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In situ localization of faba bean and oat legumin-type proteins in transgenic tobacco seeds by a highly sensitive immunological tissue print technique

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Abstract

We have used a highly sensitive immunological tissue print technique to study cell- and tissue-specific expression of heterologous genes in transgenic plants. Primary polyclonal antibodies, raised against legumin of faba bean (*Vicia faba* L.) and 12S globulin of oat (*Avena sativa* L.) were used to localize these proteins in transgenic tobacco seeds in a streptavidin-alkaline phosphatase assay in combination with biotinylated secondary antibodies producing a higher sensitivity (by several amplification steps) of the assay. Both storage protein genes were found to be expressed in a specific pattern. While legumin is preferentially accumulated in certain parts of the embryo, the oat legumin-type globulin is restricted to the endosperm. The applied technique is highly sensitive with a resolution power down to the single-cell level and allows rapid screening of large numbers of samples.

Introduction

Recombinant DNA technology allows the study of the expression patterns of a large number of genes, either in their tissue of origin or after transfer into heterologous hosts. Different techniques are available for biochemical detection of specific RNA or protein molecules in tissue extracts or at the histological level. However, these techniques are either not able to reveal data on cell-specific expression patterns or are rather tedious and time-consuming, such as *in situ* hybridization or immunohistochemistry.

During our studies on seed protein gene expression in developing transgenic tobacco seeds [1, 3] we wished to analyse a great number of different transgenic lines for spatial and temporal expression patterns of several dicot and monocot genes and derived promoter deletion constructs. To this end we improved the immunological tissue print technique to detect very low levels of foreign proteins in transgenic plants. In this communication we demonstrate the efficiency, sensitivity and resolution power of this technique for localizing both a faba bean legumin and an oat legumin-type globulin in seeds of transgenic tobacco plants.

Material and methods

Plant material

Transgenic plants of *Nicotiana tabacum* L. cv. Gatersleben were grown in the greenhouse. Ex-

periments were carried out with mature seeds of the following transformants: plant TA 14/6 containing one complete copy of the *Vicia faba* legumin B-type gene VfLeB4 [2] and plant 5/12 with an unknown copy number of the 12S globulin gene AsGlo5 from *Avena sativa* [15]. As shown by ELISA, mature seeds of plant TA 14/6 contain 0.8 ng legumin B protein per μg extracted globulins [2]. Untransformed tobacco seeds were used as control treatments.

Antibodies and immunostaining reagents

Monospecific antibodies directed against legumin of *Vicia faba* and 12S globulin of *Avena sativa* were produced in rabbits by a special immunization schedule [18]. The IgG fraction of the antisera was enriched by ammonium sulphate precipitation and further purified by affinity chromatography using homologous antigen covalently linked to glutardialdehyde activated affinity absorbents according to the instructions of the manufacturer (Boehringer, Mannheim). Depletion of unwanted cross-reactivities was achieved by antiserum absorption to tobacco globulins immobilized on affinity absorbents. Specificities of the primary antibodies were checked by dot-immunobinding assay [9] using the homologous storage protein as well as tobacco globulins as antigenic probes. The biotinylated secondary antibody (goat anti-rabbit IgG) and the streptavidin-alkaline phosphatase conjugate were purchased from Amersham. The immunostaining conditions were optimized by retitration of primary and secondary antibodies and detection reagents. A mixture of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP) from GIBCO was used as substrate for colour development.

Tissue printing

Mature seeds were mounted with a quickly hardening adhesive on a teflon block and carefully dissected by hand into about 0.2 mm thick sections using a razor blade. Sections were put on a snip of nitrocellulose paper, which was dipped using forceps into hexane for 20 s to remove lipids from the freshly cut surface. Subsequently hexane was allowed to evaporate from the sample. For tissue printing a strip of nitrocellulose paper (Schleicher and Schuell, BA 85) was briefly dipped into distilled water and subsequently laid upon filter paper to remove excess water. Afterwards the strip was gently placed over the section and pressed for 5–10 s onto the sample. The section was then carefully removed from the paper. When prints from different sections were placed successively on the same strip, the nitrocellulose was stored in a moist chamber until all prints had been made. The quality of prints mainly depended on the optimal moisture content of the nitrocellulose rather than on the printing pressure. After the prints had been made the nitrocellulose strip was baked for 30 min at 60 °C.

