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Iron Accumulation in Transgenic Red Pepper Plants Introduced Fp1 Gene Encoding the Iron Storage Protein

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ABSTRACT

The Fp1 gene, originally isolated from red pepper seedlings, encode the iron storage protein, and have a high homology with ferritin genes at DNA and amino acid level. In order to determine ferritin protein expression in vegetative tissue, Fp1 gene was constructed in plant expression vector(PIG121Hm) and introduced in red pepper(var. Bukang, Chungyang and Kalag-Kimjang 2) via *Agrobacterium tumefaciens*mediated transformation. After selection on MS media containing kanamycin(Km), putatively selected transformants were confirmed by amplification of selectable marker gene(Fp1 and NPTII) by polymerase chain reaction. Northern blot showed that transcripts of Fp1 gene were detected in mature leaves of the plants. In A6, A7 and A8 and A14 of transgenic plants, transcript of Fp1 gene was increased seven-fold to eight-fold than other transgenic plants. Also the proteins obtained from leaves of transgenic plants were immunologically detected by Western blot using rabbit anti-ferritin polyclonal antibody. The expression protein appeared as strong band of apparent mass of 23.5kDa, suggesting the iron accumulation in transgenic red pepper plants.

Key words: iron storage protein, northern blot, PCR amplification, red pepper, transgenic plant, Westwen blot.

INTRODUCTION

Over the past decade, the value of introducing foreign genes into plants has been well documented. In plants, genetic transformation has been used to study plant processes such as the action of transposable genetic elements (Finnegan et al., 1989; Spena et al., 1989), the impact of manipulating metabolic pathways(Last and Gray, 1990; van. der Krol et al., 1990), tissue specific gene expression(Benifey and Chua, 1989) as well as other aspects of gene regulation(Matzke et al., 1989; Rocha et al., 1989). The application of genetic transformation techniques to answer basic research questions will increase in frequency and sophistication as methods continue to improve. The availability of gene transfer system has already lead to several important insights into the regulation of gene expression and protein function in plants. Combined with efficient expression system for the introduced genes, gene transfer could turn plants into a suitable alternative for the production of desirable products. In living organisms, iron is of great interest because of its role in important metabolic processes such as oxygen transfer, nitrogen fixations, electron transfer and DNA synthesis (ribonucleotide reduction). However, its tendency to form insoluble salts in aqueous solutions and its potential for toxicity via free radical formation as result of specific genetic systems which control iron homoeostasis in cells. These systems include iron uptake, transport and storage (Theil, 1987). Iron storage is achieved by class of multimeric(24-mer)

proteins called ferritin(Theil, 1987). They organized in hollow spheres able to accommodate a few thousand iron atoms inside their central cavity. Ferritin are known to sequester and thus detoxify iron taken up by cells which is not utilized for metabolic requirements. Under conditions iron need, ferritin - Fe(Ⅲ) can be released by reduction for cellular used(Bienfait and van der Vriel, 1980; Laulhere et al., 1990). Therefore, ferritins are key proteins acting as a buffer for iron, protecting cells from a harmful concentration of free iron and regulating their immediate need.

In this paper, we describe stable transformation of pepper by *Agrobacterium* mediated and investigate the iron accumulation and gene expression from transgenic red pepper plants introduced Fp1 gene encoding the iron storage protein.

MATERIALS AND METHODS

plasmid construction

All procedures used for DNA manipulation were carried out according to the procedures of Maniatiatis et al(1982). The plasmid PIG121Hm, is binary vector that contains chimeric gene for hygromycin resistance and GUS in the T-DNA region, as well as for kanamycin resistance. The open reading frame of the β - glucuronidase coding sequencing (GUS)gene was removed by digestion with XbaI by making the ends blunt with the klenow fragment of *Escherichia coil* DNA polymerase I, followed by digestion with *SacI* (Fig. 1).. The ferritin open reading frame was isolated from pRF12 as a *EcoRI*(made blunt with the klenow fragment of *E. coli* DNA polymerase Ⅰ), and was inserted in place of the ferritin open reading frame.

