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Gibberellin-Responsive Elements in the Promoter of a Barley High-pI α-Amylase Gene

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Deletion analysis has previously shown that the major gibberellic acid (GA)- and abscisic acid (ABA)-responsive elements in the promoter of a high-pI α-amylase gene of barley are located downstream of −174 (Jacobsen and Close, 1991). We have used transient expression assays in barley aleurone protoplasts to identify sequences between −174 and +53 that confer GA and ABA responsiveness on expression of a β-glucuronidase reporter gene. Using α-amylase promoter fragments and synthetic oligonucleotides fused to minimal promoters, we have shown that the hormone-responsive region is located between −174 and −108. A single copy of this region fused to a minimal α-amylase promoter (−41) conferred both GA- and ABA-responsive expression on the reporter gene comparable to the positive control, Am(−174)IGN. Multiple copies of this region were able to activate even greater levels of expression. Site-directed mutagenesis was used to determine the functional importance of the conserved motifs ($^{-169}$pyrimidine box, $^{-143}$TAACAAA box, and $^{-124}$TATCCAC box) and nonconserved intervening sequences within the region between −174 and −108. Our results showed that both the TAACAAA and TATCCAC boxes play an important role in GA-regulated expression. We propose that the TAACAAA box is a gibberellin response element, that the TATCCAC box acts cooperatively with the TAACAAA box to give a high level of GA-regulated expression, and that together these motifs form important components of a gibberellin response complex in high-pI α-amylase genes. The TAACAAA box also appears to be the site of action of ABA. In addition, we have identified a sequence that acts as a repressor of GA action and that resembles a cAMP response element.

INTRODUCTION

The aleurone layer of barley has been used extensively to study the molecular mechanisms of gibberellic acid (GA) and abscisic acid (ABA) action with much of the interest focusing on the expression of α-amylase genes. Time course studies have shown that GA increases the level of α-amylase mRNA, as measured by in vitro translation, by the use of specific cDNA probes, and by primer extension analysis (Jacobsen and Chandler, 1987; Fincher, 1989; Jones and Jacobsen, 1991). Based on run-on transcription experiments using nuclei from barley (Jacobsen and Beach, 1985) and oat aleurone protoplasts (Zwar and Hooley, 1986), it is now clear that both GA and ABA exercise important control at the transcriptional level over α-amylase gene expression in aleurone cells.

Functional analyses of the promoters of α-amylase genes fused to reporter genes using transient expression assays have been initiated in several laboratories to identify gibberellin response elements (GARE) involved in hormone-regulated gene expression. Results from 5′ deletion analysis of a promoter fragment from a gene coding for a high-pI α-amylase protein (Amy pHV19) showed that there was a dramatic decrease in GA-induced transcriptional activity when sequences downstream of −174 were removed (Jacobsen and Close, 1991). A similar study of a wheat low-pI α-amylase gene (Amy 2/54) found that the GA-responsive region lies within 300 bp upstream of the transcription start site (Huttly and Baulcombe, 1989). Neither of these studies was able to separate the ABA- and GA-responsive regions. In a recent study, a chimeric promoter containing six copies of the sequence from −148 to −128 of a high-pI α-amylase gene promoter (Amy 1/6-4) fused to a minimal 35S promoter was shown to confer GA- and ABA-responsive expression in barley aleurone protoplasts (Skriver et al., 1991). This 21-bp sequence contained a conserved motif, TAACAAA, identified in sequence comparisons between α-amylase gene promoters of barley, wheat, and rice (Huang et al., 1990). The roles of this putative GARE and of other closely associated conserved motifs in cereal α-amylase gene promoters (the pyrimidine box and TATCCAC/T motifs) (Huang et al., 1990) have yet to be clarified. A recently reported functional analysis of the promoter of the Amy32b gene, which codes for a low-pI α-amylase, defined several sequences that were important in control of expression (Lanahan et al., 1992). Using deletion and mutation analysis, the study indicated that the pyrimidine box, a nonconserved sequence GCAGTG adjacent to the conserved TATCCAT box, and an Opaque-2–like sequence just upstream of the pyrimidine box were important in modulating the absolute level of gene expression but not

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the hormone response. This study did not specifically target the conserved TAACAGA and TATCCAT sequences, but mutation of the sequence AGAGTC, which partly overlaps the conserved TAACAGA box, greatly reduced the hormone response. From the above, it is evident that a clear picture of action and interaction of *cis*-acting elements involved in α-amylase gene expression is yet to emerge.

