A Technique for Producing Plants Growable in Non-Fertile Soils

September 2007
Japan Science and Technology Agency

Introduction

Sixty-seven percent of all terrestrial land areas on earth are non-fertile soils which offer marginal productivity as farmlands, and about half of which is calcareous alkaline soil (CALCISOLS) (Figure 1). In calcareous alkaline soils, iron occurs in the form of ferric hydroxide which is hardly soluble in water. This prevents plants from absorbing this essential nutrient for growth. With no iron uptake, plants suffer from iron deficiency (iron-deficient chlorosis) and die off by blighting.

If the plants were able to grow productively even in such alkaline soils, then it would be possible to not only increase food production but provide solutions to various environmental problems such as increased carbon dioxide, global warning, and desertification, as well as energy problems caused by increased production of bio-ethanol materials.

In view of this background, in the present study, a technique was developed that enables plant growth even in non-fertile soils such as alkaline soils.

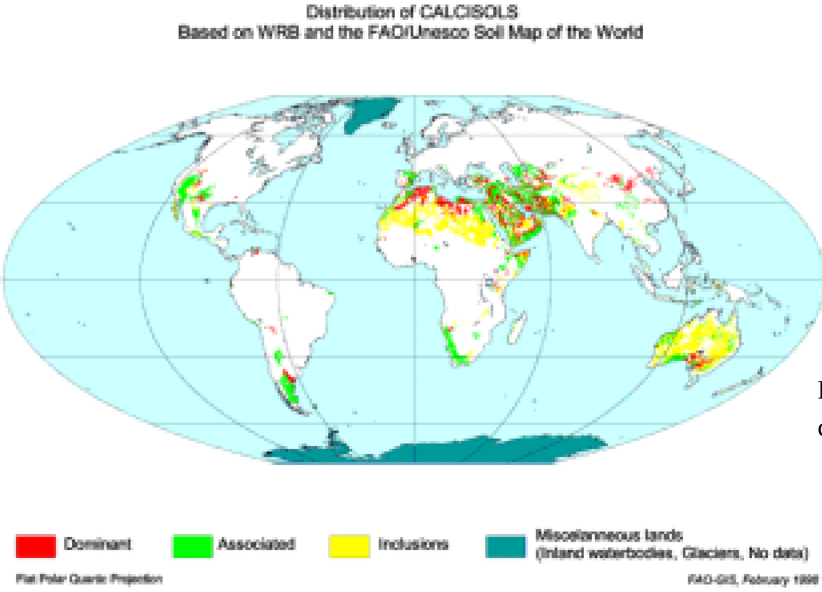

Fig. 1: Distribution of calcareous alkaline soil

Features of the Present Technique

Broadly, plants employ two iron absorption mechanisms.

(i) Rice plants including many major crops such as rice, barley, corn, and sugar cane secrete mugineic acids from the root. The mugineic acids, which are chelators, convert the iron of water-insoluble form, Fe(III), into a water-soluble chelate, “Fe(III)·mugineic acid,” for iron uptake from the soil (**chelating strategy**; Figure 2).

(ii) In non-rice plants, iron is absorbed in the form of water-soluble Fe(II) ions, which result from the reduction of water-insoluble Fe(III) in the soil by Fe(III) reductase that occur on the surface of the root (**reducing strategy**; Figure 3).

In the present study, a technique was developed that enhances the two plant iron absorption mechanisms to produce plants that can grow even in non-fertile soils such as calcareous alkaline soils. In another technique developed by the researchers, iron-deficiency responsive transcription factors (cis-elements, trans-factors) were identified, which were used to render plants tolerant to iron-deficiency.

It has been confirmed at field level that the present technique enables production of plants that can overcome iron deficiency and grow even in calcareous alkaline soils.

