Difference between ferritin genes overexpressing in transgenic tobacco

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ABSTRACT. With the development of molecular biology techniques, intron was known as playing an imperative role in gene's expression and regulation. Transgenic tobacco IN lines overexpressing InFer1 gene and NT lines overexpressing NtFer1 cDNA gene were obtained, and the exogenous gene expression were confirmed by molecular test. Then for iron content of transgenic tobacco lines and non-transformants as a physiological indicator in status of different iron concentration were measured, and results indicated that the iron content of transgenic tobacco was more than that of non-transformants. In high Fe (II) condition, the NT lines showed higher level in plant height, fresh weight and iron content then that of IN lines, while NT lines showed lower in Malondialdehyde content then IN lines. In soil condition, IN lines showed higher level in plant height, fresh weight, chlorophyll, photosynthesis rate, iron content then that of NT lines. It indicates that intron could play a vital role for improved protective enzyme activity level while reducing reactive oxygen damage and also could help to inhibit the absorption of aborting iron.

Key words: Ferritin; Intron; Iron content; Tobacco

INTRODUCTION

Iron (Fe) is a chemical element that is universally abundant and plays a vital role in biological processes (Dlouhy and Outten, 2013), such as forming complexes with molecular oxygen in hemoglobin and myoglobin. It is also present at the active sites of many important redox enzymes involved in cellular respiration (i.e., oxidation reduction processes in plants and animals). Ferritin genes have been documented in plants, animals, and microbes (Theil, 1987; Proudhon et al., 1996; Andrews, 1998; Harrison et al., 1998; Carrondo, 2003; Pham et al., 2004). In addition to storing iron, ferritin may also be involved in ferroxidase activity, immune responses, mitochondrial stress responses, yolk, and industrial applications.

Genetic engineering techniques have been applied in numerous fields, including research, agriculture, industrial biotechnology, and medicine. They can be used to increase growth rates and resistance to different diseases caused by pathogens and parasites. The modified crops would also reduce the usage of chemicals such as fertilizers and pesticides; thus decreasing the severity and frequency of the damages produced by these chemical pollutants (Carrington, 2012). For the current research, it provides a useful method for studying gene expression and functions; thus, a particular gene was introduced into a plant to test its role.

An intron opposite of the exon, often referred to as "junk DNA", is a nucleotide sequence within a gene that is removed by RNA splicing, while the final mature RNA product of the gene is being generated (Bruce, 2008). The underlying mechanisms of introns are still ambiguous; however, some of these mechanisms have been verified by encoding a specific protein or generate noncoding RNA (Rearick et al., 2011), which may be involved in the recombination of separated exons and participates in the evolution of new proteins (Darnell, 1978; Gilbert, 1978).

Few studies have focused on the intron roles in gene expression. The objectives of the current study were to: a) introduce the ferritin gene (exon plus intron and exon only) into tobacco and b) investigate the difference between these 2 transgenic tobacco lines to identify whether the intron is silent in gene expression.

MATERIAL AND METHODS

Plasmid construction

Tobacco leaves were harvested 24 h after the transfer of plantlets from a nutrient solution containing no iron to a nutrient solution containing Fe-EDTA. A pair of primers (5'-GCGACTAGTCCATGCTTCTCAAGGCGG-3' and 5'-GCGAGCTCCTTCAAGCAGCA GCTCC-3') was designed to generate a 756-bp fragment containing *Spe*I and *Sac*I restriction sites of the *NtFer1* gene via polymerase chain reaction (PCR). Another pair of primers (5'-GCGACTAGTCCTCACCACACACTGAATTTCGCC-3' and 5'-GCGCTCGAGCTTCA AGCAGCAGCTCCCTCATTC-3') was designed *via* PCR to generate a 1996-bp fragment containing the *Spe*I and *Xho*I restriction sites of the *InFer1* gene. The PCR products were digested by the restriction sites and then inserted into the plasmid PBI121. GUS was excised in the newly generated plasmid.

