The promoter of the gene *ltr1* from barley confers a different tissue specificity in transgenic tobacco

Isabel Díaz, Joaquín Royo, Aileen O'Connor and Pilar Carbonero

Abstract Tissue-specific expression of the gene coding for trypsin inhibitor BTI-CMe in barley *(ltri)* occurs during the first half of endosperm development. In transgenic tobacco, the *Itr1* promoter drives expression of the {3-glucuronidase reporter gene not only in developing endosperm but also in embryo, cotyledons and the meristematic intercotyledonary zone of germinating seedlings. A promoter fragment extending 343 bp upstream of the translation initiation ATG codon was sufficient for full transgene expression, whereas, the proximal 83 bp segment of the promoter was inactive. Possible reasons for the differences in expression patterns are discussed.

Introduction

Functional analysis of barley gene promoters is difficult owing to the lack of a routine method for stable transformation of this species. An alternative approach involves the use of a heterologous host species, such as tobacco, in which barley promoters are expressed as reporter constructs fused translationally to /3-glucuronidase (GUS). The promoters of a number of monocotyledonous endosperm specific genes, such as those of maize zeins (Schernthaner et al. 1988; Matzke et al. 1990), barley hordeins (Marris et al. 1989), and wheat

HMW glutenins (Roberts et al. 1989), have been shown to retain their tissue specificity and temporal regulation in the tobacco system. However, in other cases, such specificity is not maintained in the heterologous dicotyledonous host (Ueng et al. 1988; Schubert et al. 1994). Particularly notorious is the case of the endosperm-specific *Opaque-2* gene, which regulates zein deposition in maize (Gallusci et al. 1994).

The barley *Lys 3a* gene, which has been identified through the Ris0 1508 mutation of cv. Borní (Doll et al. 1974), seems to play a similar role to that of *Opaque-2* in the control of severa! genes expressed in barley endosperm, such as those encoding certain hordeins, {3-amylase, protein Z, and trypsin inhibitor BTI-CMe (Hopp et al. 1983; Kreis et al. 1984, 1987; Lazaro et al. 1985; Rodriguez-Palenzuela et al. 1989).

Expression of the gene for BTI-CMe (gene *ltri*) is tissue specifically and temporally controlled during endosperm development, with a maximum mRNA leve! at about 15 days after pollination (DAP) (Rodriguez-Palenzuela et al. 1989). Inthe high-lysine mutant Ris0 1508, both the BTI-CMe protein and its corresponding mRNA occur at very low levels, compared with the wild type, although the structural gene seems not to have been affected by the mutation. These observations, together with genetic evidence from appropriate crosses, imply that the *Lys 3a* locus on chromosome 5H regulates in trans the expression of the *Itr 1* gene, which is located on chromosome 3H (Rodriguez-Palenzuela et al. 1989). A functional investigation of the *cis* motifs present in the *Itr 1* promoter is of interest in connection with the ongoing characterization of the *Lys 3a* regulatory locus.

We report here that the *ltri* promoter drives GUS expression not only in endosperm but also in embryo, cotyledons and in meristematic tissues of transgenic tobacco. We also show that a 343 bp proximal fragment of the promoter is sufficient to determine the observed expression pattern.

Materials and methods

Biological materials

Tobacco plants (Nicotiana tabacum cv. SR1) used in the transformation experiments were grown axenically in sucrose-MS medium (Murashige and Skoog 1962) at 25° C under a 16:8 h photoperiod. After rooting in the presence of kanamycin, transformed plants were transferred to soil and grown in the greenhouse, where they were allowed to self-fertilize.

Barley plants (Hordeum vulgare L. cv. Bomi) were grown under greenhouse conditions or in growth chambers. Endosperms, embryos and aleurones were hand-dissected from seeds at different DAP. Coleoptiles and roots were harvested from seeds grown in the dark for 4 days at 27°C under sterile conditions. Leaves were collected after 10 days under a 16:8 h photoperiod. After harvesting, plant tissues were frozen in liquid nitrogen and stored at − 70° C until used for RNA extraction.