Transformation of pepper

The intermediate plasmid, pHF15 were introduced into *Agrobacterium tumefaciens* strain EHA101 and LBA 4404 by triparental mating using pRK2013 as helper. Introduction of the plasmids was confirmed by Polymerase Chain Reaction(PCR) method using NPT Ⅱ specific primer. Hypocotyl and cotyledon of red pepper(var. Bukang, Chungyang and Kalag-Kimjang 2) were infected with the *A. tumefaciens* strains harboring co-integrated Ti vectors by the method of Horsch et al(1985). Plant transformants were selected on MS(Murashige and Skoog, 1962) agar medium supplemented with 500mg/L Carbenicillin, 100mg/L Kanamycin, NAA 0.1mg/L, Zeatin 2mg/L and $AgNo_3$ 1mg/L, regenerated plantlets obtained from hormone free MS medium with 100mg/L of Kanamycin, were checked for correct integration of the constructs in the red pepper genome by PCR.

DNA analysis by PCR

Genomic DNA of red pepper was prepared according to the method of Hirai(1989). The presence of the recombinant gene in generated red pepper plants was confirmed using PCR with the primers 5' -TTGTCAAGACCGACCTGTCC-3' , and 5' -ACCGTAAAGCACGAGGAAGC-3' , specific for the NPT Ⅱ gene, and 5' -AGTGAGGAAGAAAGAGAGCA-3' and 5' -AAAGTGCCAAACACCGTG-3' , specific region of Fp1 gene. DNA fragments were amplified in a total volume of 100μl with 10mM Tris-HCl, pH8.3, 40mM KCl, 1.2mM $MgCl_2$, 1mM DTT, 0.01% gelatin, 1.5mM each specific primer, and 2.5 units of Taq polymerase(Takara). PCR condition was one cycle at 94℃ 3min and following 35cycles at 96℃ 1min, 55℃ 2min, 72℃ 4min, and finally 72℃ 10min. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized after

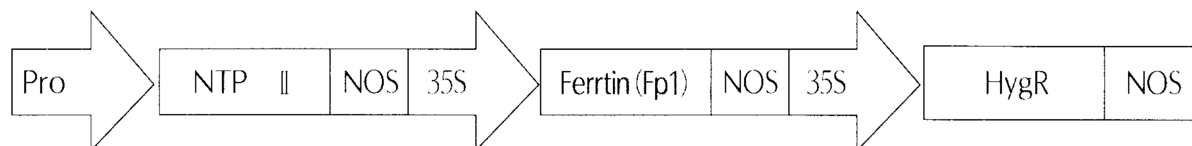

Fig. 1. PIG121hm vector construction containing Fp1 gene encoding iron storage protein of red pepper which is locate on ORF region of GUS.

staining with ethidium bromide.

Northern blot analysis

RNAs were extracted by the guanidine method with the following modification. After phenol extraction, total nucleic acids were precipitated by isoprophanol. RNAs were differentially precipitated in 2M LiCl according to SanO et al(1991). For Northern blot experiments, RNAs were electrophoresis, through formaldehyde agarose gels and blotted on to Hybond N plus membrane(Amersham) according to the manufacturers instructions. The probe was a 540bp *Sac*I-*Stu*I restriction fragment which corresponds to the mature subunit coding sequence. This fragment was subcloned in plasmid Bluescript II KS(Stratagene) which allows to prepare riboprobes. Prehybridization was performed for 4hr at 68℃ in 50% formamide, 5×SSC, 50mM Tris-HCl pH7.5 0.1% sodium pyrophosphate, 1% SDS, 0.2% PVP, 0.2% Ficoll, 5mM EDTA and 150μg/ml denatured salmon sperm DNA. Hybridizations were achieved in the same buffer containing the probe at 68℃ for 12hr. Filters were washed twice in 2×SSC, 0.1% SDS for 15min and twice in 0.1×SSC, 0.1% SDS for 15min. at 68℃ prior to exposure at -70℃ using Kodak X-ray films.

