A Gibberellin Response Complex in Cereal α-Amylase Gene Promoters

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The Amy32b gene is a representative member of a closely related family of α-amylase genes expressed under hormonal control in aleurone layers of barley grains. Transcription of this gene is induced by gibberellin (GA) and suppressed by abscisic acid. In this study, we functionally defined the promoter elements of the Amy32b gene that govern the developmental and hormonal control of its expression in aleurone. Two functionally distinct yet physically associated elements are essential: a gibberellin response element mediates regulation by GA and abscisic acid, and an Opaque-2 binding sequence (O2S) is thought to interact with a barley homolog of the maize endosperm-specific transcriptional regulator Opaque-2. An additional element CCTTTT, which with the O2S forms part of a canonical "endosperm box," is important in modulating the absolute level of expression of the Amy32b promoter, as is another separate, highly conserved element TATCCATGCAGTG.

INTRODUCTION

The plant hormones abscisic acid and gibberellin (GA) play major and opposing roles in the development and germination of cereal grains. The paradigm for their actions is found in genes for hydrolytic enzymes, such as α-amylase, which are expressed at high levels only in aleurone and scutellar epithelial cells of the grain during germination. In aleurone, GA induces expression of these genes, leading to destruction of the storage products of the grain, whereas abscisic acid prevents their expression (Higgins et al., 1982; Nolan et al., 1987). Little is known of the molecular events that connect abscisic acid or GA with activation or suppression of one of these genes, and less is known about the mechanisms that direct their tissue-specific expression.

Recently, Skriver et al. (1991) demonstrated that six tandemly repeated copies of the sequence 5'-CCGATAACA-AACTCCGG-3' from the promoter of a hormonally regulated barley α-amylase gene, Amy6-4 (Khursheed and Rogers, 1988), could confer proper induction of transcription by GA and its suppression by equimolar abscisic acid on a minimal promoter-marker gene when the chimeric construct was transiently expressed in barley aleurone protoplasts (Skriver et al., 1991). The fact that this sequence could mediate effects by both hormones showed that the signal transduction pathway for each ultimately converges on this 17-nucleotide DNA

sequence; this functionally constitutes a gibberellin response element (GARE; Skriver et al., 1991). Tandemly repeated units were required for its action to be detected (Skriver et al., 1991). Its TAACAAACTCCGG motif is highly conserved as a single-copy element in promoters of α-amylase genes from different species (Huang et al., 1990).

We wanted to define the promoter environment that permits a single copy of a GARE to function with high efficiency and to understand more about variations in the sequence that are compatible with its function. We analyzed the promoter from the low-pI α-amylase gene Amy32b (Whittier et al., 1987), which is known to be hormonally regulated by GA and abscisic acid (Rogers and Milliman, 1984; Khursheed and Rogers, 1988) but that has little apparent homology to the promoter of Amy6-4. Accordingly, we generated a series of Amy32b promoter derivatives fused to the reporter gene encoding β-glucuronidase (GUS) (Jefferson et al., 1987), and these gene constructs were expressed transiently following particle bombardment-mediated delivery (Klein et al., 1987; Bruce et al., 1989) into intact aleurone cells.

Results from linker scan mutation and deletion experiments defined three functionally distinct but closely linked elements in the promoter of Amy32b that are necessary for high levels of expression in aleurone cells. Two of these elements from this promoter are absolutely required for expression: a sequence closely related to the GARE defined by Skriver et al. (1991), which probably mediates hormonal regulation, and an Opaque-2 binding sequence (O2S) (Lohmer et al., 1991), which probably interacts with a barley homolog of

the maize endosperm-specific transcriptional regulatory protein Opaque-2 (Hartings et al., 1989; Schmidt et al., 1990). Another sequence element, CCTTTT, coupled with the O2S forms a sequence very similar to an "endosperm box" (Forde et al., 1985) and has important effects on the level of expression from the Amy32b promoter. In addition, there are two other sequences that also significantly affect the level of expression: one, located between -192 and -158, was not characterized in detail; the other, GCAGTG, is positioned immediately 3' to a sequence (TATCCAT) that is highly conserved in α-amylase promoters from different species (Huang et al., 1990). Our data indicated that the interactive effects of all of these elements contribute to proper tissue-specific, hormonally regulated transcription from this promoter.

RESULTS

All promoter analyses were performed with particle bombardment delivery of Amy32b promoter–GUS marker gene constructs into intact aleurone layers that were then treated with various combinations of hormones. The inherent variability of transfection efficiencies associated with this technique requires an effective means of normalization. Throughout this analysis, we relied on the use of an oat ubiquitin promoter/ firefly luciferase reporter construct as an internal standard (Bruce et al., 1989; Bruce and Quail, 1990), which, when introduced with the α-amylase promoter–GUS test construct, allowed reproducible comparisons between levels of expression from different test constructs. We determined that expression from the ubiquitin promoter was not significantly affected by GA treatment of the tissue and that abscisic acid treatment had an effect that was negligible in these particular experiments (see Methods).