Construction of chimeric genes and tobacco transformation

Five Itr1 gene promoter fragments were obtained extending from the ATG translation initiation codon to the indicated restriction sites at their 5' ends (S, SalI; K, KpnI; H, HaeIII; Sp, SphI; P, Pvu, II). These fragments were fused in-phase to the β-glucuronidase reporter gene (Jefferson et al. 1987) in the binary vector pBin19 (Bevan 1984), to obtain the following constructions as schematically represented in Fig. 1:pS (−2369:GUS), pK (−1969 : GUS), pH (−816 : GUS), pSp (−343 : GUS) and pP (−83 : GUS). These were introduced into Agrobacterium tumefaciens LBA4404 cells (Hoekema et al. 1983). Leaf disc transformation of axenic tobacco cultures, selection in 100 μg/ml kanamycin, and plant regeneration followed established protocols (Horsch et al. 1985).

Quantification and histochemical localization of GUS activity in transgenic tobacco

Fluorometric assays of the GUS enzyme were performed as described by Jefferson (1987). Tissues were ground in the GUS extraction buffer and, after centrifugation, the supernatants were collected and mixed with an equal volume of the same buffer containing 2 mM 4-methyl-umbelliferyl glucuronide (MUG). The reactions were carried out in a final volume of 100 μl at 37° C for 1 h and stopped with 900 μl of 0.2 M Na_2CO_3. Fluorescence was measured at 455 nm, using 4-methyl-umbelliferone (MU) as an internal control. Protein content was determined with the BioRad kit using bovine serum albumin as standard.

Histochemical GUS detection was performed essentially as described by DeBlock and Debrouwer (1992). After pretreating the tissues in 100 mM phosphate buffer, pH 7.0, 1 mM spermidine, they were vacuum infiltrated with a solution containing 2 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-GLUC) at 37° C. After staining, green tissues were cleared of chlorophyll by washing with absolute ethanol. Seeds taken at different developmental stages were hand dissected. Thin sections (10–25 μm) from stained half seeds were obtained after imbibition in 70-2218-500 Historesine (Reichert Jung) using a Jung Autocut 2055, Leica microtome.

RNA extraction and Northern blot analysis

Total RNAs were purified after phenol/chloroform extraction by LiCl precipitation as described by Lagrimini et al. (1987). For Northern analysis, mRNA was separated on 1.2% agarose, 7% formaldehyde gels and transferred to Hybond-N membranes (Amersham), according to the manufacturer's instructions. Hybridization was performed at 65° C by standard procedures (Sambrook et al. 1989), using as specific probe BTI-CMe cDNA (Rodriguez-Palenzuela et al. 1989) that was ^{32}P-labelled by the random primer labelling procedure of Feinberg and Vogelstein (1983).

Results

GUS activity in transgenic tobacco seeds directed by the Itr1 gene promoter

Chimeric genes, constructed by fusing barley Itr1 promoter fragments, extending to positions −2369, −1969, −816, −343 and −83 bp upstream of the ATG translation initiation site, to the GUS reporter gene (Fig. 1), were introduced into tobacco by Agrobacterium-mediated transformation and kanamycin selection. GUS activity was assayed in leaves, roots and mature seeds of transgenic plants and found to be significantly above background levels only in seeds (Table 1). All gene promoter constructions, except the shortest (pP, −83 bp), gave expression levels that were not significantly different from each other. A possible negative effect of the SalI-KpnI segment of the promoter (Table 1) might have been obscured by the observed scatter among the activity values for individual transformants with a given construct (Fig. 2). Such quantitative variations among transformants have been repeatedly observed and have been attributed to position effects and other factors.

Temporal regulation of the Itr1 gene was investigated in transgenic tobacco seeds harvested at different times after pollination (Fig. 3). GUS activity above background levels was first detected at 12 DAP and increased to reach a maximum at about 20 DAP for all
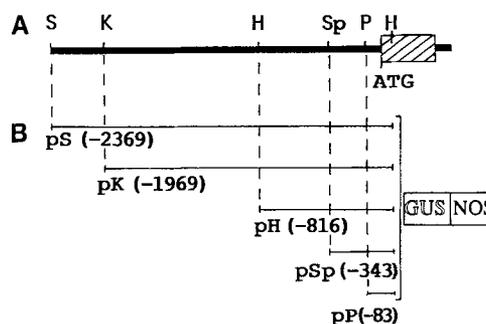