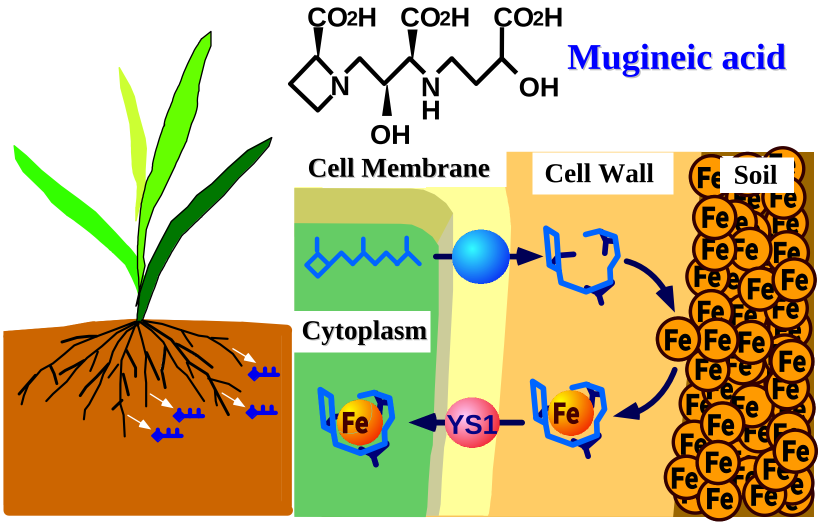

Fig. 2: Iron acquisition mechanism of rice plants (chelating strategy)

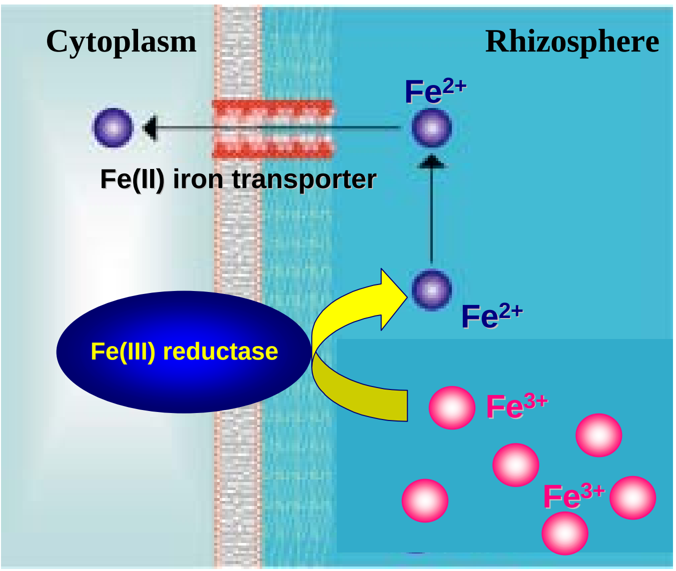

Fig. 3: Iron acquisition mechanism of non-rice plants (reducing strategy)

(i) Enhancement of Chelating Strategy

Previously, synthetic pathways of mugineic acids have been revealed by the researchers of the present study. In the pathways, deoxymugineic acid is first synthesized from methionine via S- adenosylmethionine, nicotianamine, and a keto form, and this is followed by syntheses of other mugineic acids. Further, genes of all enzymes catalyzing each step of the biosynthetic pathways of mugineic acids have been isolated from barley. Figure 4 illustrates the biosynthetic pathways of mugineic acids.

In the present study, a technique was developed that enhances the synthetic capability of mugineic acids in plants through introduction of enzyme genes associated with the synthesis of mugineic acids. The technique enables production of plants that can overcome iron deficiency and grow even in alkaline soils.