Huttly and Baulcombe (1989) and Jacobsen and Close (1991) have shown that the promoter sequences of a wheat low pI- and a barley high pI-type α-amylase gene appropriately directed high levels of GA-dependent transcription of reporter genes in aleurone protoplasts. Their results indicated that essentially all of the elements necessary for this activity were found within 300 nucleotides upstream from the transcription initiation sites. We first compared GUS expression from the construct JR248, which has 1.4 kb of promoter/ upstream DNA from Amy32b (Whittier et al., 1987), to that from ML022, a 5' deletion containing 331 bp of upstream sequence. Both the absolute level of expression from ML022 and the induction of expression by GA were twice as high compared to results from JR248, and this induction was completely inhibited by 50 μM abscisic acid (data not shown). Accordingly, ML022 was used as the parental construct for generating further deletions and mutations.

Single XbaI sites (TCTAGA) were introduced in individual constructs to replace hexanucleotides at $\sim$30-bp intervals in the ML022 promoter region, thus generating constructs X1 through X7, as shown in Figure 1 (data for X1 through X4 not
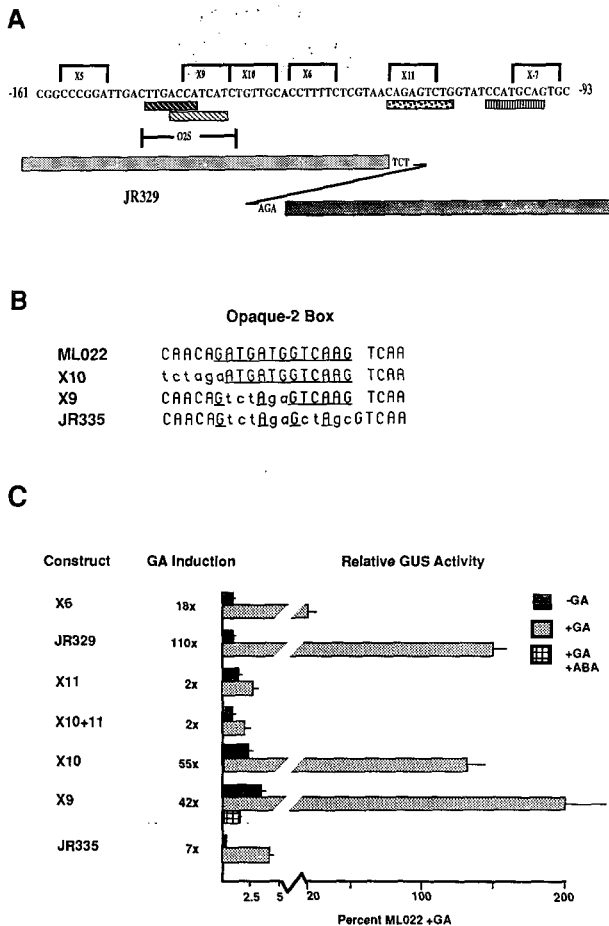
shown). Restriction fragments derived from these constructs were recombined in vitro to generate $\sim$30 bp deletions that scanned the promoter; these constructs are X1-2 through X6-7 (Figure 1). These constructs were then tested for transcriptional activity. Activities of test constructs were determined on different days, but in every experiment both the parental ML022 construct and one or two of the test constructs were introduced into four or five sets of aleurone layers. These results are shown in Figure 1. The narrow SE for the results obtained with ML022 on six different days ($n = 25$; Figure 1) demonstrates the reproducibility of the system. The results from this series of experiments, therefore, are presented together and the data are expressed as actual units of GUS activity.

Linker substitutions X1 through X5 (as illustrated by X5, Figure 1; data for X1 to X4 not shown) had little or no effect
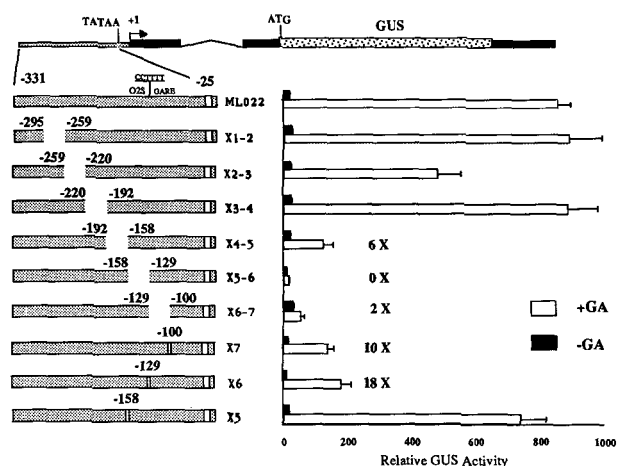
Figure 1. Linker Scan Mutation/Deletion Analysis of the Amy32b Promoter.

