M. J. Muhitch · R. G. Shatters

Regulation of the maize ubiquitin (Ubi-1) promoter in developing maize (Zea mays L.) seeds examined using transient gene expression in kernels grown in vitro

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Abstract Kernel culture was assessed for evaluating novel gene expression in developing maize (Zea mays L.) seeds by comparing the transient expression of maize ubiquitin (Ubi-1) promoter-driven β-glucuronidase (GUS) delivered by particle bombardment in kernels grown in culture with those grown in planta. With kernels from either source, GUS expression, as determined by histochemical staining, was widespread in young, actively growing kernels, but it diminished with kernel age and by 25 days after pollination was found only in the embryo. Transient expression of Ubi-1 in kernels grown in vitro was not affected by wounding, ethylene treatment, pathogen invasion, or heat shock. In contrast, the plant hormones indole-3-acetic acid and kinetin both stimulated transient Ubi-1 expression in the endosperm, particularly at the periphery. Transient gene expression in developing maize seeds grown in vitro should allow for facile and rapid evaluation of the tissue-specificity and environmental responses of novel gene constructs in developing maize seeds.

Key words Maize · Transient assay · Gene expression · Seeds

Abbreviations DAP Days after pollination · GUS β-glucuronidase · IAA indole-acetic acid

Introduction

Testing the expression of novel genes in developing maize seeds is hampered in two ways: (1) the length of time required for stably transformed cell lines to be regenerated and to set seed and (2) the anatomy of the maize cob renders it intractable to the practical experimental manipulation (e.g., heat shock) necessary for testing putative promoter constructs. Transient gene expression of constructs introduced into protoplasts and tissues has been used to overcome the first limitation (Dekeyser et al. 1990), while growing individual kernels with attached cob pieces in vitro has been used to circumvent the second (Gengenbach 1977). Maize kernel culture has proven a facile experimental system for examining the effects of nutrient supply (Singletary and Below 1989, 1990; Singletary et al. 1990), temperature (Jones et al. 1981, 1985; Afuakwa et al. 1984), inhibitors, and other treatments (e.g., Felker 1992) on kernel growth and metabolism. In this report, we demonstrate the competence of in vitro-grown kernels to duplicate the pattern of transient gene expression of the maize ubiquitin (Ubi-1) promoter-driven β-glucuronidase (GUS) found for kernels grown in planta. Furthermore, we show the utility of the in vitro kernel culture system for examining the effects of a variety of treatments that have been shown to induce the expression of various plant ubiquitin genes on the transient expression of Ubi-1/GUS in developing maize seeds.

Materials and methods

Maize (Zea mays L. A619 × W64A) plants were grown under greenhouse conditions and hand-pollinated. Kernels were cultured 5 days after pollination (or in the case of the 3 DAP developmental sample, 2 days after pollination) as previously described (Felker 1992). Briefly, cobs were cut into 6 kernel segments under aseptic conditions; 5 kernels were then removed from each segment, and the remaining kernel and associated cob piece was placed into the standard medium, 4.3 g of Murashige and Skoog Salts (Sigma), 100 g/l sucrose and 7 g/l agar (pH 5.8), with only the cob piece contacting the medium.

Transient expression of the bacterial gusA gene, driven by the maize Ubi-1 promoter, was determined after delivery of the pAHC25 plasmid to maize kernels via particle bombardment. The pAHC25 plasmid, which contains the GUS gene coding region driven by the maize ubiquitin (Ubi-1) promoter, as well as the BAR gene driven by
Fig. 1 Diagram of the pAHCl25 plasmid. GUSUid'A

- Ubi-1
- NOS
gene for β-glucuronidase, BAR
- phosphinothricin acetyltransferase gene for herbicide resistance

5' - GUS - 3'