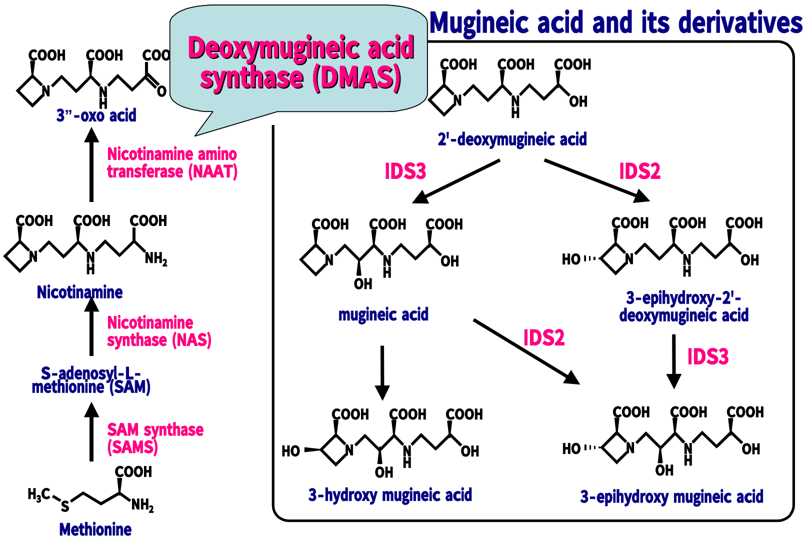

Fig. 4: Biosynthetic pathways of mugineic acid and its derivatives

(ii) Enhancement of Reducing Strategy

Non-rice plants possess Fe(III) reductase and Fe(II)-ion transporter in the root, and absorb iron in the form of Fe(II). The Fe(II)-ion transporter also occurs in the root of rice; however, there is substantially no Fe(III) reductase activity under iron deficiency conditions. Based on this information, in the present study, a technique was developed that transforms plants with genes that have been modified to encode enzymes that exhibit strong Fe(III) reductase activity even under alkaline conditions. The modification was made using genes of food yeast by evolutionary engineering. By the technique, plants with no Fe(III) reducing capability were rendered capable of reducing Fe(III). For plants that already have Fe(III) reducing capability, the reducing activity can be enhanced so that the plants can exhibit stronger activity under alkaline conditions. That is, the technique enables production of plants that can absorb iron even in alkaline soils and exhibit tolerance to iron deficiency.

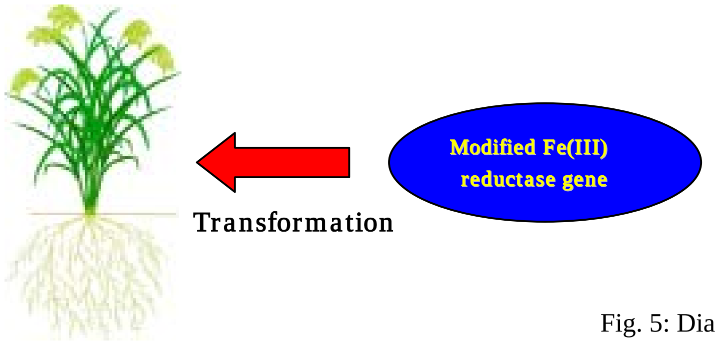

Fig. 5: Diagram schematizing enhancement of reducing strategy

(iii) Combined Enhancement of Chelating Strategy and Reducing Strategy

Tolerance to alkaline soil can be improved significantly by the combination of the techniques enhancing (i) the chelating strategy and (ii) the reducing strategy.

(iv) Use of Iron-Deficiency-Responsive Transcription Factors

Previously, there have been identified by the researchers of the present study iron-deficiency responsive cis-elements IDE1 and IDE2 that occur in the promoter region of a gene whose expression is induced under iron deficient conditions, and trans-factors IDEF1 and IDEF2 that interact with the cis-elements IDE1 and IDE2, respectively. Figure 6 illustrates the mechanism of iron-deficiency responsive expression induction by IDE1, IDE2, IDEF1, and IDEF2.

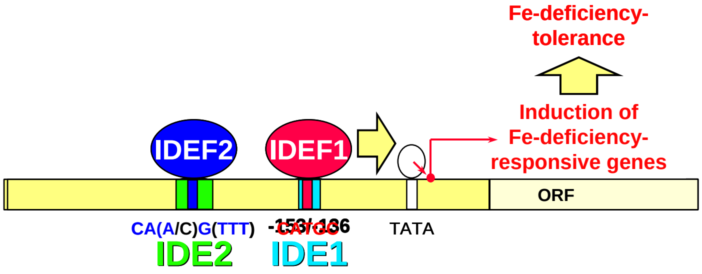

Fig. 6 Mechanism of iron-deficiency responsive expression induction

In the present study, a technique was developed that confers strong iron-deficiency responsive inductivity through introduction of a promoter fragment having one copy each of the iron-deficiency responsive cis-elements IDE1 and IDE2 into plants. In another technique developed by the researchers, genes encoding the trans-factors IDEF1 and IDEF2 were introduced into plants to produce plants that exhibit tolerance to iron-deficiency in alkaline soils.

