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TECHNICAL NOTE

# Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants

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A set of plasmids has been constructed utilizing the promoter, 5′ untranslated exon, and first intron of the maize ubiquitin (*Ubi-1*) gene to drive expression of protein coding sequences of choice. Plasmids containing chimaeric genes for ubiquitin-luciferase (*Ubi-Luc*), ubiquitin-β-glucuronidase (*Ubi-GUS*), and ubiquitin-phosphinothricin acetyl transferase (*Ubi-bar*) have been generated, as well as a construct containing chimaeric genes for both *Ubi-GUS* and *Ubi-bar* in a single plasmid. Another construct was generated to allow cloning of protein coding sequences of choice on *Bam* HI and *Bam* HI–compatible restriction fragments downstream of the *Ubi-1* gene fragment. Because the *Ubi-1* promotor has been shown to be highly active in monocots, these constructs may be useful for generating high-level gene expression of selectable markers to facilitate efficient transformation of monocots, to drive expression of reference reporter genes in studies of gene expression, and to provide expression of biotechnologically important protein products in transgenic plants.

*Keywords*: gene expression; transgenic monocots; ubiquitin

## Introduction

The general availability of strong promoters active in all or most cell types of monocotyledonous plants would be useful in a variety of applications in gene transfer studies with this plant group (McElroy and Brettell, 1994). Although the widely-used cauliflower mosaic virus (CaMV) 35S promoter is active in monocot cells, its relative strength is substantially less than in dicot cells, and it is inactive in some cell types, e.g. pollen (Bruce *et al.*, 1989; Christensen *et al.*, 1992; McElroy and Brettel, 1994). The maize *Adh1* promoter has also been used in monocot transformation studies (Fromm *et al.*, 1990), but its activity appears to be restricted to root and shoot meristems, endosperm, and pollen (Kyozuka *et al.*, 1991). Because of their expected involvement in fundamental processes in all cell types, the genes for rice actin (*Act-1*) (McElroy *et al.*, 1990) and maize ubiquitin (*Ubi-1*) (Christensen *et al.*, 1992) have been investigated as potentially useful alternatives to the CaMV 35S and *Adh1* sequences. Both of these monocot promoters have been shown to be significantly more active than the CaMV 35S promoter in monocot cells (Bruce *et al.*, 1989; McElroy *et al.*, 1990; Christensen *et al.*, 1992; Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; McElroy and Brettell, 1994) with the *Ubi-1* promoter being somewhat stronger than the *Act-1* promoter where compared directly (Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; Schledzewski and Mendel, 1994; Wilmink *et al.*, 1995).

Since our initial reports on the use of maize *Ubi-1* promoter constructs in transient (Christensen *et al.*, 1992) and stable (Toki *et al.*, 1992; Uchimiya *et al.*, 1993) cereal transformation studies, we have distributed to a large number of researchers a variety of constructs with the *Ubi-1* promoter fused to a spectrum of selectable and scorable markers. Certain of these constructs or their derivatives have been used successfully in transforming a number of different monocot species (Wilmink *et al.*, 1995), including several cereals (McElroy and Brettell, 1994) and *Lemna* (Rolfe and Tobin, 1991), with reports of transgenic plants having been generated for rice (Cornejo *et al.*, 1993), wheat (Weeks *et al.*, 1993), and

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barley (Wan and Lemaux, 1994). This report presents the structural details of the complete set of these constructs.

## Materials and methods

The cloning and sequencing of the maize ubiquitin gene and its promoter have been reported previously (Christensen *et al.*, 1992). All DNA cloning and manipulations reported here were performed according to standard protocols (Ausubel *et al.*, 1989). Restriction endonuclease digestions were carried out according to manufacturers' recommendations. GeneClean (BIO 101, La Jolla, CA, USA) was used to isolate specific restriction fragments from agarose gels. Recovery of DNA fragments was quantified by comparison of ethidium bromide fluorescence of an aliquot of the fragment with known masses of DNA on agarose gels. The *Hin*dIII linker (5′ CAAGCTTG 3′) used in the construction of pAHC27 was obtained from New England Biolabs. DNA ligations and subsequent transformations into competent *Escherichia coli* strain XL1-Blue or HB101 cells and plasmid DNA preparations were carried out using standard protocols (Ausubel *et al.*, 1989). Analysis of DNA sequences was performed using the UWGCG package of programs (Fromm *et al.*, 1990) and DNA Inspector II (Textco, W. Lebanon, NH, USA).