Plant transformation and regeneration

The newly generated plasmid was transferred into the *Agrobacterium tumefaciens*

strain EHA105 by electro-transformation; then, the transformants were obtained by the leaf disc transformation method. Transformants and regeneration of tobacco plants (*Nicotiana tabacum* L. 'Petit Havana SR-1') were cultured on Murashige and Shoog (MS) medium (100 mg/L kanamycin) and termed the T_0 generation.

PCR, Southern blot, and Northern blot analysis

PCR test primers were designed according to the *NtFer1* and *InFer1* genes (5'-CCACT ATCCTTCGCAAGACCCTTCC-3' and 5'-CATCGCAAGACCGGCAACAGGATTC-3').

Total genomic DNA was extracted from fresh leaves by the cetyl trimethyl ammonium bromide method (Murray and Thompson, 1980) and used to conduct the PCR analysis. The PCR was performed in a total volume of 20 μL containing 1.0 μL DNA, 10 pmol each primer, 1X PCR buffer, 10 μmol dNTPs, 1X $MgCl_2$, and 0.5 U *Taq* polymerase (TaKaRa, Dalian, China). The PCR reaction was carried out by denaturing the template at 94°C for 2 min followed by 30 cycles of amplification (30 s at 94°C, 30 s at 58.5°C, and 2 min at 72°C), and an extension at 72°C for 10 min.

Southern blot analysis (Engler-Blum et al., 1993) was further used to confirm the transgenic status of positive plants by PCR analysis. The complementary DNA was marked by PCR primers and the PCR DIG Probe Synthesis Kit (Roche, Switzerland) was used as the probe. RNA extraction was conducted using TRIzol (Reagent, Carlsbad, CA, USA) for Northern blot analysis; 30 μg RNA was denatured at 100°C for 5 min, then separated from 1% agarose/formaldehyde gel through electrophoresis. The methodology and probe used were the same as those in the Southern blot analysis.

Culture of the T_1 generation

The seeds of T_0 (i.e., IN: *InFer1* gene transgenic plants and NT: *NtFer1* gene transgenic plants) were collected and grown on MS medium (100 mg/L kanamycin) for further research. The resistant seedlings and nonresistant seedlings were counted separately to calculate the ratio.

After 15 days, 3 replicates of each transgenic tobacco line were transplanted to MS medium [containing 100 mg/L kanamycin, 300 μM Fe (II), and soil]. After 30 days, the plants grown in the MS medium were tested for physiological characteristics, and 60-day-old soil-grown plants were tested for physiological characteristics and photosynthetic parameters.

Iron extraction and measurements

For iron extraction and analysis, the samples were obtained from the top leaves. The leaves were dried at 60°C, ground, and mineralized (0.1 g dry matter). The Fe concentration was measured by assessing the absorbance of Fe at 248.3 nm using an Atomic Absorption Spectrophotometer (Z-2000, Hitachi, Japan).

Physiological test of transgenic tobacco under Fe (II) stress

The height and fresh weight of all samples were tested, and the leaf chlorophyll content of the top leaf was measured by the chlorophyll meter SPAD-520 (Minolta, Japan). Subsequently, the materials were prepared for other index measurements. In higher plants, super-

oxide dismutase enzymes (SODs) act as antioxidants and protect cellular components from oxidation by reactive oxygen species (ROS) (Alscher et al., 2002); SODs were measured by the nitro blue tetrazolium chloride method (Giannopolitis and Ries, 1977).

MDA is one of the final decomposition products of lipid peroxidation and has been used as an index for the status of lipid peroxidation. The levels of MDA contents were determined by the thiobarbituric acid (TBA) reaction as described by Stewart and Bewley (1980), with some modifications. One gram of leaves (FW) was homogenized in 4 mL 0.5% (w/v) TCA. The homogenate was centrifuged at 10,000 g for 10 min and 4 mL 20% TCA containing TBA 0.5% (w/v) was added to 1 mL supernatant. The mixture was heated at 95°C for 20 min, quickly cooled on ice, and then centrifuged at 10,000 g for 10 min; the absorbance of the supernatant was taken at 532 and 600 nm. After subtracting the nonspecific absorbance at 600 nm, the MDA concentration was determined by its extinction coefficient of 155 mM/cm.

