The maize streak virus coat protein transcription unit exhibits tissue-specific expression in transgenic rice

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Abstract

Maize streak geminivirus (MSV) is a single-stranded DNA virus that infects cereals and other grasses. A promoter region incorporating the MSV large intergenic region and movement protein gene sequence was ligated to the gus (β-glucuronidase) reporter gene which replaced the virus coat protein (CP) gene. The CP promoter activity was analysed in transgenic rice plants (Oryza sativa L.) and was compared with that obtained in plants transformed with the gus gene downstream of the cauliflower mosaic virus (CaMV) 35S promoter. The MSV CP promoter activity varied in the five plant lines tested, but was always less than that of the CaMV promoter. Histochemistry showed that the MSV CP promoter was active in cells of regenerating callus but in regenerated plants it provided an expression pattern restricted to the vascular tissues of the root, stem, leaf and floral organs. Expression was highest in phloem-associated tissues of the vegetative organs and was absent from the tip and elongation region of seedling roots. Thus, the MSV CP promoter shows a degree of developmental regulation and can be used to confer tissue-specific expression in transgenic rice plants. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: GUS; Tissue-specific expression; Maize streak virus; MSV; Coat protein promoter; Transgenic rice

1. Introduction

The 35S promoter of cauliflower mosaic virus (CaMV) has been widely used to provide expression of foreign genes in transgenic plants. However, there is a need for a wider range of promoters with different characteristics, for example, to obtain different levels of transgene expression, expression at different developmental stages, or in specific organs and cell types. Furthermore, although the CaMV promoter is suitable for expression of foreign genes in transgenic rice, it provides only low expression in other economically important cereals and grasses of the family Poaceae. Recent successes with the genetic transformation of maize [1–4], rice [5,6], wheat [7], oat (reviewed in [8]), and barley [9] have required the identification of appropriate promoters for the genetic modification of these crops.

Isolation of promoters from plants is ongoing but plant DNA virus promoters are now accessible and, as was the case for CaMV, may contain cis regulatory elements able to confer specific patterns of transgene expression in plants [10]. The promoters of other pararetroviruses such as cassava vein mosaic virus (CsVMV, [11]), Commelina yellow mottle virus (CoYMV, [12]), sugarcane bacilliform virus (ScBV, [13]) and rice tungro bacilliform virus (RTBV, [14,15]) have been investigated for their ability to promote gene expression in transgenic cereals. All but CsVMV naturally infect monocots, but only ScBV and RTBV infect mem-
bers of the Poaceae. The pattern of transgene expression, identified by β-glucuronidase (GUS) staining, was not identical for the promoters of these viruses, with the CsVMV and ScBV promoters being active in all organs tested and providing near constitutive expression, whereas the RTBV and CoYMV promoters were specific to the vascular tissue. Only the RTBV promoter was tested in its natural host species. Another plant DNA virus family, the Geminiviridae, contains a genus (Mastreivirus, [16]) whose species infect members of the Poaceae. These viruses include maize streak virus (MSV), wheat dwarf virus (WDV) and Chloris striate mosaic virus (CSMV). Of these, MSV has the widest host range, infecting most of the economically important Poaceae [17]. However, the activity of the Mastreivirus promoters in transgenic cereals has not yet been reported.

MSV possesses a single-stranded circular DNA genome but transcription of the double-stranded viral DNA is bidirectional, initiating mainly in the large intergenic region (LIR), and terminating in the small intergenic region (SIR) which contains polyadenylation signals [18–20]. The LIR contains the rightward promoter element (rpe1) [21] from which the virion sense genes encoding the movement protein (MP) and coat protein (CP) are expressed [20].

To study the strength and tissue specificity of the MSV CP promoter region in transgenic rice, a cp-gus reporter gene replacement construct was made. The construct contained the MSV LIR and SIR, the mp gene sequence and parts of the replication associated genes and therefore constituted an MSV CP ‘extended promoter’. This MSV promoter conferred tissue specific expression and showed some developmental regulation in transgenic rice plants. The properties of this promoter are compared with those of the CaMV 35S promoter, and other virus promoters, in the context of their use for tissue-specific expression in transgenic rice.

