The promoter of barley trypsin-inhibitor BTI-CMe, discriminates between wheat and barley endosperm protoplasts in transient expression assays

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ABSTRACT. Several promoter fragments from the barley gene coding for trypsin inhibitor, BTI-CMe, have been fused to the β -glucuronidase (GUS) reporter gene and these chimeric constructs used for transient expression in protoplasts. Transfection of developing endosperm protoplasts from barley (cv Bomi) show a maximum GUS expression of about 50% of that driven by the cauliflower mosaic virus 35S promoter, while in wheat endosperm protoplasts expression is less than 10%. No significant expression is found in transfected leaf protoplasts from barley, wheat or tobacco (<2% of the 35S control). All the information required for endosperm and barley specificity is present in the 343 bp proximal to the translation initiation site.

ABBREVIATIONS: MS: Murashige and Skoog medium, PEG: polyethyleneglycol, GUS: β -glucuronidase, MU: methylumbelliferone, MUG: 4-methylumbelliferyl- β -D glucuronide, pp: protoplasts.

INTRODUCTION

Although barley and wheat protoplasts are routinely isolated from a number of cell types such as leaf mesophyll, scutellar epithelium, aleurone and cell cultures, metabolically active protoplasts from starchy cereal endosperm have been found to be difficult to prepare. In fact, there are only two recent reports (Lee et al. 1991, Díaz and Carbonero 1992) in which reproducible procedures to obtain viable protoplasts from such a tissue have been described. Since wheat and barley are recalcitrant to stable transformation and regeneration, transient transformation of protoplasts from these species can be used to study gene expression and regulation. For these experiments chimeric constructs derived from the gene promoters under study are transcriptionally fused to reporter genes, and protoplasts from different tissues transfected.

In wheat and barley kernels a substantial fraction of the albumins and globulins is represented by a very complex multigene family of proteins that are inhibitors of heterologous enzymes (proteases/ α -amylases). These inhibitors may have a role in the plant defence mechanism against insect pests and

microbial pathogens (For reviews see Garcia-Olmedo et al. 1992, Carbonero et al. 1993). One of the best studied members from this family is the barley trypsin inhibitor, BTI-CMe. This 13 kDa protein, which is very abundant in barley endosperm, inhibits trypsin but is inactive against other proteases such as chymotrypsin, elastase, papain, pepsin or the endogenous proteases of green malt (Mikola and Soulinna 1969) and does not inhibit α -amylases (Barber et al. 1986). As its maize homologue (Mahoney et al. 1984), the BTI-CMe is also active towards plasma kallikrein and the activated Hageman factor (β -factor XII a of the blood clotting cascade) (Chong and Reeck 1987). Both cDNA and genomic clones have been characterized and BTI-CMe gene expression has been shown to be dependent on **trans** acting factor(s) (Rodriguez-Palenzuela et al. 1989, Royo 1991). A structural and functional investigation of the promoter of this gene should contribute to the identification of **cis** regulatory elements involved in its control. We report here the functional characterization of the BTI-CMe promoter through transient expression studies in protoplasts from different tissues and show that this promoter discriminates not only between endosperm and leaf protoplasts but also between wheat and barley endosperm protoplasts and that all the information required for this species and tissue specificity is condensed in the 343 bp promoter fragment close to the ATG initiation codon.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* L. cv Bomi), wheat (*Triticum aestivum* L. cv Chinese Spring) and tobacco (*Nicotiana tabacum* L. cv W38) were used throughout this study.

Isolation of protoplasts from wheat and barley developing endosperm. Wheat and barley endosperms from early developmental stages (up to 15 days after pollination) were the starting material for the isolation of metabolically active protoplasts that were prepared essentially as described by Díaz and Carbonero (1992). Wheat kernels required less drastic treatments for surface sterilization than those from barley (7.5% (w/v) versus 10% (w/v) Domestos (Lever) for 30 min). Endosperms isolated

under aseptic conditions were plasmolyzed for 1 h in CPW salts (Power and Chapman 1985) with 11% (w/v) mannitol as osmoticum. Enzymatic digestion was performed in the same medium containing 0.4% (w/v) cellulase Onozuka RS in the dark for 14 h. After filtering and washing, the protoplasts were centrifuged (60 g 5 min) onto a 50% Percoll cushion. The purified protoplasts band was collected at the Percoll interface, washed with MSL medium (MS base medium containing 9% (w/v) mannitol, 3% (w/v) sucrose and 30 ml/l coconut water, pH 5.8), centrifuged (40 g 3 min) and the pellet resuspended at a final density of 10^5 protoplasts per ml.