A schematic diagram of the ML022 construct is shown at the top; -331 indicates the 5' end of the promoter, and -25 indicates the position of the TATAA box. The promoter region (left) is stippled, the thick black lines represent the 5' and 3' untranslated sequences from Amy32b, and the thin black line indicates the position of the first intron from Amy32b that was inserted into the 5' untranslated sequence. The stippled box (right) represents the GUS coding sequence. Six nucleotides were mutated in individual constructs to change the promoter sequence at $\sim$30-bp intervals to an XbaI site. Three of these mutated constructs (X5, X6, and X7) are shown at the bottom; the number(s) above a construct indicate the 5' end of that mutation. Individual mutants were then recombined to form 30-bp deletions; these are indicated by the linker sites used to make the deletions, e.g., X1-2, X2-3, and so forth. To the right are the results obtained from expressing each construct. These are expressed as relative GUS activity; each column represents the mean (with SE) of at least four samples (black bars, layers incubated in the absence of hormones; white bars, layers incubated in the presence of GA). The numbers to the right of the bars for X4-5 through X6 represent the fold induction of expression by GA (e.g., 6x = sixfold).

A

-161 CGGCCCGGATTGACTTGACCATCATCTGTTGCACCTTTTCTCGTAACAGAGTCTGGTATCCATGCAGTGC -93

JR329

B

Opaque-2 Box

ML022   CRRCAGATGATGGTCARG TCRR
X10   tctagaATGATGGTCARG TCRR
X9   CRRCAGtctAgaGTCARG TCRR
JR335   CRRCAGtctAgaGctAgcGTCRR

C

Figure 2. Functional Analysis of Mutations in the Amy32b Promoter −158 to −95 Interval.

(A) The Amy32b sequence. The sequence of the −161 to −93 interval of Amy32b (Whittier et al., 1987) is shown. The positions of the X5, X6, and X7 mutations are identified above by brackets with the name of the specific mutation noted inside. The positions of three additional linker mutations, X9, X10, and X11, are also indicated. The X6, X7, and X11 mutations disrupt the CCTTTT, TATCCATGCAGTG, and TAACAGAGTCTGG (mutated sequences underlined) elements, respectively. The notation O2S is placed below the sequence that corresponds to the Opaque-2 box. Regions of this promoter interval protected from DNase I nicking by nuclear proteins are indicated immediately below the sequence by bars; the bars above O2S indicate the extent of the footprint when proteins from GA-treated aleurone cells are used (dark bar with diagonal stripes) and untreated cells (light bar with diagonal stripes) (Lanahan, 1991; M. Lanahan and T.-H.D. Ho, unpublished data). The two other footprints were generated with proteins from either extract (vertical striped and stippled bars, respectively) (Lanahan, 1991; M. Lanahan and T.-H.D. Ho, unpublished data). The construct JR329 was made by recombining the parts of X10 and X11 and is indicated by the gray and black bars. **(B)** Construct sequences. The sequences of constructs with the mutations that affect the Opaque-2 box (underlined nucleotides) are presented and compared to the sequence of ML022. Nucleotides that alter the normal sequence are in lowercase letters. These are

on expression, but two, X6 and X7, caused significant reductions in the level of GA-induced expression to ~20 to 25% of the control (Figure 1). The significance of these mutations is discussed below. It can also be seen that three of the deletions, X4-5, X5-6, and X6-7, caused drastic decreases in the levels of GA-induced expression (Figure 1). The X4-5 deletion permitted only a sixfold increase in expression with GA; the functionally important sequences in this interval were not characterized further. The X5-6 deletion virtually silenced the promoter (2% of ML022), whereas the effects of the X6-7 deletion were somewhat less severe (8.7% of ML022), but neither deletion permitted significant induction of expression by GA.

We further studied the functional role of sequences present in the X5 to X7 interval by constructing additional promoter derivatives with mutations in specific nucleotides. In Figure 2A, the positions of the original XbaI linker mutations (X5, X6, and X7) are depicted as are the additional XbaI linker mutations X9, X10, and X11. JR329 is a construct made by ligating the 5' XbaI fragment from X11 to the 3' XbaI fragment from X10 to generate a duplication of the central sequence containing the sequence CCTTTT. A detailed depiction of the base-pair changes induced by X9, X10, and JR335 within the region we call the "Opaque-2 box" is shown in Figure 2B. In each experiment where these constructs were assayed for transcriptional activity, the ML022 parental construct was also analyzed as a standard for comparison. To allow precise comparisons of the effects of each of the mutations, the results (presented in Figure 2C) are expressed as a percentage of the GUS activity obtained from the ML022 + GA samples analyzed on the same day. Again, the ML022 construct reproducibly showed a 40- to 50-fold increase in expression in the presence of GA (data not shown).

The X6 mutation disrupted the sequence CCTTTT, which is highly conserved in α-amylase gene promoters and in promoters of other genes expressed specifically in endosperm (see below). This mutation significantly decreased both the absolute level of expression and the effect of GA on expression (Figure 2C). It is possible that this sequence could be involved in an interaction with nuclear proteins that somehow enhances transcription in concert with hormonally regulated interactions on other sequences. JR329, which has this sequence duplicated, had a level of GA-induced expression that was significantly greater (152 ± 14%; P = 0.04; Figure 2C) than the ML022 parent promoter. Interestingly, it also doubled the amount of GA-induced transcription from the