Western blot analysis

The protein extraction procedure was done as described by Hurkman and Tanaka(1986). The mature leaves were cut from the transgenic and nontransgenic plants, immediately frozen in lipid nitrogen, and stored at -70℃ until use. Soluble proteins were extracted from 500mg tissue, homogenized on ice with a polytron homogenizer in three volumes of 10mM sodium phosphate buffer (pH 6.0) and 1% sodium bisulfite as a reducing agent, except that 0.1% o-phenanthroline and 20mM EDTA were added to the extraction buffer in order to prevent frenton reactions leading to possible ferritin degradation during sample preparation (Laulhere et al., 1990). The homogenate was centrifuged at 14000 × g for 10 min. The protein content was measured as described by the method of Bradford(1976). Prior to fractionation on SDS-PAGE, protein was denatured at 100℃ for 3min in a solution containing 100mM dithiothreitol, 2% SDS, 50mM Tris-HCl (pH6.8), 10% (v/v) glycerol, 0.1% bromphenol blue. Electrophoresis was performed at 25mA in 12% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970) and electroblotted to Immobile-P membrane (Milipore, Bedford, MA). Blots were blocked for 1hr in phosphate buffered Saline(PBS) containing 3% skim milk and incubated with polyclonal anti-ferritin antibody(1:1000 dilution) for 1hr. After washing buffer (PBS + 0.5% Triton X-100) three times for 10min each, the blot was treated with alkaline phosphatase-conjugated goat anti rabbit antiserum and developed with BCIP/NBT substrate solution.

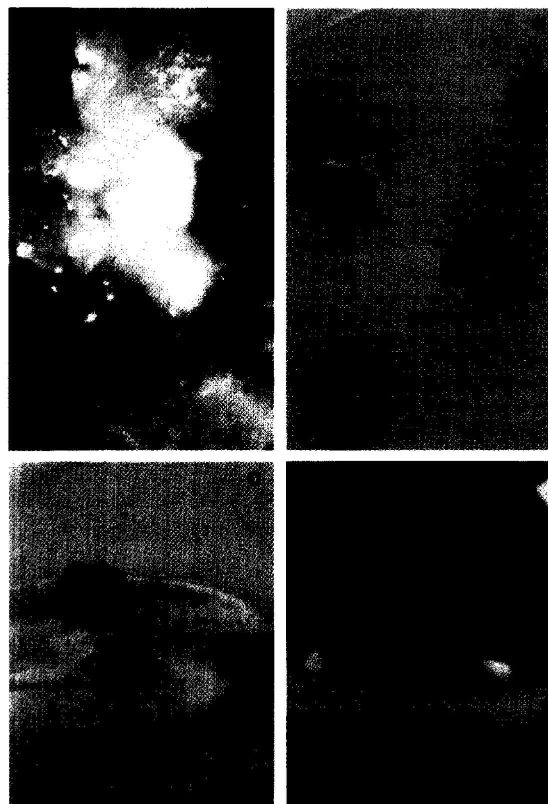

Fig. 2. Differentiation of shoots red pepper leaf tissues inoculated with *A. tumefaciens* containing PIG121Hm-Fp1 vector. Plant transformants were selected on MS agar medium supplemented with 500mg/L Carbenicillin, 100mg/L Kanamycin, NAA 0.1mg/L, Zeatin 2mg/L and AgNo3 1mg/L(A, B), regenerated plantlets obtained from hormone free MS medium with 100mg/L of Kanamycin(C, D).

RESULTS

Transgenic plant formation

Hypocotyl and cotyledon of red peppers(var. Chungyang, BuKang, Kalag-Kimjang 2) were co-cultivated with *Agrobacterium tumefaciens* strain EHA101 and LBA4404. They were further cultured on the MS medium containing NAA 0.1mg/L, Zeatin 2mg/L and $AgNo_3$ 1mg/L which contained 100mg/L kanamycin, 500mg/L carbenicillin for selecting transformed plants. All the three varieties responded but good transient expression was detected in Chungyang and BuKang and to a less extent in Kalag-Kimjang 2. After one month, the kanamycin resistant plants from all varieties were transfered to the MS medium containing NAA 0.1mg/L, Zeatin 2mg/L, 100mg/L Kanamycin, where shoot formation occurred(Fig. 2). The transformed plants were readily regenerated on the MS medium containing Kanamycin 100ppm without any hormone(Fig 3).

