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Iron accumulation and enhanced growth in transgenic lettuce plants expressing the iron- binding protein ferritin

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Abstract We have produced transgenic lettuce plants accumulating the iron storage protein ferritin. The integration of the ferritin gene and expression levels in leaves were examined by Southern- and Western-blot analysis, respectively. It was shown that transgenic lettuce plants contained iron levels ranging from 1.2 to 1.7 times that of the control plants, however, the manganese content in transgenic lettuce plants was similar to that in the control. Enhanced growth of transgenic lettuces was observed at the early developmental stages, resulting in weights 27–42% greater than those of control plants. Transgenic lettuce had photosynthesis rates superior to those of the controls, and grew larger and faster compared with the controls during the period of 3 months from germination. These results demonstrate the possibility of producing lettuce plants with high yield, high iron content and rapid growth rate.

Key words Ferritin · Iron · *Lactuca sativa* · Transgenic · Enhanced growth

Introduction

Lettuce is one of the most popular leafy vegetables in the world. The iron content of lettuce is low compared with that of spinach, known as an iron accumulator. Lettuce contains 100-times less oxalic acid than that of spinach per fresh weight (Andrews and Viser 1951). This is advantageous, since oxalic acid has a negative effect on binding to calcium which can result in kidney stones. (Fincke and Sherman 1935; Libert and Franceschi 1987). Iron is an important mineral for living organisms. In humans, iron deficiency leads to anemia, which weakens the body as a result of decreased blood-cell numbers. It is estimated that 30% of the world's population is anemic, with the highest prevalence in developing countries (Baynes and Bothwell 1990). Even in developed countries, iron deficiency in humans is a health problem. Dietary iron obtained from plant sources, including cereals, can be significant: in Japan such sources are thought to account for 70% of total iron intake. Thus, iron-fortified lettuce could be a good potential source of iron but without the negative effects of excess oxalic acid.

The iron storage protein ferritin is widely distributed throughout living organisms. Plant ferritin is composed of 24 subunits (Crichton et al. 1978), which can store up to 4000 iron atoms in its central cavity (Korcz and Twardowski 1992). Iron stored in ferritin can be used by the metal enzymes for photosynthesis processes (Briat et al. 1995). The precursor of the plant ferritin subunit is encoded in the nucleus and synthesized in the cytoplasm with a transit peptide (TP). Following synthesis, it is transported into the plastid, and finally assembled into the mature protein by cleavage of the TP. An extension peptide (EP) in the subunit is cleaved in response to iron release from ferritin (van der Mark et al. 1983; Ragland et al. 1990).

We have recently produced high iron-content plants by transferring the cDNA of soybean ferritin into tobacco and rice (Goto et al. 1998, 1999). The maximum iron content in leaves of transgenic tobacco plants was approximately 30% higher than that of non-transformants. Beard et al. (1996) re-valued the dietary iron sources and showed that iron stored in ferritin is bioavailable: in animals made anemic by dietary iron deficiency, the bioavailability of extrinsic ferritin as an iron supplement is as high as that of $FeSO_4$. Thus, from a nutritive point of view, edible vegetables accumulating ferritin can be beneficial, due to increased iron absorption.

Another important role of ferritin is thought to be in the protection of cells against oxidative stress caused by free iron (Briat 1996). Recently, genes encoding pro-

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teins which have the function of scavenging oxygen radicals have been introduced into tobacco plants. Holmberg et al. (1997) introduced the gene for bacterial hemoglobin (*VHb*) into tobacco, which resulted in an enhanced growth of transformants. Kawaoka et al. (1994) showed that an increase in the growth of tobacco plants was attributable to the introduction of the peroxidase gene *prxC1a* which is known as a scavenger of oxygen radicals. Transgenic lettuce plants expressing ferritin could benefit from a potential reduction of oxidative stress.

In this paper, we tested the hypothesis that iron accumulation in lettuce leaves and an increase in growth could occur as a result of exogenous ferritin gene expression.