presented in the strand opposite that in **(A)** for easier comparison to the Opaque-2 consensus binding sequence shown in Figure 3. **(C)** GUS activity. Relative GUS activity for each of the above constructs was compared to that for ML022 in each experiment; results are presented as percent of the mean activity obtained for the ML022 +GA samples. The numbers (e.g., 18×) indicate the increase in expression caused by GA. The ML022 construct in the multiple analyses for these experiments gave an average GA induction of 42-fold. ABA, abscisic acid.
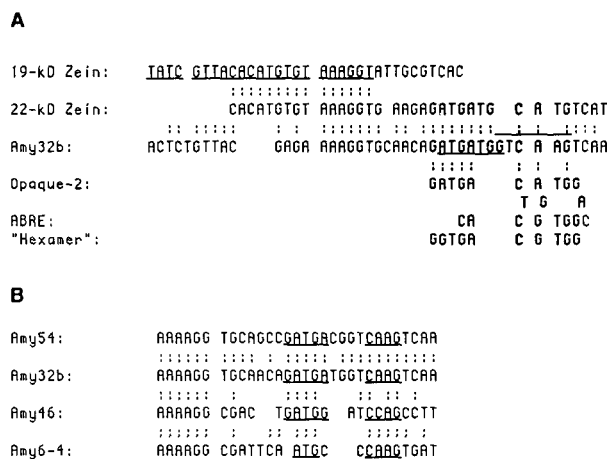
promoter (110 times for JR329 versus 42 times for ML022). We therefore conclude that the CCTTTT sequence plays an important role in the absolute level of transcription when it is present with other essential elements that are defined by additional mutations.

The most profound effects on expression were observed with the X11 mutation regardless of whether it was alone or present with the X10 mutation (Figure 2C). The X11 mutation was situated in the sequence TAAC<u>AGAGTC</u>TGG (position of mutation underlined), which is the apparent equivalent of the TAACAAACTCCGG motif found in the GARE from the Amy6-4 promoter (Skriver et al., 1991). This mutation essentially abolished GA-induced expression (Figure 2C). X10, which mutates a similar sequence (CAACAGA) on the opposite strand, had little effect on transcription (134 ± 13%, P = 0.13; Figure 2C). These results are consistent with the conclusion that X11 disrupts the functional GARE in the Amy32b promoter. This experimental strategy, based on loss of function mutations, would not distinguish between a mutation that disrupted a hormone response element and one that disrupted a sequence necessary for high levels of transcription. Accordingly, our proposed identification of this putative GARE is based solely upon the similarity of its sequence to that defined in gain of function experiments by Skriver et al. (1991) as a GARE.

Another sequence element that independently exerts profound effects on expression of the promoter is found in the interval between X5 and X10; this reads GATGATGGTCAAG on the opposite strand and corresponds to an O2S (Lohmer et al., 1991) (Opaque-2 box, Figure 2B). Mutation of the 5' half of this sequence (X9) doubled the level of expression from the promoter (P = 0.002). This increase was not due to a derangement of hormonal controls because it was completely suppressed by abscisic acid (Figure 2C). Mutation of both the 3' half and 5' half of the O2S sequence (JR335) caused a drop in the level of expression to 2% of that of X9 but retained significant albeit low levels of GA inducibility (Figure 2C).

This region of the promoter has striking homologies to two well-described motifs, as illustrated in Figure 3A (the sequence of Amy32b is written from the opposite strand for easier comparisons). The central region AAAGGTGCAACC-AGATGA is a consensus "endosperm box" sequence described by Forde et al. (1985) as being an element present in the promoters of maize, barley, and wheat endosperm storage protein genes. It is illustrated here by the sequence from a maize 22-kD zein gene promoter (Kridl et al., 1984). A related sequence is found in promoters from maize 19-kD zein genes, and Maier et al. (1987) showed that proteins from extracts of maize endosperm footprinted the underlined nucleotides in the 19-kD promoter sequence that overlaps the 5' end of the endosperm box consensus. The corresponding Amy32b sequence also has a block of conserved residues very similar to this 19 kD-zein sequence (Figure 2).

The Opaque-2 gene product (Hartings et al., 1989; Schmidt et al., 1990) is a leucine zipper protein that is necessary for transcription of the 22- but not the 19-kD family of zein genes.

A

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19-kD Zein:    TATC GTTACACATGTGT AAAGGTATTGCGTCAC
                    ::::::::::  ::::::
22-kD Zein:           CACATGTGT AAAGGTG AAGAGATGATG  C A TGTCAT
                   :: ::::::   : :  :::::::: ::  ::::::::  : : :::
Amy32b:        ACTCTGTTAC    GAGA AAAGGTGCAACAGATGATGGTC A AGTCAA
                                 :::::     : : :
Opaque-2:                         GATGA   C A TGG
                                         T G   A
ABRE:                                CA   C G TGGC
"Hexamer":                         GGTGA  C G TGG
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B

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Amy54:    AAAGG TGCAGCCGATGACGGTCAAGTCAA
          ::::::  ::::  :  :::::  :::::::::::
Amy32b:   AAAGG TGCAACAGATGATGGTCAAGTCAA
          ::::::  :    :  ::::     :: :: :
Amy16:    AAAGG CGAC TGATGG ATCCAGCCTT
          ::::::  :   :: :::    :::::  :
Amy6-4:   AAAGG CGATTCA ATGC CCAAGTGAT
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Figure 3. Comparisons of the X5-X7 Interval Sequence to Other Sequences Important for Developmental and Hormonal Regulation of Gene Expression in Cereals.

