Plant Res. 4(1) 26~30(2001)

Gene Expression and Iron Accumulation in Progeny of Transformants Introduced *Fp1* Gene Encoding the Iron Storage Protein in Red Pepper (*Capsicum annuum* L.)

Kwon-Kyoo Kang and Young-Ho Kim
Department of Horticulture, Hankyong National University, Ansung, 456-749, Korea,

ABSTRACT

To improve the iron content of red pepper, we have transferred the entire coding sequence of the ferritin gene(Fp1) into *Capsicum annuum* (L. cv. Chungyang and Bukang) by *Agrobacterium* mediated transformation. Transformants were found to contain the Fp1 gene at up to three loci, increased distinct iron content changes. In transgenic plants, iron content was as much as 7-fold to 8-folds greater than that of their untransformed counterparts. Furthermore, the R1 progenies from transformant(A7, A8) co-segregated into a 15:1 ratio for both Kanamycin resistance and genotype of high iron.

Key Words : iron content, genetic improvement, genetic analysis, *Capsicum annuum*

INTRODUCTION

When plant cells are infected with *agrobacterium tumefaciens*, a part of the Ti-plasmid is integrated in to the plant chromosomes(Benfey et al., 1989). Ferritin genes are expressed in most cells but the concentration of the protein among different cell types can vary 1000-fold(Cairo et al., 1985). For many genes, changing mRNA concentration(transcription or stability) is the major mechanism of regulation, with changing the utilization of mRNA(storage or translocational efficiency) providing modulation(White et al,. 1988). In the case of ferritin, changing the utilization of mRNA is major site of regulation, at least in cells specialized for iron storage where potential accumulation of the protein is high, ferritin mRNA is abundant, and induction of ferritin synthesis can occur throughout the life of the cell(Munro et al., 1978). Iron can regulate the synthesis of ferritin at two post transcriptional steps. The synthesis of ferritin in cells specialized for iron storage is thus a pardigm for translational control mechanisms. However because ferritin functions both as a housekeeping protein(iron stored for intracellular use) and a specialized-cell protein(iron stored for use by othe cells), mechanisms of regulation could vary depending on the cell type. The mechanisms of ferritin synthesis induced by iron have been studied fairly extensively, but little is known about other agents that induce ferritin accumulation eg. infection, inflammation, and heat shock (Furst et al., 1988, Zahriger et al., 1976). Transgenic red pepper of CaMV35S promoter-Fp1 gene are expressed more highly in certain tissues than in others and can display variable patterns(Kim et al.

Corresponding author : **Kwon-Kyoo Kang,** E-mail: kykang@hnu.hankyong.ac.kr

1998). From results of Northern and Western blot, most of transgenic red pepper lines were found to show at least some expression in all tissues with strong expression in A6, A7 and A8 lines(Kim et al. 1998). In this paper, we has approached the question of the physiological functions of these iron storage proteins by studying their effects in transgenic plants, and present here that red pepper plants transformed with Fp1 gene show genetic analysis to progenies and total iron content in development stages and various organs.

MATERIALS AND METHODS

Plant materials

Hypocotyl segment of red pepper seedlings were inoculated with *A. tumefaciens* LBA 4404 harboring pFp1(Kim et al. 1998). After one month, regenerated plantlets were sown onto a mixed bed of vermiculite and peat moss(1:1) and grown to maturity at 28℃ on an 18h day-length cycle. The regenerated plants ensured to be originated independently from individual calli, were designated numerically according to the experimental series. The first generation progeny, R1, were obtained by self-pollination of the primary transformants. These procedures have been described in detail previously (Kim et al. 1998).

Total iron content

Different organs and tissues of transformant plants were harvested at different times, frozen in liquid N_2 and stored at -70℃ Total iron concentration was measured by recording absorbance of Fe^{2+} o-phenanthroline (0.02%) at 510nm, pH6.0 (50mM acetic acid/NaOH buffer), using thioglycollic acid (Sigma) as a reducing agent. Total iron of the each tissues was determined after dry ashing according to Scott (1944).

Genetic analysis

Approximately 200 R1 seeds were sterilized in a 1%(v/v) NaOCl solution for 10min, washed with sterilized water and sown to a medium containing MS, 1% sucrose, 300μg/mL kanamycin and 1.5% agar. After 10-20 days, the ratio of surviving and dead seedlings were scored. In an additional analysis, appropriate numbers of kanamycin-resistant seedlings were randomly selected, transferred on to soil, and grown to maturity. Kanamycin-sensitive seedlings were selected before the cotyledons turned completely yellow, transferred on to a medium without kanamycin, and grown further. Although many germinated seedlings died, some recovered and grew normally. Kanamycin sensitivity was examined by directly spray with kanamycin solution(400μg/ml) on to young leaves once a day for 3days as described Benfey et al.(1989). After 2 weeks, plants turned yellow and showed necrotic symptoms were regarded as kanamycin sensitive, whereas unaffected plants were resistant.

RESULTS AND DISCUSSION

Characteristics of transgenic red pepper containing Fp1.

