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Genetic Improvement of Iron Content and Stress Adaptation in Plants Using Ferritin Gene

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Introduction

Iron deficiency resulting from an inadequate diet is a serious nutritional problem. Anaemia derived from iron deficiency causes a host of illnesses, including abortion, brain damage in infants, increased susceptibility to infection, and chronic exhaustion (Baynes and Bothwell, 1990). An estimated 30% of the world's population suffer from some level of iron deficiency, with the highest prevalence found in the developing countries. On the contrary, iron intake by people in developed countries is adequate, and the prevalence of iron deficiency is decreasing. However, anaemia derived from iron deficiency in Japanese females is a concern, and its incidence has remained constant in recent years. There are two approaches to overcome the iron deficiency: one is supplementation of iron to dairy diets, and another is fortification using biological methods. Although supplements added to food or taken in tablet form are effective in preventing and controlling iron deficiency, such treatments are difficult to implement in developing countries because of the associated high costs and lack of primary health care programmes. The other approach is the fortification using biological methods, and there are two ways. The first way is to increase the iron concentration of the hydroponic culture media or soil. This method is costly and cannot accumulate iron to a desirable part of the plant. The second way is to improve the iron content in crops genetically. This way seems better than the first one. This is

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Abbreviations: AO, active oxygen; CaMV, cauliflower mosaic virus; EP, extension peptide; IRE, iron responsive element; MA, mugineic acid; MDA, malondialdehyde; NA, nicotianamine; NAAT, nicotianamine aminotransferase; PCR, polymerase chain reaction; QTL, quantitative trait locus; RT-PCR, reverse transcriptase PCR; SAM, S-adenosylmethionine; TP, transit peptide.

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because once a plant variety with high iron content has been produced, people can obtain iron from new crops at a low cost.

We have worked on transgenic plants expressing the ferritin gene with a view to overcome the nutritional problem of iron. The iron storage protein ferritin is common to living organisms, including humans and higher plants, and consists of 24 subunits, which can store up to 4000 iron atoms. Thus, enrichment of plants with ferritin using a transgenic technology could prove of benefit for increasing the iron levels in crops. Since Hyde and co-workers' first report in 1963 (Hyde *et al.*, 1963), plant ferritin has now been identified in many species, such as soybean, French bean, cowbean, pea, maize, alfalfa, cocklebur, and whole or parts of the ferritin cDNAs have been cloned from more than ten plant species in recent years.

On the other hand, the production of stress resistant plants is also an important goal, because growth and yield of plants are always restricted by stress, whatever its cause. Such stresses in plants can be classified into two categories: biotic (pathogen, insect, weed) and abiotic (temperature, light, salinity, drought, flooding, freezing nutrition), although both categories are common in the mechanism that induces damage to inner membranes and DNA by the production of active oxygen (AO) species. Recent developments in gene transfer technology into plants has made it possible to produce transgenic plants containing genes coding anti-stress enzymes such as peroxidase and APX, with the result that these plants have obtained some resistance to stresses. Plant ferritin is thought to have an important function besides iron storage. In a plant cell, free iron is very hazardous because of its ability to generate AO species. Ferritin functions as a scavenger of AO species due to binding to free iron in the cell.

In this review we would like to consider such new ideas for the creation of novel crops expressing the ferritin gene. The structure, role, and cloned genes of plant ferritin will be explained briefly at first, followed by a summary of the iron fortification of crops by ectopic ferritin gene expression. Finally, the possibility of breeding anti-stress plants using the ferritin gene is discussed.

Overview of ferritin

STRUCTURE

Plant ferritin is synthesized as the precursor containing a unique amino acid sequence made up over 70 residues on the N-terminal, followed by the relatively conserved region among other ferritins (*Figure 14.5*). The first part of this region is called the transit peptide (TP), which is responsible for plastid targeting and processed on entry into the plastid. The second part is called the extension peptide (EP), which may be lost in the germination process (Ragland *et al.*, 1990). The TP and EP consist of about 40 and 30 amino acid residues, respectively. The conserved region neighbouring the extension peptide has been shown to have 40–50% sequence identity with mammalian ferritins.

The three-dimensional structure of almost all ferritins is highly conserved, although their primary structures vary among organisms (Harrison and Arosio, 1996). The three-dimensional structure of plant ferritin has not been determined yet, but from the computational analysis and the amino acid sequence alignment with mammalian ferritin, it is suggested that the higher-order structure of the plant ferritin is similar to

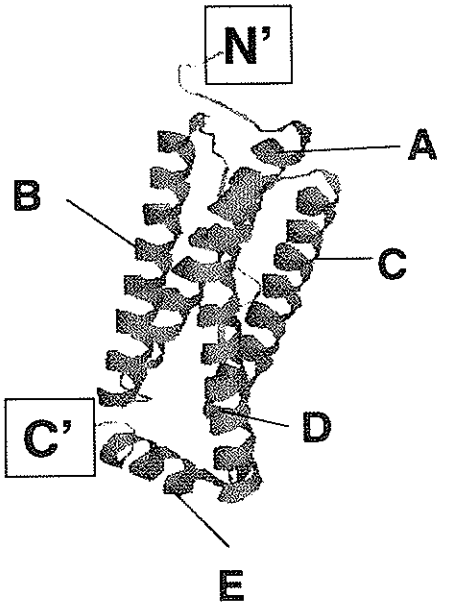

Figure 14.1. Ribbon diagram of human H ferritin (Lawson *et al.*, 1991). A–E indicates helix. N' and C' indicates an N-terminal and a C-terminal, respectively.

the animal one (Lobréaux *et al.*, 1992b). Therefore, for explaining the higher-order structure of the plant ferritin, we review here mainly the structure of mammalian H-(heavy) chain and L-(light) chain ferritins, whose crystal structure has been established (Clegg *et al.*, 1980; Lawson *et al.*, 1991; Trikha *et al.*, 1994, 1995; Hempstead *et al.*, 1997). The H-chain of ferritin possesses ferroxidase activity and is responsible for the uptake of the iron atoms into the cell (Lawson *et al.*, 1989), whilst the L-chain has no ferroxidase activity and is responsible for iron mineralization (Levi *et al.*, 1989). The subunit of ferritin is a four-helix bundle, consisting of four long helices A–D, the fifth short helix E, and a long loop connecting B and C helices (*Figure 14.1*). The A, B, C, D, and E helices of human H-chain consist of 30, 30, 31, 34, and 12 amino acid residues, respectively. In the case of the plant ferritin, the EP is supposed to form a unique helix called P-helix by computational analysis (Lobréaux *et al.*, 1992b).