(A) Sequence comparisons. The Amy32b sequence (written on the opposite strand from −111 to −152) is compared to sequences from 19-kD zein (Maier et al., 1987) and 22-kD zein (Kridl et al., 1984) "endosperm boxes," to the consensus sequence for binding of the Opaque-2 protein (Lohmer et al., 1991), the sequence of the ABRE (Guiltinan et al., 1990; Skriver et al., 1991), and a sequence from a wheat histone gene promoter that binds a leucine zipper protein (Hexamer, Tabata et al., 1989). In bold type are sequences that correspond to the Opaque-2 consensus sequence. The underlined nucleotides in the 19-kD zein sequence are footprinted by proteins in an endosperm cell nuclear extract (Maier et al., 1987). The underlined nucleotides in the Amy32b sequence are footprinted by nuclear proteins from aleurone cells that were not exposed to GA, whereas the line above indicates nucleotides that were footprinted by nuclear proteins from GA-treated aleurone cells (Lanahan, 1991; M. Lanahan and T.-H.D. Ho, unpublished data). Colons above or below the nucleotides indicate identity to the Amy32b sequence.

(B) Comparison of the endosperm box/Opaque-2 box sequences in different α-amylase gene promoters. The sequences of Amy54 (Huttly and Baulcombe, 1989), a low-pI wheat gene, and the two high-pI barley genes Amy6-4 and Amy46 (Khursheed and Rogers, 1988) are compared to Amy32b. Colons above or below nucleotides indicate identity to the Amy32b sequence.

This protein binds specifically to the consensus sequence (Lohmer et al., 1991) presented in Figure 3A. This element comprises part of the endosperm box from the 22-kD zein gene (Figure 3A, boldface type). An identical sequence (GATGACATG) is present twice in another Opaque-2-regulated maize zein gene, b-32, and these sites have been shown to interact specifically with the Opaque-2 protein (Lohmer et al., 1991). The consensus O2S, GATGA**CATC, permits a space (indicated by asterisks) between the two halves. In Amy32b, these motifs are highly conserved (Figure 3A, boldface type). The abscisic acid response element (ABRE), a sequence that mediates abscisic acid-induced transcription in cereal cells (Guiltinan et al., 1990; Skriver et al., 1991) and is bound by

a leucine zipper protein (Guiltinan et al., 1990) (Figure 3A, ABRE), and the "hexamer," a sequence from a wheat histone gene promoter that is bound by a leucine zipper protein (Tabata et al., 1989) (Figure 3A, Hexamer), also appear to be variations of portions of this Opaque-2 consensus sequence (Figure 3A).

A similar conservation of both primary sequence and spatial organization for the endosperm box (AAAAGG) with respect to the O2S (Figure 3B) is found in all four α-amylase promoters that have been shown in aleurone cell transient expression experiments to be regulated by GA and abscisic acid. A consensus for the O2S from these four sequences reads: GATGA $n_{(0-3)}$ TCAAGT (where n equals the presence of any zero through three nucleotides). In addition, these α-amylase genes share striking conservation of the putative GARE and TATCCAT elements (Huang et al., 1990). All have the same organization of closely spaced elements, which is O2S-CCTTTT-GARE. We have shown that this arrangement comprises a functionally defined "gibberellin response complex" in the Amy32b promoter.

We investigated the role of the Opaque-2 protein in mediating the functional effect of this O2S by expressing the unmodified ML022 construct and the JR335 construct with a
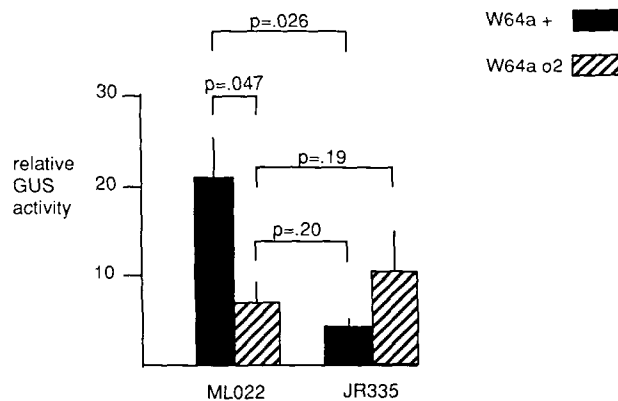
Figure 4. Effect of the Opaque-2 Gene Product on ML022 and JR335 Expression in Maize Aleurone Cells.