Examples

(1) Production of Transgenic Plants with Genes Associated with Mugineic Acid Synthesis

Transgenic rice was produced by introducing a genomic gene of barley encoding nicotianamine amino transferase (NAAT), which is a key enzyme in the biosynthetic pathways of barley mugineic acids.

Figure 7 represents a result of a pot test performed with calcareous alkaline soil in an isolated greenhouse. As can be seen from the figure, the transgenic rice with NAAT showed tolerance to iron-deficiency in calcareous alkaline soil, increasing the yield by several fold compared with the control.

Fig. 7 Pot test with calcareous alkaline soil in isolated greenhouse
Left: Vector control
Right: NAAT transgenic rice

The pot test was also performed with calcareous alkaline soil in an isolated greenhouse, using transgenic rice that had been produced by introducing enzyme genes involved in the biosynthetic pathways of mugineic acids other than NAAT, or enzyme genes involved in the methionine cycle that supplies the methionine used as a substrate. As a result, rice plants that were transformed with the following 6 different genes were shown to have tolerance to iron-deficiency in a calcareous alkaline soil.

(1) Genomic nicotianamine amino transferase (NAAT) gene
(2) Genomic nicotianamine synthase (NAS1) gene
(3) Genomic mugineic acid synthase (IDS3) gene
(4) Genomic nicotianamine synthase (NAS1) gene and genomic nicotianamine amino transferase (NAAT) gene
(5) Adenine phosphoribosyltransferase gene under control of IDS3 promoter
(6) Nicotianamine synthase (NAS1) gene, nicotianamine amino transferase gene (NAAT) gene, and adenine phosphoribosyltransferase gene, all under control of IDS3 promoter

Among the 6 kinds of transformed lines of rice, the transformants (2), (3), and (4) were subjected to a calcareous alkaline soil tolerance test in an isolated field. The line transformed with the NAS1 gene (2) was also tested using seeds (gNAS1 cal) obtained from rice that had been cultivated in a calcareous alkaline soil previous year.

One hundred tons of calcareous alkaline soil was dumped into an isolated field to construct a paddy field (10 m × 10 m × 50 cm), where transgenic and non-transgenic rice (NT) were cultivated. For comparison, the cultivation was also made in an Andisol paddy field.

As can be seen from the result shown in Figure 8, there was a significant difference in the growth of the two samples. Specifically, while some of the non-transgenic rice (NT) underwent iron deficiency chlorosis and blighted in the paddy field with calcareous alkaline soil, all transgenic rice grew desirably in the same field. Growths of transgenic rice and non-transgenic rice were not significantly different in the Andisol paddy field.

Fig. 8: Calcareous alkaline soil tolerance test in isolated field
Left: IDS3 transgenic rice Right: Non-transgenic rice

Figure 9 represents results of observation concerning plant height, leaf color, and the number of tillers at an early stage (15 days to 45 days) of growth after the transplant to the calcareous alkaline soil. As can be seen from the figure, the height of transgenic rice was higher than that of non-transgenic rice. The difference was particularly significant in gNAS1cal and gIDS3. As to the leaf color, the transgenic rice had higher SPAD scores than the non-transgenic rice. The transgenic rice and non-transgenic rice showed different numbers of tillers between 15 DAT and 25 DAT.

The foregoing results demonstrated that the transgenic rice with the enhanced ability to synthesize mugineic acids was indeed capable of exhibiting iron-deficiency tolerance in calcareous alkaline soil even at field level.

