The promoter of the gene \textit{ltr1} from barley confers a different tissue specificity in transgenic tobacco

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Abstract  Tissue-specific expression of the gene coding for trypsin inhibitor BTI-CMe in barley (\textit{ftri}) occurs during the first half of endosperm development. In transgenic tobacco, the \textit{ltr1} promoter drives expression of the \textit{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 \textit{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 \textit{Opaque-2} gene, which regulates zein deposition in maize (Gallusci et al. 1994).

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

Expression of the gene for BTI-CMe (gene \textit{ftri}) 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 Riso 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 \textit{Lys 3a} locus on chromosome 5H regulates in trans the expression of the \textit{ltr1} gene, which is located on chromosome 3H (Rodriguez-Palenzuela et al. 1989). A functional investigation of the \textit{cis} motifs present in the \textit{ltr1} promoter is of interest in connection with the ongoing characterization of the \textit{Lys 3a} regulatory locus.

We report here that the \textit{ftri} 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. Boml) 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 ltrl gene promoter fragments were obtained extending from the ATG translation initiation codon to the indicated restriction sites at their 5’ ends (SalI, Kpnl, H, HaeIII, Sp, SphI, P, PvuI, 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. p5 (−2369:GUS), pK (−1969:GUS), pH (−816:GUS), pSp (−343:GUS) and pP (−83:GUS). These were introduced into Agrobacterium tumefaciens LBA4404 cells (Horsch 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-methylumbelliferyl 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 Na2CO3. Fluorescence was measured at 455 nm, using 4-methylumbelliferone (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-D-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 Histoireine (Reichert Jung) using a Jung Autocut 2055, Leica microscope.

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 ltrl gene promoter

Chimeric genes, constructed by fusing barley ltrl 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-Kpnl 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 ltrl 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), Kpnl (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 ATO 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.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Seeds</th>
<th>Leaves</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS (−2369)</td>
<td>65 ± 49 (9)</td>
<td>3.1 ± 1.2 (9)</td>
<td>1.6 ± 0.7 (9)</td>
</tr>
<tr>
<td>pK (−1969)</td>
<td>109 ± 31 (10)</td>
<td>2.5 ± 0.4 (10)</td>
<td>2.3 ± 0.4 (10)</td>
</tr>
<tr>
<td>pH (−816)</td>
<td>120 ± 63 (8)</td>
<td>2.9 ± 0.3 (8)</td>
<td>1.9 ± 0.9 (8)</td>
</tr>
<tr>
<td>pSp (−343)</td>
<td>139 ± 99 (9)</td>
<td>3.2 ± 0.9 (9)</td>
<td>2.0 ± 0.7 (9)</td>
</tr>
<tr>
<td>pP (−83)</td>
<td>19 ± 6 (8)</td>
<td>2.3 ± 0.7 (8)</td>
<td>1.7 ± 0.6 (8)</td>
</tr>
<tr>
<td>SR1</td>
<td>14 ± 2 (7)</td>
<td>2.0 ± 0.7 (6)</td>
<td>1.3 ± 0.5 (6)</td>
</tr>
<tr>
<td>35S</td>
<td>~7000 (4)</td>
<td>~200000 (4)</td>
<td>~120000 (4)</td>
</tr>
</tbody>
</table>

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–c). During germination, after imbition 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 ltrl gene in barley

To ascertain whether the ltrl 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 BTTI-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–e Transverse sections of 10 DAP, 20 DAP and dry seeds. Endosperm (arrowhead), embryo (star). f–j Germinating seedlings after selection 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 |xg) 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 ltrl 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 ltrl promoter are much lower than those obtained for the 35S:GUS fusion, used as positive control. The possibility that the ltrl 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 10^6 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 Soulínna 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, ltrl 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 ltrl 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 ltrl 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 ltrl 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 ltrl 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 (TAACCTA 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
tobacco (Colot et al. 1987; Matzke et al. 1990; Quayle and Feix 1992). There is no clear canonical endosperm box in the \textit{Irl} promoter, although a related motif, -AATGC\textsubscript{AAAAA}, 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 \textit{Irl} and \textit{Opaque-2} promoters might result in their expression in tobacco not being restricted to the endosperm.

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\begin{itemize}
  \item DeBlock M, Debrouwer D (1992) In situ enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free \textbeta-glucomidase assay. Plant J 2: 261–266
\end{itemize}

\begin{figure}
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\includegraphics[width=\textwidth]{fig6}
\caption{Sequence analysis of the barley \textit{Irl} gene proximal promoter. The putative TATA box is framed, the CAAAAT motif is \textit{double underlined} and putative bZip-like binding sites are indicated by \textit{solid bars}. Direct and inverted repeats are indicated by \textit{arrows}. The \textit{SphI} and \textit{PvuII} endonuclease restriction sites are \textit{stippled}. Numbers on the left are with reference to the translation initiation ATG codon.}
\end{figure}


