Plant Cell Reports (1998) 17: 650–655 © Springer-Verlag 1998

S. Liu · A. Kriz · D. Duncan · J. Widholm

Abscisic acid-regulated *Glb1* transient expression in cultured maize P3377 cells

Received: 9 May 1997 / Revision received: 9 November 1997 / Accepted: 8 December 1997

Abstract In a study of the 5′-flanking sequence of the *Zea mays* L. (maize) *Glb1* gene in vitro, serial promoter deletions were generated and linked with the β-glucuronidase (GUS) reporter gene. The promoter deletion-GUS fusions were introduced into the maize P3377 cell line by particle bombardment. GUS assays indicated that treatment of the maize cultured cells with abscisic acid (ABA) was required for *Glb1*-driven GUS transient expression, and that the –272-bp sequence of the *Glb1* promoter was sufficient for ABA-regulated expression of GUS. The longest undeleted sequence used, –1391 GUS, showed relatively low expression which could be indicative of an upstream silencer element in the *Glb1* promoter between –1391 and –805. Further studies show that the *Glb1*-driven GUS activity of bombarded maize P3377 cells increases with increasing ABA concentration (up to 100–300 μM). Site-directed mutagenesis of a putative ABA response element, Em1a, abolished GUS expression in P3377 cells. This observation indicated that the Em1a sequence in the *Glb1* 5′ regulatory region is responsible for the positive ABA regulation of gene expression.

Key words Particle bombardment · Maize P3377 cells · *Glb1* gene · Abscisic acid regulation

Communicated by J. J. Finer

J. Widholm (✉)
Department of Crop Sciences,, University of Illinois,
Urbana, IL 61801, USA

S. Liu
Department of Molecular and Integrative Physiology,
University of Illinois,
Urbana, IL 61801, USA

A. Kriz
DEKALB Plant Genetics,
62 Maritime Drive, Mystic, CT 06355-1958, USA

D. Duncan
Monsanto Agricultural Co.,
700 Chesterfield Village,
Parkway N, St. Louis, MO 63198, USA

Abbreviations *ABA* Abscisic acid · *ABRE* ABA-responsive element · *BMS* Black Mexican Sweet · *GUS* β-Glucuronidase

Introduction

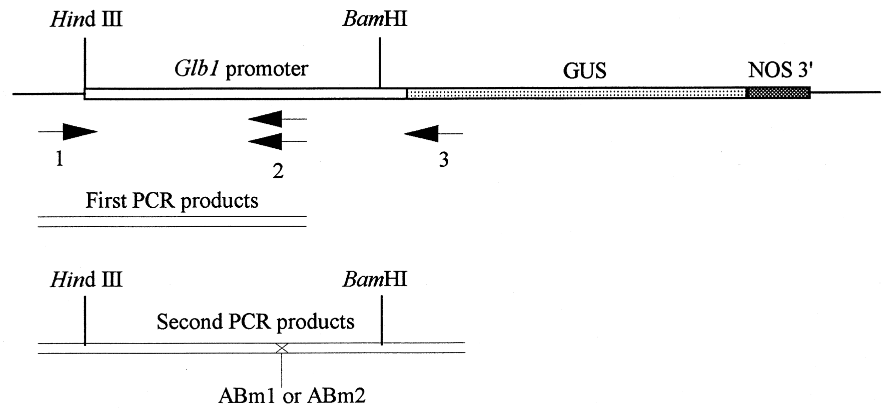
The molecular mechanisms of plant responses to plant growth regulators have been extensively studied for many years. The molecular action of abscisic acid (ABA) has been investigated in different tissues of a wide variety of plants (Hetherington and Quatrano 1991). The gene encoding maize seed storage protein GLB1 has been shown to be positively regulated by ABA (Kriz et al. 1990; Paiva and Kriz 1994). Nucleotide sequence analysis of the 5′-flanking sequence of the *Glb1* gene indicated that ABA-responsive elements (ABREs) similar to Em1a, Em1b, and Em2, which were first described in the wheat *Em* gene (Marcotte et al. 1989), are also present in the 5′ region of the *Glb1* gene at positions –118, –76, and –161 bp, respectively (Belanger and Kriz 1991).