In this study, we have used transient expression assays of promoter/reporter gene constructs in barley aleurone protoplasts to identify the sequences downstream of −174 in a high-pI α-amylase promoter from Amy pHV19 that are required to confer high levels of GA and ABA control of expression. Using oligonucleotides fused to a minimal α-amylase promoter, we have been able to locate the hormone-responsive region between 174 and 108 bases upstream of the transcription start site. Furthermore, using site-directed mutagenesis, we have analyzed the functional importance of both conserved and nonconserved sequences within this region and have identified three important elements, two exercising positive control and one exercising negative control over gene expression. We could not identify separate GA and ABA response elements.

RESULTS

The region of the high-pI α-amylase gene (Amy pHV19) promoter between −174 and +54 has been shown to be GA and ABA responsive (Jacobsen and Close, 1991). To determine whether the sequences that are involved in the GA and ABA response are upstream of the TATA box, a promoter fragment containing the sequences from −174 to −41 was inserted in both orientations, in front of a minimal cauliflower mosaic virus 35S promoter fused to the β-glucuronidase (GUS) reporter gene, as shown in Figure 1A. The expression of the chimeric promoter constructs in barley aleurone protoplasts was measured relative to that of Am(−174)IGN, which we chose as our positive control.

Figure 1B shows that the expression of the basic construct containing the 35S promoter alone linked to GUS [35S(−45)IGN] in aleurone protoplasts did not respond to GA and ABA treatments. The levels of GUS activity in these treatments were not significantly higher than those in no DNA treatments, indicating that most, if not all, of the detectable GUS activity was due to endogenous activity in aleurone protoplasts. However, when the α-amylase promoter fragment was fused in the correct orientation upstream of the 35S promoter [Am(−174/−41)/35S(−45)IGN], GUS expression in aleurone protoplasts increased twofold to threefold in response to GA. This increase was inhibited by ABA. Hormonal control of expression was lost if the α-amylase promoter fragment was inserted in the reverse orientation. Although the results shown in Figure 1B demonstrate that the region between −174 and −41 of the α-amylase promoter can confer GA and ABA responsiveness to the minimal 35S promoter in an orientation-dependent manner, the overall level of expression of the

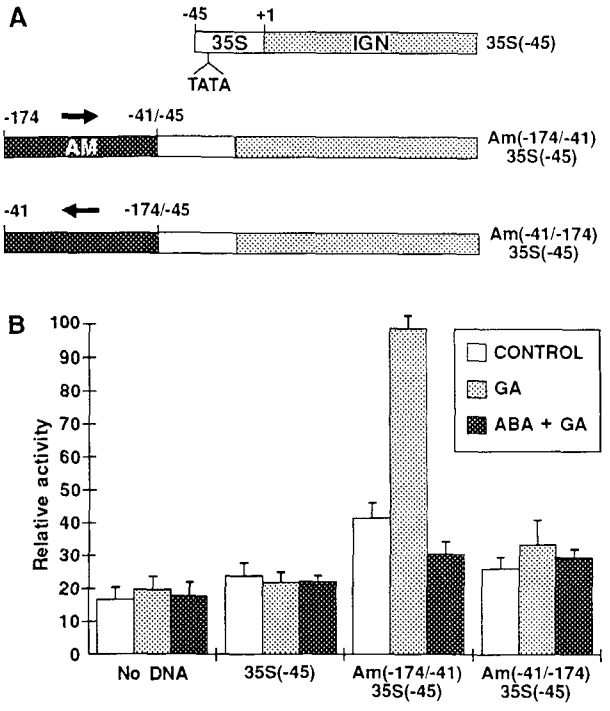

Figure 1. Effects of GA and ABA on Transient Expression of α-Amylase/35S Promoter Constructs in Barley Aleurone Protoplasts.