## Results

All of the constructs described here were generated by fusing the same 1992 bp *Pst*I fragment from the maize *Ubi-1* gene upstream of the relevant polylinker or marker sequence (Fig. 1). This *Ubi-1 Pst*I fragment contains 899 bp of promoter sequence, 83 bp of 5′ untranslated exon, and 1010 bp of first intron sequence, terminating through reconstitution of the *Pst*I site precisely at the G in the AG dinucleotide of the 3′ splice junction of the intron (Christensen *et al.*, 1992). The nucleotide sequences at the fusion junctions at the 3′ end of the *Ubi-1* DNA are shown for each construct in Fig. 2.

### *pAHC17*

This plasmid is a *Ubi-1* promoter expression vector for *Bam*HI (or *Bam*HI-compatible) cloning of protein coding regions. It contains the *Ubi-1* promoter, 5′ untranslated region and intron upstream of an unique *Bam*HI site (Fig. 1). About 250 bp of nopaline synthase (NOS) 3′ untranslated sequence and polyadenylation signals are located downstream of the *Bam*HI site. The 1992 bp *Pst*I fragment of the *Ubi-1* gene had been previously cloned into the *Pst*I site of M13mp19 for sequencing (Christensen *et al.*, 1992). A *Hin*dIII-*Bam*HI fragment from the replicative form of that clone was isolated and ligated to a 3175 bp *Hin* dIII–*Bam*HI fragment of pMF6 (Goff *et al.*, 1991) containing pUC8 sequence and 250 bp of NOS 3′ polyadenylation sequence adjacent to the *Eco*RI site.

The polylinker sequence is located between the end of the *Ubi-1* intron and the *Bam*HI cloning site and between the *Bam*HI site and the NOS sequence (Fig. 2). Thus, a reporter gene cloned into the *Bam*HI site is flanked by polylinker sequence on both the 5′ and 3′ sides. *Sal*I and *Xba*I sites from the M13mp19 polylinker are upstream of the *Bam*HI site and a *Sal*I and a *Pst*I site from the pUC8 polylinker are on the 3′ side.

### *pAHC15 and pAHC27 (pUbi-GUS)*

These plasmids contain the maize *Ubi-1* promoter, 5′ untranslated region and first intron fused to the coding region of the *E. coli uid*A gene (GUS) (Fig. 1). To produce pAHC15, *Hin*dIII–*Eco*RI fragment of pBI101.2 (Jefferson *et al.*, 1987) containing the *Hin*dIII to *Sma*I region of the pUC19 polylinker, the GUS coding sequence, and 260 bp of the nopaline synthase gene polyadenylation signal was cloned into the *Hin*dIII and *Eco*RI sites of pUC19 (pUC19-GUS-NOS). The 1992 bp *Pst*I fragment of the maize *Ubi-1* gene (Christensen *et al.*, 1992) was cloned into the *Pst*I site of the polylinker sequence upstream of the GUS coding sequence in pUC19-GUS-NOS. The construct contains the *Ubi-1* sequence in an orientation such that transcription will proceed through the ubiquitin 5′ exon, intron and the GUS coding sequence, terminating in the NOS 3′ sequence.

pAHC27 contains the same Ubi-GUS-NOS construct as pACH15 but as a *Hin*dIII fragment cloned into the *Hin*dIII site of pUC19 (Fig. 1). This construct was generated to facilitate the production of pAHC25 (see below). The *Eco*RI site at the 3′ end of the chimeric gene in pAHC15 is not unique as there is an additional *Eco*RI site in the *Ubi-1* intron. However, the *Hin*dIII site at the 5′ end of the chimaeric gene is unique. To allow the entire construct to be removed as one fragment for further subcloning, a *Hin*dIII site was introduced at the 3′ end of the chimaeric gene. This was achieved by partially digesting pAHC15 with *Eco*RI, optimizing the digestion for linear fragments. The *Eco*RI sites were filled in with dNTPs and Klenow fragment of DNA Polymerase and a *Hin*dIII linker (5′ CAAGCTTG 3′; New England Biolabs) was added. Addition of the linker also restored the *Eco*RI site. The DNA was digested with *Hin*dIII to remove excess linker and to cut at the 5′ end of the chimaeric gene. The 4.15 kb *Hin*dIII fragment containing the *Ubi-1 gus* chimaeric gene was gel-purified and subcloned into *Hin*dIII–digested pUC19. The chimaeric gene in the resultant pAHC27 is oriented such that the entire pUC19 polylinker is upstream of the *Ubi-1* promoter (Fig. 1).

