Gene specificity is maintained in transient expression assays with protoplasts derived from different tissues of barley

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In some cereal species that are still recalcitrant to stable transformation and regeneration, transient expression in isolated protoplasts is a useful tool for the study of gene expression and regulation. We have successfully applied these techniques to barley protoplasts derived from developing endosperm, aleurone, leaves and roots in order to characterize functionally cis-acting motives in two gene promoters, corresponding to trypsin inhibitor BTI-CMe and to sucrose synthase Ssl. Gene specificity is maintained in transient expression assays with protoplasts isolated from these different barley tissues and the pattern of expression parallels the mRNA levels observed for the corresponding genes in the same tissues.

Introduction

Transient gene expression in plant protoplasts has emerged as a refined method for the analysis of gene expression and regulation, as the activity of chimeric constructs of promoters fused to reporter genes, unintegrated in the cell nucleus, can be quantified within a few hours after DNA transfection. Rapidity of analysis is the major advantage of the transient assay system, particularly when compared to the time-consuming regeneration of transgenic plants. In the case of barley, regarded until very recently as recalcitrant for stable transformation (only a few references reporting fertile transgenic plants obtained by bombardment of immature embryos: Wan & Lemaux, 1994; Ritala et al., 1994), the transient transformation of protoplasts has been the method of choice for the functional analysis of gene promoters (Teeri et al., 1989; Salmenkallio et al., 1990; Diaz & Carbonero, 1992; Diaz et al., 1993; Jenes et al., 1994). However, transient expression data in cereal protoplasts derived from certain tissues such

as endosperm or roots, are scarce due to difficulties in isolating viable protoplasts from such tissues at reasonable yields.

Our data, reported here, suggests that protoplasts derived from developing endosperm, aleurone, leaves and roots, maintain their tissue specificity during transient expression, and that these assays are useful for defining the regulatory cis-acting motives in promoters such as those of genes for trypsin inhibitor BTI-CMe and sucrose synthase Ssl.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L.) cv Bomi was used throughout this study.

Abbreviations: CaMV35S – 35S-cauliflower mosaic virus promoter, GUS – β -glucuronidase, MU – 4-methylumbelliferone, NOS – nopaline synthase gene, PEG – polyethyleneglycol

Isolation of barley protoplasts

Endosperm. Developing barley endosperms up to 15 days after pollination were the optimal material for the isolation of metabolically active protoplasts, which were prepared essentially as described by Díaz & Carbonero (1992).

Aleurone. Protoplasts from aleurone were prepared from mature de-embryonated kernels after 24 h imbibition in water, following the procedure reported by Díaz (1994). The purified protoplasts were resuspended in the medium described by Jacobson et al. (1985).

Leaves. After seed germination for 6 days in the dark under axenic conditions, etiolated leaves were cut into small pieces, plasmolysed and digested in an enzyme solution described by Díaz (1994). Transfected protoplasts were cultured in the media described by Gupta & Pattanayak (1993).

Roots. Seeds were axenically germinated for 2 days in the dark and protoplasts were isolated from roots of about 1-2 cm in length, as previously described by Díaz (1994). The culture medium used after transfection was that previously described for leaf protoplasts.

Plasmids

The plasmid p35S-GUS, used as a positive control in the transient expression assays, is a pUC 19-based vector containing the GUS coding region under the control of the CaMV35S and the polyadenylation signal of the NOS. Chimeric constructs corresponding to different fragments of the barley trypsin-inhibitor (BTI-CMe) promoter and of the barley sucrose synthase (Ssl) promoter, were fused to the *gus* reporter gene in similar pUC-derived vectors and have been described in detail by Díaz et al. (1993) and Sánchez de la Hoz (1992).

Protoplast transfection

Transfection of endosperm and aleurone protoplasts was mediated by PEG following the procedure previously described (Díaz & Carbonero, 1992; Díaz et al., 1993; Díaz, 1994). 0.5 ml of culture medium containing $0.5\text{--}1.0 \times 10^6$ protoplasts were incubated with 25 μg of the corresponding plasmid and 50 μg of herring sperm DNA as carrier. After 5 min, 0.25 ml of PEG solution (40% PEG 3400, 0.4 M mannitol, 0.1 M

$\text{Ca}(\text{NO}_3)_2$, pH 8) was added, mixed and incubated at 25°C for 20 min. The mixture was gradually diluted with F solution (Krens et al., 1982) and centrifuged. Transfected protoplasts were cultured for 20 h before sampling for GUS determination.

DNA uptake into protoplasts isolated from leaves and roots was achieved by electroporation with the Electro Cell Manipulator 600 (BTX electroporation system) as has been reported by Díaz (1994). Optimal electroporation conditions were different for each kind of protoplast (leaf protoplasts: 200 μF , 500 V/cm, 10 msec; root protoplasts: 500 μF , 625 V/cm, 20 msec). Electroporated protoplasts were incubated for 20 h before harvesting.

GUS determination

Transfected protoplasts were collected by centrifugation and GUS activity was measured by the fluorometric assay of Jefferson (1987). Total cellular protein was determined using the BioRad kit.

Northern blot analysis

RNA was purified from immature endosperm, aleurone, young leaves and roots by phenol/chloroform extraction followed by lithium chloride precipitation (Lagrimini et al., 1987). Hybridization was performed by standard procedures (Sambrook et al., 1989) using specific probes derived from the 3' terminal regions of the cDNAs corresponding to BTI-CMe and Ssl (Rodríguez-Palenzuela et al., 1989; Sánchez de la Hoz, 1992).

Results and discussion

Isolation and transfection of barley protoplasts

Procedures previously described by us allowed the routine isolation of metabolically-active protoplasts from different tissues of barley. Both the state of development of the tissue and the growth conditions affected the yield and viability of the preparations.