(A) Schematic diagram of α-amylase/35S promoters fused to a reporter gene cassette (IGN) containing the maize *Adh1* intron1 (I), the β-glucuronidase coding region (G), and 3′ sequence from the nopaline synthase gene (N).

(B) Relative GUS activities in barley aleurone protoplasts transfected with the α-amylase/35S constructs and incubated with no hormone (control), 10^{-5} M GA_3 (GA), and 10^{-5} M GA_3 plus 10^{-4} M ABA (ABA + GA) for 43 hr. All values have been shown relative to the activity of Am(−174/−41)/35S(−45)IGN, which was given a value of 100 in GA treatments. The error bars represent standard error of the mean (n = 9).

chimeric promoter construct was low in comparison to Am(−174)IGN (about 2%), indicating that sequences downstream of −41 in the α-amylase promoter are required for high level of expression in aleurone protoplasts. Consequently, all subsequent constructs were made using an α-amylase minimal promoter instead of the 35S promoter sequences.

Activity of Oligonucleotide/Am(−41)IGN Constructs

To locate the GA- and ABA-responsive element(s) within the region of −174 and −41 of the α-amylase promoter, synthetic oligonucleotides covering various regions were synthesized and fused upstream to an α-amylase minimal promoter construct, Am(−41)IGN, as shown in Figure 2A. Expression analyses of these constructs relative to the Am(−174)IGN are shown in Figure 2B. The basic construct containing the α-amylase minimal promoter exhibited weak GA responsiveness.

Experiments with oligos 1 (−174 to −108), 2 (−107 to −41), and 3 (−131 to −84) fused to the minimal promoter showed that oligo 1 restored strong GA- and ABA-dependent expression on the truncated α-amylase promoter to levels comparable to that driven by the positive control, Am (−174)IGN. GA induced a fivefold to sixfold increase in the level of expression of both oligo 1/Am(−41)IGN and Am(−174)IGN. This effect was reversed by ABA. Oligo 2 had little effect on the expression of the truncated α-amylase promoter. Oligo 3, which partly overlaps with oligo 1, conferred high levels of expression in both control and hormone treatments, indicating that it contained positive *cis*-acting elements which conferred high levels of expression but which were poorly responsive to GA and ABA.

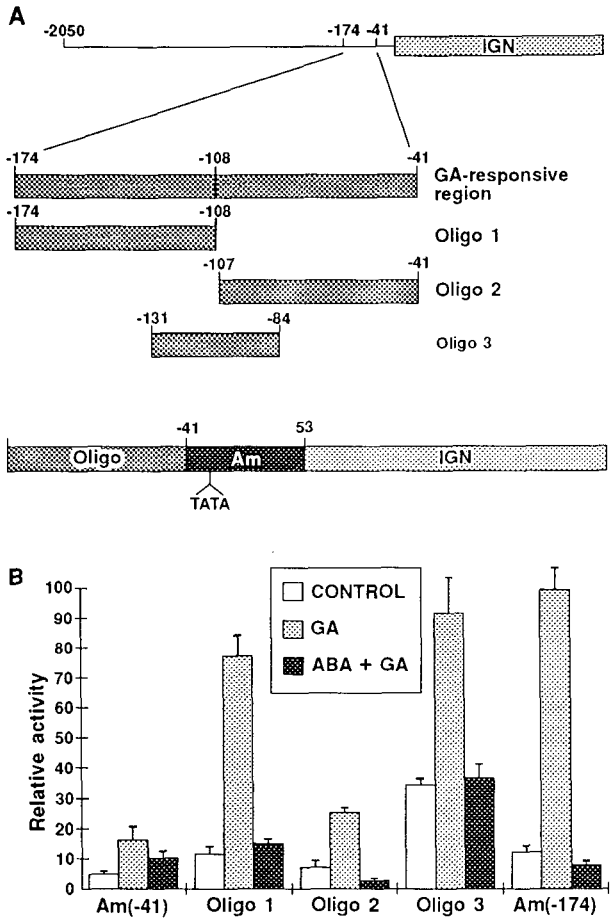

Figure 2. Activity of 5′ Upstream Sequences Spanning Various Regions between −174 and −41 on the Expression of Am(−41)IGN.