Physiological test of transgenic tobacco planted in soil

The leaf chlorophyll content was measured by the chlorophyll meter SPAD-520 (Minolta, Japan), Net photosynthetic rate, transpiration rate, and stomatal conductance were measured by LI6400 (LI-COR, USA), and the iron content of the leaves was measured by atomic absorption spectrophotometer.

RESULTS

Analysis of putative transgenic plants

After transformation by the *Agrobacterium tumefaciens* infection method, the *InFer1* gene transgenic plant (IN) and *NtFer1* gene transgenic plant (NT) transformants were selected with kanamycin (100 mg/L). The T_0 generation of the IN and NT lines were selected for PCR confirmation and then verified. The seeds of the T_0 transgenic tobacco were collected and germinated on MS medium (100 mg/L kanamycin). The 5 InFer1 transgenic tobacco lines (IN 1-5) and 5 NtFer1 transgenic tobacco lines (NT 1-5) were selected for further research. As shown in Table 1, the separation ratio of IN-6 and NT-6 was 15:1, which may be caused by the cloning of the 2 fragments into 2 nonhomologous chromosomes; this was not our research objective. IN 1-5 and NT 1-5 were separated into a ratio of 3:1, which showed that a single clone was integrated within the genome of interest.

Table 1. Kanamycin-resistance analysis of the transgenic tobacco T_1 generation.

Lines	Total plants (No.)	Resistant plants (A)	Sensitive plants (a)	Expected rate	χ^2 value
IT-1	67	51	16	3:1	0.0049
IT-2	42	32	10	3:1	0
IT-3	59	44	15	3:1	0.0059
IT-4	54	38	16	3:1	0.2960
IT-5	47	36	11	3:1	0.0071
IT-6	77	68	9	15:1	3.0138
NT-1	99	77	22	3:1	0.3367
NT-2	60	48	12	3:1	0.5203
NT-3	87	68	19	3:1	0.3103
NT-4	85	67	18	3:1	0.4745
NT-5	45	34	11	3:1	0.0056
NT-6	72	65	7	15:1	0.9481

$\chi^2_{0.05} = 3.841$; $\chi^2_{0.01} = 6.635$. $\chi^2 = [|A - ra| - (r + 1) / 2]^2 / rn$.

PCR test, Southern blot, and Northern blot

Total DNA was extracted from the mature leaves of kanamycin-resistant plants and used in the PCR to analyze the presence of the introduced ferritin gene (IN and NT). A set of PCR primers was designed to amplify a 1996-bp sequence that contained InFer1, and a 756-bp sequence that contained NtFer1. Figure 1a shows the specific bands of the *InFer1* and *NtFer1* genes in 5 tobacco lines (one individual per line, identical results were obtained from the other individuals of each line, data not shown). Southern blot analysis was carried out with the genomic DNA of transformants and the nontransgenic plants (SR) (Figure 1b). Northern blot was carried out to verify gene expression in the transformants (Figure 1c, d). The results showed that the exogenous gene was expressed in the transformants.
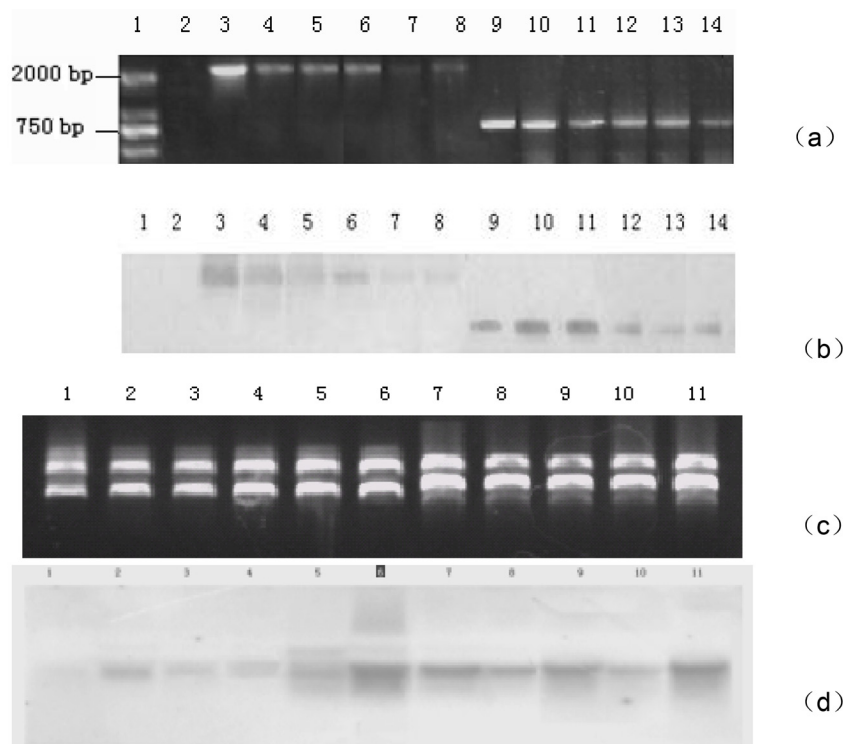