Protoplasts derived from developing endosperm were difficult to handle due to the large amount of starch granules present in their cytoplasm which can easily damage cell membranes during isolation and transfection. No viable protoplasts could be obtained beyond 20 days after pollination (Lee et al., 1991; Díaz & Carbonero, 1992). PEG-mediated DNA uptake

Fig. 1. Transient gene expression after transfection of barley protoplasts with chimeric constructs made with promoter fragments of BTI-CMe and Ss1 genes fused to the *gus* reporter gene. Digestions with different restriction endonucleases were used to obtain the different promoter fragments. B: Bam HI, H: Hae III, K: Kpn I, N: Nco I, P: Pvu II, S: Sal I, Sp: Sph I. The 3' non-coding region comes from the *nos* gene. A) Expression of constructs derived from the gene promoter of trypsin inhibitor BTI-CMe (pS: - 2369 bp; pK: - 1969 bp; pH: - 816 bp; pSp: - 343 bp; pP: - 83 bp preceding the ATG site). B) Expression of constructs derived from the gene promoter of sucrose synthase Ss1 (pN: - 1190 bp; pS: - 695 bp; pB: - 252 bp preceding the ATG site). Values are expressed as % of the expression obtained with the 35S cauliflower mosaic virus promoter in protoplasts of the same tissue. Means and standard errors of five independent experiments. Nucleotide sequences of these promoters have EMBL-GenBank accession numbers X65875 (BTI-CMe) and X73221 (Ss1) respectively.

was the only successful approach for endosperm protoplast transfection, since even the mildest electroporation conditions assayed (250 V/cm, 200 μ F, 10 msec) produced a high level of cell mortality (Díaz, 1994).

Aleurone layers yielded protoplasts which were more resistant to manipulation, probably due to their smaller size compared with endosperm protoplasts and to their lack of starch granules. GUS activity per mg of protein after PEG transfection with the plasmid p35S-GUS was approximately 30% of that measured for endosperm protoplasts using the same conditions (data not shown). Numerous groups have reported the

use of barley aleurone protoplasts in transient expression studies, especially in studies of hormonal regulation of gene expression (Huttley & Baulcome, 1989; Salmenkallio et al., 1990).

The protoplast yield was higher when etiolated leaves were used as the starting material as opposed to green leaves with well-developed chloroplasts (Díaz, 1994). After electroporation, the level of GUS activity in etiolated protoplasts was about twice the activity obtained with green protoplasts, using the plasmid p35S-GUS (data not shown).

Protoplasts from barley roots could be obtained only from the apical portion of young plants. Yield sharply decreased when roots were older than 48 h. These protoplasts, very small in size, were quite tolerant to physical treatments such as centrifugation and electrical discharge (Díaz, 1994). No previous reports had been published on transient expression with root protoplasts from barley, or from other small-grain cereals. Maize root protoplasts have been successfully electroporated by Sheen (1991).

Functional analysis of the barley trypsin inhibitor BTI-CMe promoter by transient GUS expression

Deletion analysis of the gene promoter for barley trypsin inhibitor BTI-CMe, using five chimeric constructs (Díaz et al., 1993) in protoplasts derived from different barley tissues, is summarized in Fig. 1A. The plasmid p35S-GUS and the promoterless GUS construct were used as positive and negative controls respectively. Only protoplasts derived from developing endosperm were able to support GUS expression driven by the BTI-CMe promoter ($\approx 50\%$ of that obtained with the 35S promoter), and the expression detected in protoplasts isolated from leaves and roots was less than 5% of that obtained with the 35S promoter in the same tissues (Fig. 1A). No expression was detected in aleurone protoplasts (data not shown). This pattern of transient gene expression parallels the mRNA levels observed by Northern blot analysis in the same tissues from which the protoplasts were obtained (Fig. 2), and corroborates that the BTI-CMe gene is endosperm-specific. Transient expression assays also showed that all the information required for this specificity was present in the 343 nt region preceding the ATG translation initiation site (construct pSp, Fig. 1A). In similar experiments with wheat endosperm protoplasts, GUS activity under the BTI-CMe promoter was less than 20% of that obtained in barley, which reflects the species and endosperm specificity of the barley BTI-CMe gene and might indicate that a positive transacting factor(s) needed for the trypsin inhibitor expression is (are) absent in the wheat endosperm cells, as well as in the leaves and roots of wheat and barley (Díaz et al., 1993).

Functional analysis of the barley sucrose synthase Ss1 promoter

Three promoter deletions of sucrose synthase type 1 gene (Ss1) fused to the GUS coding region (Sánchez

Fig. 2. Northern blot analysis using total RNA from the indicated tissues hybridized to specific probes for trypsin inhibitor BTI-CMe and for sucrose synthase Ss1. E: developing endosperm 15 days after pollination, A: immature aleurone, L: 4-day old green leaves, R: 4-day old roots. 2 μ g of total RNA were applied per slot.

de la Hoz, 1992) were used in transient expression assays (Fig. 1B).

The strength of the complete Ss1 promoter (pN construct) was of the same order as that of the 35S promoter in endosperm and leaf protoplasts, less than 5% in aleurone protoplasts, and intermediate (50–70%) in root protoplasts (Fig. 1B). Full expression was not obtained when only 269 bp of the promoter (construct pB) were used, indicating the existence of enhancer elements between positions -269 and -695 (construct pS). Again, the levels of Ss1 mRNA 'in planta' (Fig. 2) showed a similar relative expression pattern as that observed in protoplasts (Fig. 1B).

We can conclude that gene specificity is maintained in transient expression assays with protoplasts derived from different tissues of barley, at least 24–48 h after transfection, which allows for the assay of gene expression with large numbers of constructions in a short period of time.

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