(A) Diagram showing construction of oligo/Am(−41)IGN genes. Oligos spanning various domains of the hormone-responsive region between −174 and −41 (1 to 3) were fused upstream of a truncated α-amylase promoter AmIGN construct.

(B) Effects of GA and ABA on expression of oligo/Am(−41)IGN constructs in protoplasts. GA and ABA concentrations are as for Figure 1. The vertical bars give the standard error of the mean (n = 6).

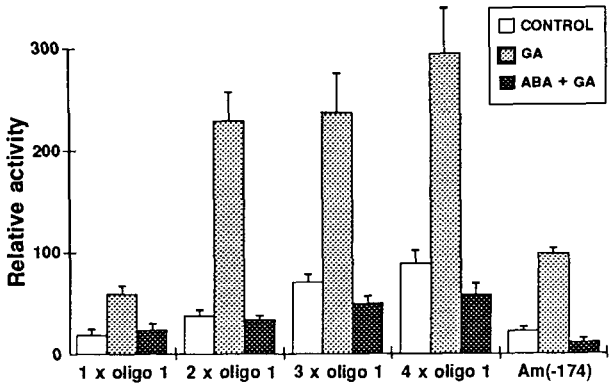

Figure 3. The Effect of Multiple Copies of Oligo 1 on the Expression of Am(−41)IGN in Barley Aleurone Protoplasts.

The error bars represent standard error of the mean (n = 21).

Addition of GA resulted in only a twofold to threefold induction of expression similar to that found with the minimal α-amylase promoter. It is clear from these results that the oligo 1 region contains all of the *cis*-acting elements required to confer strong GA- and ABA-dependent expression on the minimal α-amylase promoter. It would also appear that elements contained within oligo 1 can function, at least to some extent, in a position-independent manner because the oligo 1 sequence is 66 bases closer to the TATA box than in the native promoter.

To determine whether activation effects of oligo 1 on the minimal α-amylase promoter were additive, multiple tandem copies of oligo 1 were fused to the minimal promoter. The level of expression in response to GA increased dramatically with two copies of oligo 1, but further additional copies had little effect, as shown in Figure 3. In contrast, expression in control and GA plus ABA treatments increased gradually with oligo 1 copy number.

Effects of Site-Directed Mutations in the Oligo 1 Region on Hormone Responsiveness

The results from oligonucleotide constructs (Figure 2) demonstrated that sequences between −174 and −108 were important for hormone-regulated expression. This region contains three sequences that are conserved in promoters of other GA-responsive α-amylase genes (Huang et al., 1990), as highlighted in Figure 4A. To test the function of these conserved sequences and adjacent nonconserved sequences, clustered point mutations were introduced into the oligo 1 region of Am(−174)IGN. Expression analyses of these constructs are shown in Figure 4B. Mutation of the conserved TAACAAA box strongly reduced GA-induced expression, and nearby mutations, especially of the TATCCAC box, also affected expression but less so than for TAACAAA. Mutations of nonconserved sequences between these two conserved boxes also caused

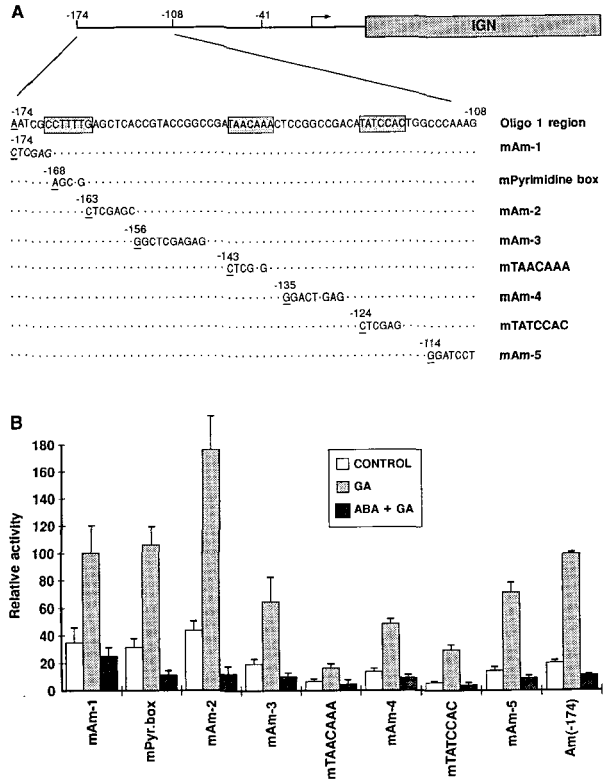

Figure 4. Effect of Site-Directed Mutagenesis of Sequences between −174 and −108 (Oligo 1 Region) on Expression of Am(−174)IGN in Barley Aleurone Protoplasts.