Plant ferritins are huge oligomeric proteins, of oligomeric molecular weight estimated to be 540,000 (Laulhère *et al.*, 1988), and assembled from 24 subunits into a spherical shell, i.e. the same as for the other ferritins (Van der Mark *et al.*, 1983b). The external and internal diameters of the ferritin are about 130Å and 80Å, respectively (Trikha *et al.*, 1994). Iron atoms are stored in the inner cavity of the protein shell. In the assembled protein shell, subunits can be related by 4-fold, 3-fold, and 2-fold symmetry (*Figure 14.2*), and there are channels around the 3-fold and 4-fold axes, individually. The channel around the 3-fold axis is composed of relatively hydrophilic residues and it is supposed that iron atoms are passed through this channel to enter the cavity (Yablonski and Theil, 1992). This channel is thought to be well conserved among plant and mammalian ferritins. The other channels existing around the 4-fold inter-subunit symmetry axes are narrower than the 3-fold channel. The

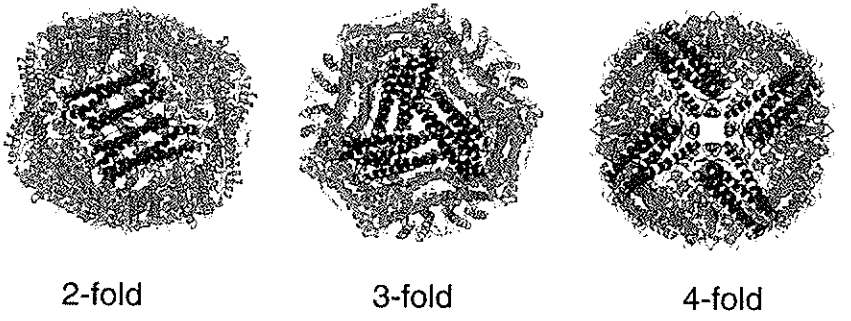

Figure 14.2. Subunit packing in the protein shell of human H ferritin. (a) 2-fold, (b) 3-fold, and (c) 4-fold inter-subunit symmetry axes exist on the assembled protein shell (Lawson *et al.*, 1991).

4-fold channel is also predicted to be hydrophilic (Lobréaux *et al.*, 1992b), although, in the case of human and other mammalian ferritins, side-chains of hydrophobic residues, such as leucine, are positioned around this channel (Hempstead *et al.*, 1997).

In general, the sequence identity of residues forming the inter-subunit interface is remarkably high, e.g. the sequence identity of interface residues is 71%, while the identity of overall sequence of the subunits is 53% between human H-chain and horse L-chain (Hempstead *et al.*, 1997). In the sequence of plant ferritin, the residues forming the inter-subunit interface across the 4-fold symmetry axes are less conserved than that of 2-fold and 3-fold axes. In particular, the apolar residues in the E-helix, which contacts with the neighbouring 4-fold axis-related subunit by hydrophobic interaction, are substituted by polar residues such as histidine in the plant ferritin. In short, there are two unique structural points of plant ferritin, first the existence of the N-terminal extension peptide, second the hydrophilicity of the channel around the 4-fold inter-subunit symmetry axes. However, the elucidation of the crystal structure of plant ferritin is needed for further understanding of iron metabolism in plants.

ROLE

Ferritin is thought to play at least two important roles in plants. The first is storage of iron, an element required by metal enzymes involved in photosynthesis and other respiratory processes. Ferritin can store up to 4000 iron atoms in its central cavity (Korcz and Twardowski, 1992) and is usually observed in seeds during maturation or in nodule development in legumes (Kimata and Theil, 1994; Lucas *et al.*, 1998). However, under normal nutritional conditions, ferritin is hardly detectable in vegetative organs. Iron in soil usually exists as a part of water-insoluble compounds and is thus difficult for plants to absorb at rates required for growth. Storage of iron by ferritin alleviates this problem but a small amount of ferritin seems to be enough to store iron for this role. Therefore, the plant ferritin mainly functions as an iron reservoir for early development of juvenile plants and for the formation of legume nodules, relating to nitrogenase and leghaemoglobin (Huang and Barker, 1983; Ko *et al.*, 1987). On the other hand, ferritin is induced by iron overload in soybean, French bean and maize plantlets (Lobréaux *et al.*, 1992a; Proudhon *et al.*, 1989; Van der Mark

et al., 1983a). The second role of ferritin is thus thought to be protection of cells against the toxic effects of iron (or other metals) overload. Fe (II) in the presence of dioxygen can catalyze the production of active oxygen species, known as the Fenton reaction, which accelerates the peroxidation of lipids, inactivation of enzymes, and DNA damage. Ferritin protects the cell from such chemistry derived from free iron (Theil, 1987).

FERRITIN GENES AND THEIR EXPRESSION

In 1990, a part of a plant ferritin gene was first cloned from soybean hypocotyl by Ragland *et al.* using a frog ferritin cDNA as a probe. Her group completed the cloning of the whole cDNA of soybean ferritin from iron-induced cell suspensions (Lescure *et al.*, 1991). Since this work, other ferritin genes of beans have been cloned from French bean (Spence *et al.*, 1991), pea (Lobréaux *et al.*, 1992b), cowpea (Wicks and Entsch, 1993), and *Acacia mangium* (Hoya *et al.*, 1997). More recently, many cDNA sequences of ferritin genes have been registered on the databases, for example, watermelon (Shin, 1999), the common ice plant (Cushman, 1997), sugar beet (de los Reyes *et al.*, 2000) and rice (Uchimiya, 1993). Buchanan-Wollaston and Ainsworth (1997) have cloned a ferritin gene as a senescence related gene from *Brassica napus*. The gene was expressed in young green leaves, transcript levels falling in mature leaves. However, the expression increased significantly at senescence stage. Hortensteiner *et al.* (2000) reported a putative ferritin gene of *Chlorella protothecoides* during the degreening process. In addition to the genes mentioned above, ferritin genes have been reported in *Medicago truncatula* (Gyorgyey *et al.*, 2000), loblolly pine (Li *et al.*, 1998) and tobacco (Yoshihara *et al.*, 2000). At first, the ferritin gene was thought to be a single copy gene, however recent observations have indicated some evidence indicating the ferritin gene exists as a gene family. For instance, Wicks and Entsch (1993) have indicated that some different ferritin genes exist in cowpea. Recently, they showed four members of the ferritin gene family in cowpea (Wardrop *et al.*, 1999). In *Arabidopsis*, Gaymard *et al.* (1996) have mentioned the existence of some ferritin genes apart from the gene they cloned in comparison with the expressed sequence tags (ESTs). Lobréaux *et al.* (1992a) have isolated two ferritin cDNAs from maize (this was the first clone from monocot plants).