2. Methods

2.1. Constructs for gene transfer

Two plasmids were used for transformation of rice immature embryos. Plasmid WRG4517 [22] contained the hpt gene (conferring hygromycin resistance) and was used to select transgenic callus. Plasmid MSVCPp-gus contained the gus reporter gene, encoding GUS, in place of the MSV CP coding region. This construct was designed to allow GUS to be produced from a MSV expression unit that contained all identified MSV tran-

![Fig. 1. Construction of pMSVCPp-gus. Plasmid MSVCPp-gus used for bombardment of rice cultures was constructed from pMSVV2 [23] and a clone containing a tandem repeat of the Maize streak geminivirus (MSV)-Ns genome. The position of restriction enzyme sites used for the construction of pMSVV2 and pMSVCPp-gus are shown above the plasmids, the MSV co-ordinates are those reported in Ref. [18]. Restriction sites destroyed during the cloning procedure are suffixed by *. The position of the MSV and gus genes are shown by boxes, below these the MSV coat protein (CP) extended promoter region, and the proteins likely to be translated in transgenic plants, are shown. Virion-sense transcript initiation sites mapped to the MSV-Ns genome [20] are indicated by * and curved arrows show the direction of transcription. TATA boxes are marked ‘T’. The cl gene product is also known as RepA, and the C2 open reading frame (ORF) product forms the C-terminus of the C1:C2 (Rep) protein. mp, movement protein gene; LIR, large intergenic region; SIR, small intergenic region.](image-url)
scription regulatory sequences, thereby comprising an 'extended' CP promoter (Fig. 1). Upstream of the gus gene there is the 5' region of the MSV c1 (replication-associated protein A, repA) gene, the MSV LIR and the MSV mp gene (in which the MSV virion sense intron is situated, [20]). The SIR and the MSV C2 open reading frame (ORF) are downstream of gus. Plasmid MSVCPp-gus was produced from a previously constructed plasmid pMSVV2-GUS ([23] and Fig. 1). Plasmid MSVV2GUS, based on pUC19, contained MSV (strain Ns, [24]) sequences between co-ordinates 2433–1469, according to [18]), except that the cp sequence was replaced by the NcoI–PstI gus gene fragment (a gift from V. Citovsky). This replacement was facilitated by site-directed mutagenesis at the cp translation initiation and termination sites to produce NcoI and PstI sites, respectively. As pMSVV2GUS did not contain the entire LIR, a 2 kbp AffIII–XhoI MSV fragment (comprising MSV sequences between co-ordinates 2323 and 1682) was introduced into AffIII and SalI-digested pUC19 to generate construct pMSVPUC19. The SalII–BglII fragment from pMSVV2GUS, which contained the gus replacement, was then introduced in place of the homologous segment of pMSVPUC19 to generate pMSVCPp-gus.

2.2. Particle bombardment and regeneration of transformed plants

Immature rice embryos (Oryza sativa L., genotype ITA212) were transformed by particle bombardment and cultured as described [5] and modified [25]. Plasmids pMSVCPp-gus and pWRG4517 were co-precipitated onto 0.95 μm gold particles at a ratio of 3:1 by weight. Transformed callus was selected on CCM3 medium containing 35 mg l⁻¹ hygromycin. Regenerated rice plants were transferred to a loam-based compost in a controlled-environment growth chamber (80% humidity, 27°C) before transfer to a glasshouse maintained at 25°C (± 5°C) and approximately 80% humidity. Daylight was supplemented to a 16-h photoperiod. Plants regenerated from tissue culture were designated as the T0 generation. Plants regenerated from the same immature embryo-derived callus (each callus was designated a ‘clone’) were given the same line number. T1 plants were derived by self-pollination of the T0 plants.