Isolation of leaf protoplasts. Wheat and barley seeds were axenically germinated for 7 d at 27°C under a 14 h light period. The young green leaves (6 to 7 cm long) were cut into small pieces and plasmolyzed for 1 h in CPW salts with 13% (w/v) mannitol. Digestion with the enzyme solution containing 1.5% (w/v) cellulase Onozuka RS and 0.37% (w/v) Macerozyme Onozuka R10 was for 14 h at 27°C. After filtration (500 and 64 µm pore size sieves) and washing with CPW 13% mannitol, the protoplasts were resuspended in CPW 21S medium (CPW salts with 21% (w/v) sucrose) and centrifuged (80 g 5 min). The band of floating viable protoplasts was diluted in the transfection buffer and resuspended at the final density required ($1-2.5 \times 10^6$ /ml for leaf protoplasts to be electroporated or $0.5-1 \times 10^6$ /ml for those to be PEG transfected).

Tobacco leaf protoplasts were isolated following the procedure described by Power et al. (1989) and resuspended at $1-2.5 \times 10^6$ /ml in the corresponding transfection solution.

Construction of chimeric genes containing different fragments of the BTI-CMe promoter fused to the GUS reporter gene. Chimeric gene constructs used for protoplast transfection are schematically represented in Figure 1. From a Sall-EcoRI DNA fragment of approximately 3 kbp (Fig. 1A) including the promoter, the coding region and the 3' non-translatable region of the barley trypsin inhibitor BTI-CMe gene (Royo 1991), five different promoter regions were obtained spanning from the indicated restriction sites in its 5'-end (Sall, KpnI, HaeIII, SphI, PvuII) to the first HaeIII site after the translation initiation ATG codon. These were subcloned in the SmaI site of the polylinker in the pHW9 GUS containing plasmid (gift from Plant Genetic Systems, Gent) which produced an in-phase fusion with this reporter gene. The 3'-non coding region was from the nopaline synthase gene (NOS) (Fig. 1B).

DNA transfection of protoplasts. Two transfection procedures were used for plasmid uptake into barley and wheat protoplasts. The protoplast transfection mediated by PEG was adapted from Negrutiu et al. (1987) and has been described in detail in Diaz and Carbonero (1992).

The second procedure involved electroporation of protoplasts with the Electro Cell Manipulator 600 (BTX electroporation system). Aliquots of 400 µl, containing 1×10^6 pp/ml, in 0.65M mannitol were mixed with 30 µg of the plasmid DNA together with 50 µg of herring sperm DNA, as a carrier, in prechilled cu-

Figure 1. A) Partial restriction map of the gene for barley trypsin inhibitor BTI-CMe. The coding region is dashed (▨). The numbering of the promoter region refers to the ATG translation initiation codon. The positions of the TATA and CAAT boxes as well as the -300 endosperm box are indicated. The nucleotide sequence of this promoter will appear in the EMBL-Gene Bank under the accession number X65875. **B)** Chimeric constructs derived from different endonuclease restriction fragments of the promoter in 1A fused in phase to the GUS reporter gene (TATA box ■; -300 endosperm box □). The 3' non-coding region derives from the nopaline synthase gene (Nos). The strong constitutive 35S promoter construct was used as a control. S = Sall; K = KpnI; H = HaeIII; Sp = SphI; P = PvuII; E = EcoRI.

vettes with a 2 mm gap between electrodes. Electrical fields ranging from 300 up to 850 V/cm were applied by discharge of a 200 µF capacitor. Various discharge conditions were used with protoplasts from the different tissues and cereal species. Following electroporation, protoplasts were diluted 20 times with the appropriate culture medium, centrifuged and incubated for 20-24 h at 27°C before evaluation of GUS activity.

β-Glucuronidase (GUS) assay. This assay was performed using 4-methylumbelliferyl β-glucuronide (MUG) as a substrate, by the fluorometric method described by Jefferson (1987). Protein content was determined using the Bio-Rad kit, with bovine serum albumin as standard. A Neubauer chamber was used to count protoplasts.

RESULTS AND DISCUSSION

Protoplast isolation and transfection. The procedure described here produces routinely good yields ($\sim 3 \times 10^6$ pp per 100 kernels) of metabolically active protoplasts from developing endosperms of wheat and barley. From young leaves of both species yields are $\sim 3.5 \times 10^6$ pp per gram of fresh tissue (Table 1).