5' - Ubi-1 - NOS term

3' - BAR - NOS term

a separate Ubi-1 promoter (see Fig. 1), was obtained from Peter Quail, UC-Berkeley/USDA Plant Gene Expression Center. Plasmid DNA was coated onto gold particles (average diameter of 1 μm), Bio-Rad Laboratories, Hercules, Calif.) following the procedure of Gordon-Kamm et al. (1990). The precipitation mixture contained 2.1 mg of gold particles, 25 μg of plasmid DNA, 1.1 M CaCl₂, and 8.1 mM spermidine in a total volume of 545 μl. After vortexing, the mixture was centrifuged at 500 g for 5 min. The pellet was washed with 600 μl of ethanol, centrifuged, resuspended in 56 μl of ethanol, and then 10-μl samples were pipetted onto each of three macrocarriers. Kernels were bisected along the longitudinal axis immediately before bombardment. Particle bombardment was accomplished using a PDS-1000 helium system (Bio-Rad), with 1550 p.s.i. rupture disks, 16 mm between the rupture disk and the macrocarrier, 6 mm between the macrocarrier and the stop screen, and 7 cm between the stop screen and the target tissue, under a 28- to 29-in. Hg vacuum. Unless noted otherwise, kernel halves were subsequently incubated by placing their cob portions in the standard agar-based kernel culture medium overnight (up to 24 h) before assays for GUS activity. Longer incubation periods (up to 48 h) did not result in the appearance of additional GUS activity; however, kernel halves began to desiccate after 24 h. Histochemical and spectrophotometric GUS assays, incubated for 15 and 20 h, respectively, were performed as described by Jefferson et al. (1987) and Jefferson et al. (1986), respectively.

Wounding was administered by making six approximately 2-mm slices in the upper endosperm of 14 or 24 DAP in vitro-grown kernels using a sterile scalpel. Ethylene treatment consisted of incubation overnight (16 h) with six 2.5-cm square pieces of ripe apple skin in the culture plates of 14 and 24 DAP kernels at 28°C prior to particle bombardment. Ethylene evolution by the apple slices was verified by the growth reduction and loss of geotropic response of roots on parallel plates containing germinating maize seeds, in contrast to cells undergoing division and correlates well with known kernel growth patterns (Lur and Setter 1993). These results are similar to those of Cornejo et al. (1993) where the maize Ubi-1 promoter also exhibited a meristematic cell-restricted pattern of expression in stably transformed rice plants.

Results and discussion

Transient expression of the Ubi-1-driven GUS (Ubi-

1/GUS) in developing maize kernels grown in planta was examined as a function of kernel age (Fig. 2). In many of the kernels, staining for GUS activity, an endogenous kernel GUS activity was noted in the basal endosperm and/or maternal pedicel tissues; however, the diffuse endogenously generated staining was readily distinguished from the discrete, intense staining that resulted from the expression of the bacterial GUS protein. In the youngest kernels examined (3 DAP), extensive GUS staining was evident, with intense staining of the nucellus/endosperm tissues and the surrounding integumentary/pericarp layers being particularly notable (Fig. 2A). In contrast, in kernels sampled at 8 DAP, staining was essentially absent from the central endosperm, being confined to the periphery of this tissue and to the surrounding maternal seed and cob tissues (Fig. 2B). By 18 DAP, GUS expression had become further limited, being confined mainly in the embryo tissues, with sporadic staining in the endosperm, the pedicel, and pericarp tissues (Fig. 2C). Finally, in the oldest kernels sampled (25 DAP), GUS staining was almost completely to the embryo and the scutellum (Fig. 2D). Thus, Ubi-1 promoter-driven transient GUS expression in developing maize kernels appears to be confined to cells undergoing division and correlates well with known kernel growth patterns (Lur and Setter 1993). Where the maize Ubi-1 promoter also exhibited a meristematic cell-restricted pattern of expression in stably transformed rice plants.

In order to establish that maize kernels grown in vitro duplicated the gene expression patterns exhibited by kernels grown in planta, transient expression of the Ubi-

1/GUS gene was also examined for a developmental series of in vitro-grown kernels (Fig. 3). The resultant expression patterns found for the cultured kernels were essentially the same as those found for the in planta-grown kernels; that is, strong generalized GUS staining in young, actively growing kernels becoming confined to the embryos as the kernels matured. These results demonstrate that the transient expression of the Ubi-1 promoter in developing maize seeds is not affected by growing the seeds under in vitro culture conditions.

Transient expression of Ubi-1/GUS was not affected by wounding, ethylene treatment, fungal invasion, or heat shock (data not shown). Because the degree of the heatshock response in plants can be highly influenced by the time between tissue preparation and transformation, by the duration of the heat treatment, or by the time period
between the treatment and the analysis (e.g., Cornejo et al. 1993), we tried numerous combinations of these variables (see Materials and methods) as well as incubating post-bombarded kernels in liquid culture medium to lessen post-bombardment stress. In no case, however, was there any increase in GUS staining due to incubation at higher temperature. Cornejo et al. (1993) have suggested that the lack of heat-shock response in transient assay systems may be due in some cases to the plant tissues having not sufficiently recovered from the stress of tissue preparation and/or DNA delivery prior to the application of heat-shock treatment. Certainly, this possibility cannot be ruled out in the present case. On the other hand, transient expression of Ubi-1/GUS was significantly increased when kernels were transferred at 7 DAP to media containing either 1 mg/l IAA or 2 mg/l kinetin for 72 h prior to transformation (Table 1). Histochemical staining (Fig. 4) revealed the tissue-specific nature of the hormone-induced increases in GUS activity staining, as most of the increased activity was associated with the sub-aleurone portions of the endosperm where a significant portion of cereal seed storage protein synthesis takes place. It should be noted that the diminished GUS staining in the control kernels for the hormone treatments (Fig. 4A) when compared to that found for kernels of comparable age grown on standard agar-based media (Fig. 3B) reflects the more advanced physiological age of liquid medium-incubated kernels, which develop at an accelerated rate (data not presented).