Materials and methods

Plant transformation

The binary plasmid pBG1 (Fig. 1A) vector, consisting of CaMV 35 S promoter-soybean ferritin cDNA and the kanamycin (Km) resistance gene, was transferred into *Agrobacterium tumefaciens* strain LBA4404 (Goto et al. 1998). Transformation and regeneration of lettuce plants (*Lactuca sativa L*. cv Green leaf) was carried out according to the modified methods of Enomoto et al. (1990). Claforan instead of cephaloridin was included in the selection medium to remove *A. tumefaciens*. One hundred milligrams per litre of Km was used for the selection of transformants during in vitro culture. After acclimation, the transformants were grown in soil in a greenhouse. Total genomic DNA extracted from regenerated plantlets was used as a template for the polymerase chain reaction (PCR) to confirm the existence of the foreign ferritin gene in the plant genome.

Southern-blot analysis

Total DNA was isolated from leaf tissue of progenies (T1) and nontransformed control plants using a modified procedure (Shure at al. 1983). DNA (10 µg) was digested with the restriction endonucleases *Eco*RI or *Hin*dIII. Digested DNA was applied to a 1% agarose gel, electrophoresed, and transferred to a nylon membrane. Hybridization and detection were carried out using digoxigenin-labelled ferritin cDNA as a probe by the method of Engler-Blum et al. (1993).

Protein extraction and immunological analysis

Total protein was extracted from 0.1 g of leaves of each progeny plant by homogenizing with extraction buffer [80 mM Tris-HCl (pH 7), 2% SDS, 2% 2-ME, 20% glycerol] and sea sand. The protein concentration of the extract was determined by the Bradford method (Protein assay kit; Bio Rad, USA) using bovine-globin as a standard. The extract was boiled for 3 min and clarified by centrifugation at 12000 rpm for 10 min. The supernatant was separated by SDS-PAGE and electroblotted to a polyvinylidene difluoride (PVDF) membrane. Immunodetection of protein was performed essentially according to the method of Matudaira (1987). Antiserum was raised against the soybean ferritin subunit expressed in *E.coli* using an expression vector containing a soybean ferritin cDNA insert (Goto et al. 1998). Probing of blots with soybean ferritin antibody was carried out using anti-rabbit IgG sheep immunoglobulin coupled with biotinylated horseradish peroxidase (Vectastain ABC kit; Vector Labs., USA). Immunostain HRP-1000 (Konica, Japan) was used for visualizing the signal.

Metal extraction and measurements

Seeds harvested from transgenic lettuce plants were germinated on MS medium (Murashige and Skoog 1962) containing 200 mg/l of Km. Resistant plants were grown in a culture solution containing (mg/l) KNO_3, 404; $Ca(NO_3)_2 \cdot 4H_2O$, 472; $MgSO_4 \cdot 7H_2O$, 246; $NH_4H_2PO_4$, 76; Fe-EDTA, 24; H_3BO_3, 1.5; $MnSO_4(4–7) \cdot H_2O$, 1; $ZnSO_4 \cdot 7H_2O$, 110; $CuSO_4 \cdot H_2O$, 25; $Na_2MoO_4 \cdot 2H_2O$, 10. Cultures were maintained by changing with fresh solution every 2 weeks. The leaves of transformant-progenies were harvested 1 week after the last re-inoculation. Collected leaves were dried at 60°C for 1 week, ground to fine powder, and 0.1 g of dry matter was mineralized. Metal concentration was measured by the absorbance of Fe and Mn at 238.2 and 257.6 nm, respectively, using inductively coupled plasma (ICP) spectrometry (P-4000; Hitachi, Japan). *T*-tests were performed to examine the difference in the mean contents of three individuals between seven transgenic progeny lines and non-transgenic controls. Plants containing maximum and minimum metal contents were excluded from these tests.