(A) Diagram showing native and mutated sequences in the oligo 1 region of Am(−174)IGN constructs.

(B) Effects of GA and ABA on expression of wild-type and mutant Am(−174)IGN constructs in barley aleurone protoplasts. Hormone concentrations are as for Figure 1. Activities are relative to Am(−174)IGN, which was given the value 100. The error bars represent standard error of the mean (n = 9 to 18).

reductions in the GA response, but the effects were not as marked as seen when the conserved boxes were mutated. In contrast, mutation of sequences between −174 and −147, which includes the pyrimidine box, and between −114 and −108 had little or no negative effect on GA-induced expression. On the contrary, mutation of a cAMP-like response element, TGAGCTC (Deutsch et al., 1988), which partly overlaps with the pyrimidine box, resulted in a significant increase of about 70% in the level of expression in response to GA (P = 0.006). It is important to note that the mutation does not introduce a sequence that we recognize as a GARE. In addition, introduction of similar sequences elsewhere in the oligo 1 region either had no effect or inhibited GA activity. These results indicated that sequences important for GA-responsive promoter activity occur between −146 and −115. One of the sequences, the cAMP-like element, appears to repress the GA response, and two other sequences, TAACAAA and TATCCAC, appear to be required for the GA response.

All of the constructs, with the possible exception of the mTAACAAA box, exhibited sensitivity to ABA. The dramatic loss of GA induction associated with the mutation of the TAACAAA box made it difficult to ascertain the effect of ABA on down-regulating reporter gene expression.

DISCUSSION

Previously, we have shown that the major GA and ABA response elements occur in the promoter of the high-pI α-amylase gene, Amy pHV19, downstream of −174 (Jacobsen and Close, 1991). To more closely locate the *cis*-acting elements involved in hormone action, we have now screened the important region of the promoter using gain-of-function experiments and site-directed mutagenesis coupled with functional analysis. Our results from transient expression assays in barley aleurone protoplasts show that (1) the GA-responsive elements occur between −146 and −115, (2) sequences that are highly conserved among GA-regulated genes occur in this region, (3) two of these sequences, TAACAAA and TATCCAC, act as positive control elements in GA action, (4) a cAMP-like response element acts like a repressor of GA action, and (5) ABA appears to act via the same sequence as GA (viz. TAACAAA). Therefore, transcriptional control by this promoter appears to be exercised by a cluster of elements centered about 120 bases upstream of the TATA box, the action of which depends on the order of the elements within the promoter.

The sequence TAACAAA appears to play a central role in GA action because mutation of it caused a large decrease in GA-driven gene expression. Skriver et al. (1991) showed that multiple (six) copies of a 21-bp promoter sequence containing TAACAAA (but not TATCCAC) conferred GA (and ABA) responsiveness on a reporter gene when transfected into barley aleurone protoplasts. Taken together, these results indicated that TAACAAA is a GARE. Mutation of another conserved sequence, TATCCAC, also caused loss of GA-induced gene expression but less so than for TAACAAA. We propose that TAACAAA and TATCCAC act cooperatively in controlling gene expression, and together they form important components of a GA response complex in high-pI α-amylase genes. Mutation of the pyrimidine box and sequences upstream of it within the oligo 1 region had little effect on GA-regulated expression, indicating that these sequences do not play a role in regulation of high-pI α-amylase gene expression in barley aleurone protoplasts.