The expression of the animal ferritin gene which has an iron responsive element (IRE) is regulated at the translational (post transcriptional) level in the cells. IRE has a stem loop structure and is located within the 5' untranslated region of ferritin mRNA (Theil, 1990). When the cellular iron level is low, iron regulatory protein binds to the stem loop of the IRE, resulting in inhibition of the translation of ferritin. In contrast, there is no IRE in the plant ferritin genes and the expression of plant ferritin is just controlled at the transcriptional level (Van der Mark *et al.*, 1983a; Proudhon *et al.*, 1989; Lobréaux *et al.*, 1992a). However, Lobréaux *et al.* (1993) have indicated that exogenous plant hormone abscisic acid (ABA) induces ferritin mRNA in leaves of iron-starved maize even though ferritin abundance is much lower in contrast to the situation of iron overloading. The implication of this finding was that a possibility exists for translational control in plant ferritin. In fact, soon after, Loisy *et al.* (1996) showed the translational regulation of plant ferritin gene in a maize mutant in response to iron overloading. The mechanism(s) without IRE to

regulate the expression of plant ferritin is still unclear, however Wei and Theil (2000) have reported that a novel iron regulatory element (FRE) controls iron-mediated depression of the ferritin gene.

Development of iron-rich crops

STRATEGY FOR INCREASING IRON CONTENT IN PLANTS

Two strategies have been considered for increasing the iron content in plants or to avoid the suppression of plant growth derived from iron deficiency. The first is an *agricultural method*. Iron in the soil exists as part of water-insoluble compounds, especially in alkaline soils, and it is difficult to absorb at the rates required for growth of plants. Therefore to solve this problem, spraying iron onto the leaf surface is effective for reducing chrolosis and restoring growth. Inoue *et al.* (1995) have demonstrated that iron-rich vegetables without injury could be guaranteed by controlling the iron concentration in the treatment solution and also the soaking time. However, to increase the iron content of plants without injury is difficult in general.

The second strategy is a *breeding method*. Researchers have selected crops grown in alkaline soils or high iron content crops. Gregorio *et al.* (2000), for example, report the selection of high iron content rice to reduce anaemia derived from iron deficiency and have mapped three QTLs for high iron trait on three chromosomes. Variety breeding biotechnology, including genetic modification of plants, has become a strong tool for breeding iron-fortified crops, and we consider below the target gene(s) to be used for these crops.

Plants can be classified according to iron uptake strategies (Romheld and Marschner, 1986) (*Figure 14.3a,b*). Most plants, except for grasses, use 'strategy I': Under iron-deficient conditions, plants of this type secrete H^+ to acidify the soil using a H^+-ATPase (Harper *et al.*, 1989, 1990; Campos *et al.*, 1996), reduce Fe(III) to the more soluble Fe(II) form with a Fe(III) chelate reductase bound to the plasma membrane, and take up the Fe(II) form by an Fe(II) transporter. The iron form of translocation via the xylem is known as the *ferric citrate complex*. By contrast, minerals including iron may be transported at least in a chelated form in the phloem. Interestingly, the non-proteinaceous amino acid, nicotianamine (NA) – which is ubiquitous in the plant kingdom – could be a candidate for a mineral chelator in the phloem (Scholz *et al.*, 1992). NA is a precursor of mugineic acid, which is a key compound for iron uptake system in the 'strategy II' plants. The iron taken up from the soil is transported to tissues and excess iron is then isolated in the ferritin as the Fe(III) form. The structure, role and gene of ferritin are explained in detail in the section titled 'overview of ferritin'.

We have at least four target proteins to increase the iron content in plants so far. However, it may be difficult to use a modified gene of the H^+-ATPase alone because the ability to acidify the rhizosphere depends not only on iron deficiency but to some extent on the ion uptake balance. Yi and Guerinot (1996) have confirmed that iron must be reduced before it can be transported into the cell and have concluded that Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency: this conclusion derives from the analysis of *Arabidopsis* mutants (*frd1*) which did not show induction of Fe(III) chelate reductase activity under iron-deficient conditions.

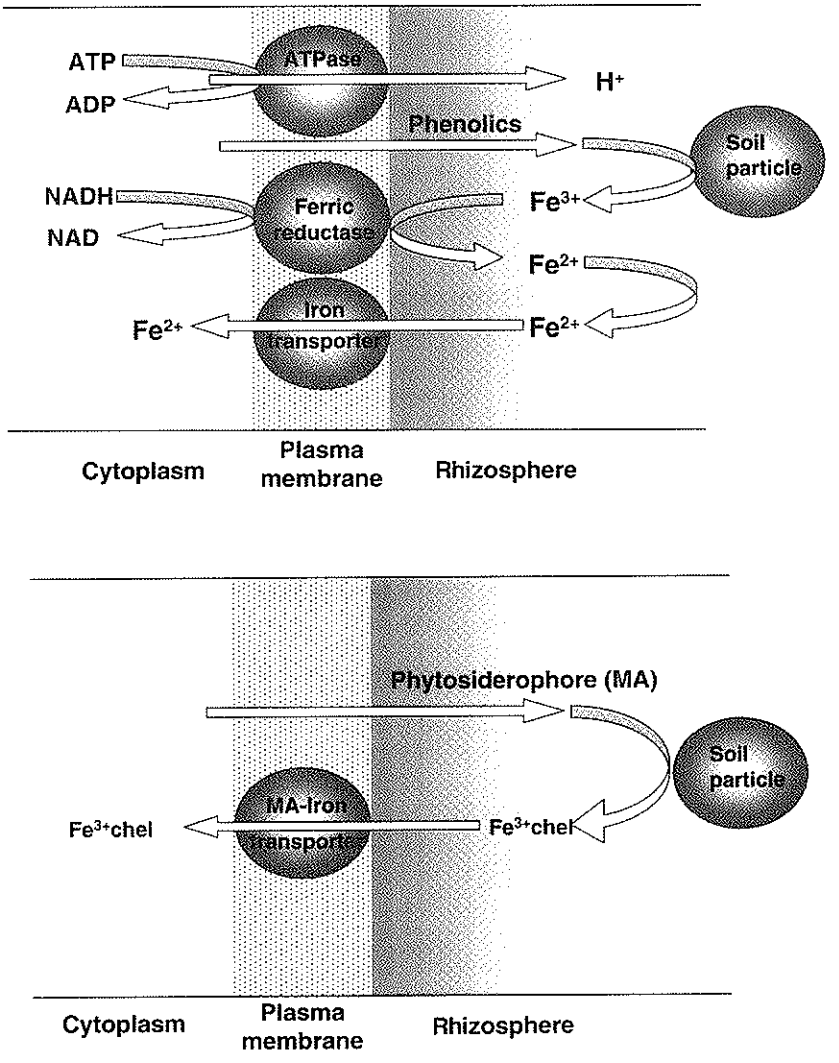

Figure 14.3. Iron absorption strategies of plants. (a) Model for iron acquisition of strategy 1 in higher plants except *Gramineae*. (b) Model for iron acquisition of strategy 2 in *Gramineae*.

