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 *ltr1* promoter drives expression of the β -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 β -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. Borni (Doll et al. 1974), seems to play a similar role to that of *Opaque-2* in the control of several genes expressed in barley endosperm, such as those encoding certain hordeins, β -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 level at about 15 days after pollination (DAP) (Rodriguez-Palenzuela et al. 1989). In the 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 *ltri* gene, which is located on chromosome 3H (Rodriguez-Palenzuela et al. 1989). A functional investigation of the *cis* motifs present in the *ltri* 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, *Sall*; K, *KpnI*; H, *HaeIII*; Sp, *SphI*; P, *PvuII*, 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 μ g/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 μ l at 37°C for 1 h and stopped with 900 μ l of 0.2 M Na₂CO₃. 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 μ m) from stained half seeds were obtained after imbibition in 70-2218-500 Histoiresine (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 ³²P-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 *Sall*-*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 *Sall* (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 constructs 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 $\pm$ 49 (9)	3.1 $\pm$ 1.2 (9)	1.6 $\pm$ 0.7 (9)
pK (-1969)	109 $\pm$ 31 (10)	2.5 $\pm$ 0.4 (10)	2.3 $\pm$ 0.4 (10)
pH (-816)	120 $\pm$ 63 (8)	2.9 $\pm$ 0.3 (8)	1.9 $\pm$ 0.9 (8)
pSp (-343)	139 $\pm$ 99 (9)	3.2 $\pm$ 0.9 (9)	2.0 $\pm$ 0.7 (9)
pP (-83)	19 $\pm$ 6 (8)	2.3 $\pm$ 0.7 (8)	1.7 $\pm$ 0.6 (8)
SR1	14 $\pm$ 2 (7)	2.0 $\pm$ 0.7 (6)	1.3 $\pm$ 0.5 (6)
35S	$\sim$ 7000 (4)	$\sim$ 200 000 (4)	$\sim$ 120 000 (4)

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

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 $\circ$ pP (-83), $\bullet$ pSp (-343), $\square$ pH (-816), $\blacksquare$ pK (-1969), $\blacktriangle$ pS (-1369), $\triangle$ non transformed SR1 control

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 selling of the primary transformants and selection on kanamycin. **k, l, m** Details from **h, i** and **j** respectively

Fig. 5 Northern blot analysis of total RNA (2 µg) extracted from the indicated organs of barley cv. Bomi. (*E* 18 DAP endosperm, *A* aleurone *Em* embryo, *R* and *C* roots and coleoptiles from 4 day old dark-grown seedlings; *L*, leaves from light-grown 10 day old plants). The cDNA clones for barley trypsin inhibitor (*CMe*) and for ribosomal RNA (*rib*) were used as probes. Hybridization was under stringent conditions

Discussion

Transgenic expression in tobacco of GUS directed by the *Itr1* promoter appears in seeds during the first half of their developmental process, which is in line with the observed time course of expression of this gene in barley kernels (Rodriguez-Palenzuela et al. 1989) as the times from pollination to maturity are very similar in SR1 tobacco and in Bomi barley (32 and 35 days, respectively). However, transgenic GUS expression levels under the *Itr1* promoter are much lower than those obtained for the 35S:GUS fusion, used as positive control. The possibility that the *Itr1* promoter is intrinsically weak can be excluded, in view of the high transient expression levels previously obtained with these same constructions (Diaz et al. 1993) in barley protoplasts (over 2000 pmol MU per 10⁶ protoplasts per minute, or 50% of the activity observed for the 35S:GUS fusion), as well as by the significant histochemical staining observed in the embryos of transgenic tobacco (Fig. 4). A high proportion of the GUS activity determined in developing seeds (Fig. 3) and all the activity in mature seeds (Fig. 2) was contributed by the embryos. GUS activity did not persist in the mature tobacco endosperm, while BTI-CMe is an abundant protein in the same tissue of barley (Mikola and Soulinna 1969; Salcedo et al. 1984), which may reflect differences in inherent turnover rates of the two proteins.