Growth test of T_1 plants

Thirty five seeds from individual transgenic lettuces and non-transgenic controls were germinated in MS medium without organic elements and plant hormones, in the presence (transformants) or absence (controls) of 200 mg/l of Km. The fresh weight of successfully grown seedlings was measured after 2 weeks of sowing (non-germinated or very weak seedlings were ignored). Ten plants of each transgenic line were transplanted to a pot (0.2 m^2) after 3 weeks of germination and grown in a greenhouse with an automatic water supply system. Approximately 3 months later the height of transgenic lettuce plants was measured on three separate occasions. The weight of 50 self-pollinated seeds (T_2) produced for all lines were also measured on five separate occasions. Comparisons of growth data were analysed using a *t*-test.

Measurement of photosynthesis

Photosynthesis rates (per leaf square per min) were determined for three lines of 8-week-old transgenic plants. Three plants of each were transferred from an air-conditioned greenhouse to an experimental room 1 h prior to measurement for acclimation. The rate of photosynthesis of each sample was measured at three times under the following conditions: a photon flux density of approximately 200 µmol $m^{-2}s^{-1}$, a relative humidity of 30% and a temperature of 25°C.

Results

Production of transgenic lettuce containing the ferritin cDNA

The plasmid pBG1 (11.9 kbp) was used for the transformation of lettuce plants (Fig. 1A). The soybean ferritin cDNA (780 bp) was under the control of the CaMV 35S promoter. After transformation mediated by *A. tumefaciens*, transformants were selected for kanamycin resistance (Km; 100 mg/l). At first, we attempted to select transformants with 50 mg/l of Km according to previous reports (Michlmore et al. 1987; Enomoto et al. 1990); however, these experiments resulted in failure. The exogenous gene in transformed lettuce disappeared during either in vitro culture or culture in soil. These results indicate that a higher concentration of Km (100 mg/l) and a longer growth period (approximately 40 days) was re-

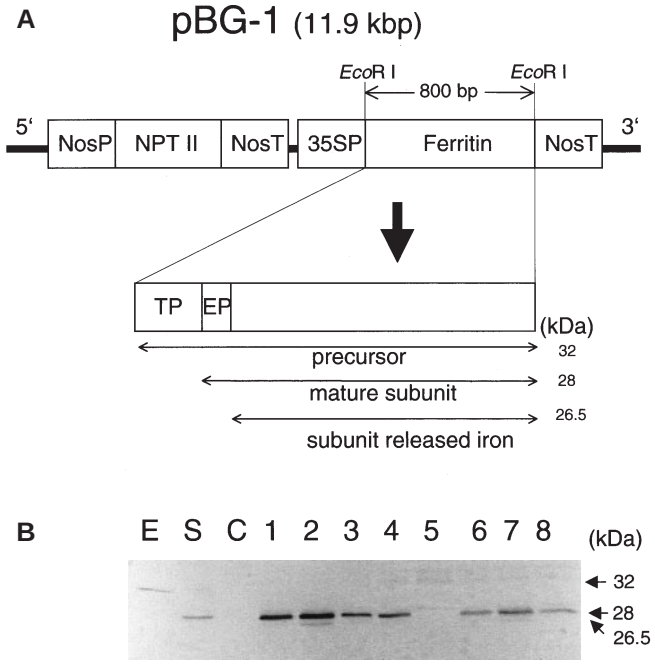

Fig. 1A Construction of the binary vector pBG1 (Goto et al. 1998) and its product. pBG1 has a soybean ferritin cDNA insert and the *NPT II* gene as a selection marker. *Nos-P*=promoter of nopaline synthase gene; *NPT II*=Neomycin phosphotransferase II structural gene; *Nos-T*=terminator of the nopaline synthase gene; *35S-P*=35S promoter of cauliflower mosaic virus; *TP*=transit peptide; *EP*=extension peptide. **B** Immunoblot analysis of ferritin in leaves of transgenic progenies. Total soluble protein was extracted from leaves and an *E. coli*-containing expression vector, resolved by SDS-PAGE, and electroblotted onto a PVDF membrane. *Lanes 1–8* transformants (30 µg crude protein loaded in each lane); *lane C* non-transformed control plant (30 µg crude protein); *lane E E. coli* (50 ng purified protein); *lane S*: soybean (20 µg crude protein) *Arrows* indicate size of bands in kDa