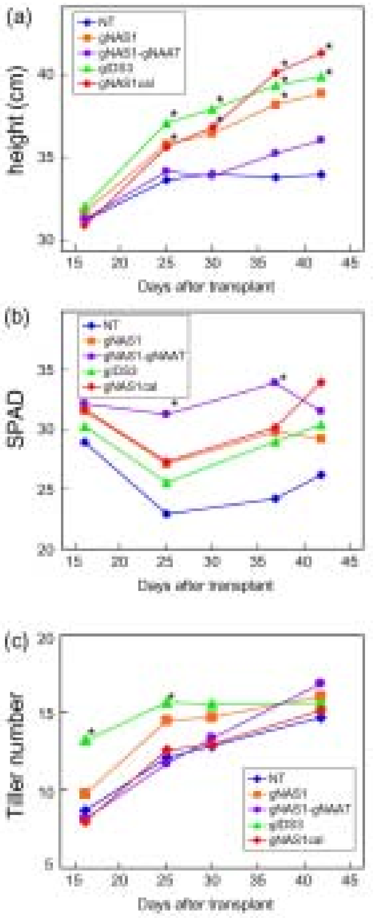

Fig. 9 Growth of rice at an early stage of growth after transplant to calcareous alkaline soil
(a) Plant height
(b) SPAD score
(c) Number of tillers

(2) Production of Transgenic Plants with Modified Fe(III) Reductase Gene

A gene of food yeast was modified by evolutionary engineering to fully synthesize a modified Fe(III) reductase gene (*refre1-372*) that can exhibit strong enzyme activity even at high pH. The modified Fe(III) reductase gene (*refre1-372*) was ligated to the promoter of Fe(II)-ion transporter and introduced into rice to produce transgenic rice. A growth of the transgenic rice was examined in alkaline soil.

As can be seen from Figure 10, the transgenic rice was shown to have tolerance to iron deficiency in calcareous alkaline soil.

Fig. 10: Tolerance to iron deficiency in rice transformed with modified Fe(III) reductase gene

Left: Cultivation of non-transgenic rice in ordinary soil

Middle: Cultivation of non-transgenic rice in calcareous alkaline soil

Right: Cultivation of transgenic rice in calcareous alkaline soil

(3) Production of Transgenic Plants Using Iron-Deficiency Responsive Transcription Factors

The cis-elements and trans-factors that induce iron-deficiency responsive expression were used to produce plants that are tolerant to iron deficiency in alkaline soil.

First, IDE1 and IDE2 were ligated to an enhancer-like sequence at various different positions or in redundancy and introduced into rice for expression analysis. As a result, notable expression induction was detected in roots and leaves under iron deficient conditions. This confirmed that a promoter sequence having one copy each of IDE1 and IDE2 was sufficient to render strong iron deficiency inductivity also in rice.

Further, transgenic rice was produced by introducing an IDEF1 coding gene (*IDEF1*), and the state of leaf and the plant height under iron deficient conditions were examined. As shown in Figure 11, chlorosis (yellowing) in leaves was less apparent in the transgenic rice than in the non-transgenic rice (NT), and the transgenic rice has a significantly higher plant height than the non-transgenic rice. The result therefore showed that the introduction of the trans-factors rendered the plant tolerant to iron deficiency in alkaline soil.

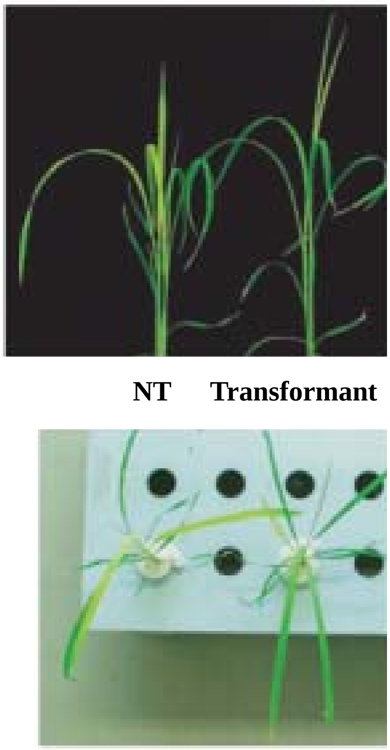

Fig. 11: Iron-deficiency tolerance to IDEF1 transgenic rice
Left: Non-transgenic rice (NT)
Right: Transgenic rice (Transformant)

Intellectual Property Rights

The following patent applications have been filed for the present technique.