Figure 1. Test analysis of InFer1 transgenic tobacco lines (IN) and NtFer1 transgenic tobacco lines (NT) transgenic tobacco plants. **a.** Polymerase chain reaction (PCR) analysis of transgenic tobacco plants. **b.** PCR-Southern blot analysis of IN and NT generation plants. **c.** Electrophoresis of total RNA treated with RNase-free DNase. **d.** Northern blot analysis of ferritin expression. **a. b.** *Lane 1* = DNA molecular weight marker DL2000; *lane 2* = non-transformant; *lane 3* = IN plasmid positive control; *lanes 4-8* = IN1-IN5; *lane 9*, NT plasmid positive control; and *lanes 10-14*, NT1-NT5. **c. d.** *Lane 1* = non-transformant; *lanes 2-6* = IN1-IN5; and *lanes 7-11* = NT1-NT5.

Test of transgenic tobacco under Fe (II) stress

Transformants and nontransgenic plants were grown in MS medium containing 300 μmol/L Fe (II). Thirty days later, growth and physiology were evaluated; transgenic lines and

SR test results are shown in Figure 1. The average height of the IN transgenic tobacco lines was 25.7 and 31.1% less than that of the SRs and NT transgenic lines, respectively. The average fresh weight of the IN transgenic tobacco lines was 18.9 and 30.3% less than that of the SRs and NT transgenic lines, respectively. The height and weight were significantly different between SR, IN, and NT (Figure 2a, b). Differences in the relative chlorophyll content were not significant between all lines (Figure 2c).
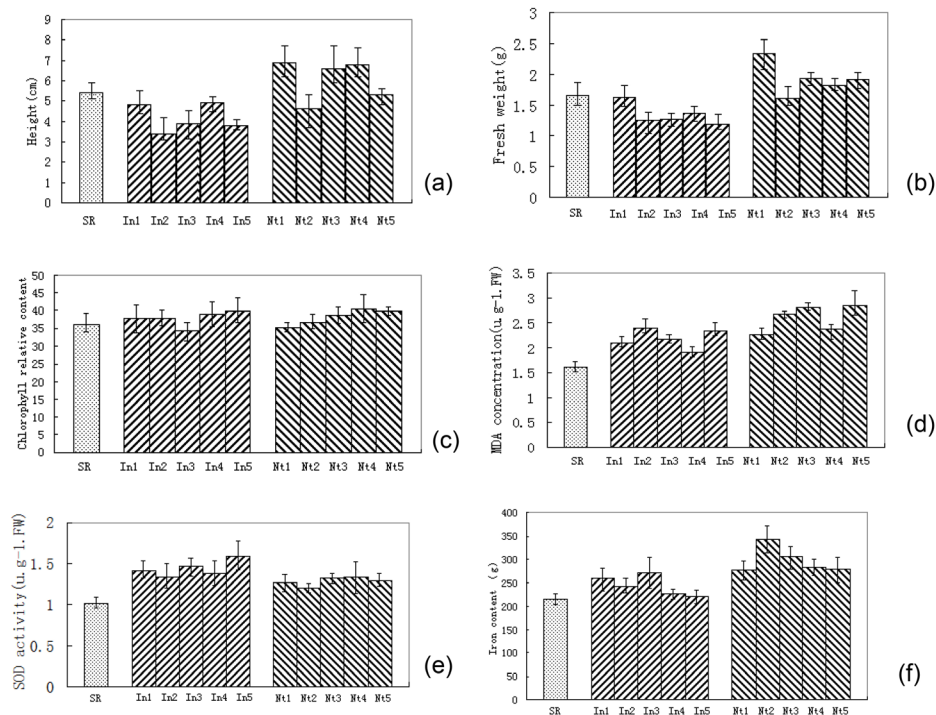