Immunostaining of tissue prints

In order to block non-specific antibody binding the tissue prints were placed in Tris-HCl-buffered saline (TBS) pH 7.4 containing 5% (w/v) non-fat dried milk and 10% (v/v) horse non-immune serum for 12 h at 4 °C. After the blocking procedure, the tissue prints were rinsed briefly with TBS and immediately transferred to a specific primary antibody dilute 1:5000 in TBS supplemented with 1% (w/v) bovine serum albumin (BSA), 10% (v/v) horse serum and 0.05% (v/v) Tween 20 for 1 h at room temperature. After the primary antibody treatment, unbound antibodies were removed by three 10 min washes with TBS containing 0.5% (v/v) Triton X-100 (TBS-T). Tissue prints were then subjected to a secondary antibody treatment by incubation with biotinylated goat anti-rabbit IgG diluted 1:1000 in TBS supplemented with BSA, horse serum, and Tween 20 for 1 h at room temperature. After the tissue prints were washed in three changes of TBS-T over a period of 30 min, the biotin moieties from the biotinylated secondary antibody were labelled by treatment with a streptavidin-alkaline phos-

phatase conjugate, diluted 1:300 in TBS supplemented with 0.5% (w/v) BSA, 1 mM $MgCl_2$. Colour development was examined under the stereomicroscope and stopped by the addition of 20 mM EDTA dissolved in phosphate-buffered saline.

Results and discussion

Efficiency of the method

Immunostaining of tissue prints has been used to visualize the distribution pattern of abundant proteins in several plant tissues [4, 17]. We improved this method to detect very low levels of heterologous proteins in transgenic cells by introduction of a biotin-streptavidin based detection system. In contrast to the method of Cassab and Varner [4], we used a primary antibody with a high avidity (diluted 1:5000) in combination with a biotinylated secondary antibody and a streptavidin-alkaline phosphatase assay for immunolabelling of the antigen. The application of the biotin-streptavidin reaction increased the efficiency of the labelling procedure by the introduction of several amplification steps. Thereby the technique became increased in sensitivity (detection of a few picograms). The high sensitivity of the labelling procedure did not allow localization of the distribution pattern of the abundant storage proteins in tissue prints of faba bean and oat seeds. For this purpose a shorter detection chain with a lower sensitivity is recommended (unpublished results). The high sensitivity of the described immunolabelling procedure was also borne out by a comparative analysis of legumin expression in several transgenic tobacco seeds estimated by an enzyme immunoassay [2] as well as by immunostaining of their prints. Transgenic tobacco seeds, containing a very low legumin level of 0.8 ng legumin per μg seed globulin [2], were also found to produce a significant staining reaction in tissue prints of single seeds. An additional advantage of the immunostaining method is its high degree of specificity. Under appropriate conditions, which vary with the primary antibody used, anti-storage globulin IgG cross-absorbed with tobacco globulins showed no background reaction to tobacco tissues. Furthermore, the technique is simple; it neither requires extraordinary technical expertise, manipulation and equipment, nor production of specific antibody-enzyme conjugates. All reagents, except for the primary antibody, are commercially available.

Although the method prescribes an overnight incubation with the blocking reagent, the actual time needed to set up and process immunostaining of tissue prints is considerably less than that needed for ELISA, western blotting, or even immunohistology. Thus, for immunohistology each sample must be fixed, embedded, sliced and mounted prior to immunolabelling. The technique used here effectively complements quantitative techniques such as ELISA and western blotting. Furthermore, immunological tissue printing can be used with success if in the case of an apparent insolubility of the protein, as shown for oat 12S globulin [15], the foreign protein is not detectable by ELISA technique. However, the method cannot be a substitute for electron microscopic immunolocalization of proteins, which has its main advantage in the visualization of protein distribution at the subcellular level.