(1) Japanese patent 3920453 (Application No. 10-096637, filing date: March 24, 1998)
Title of the Invention: Method for transforming plant, the resultant plant and gene thereof
International application: PCT/JP99/01481 (filing date: March 24, 1999)
US patent: 6849724
CA patent: 2329273
AU patent: 754079
EP patent: 1080633 (DE, ES, FR, GB, IT, and NL)

(2) International Application: PCTJP99/02305 (filing date: April 30, 1999)
Title of the Invention: Nicotinamine synthase and gene encoding the same
JP patent: 3893024
US patent: 7192755
US patent: 7157260
CA: Being prosecuted

AU patent: 759256
EP patent 1077255 (DE, ES, FR, GB, IT, and NL)

(3) JP application 11-190318 (filing date: July 5, 1999); being prosecuted
Title of the Invention: Construction of rice tolerant to iron deficiency
International application: PCT/JP00/04425 (filing date: July 4, 2000)
US patent: 7259292
IN patent: abandoned
AU patent: 772529
CN patent: 008100047
KR patent: 454083
EP patent: being prosecuted

(4) JP application 2002-177943 (filing date: June 19, 2002)
Title of the Invention: Tissue-specific/environmental stress-specific promoter
International application: PCT/JP2003/007784 (filing date: June 19, 2003)

(5) JP application 2002- 233949 (filing date: August 9, 2002)
Title of the Invention: Adenine phosphoribosyltransferase (APRT) agent for improving acquisition rate of transformant, and agent for improving early growth of plants

(6) JP application 2003-70926 (filing date: March 14, 2003)
Title of the Invention: Novel nicotinamine synthase and genes encoding same

(7) JP application 2003-177063 (filing date June 20, 200)
Title of the Invention: Cis-element for inducing iron deficiency responsiveness and/or root-specific expression of plants
Co-Applicant: Central Research Institute of Electric Power Industry

(8) JP application 2005-9600 (filing date: January 17, 2005)
Title of the Invention: Transformation vector for plants

(9) International application: PCT/JP2004/014064 (filing date: September 27, 2004)
Title of the Invention: Transporter participating in the absorption and transportation of complex of metal such as iron in rice and gene thereof

(10) JP application 2006-218548 (filing date: August 10, 2006)
Title of the Invention: Deoxymugineic acid synthase and use thereof

(11) JP application 2007-117725 (filing date: April 26, 2007)
Title of the Invention: Polypeptides for improving tolerance to iron deficiency in plants and use thereof

Closing Remarks

Due to the explosive population growth, the crop production per capita has been declining since the peak year 1985. In this connection, many productive farmlands have been disappearing by urbanization. There has also been destruction of environment, ranging from desolation of soil to desertification. Taken together, the number of farmlands has been decreasing worldwide every year. Meanwhile, there is a worldwide distribution of many farmlands known as non-fertile soils, which offer only marginal productivity. About 25% of all terrestrial land areas are such non-fertile soils, or more specifically calcareous alkaline soils. The cause of poor agricultural productivity in calcareous alkaline soils is iron deficiency.

In this conjuncture, the present study developed a technique that can dramatically increase productivity of crops and trees, by overcoming iron deficiency in calcareous alkaline soils using a group of genes associated with iron absorption in plants (genes involved in the synthetic pathways of mugineic acids, Fe(II) transporter genes, Fe(III) reductase genes, genes encoding cis-elements and trans-factors, etc.). Specifically, transgenic rice was produced by introducing such a group of genes, and tolerance to iron deficiency was demonstrated at field level.

The world expects increased production of non-rice crops such as corn, sugar cane, barley, and wheat not only as a food source but as bio-ethanol materials for energy applications. Japan Science and Technology Agency intends to license the present technique to those interested.

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