### *pAHC18 – pUbi-LUC*

This plasmid contains the *Ubi-1* promoter-5′ exon-first

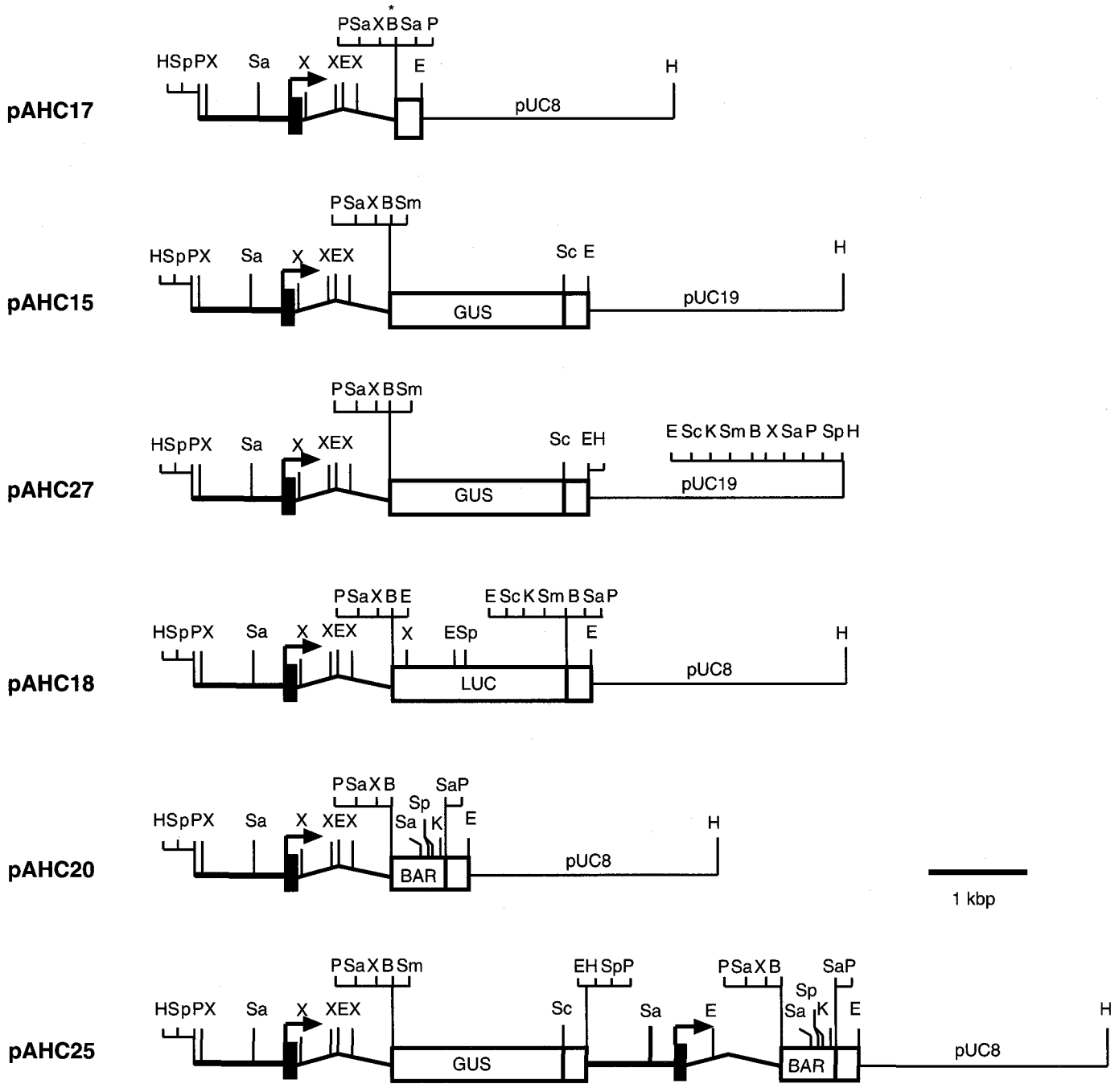


**Fig. 1.** Schematic diagrams of expression vectors based on maize *Ubi-1* sequences. The relative sizes of the various segments of the linearized plasmids are drawn to scale. Bold straight line, *Ubi-1* promoter sequences; filled box, *Ubi-1* exon; angled line, *Ubi-1* intron; labelled open boxes, reporter gene sequences; blank open box, nopaline synthases 3′ untranslated sequence; thin straight line, pUC8 (pAHC17, 18, 20, and 25) or pUC19 (pAHC15 and 27) sequence. Arrow at the Ubi-1 exon signifies transcription start site and direction. GUS, β-glucuronidase (Jefferson *et al.*, 1987); LUC, firefly luciferase (Ow *et al.*, 1986); BAR, phosphinothricin acetyltransferase (De Block *et al.*, 1987). Restriction sites used in construction of the chimaeric genes and in adjacent polylinker sequences are shown. The *Bam* HI site marked with an asterisk in pAHC17 is an unique site for cloning *Bam* HI or *Bam* HI-compatible fragments. (Note: The *Xba* I sites located in the Ubi-1 intron are subject to methylation interference in *dam* + *E. coli* strains. Also, although the *Eco* RI site in the *Ubi-1* intron upstream of the *gus* sequence in pAHC15 is cleaved efficiently, in both pAHC27 and pAHC25 the corresponding *Eco* RI site is cut very inefficiently). B, *Bam* HI; E, *Eco* RI; H, *Hin* dIII; K, *Kpn* I; P, *Pst* I; Sa, *Sal* I; Sc, *Sac* I; Sm, *Sma* I; Sp, *Sph* I; X, *Xba* I.

**pAHC17**

**UBI1 Intron** **NOS 3'**

**Pst I Sal I Xba I Bam HI Sal I Pst I**

.....ctgcagGTCGACTCTAGAGGATCCGTCGACCTGCAG.....