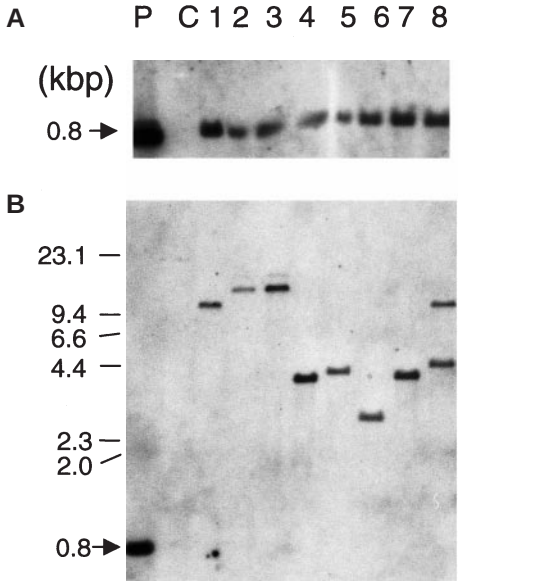

Fig. 2A, B Southern-blot analysis of eight independent progenies of transgenic lettuce plants (*lanes 1–8*) and a non-transformed control (*lane C*). **A** Total DNA was digested with *Eco*RI. The *arrow* represents the expected 0.8-kb band, which appeared only in the positive control (*lane P*) and transgenic plants. **B** Total DNA was digested with *Hin*dIII. *Numerals* indicate size in kbp

quired for the selection of transformants. When shoot formation was performed with 0.5 mg/l of 1-naphthaleneacetic acid (NAA) and 0.5 mg/l of benzyladenine (BA) in culture medium, the transformation efficiency improved to 32%.

The presence of the introduced ferritin gene was confirmed by PCR with total DNA prepared from mature leaves of Km-resistant plants (data not shown). Southern-blot analysis was carried out with the genomic DNA of self-pollinated progenies (T_1 generation) from eight independent transformants (T_0) and a non-transformed control plant. The results of Southern hybridization using ferritin cDNA as a probe are shown in Fig. 2. When DNA was digested with *Eco*RI, all eight plants possessed an 800-bp band corresponding to the ferritin cDNA introduced into the lettuce genome (Fig. 2A). No other bands were detected. When total DNA was digested with *Hin*dIII, having no restriction site in the pBG-1 vector, one or two bands were observed for all plants examined (Fig. 2B). These results indicate that at least one or two intact ferritin cDNAs have been integrated into the lettuce genome. T_1 progeny lines Nos.1–8 were employed in the following experiments.

Expression of soybean ferritin in lettuce leaves

Ten micrograms of total protein from T_1 transformants and non-transgenic controls were electrophoresised on SDS-PAGE, transferred to a PVDF membrane, and incubated with polyclonal antiserum raised against soybean ferritin. Figure 1A shows that the ferritin subunit is produced by two steps through the excision of the transit peptide (TP) and the extension peptide (EP) after translation from soybean mRNA expressed in the transgenic lettuce leaf. The 28-kDa peptide including the EP was detected in total protein extracted from T_1 progenies (Fig. 1B). The 32-kDa peptide was not detected in total protein from transgenic lettuce and normal soybean; however, it was detected in total protein from *E. coli* harboring the soybean ferritin cDNA within pBG-1. The signal from the 26.5-kDa peptide was either very weak (e.g. No. 2) or non-existent (e.g. No. 1 or No. 3). No peptide was detected in protein from controls (non-transformants). The levels of ferritin expression were variable among individual plants.

