protein in maize roots. (a) Silver staining of protein gel on day 14 in maize roots; (b) Western hybridization after transferring protein to PVDF filter. +, Iron-added treatment; −, iron-deleted treatment.

Biologists have made the big progress in biosynthesis of phytosiderophores in recent years. Okamura et al.[10] have isolated 7 specific clones from the iron-deficient cDNA library of barley roots by differential screening and named them *ids1* — *7* respectively (iron-deficiency specific clone). They suggested that the genes were related to those of biosynthesis of PS and regulation for Fe^{3+}-PS uptake.

Iron-deficient induced genes in graminaceous plants fall into 3 types. The first one[6] is the genes related to the biosynthesis of phytosiderophores including *nas* (nicotianamine synthetase), *naat* (nicotianamine amino transferase), 36 ku peptide gene, *Ids3* and *aprt* (adenine phosphoribosyl transferase) which plays a role in the production of AMP from adenioe. AMP is a precursor of ATP, and the ribose moiety of ATP can be a procurer of methionine throughout the Yang cycle resulting in the release of adenine under Fe-deficiency[11].

The second is the genes in the presence of excess Cu such as *Ids1* and *Ids2* which may be indirectly regulated by Fe-deficiency. *Ids1* is a metallothionein-like gene. The amino acid sequence of *Ids2* resembles that of oxoglutarate-dependent dioxygenase gene. Both genes have an upstream Cu responsive element.

The third is the genes involved in complementary energy through the secondary anaerobic energy production metabolism. For example, gene expression of *fdh* (formate dehydrogenase gene) and *adh* (alcohol dehydrogenase gene) had enhanced under the iron-deficient condition.

Fdr3 sequence has no homology with MAS-biosynthesis related genes which were reported in recent years. So *Fdr3* perhaps encodes other genes of Fe uptake or transport protein. So far the new Fe-deficiency related genes have not been reported in the world. Uncovering the physiological process controlled by *Fdr3* gene will be of benefit to explaining the fact of maize sensitivity to Fe-stress. Furthermore, the resistance to Fe-stress in maize will be improved by the biochemistry and molecular biology technique.

Acknowledgements This work was supported by Beijing Municipal Natural Science Foundation (Grant No. 5942004) and the National Natural Science Foundation of China (Grant No. 39770074).

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(Received October 18, 1999)