Figure 2. Comparison between transgenic tobacco plants and non-transformants grown in supplemented MS media. **a.** Comparison of the heights between IN, NT, and SR. **b.** Comparison of the fresh weights between IN, NT, and SR. **c.** Comparison of the chlorophyll relative contents of IN, NT, and SR. **d.** Comparison of malondialdehyde (MDA) contents in the leaves of IN, NT, and SR. **e.** Comparison of superoxide dismutase (SOD) contents in the leaves of IN, NT, and SR. **f.** Comparison of total iron relative contents in the leaves of IN, NT, and SR.

The MDA content reflected varied grades of cellular damage. The average MDA content of IN transgenic tobacco lines was 36.5% higher than that of the SRs and 15.9% less than that of the NT transgenic lines (Figure 2d). SODs catalyze the production of O_2 and H_2O_2 from superoxide (O^{2-}), which resulted in less harmful reactants. The average SOD content of IN transgenic tobacco lines was 41.0% more than that of the SRs and 12.0% higher than that of the NT transgenic lines (Figure 2e).

The average iron content of the controls (SRs) was 216.0 μg/g dry weight. The average iron content of the transformants (NT2) was 1.6- and 1.4-fold higher than that of the controls and IN lines, respectively. The average iron content of the IN transgenic tobacco lines was 12.9% higher than that of the SRs and 17.8% lower than that of the NT transgenic lines (Figure 2f).

Test of transgenic tobacco planted in soil

After 60 days, when the SR, IN, and NT transgenic lines were transplanted to soil, they were tested for height, fresh weight, chlorophyll, net photosynthetic rate, transpiration rate, H_2O conductance, and total relative iron content.