Barley endosperm protoplasts vary in size from 25 to 45 μm in diameter while those derived from wheat endosperm are between 25 and 35 μm . Both types show a dense cytoplasm containing abundant starch granules. Protoplasts from developing endosperms of more than 15 days after pollination are difficult to isolate and transfect, and the yield of metabolically active transfected protoplasts decreases sharply with starch content.

Protoplasts obtained from young leaves of wheat and barley present no species differences in size (15 to 25 μm in diameter), morphology or viability.

The optimal conditions for PEG and electroporation mediated DNA uptake into wheat and barley protoplasts have been established using the cauliflower mosaic virus 35S promoter fused to the GUS reporter gene. To optimize the electroporation procedure, we varied the input voltage between 300 and 850 V/cm keeping the capacitor to 200 μF and the pulse length at 10 milliseconds. The last two parameters (200 μF and 10 msec) have been described as the optimal for cereal mesophyll protoplasts by Teeri et al. (1989). In our hands, maximal GUS expression in transfected wheat and barley leaf protoplasts is at 625 V/cm. At lower initial voltages, transfection diminishes, and at higher ones, very few protoplasts remain alive. This electroporation method compared with the PEG mediated transfection procedure (Diaz and Carbonero 1992), is approximately three times as efficient in terms of final GUS activity per μg of plasmid DNA transfected (Table 1). However, with endosperm protoplasts, electroporation can not be used because even the gentlest conditions used (300 V/cm, 200 μF , 10 msec) produce a high cell mortality (<10% viable cells in barley; <2% in wheat; Table 1). The PEG transfection method previously described (Diaz and Carbonero 1992) remains the protocol of choice for endosperm protoplasts while electroporation (625 V/cm, 200 μF , 10 msec) has been routinely used from now on for leaf protoplast transfection.

Figure 2. GUS expression (pmoles MU/10⁶ pp x min) after PEG transfection of endosperm protoplasts (30 μg of plasmid DNA/10⁶ pp) with the chimeric constructs described in Fig. 1. Transient expression after electroporation of leaf protoplasts with 20 μg of plasmid DNA/10⁶ pp, is shown in the insert. Means and standard error of five independent experiments with each construct are shown.

Table 1. Yield, viability and GUS activity after transfection of cereal protoplasts

SPECIES	TISSUE	PROTOPLAST YIELD ^a	% PROTOPLAST VIABILITY ^b		GUS ACTIVITY ^c
		x $\pm$ s.e.m.	before transfection	after transfection	pmol MU/mg protein x min
BARLEY	Endosperm	2.91 x 10 ⁶ $\pm$ 0.62	75	43 (PEG) <10 (E)	652 $\pm$ 23
	Leaf	3.61 x 10 ⁶ $\pm$ 0.39	89	45 (PEG) 43 (E)	824 $\pm$ 13 1423 $\pm$ 16
WHEAT	Endosperm	3.10 x 10 ⁶ $\pm$ 0.70	70	41 (PEG) <2 (E)	569 $\pm$ 17
	Leaf	3.53 x 10 ⁶ $\pm$ 0.31	83	47 (PEG) 42 (E)	783 $\pm$ 11 1507 $\pm$ 13

^aProtoplast yield for endosperm is referred to 100 developing kernels. Yield for leaf protoplasts is referred to 1 gram of fresh tissue. Values are means $\pm$ standard error of 5 experiments.

^bProtoplast viabilities before and after PEG or electroporation (E) are expressed as % of protoplasts that are excluded by the Evans blue dye (Gaff and Okong'O-ogola 1971).

^cGUS activity after transfection with p35S:GUS (30 μg and 20 μg of plasmid DNA were used in the PEG and in the electroporation transfection treatments respectively) is expressed as picomoles MU per mg total protein per minute (average of 5 experiments). GUS activity could not be determined in the electroporated endosperm protoplast.

Promoter deletion analysis of the barley trypsin inhibitor BTI-CMe gene. Wheat and barley protoplasts were transfected with the five chimeric constructs shown in Fig. 1B, using as a control the 35S promoter used to the GUS reporter gene (Hauptman et al. 1987). Results are presented in Fig. 2. The value obtained for the promoterless GUS construct expression was subtracted from all data.