Fig. 1A Schematic representation of the gene for barley trypsin inhibitor BTI-CMe Restriction endonuclease sites for SalI (S), KpnI (K), HaeIII (H), SphI (Sp), PvuII (P) are indicated. The coding region has no introns and is hatched. **B** Chimeric constructs derived from different endonuclease restriction fragments of the promoter in **A**, fused to the β-glucuronidase/nopaline synthase (GUS/NOS) reporter gene. Numbers at the 5' ends of the constructs refer to nucleotide positions relative to the ATG translation initiation site. This nucleotide sequence appears in the EMBL database under the accession number X65875

Table 1 Average GUS specific activity [picomoles 4-methylumbelliferone (4-MU) per minute per microgram protein] of dry seeds, leaves and roots of tobacco stably transformed with chimeric contructs derived from the promoter of barley trypsin inhibitor BTI-CMe fused to the β-glucuronidase reporter gene. Data are average values with their standard deviations. The number of plants analysed appears in brackets

Construct	Seeds	Leaves	Roots
pS (−2369)	65 ± 49 (9)	3.1 ± 1.2 (9)	1.6 ± 0.7 (9)
pK (−1969)	109 ± 31 (10)	2.5 ± 0.4 (10)	2.3 ± 0.4 (10)
pH (−816)	120 ± 63 (8)	2.9 ± 0.3 (8)	1.9 ± 0.9 (8)
pSp (−343)	139 ± 99 (9)	3.2 ± 0.9 (9)	2.0 ± 0.7 (9)
pP (−83)	19 ± 6 (8)	2.3 ± 0.7 (8)	1.7 ± 0.6 (8)
SR1	14 ± 2 (7)	2.0 ± 0.7 (6)	1.3 ± 0.5 (6)
35S	~ 7000 (4)	~ 200 000 (4)	~ 120 000 (4)

Fig. 3 Temporal expression of GUS activity (picomoles MU per minute per milligram protein) during seed development of transgenic tobacco. (*DAP* days after pollination) One representative plant is shown for each construct ○ pP (−83), ● pSp (−343), □ pH (−816), ■ pK (−1969), ▲pS (−1369), △ non transformed SR1 control
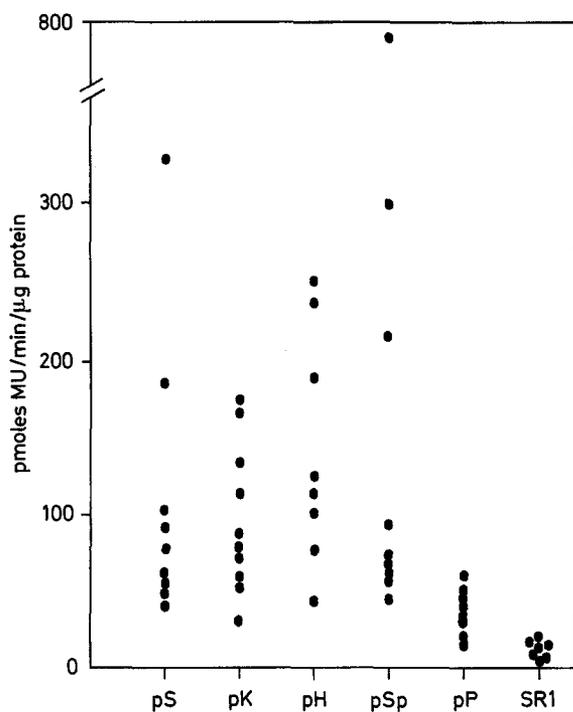

Fig. 2 Expression of GUS activity in mature seeds of transgenic tobacco plants. Each point represents the GUS activity of one independent transformant (50 seeds pooled from 3 to 4 independent pods), expressed as picomoles of 4-MU per min per microgram of total protein *SR1*, untransformed seeds

gene constructions, except for the minimal one (pP, − 83 bp).