In 1999 their group (Robinson *et al.,* 1999) isolated the iron reductase gene (*FRO2*) which was allelic to the *frd1* mutation. Transgenic *frd1* mutant containing *FRO2* showed that *FRO2* complemented the *frd1* mutant phenotype. Namely, the low-iron-inducible ferric chelate reductase activity was recovered by gene transformation. These results indicate that *FRO2* must be one of the useful genes for the iron uptake system in transgenic plants. Eide *et al.* (1996) identified an iron transporter gene, called iron-regulated transporter (*IRT1*), in *Arabidopsis* using functional expression in yeast. The *IRT1* was a member of a gene family, expressed in roots and induced by iron deficiency. Recently, the IRT1 protein has been understood to be a broad-range metal ion transporter in plants because it mediated uptake of manganese, zinc, cobalt,

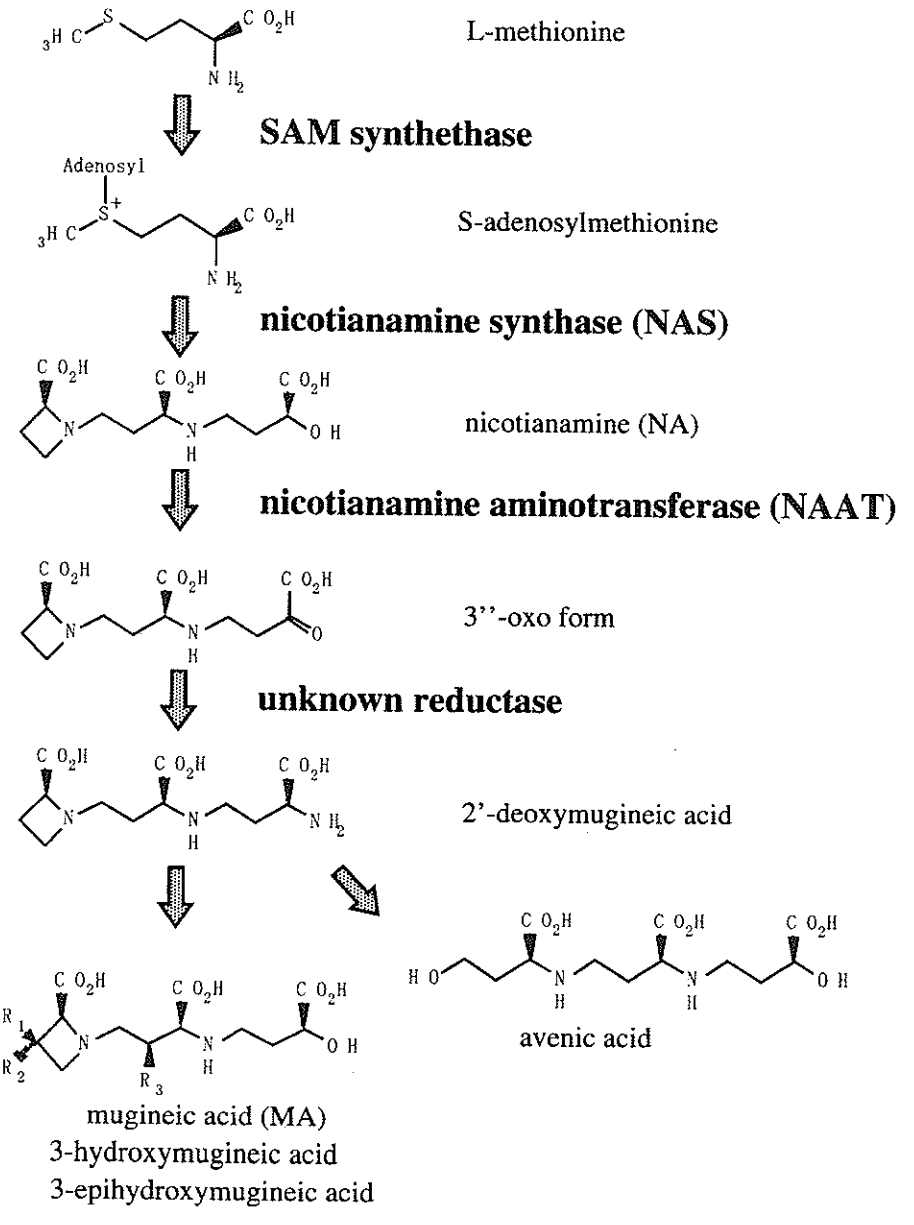

Figure 14.4. Biosynthetic pathway of the MAs of phytosiderophores (Higuchi *et al.*, 1999).

in addition to iron under iron-deficient conditions (Korshunova *et al.*, 1999). If we were to use the *IRT1* for iron fortification of crops, there would be a danger of over-uptake of metals hazardous for human health.

Strategy II plants, including major cereal crops such as rice, wheat, barley and oat, secrete low-molecular-weight compounds called phytosiderophores, e.g. mugineic acid (MA), which binds to Fe(III) (*Figure 14.3b*). The conjugate of Fe(III)-MA is probably taken up through an as yet unknown transport system. Another probable

system of iron uptake is that the conjugate of Fe(III)-MA splits at the outer face of the plasma membrane and ferric iron could be separately absorbed into cytoplasm. The biosynthetic pathway of the MA family has now been determined and the related genes have been cloned (*Figure 14.4*). *S*-Adenosylmethionine (SAM) is synthesized from methionine by SAM synthetase. Subsequently, one molecule of NA is formed with three molecule of SAM by NA synthase. NA is then converted to [3'-keto acid] by nicotianamine aminotransferase (NAAT). The MA family is produced from deoxymugineic acid which is formed from [3'-keto acid] by an as yet unknown reductase. The genes of SAM synthetase, NA synthase, NAAT have all been cloned (Mori and Takizawa, 1996; Higuchi *et al.*, 1999; Takahashi *et al.*, 1999). These genes, including the gene for the unknown reductase that catalyzes [3'-keto acid] to deoxymugineic acid, should be effective for adaptation to alkaline soils rather than for breeding of iron-fortified crops.