The frequency of regeneration varied from 5-20% of

Fig. 3. Regenerated whol plant of transgenic red pepper(A, B) and wild type(C). Transgenic plants were transplanted in to the plastic pot and maintained in a greenhouse.

Table 1. Number of inoculated tissue with *A. tumefaciens*, regenerated plant and transgenic plant in red pepper

	Number of		
Variety	Inoculated tissue	Regeneration plant	transgenic plant
ChungYang	196	3	1
BuKang	113	15	5
Kalag-KimJang 2	106	19	6

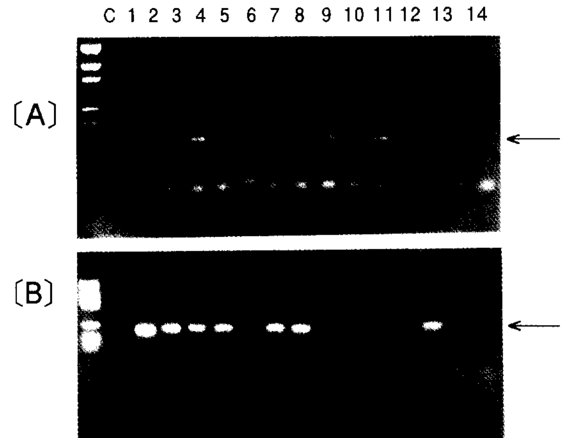

Fig. 4. Amplification of Fp1 gene(A) and NPTII gene(B) in putative transgenic red pepper plants by PCR(C: wild type control, 1-14: putative transgenic plants).

selected plants. BuKang showed a transformation frequency of 16.5% (Table 1). These transgenic plants were grown to a greenhouse(Fig. 2d), and investigated for integrate of foreign DNA in the genome of transgenic plants by PCR and the expression of ferritin protein by Northern and Western blot analysis. To determine the correct integration of the foreign DNA in the red pepper genome, we checked by PCR using of Fp1 and NPT Ⅱ gene specific primers on the open reading region. As shown in Fig. 4, the DNA fragment of 380bp size(Ferritin) and 590bp (NPT Ⅱ) were identified in transgenetic plants. However, these foreign DNA was not detectable in the nontransformed plant.

Northern blot analysis

The expression of Fp1 gene in wild type and transgenic plants of pepper was analysed by Northern hybridization. Thirty microgram of total RNA was applied to agarose-formaldehyde gel electrophoresis and transferred to a Hybond N plus membrane. The amounts of total RNA loaded were normalized with plant acting gene(Sano et al., 1993). Transcripts of Fp1 gene was detected in all transgenic plants integrated with Fp1 gene, indicating that mRNA of the proper size was accumulated in the transformed plants. However, the hybridization signal intensively differed among all transformants, in some of

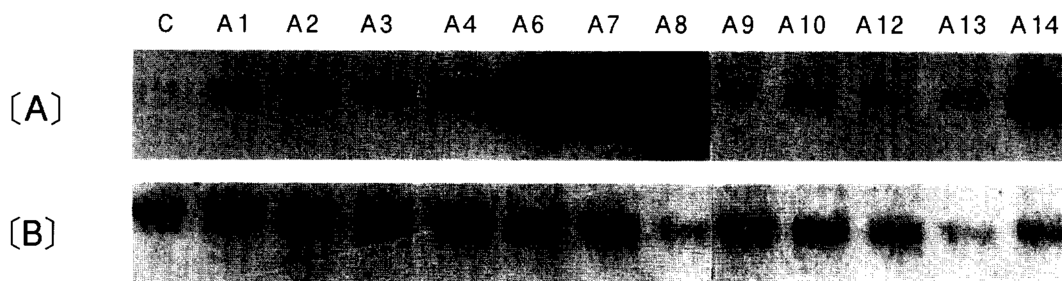

Fig. 5. Profile of Northern blot analysis in transformants(A1, A2, A3, A4, A6, A7, A8, A9, A10, A12, A13, A14) and wild type control(C). 40g of total RNA extracts from transgenic and wild type plant. Hybridizations were performed in 6 × SSPE (0.1% SDS, 0.02% PVP, 0.02% Ficoll, 50㎍/ml salmon sperm DNA) with a partial pepper ferritin cDNA labelled with 32P-dCTP(Amersham 18.5 Bq/mmol) and a random priming kit from Pharmacia, at 52℃.

which ferritin gene was expressed with small amount despite its integration(Fig. 5). In A_6, A_7 and A_8 and A_{14} transgenic plants, increase in the ferritin transcript was observed seven-fold to eight-fold than other transgenic plants(Fig. 5).