The average height and fresh weight of IN transgenic tobacco lines increased at the highest rate after 60 days. The height of IN transgenic tobacco lines were 12.8 and 4.8% greater than that of the SR and NT transgenic lines, respectively. The fresh weight of IN transgenic tobacco lines was 12.0 and 4.9% greater than that of the SR and NT transgenic lines, respectively (Figure 3a, b).
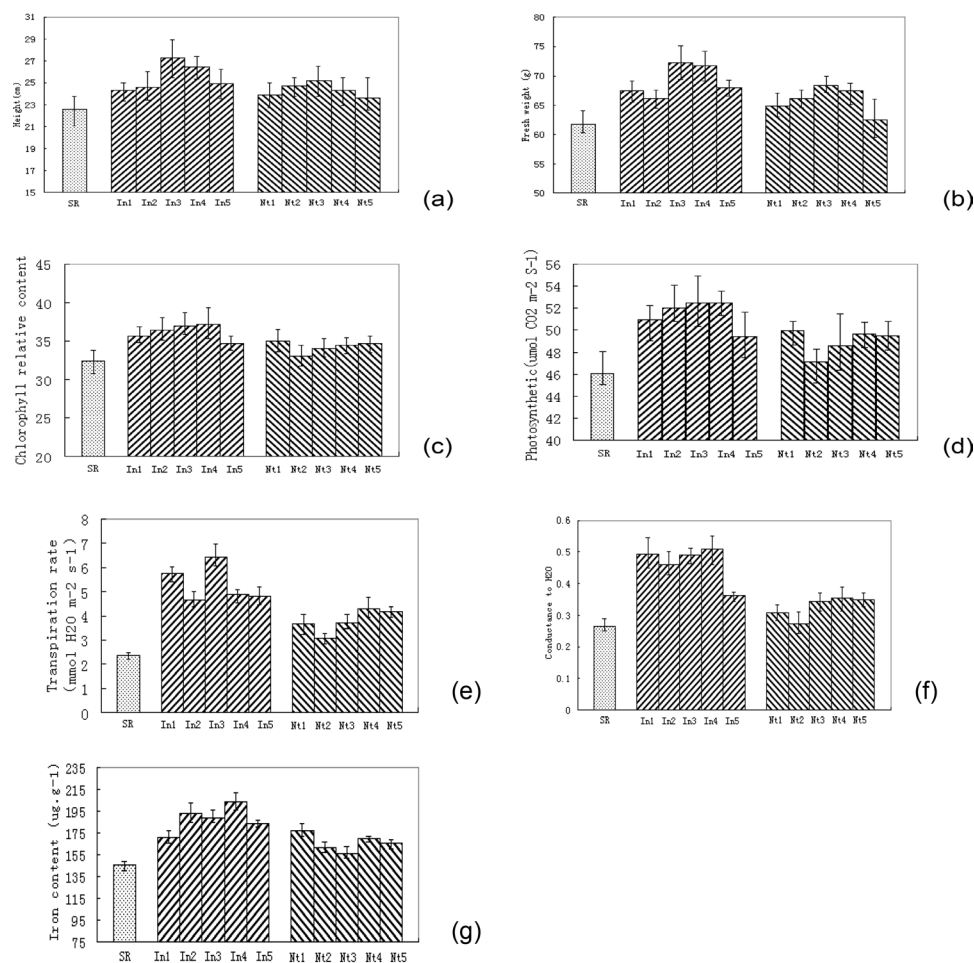

Figure 3. Comparison between transgenic tobacco plants and non-transformants grown in soil. **a.** Comparison of the heights between IN, NT, and SR. **b.** Comparison of the fresh weights between IN, NT, and SR. **c.** Comparison of the chlorophyll relative contents of IN, NT, and SR. **d.** Comparison of the net photosynthetic rates of IN, NT, and SR. **e.** Comparison of the transpiration rates of IN, NT, and SR. **f.** Comparison of the conductance to H_2O of IN, NT, and SR. **g.** Comparison of total relative iron content in the leaves of IN, NT, and SR.

Chlorophyll is vital for photosynthesis; it allows plants to absorb energy from light (Stein Carter, 1996). Iron is needed to produce chlorophyll; hence, its deficiency causes chlorosis. The average chlorophyll content of the IN transgenic tobacco lines was 12.4 and 5.8% greater than that of the SR and NT transgenic lines, respectively (Figure 3c). The net photosynthetic rate of the IN transgenic tobacco lines was 11.8 and 5.1% greater than that of the SR and NT transgenic lines, respectively (Figure 3d). Photosynthesis, transpiration rate, and conductance are closely related. The average transpiration rate of the IN transgenic tobacco lines was 123.3 and 39.7% greater than that of the SR and NT transgenic lines, respectively. The H_2O conductance to the IN transgenic tobacco lines was 74.5 and 42.1% greater than that of the SR and NT transgenic lines, respectively (Figure 3e, f).

The average iron content of the control samples (SRs) was 145.7 μg/g dry weight. The highest iron content of the transformant (IN4) contained 1.4-fold higher amounts of iron than the controls and 22.46% more than the average of the NT lines. The total relative iron content of the IN transgenic tobacco lines was 28.9% more than that of the SRs and 13.2% more than that of the NT transgenic lines (Figure 3g).