We have noticed that the ferritin gene offers a great advantage for increasing iron in plants among the candidate genes for the following reasons: (a) Ferritin can markedly store iron atoms in contrast with other iron binding proteins, which bind to few irons. (b) Ferritin is formed from a single subunit. Therefore there is no need to introduce more than one gene for inducing the function of the translated production. (c) Since ferritin exists in both monocot and dicot plants, the ferritin gene is useful for both types of plants. (d) Iron stored in ferritin is bioavailable. Iron form and iron absorption in the human body are important factors whatever strategy we choose for increasing iron content of plants (Schümann *et al.*, 1998). The various dietary iron sources have been examined by Beard *et al.* (1996): the bioavailability of extrinsic ferritin as an iron supplement was almost the same as that of $FeSO_4$ in rats which had been made anaemic by dietary iron deficiency. (e) Even if the other genes can introduce the uptake of iron into the cell, such iron has to be isolated in order to avoid the generation of oxidative stress mediated by free iron. (f) Transgenic plants

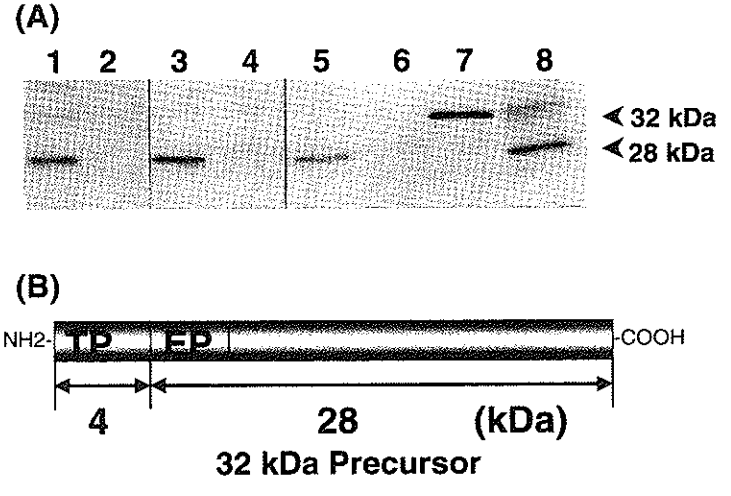

Figure 14.5. (A) Western blot analysis of transformants reproducing the soybean ferritin subunit. Rice (lane 1, 2), Tobacco (lane 3, 4), Lettuce (lane 5, 6), Soybean ferritin subunit produced by *E. coli* (lane 7), Soybean (lane 8). Transgenic plants (lane 1, 3, 5), Control plants (lane 2, 4, 6). (B) Schematic representation of the ferritin subunit. TP: transit peptide, EP: extension peptide.

possessing the ferritin gene should be acceptable by the public because ferritin exists in host plants already.

For future work, it will be more important for the effective improvement of crops to combine two or more relevant genes into a plant. Controlling two or more genes systematically would, however, be difficult because the expression of these genes would inhibit the function of each other. However, it may still be possible to breed 'iron super-rich crops', for instance, if the ferritin gene and IRT1 gene were successfully co-transferred and expressed independently in plants.

TRANSGENIC TECHNOLOGY WITH THE FERRITIN GENE

In this section, we summarize the transgenic plants containing the ferritin gene related to iron accumulation. Goto *et al.* (1998) have indicated that the expression of exogenous ferritin gene driven by cauliflower mosaic virus 35S promoter leads to iron accumulation in host tobacco plants. In that study, ferritin cDNA was synthesized using the RT-PCR technique with poly(A)+ mRNA extracted from soybean (*Glycine max* Merr.) leaves which were harvested 24 hours after transfer of plantlets from a nutrient solution without Fe-EDTA to that with Fe-EDTA. The ferritin subunit derived from soybean cDNA in transgenic tobacco plants was cleaved of the TP and EP in a fashion similar to that of the native ferritin subunit in soybean. *Figure 14.5* indicates that this finding is common among the transgenic plants. In the best case, the transformant contained 1.3-fold greater amount of iron than the control plants. There was a linear relationship between the iron content in the leaves of the transformants and the amount of expressed ferritin, as calculated from the immunoblot signals using a densitometer. In that study, Goto *et al.* (1998) also mentioned that, under conditions of increased iron supply to culture medium or soil, the iron content of transgenic plants could be increased. They had estimated that one molecule of exogenous ferritin contained less than 1000 iron atoms, based on the assumption that the molecular weight of ferritin is 540,000 (Laulhère *et al.*, 1988).

Iron in transgenic tobacco plants expressing the native or TP-less soybean ferritin gene accumulates in the cytoplasm and the plastid, respectively (Wuytswinkel *et al.*, 1998). In that study, Wuytswinkel *et al.* (1998) used two types of transgenic plants: One (P) had normal ferritin which accumulated in the plastid. The other (C) contained the TP-less ferritin, which could not target to the plastid, resulting in accumulation of ferritin in the cytoplasm. Both transgenic plants were able to accumulate iron. They showed an increasing iron storage capacity with the concomitant increase in iron uptake mechanism. The root ferric reductase activity was 2- to 5-fold higher in the P and C plants than that in the control plants. The iron content of the leaves was 2- to 3-fold greater in the transgenic plants than that in the control plants. Grusak and Pezeshgi (1996) observed similar phenomena in an Fe-hyperaccumulating mutant of pea. However, the mechanism of shoot-to-root signal transmission was not clear.

The endosperm, edible part of rice is ideal for expressing the exogenous ferritin gene for the following reasons. First, rice is one of the most important foods in the world, especially for Asia, Africa and South America. More than 50% of the world's population depends on rice. However, the nutritional quality of rice is not always better than that of the other major food crops, such as wheat or corn. In addition, the seed-specific expression of exogenous ferritin is useful for reducing the influence

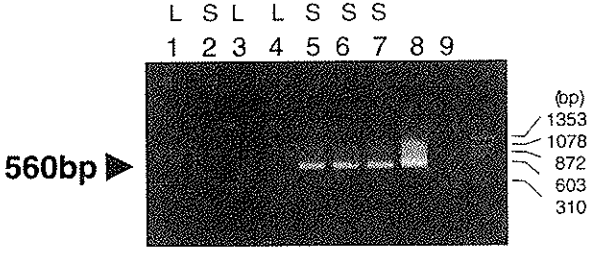

Figure 14.6. RT-PCR analysis of total RNA from transgenic rice plants expressing ferritin gene. Non-transformant (lanes 1, 2), Transformants (lanes 3–7), Positive control (lane 8), No template (lane 9), Molecular marker (lane 9). L: leaf, S: seed.