Western blot analysis

To investigate whether ferritin protein is expressed in the transgenic plants, soluble proteins were extracted from mature leaf tissue and extract corresponds to 10mg of leaf were fractionated on 12% SDS-PAGE. After transferred to membrane, Western analysis was carried out using anti-ferreting polygonal antibody. Although the ferritin proteins were not detected on SDS-polyacrylamide gel with silver staining, the immunoreactive bands were recognized in all transgenic plants tested by the anti-ferritin antibody except for A3, but the extract of the control plant did not react with the antibody (Fig. 6). The difference of intensity among transgenic plants showed the ferritin expression at different levels, which was roughly proportional to the levels of specific ferritin mRNA. The ferritin protein produced in transgenic plants migrated in the SDS-PAGE with a molecular mass of 23.5kDa.

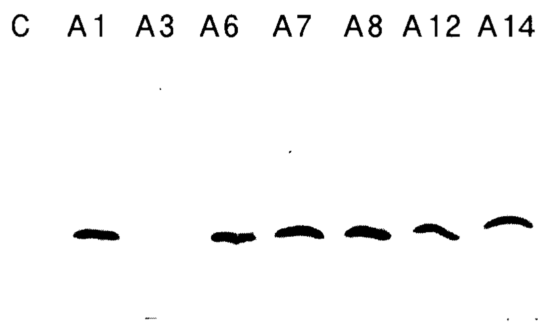

Fig. 6. Immunodetection of ferritin protein in transgenic plants. Total protein extract corresponded to 10mg of mature leaf tissue was fractionated by SDS-PAGE and blotted ontoa membrane, probed with anti-ferritin polyclonal antibody, and developed with BCIP/NBT reagents. C: Wild type control. A1, A3, A6, A7, A8, A12, A14: Transgenic red pepper plants.

DISCUSSION

This experiment have effectively disassociated ferritin protein overproduction in vegetative tissue of red pepper via a combination of molecular genetic approaches. The advantages of *Agrobacterium* mediated gene transfer over other methods that can be used for the transformation of higher plants include the high efficiency of transformation, the transfer of pieces of DNA with defined ends, the transfer of relatively large segments of DNA, and the absence of requirement for protoplast culture techniques. Therefore, this type of gene transfer is normally the method of choice when more than one method is available. In the case of red pepper, information of culture medium for successful gene transformation is still scanty on the media used red pepper(Gumay et al., 1980). The most balanced mixture containing macro- and microelements is the basic medium of Murashige and skoog(1962) completed with vitamins, carbon sources and growth substances. Some

modified variations of MS have also been applied for regeneration, demonstrated direct induction of adventitious meristems from cotyledon and hypocotyl in pepper(Gunay and Roa, 1980). The frequency of regeneration varied from 5-20% of selected plants, which was as high as reported by Yang et al.(1997). Also, this experiment used a binary vector, pIG212Hm-Ferritin which was derived from one of the most common binary vector, pBI121(Ohta et al.,1990). Previous some papers reported that a super binary vector, in which a DNA fragment from the virulence region was introduced into a binary vector, was more effective for gene transformation(Hiei et al., 1994; Ohta et al. 1990). In plant, ferritin gene regulation has common features, since excess iron leads to the accumulation of ferritin protein to store iron. Also, translational control of ferritin mRNA leads to changes in ferritin synthesis that is dependent on intracellular iron concentration. Lescure et al.(1991) and Stephane et al. (1991) have reported that ferritin mRNA leads to a rapid and transient accumulation by iron loading. CaMV35S promoter used in this experiment are expressed more highly in certain tissues than in others and can display variable patterns. From results of Northern and Western blot, transgenic red pepper lines indicate that most lines show at least some expression in all tissues and that strang expression in A6, A7 and A8 and A14 line.

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