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In situ Western hybridization: a new, highly sensitive technique to detect foreign and endogenous protein distribution in rice seeds

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Abstract We have developed a highly sensitive in situ Western hybridization technique to study tissue-specific expression of foreign and endogenous genes in transgenic and non-transformed rice seed. The expression pattern of the soybean ferritin gene directed by a rice glutelin gene promoter, *GluB-1*, in transgenic rice seed revealed by this method was exactly the same as that revealed by immunological tissue printing but much clearer than the latter, and corresponded well to the results of *GluB-1* promoter characterization studies. This method provides an alternative choice for studying the tissue-specific expression of a promoter, omitting the complicated transgenic procedure. The method can also be used to study the expression and accumulation pattern of endogenous genes, such as glutelin and prolamine genes, in non-transformed plants.

Keywords Foreign and endogenous proteins · In situ Western hybridization · Protein detection · Tissue-specific expression

Abbreviations *DAF:* Days after flowering · *GUS:* β-Glucuronidase · *GFP:* Green fluorescent protein · *TBS:* Tris-HCl-buffered saline · *TBST:* TBS with Tween-20

Introduction

The study of protein distribution in seed tissue is of great interest for the plant physiologist in the study of protein biosynthesis and storage, and for the molecular biologist studying the expression pattern of promoters in transgenic plants. Many approaches are now available for biochemical detection of a specific RNA or protein molecule in tissue extracts or at the histological level. Traditional Western hybridization is sensitive enough to analysis protein after electrophoresis and trans-blotting onto a nitrocellulose membrane, but fails to reveal the protein expression and accumulation pattern under natural conditions. Immunological electron microscopy has been used commonly in studying protein distribution in cells, and β-glucuronidase (GUS) (Jefferson 1987) and green fluorescent protein (GFP) are the commonest strategies used for investigating tissue-specific expression of promoters. Due to the equipment necessary, not every researcher can use electron microscopy. Though GUS and GFP can be easily detected in transgenic plants, the position effect of the inserted chimeric gene often leads to different and atypical expression patterns for the foreign genes in the individual transformants even though the inserts have the same construction. Therefore, it is necessary to develop a convenient method to detect the expression pattern of genes in the native organism. Tissue print is a successful approach for achieving this (Manteuffel and Panitz 1993). However, the results of tissue print are not always satisfactory since this method often results in unclear figures and varies tremendously with the moisture content of the nitrocellulose. In this communication, we report a sensitive and convenient method of in situ Western hybridization for localizing both native and foreign proteins in maturing and mature seeds.

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Materials and methods

Plant materials

Rice (*Oryza sativa* cv. Kitaake) seeds, both maturing and mature, were used for detecting the distribution of the native proteins glutelin and prolamine. The mature seeds of FK22 (Goto et al. 1999), which is a transgenic plant containing the soybean ferritin gene directed by a rice glutelin gene promoter, *GluB-1*, were used for detecting the foreign protein.

Antibody preparation

The antiserium against the soybean ferritin subunit was produced according to Goto et al. (1999). The preparation of anti-proglutelin *GluA-1* and *GluB-1* sera has been described previously (Katsube et al. 1999). The antiserium against the rice 13-kDa prolamine subunit was a gift from Professor K. Tanaka, Kyoto Prefectural University.

In situ Western hybridization

The dry seeds were soaked in distilled water at 4°C overnight. The softened mature seeds and maturing seeds were sectioned longitudinally with a razor blade into two parts containing both embryo and endosperm. After being soaked in acetone for 20 s, the seed sections were washed with deionized water and then soaked in 125 m*M* Tris-HCl, pH 6.8, with 2% sodium dodecyl sulfate for 30 min followed by washing with Tris-HCl-buffered saline (TBS) (10 m*M* Tris-HCl, 500 m*M* NaCl, pH 7.5) containing 0.1% Tween-20 (TBST) for 15 min and with TBS for 5 min, 3 times, respectively. In order to block non-specific antibody binding, the sections were placed in TBS, pH 7.5, containing 3% skimmed milk for more than 3 h. After the blocking procedure, the sections were allowed to react with the first antibody (against soybean ferritin, rice glutelin GluA-1, GluB-1 and rice 13-kDa prolamine) overnight in TBS containing 1% skimmed milk. The unbound antibodies were removed by washing with TBST for 15 min, and with TBS for 5 min, 3 times, respectively. The sections were then subjected to a second antibody [anti-rabbit IgG (Fc) alkaline phosphatase conjugate (Promega)], diluted 1: 2,500 in TBS containing 1% skimmed milk, for 2 h. They were again washed with TBST for 15 min, and with TBS for 5 min, 3 times, respectively. The protein signals were detected by staining with western blue stabilized substrate for alkaline phosphatase (Promega). Staining was stopped with distilled water. All of the treatments were performed at room temperature.

