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RESEARCH NOTE

Tissue-specific histochemical localization of iron and ferritin gene expression in transgenic *indica* rice Pusa Basmati (*Oryza sativa* L.)

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Introduction

Several molecular-biological techniques are employed to characterize transgene expression in transgenic plants and animals. However, no technique is available to detect expression of a transgene product in a tissue-specific manner. Histochemistry is a powerful technique for localization of trace quantities of substances present in biological tissues (Pearse 1972, 1988; Krishnamurthy 1998). Histochemical techniques have been employed to characterize structure and development, and to study time course of deposition and distribution of major storage compounds such as protein, lipid, starch, phytin, and minerals such as calcium, potassium and iron in rice grains (Krishnan *et al.* 2001; Krishnan and Dayanandan 2003).

Iron is found in haemoglobin, which transports oxygen in the blood of vertebrates. When iron levels are low, the amount of available oxygen declines, causing a common symptom of iron deficiency called anaemia. Iron deficiency causes a range of health problems in humans, including poor pregnancy-related complications, brain damage in infants, chronic hypoxia and reduced work performance (Goto and Yoshihara 2001). Dietary iron deficiency owing to an insufficient and inappropriate diet is a severe nutritional problem (Goto *et al.* 2001) that affects 30% of the world's population (WHO 1992).

Plant-based foods are potential sources of all essential minerals and organic nutrients that are directly or indirectly required by humans. Unfortunately, mineral contents are low in many of the staple food crops. Hence efforts to increase the mineral content of food crops are required as a means to ensure sufficient dietary minerals for all individuals (Grusak 2002). Rice is the most important staple food crop in the world; nearly half of the world's population depends on rice as the source of their calories. Therefore, increasing iron content in rice is a viable option in alleviation of micronutrient malnutrition anaemia (Coffman and Juliano 1987).

Ferritin is an iron storage protein which stores 4500 iron atoms in its central cavity in nontoxic form (Korcz and Twardowski 1992). Iron stored in ferritin is completely bioavailable (Goto *et al.* 2001) and is released when need arises for metabolic functions (Briat 1996). There are reports of increasing iron content by overexpressing ferritin gene in transgenic rice (Goto *et al.* 1999; Vasconcelos *et al.* 2003) and wheat (Drakakaki *et al.* 2000). Since Asian countries, where most of the world's rice is grown, have different climatic and agroecological zones, it is important to enhance iron content in highly adapted local-specific native *indica* rice varieties (Krishnan *et al.* 2003). Our study is aimed at increasing iron content in a high-economic-value *indica* rice variety (*Oryza sativa* L. cv. Pusa Basmati) by manipulating ferritin gene expression. Here, we report expression of soyabean ferritin under the control of rice endosperm-specific glutelin promoter (GluB-1) (Takaiwa *et al.* 1991) and iron accumulation in the endosperm of transgenic rice by iron-specific histochemical staining reaction.

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Keywords. ferritin; *indica* rice; *Oryza sativa*.

Materials and methods

Plant material and transformation

Ferritin cDNAs from *Glycine max* L. cv. Kitano-shiki (Lescure *et al*. 1991) and *Avicennia marina* (Jithesh *et al*. 2006) were used for transformation. We report our findings on overexpression of *Glycine max* ferritin in rice plants. Six independent integration events of ferritin transgenic lines were developed by *Agrobacterium*-mediated transformation (Rashid *et al*. 1996) in the *indica* rice *Oryza sativa* L. cv. Pusa Basmati at the M. S. Swaminathan Research Foundation, Chennai, India. Matured nontransgenic and T_2 homozygous transgenic seeds were used for histochemical and immunoblot analysis.

Localization of iron

Perl's Prussian blue technique was employed for localization of iron (Pearse 1972). This is an extremely reliable and sensitive technique that can detect even small quantities of iron microscopically (Krishnan *et al*. 2003). Transgenic and control rice caryopses were soaked in distilled water for about 2–3 h and sectioned at 15 μm using a Vibratome series 3000 sectioning system (Intracel, Royston, UK) (Krishnan *et al*. 2003). Thin transverse sections of transgenic and nontransgenic rice grains were treated with a mixture of freshly prepared 2% potassium ferrocyanide and 2% hydrochloric acid for 20–30 min. The treatment with acid was done to release ferric ions from the tissue, which immediately reacted with the cation of potassium ferrocyanide to produce a blue insoluble compound, ferric ferrocyanide. Observations and photographs were made using a Nikon E-800 microscope with bright-field optics.