DISCUSSION

In the current experiment, materials for indicator measurements were obtained from the top part of the tobacco plant because iron mostly presents as a polymer that exhibits decreased mobility and does not transfer from the old leaves to the new tissues easily. Subsequently, because of the iron deficiency in the young leaves, the leaves turn yellow, resulting in a disease known as chlorosis.

Iron is the one of the most important micro minerals for the maintenance of good health. Ferritin is thought to play at least 2 important roles in plants. The first is the storage of iron (van der Mark et al., 1981; Briat et al., 1995). Iron in the soil is difficult for a plant to absorb because it is a water-insoluble compound; thus, ferritin is needed to increase the iron storage for normal growth. The second main role of ferritin is the protection of cells against the toxic effects of iron due to stress. Iron distribution is heavily regulated in mammals, partly because iron ions have a high potential for biological toxicity (Nanami et al., 2005). Ferritin expression is involved in induction by iron-overload (van der Mark et al., 1982) and, hence, protects the cell from such chemistry stress (Theil, 1987). In the MS medium [excessive stress cause by a high Fe (II) concentration, defined as Fe (II) concentration >120 μmol/L] or in soil [appropriate Fe (II) concentration, defined as Fe (II) concentration of 30-120 μmol/L). The iron content of the transgenic plants was more than that of the SRs; the ferritin gene showed enhanced iron absorption power, and similar results were obtained in potato, rice, and tobacco plants (Goto et al., 1998; Mata et al., 2001; Ye et al., 2008).

In MS medium, the average iron content was NT > IN, while in soil, it was IN > NT. The iron content increased by 78.87 and 29.85% in the NT and IN lines, respectively, with differences in the Fe (II) concentration. Previous research showed that the introns were not "silent"; they always functioned with regard to gene expression. They enhanced the activity of the promoter (Cabello et al., 2007), participated in the quantitative determination expression of the chimeric gene (Norris et al., 1993) and controlled gene expression in cells (Weise et al., 2008). Introns can affect both the level of gene expression and its pattern, but the action mechanisms in which plant introns are involved in enhancing transcriptional and/or translational

activity of promoters is still unknown. Thus, according to our research results, the intron of the ferritin gene appears to regulate iron absorption by a rapid increase in the Fe (II) concentration and maintaining the iron content at an appropriate level in order to avoid damage caused by iron overload. The previous research results showed that iron uptake is tightly regulated in the human body, with no regulated physiological means of its excretion. Only small amounts of iron are lost daily; thus, iron levels are mostly controlled by uptake regulation. For this reason, it is advised that people do not take iron supplements unless they suffer from severe iron deficiency (Durupt et al., 2000).

In our research, iron content of the NT transgenic lines was greater than that of the others grown in the MS medium; the IN transgenic lines exhibited the highest iron content. The difference between these 2 results may be caused by the differences in iron content between the medium and the soil. When the Fe (II) concentration was <30 μmol/L, it was caused by a lack of iron stress; when the Fe (II) concentration was >120 μmol/L, it was caused by iron over stress, and both of these conditions negatively affected plant growth. The iron concentration in the MS medium was 300 μmol/L, the height and fresh weight of the IN transgenic lines were lower than those of the NT transgenic lines and non-transformants, thus indicating that the intron plays an inhibitory role when Fe (II) is overexpressed.

In addition to iron content, the height and fresh weight of the NT lines were greater than those of the IN lines when cultivated in the MS medium but less than those of the soil-cultivated IN lines. These results confirm that the Fe content was very important for plant growth and integrity, and the intron inhibits the Fe (II) absorption rate when available at high concentrations. However, in the MS medium, the MDA content results showed that NT > IN > SR, whereas MDA levels reflected the degree of peroxidation of the membrane lipids. H_2O_2 acts as an ROS and can damage membrane lipids, proteins, and DNA (Foyer and Noctor, 2009); thus indicating that the damage level of the IN transgenic line membrane lipids was less than that of the NT lines, while SOD activity of the IN lines was greater than that of the NT lines. Moreover, the results confirm that the IN transgenic lines suffered less damage overall throughout the transgenic process.

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