Results and discussion

Figure 1 shows the in situ Western hybridization analysis of the distribution of soybean ferritin in transgenic rice seeds directed by a rice glutelin gene promoter *GluB-1*. The expression of ferritin in transgenic plants was restricted to the endosperm and was more intensive in the outer portion of the endosperm; it was not detected in the embryo (Fig. 1). Non-transformed rice seeds used for control remained essentially unstained. The ferritin distribution and accumulation pattern revealed by in situ Western hybridization was exactly the same as that detected by immunological tissue printing (Goto et al. 1999) but was much clearer than the latter. In situ Western hybridization detected a pattern of protein accumulation corresponding to the tissue-specific expression of its promoter. The soybean ferritin gene in the transgenic plant was directed by the *GluB-1* promoter. This promoter directs the foreign gene to express and accumulate its product in the aleurone, subaleurone and starchy endosperm, especially in the outer portion of the endosperm (Takaiwa et al. 1991a; Wu et al. 1998). The ferritin expression pattern in the transgenic plant revealed by in situ Western hybridization conformed to the result of *GluB-1* promoter studies.

To ascertain the usefulness of this method, we used it to investigate endogenous protein expression in non-transgenic plants. Rice glutelin and prolamine are the main seed storage proteins accounting for about 70% and 15% of total protein on a weight basis, respectively (Li and Okita 1993; Ogawa et al. 1987). Rice glutelins have been classified into two subfamilies, GluA and GluB, based on their nucleotide and amino acid sequences (Takaiwa et al. 1987, 1991b). Figure 2 shows the results of experiments to investigate the expression and accumulation of the endogenous proteins of rice, glutelin and prolamine, in mature and maturing seeds. Rice glutelin *GluA* and *GluB* subunits were expressed in the aleurone, subaleurone and outer portion of endosperm but not in the embryo (Fig. 2a, b). These results correspond to GUS expression patterns directed by these promoters obtained by transgenic strategy (Takaiwa et al. 1991a; Wu et al. 1998). The rice 13-kDa prolamine subunit was expressed in all parts of the endosperm (Fig. 2c). The expression pattern of the 13-kDa prolamine subunit revealed by this

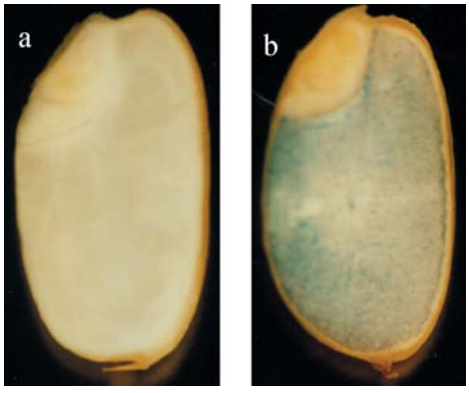

Fig. 1a, b In situ Western localization of soybean ferritin in transformed rice seeds. **a** Non-transformed seed of cv. Kitaake. **b** Western hybridization from a median-longitudinal section showing the distribution of ferritin directed by the *GluB-1* promoter in the transgenic seed of FK22. The ferritin is localized exclusively in the aleurone, subaleurone and outer portion of starchy endosperm; it is not found in the embryo. The non-transformed seed remained unstained

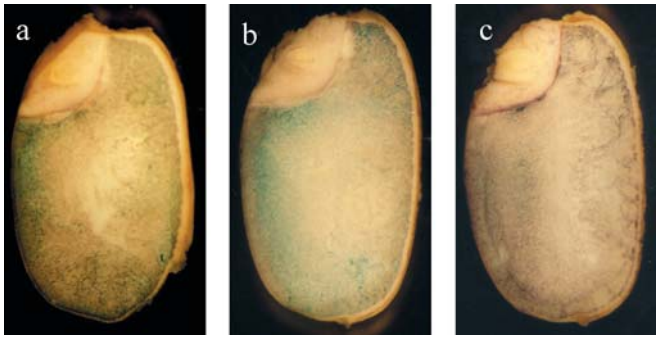

Fig. 2a–c In situ Western localization of endogenous protein in rice seeds. **a** Rice glutelin *GluA* subunit. **b** Rice glutelin *GluB* subunit. **c** Rice 13-kDa prolamine subunit. The rice glutelin and prolamine accumulated at high levels in the aleurone, subaleurone and outer portion of endosperm, but was less expressed in the inner portion of the endosperm, though not in the embryo