Protein extraction and immunoblotting

Total protein was extracted from matured nontransgenic and T_2 homozygous transgenic seeds. The seeds were homogenized with mortar and pestle with 300 μl of extraction buffer (80 mM Tris-Cl pH 7.0, 2% SDS, 20% glycerol, 2% mercaptoethanol, 10 mM PMSF). The samples were then spun at 10,000 g for 15 min, and the supernatant was transferred into a fresh tube. Total protein in these extracts was measured with Genei protein estimation kit (Bangalore Genei, Bangalore, India). The protein samples (20 μg each) were then boiled for 3 min with 2× sample buffer (100 mM Tris.Cl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and electrophoresed on 12% (w/v) SDS-PAGE gel with appropriate protein marker (New England BioLabs, Ipswich, USA). The gel was transferred to PVDF membrane (Westran S; Schleicher and Schuell Bioscience, Dassel, Germany) according to the method described in Sambrook *et al*. (1989).

For Western blot analysis, the membrane was blocked for 2 h to prevent nonspecific binding by incubating in blocking solution (3% BSA in 1× Tris HCl buffered saline (TBS) (10 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBST)) at room temperature with gentle agitation, and then washed with 1× TBST thrice. After blocking, probe of pea ferritin primary antibody (1/500 dilution in 1× TBST) was added and the membrane incubated for 2 h with gentle agitation. Unbound antibody was removed by washing the membrane thrice with 1× TBST. The membrane was then treated with alkaline-phosphatase-conjugated secondary antibody (goat anti-rabbit IgG, 1/2000 dilution in 1× TBST; Pierce, Rockford, USA) for 2 h. After preincubation in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris.Cl pH 9.5) for 10 min the membrane was stained with substrate for alkaline phosphatase (1-step NBT/BCIP mix; Pierce, Rockford, USA) to detect protein. The staining reaction was stopped by adding distilled water.

Results and discussion

Southern blot analysis was carried out with genomic DNA from nontransgenic and T_2 homozygous PCR-positive plants. Genomic DNA digested with *Eco*RI and probed with ferritin cDNA showed the expected hybridization signal of 800 bp in all the transformed plants (figure 1). This confirmed integration of the ferritin cDNA in the rice genome.

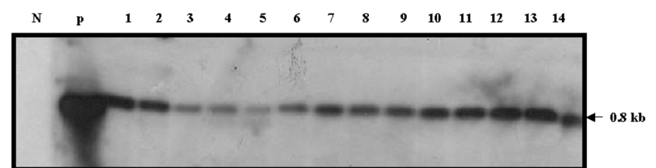

Figure 1. Southern blot analysis of T_2 homozygous transgenic rice plants. Genomic DNA (15 μg) was digested with *Eco*RI (MBI Fermentas, Hanover, USA), fractioned on 0.8% agarose gel, transferred to Hybond N+ membrane (Amersham Biosciences, Piscataway, USA), and subjected to hybridization using an 800 bp ferritin probe. N Nontransformed control; lanes 1–14, transgenic plants; P, positive control (binary vector pFer 4.0 digested with *Eco*RI to release 0.8 kb ferritin cDNA).

Perl's Prussian blue staining of transgenic rice grain sections showed distribution of iron accumulation (blue compound of ferric ferrocyanide) throughout the alureone and subaleurone layers and also in the central region of starchy endosperm (figure 2,b). However, in nontransgenic grains, blue colour formation indicating iron accumulation was restricted only to aleurone layer and the intensity of colour was also very low (figure 2,a). Perl's Prussian blue staining method has been recommended for locating Fe(III) in animal tissues since it is a highly reproducible and fast method and the reagent can penetrate into bulky tissues to give a distinctive blue colour reaction (Baker 1958). This technique has been used in nontransgenic rice to localize iron in seed materials (Krishnan *et al*. 2001).

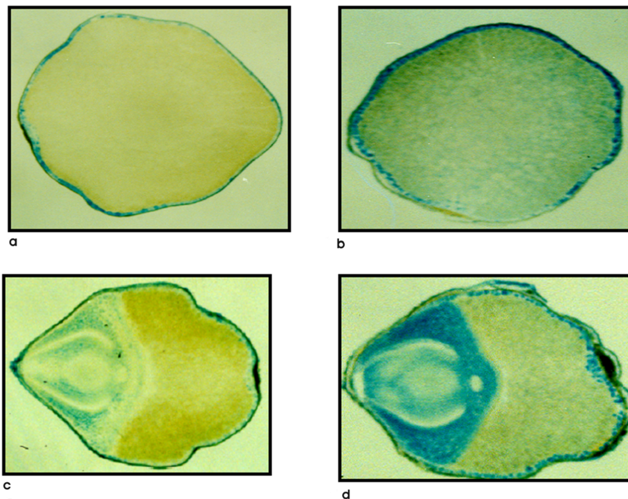

Figure 2. Transverse section of matured nontransformed (a, c) and transgenic (b, d) rice grains; blue colour indicates presence of iron. In control, iron is restricted only to aleurone and embryo, and is not found in the endosperm. However, in transgenic rice grain, in addition to aleurone and embryo, iron is also strongly present in the endosperm cells. Also note the higher accumulation of iron in embryo of transgenic rice, compared to control, as shown by intensity of colour.