Sterilized maize seeds from which the pericarp and testa had been removed were imbibed, bombarded, and incubated in the absence of GA as previously described for experiments with barley grains. The average relative GUS activity (with SE bars, and P values derived from Student's t test indicated above) obtained when seeds from W64a and W64a-o2 (Aukerman et al., 1991) were bombarded with either ML022 or JR335 is presented. The construct used in each experiment is indicated below the appropriate pair of bars; solid black bars, W64a; hatched bars, W64a-o2. (For ML022 [+] versus JR335 [o2], P = 0.083; for JR335 [+] versus JR335 [o2], P = 0.298.) The level of expression of the luciferase internal standard in these aleurone cells was indistinguishable from that obtained with barley (based on approximate cross-sectional area of target tissue), whereas relative GUS activity values were approximately 30-fold lower than in comparable experiments in barley.

mutated O2S sequence in maize aleurone cells. (We cannot exclude the possibility, however, that some viable subaleurone cells contributed to expression of these constructs.) We compared their expression in the inbred W64a and in the isogenic W64a-o2, a spontaneous Opaque-2 (o2) null mutant (Aukerman et al., 1991); these results are presented in Figure 4. It can be seen that ML022 was expressed at a significant threefold to fourfold lower level in W64a-o2 when compared with W64a (P = 0.047). This lower level of expression was the same as that obtained when JR335 was expressed in aleurone cells of either genotype. These results demonstrated that the Opaque-2 gene product is responsible for the elevated level of expression of ML022 in W64a and suggest that interaction of a barley protein homologous to Opaque-2 with the O2S sequence is essential for high-level expression in barley aleurone cells.

DISCUSSION

The effects of GA and abscisic acid on α-amylase gene transcription were established from runoff transcription experiments using nuclei from oat or barley aleurone protoplasts (Jacobsen and Beach, 1985; Zwar and Hooley, 1986) and from transient expression experiments using α-amylase promoter–reporter gene constructs in aleurone protoplasts (Huttly and Baulcombe, 1989; Salmenkallio et al., 1990; Jacobsen and Close, 1991; Skriver et al., 1991). Huttly and Baulcombe (1989) and Jacobsen and Close (1991) made progressive 5' deletions on a low-pI and a high-pI α-amylase promoter, respectively, and found that sequences responsible both for high-level expression and for GA response were located within ~200 bp from the transcription initiation sites. Their deletion strategies did not allow them to identify the functionally important sequences. The promoter from the wheat low-pI gene Amy54 (Huttly and Baulcombe, 1989) is very similar to our Amy32b promoter, and our results are consistent with those of Huttly and Baulcombe (1989) in that the deletions that caused profound loss of expression (X4-5, X5-6, and X6-7) were all positioned within −192 (Figure 1). The functionally important sequences within the X4-5 interval have not yet been characterized, but it is interesting that this interval contains an inverted repetitive sequence element (identified as "A" in Whittier et al., 1987) also present in the promoter of a thiol protease gene expressed at high levels in aleurone cells.

The X7, X11, X9, and JR335 mutations were positioned to be centered on sequences that bound nuclear proteins from aleurone cell nuclei and were protected by them from DNase I digestion (Lanahan, 1991; M. Lanahan and T.-H.D. Ho, unpublished data). For reference, the positions of these footprints are indicated in Figure 2 by bars beneath the DNA sequence. These data suggest, in fact, that the footprint on the O2S is affected by GA treatment of the cells from which nuclear proteins are prepared (Lanahan, 1991; M. Lanahan

and T.-H.D. Ho, unpublished data). At a minimum, these results demonstrate that nuclear proteins interact with the sequences that are defined here as important in directing high-level expression from the promoter.

Other hormonally and developmentally regulated gene systems provide precedents to which the roles of the O2S and GARE sequences can be compared. Perhaps the best-studied system is transcriptional activation of certain genes by abscisic acid (Marcotte et al., 1989; Guiltinan et al., 1990; Mundy et al., 1990), where a highly conserved element is sufficient to confer abscisic acid responsiveness on a minimal promoter (Skriver et al., 1991), and mutations abolish abscisic acid responsiveness of model promoters (Guiltinan et al., 1990). A leucine zipper protein that binds to this ABRE has been cloned from wheat (Guiltinan et al., 1990). There is no published information regarding the mechanism(s) by which this protein might mediate abscisic acid effects on transcription.

The ABRE sequence conforms to part of the O2S. Opaque-2 itself is a leucine zipper protein (Hartings et al., 1989; Schmidt et al., 1990), and the amino acid sequence that corresponds to much of its DNA binding domain (the basic α-helical domain) is very similar to that of the ABRE binding protein EmBP-1 and to a protein (HBP-1; Tabata et al., 1989) that binds to TGACGT (also part of the canonical O2S) in wheat histone gene promoters. It should be emphasized that only Opaque-2 has been proven to be responsible for regulating expression of its target genes. EmBP-1 and HBP-1 are implicated because they bind to a DNA sequence of interest; in addition, a 2-bp mutation in the ABRE abolished abscisic acid response in transient expression and abolished EmBP-1 binding (Guiltinan et al., 1990), thus suggesting that EmBP-1 may be involved in the abscisic acid response.

When considering binding sites and transcriptional effects of leucine zipper proteins, it is important to recognize that the DNA binding region of each protein recognizes half a target binding site (e.g., Gentz et al., 1989; Turner and Tjian, 1989). Dimerization is essential for high-affinity binding to the site, and the ability to dimerize is governed by the leucine zipper sequence. All of these proteins have additional nonhomologous sequences—both N-terminal to the DNA binding domain and C-terminal to the leucine zipper—that probably are important in determining other interactions (e.g., with other members of a transcription complex).