Histochemical analysis of developing endosperm and young seedlings

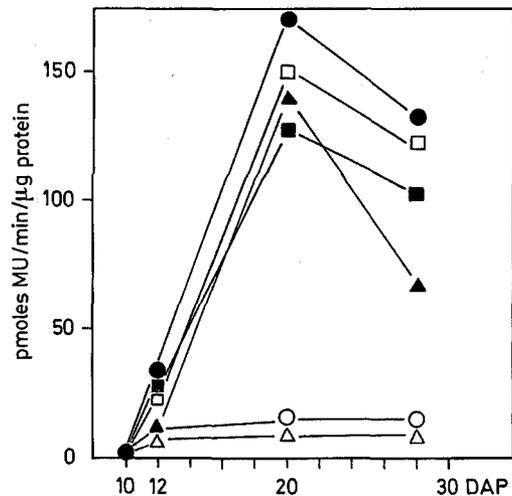GUS expression during seed development was investigated in all transformants by histochemical staining. Again, all constructs, except the shortest (pP), gave the same expression pattern, which is illustrated in Fig. 4. Activity was detected both in the embryo and in the endosperm. In the endosperm, it declined after 20 DAP and became undetectable at maturity. This was checked by staining hand-dissected endosperms (not shown). In the embryo, GUS activity persisted into the dry stage (Fig. 4a–e). During germination, after imbibition of seeds, when fast growth of root and cotyledons began, activity was detected in the cotyledons (Fig. 5f–h). At later stages (6–8 days of germination), when cotyledons began to expand, GUS activity was localized in the vascular region and in the area between the intercotyledonary zone and the hypocotyl, where the shoot meristem is located (Fig. 5i, j, l, m). GUS expression also appeared in the region that separates root cells from hypocotyl cells (Fig. 5k). No activity was detected anywhere when the first true leaves began to emerge (10 to 12 days of germination).

Expression of the *Itr1* gene in barley

To ascertain whether the *Itr1* gene is expressed in barley tissues other than endosperm, Northern blot analysis of total RNAs from barley embryo, endosperm, aleurone, coleoptile, roots and leaves, was carried out using the complete BTI-CMe cDNA as a probe. As shown in Fig. 5, expression was detected only in the endosperm and not in the other parts analysed, even after long exposure times. A ribosomal DNA probe was used to ascertain that equal amounts of RNA had been applied in each lane.
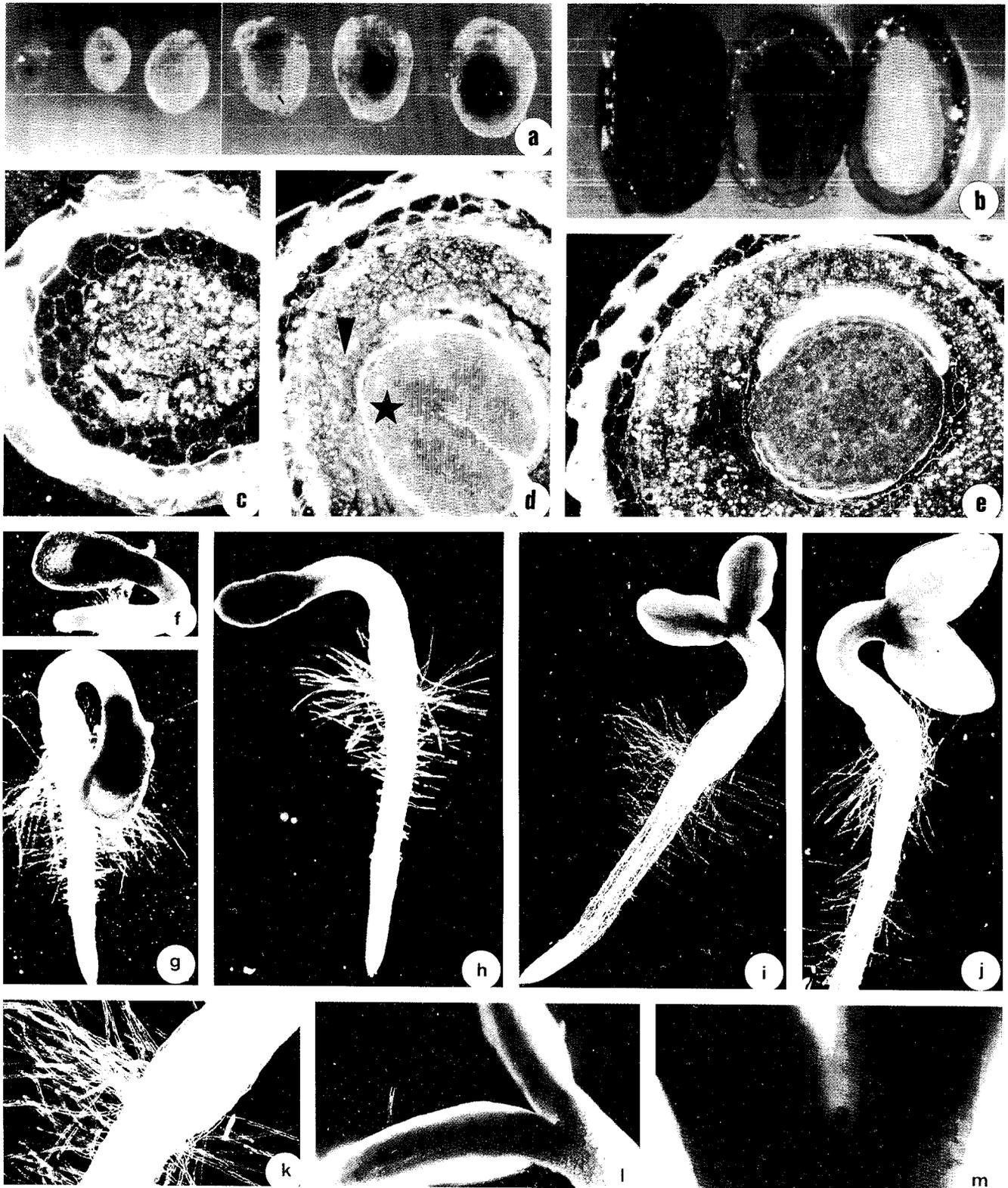
Fig. 4a–m Histochemical localization of GUS activity in transgenic pSp (− 343) tobacco seeds and R1 seedlings. **a** Developing seeds: 4, 8, 10, 14, 18, 25 DAP. **b** Transgenic dry seed between positive (35S) and negative (non-transformed SR1) controls. **c, d, e** Transverse sections of 10 DAP, 20 DAP and dry seeds. Endosperm (*arrowhead*), embryo (*star*). **f**–**j** Germinating seedlings after selfing of the primary transformants and selection on kanamycin. **k, l, m** Details from **h, i** and **j** respectively