In this study, a series of *Glb1* promoter deletions, as well as mutations of the Em1a element, were generated and linked with a β-glucouronidase (GUS) reporter gene to identify ABREs in the *Glb1* promoter region which are responsible for ABA regulation of gene expression. These constructs were introduced into cultured maize P3377 cells by particle bombardment, and transient assays were performed to determine *Glb1* promoter activity. We provide evidence that ABREs are responsible for the positive regulation of *Glb1* gene expression by ABA, and that an upstream silencer between –805 and –1391 bp may negatively regulate *Glb1* gene expression.

Materials and methods

Maize cultured cells

Maize P3377 suspension cultures were developed from pooled immature embryos of the self-pollinated hybrid Pioneer 3377 (Duncan



Em1a of *Em* gene:	ACGTGGCGC	Marcotte et al., 1989
Em1a of *Glb1* gene:	ACGTGGCGA	Belanger and Kriz, 1991
ABm1 of *Glb1* gene:	CCATGGCGA	
ABm2 of *Glb1* gene:	ACGTGGTAC	

Fig. 1 Schematic presentation of the method for site-directed mutagenesis using PCR (Landt et al. 1990). Primer *1* and primer *2* were used for the first PCR; primer *3* and the product of the first PCR were used as primers for the second PCR

and Widholm 1991). Suspension cultures were maintained in AMCF-ARM medium (Duncan et al. 1985) with 7-day subcultures in the dark at 28°C with 115 rpm continuous shaking. For ABA treatment, the cells were collected by suction filtration on 4.25-cm Whatman no. 1 filter paper and precultured on CSM medium [D medium (Duncan et al. 1985) with 531 mM mannitol], plus or minus ABA (100 µM) in the dark for 24 h at 28°C (ABA was filter sterilized and added to the autoclaved medium after partial cooling).

RNA extraction and northern blot analysis

Total cellular RNA was prepared using the method of Shirzadegan et al. (1991), with slight modifications. The P3377 suspension cells were collected and precultured on CSM medium either treated with 100 µM ABA for 24 h or without ABA. About 100 mg of the cells was frozen in liquid nitrogen and homogenized using 400 µl of hot phenol extraction buffer, which is phenol containing 0.1 M LiCl, 0.1 M Tris-HCl (pH 8.0), 0.01 M EDTA, and 1% SDS preheated at 80°C. An equal volume of chloroform/isoamyl alcohol (24:1) extraction followed and 4 M LiCl was used to precipitate the RNA. Ten micrograms of total denatured RNA from both ABA-treated and untreated tissues was separated on a 1.2% agarose gel containing formaldehyde (Maniatis et al. 1982). Northern blot analysis using the fragment of *Glb1* cDNA clone as probe was performed as described earlier (Paiva and Kriz 1994).

Promoter deletions

To generate promoter deletions, the EXO/MUNG sequencing kit from Stratagene (La Jolla, Calif.) was used to produce unidirectional deletions of different sizes. Twenty-five micrograms of the *Glb1* promoter ppGlb1P4 (Liu and Kriz 1996) was digested with 200 units of *Cla*I and then subjected to thioderivative fill-in to produce blunt ends with deoxy-thioderivatives. After fill-in, DNA was cut with 200 units of *Hin*dIII to produce 5′ overhangs. The 5′ overhangs were treated by EXO and a series of deletions were produced in one of the DNA strands. The deletion reactions were followed by mung bean nuclease treatment to produce double-stranded deletions, and the resulting blunt-ended DNA was ligated with T4 DNA ligase. Single colonies from the ligation mixture were screened by *Bam*HI digestion and the desired deletions were selected. Double-stranded DNA sequencing was performed by the dideoxy-mediated chain termination method described by Maniatis et al. (1982) to determine the exact site of deletions. All the selected deletion constructs were digested with *Sal*I/*Bam*HI to release the corresponding fragments and these were cloned into a GUS vector pUC19GUS at *Sal*I/*Bam*HI sites (pUC19GUS is a pUC19 vector containing the GUS reporter region constructed by subcloning the *Xba*I/*Eco*RI fragment of pBI101 from Clonetech Laboratories, Palo Alto, Calif. into pUC19 at the *Xba*I/*Bam*HI sites.).