Iron and manganese contents in transgenic lettuce plants

We measured iron and manganese contents in leaves of transgenic plants. All transgenic lettuce lines contained at least 1.2-times more iron than controls (Fig. 3). The transgenic line exhibiting maximal iron content contained 398 µg/g of leaves (No. 8; dry weight) which was approximately 1.7-times that of the non-transformed leaves (227 µg/g of leaves). However, with the exception of line No. 4, the manganese content in transgenic lettuces was

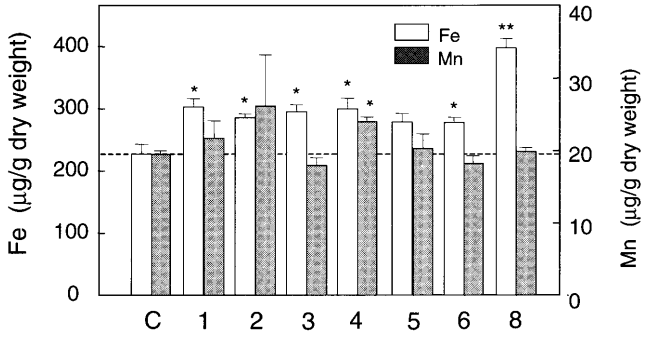

Fig. 3 Iron and manganese content in leaves of transgenic progenies (Nos. 1–6, 8) and controls (*C*). A *horizontal dotted line* shows the amount of each metal in controls. *Bars* indicate standard error. *One asterisk* and *two asterisks* indicate a significant difference from controls at 95% and 99% confidence levels, respectively

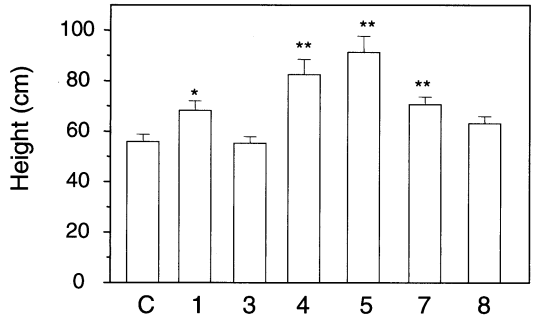

Fig. 5 Height of transgenic progenies (Nos. 1, 3–5, 7, 8) 3 months after sowing. *Bars* indicate standard error. *One and two asterisks* indicate a significant difference from controls at the 95% and 99% confidence level, respectively

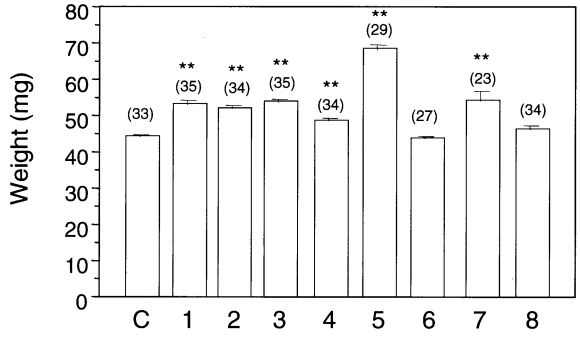

Fig. 4 The seedlings of the transgenic lettuce lines (Nos. 1–8) and controls were weighed at early developmental stages. *Bars* indicate standard error. *Two asterisks* indicate a significant difference from controls at a 99% confidence level. *Numerals* indicate the number of measured seedlings for each transgenic line and the control (*C*)

not significantly different from that in controls. Most samples, including non-transformed controls, had manganese contents ranging from 18 to 24 μg/g of leaves.