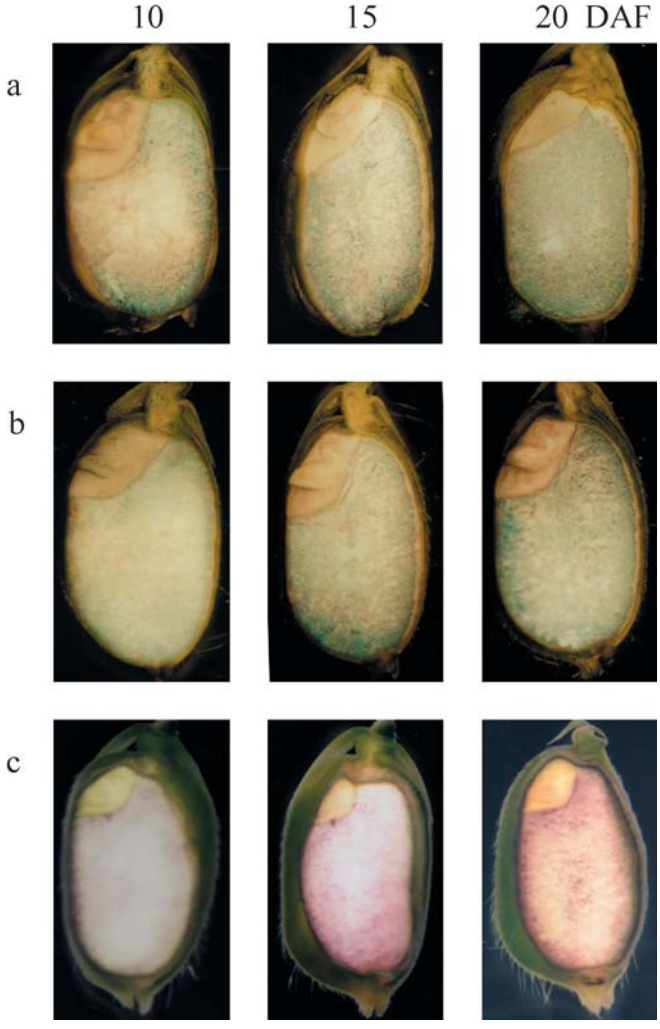

Fig. 3a–c Initiation and accumulation of rice endogenous protein during maturing. Longitudinal median sections of transgenic rice seeds at 10, 15 and 20 days after flowering were hybridized with the relevant antibody. **a** Rice glutelin *GluA* subunit. **b** Rice glutelin *GluB* subunit. **c** Rice 13-kDa prolamine subunit

method was similar to that obtained by transgenic study (Wu et al. 1998).

In the biological study of protein biosynthesis and accumulation, it is desired to understand the initiation and the developmental pattern of protein accumulation. To test if in situ Western hybridization can achieve this, we used this method to detect the protein at the seed-development stage. Maturing seeds were collected and treated at 10, 15 and 20 days after flowering (DAF). The developmental time course for each storage protein is shown in Fig. 3. Rice glutelin were initiated in early development and were restricted to the aleurone, subaleurone and outer part of the endosperm, but then spread throughout the starchy endosperm by 20 DAF, but less was observed in the central part of the endosperm (Fig. 3a, b). The expression of the rice 13-kDa prolamine subunit was initiated in all parts of the endosperm and accumulated as the seed matured (Fig. 3c). Rice prolamine synthesis is first detectable approximately 8–10 DAF (Li and Okita 1993) and that of glutelin can be detected earlier. Prolamine can be observed at the early stage of 10 DAF, indicating the sensitivity of in situ Western hybridization in detecting endogenous proteins.

Western blotting hybridization is a powerful tool for detecting protein whose expression is not high enough to be detected by Coomassie brilliant blue and silver staining after electrophoresis. The traditional method is to transfer the protein onto a polyvinylidene difluoride membrane and then to hybridize with a specific antibody giving signals to be measured qualitatively and quantitatively. However this result is usually obtained under artificial conditions. The physiological study of protein biosynthesis and function necessitates detecting the distribution of protein under native conditions. Immunological electron microscopy can be used for this kind of study, but it is not convenient for every researcher. GUS and GFP are the most commonly used reporter genes for investigating the expression pattern of promoter under native conditions, but, due to the random insertion of the chimeric gene into the genome, the expression patterns varies tremendously among individual transgenic plants even though the inserts have the same construction. This makes it difficult to evaluate the expression pattern precisely under natural condition.

Rice glutelin accumulates in the aleurone, subaleurone and outer portion of the starchy endosperm, but not in the embryo. This is in agreement with the results of GUS endosperm-specific expression directed by glutelin gene promoters in transgenic tobacco and rice (Takaiwa et al. 1991a, 1996; Zheng et al. 1993, 1995; Yoshihara and Takaiwa 1996; Yoshihara et al. 1996; Wu et al. 1998). However, compared to glutelin, the expression of rice 13-kDa prolamine in endosperm was relatively uniform. In situ Western hybridization revealed that 13-kDa prolamine expressed in all parts of the endosperm, with stronger expression in the aleurone and subaleurone cells. This result was similar to that obtained from transgenic study in rice (Wu et al. 1998).

This is the first time that this level of precision has been achieved in investigating protein expression patterns and evaluating promoter activity under native conditions. It highlights the usefulness of in situ Western hybridization experiments in transgenic and non-transgenic plants. It makes it possible to investigate the tissue-specific expression of promoters with no necessity to produce transgenic plants, especially for single copy genes.

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