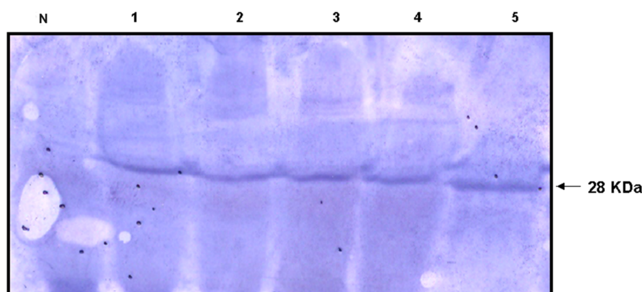

Figure 3. Immunoblot analysis of ferritin protein in transgenic seeds. Total protein was extracted from matured seeds of transgenic and nontransgenic plants. Twenty μg of total protein was loaded in each lane of 12% SDS-PAGE gel. Seperated protein was transferred onto a PVDF membrane. N, Nontransformed control; lanes 1–5, transformed seeds; molecular mass of ferritin protein (28 kDa) is indicated.

Transverse section of mature transgenic rice grains passing through the embryo region showed high iron accumulation in embryo as well as in the endosperm (figure 2,d). However, in nontransgenic rice grains iron was restricted to the embryo and aleurone layer. Further, compared to transgenic grains, the intensity of colour development in the embryo region was very low (figure 2,c). This histochemical analysis of iron in rice specifically showed temporal and spatial deposition of storage iron.

In all the transgenic Southern-positive plants (seeds), a 28-kDa ferritin protein was detected (figure 3), confirming that ferritin protein accumulates in rice seeds. The polyclonal antibody raised against pea ferritin did not cross-react with endogenous rice ferritin. Hence, we did not observe any endogenous ferritin signal in the negative control (untransformed seeds). Similar observations have been made in *japonica* (Goto *et al*. 1999; Lucca *et al*. 2001) and *indica* rice varieties (Drakakaki *et al*. 2000; Vasconcelos *et al*. 2003) as well as in tobacco (Goto *et al*. 1998). Endogenous ferritin genes are developmentally regulated so that the protein is nearly undetectable except under stress or iron overload (Proudhon *et al*. 1989).

Most of the minerals in rice seeds, including iron, are completely removed during commercial milling (Krishnan *et al*. 2003; Bajaj and Mohanthy 2005). Genetic modification of quality of rice grains should focus on the subaleurone layers or endosperm as the major targets for controlling expression of relevant genes to get higher-quality transgenic plants. Manipulations of the deeper layers of endosperm to enhance proteins in rice grains were emphasized in an earlier study (Krishnan *et al*. 2001). Here we have shown increase of iron content in endosperm cells by expression of a foreign ferritin gene under control of endosperm-specific promoter.

We have shown that histochemical analysis is a powerful tool and can be used for preliminary screening of transgenic lines with high iron content (qualitatively) to select the desired line for further analysis. The method revealed the tissue-specific nature of expression of transgene ferritin, and temporal and spatial deposition of iron in the rice grains. This technique does not require antibody or radiolabelled probe. Previously, iron content of grains was measured only by chemical analysis. But this method is beset by technical problems when dealing with a large germplasm screening and limited amounts of sample materials. It has also been reported that iron content could vary widely among rice genotypes (Prom-u-thai *et al*. 2003). The histochemical technique can easily be used in large germplasm screenings to select the right background material for genetic engineering or for breeding programmes. Further studies are in progress on quantitative estimation of iron content in transgenic rice seeds using atomic absorption spectroscopy.

Acknowledgements

We thank Department of Biotechnology, Government of India, for financial support. The first author thanks Harvest Plus, USA, for an overseas fellowship to work at IRRI, Manila, The Philippines. We thank Dr F. Goto for providing *Glycine max* ferritin cDNA and GluBI promoter and Dr J. F. Briat for donating polyclonal antibodies against pea ferritin. We also thank Prof. Dayanandan (MCC, Chennai), Dr Jithesh and Prashanth (MSSRF) for very valuable suggestions and for reading this manuscript critically.

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Received 28 November 2005; in revised form 21 February 2006