Enhanced growth of transgenic lettuce

The fresh weight of all T_1 transformed lines was heavier than that of controls 2 weeks after sowing (Fig. 4). The heaviest transgenic line (No. 5) was 1.4-times the weight of the controls. Figure 6A shows that growth of a transgenic line grown in a medium containing Km (200 mg/l) was superior to controls grown in a medium without Km. These transgenic lines were subsequently grown for 3 months. Five of the six transgenic lines tested were taller than the controls (Fig. 5). The height of line No. 3 was essentially the same as the height of the control. The average of the tallest line (No. 5) was 91 cm, which was over 30-cm taller than that of controls (56 cm). Flower buds were observed in transformants, but had not yet formed in the controls (Fig. 6B). There was no apparent relationship between iron content and growth (data not shown). A similar tendency towards greater growth was obtained from the seeds (T_2 generation) of transformed lines (Fig. 7). Figure 8 shows the photosynthesis rate of three transgenic lines and non-transformed controls. The rate of photosynthesis in transgenic lines was approximately 1.5–1.8-times greater than that of controls with a wider distribution. The rate in controls was 1.55±0.11 mg-CO_2/s·m^2. The maximum rate of photosynthesis was 2.76±0.39 mg-CO_2/s·m^2, observed in transgenic line No. 3.

Discussion

In this report we showed that transgenic lettuces expressing the ferritin gene have a higher iron content compared to non-transformed control plants. The molecular weight of the ferritin subunit extracted from transformants (28 kDa) was different from that deduced from the soybean ferritin cDNA sequence (32 kDa, Fig. 1), which was incorporated intact into the lettuce genome (Fig. 2). A similar result has been observed in a transgenic tobacco plant, where the ferritin subunit was post-translationally processed into the mature size of 28 kDa. The 28-kDa subunit was subsequently converted to a 26.5-kDa subunit (Goto et al. 1998). The results of this study show that the ferritin subunit derived from soybean cDNA in transgenic lettuce is cleaved to release the TP and EP, resulting in iron accumulation in transgenic lettuce plants. Quantitatively, the iron-content in transgenic lettuce plants was 1.2–1.7-times that of controls (Fig. 3). This increase in iron content is significant, and similar results were obtained from the transgenic tobacco plant, the maximum rate of which was 1.3-fold that of controls. The ferritin gene in both types of transgenic plant was driven by a CaMV 35S promoter, which is known to promote genes constitutively (Benfey and Chua 1990). Thus, the limit of iron accumulation in leaves of the transgenic plants expressing the ferritin gene under the control of the CaMV 35S promoter is up to approximately 1.7-times that of controls when these plants are grown in a normal nutrient solution.