The almost uniqitous occurrence of Fp1 gene among higher plants suggest that they may play important physiological roles in the regulation of the life cycle. To examine the phenotypic effects of tansgenic red pepper containing Fp1 gene, the coding region was introduced into red pepper genomes in sense orientations, in the expectation that the normal expression of endogenous Fp1 homologue(s) of red pepper would be disturbed. Approximatly 2 month after transformation, young plants exhibited the symptoms of phenotypic alteration of reduced apical dominance. This change, observed in 4 out of 12 transformants, was clearly distinguishable from wild type plants(Table 1). Among 12 transformants, 4 plants were dwarf, with average height of 45cm which is equivalent to a 42% reduction in height in comparison with controls (74cm).

However all transformants showed normal flower development and produced viable seeds. Introduction of the Fp1 gene into the recipient red pepper genome was confirmed by Southern hybridization assay. Genomic red pepper DNA was simultaneously digested with Hind Ⅲ and *Eco*RI, cleaving 2.1kb internal fragment of the insert DNA, and was then hybridized with the ^{32}P-labelled *Fp*1 cDNA probe. Estimations from singnal intensity showed that transformants contained the *Fp*1 gene at up to three loci(Fig. 1, Table 1). Northern hybridization analysis showed many transformed plants integrated *Fp*1 gene. However the hybridization signal intensity differed among transformants and in some of which *Fp*1 were not expressed desipte it's integration.

Table 1. Properties of normal and transgenic red pepper plants.

Plants	Height (cm)	Fertility[a]	Apparent	Copynumber of Fp1 gene	Expression of Fp1 gene gene	Copy number of NPT Ⅱ [b]
WT	76	+	N			
WT	70	+	N			
Transformant						
A1	59	+	N	1	+	nt
A2	40	+	N	nt	+	nt
A3	67	-	N	1	+	nt
A4	48	+	N	1	+	1
A6	67	+	N	2-3	+	2
A7	60	+	N	2-3	+	2
A8	71	+	N	2-3	+	2
A9	70	-	N	nt	+	1
A10	70	+	N	1	+	1
A12	48	+	N	nt	+	1
A13	46	+	N	nt	+	1
A14	58	+	N	1	+	1

WT: wild type ; N: normal type ; nt: not test.

a: Formation of viable seeds. b: Estimated from the segregation of R1 progeny whenever available.

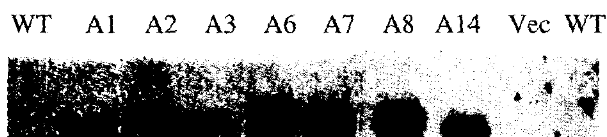

Fig. 1. Estimation of Fp1 gene numbers in transgenic plants by Southern hybridization analyis. A 5㎍ sample of total genomic DNA was simultaneously digested with ECORI and Hind Ⅲ. Sample, are from wild type control(WT), transgenic plants, A1, A2, A3, A4, A6, A7, A8, A10, A14, vector, and wild type control 2(WT2).

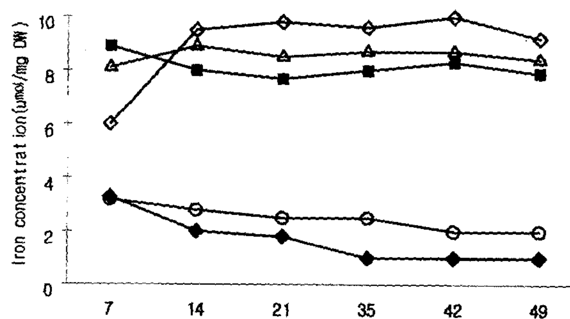

Fig. 2. Total iron content on the basis of dry weight in growing leaves of transgenic red pepper at different time after germination.

■: A8, △: A6, □: A7, O: A11, ◆: wild type

Table 2. Iron content of the various organs at 42days and 63days after germination.

Plant type / Growth(days)	WT			A7			A8		
	L	N	F	L	N	F	L	N	F
42	0.3±0.1	0.3±0.1	1.7±0.2	11.7±0.9	11.0±1.2	14.9±0.4	8.1±0.2	8.7±0.2	9.4±1.4
63	0.39±0.2	0.91±0.3	1.9±0.4	13.1±0.4	13.9±1.1	14.0±1.3	10.4±0.7	9.6±0.9	12.1±0.8

WT: Wild type; A8 and A14: Transgenic red pepper; L: Leaf; N: Node; F: Fruit.

Table 3. Segregation of kanamycin-resistant and genotype of iron content in R1 progenies.