Ubiquitins have been implicated in many varied functions such as selective protein degradation, chromatin
structure, heat-stress response, and ribosome biogenesis (Monia et al. 1990). Ubiquitin is a highly conserved protein encoded either as tandem repeats (polyubiquitins) or as monomeric extension fusion proteins. Generally, the monomeric ubiquitin genes are strongly expressed in tissues undergoing rapid growth (Monia et al. 1990; Gausing and Barkardottir 1986; Callis et al. 1990; Garbarino et al. 1992), while the polyubiquitins are often either constitutively expressed (Christensen et al. 1992) or induced by heat shock (Monia et al. 1990), wounding (Didierjean et al. 1996; Garbarino et al. 1992), ethylene exposure (Garbarino et al. 1992), or pathogen invasion (Basso et al. 1996). The ubiquitin promoter used in the present study was derived from a maize polyubiquitin gene (Ubi-1) that is constitutively expressed in maize seedlings, but whose expression is increased by growth of seedlings at elevated temperatures (Christensen et al. 1992). The Ubi-1 promoter has been shown to drive transient or stable gene expression in a number of monocot species (Christensen et al. 1992; Hanch et al. 1995; Cornejo et al. 1993; Taylor et al. 1993). However, despite its polyubiquitin gene origin, the Ubi-1 promoter-driven GUS expression appeared confined to meristematic cells when transiently expressed in developing maize kernels (Figs. 1 and 2) and was unaffected by wounding, heat shock, or fungal infection. These results are more similar to those expected for a monomeric ubiquitin gene than a polyubiquitin gene (Monia et al. 1990); however, more recent evidence suggests that the stress response of a specific ubiquitin gene is both tissue- and developmentally dependent (Bouchard et al. 1993; Garbarino et al. 1995; Liu et al. 1995, 1996). Therefore, while it is possible that we have not yet found the particular combi-
Table 1  Effects of IAA and kinetin treatment on Ubi-l-driven GUS activity of maize kernels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nmol product h⁻¹ kernel⁻¹</th>
<th>t-test</th>
<th>probability b</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>3.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mg/l IAA</td>
<td>4.41</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>1 mg/l IAA</td>
<td>5.26</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>0.25 mg/l kinetin</td>
<td>3.27</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>2 mg/l kinetin</td>
<td>7.68</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

a Kernels were placed in control medium at 5 DAP, then moved at 7 DAP to liquid medium containing indicated amounts of hormone for 72 h before particle bombardment. Kernel halves were incubated in appropriate hormone-containing agar-based media overnight before extraction and spectrophotometric assay of GUS activity. Each assay result is from three separate extractions of triple kernels samples.
b Probability of a larger deviation by chance between control and treatment means.

tially useful promoters. While protoplasts have proven to be a relatively facile system for transient gene expression, there is always a concern that the results do not necessarily reflect those that would be obtained with the corresponding tissues and organs. In the case of maize endosperm, transient gene expression experiments can be readily carried out using endosperm-derived tissue culture cells (Ueda et al. 1992; Unger et al. 1993). However, biochemical studies reveal that, depending on the particular metabolic pathway or process of interest, the results are not necessarily the same as those obtained from intact tissues (Muhitch and Felker 1994). For maize though, the intact seed is not readily amenable to the kinds of experimental manipulations that may be necessary for testing transiently induced gene expression. Maize kernel culture appears to be a suitable method for overcoming this obstacle. Given that the transient expression pattern of the Ubi-1/GUS gene in a developmental series of maize kernels grown in culture was the same as that for kernels grown in planta and the ease with which kernels grown in vitro can be subjected to experimental manipulation (i.e., heat shock, hormone treatment, etc.), this approach should provide a system for the relatively facile and rapid evaluation of the tissue-specificity and environmental response of novel gene constructs in developing maize seeds.

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