Interesting differences are emerging between controlling elements within the promoters of the high- and low-pI α-amylase genes. Although there is considerable promoter sequence similarity within groups, there is little between groups except for the highly conserved sequences mentioned previously. Even so, it would appear that even the conserved elements do not necessarily have the same functions. In the Amy pHV19

promoter, the pyrimidine box appears to play no role in transcription control, but in the low-pI gene promoters, it seems to be very important. Deletion analysis of a low-pI α-amylase gene promoter from the wheat gene α-Amy 2/54 showed that deletion from −289 to −217, which removed two pyrimidine boxes leaving the (downstream) TAACAGA and TATCCAC sequences intact, resulted in a fivefold reduction in the level of GA-regulated expression (Huttly and Baulcombe, 1989). The remaining promoter was still GA responsive albeit at a much lower level. In the Amy32b gene promoter, the pyrimidine box was also of crucial importance to function (Lanahan et al., 1992). Also, whereas in Amy 32b upstream of the pyrimidine box, there is an opaque-2–like sequence and another sequence (from −192 to −158), both of which are important in gene expression (Lanahan et al., 1992), an opaque-2–like sequence (from −177 to −185) upstream of the pyrimidine box in Amy pHV19 appears to be unimportant in transcription control (Jacobsen and Close, 1991). Conversely, the cAMP-like response element in Amy pHV19 does not occur in the low-pI promoter sequences. On the other hand, the available evidence indicates that the TAACAA/GA sequence is the GARE in both high- and low-pI α-amylase gene promoters (Skriver et al., 1991; Lanahan et al., 1992; this report). Our data show TATCCAC is also important for controlling expression of high-pI α-amylase genes, but this is yet to be confirmed for low-pI α-amylase genes. There are many differences between the two α-amylase groups with regard to enzyme characteristics and induction kinetics (reviewed in Jacobsen and Chandler, 1987; Fincher, 1989; Jones and Jacobsen, 1991). Differences in promoter structure may provide a rational basis for the latter. Clearly, considerably more promoter analysis is required before a clear picture of the elements involved in hormonal control of these genes can be expected to emerge.

Mutation of the mammalian cAMP-like response element TGAGCTCA (correct sequence is TGACGTCA; Deutsch et al., 1988) caused a significant increase in the level of expression in response to GA, which raises the question of whether cAMP is involved in α-amylase gene expression. Despite early reports that cAMP (and cGMP) plays a role in α-amylase gene expression in aleurone layers (Kessler, 1973), we (F. Gubler and J.V. Jacobsen, unpublished results) have not been able to obtain high level expression of α-amylase in aleurone cells with added cAMP or cAMP derivatives. It remains to be seen whether this sequence exerts its effect through local position-dependent interference with positive regulatory effects on factors binding to TAACAAA and TATCCAC or whether it has some autonomous suppressor effect. It is possible that in other barley tissues the sequence plays a role in suppressing (much more than in aleurone cells) GA-induced expression of high-pI α-amylase genes. High-pI genes are expressed at high levels in aleurone but apparently less so in scutellum (Chandler and Mosleth, 1990) and not at all in leaf tissue (Jacobsen et al., 1986). In contrast, low-pI genes, which do not have the same cAMP-like element, are expressed more widely in pericarp (MacGregor and Dushnicky, 1989) and leaves (Jacobsen et al., 1986; Mares, 1987).

Functional analysis of α-amylase promoter sequences revealed that the TAACAAA box is also the likely site of ABA action in repressing GA promotion of gene expression. This is supported by two lines of evidence. First, deletion analyses of both high-pI (Jacobsen and Close, 1991) and low-pI (Huttly and Baulcombe, 1989) α-amylase promoters show that the site of ABA action occurs in the same region as the GARE. This is further supported by the results of Skriver et al. (1991) and our present study, which show that promoter fragments containing the TAACAAA box can confer both GA- and ABA-responsive expression on minimal promoter/reporter gene constructs. Second, our mutation analysis showed that mutation of sequences between −174 and −108, other than the TAACAAA box, failed to abolish the inhibitory effect of ABA on GA-induced expression. By elimination, this leaves the putative GARE, the TAACAAA box, as the most likely candidate for the site of ABA action. This contrasts with what is known about ABA response elements in promoters of genes that are induced by ABA. Recent functional studies of the promoters of a wheat Em gene (Marcotte et al., 1989; Guiltinan et al., 1990) and rice *rab* genes (Yamaguchi-Shinozaki et al., 1989; Mundy et al., 1990) have identified a conserved sequence motif, $_{C}$\TACGTGGC, as an ABA response element. A 2-bp mutation in this sequence abolished the ABA response (Guiltinan et al., 1990). A similar sequence adjacent to the TATA box in our high-pI gene promoter was found not to be involved with ABA action (Jacobsen and Close, 1991).