2.3. Southern hybridisation analysis of transgenic plants

Nuclear DNA was isolated from the leaves of rice plants using the Nucleon-Phytopure™ DNA Extraction Kit (Amersham). Purified DNA was estimated spectrophotometrically and also by comparison with ethidium bromide-stained control DNA samples, following electrophoresis in a TBE agarose gel [26]. Genomic DNA (5 μg) was digested with SacII, which cuts at a single site in pMSVCPp-gus. Southern transfer of the separated DNA fragments to Hybond N+ membranes (Amersham) and hybridisation to a high specific activity α-[32P]dCTP-labelled NeoI–PstI gus fragment from pMSVV2GUS (Fig. 1), were carried out according to the method of Ref. [27]. Hybridisation was visualised using phosphor-imaging.

2.4. Aseptic germination of seeds

Rice seeds (T1 generation or non-transformed) were dehusked by rubbing laterally with sandpaper (grit number 50, English Abrasives). After surface-sterilisation, the seeds were placed on sterile, damp filter paper in Petri plates. Incubation was in the dark at 37°C for 16–24 h and then germination was completed at 25–27°C for up to 8 days. Seeds were used for GUS assays or were transferred to compost if mature plants were required.

2.5. Analysis of GUS activity in rice plants

2.5.1. Chemiluminescent analysis

Quantitative detection of GUS in the young (approx 5 cm long) leaves of T0 plants was carried out using the GUS-light™ chemiluminescent reporter gene assay system (Tropix) as described by the manufacturer. Freshly prepared plant tissue lysate (30 μl) was used and each sample was assayed in duplicate. Light emission was measured in a luminometer (Lumat LB905, Bertold) 5 s after the addition of 100 μl light emission accelerator. In order to express the GUS activity in relative light units (RLU) per μg of protein, the total protein concentration of each lysate was determined by the Bradford method using a Bio-Rad protein estimation kit and the microplate assay. The protein concentration was determined by comparison to bovine serum albumin (BSA) stan-
Table 1

<table>
<thead>
<tr>
<th>Line #</th>
<th>Mean RLU&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Seed production/plant (and fertility)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>76</td>
<td>$1003 \pm 5$</td>
<td>108 $\pm$ 15 (2:3)</td>
</tr>
<tr>
<td>77</td>
<td>$58 \pm 4$</td>
<td>431 $\pm$ 28 (3:1)</td>
</tr>
<tr>
<td>78</td>
<td>$36 \pm 3$</td>
<td>492 $\pm$ 144 (4:1)</td>
</tr>
<tr>
<td>79</td>
<td>$24 \pm 1$</td>
<td>55 $\pm$ 0 (1:0)</td>
</tr>
<tr>
<td>80</td>
<td>$61 \pm 2$</td>
<td>587 $\pm$ 58 (2:0)</td>
</tr>
<tr>
<td>CaMV 35S-gus</td>
<td>54 132</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>70d</td>
<td>15</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative light units (RLU) are presented as the mean for each line ($n = $ number of siblings tested) and are expressed per 10 μg protein min<sup>−1</sup>. Data were analysed using an analysis of variance test (95% confidence; Microsoft Excel 1997©).

<sup>b</sup> Fertility is shown as the number of plants producing seed: number of sterile plants.

<sup>c</sup> ND, not determined.

<sup>d</sup> This line was transformed with pWRG4517.

2.5.2. Histochemical staining and microscopical analysis of tissues

Histochemical staining of rice tissue for GUS activity was done essentially as previously described [28,29] in stain containing 0.05 mM potassium ferricyanide and 0.05 mM potassium ferrocyanide. In order to wet the tissues and aid absorption of the stain, a drop of Nonidet-P40 (Sigma) was added to each 10 ml of stain. The stain was vacuum-infiltrated for five minutes to aid penetration into all tissues and samples were incubated for 12–16 h at 37°C. When necessary, stained tissues were cleared of pigment by incubation in chloralactophenol for 24–48 h as described [30]. For chemiluminescent and histochemical staining, rice plants (Koshihikari, [31] or ITA 212 plant 15-1, Duc Le Tan, John Innes Centre, unpublished data) transformed with a CaMV 35S-gus construct served as positive controls for assay techniques.