Motto M, DiFonzo N, Hartings H, Maddaloni M, Salamini F, Soave C, Thompson RD (1989) Regulatory genes affecting maize storage protein synthesis. In: Shewry PR (ed) Oxford surveys plant molecular and cell biology, vol 6. IRL Press, Oxford, 87-144

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Quayle T, Feix G (1992) Functional analysis of the −300 region of maize zein genes. Mol Gen Genet 231: 369-374

Reeves CD, Okita TW (1987) Analysis of $\alpha/$ {3-type gliadin genes from diploid and hexaploid wheats. Gene 52: 257-266

Roberts LS, Thompson RD, Flavell RB (1989) Tissue-specific expression of a wheat high molecular weight glutenin gene in transgenic tobacco. Plant Cell 1:569-578

Rodriguez-Palenzuela P, Royo J, Gómez L, Sánchez-Monge R, Salcedo G, Molina-Cano JL, Garcia-Olmedo F, Carbonero P (1989) The gene for trypsin inhibitor CMe is regulated in trans by the *lys 3a* locus in the endosperm of barley *(Hordeum vulgare L)*. Mol Gen Genet 219:474-479

Salcedo G, Fra-Mon P, Molina-Cano JL, Aragoncillo C, Garcia-Olmedo F (1984) Genetics of CM-proteins (A-hordeins) in barley. Theor Appl Genet 68: 53-59

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schernthaner JP, Matzke MA, Matzke AJM (1988) Endosperm specific activity of a zein gene promoter in transgenic tobacco plants. EMBO J 7: 1249-1255

Schmidt RJ, Burr FA, Aukerman MJ, Burr B (1990) Maize regulatory gene *opaque2* encodes a protein with a "leuzine-zipper" motif that binds to zein DNA. Proc Natl Acad Sci USA 87:46-50

Schubert R, Panitz R, Manteuffel R, Nagy I, Wobus U, Baumlein H (1994) Tissue-specific expression of an oat 12S seed globulin gene in developing tobacco seeds: differential mRNA and protein accumulation. Plant Mol Biol 26 :203-210

Thompson GA, Boston RS, Lyznik LA, Hodges TK, Larkins BA (1990) Analysis of promoter activity from an a-zein gene 5' flanking sequence in transient expression assays. Plant Mol Biol 15:755-764

Ueng P, Gallili G, Sapanara V, Goldsbrough PB, Dube P, Beachy RN (1988) Expression of a maize storage protein gene in petunia plants is not restricted to seeds. Plant Physiol 86: 1281-1285