Deletion analysis has previously shown that the major gibberellic acid (GA)- and abscisic acid (ABA)-responsive elements in the promoter of a high-pl 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 −143 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-pl 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-pl 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-pl α-amylase gene (Amy 2/54) found that the GA-responsive region lies within 300 bp upstream of the transcription start site (Huttley 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-pl α-amylase gene promoter (Amy 1/6-4) fused to a minimal 3S 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 of barley, wheat, 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-pl 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 GCAAGTG 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...
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-pl 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 non-conserved 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-pl 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/-41)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 inserted 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 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.

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...
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-pl 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-pl 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-pl α-amylase gene expression in barley aleurone protoplasts.

Interesting differences are emerging between controlling elements within the promoters of the high- and low-pl 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-pl gene promoters, it seems to be very important. Deletion analysis of a low-pl 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-pl promoter sequences. On the other hand, the available evidence indicates that the TAACAAA/GA sequence is the GARE in both high- and low-pl 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-pl α-amylase genes, but this is yet to be confirmed for low-pl 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-pl α-amylase genes. High-pl 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-pl 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-pl (Jacobsen and Close, 1991) and low-pl (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, \(\gamma TACGTGGC\), 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-pl 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-pl 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 reaggregating the cut ends. The 3SS promoter is a minimal promoter containing only a functional TATA box. To construct α-amylase/3SS promoter fusions, a high-pl 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 3SS promoter in plasmid p3SS(−45)IGN. Plasmids were isolated that contained the α-amylase promoter insert in the two possible orientations, pAM(−174/−41)/3SS(−45)IGN and pAM(−41/−174)/3SS(−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-pl α-amylase promoter were synthesized on an Applied Biosystems DNA synthesizer. Oligos designated 1, 2, and 3 spanned the region from −174 to −106, −107 to −41, and −131 to −84, respectively. The oligos were synthesized with BamHl sites at the 5’ ends and BglII sites at the 3’ ends. Oligos 1, 2, and 3 were cloned into the BamHl site of the plasmid pUC18. The oligos were excised from the plasmid as PstI-Smal 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 Δmut ung strain of E. coli JH1200 (New England Biolabs) 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 Oozukka digestion, protoplasts from 13 flasks (∼130 α-amylase 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 10g 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 x 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 10g to pellet the protoplasts. The protoplasts were then gently resuspended in 95 mL of Gamborg’s solution containing 150 µg mL−1 cefotaxime and 50 µL mL−1 nystatin, and then 1-mL aliquots were transferred into sterile 30-mL Erlenmeyer flasks.

Where required, 100 µL 10−3 M gibberellic acid and 10−3 M abscisic acid were added to the flasks. Control flasks received the equivalent volume of H2O. 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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