In barley endosperm protoplasts, GUS expression is from 2000-2250 pmoles MU/10⁶ pp x min (~50% of that driven by p35S:GUS), when using four out of the five BTI-CMe constructs (pSp, pH, pK, pS), while deletion of the nucleotides between bases at -343 and -83 (construct pP), reduces this expression to <300 pmoles MU/10⁶ pp x min (~8% of p35S:GUS). This construct still contains the TATA and the CAAT boxes, but is devoid of the -300 endosperm box (Fig. 1A). In wheat endosperm protoplasts, all the chimeric BTI-CMe derived genes gave a very low GUS expression (<200 pmoles MU/10⁶ pp x min), while the 35S expression is of the same order to that obtained in barley (4000-4250 pmoles MU/10⁶ pp x min).

In cereal leaf protoplasts, transient expression with the BTI-CMe constructs is almost undetectable: ~100 pmoles MU/10⁶ pp x min, versus 5000-7000 pmoles MU/10⁶ pp x min when the p35S:GUS is used (insert in Fig. 2). Similarly transfected tobacco leaf protoplasts, that were extremely efficient in transient expression with the p35S:GUS (37000 pmoles MU/10⁶ pp x min), give <600 MU units when transfected with any of the BTI-CMe promoter-derived constructs.

All these data indicate the species and endosperm specificity of the barley trypsin inhibitor BTI-CMe gene, and demonstrate that all the information required for it, is present in the 343 nt preceding the ATG translation initiation codon. The observation that in wheat endosperm protoplasts, which physiologically are quite similar to those from barley, the GUS activity driven by the BTI-CMe promoter is only ~20% of that obtained in barley, may indicate that a positive trans-acting factor(s) needed for this trypsin inhibitor expression is(are) present in the endosperm of barley cv Bomi and absent in that of wheat cv Chinese Spring, as well as in the leaves of both species and of tobacco. Previous BTI-CMe mRNA data from our group show that this gene is specifically expressed early during endosperm development in barley cv Bomi while in wheat this mRNA is not detected (Rodríguez-Palenzuela et al. 1989).

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REFERENCES

- Barber D, Sanchez-Monge R, Mendez E, Lazaro A, García-Olmedo F, Salcedo G (1986) *Biochim Biophys Acta* 869:115-118
- Carbonero P, Salcedo G, Sanchez-Monge R, García-Maroto F, Royo J, Gomez L, Mena M, Medina J, Diaz I (1993) In: Aviles FX (ed) *Innovations on proteases and their inhibitors*. Walter de Gruyter, Berlin, New York (in press)
- Chong GL, Reek GR (1987) *Thrombosis Res* 48: 211-221
- Diaz I, Carbonero P (1992) *Plant Cell Rep* 10: 595-598
- Gaff DF, Okong'O-ogola O (1971) *J Exp Bot* 22: 756-758
- García-Olmedo F, Salcedo G, Sanchez-Monge R, Hernandez-Lucas C, Carmona MJ, Lopez-Fando JJ, Fernandez JA, Gomez L, Royo J, García-Maroto F, Castagnaro A, Carbonero P (1992) In: Shewry PR (ed) *Barley, genetics, biochemistry, molecular biology and biotechnology*. CAB International pp 335-350
- Hauptman RM, Ozias-Aking P, Vasil V, Tabeizadesh Z, Rogers SG, Horsch RB, Vasil IK, Fraley RT (1987) *Plant Cell Rep* 6:265-270
- Jefferson RA (1987) *Plant Mol Biol Rep* 5:387-405
- Lee BT, Murdoch K, Topping J, Jones MGK, Kreis M (1991) *Plant Sci* 78:237-246
- Mahoney WX, Hermodson MA, Jones B, Powers DD, Corfman RS, Reek GR (1984) *J Biol Chem* 359:8412-8516
- Mikola J, Soulinna EM (1969) *Eur J Biochem* 9:555-560
- Negrutiu I, Shillato R, Potrykus I, Biasini G, Sala F (1987) *Plant Mol Biol* 8:363-373
- Power JB, Chapman JV (1985) In: Dixon RA (ed) *Plant Cell Culture*. IRL Press, Oxford, Washington DC pp 37-66
- Power JB, Davey MR, McLellan M, Wilson D (1989) *Laboratory Manual plant tissue culture*. University of Nottingham, UK
- Rodríguez-Palenzuela P, Royo J, Gomez L, Sanchez-Monge R, Salcedo G, Molina-Cano JL, García-Olmedo F, Carbonero P (1989) *Mol Gen Genet* 219:474-479
- Royo J (1991) *Doctoral Thesis*. ETS Ingenieros Agrónomos. Universidad Politécnica de Madrid, Spain
- Teeri TH, Patel GK, Aspegren K, Kauppinen V (1989) *Plant Cell Rep* 8:187-190