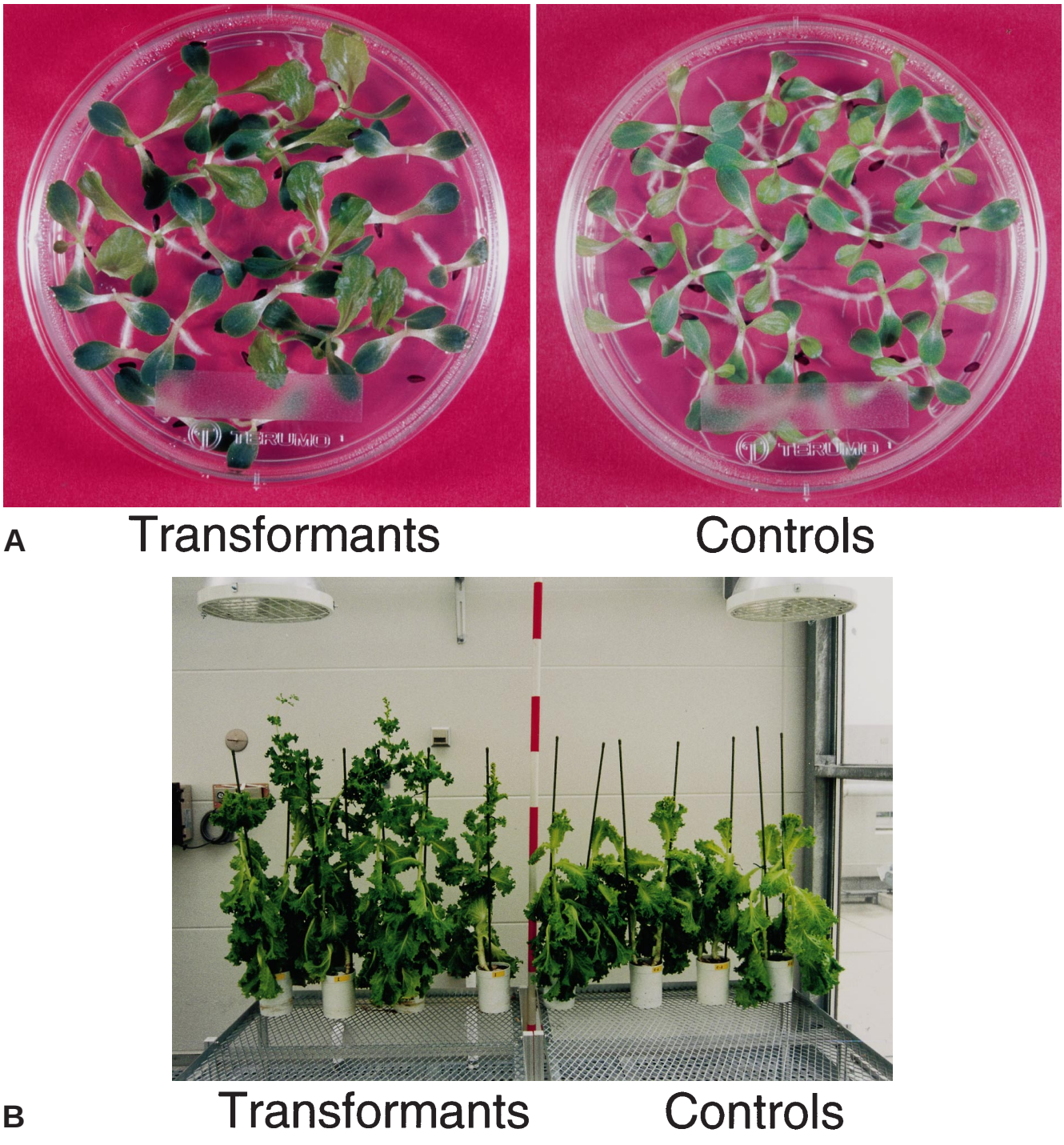

Fig. 6A, B Comparison of the growth between transformants and controls. **A** Photos were taken 2 weeks after sowing. Transgenic progeny (No. 2) were sown on agar medium containing 200 mg/l of kanamycin (left). Control plants were sown on agar medium without Km (right). **B** Photo taken 3 months after sowing indicating transgenic line No. 5 and controls

Although transgenic lettuce plants accumulated iron, they did not accumulate manganese. We measured the manganese content of leaves in order to investigate whether ferritin gene expression affects the accumulation of heavy metals other than iron. Manganese is a good indicator of the potential effects of ferritin expression, because the chemical and biological nature of manganese is similar to iron. Both metals are transition elements and belong to the same chemical family. It is known that manganese absorption from roots decreases during increases in iron absorption (Zaharieva 1995). If iron is accumulated in cells of transgenic lettuce plants, the manganese content would be expected to decrease. As shown in Fig. 3, except for line No. 6 the, manganese content of transgenic lettuce plants remained almost constant irrespective of the increase in iron content. These results demonstrate that iron ac-

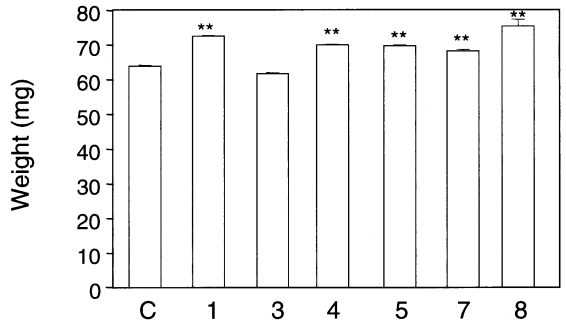

Fig. 7 Weight of seeds obtained from eight T1 self-pollinated plants (Nos. 1, 3–5, 7, 8) and a control. *Two asterisks* indicate a significant difference from controls at a 99% confidence level