A high level of GUS expression was not only observed in the developing and mature embryo of transgenic tobacco, but also in cotyledons and meristematic tissues of seedlings. In contrast, *Itr1* mRNA was only found in the developing endosperm and could not be detected in the embryo or coleoptile or in the aleurone, roots or leaves of barley. The difference in tissue specificity might reflect the prominent storage role played by dicotyledonous embryos, especially by the cotyledons,

in addition to the endosperm (see Goldberg et al. 1989; Lopes and Larkins 1993), which represents the main reserve tissue in monocotyledonous species. This makes plausible, the suggestion that at least some of the regulatory factors driving the expression of seed proteins in tobacco are present both in the endosperm and in the embryo, thus accounting for the observed transgenic activity, while in barley, factors with similar activity are present only in the endosperm. Other factors might be responsible for the persistence of GUS in cotyledon meristems after they have ceased to serve as a sink tissue.

Temporal control and seed specificity in transgenic tobacco were essentially the same for all tested constructs (except for the inactive pP), which indicates that positive-*cis* regulatory motifs in the proximal 343 bp of the promoter are responsible for the expression pattern in transgenic tobacco. These results are in agreement with those of transient expression experiments in endosperm protoplasts from barley using the same set of 5' deletions of the *Itr1* promoter (Diaz et al. 1993).

A number of barley endosperm-specific promoters, both from early-expressed genes, such as those for thionins (Fernandez et al. 1993), or from late-expressed genes, such as those coding for hordeins (Marris et al. 1989), have been functionally tested in tobacco and found to retain their tissue specificity and temporal control. The exception represented by the recently reported case of the *Opaque-2* promoter from maize (Gallusci et al. 1994) is of particular interest because it shows an activity pattern in tobacco that is quite similar to that of the *Itr1* promoter. The functional similarity between the maize *Opaque-2* regulatory gene and the barley *Lys 3a* gene, which controls in *trans* the expression of the *Itr1* gene (Motto et al. 1989; Rodriguez-Palenzuela et al. 1989; Schmidt et al. 1990), suggests that a tobacco equivalent to these regulatory genes might be responsible for the activity pattern of the *Itr1* promoter in this species. It has been suggested (Gallusci et al. 1994) that the *Opaque-2* promoter activity in the endosperm and embryo of transgenic tobacco seed might be due to the presence of the legumine box (CATGCATG) and G-box (CACGTG) motifs. However, there are no legumine or G-box motifs in the 343 bp of the BTI-CMe promoter (Fig. 6), although two putative bZip binding sites are found (TAACTCA and ACATGT). In fact, there are no clear similarities between the sequences of both promoters, with the exception of the motif CAAAT which has also been described in other cereal endosperm storage gene promoters as a transcriptional enhancer (Forde et al. 1985; Reeves and Okita 1987; Cameron-Mills and Brandt 1988; Maier et al. 1990; Thompson et al. 1990).

A highly conserved motif, the so-called endosperm box, is present in the promoters of the cereal endosperm storage proteins (Motto et al. 1989) and it has been suggested that it is responsible for their endosperm specificity both in cereals and in transgenic

SphI

-352 AATGGCATGCAGATGAACCAGGGATGTAGCGTAGTATGATAAGATAACTCATGCCAAGCA

-292 CACTGAGAAAGGTAGGAACCGAGGAAAATATGGTTATTCTTCTACTCAAACCCCACTTTT

1

-232 GCATTCCCCTCCGAAATATTGTTAGCACAAAGCTCCAAATTCGTCTAGCAAAAAAGGTTAT

1

-172 CAAAGCTACCCTTTAATCAAAATTTCCATGCAGCGGCACCTCCAACACTAACAGAAAGTC

PvuII

-112 AGAAAGCACTTCGGTACTTACGATGCCAGCTTGCTCACTTCACATTTCACTATATATA

2

-52 TACATGTGTACGCCTCGCTTGCTCCAACAACATCCCTTCAATTTGATAACA ATG GCG

2 M A

tobacco (Colot et al. 1987; Matzke et al. 1990; Quayle and Feix 1992). There is no clear canonical endosperm box in the *Itr1* promoter, although a related motif, -AATGCAAAA-, is present in the non-coding strand between positions -228 and -236. An accurate endosperm box could be important for the activity of the transgenes in tobacco and its absence in the *Itr1* and *Opaque-2* promoters might result in their expression in tobacco not being restricted to the endosperm.

Acknowledgements We thank L. Lamonedá and J. García for technical assistance, Drs. F. García-Olmedo and J. Vicente-Carabajosa for stimulating discussions and Dr. J. Guerin and F. Acevedo for critical reading of the manuscript. This work was financed by grant Bio91-0782 from Comisión Interministerial de Ciencia y Tecnología (Spain).

- Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* 12: 8711–8721
- Cameron-Mills V, Brandt A (1988) A γ -hordein gene. *Plant Mol Biol* 11: 449–461
- Colot V, Robert LS, Kavanagh TA, Bevan MW, Thompson RD (1987) Localization of sequences in wheat endosperm protein genes which confer tissue-specific expression in tobacco. *EMBO J* 6: 3559–3564
- DeBlock M, Debrouwer D (1992) In situ enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free β -glucuronidase assay. *Plant J* 2: 261–266
- Díaz I, Royo J, Carbonero P (1993) The promoter of barley trypsin-inhibitor BTI-CMe discriminates between wheat and barley endosperm protoplasts in transient expression assays. *Plant Cell Rep* 12: 698–701
- Doll H, Kœie B, Eggum BO (1974) Induced high lysine mutants in barley. *Radiation Bot* 14: 73–80
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6–13
- Fernández JA, Moreno M, Carmona MJ, Castagnaro A, García-Olmedo F (1993) The barley α -thionin promoter is rich in negative regulatory motifs and directs tissue-specific expression of a reporter gene in tobacco. *Biochim Biophys Acta* 1172: 346–348
- Forde BG, Heyworth A, Pywell J, Kreis M (1985) Nucleotide sequence of a β -1 hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. *Nucleic Acids Res* 13: 7327–7339
- Gallusci P, Salamini F, Thompson RD (1994) Differences in cell type-specific expression of the gene *Opaque2* in maize and transgenic tobacco. *Mol Gen Genet* 244: 391–400

Fig 6. Sequence analysis of the barley *Itr1* gene proximal promoter. The putative TATA box is framed, the CAAAAT motif is doubly underlined and putative bZip-like binding sites are indicated by solid bars. Direct and inverted repeats are indicated by arrows. The *SphI* and *PvuII* endonuclease restriction sites are stippled. Numbers on the left are with reference to the translation initiation ATG codon

- Goldberg RB, Barker SJ, Pérez-Grau L (1989) Regulation of expression during plant embryogenesis. *Cell* 56: 149–160
- Hoekema A, Hirsch PR, Hooykass PJJ, Schilperoot RA (1983) A binary plant vector strategy based on the separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti plasmid. *Nature* 303: 179–180
- Hopp HE, Rasmussen SK, Brandt A (1983) Organization and transcription of B1 hordein genes in high lysine mutants of barley. *Carlsberg Res Commun* 48: 201–210
- Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231
- Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5: 387–405
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901–3907
- Kreis M, Shewry PR, Forde BG, Rahman S, Mifflin BJ (1984) Molecular analysis of the effects of the mutant *lys 3a* gene on the expression of *Hor* loci in developing endosperms of barley (*Hordeum vulgare*). *Cell* 34: 161–167
- Kreis M, Williamson M, Buxton B, Pywell J, Heijgaard J, Svendsen I (1987) Primary structure and differential expression of β -amylase in normal and mutant barleys. *Eur J Biochem* 169: 517–525
- Lagrimini LM, Burkhart W, Moyer M, Rothstein S (1987) Molecular cloning of complementary DNA encoding the lignin forming peroxidase from tobacco: molecular analysis and tissue-specific expression. *Proc Natl Acad Sci USA* 84: 7542–7546
- Lazaro A, Barber D, Salcedo G, Mendez E, García-Olmedo F (1985) Differential effects of high lysine mutations on the accumulation of individual members of a group of proteins encoded by a disperse multigene family in the endosperm of barley (*Hordeum vulgare* L.). *Eur J Biochem* 149: 617–623
- Lopes MA, Larkins BA (1993) Endosperm origin, development and function. *Plant Cell* 5: 1383–1399
- Maier UG, Grasser KD, Haass MM, Feix G (1990) Multiple proteins bind to the P2 promoter region of the zein pMS1 of maize. *Mol Gen Genet* 221: 164–170
- Marris C, Gallois P, Copley J, Kreis M (1989) The 5' flanking region of a barley B hordein gene controls tissue and developmental specific CAT expression in tobacco plants. *Plant Mol Biol* 10: 359–366
- Matzke AJM, Stöger EM, Schrenthaler JP, Matzke MA (1990) Deletion analysis of a zein gene promoter in transgenic tobacco plants. *Plant Mol Biol* 14: 323–332
- Mikola J, Soulinna EM (1969) Purification and properties of a trypsin inhibitor from barley. *Eur J Biochem* 9: 555–560

- 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 α / β -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 *hys 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
- Schernthaler 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 "leucine-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 α -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