The precedents from animal systems show that different proteins, whose expression is controlled by different extracellular signals, can interact to bind to a single canonical sequence; the effects of binding (i.e., activating or suppressing transcription) will depend upon which proteins form the dimer that recognizes the sequence. A wide range of possibilities exists, limited only by the number of different leucine zipper proteins possessing a proper DNA binding domain. In this regard, it is interesting that the similarities in DNA binding domains among the members of the c-fos and c-jun gene families and the yeast gene product GCN4 (Turner and Tjian, 1989) (all of which bind the same sequence) are less than the

similarities among Opaque-2, EmBP-1, and HBP-1 (Aukerman et al., 1991; Guiltinan et al., 1990). It is therefore likely that these plant proteins are members of a family, each of which can bind to variations of what we have called the canonical O2S (consistent with the observation that their established binding sites share that motif). It is also likely then that the effects of these proteins on transcription will depend upon whether they form homodimers or heterodimers to bind to that sequence. We have shown that a canonical O2S participates in the transcriptional response to GA and that expression of the promoter in maize aleurone depends upon the presence of the Opaque-2 protein.

We therefore propose a hypothesis to explain our findings. Because disruptions of the CCTTTT, TATCCAT<u>GCAGTG</u> (mutated sequence underlined), and −192 to −158 elements caused a reduction of overall activity but maintained GA responsiveness, it is likely that these elements are not essential for GA responsiveness but somehow enhance the interaction or the effects of the O2S and putative GARE. We suggest that the CCTTTT sequence may facilitate interactions between the Opaque-2 binding proteins and the GARE binding proteins; the system works less efficiently without it (as evidenced by X6, Figure 1) and more efficiently if it is duplicated (JR329, Figure 2). For the minimal promoter in a protoplast system, a GARE alone works but only if tandemly repeated (Skriver et al., 1991). This is consistent with the hypothesis that protein binding and interaction between two separate binding sites are required for high-level transcription and proper hormonal regulation. Such interactions would explain the ability of multiple tandemly repeated GARE units to function in the protoplast system (Skriver et al., 1991); these results suggest that the O2S and GARE units may be functionally interchangeable under certain circumstances. It is reasonable to propose that the O2S will be found to be a site of interaction of leucine zipper proteins related to Opaque-2. The nature of the protein(s) interacting with the sequences mutated by X11 in the putative GARE remains to be elucidated.

According to this model, the effects on transcription would depend upon which members of the Opaque-2 family of leucine zipper proteins bind to the O2S and perhaps the GARE sequences. In the absence of GA (or possibly even in the presence of abscisic acid), one set of proteins might interact to bind to the sequences; this pattern would not promote transcription and therefore would give the same result as when the sequences are completely deleted. In the presence of GA, however, a different set of proteins would interact to form heterodimers that bind to the sequences and promote transcription. In fact it is possible that the 5′ and 3′ halves of the O2S represent separate conserved motifs. Our finding that the X9 mutation (in the 5′ end of the O2S) significantly increased transcription whereas the JR335 mutation drastically lowered it (Figure 2) demonstrates that there are two distinct functional units within the sequence.

Our experimental results and this model provide a common focal point for the convergence of pathways that mediate tissue-specific and developmental controls on gene expression

in cereals with those that mediate directly opposing, developmentally regulated effects of two hormones. They suggest that the endosperm box sequences (CCTTTT plus the O2S) are responsible for endosperm-specific expression of the genes; this would allow aleurone-specific expression but would not explain why the genes are apparently silent in starchy endosperm during grain development (Mundy and Rogers, 1986). The interaction, however, of an endosperm box with a GARE would provide the proper balancing controls. Functions of the GARE would ensure that the genes would not be expressed in the presence of meaningful levels of abscisic acid, as might be expected to be present in developing endosperm. Conversely, it would ensure that the genes would be actively transcribed in mature aleurone where abscisic acid levels were low and where active gibberellins were provided by the embryo. As such, the model is testable. Its proposed regulatory system is unique in plant systems and may provide new insights into molecular mechanisms by which hormones affect plant development.

METHODS

Promoter Construction and Transient Expression

The 1.4-kb Amy32b promoter (Whittier et al., 1987) was attached with its entire 5' untranslated sequence (Khursheed and Rogers, 1989) to the coding sequence for the *Escherichia coli* β-glucuronidase (GUS) gene (Jefferson et al., 1987) with a modified ATG initiation codon. The first intron from Amy32b was inserted into the 5' untranslated sequence; this construct is identified as JR248. A truncated form, deleted 5' to −331, is identified as ML022 and used as a template for all mutations described here. (The DNA sequences of these constructs are available from the authors upon request.) XbaI mutations were introduced into this truncated promoter using single-stranded oligonucleotide mutagenesis, and subsequent deletions were generated by recombining the appropriate restriction enzyme fragments.