Expression patterns of dicot and monocot legumin-type genes in transgenic tobacco seeds

We used the improved technique to examine the tissue-specific expression pattern of two seed storage protein genes, the *Vicia faba* legumin gene LeB4 [1] and the evolutionary related *Avena sativa* 12S globulin gene AsGlo5 [15], in mature transgenic tobacco seeds. As shown previously, the transgenic tobacco line TA 14/6 contains one copy of gene LeB4 [2], which causes considerable accumulation of LeB4 mRNA preferentially in the cotyledons and the hypocotyl of torpedo stage embryos as revealed by *in situ* hybridization [3]. Tissue-print immunoblots detected a corresponding pattern of protein accumulation. In median-longitudinal sections the highest legumin B4 concentration was found in cotyledons and

hypocotyl; no protein was detected in embryonic root tissue, provascular tissue, and shoot meristem (Fig. 1a). Staining of endosperm was restricted to single cells or cell groups. Endosperm expression, as detected by RNA *in situ* hybridization, seemed to be more uniform but GUS staining in seeds expressing LeB4 promoter/*gus* fusion genes also revealed patches of endosperm cells stained darkly blue within rather weakly stained regions (see Fig. 3 in [3]). The spatial distribution of *Avena sativa* 12S globulin was essentially different from that of legumin. As shown in Fig. 1b the expression of the 12S globulin gene was confined to the endosperm, especially to that part surrounding cotyledons and hypocotyl; in the embryo no appreciable expression was observed. Untransformed tobacco seeds used for control and incubated with the legumin and 12S globulin antisera remained essentially unstained (Fig. 1c).

An impression of the high resolving power of the technique, reaching down to the level of single cells, provides Fig. 2. In a sagittal section passing the endosperm, the cells belonging to the upper part of the endosperm can easily be distinguished from those of the lower part. Cells of this region apparently did not appear to accumulate 12S globulin (Fig. 2a). When endosperm cells are examined in greater detail (higher magnifications), 12S globulin deposition was found to be more pronounced within the peripheral zone than within the inner region (Fig. 2b, c). Likewise, a heterologous zein protein was detected close to the wall of endosperm cells in mature transgenic petunia seeds [16]. This kind of zonation may be caused by an unequal distribution of cytoplasm in endosperm cells and is consistent with the finding that, as shown by *in situ* hybridization, Glo5-mRNA follows the same distribution pattern (R. Panitz, unpublished result).

Our studies revealed striking differences in the histological expression pattern of a dicot and a monocot 12S globulin gene in the dicot host *Nicotiana tabacum*. While the *Vicia faba* legumin B gene is mainly expressed in the embryo, *Avena sativa* 12S globulin accumulation has been found exclusively in the endosperm. This behaviour of both seed protein genes appears to reflect different expression types in the monocot and dicot gene donor species. Thus, in *Avena sativa* the 12S globulin gene expression is restricted to the aleu-