	Kanamycin sensitivity			Genotype		
	Resistant	Sensitive	x^2-test	High iron*	Normal iron*	x^2-test
Wild-type	0	217		0	10	
Transgenic						
A6	219	10	1.32 (15:1)	17	2	2.12 (3:1)
A7	263	11	2.34 (15:1)	27	5	4.8 (15:1)
A8	176	16	1.42 (15:1)	12	1	0.05 (15:1)
A14	230	74	0.07 (3:1)	8	5	1.25 (3:1)

The transgenic plants with high transcript were used to determine iron content on the basis of dry weight during development stage and from various organs (Fig. 2). In A6, A7 and A8 transgenic plants during development stages, iron content was clearly distinguishable from untransformed control plants. In untransformed control plants, total iron content was almost constant with a mean value of 2.5μM. However A6, A7 and A8 showed almost 8-fold of content with 9.6, 10.1 and 9.7μM (Fig. 2). These results indicate that *Fp*1 gene showed high expression because stably integrated in the red pepper genome. Also iron content of A7, A8 was almost constant, except at fruit, for which it increases slightly(Table 2).

Transmission of Fp1 gene to progenies

In order to examine whether iron content in the various organs in transgenic red pepper were heritable, R1 progenies of selfed plants were tested for kanamycin resistance (Kan^R) and iron content trait. As the integrated *Fp*1 gene was located down-stream of the NPTII gene and uper-stream of the hygromycin, which confers Kan^R and Hyg^R to the pBI 121 Hm vectors. The wild-type control seeds germinated normally on agar plates containing kanamycin and hygromycin, but soon turned yellow and died during about 2 weeks after sowing. In contrast, R1 progenies of most transformants segregated in a 15:1 ratio into resistant and sensitive seedlings, indicating that one copy of the NPTII was stably transmitted(Table 2). The exception was the A14 plants, which segregated into a 3:1 ratio, and thus presumably contained one copy of the NPTII gene. As the number of intergrated NPTII genes, estimated genetically, was in agreement with the number of *Fp*1 gene, estimated by Southern hybridization analysis(Table 1). From these results conclude that the NPTII, HygR and *Fp*1 genes must have been co-integrated. Plants sown in soil without kanamycin and hygromycin and grown for 2 months segregated into high and low iron content in approximately a 3:1 ratio in A14 plant and a 15:1 ratio in A6, A7 and A8, as would be expected from one and two integrated copies of *Fp*1, respectively. When these young plants were sprays with kamamycin solution, treated leaves of the

normal phenotype plant turned yellow and exhibited necrotic symptoms while leaves of the transformants were totally resistant to the drug. These results confirmed that the NPT-II and *Fp*1 genes were co-intergrated, and that the dwarf phenotype was indeed caused by in vitro culture.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Hankyong National University in 1998.

REFERENCES

Benfey, P. N. and Chun, N. H., 1989. Regulated genes in transgenic plants. Science 244:174-181.

Cairo G, Bardella L, Schiaffonati L, Arosio P, Levi S, Bernelli-Zazzera A., 1985. Multiple mechanisms of iron-induced ferritin synthesis in HeLa cells. Biochem Biophys Res Comm 133: 314-321(1985).

Esau. K., 1968. Viruses in Plant Hosts: Form, Distridution, and Pathologic Effects. pp. 58-60. Univ. of Wisc. Press, Madison.

Furst P. Hu S, Hackett R, Hamer D, 1988. Copper activates methalothionein gene transcription by altering the conformation of a specific DNA binding protein. Cell 55: 705-717.

Kim YH., YO. Lee, IS. Nou, IY. Shim, T. Kameya, T. Saito and KK. Kang, 1998. Iron accumulation in transgenic red pepper plants introduced Fp1 gene encoding the iron storage protein. Plant Res. 1(1) 6-12.

Lobreaux S, Briat JF., 1991. Ferritin accumulation and degradation in different organ of pea(*Pisum sativum*) during development. Biochem J 274: 601-606.

Munro. H. N. Linder, M.C., 1978. Ferritin: Structure, biosynthesis and role in iron metabolism. Physiol. Rev. 58. 317-396.

Rouault TA, Hentze MW, Haile DJ, Harford JB, Klausner RD, 1989. The iron-responsive element binding protein: a method for the affinity purification of a regulatory RNA binding protein. Proc Natl Acad Sci USA 86: 5768-5772.

Scott EM, 1944. The determination of iron in tissues. Arch. Birched. 6: 27-32.

Spence MJ, Henzl MT, Lammers PJ, 1991. The structure of a Phaseolus vulgaris cDNA encoding the iron storage ferritin. Plant Mol Biol 117: 499-504.

Sprey, B. G. Gliem, and A. G. S. Janossy, 1977. Changes in the iron and phosphorus content of stroma inclusions during etioplast-chlorolast development in *Nicotiana*. Z. Naturforsch. 32c: 138-139.

van der Mark F, de Langer T, Bienfail HF, 1981. The role of ferritin in developing primary bean leaves under various light conditions. Planta 153: 338-342.

White K, Munro HN, 1988. Induction of ferritin subunit synthesis by iron is regulated at both the transcriptional and translational levels. J Biol Chem 263: 8938-8942.

Zahriger J, Baliga BS, Munro HN, 1976. Novel mechanism for translational control in regulation of ferritin synthesis by iron. Proc Natl Acad Sci USA 73: 857-861.

Received 2001. 1. 1

Accepted 2001 4. 1