Site-directed mutagenesis using PCR

To mutate the Em1a-element, two primers were designed as follows: ABm1 (5′ ACGGGTCGCCATGGCCCGCCG 3′); ABm2 (5′ CACACGGGGTACCACGTCCCG 3′). The oligonucleotides were synthesized on an Applied Biosystem Model 380A DNA synthesizer at the Genetic Engineering Facility of the University of Illinois Biotechnology Center. For rapid site-directed mutagenesis using PCR, a method described by Landt et al. (1990) was modified. Mutagenesis was performed in a two-step PCR (Fig. 1). In the first step, a 5′ universal primer (reverse primer from bluescript 5′ AAC AGC TAT GAC CAT G 3′) and the 3′ mutagenic primer (ABm1 and/or ABm2) were used to generate a 1300-bp double-stranded mutated fragment. This intermediate fragment was then purified with a Geneclean II kit (Bio 101, La Jolla, Calif.). Then the mutated fragment was then used as the 5′ mutagenic primer in the second PCR together with a 3′ universal primer (the GUS primer from Clontech, 5′ TCACGGGTT GGGGTTTCTAC 3′). The 1500-bp product of the second PCR fragment was purified as above, and digested with *Bam*HI and *Hin*dIII. The 1400-bp *Bam*HI/*Hin*dIII fragment, which contains the mutated Em1a element, was then ligated into the pUC19GUS vector which was also cut with *Bam*HI and *Hin*dIII. Double-stranded DNA sequencing was performed by the dideoxy-mediated chain termination method described by Maniatis et al. (1982) to confirm the mutations.

Particle bombardment

Particle bombardment was carried out with the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Richmond, Calif.) as recommended by the manufacturer's instructions. Five micrograms of each plasmid DNA was used for particle bombardment. To prepare samples, the suspension cells were collected onto sterile 4.25-cm Whatman no. 1 filter paper disks that were transferred to CSM agar plates with or without ABA for 24 h in the dark

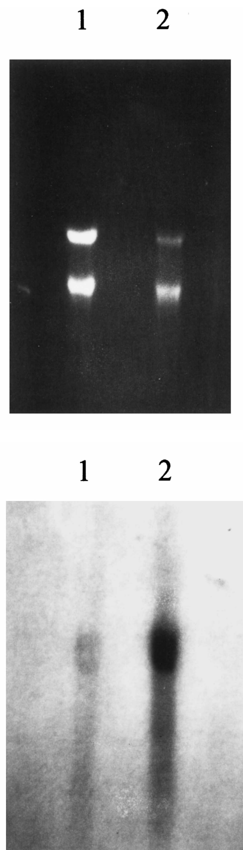


Fig. 2 **Upper panel** Agarose gel analysis of 10 µg of total RNA extracted from P3377 maize suspension cells treated with ABA for 24 h showing relative amounts of rRNA (*lane 1* – ABA; *lane 2* +ABA, 100 µM). **Lower panel** Northern blot of total RNA extracted from maize P3377 cell culture as shown in the upper panel using the *Glb1*-specific cDNA fragment as probe (*lane 1* – ABA; *lane 2* +ABA, 100 µM)

at 28°C. On the day of bombardment, the precultured calli on the disks were transferred onto three pieces of Whatman no. 3 filters presoaked in a sterile Petri dish with 5 ml CSM liquid medium. For one experiment, each construction had two duplicate plates and each plate was bombarded twice. Two experiments were performed separately for every construction. After bombardment, the calli on the filter disks were transferred back to CSM in the dark with or without ABA at 28°C for 24 h and then subjected to GUS assay after 24 h (Jefferson et al. 1987).