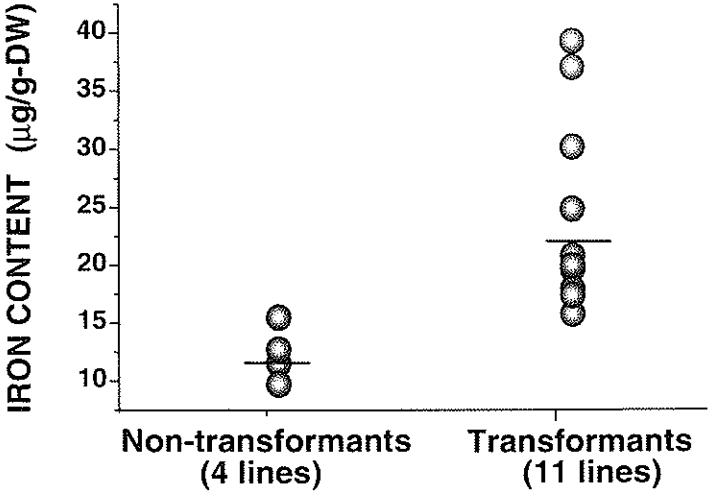

Figure 14.7. Comparison of the iron content in transgenic rice seeds expressing ferritin gene.

of it on endogenous metabolism in the transgenic rice plant, since seeds usually set after the vegetative growth period. To improve the iron content in rice, Goto *et al.* (1999) transferred the soybean ferritin cDNA into *Oryza sativa* (cv. Kita-ake) by *Agrobacterium*-mediated transformation. They obtained thirty independent transgenic rice lines. All lines produced self-pollinated seeds. The ferritin cDNA was expressed under the control of a rice seed storage protein glutelin promoter (*GluB-1*) to accumulate iron specifically in grain. These researchers recognized that ferritin gene expression was controlled by *GluB-1* promoter using at first reverse transcriptase PCR. The partial fragment of ferritin cDNA was amplified from mRNA extracted from seeds, but not leaves, of transgenic plants (*Figure 14.6*). Subsequent analysis of the protein level was carried out to confirm whether the ferritin can accumulate in the grain. The ferritin was detected from most of the transgenic grains by Western blotting. The detection of a 28 kDa mature subunit, corresponding to the ferritin subunit in soybean and in transgenic tobacco plants, meant that the ferritin subunit derived from soybean cDNA in monocot plants had been cleaved to release the TP in a fashion similar to that for the case of dicot plants. Immunological tissue printing was

Table 14.1. Percentage of iron content in some tissues from transgenic rice line expressing the ferritin gene. The iron content of each tissue of the control plant has been normalized to 100%. There is no significant difference between the tissues of the transgenic rice and that of the controls, except seed

	Seed	Leaf	Stem	Root
Transformants				
(1)	266.4	87.8	95.8	100.6
(2)	251.0	82.8	103.2	101.1
Nontransformant	100	100	100	100

also carried out to visualize the distribution of soybean ferritin in grain. The subaleurone layer of the endosperm stained most intensely, indicating a high accumulation of ferritin in this tissue. These results were consistent with the β-glucuronidase staining pattern directed by the *GluB-1* promoter (Takaiwa *et al.*, 1991). In addition, those researchers examined the iron content in seeds, leaf, stem and root. Iron levels varied from 13.3 to 38.1 and 8.6 to 14.3 μg/g dry weight in seeds of transformants and non-transformants, respectively (*Figure 14.7*). There was no specific difference of iron content in non-seed tissues between transformants and non-transformants (*Table 14.1*). The results for the iron distribution in some tissues are reasonable because these distributions correspond with the pattern of the ferritin expression driven by the *GluB-1* promoter. The authors have evaluated that 30–50% of the adult iron requirement a day would be provided with the ferritin-rice.

Goto *et al.* (2000) have also transferred the ferritin gene to lettuce (*Lactuca sativa* cv. Green leaf) plants, which is a popular vegetable in the world. To enrich popular vegetables with iron is an effective measure to overcome iron deficiency. The iron content of lettuce is appreciably lower than that of spinach, though lettuce contains 100 times less oxalic acid. Since oxalic acid in the spinach binds to calcium, resulting in kidney stones and also inhibits iron absorption, iron-fortified lettuce would be advantageous to human health as a vegetable free of harmful substances. In that study by Goto *et al.* (2000), the transformation of the lettuce plants was mediated by *Agrobacterium tumefaciens* with the soybean ferritin gene driven by the 35S promoter. One or two copies of the ferritin gene were integrated into the host genome. These researchers detected ferritin gene expression by Western blot analysis. Two different sizes of bands were observed. One was a 28 kDa major band and the other was a 26.5 kDa minor band, which was not always detected in some transgenic plants. These phenomena were also observed in transgenic tobacco plants (Goto *et al.*, 1998). These results had indicated that the ferritin subunit was produced by two steps through the excision of the TP and EP after translation from soybean mRNA expressed in the transgenic plants. They discovered a constant manganese content in leaves of transgenic plants, irrespective of the increase in iron content.

Manganese had been selected for preliminary evaluation of the specific accumulation of heavy metals in transgenic plants. It seems that expressing the soybean ferritin gene in transgenic lettuce can affect the accumulation of iron, but not the accumulation of the other harmful metals such as Cd, Cu, at least in normal crop fields.

Protection of plants by ferritin against a wide range of stresses

Active oxygen (AO) is usually formed in the chloroplast by two reactions. One is from

the direct transfer of excitation energy from chlorophyll to oxygen which produces singlet oxygen. The other is the single electron reduction, resulting in the superoxide radical. It is also known that various stresses (e.g. high and low temperature, draft, flooding, ozone, heavy metals, infection with microorganisms) induce production of AO species that are key deleterious chemicals producing damage to plant cells. Free iron in cells can mediate the generation of AO species (Becana *et al.*, 1998). Therefore, to elevate plant productivity, decreasing free iron in cells should be an important strategy. Ferritin probably participates in the protection of cells by storing excess free iron. Ferritin synthesis is induced by an iron-mediated oxidative stress (Lobréaux *et al.*, 1995; Savino *et al.*, 1997) and ferritin is located in the chloroplast where AO is mostly generated. In the section that follows, we now consider the benefit of transgenic plants possessing the ferritin gene against the stress generated by heavy metals and infection by pathogen. Furthermore, we show growth stimulation by expression of the introduced ferritin gene.

HEAVY METAL ACCUMULATION IN PLANTS

Contamination of soils or water by heavy metals has been a continuous problem worldwide. The contamination by Cd, Cu, Pb, As and Hg is well known; however, unfamiliar metals have also been propagated, such as Be, Cr(IV), Sb, Ti, arguably a result of the affluent age. Recent research has provided a new technology called phytoremediation for cleaning soils or water. Moreover, genetically modified plants have been adapted for phytoremediation. For example, transgenic plants expressing the gene of metallothionein which can bind to Cd strongly have been developed (Misra and Gedamu, 1989; Maiti *et al.*, 1991; Pan *et al.*, 1994; De Borne *et al.*, 1998). These plants have tolerance to Cd and ability of Cd accumulation. Zhu *et al.* (1999) showed Cd accumulation and tolerance of Indian mustard which over-expressed the glutathione synthetase gene.