.....gacgtcCAGCTGAGATCTCCTAGGCAGCTGGACGTC.....

**pAHC15 / 25 / 27**

**UBI1 Intron** **GUS (from pBI101.2)**

**Pst I Sal I Xba I Bam HI Sma I**

.....ctgcagGTCGACTCTAGAGGATCCCCGGGTAGTCAGTCCCTTATG.....
.....gacgtcCAGCTGAGATCTCCTAGGGGCCCATCAGTCAGGGAATAC.....

**pAHC18**

**UBI1 Intron** **LUC (from pDO432)**

**Pst I Sal I Xba I Bam HI**

.....ctgcagGTCGACTCTAGAGGATCCGAGCTTGGAATTCCTTTGTGTTACATTCTTGAATGTCGCTCGCAGTGACATTAGCATTCCGGTACTGTTGGTAAAATG.....
.....gacgtcCAGCTGAGATCTCCTAGGCTCGAACCTTAAGGAAACACAATGTAAGAACTTACAGCGAGCGTCACTGTAATCGTAAGGCCATGACAACCATTTTAC.....

**pAHC20 / 25**

**UBI1 Intron** **BAR (from pUC/BASTA)**

**Pst I Sal I Xba I Bam HI**

.....ctgcagGTCGACTCTAGAGGATCCATCGATTAGGAAGTAACCATG.....
.....gacgtcCAGCTGAGATCTCCTAGGTAGCTAATCCTTCATTGGTAC.....

**Fig. 2.** Nucleotide sequence of the polylinker region comprising the junction between the *Ubi-1* intron and the reporter gene or NOS 3′ sequence. The *Ubi-1* intron sequence (lower case) ends with a *Pst*I site containing the 3′ splice junction. The reporter gene sequences shown downstream of the polylinker are those upstream of the respective coding sequence and end with the ATG translation start codon (italicized) shown for each.

intron fused to a luciferase (LUC) reporter coding sequence (Fig. 1). An 1892 bp *Bam*HI fragment of pDO432 (Ow *et al.*, 1986) containing 80 nucleotides of 5′ untranslated sequence, the luciferase coding region (1649 nucleotides) and 163 bp of 3′ untranslated sequence was cloned into the unique *Bam*HI site of pAHC17. This construct contains the luciferase coding sequence in the same orientation as the ubiquitin promoter.