In conclusion, our results confirmed the proposal by Skriver et al. (1991) that the TAACAAA box plays a central role in both GA and ABA regulation of α-amylase gene expression. In addition, we showed that the TATCCAC box is important for high level expression. Revealing the precise mechanism of action of GA and ABA on α-amylase gene transcription mediated via the TAACAAA and TATCCAC motifs must await the detection and characterization of hormonally regulated transcription factors that interact with these elements.

METHODS

Plasmid Construction

The construct Am(−174)IGN, which has been described previously (Jacobsen and Close, 1991), contains an exonuclease III–generated deletion of a high-pI α-amylase gene promoter from barley fused to a reporter gene cassette that includes the maize *Adh1* intron1, the bacterial β-glucuronidase (GUS) gene, and the 3′ sequence of the nopaline synthase gene. The deleted α-amylase promoter contains 174 bp upstream of the transcription initiation point plus the complete 5′ untranslated region up to the position +54.

α-Amylase/35S Promoter Constructs

The construct p35S(−45)IGN, containing the cauliflower mosaic virus 35S promoter truncated to −45, was derived from the plasmid p4ARE/35S(−45)IGN (obtained from R. Dolferus) by removing the

Arabidopsis putative anaerobic response elements by PstI digestion and religating the cut ends. The 35S promoter is a minimal promoter containing only a functional TATA box. To construct α-amylase/35S promoter fusions, a high-pI α-amylase promoter fragment containing the sequence from −174 to −41 was excised from the pAm(−174)IGN as a PstI fragment and cloned into the PstI site 5′ to the 35S promoter in plasmid p35S(−45)IGN. Plasmids were isolated that contained the α-amylase promoter insert in the two possible orientations, pAm(−174/−41)/35S(−45)IGN and pAm(−41/−174)/35S(−45)IGN. Insert orientation was verified by restriction analysis and sequencing.

Synthetic Oligonucleotide Promoter Constructs

Plasmid pAm(−41)IGN was derived from pAM(−174)IGN by deleting the region −174 to −41 of the α-amylase promoter using PstI. This truncated α-amylase gene promoter-IGN construct contains a TATA box just downstream of −41 but is missing a putative CAAT box. Oligonucleotides identical to various regions between −174 and −41 of the high-pI α-amylase promoter were synthesized on an Applied Biosystems DNA synthesizer. Oligos designated 1, 2, and 3 spanned the region from −174 to −108, −107 to −41, and −131 to −84, respectively. The oligos were synthesized with BamHI sites at the 5′ ends and BglII sites at the 3′ ends. Oligos 1, 2, and 3 were cloned into the BamHI site of the plasmid pUC18. The oligos were excised from the plasmid as PstI-SmaI fragments. Following addition of PstI linkers to the blunt ends, the fragments were then cloned into the PstI site at the 5′ end of a truncated α-amylase gene promoter (−41) in the pAm(−41)IGN. Orientations of the single and multiple oligonucleotide inserts were determined by restriction analysis and sequencing. Only plasmids that contained the inserts in the correct orientation were retained.

Mutant Promoters

Site-directed mutagenesis was used to introduce mutations in the oligo 1 region (−174 to −108) of the pAm(−174)IGN following the method of Kunkel (1985) and Kunkel et al. (1987). Am(−174)IGN was transformed into the *dut*⁻ *ung*⁻ strain of *Escherichia coli* (BW313). Single-stranded template DNA was prepared by infecting the transformed cells with the helper phage M13K07. The following mutagenic oligonucleotides synthesized on an Applied Biosystems DNA synthesizer were used as primers for synthesis of the complementary strand of DNA:

mAm-1 GTCGACTCTAGAGCTCGAGCTTTTGAGCTCA

mPyrimidine box CTCTAGAGAATCGCAGCTGGAGCTCACCGTAC

mAm-2 AGAATCGCCTTTTCTCGAGCCCGTACCGGC

mAm-3 CCTTTTGAGCTCAGGCTCGAGAGCGATAACAAACTC

mTAACAAA GTACCGGCCGACTCGAGACTCCGGCCG

mAm-4 GCCGATAACAAACGGACTCGAGCATATCCACTGG

mTATCCAC CCGGCCGACACTCGAGCTGGCCCAAAG

mAm-5 CATATCCACTGGGGATCCTGAGCATTCAAGG

To facilitate screening of putative mutants, PvuII or XhoI restriction sites were included in the mutagenic sequence. After the primer extension reaction was complete, the hybrid DNA was transfected directly into Jm109 cells, and cell lines carrying mutant plasmids were screened by restriction analysis. The regions from −174 to −41 containing the mutations in the mutant Am(−174)IGN plasmids were excised as PstI fragments and used to replace the homologous segments in wild-type Am(−174)IGN. The presence of mutations in the reconstructed Am(−174)IGN plasmids was confirmed by restriction analysis and sequencing.

Preparation of Plasmid DNA

Plasmid DNA used in the transient expression assays was prepared from overnight cultures of *E. coli* Jm109 or AC001 (Last et al., 1991) and purified by fractionation on a Bio-Rad Bio-Gel A-50m (100 to 200 mesh) column. The purified DNA was resuspended in TE buffer and stored at −20°C.

Transient Expression Assays

Aleurone protoplasts were prepared from aleurone layers of *Hordeum vulgare* cv Himalaya (1985 harvest, Washington State University, Pullman) as described previously (Jacobsen and Close, 1991). To improve the efficiency and reduce variability of protoplast transformation, protoplasts were first purified on Percoll gradients prior to their use for transformation. This step removes starch grains and much of the Cellulase Onozuka R10 enzymes. After completion of Onozuka digestion, protoplasts from 13 flasks (~130 aleurone layers) suspended in Gamborg's medium were loaded onto two Percoll step gradients consisting of 5-mL 80% Percoll in Gamborg's medium below 5-mL 15% Percoll in Gamborg's medium. After centrifugation at 3000 rpm in an HB4 rotor for 10 min, the protoplasts were recovered from the top and bottom of the 15% Percoll layers and combined in a sterile 50-mL Falcon tube.

Transformation of the purified protoplasts was carried out essentially as described by Jacobsen and Close (1991) with a number of modifications. The combined Percoll fractions were diluted to 50 mL with Gamborg's medium and gently centrifuged at 10*g* for 10 min. The supernatant was discarded, and the protoplast pellet was resuspended in 10.5 mL of MaMgMES (Negrutiu et al., 1987); 2.1 mL aliquots were dispensed into each of 5 × 50 mL Falcon tubes. To each tube, 525 μg of calf thymus DNA (in 525 μL TE buffer containing 10 mM Tris, pH 8.0, and 1 mM EDTA) and 105 μg of plasmid DNA (in 105 μL TE) were added, and the tube contents were mixed gently for 1 min. After mixing, 1.58-mL PEG CMS 4000 solution (containing 40% PEG 4000, 0.4 M mannitol, and 0.1 M calcium nitrate) was added to each tube, and the contents were mixed gently for 1 min. The tubes were allowed to stand for 30 min and then centrifuged for 5 min at 10*g* to pellet the protoplasts. The protoplasts were then gently resuspended in 9.5 mL of Gamborg's solution containing 150 μg mL^{-1} cefotaxime and 50 IU mL^{-1} nystatin, and then 1-mL aliquots were transferred into sterile 30-mL Erlenmeyer flasks.

Where required, 100 μL 10^{-4} M gibberellic acid and 10^{-3} M abscisic acid were added to the flasks. Control flasks received the equivalent volume of H_2O. Flasks were then allowed to stand without shaking at room temperature for 43 hr. After the incubation with hormones, the protoplasts were lysed and extracts were assayed for GUS activity using a fluorometer, essentially as described by Jacobsen and Close (1991), with the modification that protoplasts were lysed in 0.5-mL 0.25 M Tris-HCl buffer, pH 7.5. Student's *t* test was used for statistical analysis of results.

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