By contrast, Sczekan and Joshi (1989) have investigated whether the soybean ferritin is capable of binding cations such as aluminum, beryllium, cadmium and zinc. All of the metals were bound by the protein with similar affinities, but smaller metals (Al and Be) were bound in much higher amounts than the heavier metals. The authors indicated that the phosphate anion in the iron core of ferritin is needed to bind non-ferrous metals. The iron core of ferritin from pea seed was shown to be amorphous (Wade *et al.*, 1993), which correlated with the high phosphate content (1800 Fe, 640 P atoms/ferritin molecule). A similar result was obtained by Barcelo *et al.* (1997) with alfalfa ferritin. There is a possibility of the existence of a novel ferritin which can bind easily to heavy metals except for iron, since the plant ferritin gene exists as gene family. In animal ferritins, some metals (Be, Cu, VO, Cd, Tb, Zn) have been reported to bind to ferritin (Zaman and Verwilghen, 1981; Price and Joshi, 1982, 1983; Wardeska *et al.*, 1986). The introduction of the animal ferritin gene into plants should be an interesting trial for phytoremediation. Ferritin can store much more iron than its subunit can bind to iron due to a ball-shaped structure. Therefore, if the amino acids of a ferritin subunit binding to iron were changed by genetic manipulation to other amino acids which bind to other heavy metals, and the ferritin kept a three-dimensional structure, transgenic plants possessing the modified ferritin gene would provide a strong 'hyper-accumulater' tool against heavy metals.

OXIDATIVE DAMAGE AND PATHOGEN-TOLERANT PLANTS

Deák *et al.* (1999) have shown that transgenic tobacco plants containing an alfalfa ferritin gene were tolerant to oxidative stress and pathogens. They first compared how photosynthesis was affected with excess iron treatment or the herbicide paraquat (methylviologen) which generated oxidative stress in the cells of the transformants and non-transformants. The transgenic plants tolerated oxidative injury induced by light irradiation and had chlorophyll levels 3 times higher than that of controls at 3 days after the paraquat treatment. It is known that plants produce AO species when they recognize invading pathogens, resulting in necrosis of the host plants (Baker and Orlandi, 1995; Lam and Dixon, 1997). Then, secondly, they tested whether plants whose antioxidant capacity was elevated by the expression of ferritin obtained resistance to pathogens. The transgenic plants were shown to obtain resistance against 3 microorganisms (tobacco necrosis virus, *Alternaria alternata* and *Botrytis cinerea*). Similar results concerning oxidative stress were obtained from another transgenic experiment with the ferritin gene (Wuytswinkel *et al.*, 1998), which investigated the influence of paraquat treatment on total protein concentration and malondialdehyde (MDA) content of leaf discs. MDA is an indicator of lipid peroxidation. Protein concentration of control plants decreased by 63% in response to the paraquat treatment while almost no proteolysis was observed in transgenic plants. MDA concentration of control plants was increased twofold, whereas the concentration was not affected by the paraquat treatment in transgenic plants.

HIGH GROWTH RATE PLANTS

Transgenic tobacco expressing bacterial haemoglobin (VHb), known as iron binding protein, was found to enhance growth (Holmberg *et al.*, 1997). The transgenic plants showed superior growth rate to that of controls at any time in their growth. Namely, the growth was greater during 35 days after germination and the time to the onset of flowering was faster in the transgenic plants compared to the control plants. The chlorophyll content of the transgenic plants increased by 30–40% compared to the controls. The authors mentioned that the possible function of VHb, such as scavenging of oxygen radicals, may contribute to the enhanced growth of the transgenic plants. Kawaoka *et al.* (1994) introduced the horseradish peroxidase gene, *prxC1a*, into tobacco plants which subsequently exhibited an increased growth rate in the transformants. They observed elongation of the cells in the stem of the transformants and deduced that it might have a relation to the oxidation of auxin by the induced peroxidase activity.

Ferritin is thought to be a kind of scavenger for oxygen radicals due to its ability of iron binding, therefore transgenic plants expressing ferritin are likely to exhibit enhanced growth, like transformants expressing VHb. In fact, Goto *et al.* (2000) demonstrated enhanced growth of lettuce plants expressing the soybean ferritin gene. The mean value of the T1 generation of transformed plants was heavier than that of controls 2 weeks after sowing (*Figure 14.8*). The heaviest transgenic line was 1.4-times that of the controls. *Figure 14.9* indicates that the enhanced growth rate continued for at least 3 months. Five of the six transgenic lines tested were taller than

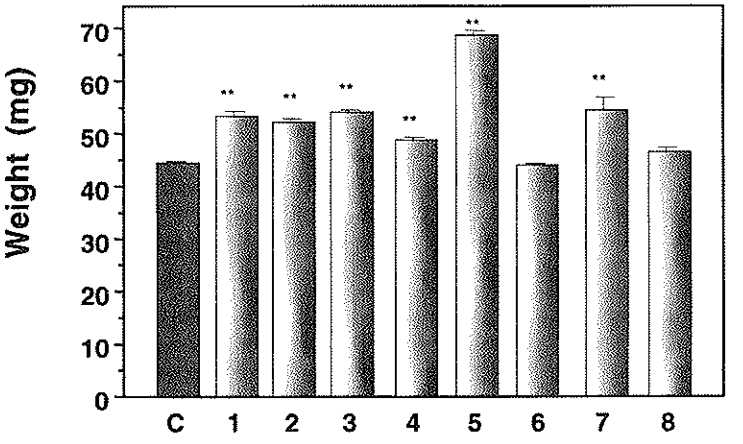

Figure 14.8. The weight of the transgenic lettuce seedlings. The seedlings of the transgenic lettuce lines and controls were weighed at early developmental stages. Bars indicate standard error. Two asterisks indicate a significant difference from controls at a 99% confidence level. Numerals indicate the number of measured seedlings for each transgenic line and a control.