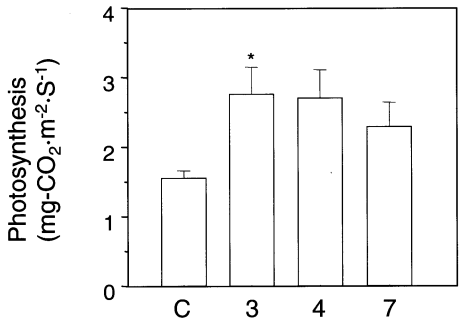

Fig. 8 Photosynthetic rate in three transgenic lines (Nos. 3, 4 and 7) and a control. *One asterisk* indicates a significant difference from controls at a 95% confidence level

cumulation can occur without a change in the level of manganese, even if ferritin is expressed constitutively in transgenic lettuce plants. There have been reports that elements other from iron (phosphate, beryllium and aluminum) are stored in ferritin (Sczekan and Joshi 1989; Wade et al. 1993). However, Sczekan and Joshi (1989) demonstrated that heavier metals such as zinc and cadmium (ionic radii of 0.74 Å and 0.97 Å, respectively) were bound to ferritin at much lower levels than smaller metals are (Be, 0.35 Å; Al, 0.51 Å). Thus, expressing the soybean ferritin gene in transgenic lettuce can affect the accumulation of iron, but not necessarily induce the accumulation of other heavy metals.

Transgenic lettuces expressing the ferritin gene showed not only the ability to accumulate excess iron but also to increase their own growth. Most of the transgenic progenies grew faster than controls at early developmental stages (Figs. 4, 6A). This enhanced growth rate continued for 3 months (Figs. 5, 6B). The seeds of the next generation were also heavier than control seeds (Fig. 7). Why did these plants grow better than controls? One explanation for enhanced growth is that oxidative damage to plastids by the effects of photosynthesis can be reduced by the presence of ferritin in transgenic lettuce plants. Fe ions that are not bound in storage or transport proteins are hazardous because they can stimulate the production of damaging free-radical reactions (Halliwell and Gutteridge 1988). For example, oxidative stress generated by iron had the effects of destroying the protein Rubisco (Desimone et al. 1996), increasing catalase activity, and increasing ascorbate peroxidase activity (Kampfenkel et al. 1995). It was hypothesized that plant ferritin played a role in de-toxification during oxidative stress (Briat et al. 1995); however, there was no clear evidence in contrast with animal ferritin. Lobréaux et al. (1995) indicated that ferritin synthesis in maize leaves was induced by an iron-mediated oxidative stress. Ferritin located in the chloroplasts of transgenic lettuce plants could have the function of de-toxification, since the rate of photosynthesis in transgenic lettuce plants was larger than that of controls (Fig. 8). Thus, it is possible that transgenic lettuce plants grow larger and faster because the expressed ferritin in transformants acts as an indirect scavenger for active oxygen species. Further work is needed to clarify the question of the increased growth. It has recently been demonstrated that transgenic tobacco expressing a foreign ferritin gene was tolerant to oxidative stress. The photosynthetic function of transformants was in contrast with that of the controls (Deák et al. 1999). These results are in strong agreement with the results of the present study.

In conclusion, the expression of soybean ferritin in transgenic lettuces resulted in iron accumulation and enhanced growth. The improvement of crops by genetically increasing their iron content could play an important role in resolving the problem of anemia. In addition, a high yield or an early harvesting lettuce variety with high commercial value could be engineered by transfer of the ferritin gene.

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