Results

Northern analysis

When Northern analysis was carried out with RNA prepared from P3377 cells treated for 24 h with 100 µM ABA, the *Glb1* and *Glb2* transcripts accumulated to high levels, while the mRNAs were present at very low levels in untreated cells (2.4- and 1.7-kb bands, respectively; Belanger and Kriz, 1991; Paiva and Kriz , 1994 (Fig. 2). This indicated that the maize P3377 cells can be used for transient studies of *Glb1* gene expression, since the *Glb1* gene is expressed highly in the cell line upon treatment with exogenous ABA.

Transient GUS assays of deleted Glb1promoter-GUS fusions

A set of nested promoter deletion constructs were generated from ppGlb1P4 (Liu and Kriz 1996) and subcloned into the pUC19GUS vector. Of the seven deletions selected (Fig. 3), –86 was the shortest construct and did not contain the Em2 and Em1a sequences, while –1391 is the longest complete promoter without deletion. The deletion constructs (Fig. 3) and a 35S GUS (pBI221,Clonetech Laboratories) control plasmid were introduced separately into the maize P3377 cells by particle bombardment. Transient GUS assays indicated that the treatment of the cells with ABA is required for *Glb1*-driven GUS expression (Fig. 4) because little or no GUS activity was observed in the absence of ABA (Fig. 5). Sequences between –86 and –385 are necessary for *Glb1* expression since the highest GUS activity in ABA-treated callus was observed with the –295 GUS, and no expression was detectable with –86 GUS even in the ABA-pretreated cells. It is interesting to note that the intact 1391-bp promoter construct, ppGlb1GUS, has lower expression compared to pp–295GUS.

The effects of ABA levels on *Glb1* gene expression

To determine the optimum ABA concentration for *Glb1* gene regulation, the maize P3377 suspension cells were precultured on CSM medium supplemented with ABA lev-

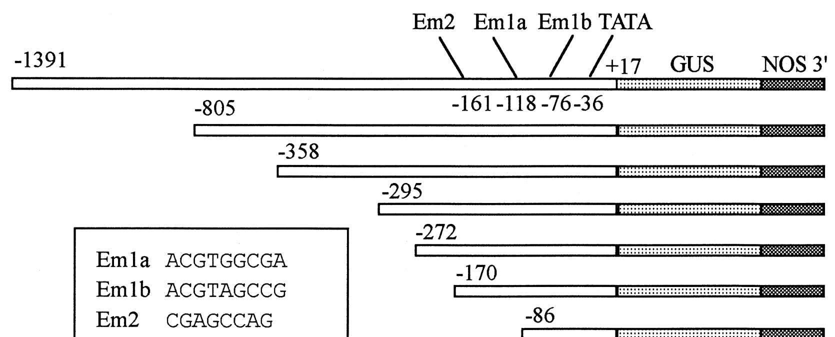


Fig. 3 Deletions of the *Glb1* gene 5′-flanking sequence were generated by ExoIII/mung bean nuclease treatment and cloned into a plasmid containing the GUS cDNA sequence and the 3′ nopaline synthase (*NOS*) terminator region. The *Em*-labelled sequences correspond to those described by Marcotte et al. (1989) as being ABREs in the wheat *Em* gene. The *Em2*, *Em1a*, *Em1b* and *TATA* sequences are at –161, –118, –76, and –36, respectively. The lengths are not drawn to scale