*pAHC20 – pUbi-BAR*

The Ubi-BAR chimaeric gene in this plasmid provides selection of transformants resistant to Basta™ herbicide (phosphinothricin) (De Block *et al.*, 1987). The Ubi-BAR construct was formed by ligating a 570 bp *Bam*HI–*Bcl*I fragment containing the *bar* gene into the *Bam*HI site of pAHC17. The *bar* gene fragment was excised from a plasmid (pUC8/BASTA) obtained from Dr M. Fromm (Fromm *et al.*, 1990). The resultant pAHC20 plasmid has *bar* in the same orientation as the maize Ubi-1 promoter (Fig. 1). The construct contains 18 bp of sequence between the *Bam*HI site and the translation start codon of the *bar* gene (Fig. 2). The *Bcl*I site is 11 bp downstream of the TGA stop codon.

The unique *Hin*dIII site at the 5′ end of the Ubi-1 sequence makes this plasmid very adaptable. This restriction site is suitable for insertion of a second chimaeric gene, such as a scorable marker also driven by a second *Ubi-1* promoter, as detailed below for pAHC25, or for any other desired promoter–gene combination.

*pAHC25 – pUbi-GUS/Ubi-BAR*

pAHC25 contains both a selectable marker (*bar*) and a scorable marker (GUS), each under the transcriptional control of a separate *Ubi-1* promoter (Fig. 1). The two chimaeric genes were first assembled separately in pAHC20 and pAHC27 and then the double construct was formed. This was achieved by excising the Ubi-GUS-NOS-containing *Hin*dIII fragment from pAHC27 and subcloning it into *Hin*dIII-digested pAHC20. The resultant pAHC25 plasmid has both Ubi-BAR and Ubi-GUS chimaeric genes in the same orientation.

## Discussion

The high activity of the maize *Ubi-1* promoter has now been documented in transient and/or stable transformation configurations in a number of monocot systems including rice (Bruce *et al.*, 1989; Toki *et al.*, 1992; Cornejo *et al.*, 1993; Uchimiya *et al.*, 1993; Takimoto *et al.*, 1994), wheat (Taylor *et al.*, 1993; Weeks *et al.*, 1993), barley (Wan and Lemaux, 1994), sugarcane (Gallo-Meagher *et al.*, 1993; Taylor *et al.*, 1993), maize (Christensen *et al.*, 1992; Gallo-Meagher *et al.*, 1993) *Pennisetum* (Taylor *et al.*, 1993), *Panicum* (Taylor *et al.*, 1993) and *Lemna* (Rolfe and Tobin., 1991). Whether or not the high level of expression of selectable marker genes fused to *Ubi-1* actually increases the efficiency of recovery of fertile transgenic plants relative to less active promoters like that from the CaMV 35S gene is yet to be rigorously examined (see Wan and Lemaux, 1994). However, the high level of GUS expression provided by the Ubi-GUS constructs has proven valuable in enabling rapid histochemical screening of transformants for transgene activity (Cornejo *et al.*, 1993).

The original intron present in the 5′-untranslated region of the Ubi-1 gene (Christensen *et al.*, 1992) was retained in all the constructs here because of numerous previous studies showing that introns frequently strongly enhance transgene expression in cereals (Callis *et al.*, 1988; Bruce and Quail, 1990; McElroy *et al.*, 1990; Vasil *et al.*, 1993). The influence of the *Ubi-1* intron has not been tested directly, but there is evidence that this maize sequence is spliced correctly in transgenic rice cells (Toki *et al.*, 1992).

Detailed examination of the spatial and temporal expression patterns of the *Ubi-1* promoter in transgenic plants is yet to be reported. However, initial data with a *Ubi-gus* construct indicate expression in all organs of transgenic rice consistent with a potential for targeting a wide spectrum of cells (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994).

An additional potentially usefull feature of the *Ubi-1* promoter is that it is stress-inducible. Both thermal and mechanical stress have been shown to cause a strong enhancement of the *Ubi-gus* transgene activity in transformed rice (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994). It is possible that this fact may result in stronger expression of selectable marker fusion genes during the early stages of transformation, where recipient cells are exposed to a variety of stresses such as high osmotic pressures, particle bombardment and growth on toxic compounds. A subsequent decrease in expression level is expected upon removal of the selective conditions so that regenerated transgenic plants would presumably not continue to express the marker at high levels when it is no longer needed. The stress-inducibility of the *Ubi-1* promoter might also be useful for driving conditional expression of genes that confer tolerance or resistance to various biotic and abiotic stresses such as pathogen attack, heat and water deficit (Takimoto *et al.*, 1994).

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