For examination by microscopy, tissue samples (1–2 cm long) were vacuum infiltrated with fixative (1% glutaraldehyde in sodium phosphate buffer pH 7.2) for 5 min and left overnight in fresh fixative. Tissue was then dehydrated for 10 min in each of a series of ethanol solutions (20, 50, 70, 85 and 95% v/v ethanol/SDW) after which it was embedded in Historesin™ (Leica) prepared according to the manufacturer’s instructions. Samples were infiltrated for 5 min under vacuum and incubated for a further 4 h with 50% Historesin™, 50% absolute ethanol, before transfer to 100% resin for 24 h. The resin was polymerised overnight at 4°C. Samples were sectioned with glass knives using a Leica RM2055 microtome. Sections of 2 μm (root) or 7 μm (leaf) tissue were taken, viewed under a Nikon microscope with bright field illumination, and photographed with a Nikon camera using Kodak Ektachrome 160 T film. Identification of cell type and tissue was according to Refs. [32,33].

3. Results

3.1. Regeneration of rice transformed with pMSVCPp-gus

Sixteen clones of ITA212 hygromycin-resistant callus were obtained following co-bombardment of 200 immature embryos with pWRG4517 (carrying the hygromycin resistance gene) and pMSVCPp-gus. To determine whether the MSV CP promoter was active in callus tissue, clones were subjected to GUS histochemical assay 6 weeks after the start of selection. After staining for 16 h, all but five of the clones showed some blue colouration although the intensity of staining varied considerably and in most clones there were unstained regions (examples of the staining are shown in Fig. 3A). Seventeen plants (derived from five clones) were regenerated (Table 1); sibling plants were identified by their line number followed by an individual number (e.g. 76-1–76-5). Plants were not obtained from the two clones exhibiting the highest levels of GUS expression. Southern blot hybridisation analysis of selected regenerants from each clone confirmed that all plants were transformed. All lines showed complex patterns of transgene integration and the pattern for each line was unique. However, the patterns for siblings were identical (Fig. 2), showing that each line resulted from a single transformation event. GUS activity, measured by chemiluminescent assay of leaf tissue, varied between plant lines, but similar levels were observed in sibling plants. GUS activity from plants transformed with the pMSVCPp-gus construct was markedly below that in plants transformed with the CaMV 35S
promoter (Table 1). Subsequent histochemical assay showed that this was caused, at least in part, by the restriction of activity to the vascular tissues compared with ubiquitous expression from the CaMV promoter. Line 76 had high GUS activity relative to the very low expression seen in lines 77–80.

Plants had a normal phenotype and seeds were obtained from all lines, although three of the five line 76 plants and one each from lines 77 and 78 were sterile. Lines 76 and 80 produced fewer seeds than lines 77–79. Thirty-five seeds each from plants 76-2 (high GUS) and 77-2 (low GUS) were germinated aseptically and stained to determine whether GUS activity was reproducible and heritable. All of the T1 seedlings of line 76-2 and 33 of line 77-2 showed GUS staining (most easily assessed in the roots). These data are indicative of integration of the transgene into more than one locus. The level of GUS expression remained higher for the line 76 derived seedlings than for the remaining lines suggesting that expression from the MSV CP promoter was stable through at least one generation. Unlike plants transformed with CaMV 35S-\textit{gus} which showed staining within 2–4 h, tissues transformed with pMSVCPp-\textit{gus} required considerably longer (sometimes overnight) for the staining pattern to be clear.