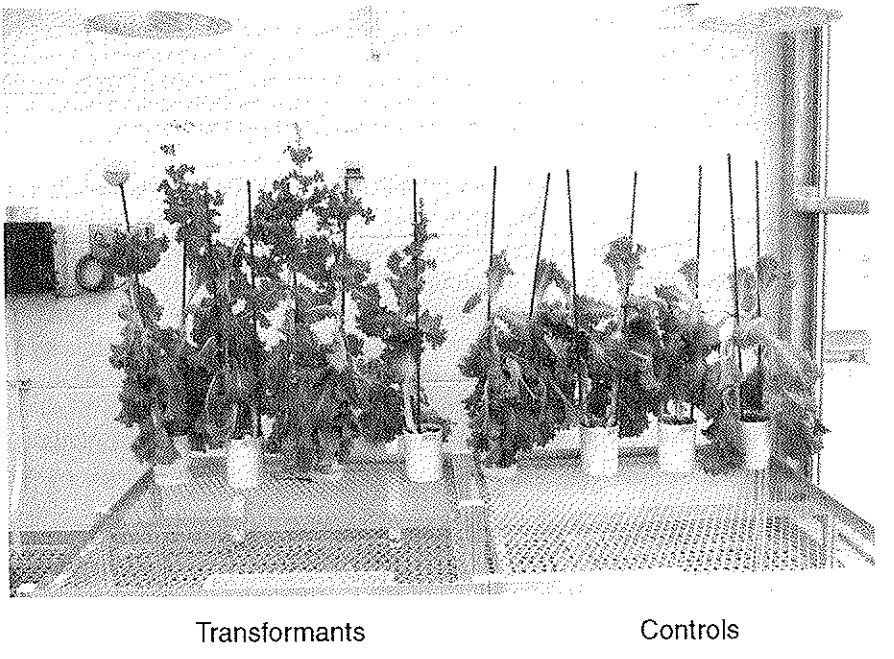

Figure 14.9. Comparison of the growth between transformants and controls. Photo taken 3 months after sowing indicating transgenic line and controls.

the controls. The average height of the tallest line was 91 cm, which was over 30 cm taller than that of the controls. When the transgenic plants generated flower buds, the control plants were still in the vegetative growth condition. The greater growth was also observed in the next generation of the transformed lines. They observed that the rate of photosynthesis in the transgenic line was 1.5–1.8 times greater than that of the

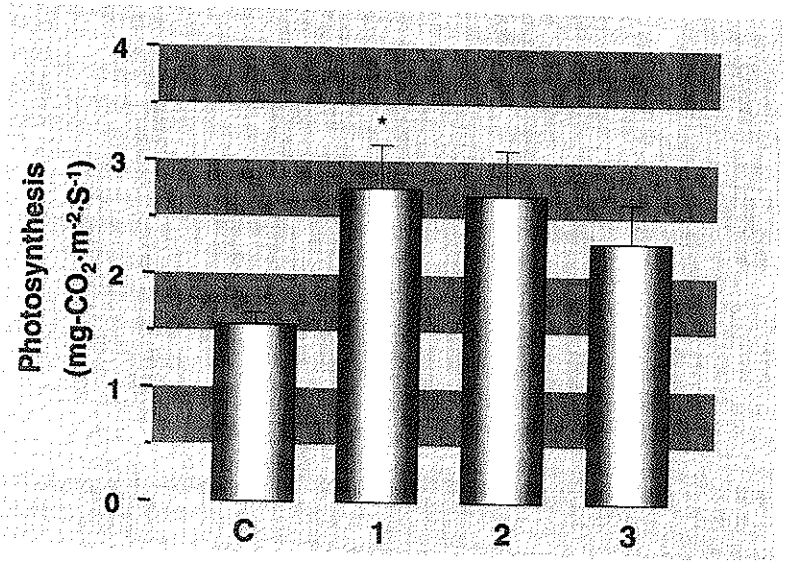

Figure 14.10. Photosynthetic rate in transgenic lettuce line and controls. Photosynthesis rates (per leaf area per minute) were determined for 3 lines of 8-week-old transgenic lines (No. 3, 4 and 7) and a control (C). Three plants of each were transferred from an air-conditioned greenhouse to an experimental room 1 hour prior to measurement for acclimation. The rate of photosynthesis of each sample was measured at 3 times under the following conditions: photon flux density of approx. 200 μmol $m^{-2}s^{-1}$, relative humidity 30% and temperature 25°C. One asterisk indicates a significant difference from controls at 95% confidence level.

controls (*Figure 14.10*). These results contradict the report by Wuytswinkel *et al.* (1998), showing decrease of chlorophyll concentration, yellow zones on leaves as observed in control plants grown in no iron medium, and decrease of fresh weight, in transgenic tobacco plants. These different observations may be explained by the difference of observation time and iron content in leaves. These authors indicated that the iron content of a young leaf was two- to three-fold greater in the transgenic tobacco than in the controls at three weeks after sowing. On the other hand, the mature leaf of the transgenic lettuce contained iron levels ranging from 1.2 to 1.7 times that of the control plants. They speculated that illegitimate ferritin accumulation in transgenic plants disturbed the photosynthetic apparatus, resulting in such physiological phenomena.

Let us return to our main subject. A reason for the enhanced growth of transgenic lettuces is presumably that oxidative stress to plastids by the effects of photosynthesis can be reduced by the presence of ferritin. Another explanation is that the constitutively expressed soybean ferritin may give a larger pool of available iron to some iron-dependent enzymes such as catalase, especially during the early growth of the seedlings. Normally, a large percentage of iron taken up by plants is precipitated (e.g. at cell walls) in its highly insoluble form, $Fe(OH)_3$. This Fe is hardly available to the plants. Young plants need relatively high amounts of Fe for optimal growth. Even plants grown in a nutrient solution may suffer from sub-optimal Fe availability after one or two weeks, resulting in a decreased growth rate. By expressing the soybean ferritin gene, the increased levels of ferritin may enhance the total amounts of available Fe resulting in an enhanced growth, compared to the control plants.

Conclusion

Iron is an essential element for almost all organisms including animals, higher plants, even bacteria. For mankind, the state of iron nutrition is not always good, especially for people in developing countries. Disorder derived from iron deficiency leads to more serious disease, such as abortion. Increasing iron content in crops is of benefit to people suffering from iron deficiency because many people receive their daily iron supply from vegetable diets. There have been many attempts to raise the iron content in plants, for example, by traditional breeding, by improved cultivation methods and also by genetic modification. The gene transfer technique has been a revolutionary tool for creating novel varieties quickly. The plants which have had the ferritin gene introduced possess two particular advantages: that is to store excess iron and the other elements, and to cancel oxidative stress. We have described the two possibilities to create useful plants using the exogenous ferritin gene in this review. One is the iron fortified plants for overcoming iron deficiency in humans and the other is the plants which have resistance to heavy metals, pathogens and other stresses, or which exhibit enhanced growth. These genetic improvements could exhibit an increase in the iron content or stimulated growth; however, the mechanism of iron storage and the scavenger system for oxidative stress using ferritin gene expression have not been determined. Understanding at a physical and molecular level is now necessary to take advantage of the ferritin gene at an advanced stage.

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