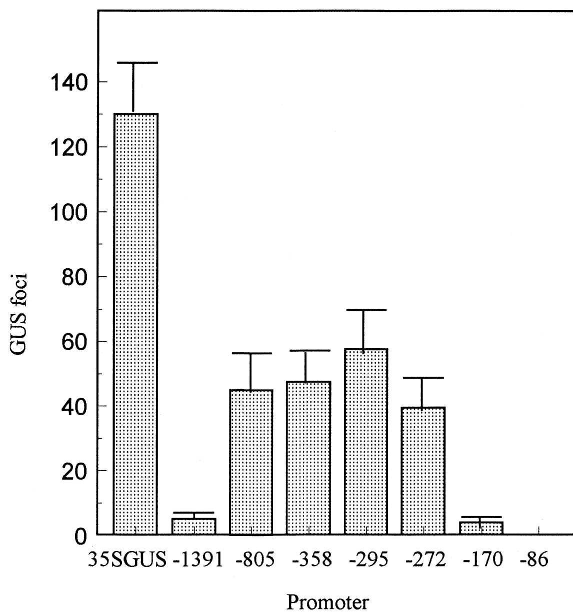


Fig. 4 The effects of promoter deletions on the GUS activity of maize P3377 suspension cells pretreated for 24 h with 100 μM ABA. GUS activity was expressed as numbers of blue spots per Petri dish after each bombardment. The *bars* represent results from two individual experiments with standard derivation

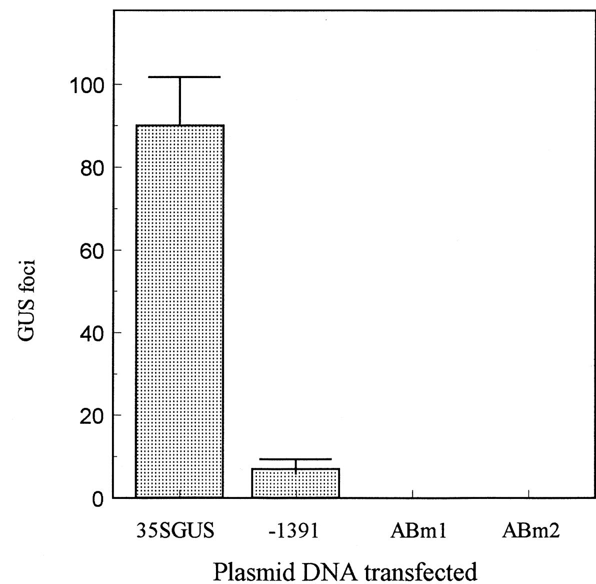


Fig. 6 The effects of specific mutations of the Em1a element as shown in Fig. 1 on the GUS activity of maize P3377 cells after bombardment with Abm1 and Abm2 as measured by the number of blue spots per plate. The *bars* represent results from two separate experiments with standard derivation

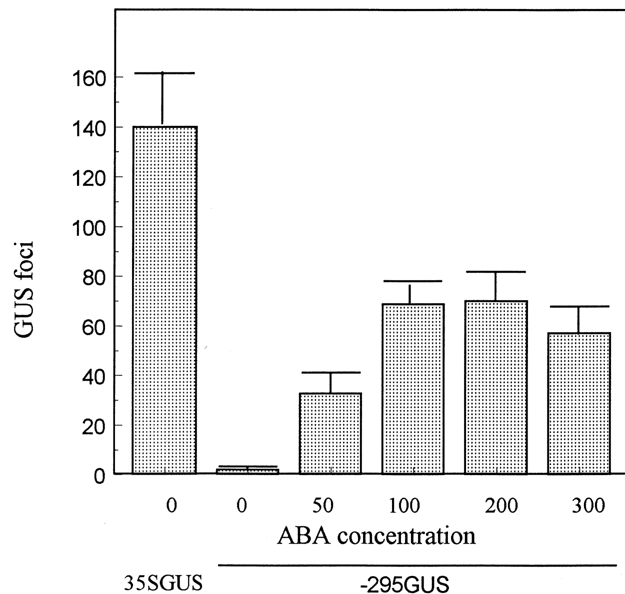


Fig. 5 The effects of pretreatment for 24 h with various ABA concentrations on GUS activity of the maize P3377 suspension cells after bombardment with –295 GUS. GUS activity was expressed as numbers of blue spots per Petri dish after each bombardment. The *bar*s represent results from two separate experiments with standard derivation

els of 0, 50, 100, 200, and 300 μM for 24 h, and the calli were then bombarded with the –295 plasmid which had the highest *Glb1*-driven GUS activity with the 100 μM ABA treatment (Fig. 4). GUS assays performed 24 h after bombardment (Fig. 5) showed that the *Glb1*-driven GUS expression in maize P3377 cells is stimulated by ABA concentrations up to about 100 μM and the activity remains high at 200 and 300 μM.