For transient expression experiments, each test construct was mixed with plasmid pAHC18 (ubiquitin promoter–luciferase fusion; Bruce et al., 1989) in a mass ratio of 2:1 (final plasmid concentration was 0.6 μg/μL). Ten microliters of this DNA mixture was precipitated onto tungsten particles using $CaCl_2$ and spermidine (free base) and introduced into intact aleurone layers using particle bombardment (Bruce et al., 1989; Bruce and Quail, 1990).

Plasmid pAHC18 served as an internal standard. We first determined that expression from the ubiquitin promoter was not affected by either gibberellin (GA) or abscisic acid. Huttly and Baulcombe (1989) and Skriver et al. (1991) both found that GA had little or no effect on expression of a cauliflower mosaic virus (CaMV) 35S promoter/GUS chimeric gene in aleurone protoplasts. We therefore introduced pAHC18 mixed with pBI221 (CaMV 35S-GUS; Jefferson et al., 1987) and used the GUS activity expressed from the latter to standardize luciferase activity in aleurone extracts from GA-treated and control layers. These results showed that extracts from control layers expressed $2.2 \pm 0.8 \times 10^6$ relative light units (RLU)/10 sec ($n = 5$) in the standard assay, whereas extracts from GA-treated layers expressed $1.6 \pm 0.4 \times 10^6$ RLU/10 sec ($n = 5$); these were not significantly different ($P = 0.215$). Thus, GA treatment has little

effect on transcription from the ubiquitin promoter. We did not use the same method to assess abscisic acid effects; rather, we used raw luciferase assay values from extracts of layers treated with GA alone, or with GA + abscisic acid, and compared the two sets from two separate experiments: experiment 1 (GA versus GA + abscisic acid; $n = 5$ for each), $0.52 \pm 0.18 \times 10^6$ versus $0.93 \pm 0.30 \times 10^6$ RLU/10 sec, P = 0.045; experiment 2 ($n = 6$ for each) $1.05 \pm 0.47 \times 10^6$ versus $2.26 \pm 1.21 \times 10^6$ RLU/10 sec, P = 0.061. We concluded that abscisic acid may increase expression from the ubiquitin promoter but that the effect is only approximately twofold. Because the observed effect of abscisic acid on expression from the α-amylase promoters in the two experiments described here was on the order of 50-fold and because the focus of the work did not involve a search for small effects of abscisic acid on transcription from different constructs, we did not pursue the matter further.

For individual bombardments, 10 sterile deembryonated half grains of Himalaya barley, which had been imbibed for 2 days and had their pericarp/testa layers removed, were arranged in a small circle (~2.5 cm in diameter) so that all would be directly in the path of the tungsten particles. From each shot, five half grains were then incubated in 5 mL of 20 mM $CaCl_2$, 20 mM Na succinate, pH 5.0, buffer containing 10 μg/mL of chloramphenicol (barley buffer) and no hormones (−GA), or 2×10^{-6} M GA_3, or 2×10^{-6} M GA_3 + abscisic acid (5×10^{-5} M) for 36 to 40 hr in a Petri plate. The five half grains were then homogenized using a mortar and pestle in 1 mL of 100 mM $NaPO_4$, pH 7.2, 5 mM DTT, and 20 μg/mL leupeptin. The homogenates were centrifuged (10,000g) for 10 min and the supernates were retained for enzyme assays. Bombardment of maize seeds was essentially the same with the exception that they were not previously deembryonated, and the face of the maize kernel that had been hit with the tungsten pellets was removed with a razor blade and homogenized.

For luciferase assays, 100-μL samples of the extract were placed in luminometer cuvettes (Analytical Luminescence Laboratory, San Diego, CA) and then 100 μL of 2 × luciferase assay buffer (1 × is 30 mM $Tris-SO_4$, pH 7.7, 10 mM $MgCl_2$, 10 mM DTT, 1 mM EDTA) with 2 mM ATP added immediately before placing into the luminometer. Placing the tube in the luminometer (Monolight 2010; Analytical Luminescence Laboratory) automatically activated the machine so that it would inject 100 μL of 1 × luciferase assay buffer containing 1 mM luciferin, and then count emitted photons for 10 sec. Typical values for luciferase activity were between 500,000 and 5,000,000 RLU, and background activity, i.e., assays using extract from tissue that had not been bombarded, was normally 200 to 300 RLU. GUS assays were performed essentially according to Huttly and Baulcombe (1989) except that extracts were not first purified by column chromatography. Fifty microliters of protein extract in 250-μL reaction mixtures was used to assay GUS using the fluorescent 4-methylumbelliferyl β-D-glucuronide substrate (Jefferson et al., 1987). Care was taken to ensure that all assays were done within a linear range. Results of the GUS assays were expressed simply as fluorescent units (minus the value obtained from control extracts) generated in 4 hr at 37°C. To standardize relative expression of GUS from the various promoter constructs, the GUS assay result from one sample was multiplied by a normalizing constant and the product divided by the luciferase value obtained from that particular sample. This relative GUS activity essentially expresses how efficiently the test promoter construct drove transcription of the reporter gene as compared to the ubiquitin/luciferase construct transcription efficiency. The average GUS activity (and SE) were calculated using four to six replicate bombardments per construct. Statistical comparisons of results from different constructs were made using Student's *t* test.

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