3.2. The MSV ‘extended’ CP promoter confers a vascular expression pattern in transgenic rice

The gross GUS expression pattern conferred by the MSV extended CP promoter in mature leaf tissue and primary roots was determined for one plant from each T0 line when plants were 2 months old. All showed GUS staining associated with the vascular tissue, although the intensity varied, with plant 76-1 showing the most intense staining (Fig. 3C,D). No endogenous GUS activity was seen in leaves or roots of plants transformed with pWRG4517 alone, or in tissue culture-derived non-transformed plants (not shown). Since the leaves and roots of all lines transformed with pMSVCPp-\textit{gus} revealed a vascular-specific expression pattern, only plants 76-1 and 77-2 were subjected to further histochemical analysis. The pattern of expression controlled by the MSV CP promoter in plant 76-1 is summarised in Fig. 3. A similar expression pattern was seen for plant 77-2, but the lower expression level made detection of staining difficult, especially in cells with little cytoplasm. In thin sections of leaf tissue, GUS activity was strongest in phloem and phloem-associated cells (phloem parenchyma and companion cells, Fig. 3E) although stain could also be seen in xylem parenchyma cells. Apparent staining of the epidermal cells was also seen in non-transformed controls and therefore was not attributed to expression from the CP promoter. No expression was detected in outer bundle sheath, mesophyll or sclerenchyma cells.

The expression pattern in the vascular bundles of the stem was similar to that found in the leaf, except that the xylem parenchyma was more strongly stained (Fig. 3F). The MSV CP promoter was also expressed in flowers; staining was limited to the vascular tissue of the palea and lemma, which are considered to comprise tissues similar to the leaf sheath [33]. Approximately 50% of pollen grains showed GUS activity (not shown).

In primary roots, GUS expression was limited to the vascular cylinder of the mature region; no activity was detected in the meristematic (root tip) or elongation regions despite the presence of vascular tissue in the elongation region (Fig. 3C). However, these regions were stained strongly in plants transformed with CaMV 35S-\textit{gus} (Fig. 3B) even after a short staining period, showing that stain was able to penetrate these regions. The

Fig. 2. Southern hybridisation of genomic DNA from T0 plants transformed with pMSVCPp-\textit{gus} or pWRG4517. Lanes M and (-) contain the Kb DNA ladder (Gibco BRL) or DNA from a plant transformed with pWRG4517, respectively. The remaining lanes each contain DNA from plants transformed with pMSVCPp-\textit{gus}. DNA (5 µg) was digested with \textit{Sac}II. The plant lines are shown above the lanes, sibling plants are identified by numbers below the plant line designation. The membrane was hybridised with a radiolabelled probe specific to the \textit{gus} gene to which a small amount of probe prepared using Kb ladder DNA was added.
vascular-specific expression of the MSV CP promoter was maintained in lateral roots of older seedlings (not shown).

4. Discussion

Although following co-bombardment of rice immature embryos with pMSVCPp-gus and pWRG4517 16 hygromycin-resistant callus clones were obtained, plants could be regenerated from only five of the 11 clones that showed GUS activity, and plants could not be obtained from the two lines which exhibited the highest activity. Furthermore, the two fertile T₀ plants of line 76, which showed highest GUS activity, produced relatively few seed (Table 1). It is unlikely that it was the GUS activity per se that prevented regeneration, as rice plants having high levels of GUS expression directed by the CaMV 35S promoter have been regenerated in this laboratory [31]. Thus, it may be that sequences within the MSV-based construct affected the ability of the plants to regenerate. Parts of the c1 (repA) gene and the C2 ORF,
as well as the entire \( mp \) gene, are present within pMSVCp-p-gus, but only the first 17 amino acids of the C1 protein may be produced and C2 ORF should not be transcribed. It may be that expression of the MSV \( mp \) gene, situated immediately upstream of the \( cp \) gene in MSV, could affect regeneration efficiency if expressed at ‘high’ level. For each line, the level of GUS is likely to reflect that of the MP as both genes are expressed from the same transcription unit. However, it is clear that the variability in GUS expression levels between different transformed rice lines is influenced by factors other than the promoter sequences in the MSV LIR. Such variation in expression is generally related to the chromosomal location of the transgene, the extent to which it is methylated and the transgene copy number [34]. It has been shown that the MSV MP is toxic when over expressed in transgenic \( N. benthamiana \) (Pitaksutheepong, Davies and Boulton, unpublished). If failure to regenerate plants with high GUS activity is determined by the toxicity of the MP, then disruption of the \( mp \) may result in transgenic plants with higher levels of expression from the MSV CP promoter.