The effects of mutations of the Em1a sequence on *Glb1* expression

The ABRE element consensus sequence at –118 was mutagenized as shown in Fig. 1. The base changes in the mutated element denoted Abm1 were in the core ACGT sequence that was changed to CCAT, while the changes in Abm2 were in the 3′ end changing CGA to TAC. The mutated plasmids and 35S GUS and –1391 GUS were bombarded into maize P3377 calli which had been precultured with 100 μM ABA for 24 h. Transient GUS assays showed that both mutations of the Em1a element abolished the responsiveness of the *Glb1* promoter to exogenous ABA because no GUS activity was detectable after bombardment with either mutated Em1a element (Fig. 6).

Discussion

Previous studies have shown that the results of expression analyses of 5′ regulatory sequences in transient assays are usually similar to those found in stably transformed tissues (Walker et al. 1987; Ellis et al. 1987). Thus, many studies of gene regulation have been conducted using transient rather than stable transformation assays due to speed and ease. The maize P3377 suspension cell cultures were derived from P3377 immature embryos, and could be similar to the embryo tissue where

the *Glb1* gene is normally expressed. Northern analyses (Fig. 2) and GUS assays (Fig. 5) showed that, in the presence of exogenous ABA, high levels of *Glb1* transcripts are detected and that the expression is ABA dependent. This indicates that the *Glb1* gene is expressed in this cell line upon exogenous ABA treatment as in developing seeds. GUS expression was not seen when this fusion construct was bombarded into the more friable, fine-textured Black Mexican Sweet (BMS) suspension cells (data not shown). This may indicate that the trans-acting factors which are responsible for embryo-specific and/or ABA-responsive expression are present in the P3377 cell line upon ABA treatment but not in the BMS cell line. Thus the P3377 system is ideally suited for the study of transient *Glb1* gene expression. No GUS expression was found with the shortest deletion construct, –86GUS, which lacks the Em1a and Em2 elements, even in the presence of ABA. The –272 bp sequence of the *Glb1* promoter, that does contain the Em1a and Em2 elements, is sufficient for ABA-regulated expression of GUS in our transient assay. These results indicate that the putative ABREs are required for the positive ABA regulation of *Glb1* gene expression. The –1391 GUS construct shows relatively low expression compared to most of the other constructs including –805, which indicates that there may be an upstream silencer in the *Glb1* promoter region between –805 and –1391. A similar result was reported for the wheat *Em* gene when deletion of the *Em* promoter to –650 bp showed an increase in GUS activity over the –1800-bp whole promoter in a rice protoplast culture system (Marcotte et al. 1988). They suggested that a silencer element (Marcotte et al. 1988) is present in the *Em* promoter between –1800 and –650 bp.

When the –1391 *Glb1* promoter was used to drive GUS expression in stably transformed tobacco, the expression was also found to be embryospecific and to be inducible in mature seed (Liu and Kriz 1996). Since none of the deletion constructs were used in the tobacco transformation experiments, we do not know if the –295 GUS construct would be more active in tobacco than the whole promoter as found in transient assays here with the P3377 cells. It appears, however, that the *Glb1* promoter does work properly in the dicot tobacco, although we found the whole promoter to have lower expression in the P3377 cells. Further experiments are needed to more fully understand the regulation of *Glb1* expression, especially the low expression noted with the –1391 promoter fragment.

We have found a clear and saturable effect of ABA on *Glb1*-driven GUS activity of bombarded maize P3377 cells. Similar results were reported by Bostock and Quatrano (1992) who used ABA and NaCl as inducers in their studies of regulation of *Em* expression in rice suspension culture cells. When NaCl was added in combination with ABA, it was determined that salt interacts with ABA synergistically in the regulation of *Em* gene expression by increasing the sensitivity of rice cells to ABA. The P3377-*Glb1* transient expression system could also be useful for studying the regulation of the *Glb1* gene by salt, ABA, and other stresses.