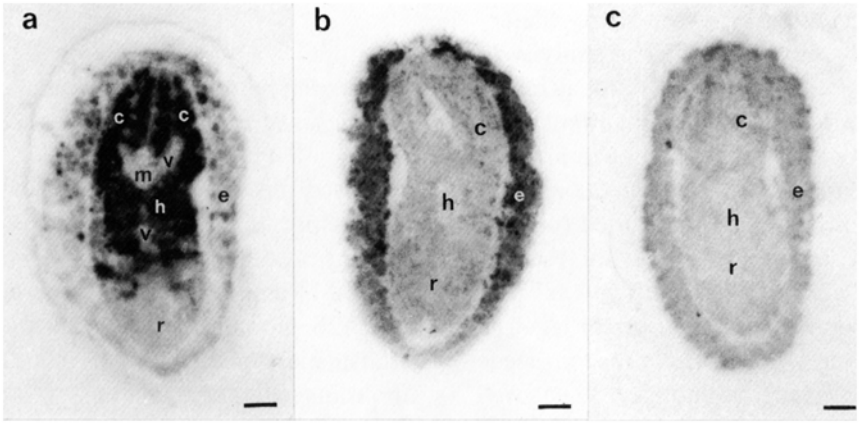

Fig. 1. Immunological tissue print localization of heterologous storage proteins in transformed tobacco seeds. a. Tissue print from a median-longitudinal section showing the distribution pattern of the *Vicia faba* legumin B4 protein. Legumin B4 is localized in cotyledons and hypocotyl, but not in embryonic root and provascular tissue. Only single cells of endosperm are labelled. b. Tissue print from a median-longitudinal section showing the distribution pattern of the ASGlo5 protein. The *Avena sativa* 12S globulin is localized exclusively in that part of the endosperm which covers the cotyledons and hypocotyl. c. Tissue print of a section from an untransformed tobacco seed treated with antibodies against legumin as a control. The control does not show any signal. c, e, h, m, r and v refer to cotyledon, endosperm, hypocotyl, shoot meristem, root and provascular tissue, respectively. Bars indicate 100 μm.

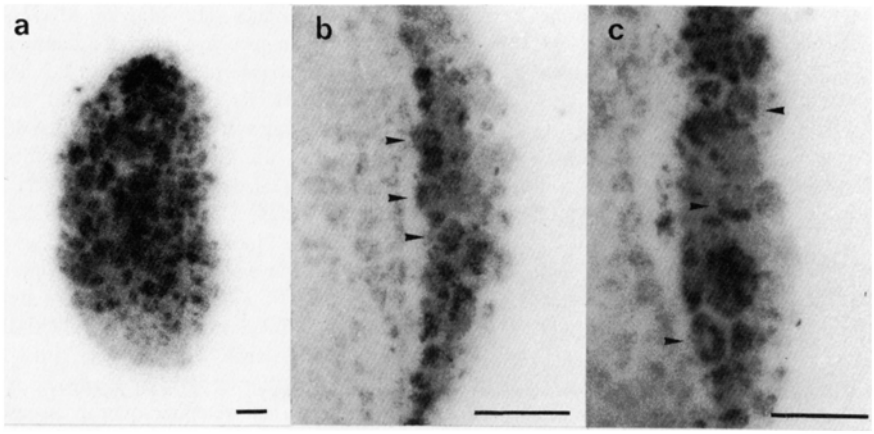

Fig. 2. Immunological tissue print localization of the ASGlo5 protein in endosperm cells of transformed tobacco seeds. a. Tissue print from a sagittal-longitudinal section cut on the plane passing the endosperm showing the multicellular composition of the tissue. b, c. Tissue prints of longitudinal sections. Single endosperm cells are conspicuous by stronger staining of their peripheral zone (arrowheads). Bars indicate 100 μm.

rone and subaleurone layer of the starchy endosperm (Manteuffel, Rudolph and Panitz, unpublished results). This is in conformity with the endosperm-specific expression in transgenic tobacco seeds as shown in this paper. Endosperm-specific expression of monocot genes in tobacco is also known for other monocot seed protein promoters including wheat [5, 13] maize [14] and barley [12]. On the other hand, seed storage proteins of legumes are accumulated in the large cotyledonary parenchyma cells of the embryo [6], which is consistent with the deposition pattern of *Vicia faba* legumin in tobacco seeds. Analogous cases of a dominating embryo-specific expression of heterologous dicot seed protein promoters in tobacco seeds have been reported for French bean [8, 11] and pea [10, 7]. Taken together, the data allow the conclusion that heterologous seed protein genes introduced into the dicot host *Nicotiana tabacum* do function in an origin-specific manner.

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