The complex transgene integration pattern present in the pMSVCp-p-gus transformed rice is similar to that reported in other studies [35] and also was seen in the CaMV35S-gus transformed rice used as controls in the current study [31]. It is ascribed to the presence of multiple copies of both intact and fragmented plasmid sequences. The complexity does not prevent comparison of the strength or expression pattern of the MSV CP promoter, as all lines produced the same tissue-specific expression pattern. Furthermore, many other studies of promoter expression in cereals have been carried out using plants with multiple transgene insertions or in plants for which the integration patterns were not characterised [13,36,37].

Unlike the CaMV promoter that directs expression in most cell types, the MSV CP promoter directed vascular-specific expression in all plant tissues examined. However, expression was also seen in undifferentiated callus tissue (Fig. 3A). A number of viral promoters have been reported to direct vascular-specific expression in monocots (e.g. RTBV [14,15], CoYMV, [12] and banana bunchy top virus (BBTV, [38]), although in the case of RTBV and BBTV expression was also seen in the actively dividing cells of the root tip. Strong activity of the BBTV DNA-6 promoter in undifferentiated, actively dividing cells (root and callus) was suggested to be a result of a motif with homology to the hexamer motif of plant histone promoters which confers S-phase specific expression. A motif with homology to the octamer motif of the maize histone 4 gene (H4C14) was identified in MSV and other mastreviruses [39]. Although this motif is situated at MSV co-ordinates 181–197, within the \( mp \) gene (and the CP promoter), it clearly does not direct S-phase expression in rice roots. However, the differential activity of the MSV CP promoter in developmentally distinct tissues of the same cell lineage in the root, suggests that the MSV CP promoter exhibits a degree of developmental regulation in transgenic rice.

The vascular-specific gene expression from the MSV CP promoter was not expected, as MSV is present in almost all cell types in maize leaves [40]. However, whereas MSV-Ns infection in maize results in chlorotic streaks which almost entirely cover the leaf [41], MSV-Ns-infected ITA212 rice shows only discontinuous narrow streaks [42] and it cannot be discounted that the narrow streaks reflect vascular limitation of the virus in this species. However, tissue specificity of the CoYMV promoter did not correlate with virus invasion patterns, although in this case the promoter was not expressed in a natural host of the virus [43]. Other factors may account for the lack of correspondence, for example, the MSV CP promoter region used here may not comprise the entire promoter although it encompasses all identified transcription regulatory signals and initiation sites [20,23]. Sunter and Bisaro [44] reported vascular-specific expression from the CP promoter of the dicot-infecting geminivirus tomato golden mosaic virus (TGMV) in transgenic \( N. benthamiana \), despite the fact that TGMV infects mesophyll and other tissues in this host. However, mesophyll expression was activated when the viral AC2 product (TrAP, transcriptional activator protein) was co-expressed in the tissues. The extent of the TGMV promoter sequence was comparable to the MSV CP promoter used in the current study. Furthermore, the CP promoters of the mastreviruses WDV, CSMV [45–47] and MSV [42] are activated by virus replication-associated proteins; therefore, expression of the MSV CP in mesophyll tissue may require the presence of additional virus-encoded proteins.
Further work will be required to determine whether expression from the MSV CP promoter can be enhanced, and to identify signals conferring tissue and developmental specificity. Nevertheless, this study shows that the MSV CP promoter provides unique gene expression patterns of use for the genetic manipulation of a wide range of economically important cereals and grasses.

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