Additional evidence to suggest that the Em1a sequence in the *Glb1* 5′ regulatory region is responsible for the positive ABA regulation of gene expression was obtained following site-directed mutagenesis of the Em1a of *Glb1* to make the Abm1 and Abm2 constructs (Fig. 1). When these mutated sequences were used in transient expression assays, GUS activity was undetectable (Fig. 6). Since the Em1a element contains the highly conserved ACGTGGCGC motif sequence, any change in the motif sequence may lead to changes in the specific binding activity of the trans-acting factors and therefore prevent expression of the *Glb1* gene.

Guiltinan et al. (1990) identified a protein (EmBP-1) that interacts specifically with the ABRE in wheat embryo nuclear extracts and found that a 2-bp mutation from ACGTGGCGC to CCGGGGCGC within the Em1a element prevents the in vitro binding. We can anticipate a similar sequence-specific binding of this type of protein to the Em1a element in the *Glb1* promoter region.

The cultured P3377 cells have been used for stress-induced gene expression studies (Paul and Ferl 1991; Subbaiah et al. 1994) and we found that this cell line is ideal for ABA-induced gene regulation studies. Since the cultured P3377 cells have the advantages of rapid growth, high yield, easy collection, and good responses to external stimuli, this system provides a rapid, sensitive means for analyzing *Glb1*-GUS fusions in cells receiving different treatments. This system could also be used for other studies involving ABA-regulated gene expression.

References

Belanger FC, Kriz AL (1991) Molecular basis for allelic polymorphism of the maize *Globulin-1*gene. Genetics 129:863–872

Bostock RM, Quatrano RS (1992) Regulation of *Em* gene expression in rice. Plant Physiol 98:1356–1363

Duncan DR, Widholm JM (1991) A note of clarification concerning P3377 suspension culture. Maize Genet Coop Newslett 65:114

Duncan DR, Williams ME, Zehr BE, Widholm JM (1985) The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. Planta 165:322–332

Ellis JG, Llewellyn DJ, Dennis ES, and Peacock WJ (1987) Maize *Adh-1* promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco. EMBO J 6:11–16

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250:267–270

Hetherington AM, Quatrano RS (1991) Mechanisms of action of abscisic acid at the cellular level. New Phytol 119:9–32

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kriz AL, Wallace M S, Paiva R (1990) Globulin gene expression in embryo of maize viviparous mutants: evidence for regulation of the *Glb1* gene by ABA. Plant Physiol 92:538–542

Landt O, Grunert H-P, Hahn U (1990) A general method for rapid site-directed mutagenesis using the polymerase chain reaction. Gene 96:125–128

Liu S, Kriz AL (1996) Tissue-specific and ABA-regulated maize *Glb1* gene expression in transgenic tobacco. Plant Cell Rep 16:158–162

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Marcotte WR, Bayley CE, Quatrano RS (1988) Regulation of a wheat promoter by abscisic acid in rice protoplasts. Nature 335:454–457

Marcotte WR, Russell SH, Quatrano RS (1989) Abscisic acid-responsive sequences from the *Em*-gene of wheat. Plant Cell 1:969–979

Paiva R, Kriz AL (1994) Effect of abscisic acid on embryo-specific gene expression during normal and precocious germination in normal and *viviparous* maize (*Zea mays*) embryos. Planta 192:332–339

Paul AL, Ferl RJ (1991) *Adh1* and *Adh2* regulation. Maydica 36:129–134

Shirzadegan M, Christie P, Seemann JR (1991) An efficient method for isolation of RNA from tissue cultured plant cells. Nucleic Acids Res 19:6055

Subbaiah CC, Bush DS, Sachs MM (1994) Elevation of cytosolic calcium precedes anoxic gene expresion in maize suspension-cultured cells. Plant Cell 6:1747–1762

Walker JC, Howard EA, Dennis ES, Peacock WJ (1987) DNA sequences required for anaerobic expression of the maize alcohol dehydrogenase 1 gene. Proc Natl Acad Sci USA 84:6624–6628